

## **Cupid's Epigenetics:**

***How experience with sexual pleasure molds  
sexual motivation and partner preference  
in the rat***

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sexual motivation and partner preference  
in the rat***

Habilitation thesis extended commentary

James G. Pfaus, Ph.D.

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#### Attachments

- A. Original review papers on conditioning and sexual behavior.
- B. Original studies on the conditioning of sexual behavior in male rats.
- C. Original studies on the conditioning of sexual behavior in female rats.

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The seeds for the studies that form the basis of this habilitation thesis were planted in the early morning hours in a bar in Philadelphia, PA (USA) in the spring of 1983 with another mentor and friend, Dr. W. Jake Jacobs, who listened to my fuzzy idea of “post-orgasmic learning” and helped me formalize it into the first study showing Pavlovian control of sexual partner preference in male rats. This finding was replicated years later by my former graduate student Dr. Tod Kippin (now a professor at the University of California Santa Barbara), and extended to female rats by another former graduate student, Dr. Genaro Coria-Avila (now a professor at the Universidad Veracruzana in Xalapa, Mexico). Their findings formed the second major research thrust in my laboratory on the role of learning in sexual behavior and how it is modulated critically by sexual reward or pleasure. These studies were funded continuously from the late-1990s to the present by the Canadian Institutes for Health Research (CIHR).

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## Foreshadows:

*Is it surprising that he prefers the features of his mistress, which promise a hundred units of happiness for him? Even the minor defects of her face, a small pox mark, for example, touches the heart of the man who loves, and, when he observes them even in another woman, sets him dreaming...*

—Marie-Henri Beyle (Stendhal) in *De l'Amour* (1822)

*Erotic fetichism makes an idol of physical or mental qualities of a person or even merely of objects... because they awaken mighty associations with... sexual pleasure.*

—R. v. Krafft-Ebing in *Psychopathia Sexualis* (1886/1929)

*The first cut is the deepest...*

—Cat Stevens (Yusuf Islam) in *New Masters* (1967)



***“Cupid and Psyche” (1817) by Jacques-Louis David***

The story of Cupid (Eros) and Psyche (Soul) comes from Lucius Apuleius Madaurensis’ book *Metamorphoses* (2<sup>nd</sup> Century CE). Cupid is Venus’ (Aphrodite’s) son, and she is none too pleased with his love for Psyche. Psyche endures many trials and tribulations imposed by Venus, but eventually she reunites with Cupid and they have a child, Volupta (Hedone, or pleasure). As I.P. Pavlov wrote, “If you want new ideas, read old books” (1926/1955).

## Food for thought:

*What do we or other animals really need to LEARN about sex?*

*Aren't our preferences, desires, and behaviors innate?*

*Don't we all just DO it?*



***Clearly, the answer is Yes and No...***

## List of original articles from my laboratory on the conditioning of sexual behavior. Articles in red are included in the habilitation thesis

### Reviews:

1. **Pfaus J.G.**, Kippin T.E., Centeno S. (2001). Conditioning and sexual behavior: A review. *Hormones and Behavior*, 40(2): 291-321.
2. **Pfaus J.G.**, Kippin T.E., Coria-Avila, G.A. (2003). What can animal models tell us about human sexual response? *Annual Review of Sex Research*, 14: 1-63.
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Modelos para entender vínculos afectivos en humanos. *Revista de Neurologia*, 47: 209-214.

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## Extended commentary on the articles included in the habilitation thesis

### Introduction

Sexual behavior serves two masters in all mammals, and perhaps in all sexually-reproducing species: reproduction and reward. Although the former is considered an “ultimate” cause of behavior, (e.g., Buss, 1994; Ghiselin, 1973; Meston & Buss, 2007; Symons, 1979), whereas the latter is considered a “proximate” cause, the intense pleasure of sexual stimulation to orgasm links the two together (Georgiadis et al., 2012; Pfaus et al., 2012). In fact, pleasure and reward overall form an important state of the brain in which regulatory and incentive systems and their associated behaviors are positively reinforced. Pleasure guides behavior toward optimality, which helps define utility and value (Cabanac, 1992; Heath, 1972; Kahneman, 2000; Young, 1966). Pavlovian association of features of beauty with sexual arousal and pleasure, for example, comes to reinforce those features by positive feedback. This, in turn, creates a kind of sexual “Gestalt” of preferred partner-related features (both physical and interpersonal) that comes to the foreground of attention against a background of less-preferred features, thus making individuals have a “type” that they are attracted to. But pleasure can also trick the brain by positively reinforcing Pavlovian associations between neutral or even aversive stimuli and pleasurable sexual outcomes. This can result in a preference for, and arousal toward, non-reproductive stimuli, as in the formation of fetishes and paraphilias. These associations are laid down during initial experiences with sexual arousal and pleasure through a process of reward-related imprinting, though they can be iterative throughout the lifespan with new experiences of sexual reward.

Animals engage in species-specific copulatory behaviors that appear as relatively fixed motor responses or action patterns (Dewsbury, 1972; Dewsbury & Pierce, 1989; Madlafousek & Hlíňák, 1977; McClintock, 1984). They also display an optimal balance between inbreeding and outbreeding (e.g., Bateson, 1983) to maximize fitness. However, there is a great deal of flexibility in the way those behaviors and preferences are manifest in different contexts. With experience, animals learn to differentiate those contexts and how to optimize their appetitive precopulatory behaviors toward pleasurable ends. This requires a complex interplay of

hormonal and neurochemical systems in the brain that subserve sexual excitation and attention, and that sensitize the incentive qualities of a sex partner and the motor responses necessary to move from distal to proximal to interactive (Pfaus, Safron, & Zakreski, 2023). All animals that engage in sexual behavior share a common set of principles and end-points that define the behavior. They must be able to respond to hormonal and neurochemical changes that signal their own sexual arousal and desire (Whalen, 1966), or what is commonly referred to as sexual motivation (Ågmo, 1999; Everitt, 1990; Pfaus, 1999; Toates, 2014). They must be able to identify external stimuli that predict where potential sex partners can be found, and then seek out, solicit, court, or otherwise work to obtain sex partners. They must be able to distinguish cues and behavioral patterns of potential sex partners from those that are not sexually receptive, and to pursue sex partners once sexual contact has been made. Neural systems exist that allow both the arousal and stimulation received during sexual contact to be perceived as rewarding or pleasurable, especially orgasm (Pfaus, 2009, 2025). Reward during orgasm leads to a state of sexual satiety in which inhibitory neural systems are activated. But it also contributes to the formation of preferences for salient stimuli associated with sexual pleasure. The most obvious examples of sexual motivation are found in behaviors that denote sexual desire. Clinicians and motivational theorists alike view sexual desire as distinct from sexual arousal in humans and other animals, a distinction that generally reflects blood flow to the genitals and erectile tissues versus a “psychological” sexual interest in which individuals “want” or “crave” sex. When an individual expresses sexual desire, attention and behavior focus on obtaining some form of positive sexual reinforcement. This can occur alone in fantasies or together with others in goal-directed social and sexual behaviors. Thus, in addition to an individual’s awareness of sexual desire (with or without corresponding arousal), desire can be inferred from the work individuals will perform to obtain sexual rewards, the excitement displayed in anticipation of such rewards, and the strength of the incentive value ascribed to a particular sexual stimulus that generates sexual arousal and/or the vigor of copulatory responses. Sexual desire and reward typically go hand-in-hand, and the strength of desire is usually directed by the anticipation of reward. Many behaviors that reflect the reward state, e.g., conditioned arousal, place, or partner preferences, are typically tested using measures of

sexual arousal (clitoral or penile erection, genital blood flow, rate of copulation, ease or delay of ejaculation or orgasm) or sexual desire (e.g., anticipatory locomotion, movements into a preferred place, selective solicitations of the partner associated with greater sexual reward, choice of partner for ejaculation, etc.).

The 27 papers included in this habilitation thesis present original research from my laboratory on the behavioral, neuroanatomical, neuropharmacological, and molecular basis of Pavlovian conditioned sexual responses in male and female rats. The conditioning effects are divided into two major classes of behavior: 1) sexual place and partner preferences that reflect incentive motivation and sexual reward states, and 2) copulatory responses that reflect conditioned sexual arousal. Both depend critically on first experiences of sexual reward that seem to fix into place, or “crystallize” (Stendhal, 1822) a hierarchy of preferred partner-related incentive features, place cues, and appetitive motor responses. This produces a preference for familiarity (i.e., cues that are associated with, and thus predict, a sexual reward state), and forms a counterweight to putative innate mechanisms that drive animals toward promiscuous partner and mate preferences.

### **Mating strategies of rodents**

Different mammalian species display different types of mating strategies, each with their own sets of defining behaviors. The sexual behavior of rodents (e.g., rats, hamsters, gerbils, voles, mice, guinea pigs) have been studied extensively in this regard. For example, in nature, rats (*Rattus rattus* and *Rattus norvegicus*) and meadow voles (*Microtus montanus*, *Microtus pennsylvanicus*) live in large natal groups and are described as having promiscuous mating strategies that are characterized by cooperative and promiscuous group mating in both males (polygamy) and females (polygyny) (Barnett, 1963). In contrast, prairie voles (*Microtus ochrogaster*) live in far smaller population densities and display social monogamy, with females and males forming pair bonds during their first sexual experiences that result in biparental care of the offspring, nest sharing, and mate-guarding behavior (Carter, DeVries, & Getz, 1995; Klug, Heuschele, & Kokko, 2010; Wang et al., 1998; Young & Wang, 2004). Thus, although voles and rats are closely related, they have adapted very different mating strategies, presumably due to

differences in environmental factors (e.g., population density, sex ratio, predator range, circannual day length) that are critical for the development of promiscuity or monogamy. However, the story is far more complex. Social monogamy does not equate to sexual fidelity. For example, both male and female prairie voles sneak copulations while foraging (Donnelly, Bergin, & Ihrig, 2015; Ophir, Wolff, & Phelps, 2008; Wolff et al., 2002), giving rise to a significant amount of cuckolding among bonded pairs.

In keeping with notions of promiscuous mating strategies, sexual partner preferences in rats are often associated with the introduction of novel versus familiar partners. Male rats copulating to sexual exhaustion with the same partner can become re-aroused by the introduction of a novel partner (Beach & Jordan, 1956). Similarly, male rats display more ejaculations (and more vigorous copulatory behaviors) when a new female partner is introduced after each ejaculatory series, relative to copulation with the same partner (the so-called “Coolidge effect”<sup>1</sup>; Bermant, Lott, & Anderson, 1968; Ford & Beach, 1951). Both are often taken as evidence that male rats “prefer” novel partners, though an alternate hypothesis is that the novel, uncopulated female possesses more potent vaginal odors, which stimulates greater sexual arousal in the male, compared to the partner that has received multiple ejaculations. Female Golden hamsters or rats copulating with the same male also show sexual satiety, including a decrease in sexual responsivity and increased rejection responses. Introduction of a novel male reverses this relative to the introduction of the same male (Lester & Gorzalka, 1988; Lisk & Baron, 1982; Ventura-Aquino et al., 2016). However, in some group mating studies, dominant female rats have been observed to show sexual/mate preferences for a particular male, and to intercept that male and take his ejaculations selectively after the subordinate

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<sup>1</sup> Named after Calvin Coolidge (1872-1933), the 30<sup>th</sup> US President (1923-1929). The story goes that “Silent Cal” was visiting a farm in New Jersey with his wife Grace in the mid-1920s. In the hen house, Grace was astonished at the number of eggs, especially given that the farmer had only one rooster. She asked the farmer, “Be sure to let Mr. Coolidge know that this was the work of ONE rooster!” When the President walked through the hen house, the farmer relayed the message. Coolidge, who had grown up on a farm, quickly replied, “Different hen every time?” The farmer nodded yes, to which Coolidge quipped, “Tell THAT to Mrs. Coolidge!” No one knows any more if this actually happened. Some have suggested that the story was made up by Frank A. Beach (1911-1988), one of the founders of behavioral endocrinology, as a sarcastic joke about the enhancing effect of novel partners on male sexual arousal, a phenomenon well known to farmers and animal breeders, and the fact that Coolidge was regarded as the most boring and un-sexy President of the United States up to that point.

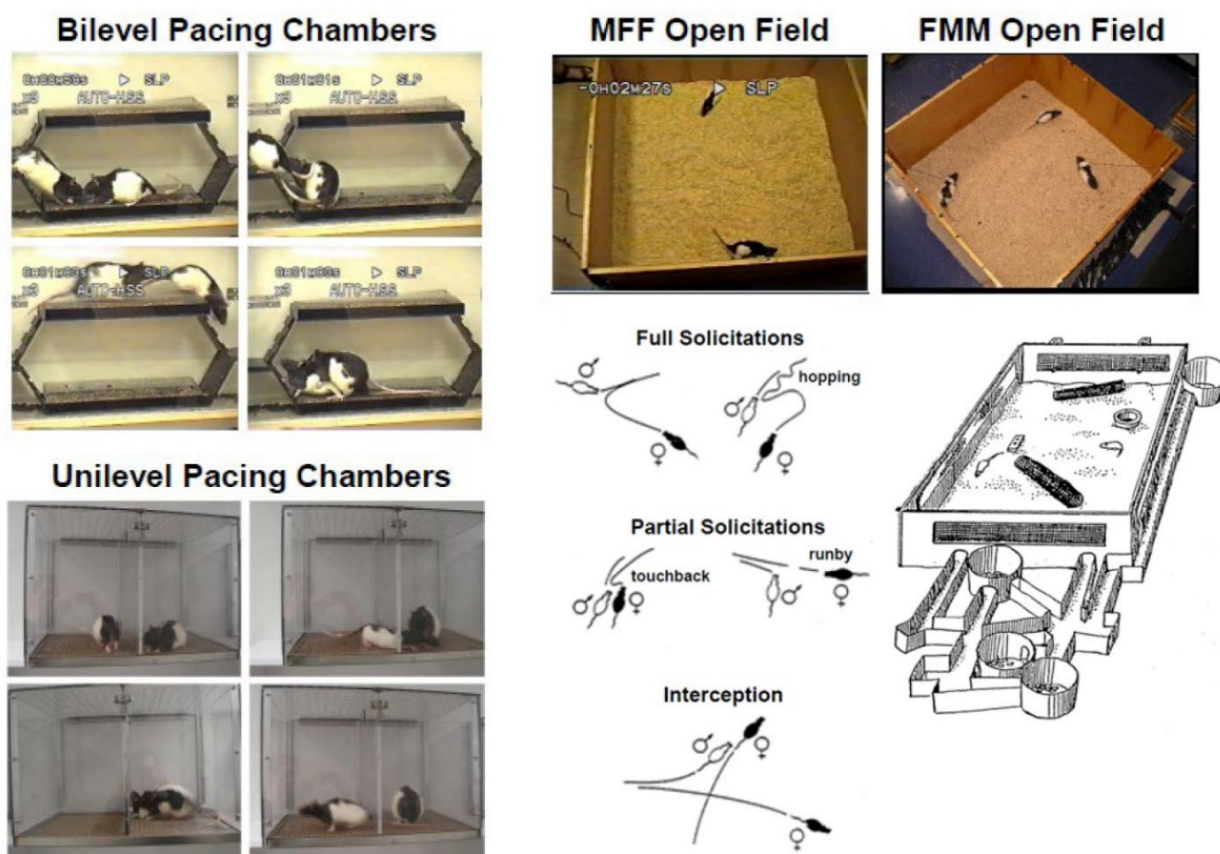
females have done all the work taking his multiple penile intromissions (see Chu, Guarraci, & Ågmo, 2015; Figure 2 in McClintock, 1984). Male rats emit a long 50 kHz ultrasonic call prior to ejaculating, which typically increases female competition and approach behaviors.

### **Rodent sexual behaviors**

All rodent species display sexual behaviors that are sex stereotyped. Females display different patterns of sexual attractivity, proceptivity, and receptivity (Beach 1976, Pfaus et al., 2015). Attractivity can include scent marking to have males move from distal to proximal. Proceptivity can include solicitations that initiate bouts of sexual interaction and pacing behaviors that control the rate of sexual interaction and force males to chase the females. Receptivity is typically denoted by lordosis, the concave arching of the back that raises the rump to allow penile intromission, and in some species like hamsters, an additional lateral tail deflection that un-obstructs the vagina. Thus, a bout of copulatory activity in female rats begins with a solicitation, then a runaway during which males chase them, then lordosis which allows the male to mount (**Figure 1**). In turn, females receive olfactory, auditory, and somatosensory stimulation from the male. Such stimulation includes odors and MHCs that allow females to identify and differentiate males and their proximity, ultrasonic vocalizations that allow females to determine the moment-to-moment sexual status of different males (and also potentially their proximity), flank stimulation from male palpation, clitoral stimulation (CLS) from pelvic thrusting, and vaginocervical stimulation (VCS) from penile intromissions and ejaculations (Pfaff, Montgomery, & Lewis, 1977; Pfaus et al., 2015). When female rats are sexually receptive, they also display appetitive behaviors, such as increased locomotion in anticipation of sex, and operant behaviors, such as nose pokes, learning to run mazes, and overcoming obstructions, to gain access to males (Cummings & Becker, 2012; Pfaus, Kippin, & Centeno, 2001). When presented with sexually naïve or sluggish males, females will display super-solicitations. In female rats, these typically involve mounting the male, palpating his flanks, then abruptly dismounting, hopping over him, and holding a pre-lordosis crouch (Afonso & Pfaus, 2006). This behavior eventually stimulates the male to mount, after which the female displays a normal pattern of solicitations and pacing behaviors.



Female sexual behaviors are typically expressed during and immediately after ovulation, when plasma levels of estradiol have peaked and are coming down, levels of testosterone have peaked as a bolus, and levels of progesterone are rising. Although lordosis can be primed in ovariectomized (OVX) rats with estradiol alone, the full complement of attractivity, proceptivity,



**Figure 1: Rat copulation in several chambers.** Top left: Bilevel pacing chambers (Mendelson & Gorzalka, 1987; Pfaus, Mendelson, & Phillips, 1990). Bottom left: Unilevel pacing chambers (Erskine, 1985; Paredes & Alonzo, 1997; Paredes & Vazquez, 1999). Bouts of copulation are typically initiated by the female through solicitations. In bilevel chambers the female solicits as she would in the wild by making a headwise orientation to the male and then either darting or hopping away on the same or other level. This forces the male to chase her and females regulate the rate of copulation by running from level to level. The endpoint of each solicitation and runaway is lordosis, allowing the male to mount and gain vaginal intromission, after which the male dismounts and grooms his penis. This is repeated several times until the male ejaculates. Unilevel pacing chambers are bisected by a Plexiglas or mesh partition with one or more holes cut out of the bottom that are big enough to allow the female to pass through but too small for the male. This restricts the male to one side. Females initiate copulatory contact by moving to the male's side, and regulate the rate of contact by running from side to side. Top right: Open fields used for tests of conditioned sexual partner preference for one male choosing among two free-ranging females (MFF) or for one female choosing between two tethered males (FMM). Bottom right: Large open field used by McClintock (1984) to examine group mating patterns and preferences in female rats. From Pfaus et al., 2015.

and receptivity requires the sequential administration of estradiol and progesterone (Albert et al., 1991; Pfaus et al., 2015; 2025), estradiol and testosterone (Jones, Ismail, & Pfaus, 2017), or estradiol and dihydrotestosterone (Maseroli et al., 2020). During estrus termination, attractiveness and proceptivity are the first behaviors to diminish with repeated VCS, whereas flank stimulation can continue to induce lordosis for several hours after (Pfaus, Smith, & Coopersmith, 1999; Pfaus et al., 2000). However, as proceptivity wanes, females show rejection responses to male mounts, and often take themselves back into the burrow systems and out of sexual interaction entirely (Calhoun, 1962; McClintock, 1984).

Male sexual behaviors involve precopulatory investigation (e.g., sniffing and licking the anogenital area of females) and chasing females once sex is solicited. Precopulatory behaviors can also involve appetitive locomotion in anticipation of sex (Mendelson & Pfaus, 1989), noncontact erections in the vicinity of females (Sachs, 1996; Sachs et al., 1994), and operant responses to gain access to females (Everitt, 1990; Pfaus, Kippin, & Centeno, 2001). Copulatory behaviors include mounts, intromissions, and ejaculations. The male typically mounts females from behind, palpating their flanks (which induces lordosis in the female), then pelvic thrusts his erect penises into the vagina for a brief period followed either by a short period of rest while still maintaining vaginal intromission (e.g., mice), or a dismount and genital grooming (e.g., rats), followed by another bout of intromission initiated typically by female solicitations or hops and darts. After several bouts and a requisite amount of sensory stimulation of the penis, a particular sensory threshold is reached for ejaculation (Truitt & Coolen 2002). The ejaculatory pattern of male rodents includes a deep and protracted penile intromission, along with a hunched back (a convex kyphosis posture), after which male dismounts. Ejaculation results in a refractory period of variable time. In many rodents, this includes an absolute refractory period in which the male displays behavioral quiescence and/or sleep, followed by a variable refractory period during which the male grooms his genitals and female solicitations or other tactile stimuli such as tail-pinch, can stimulate the male to reinitiate copulation. Together, the time from first intromission before an ejaculation to the first intromission after an ejaculation is termed an “ejaculatory series” (Hull & Dominguez, 2015; Sachs & Barfield, 1976) and male rats can achieve several ejaculatory series before becoming sexually exhausted (Beach & Jordan,

1956; Beach & Rabedeau, 1959; Rodríguez-Manzo & Fernández-Guasti, 1994). In the male rat, appetitive sexual behaviors and the pattern of mounts, intromissions, and ejaculations is driven by androgens (testosterone and dihydrotestosterone), and also by estradiol through the conversion of testosterone by aromatase in preoptic and hypothalamic neurons (Balthazart & Foidart, 1993; Södersten et al., 1986; Södersten, Eneroth, & Hansson, 1988).

### **What is rewarding about sex to a rat?**

Reward, also known as positive reinforcement, describes an event or state that increases the probability or rate of a response when the event is contingent on that response (Ferster & Skinner, 1957; Skinner, 1938). Reward is also synonymous with pleasure, and pleasure is a state of the brain that, in Pavlovian terms, is an unconditioned stimulus (UCS; Pavlov, 1927). Neutral stimuli that are associated in temporal contiguity with pleasure become conditioned stimuli (CSs) that predict the pleasurable state. Thus, in all motivational systems, both operant and Pavlovian contingencies become associated with reward (Toates, 1986). Brain plasticity allows this to occur similarly or differently in different individuals given environments in which they have similar or different experiences. Somewhat counterintuitively, the very mechanisms underlying neural plasticity help to create behavioral habits (i.e., automated motor outputs) in the brain's motor systems (e.g., basal ganglia and cerebellum; Costa, 2007; Graybiel, 1998), and "crystallize" both environmental, social, and interpersonal cues as preferred stimuli by activation of sensory cortical, limbic (e.g., amygdala, nucleus accumbens (NAc), and basal forebrain structures like the medial preoptic area (mPOA; Hermans et al., 2014; Johansen et al., 2011; Lechner & Byrne, 1998).

In all motivational systems there are two types of rewards: 1) appetitive rewards that typically increase responding; and 2) consummatory rewards that bring responding to maximal rates before inducing a satiety state that terminates responding. The appetitive state is one of arousal, desire, and wanting (Berridge & Robinson, 1998; Fibiger & Phillips, 1988; Koob, 1996). It is derived from the anticipation of consummatory reward. The two are linked together as a behavioral cascade in time, and driven by differential activation and sensitization of mesolimbic, preoptic, and hypothalamic dopamine systems that coordinate attention, arousal,

and movements from distal to proximal to interactive (Pfaus, 2009; Pfaus, Safron, & Zakreski, 2023). The appetitive period corresponds to phasic dopamine transmission in mesolimbic terminal regions, such as the NAc, which is disinhibited by the specific actions of endogenous opioids like  $\beta$ -endorphin or enkephalins acting on  $\mu$  opioid receptors (MORs) that inhibit the influence of inhibitory GABA interneurons on dopamine cell bodies in the ventral tegmental area (VTA). This disinhibition grows during the consummatory period, which corresponds to tonic and continuous dopamine transmission (Grace, 2000; Goto & Grace, 2005). Satiety, in turn, inhibits the appetitive state. This occurs by mass activation of endogenous opioids and serotonin, which inhibit mesolimbic dopamine neurons in the VTA and dopamine inputs to the hypothalamus (e.g., Hoebel, 1985; Hoebel et al., 1989; Pfaus, 2009).

Take an example from feeding. To a hungry rat, small consummatory rewards (e.g., small food pellets) sustain or increase operant responding for food (e.g., bar-pressing, faster time to the goal in a maze), whereas a large consummatory reward (e.g., a full meal) induces satiety that terminates the feeding bout. However, the availability of food can also be signaled using Pavlovian conditioned stimuli (CSs, e.g., tone, light, or a neutral odor), and once paired, the signal can induce appetitive food seeking behavior even in a sated rat (Weingarten, 1983). This occurs through a small, phasic increase in mesolimbic dopamine release that counteracts some of the inhibition induced by satiety (Blackburn, Pfaus, & Phillips, 1992).

Sexual responding follows the same patterns of mesolimbic and preoptic dopamine transmission as feeding. In male rats, appetitive mate-seeking is driven by phasic dopamine transmission in the NAc and mPOA. Consummatory copulation is driven by tonic transmission in these regions, whereas ejaculation causes an immediate decrease in dopamine transmission which stays inhibited during the absolute refractory phase and returns to baseline during the relative refractory phase (Blackburn, Pfaus, & Phillips, 1992; Damsma et al., 1992; Pfaus et al., 1990). A similar pattern is observed in female rats during appetitive mate-seeking and copulation (Pfaus et al., 1995). Endogenous opioids are released during ejaculation in male rats (Coolen et al., 2004; Szechtman, Hershkowitz, & Simantov, 1981), as is serotonin (Lorrain et al., 1999; Pfaus, 2009), although these latter effects have not been examined in female rats.

The pattern of appetitive and consummatory sexual behaviors also corresponds to changes in ultrasonic vocalization (USV) patterns. During vaginal intromissions, male rats vocalize trills and flats in the 50-kHz range, with a long sustained 50 kHz call prior to ejaculation (Biały, Rydz, & Kaczmarek, 2000; McGinnis & Vakulenko, 2003; White et al., 1990). However, immediately upon ejaculation, males vocalize in the 22-23 kHz range, which lasts throughout the beginning of the absolute refractory period (Barfield & Geyer, 1972; White et al., 1990). Although calls in this range have been regarded as aversive (e.g., trills that appear as growls), the post-ejaculatory call is syntactically different, consisting of a flat with a downward deflection and aspiration that is clearly linked to a reward state (e.g., Burgdorf et al., 2008). Females emit similar calls during copulation with males, although approximately 5 to 10 kHz higher in range. During distributed CLS with a soft paintbrush applied in such a way that mimics the pelvic thrusting pattern of a male rat, females emit 60 kHz trills and flats both upon receipt of the CLS and as they solicit more CLS from the experimenter. However, this changes abruptly in different females after different amounts of CLS, wherein they show 25 kHz trills and flats indicative of an aversive state when the experimenter attempts further stimulation. After a variable period of approximately 20 to 30 sec, the females begin emitting 60 kHz calls again and solicit more CLS (Gerson et al., 2019). Thus, in both male and female rats, there are indications of an orgasm-like response (OLR) in the structure of neurotransmitter activation and USV patterns. Both suggest a consummatory reward state and the induction of sexual satiety (Pfaus et al., 2016). However, despite these indications, does reward actually alter behavior?

*Conditioned appetitive and copulatory behaviors.* Both male rats and sexually receptive female rats will work to obtain sex partners. This includes learning to bar-press, nose-poke, cross electrified grids, and navigate obstruction boxes and complex mazes (reviewed in Pfaus, Kippin, & Centeno, 2001). Male rats also show increased motor excitation (level changes in bilevel pacing chambers) in anticipation of copulation to ejaculation with sexually receptive females, but not in anticipation of interacting with sexually non-receptive females or other males (Mendelson & Pfaus, 1989). The effect of allowing incomplete copulation (i.e., intromission without ejaculation) on several operant behaviors has been examined. In contrast to males allowed to mount and intromit only, males allowed to copulate to ejaculation made

more consistent choices in a T maze (Kagan, 1955) and developed faster running speeds in T-mazes and hurdle climbing boxes (Sheffield et al., 1951; Whalen, 1961).

Copulatory behavior in male rats is dependent on genitosensory feedback. Mounts without intromission are not sufficient to sensitize baseline rates of copulatory behavior, whereas sensory feedback from penile stimulation, even of a male rat's first intromission, is sufficient to crystallize the stereotyped behavioral pattern of intromissions leading to ejaculation (Beach, 1956; Larsson, 1956). It is also dependent on the copulatory context and what male rats are "allowed" to do in different contexts. For example, a male rat's copulatory pattern can be modified by operant contingencies that increase arousal. Silberberg and Adler (1974) reported that male rats can learn to control their intromission frequency under a negative punishment schedule of responding. Rats were limited to seven intromissions per copulatory session. Over five trials, the rats spontaneously decreased the number of intromissions before ejaculation to six (essentially rendering them rapid or premature ejaculators). In contrast, control rats showed no alteration in intromission frequency over the five trials. This suggested that intromissions without ejaculation over time induced an aversive state that facilitated sympathetic arousal, since ejaculation is under sympathetic control. Ismail, Zhao, and Pfaus (2008) compared male rats that learned to copulate in unilevel pacing chambers with a one-hole versus four-hole divider. In the one-hole condition, female exits lead the male to poke his head through the hole or place his body near the hole, both of which obstruct the hole and delay the female's return (**Figure 2**). In contrast, in the four-hole



**Figure 2:** Female availability is altered in unilevel pacing chambers bisected by a one-hole (left, middle) or four-hole (right) divider. Males obstruct the one-hole divider when the female exits, and must learn to get out of the way for the female to return. In the four-hole divider, the female has unobstructed access by darting through another hole.

condition, females can easily cross the divider in and out of the male's side by going through one of the holes that the male is not obstructing. Males trained in the four-hole condition had significantly shorter ejaculation latencies compared to males trained in the one-hole condition. Importantly, those differences persisted when males were switched into the other pacing condition, suggesting that the pattern of copulation in these males had become fixed based on their early experience with different temporal patterns of female availability.

Anticipatory sexual behaviors can come under Pavlovian control. For example, sexual approach and courtship behaviors in male Japanese quail (*Coturnix coturnix japonica*) can be displayed in response to a buzzer (Farris, 1967) or a toy dog (Domjan, O'Vary, & Greene, 1988) paired with subsequent access to a female copulatory partner. Similar conditioned courtship behavior has been observed in male ring doves (*Streptopelia risoria*) in response to a light associated with access to a female copulatory partner (Burns-Cusato, Cusato, & Daniel, 2005). Sachs and Garinello (1978) reported that placing male rats into a chamber in which copulation had previously occurred dramatically reduced the latency for males to display spontaneous penile erections. Anticipatory sexual arousal in males also increases plasma testosterone levels (Anonymous, 1970; Bonilla-Jaime et al., 2006). Graham and Desjardins (1980) found that male rats that had their first copulatory experiences to ejaculation with females scented with a neutral odor (methyl salicylate; wintergreen) showed increased plasma luteinizing hormone and testosterone levels when presented with the odor alone. Males that copulated with unscented females did not, indicating that sexual arousal and hormone release had come under Pavlovian control by a stimulus associated with copulation to ejaculation.

Classically conditioned stimuli are also capable of increasing sexual arousal as reflected by copulatory rate measures. For example, Zamble and colleagues (Zamble et al., 1985; Zamble, Mitchell, & Findlay, 1986) used placement of male rats in a holding cage as a conditioned stimulus to signal noncopulatory exposure to a receptive female during training trials. On test trials, they found that placing the males into the holding cage prior to copulation resulted in significantly shorter latencies to intromit and ejaculate than if the conditioned stimulus was omitted. Subsequent studies found that a second-order conditioned stimulus (a plastic fish toy placed into the holding cage) was effective at eliciting arousal (Zamble et al., 1985). Similarly,

male rats that wear a rodent tethering jacket (**Figure 3**) during their first nine copulatory experiences to ejaculation with receptive females copulate normally with the jacket on, but show disrupted copulation with the jacket off, including significantly fewer anticipatory level changes, longer intromission latencies, and fewer ejaculations (see Pfaus, Erickson, & Talianakis, 2013). In contrast, males that had their first nine multiejaculatory experiences without the jacket on did not show any enhancement or debilitation of copulatory behavior with the jacket on or off, indicating that the jacket itself had no inherent sexually-arousing properties. In fact, in that study if the jacket on was paired with noncopulatory access to sexually non-receptive females, and the jacket off paired with copulation to ejaculation with sexually receptive females, the jacket came to possess conditioned inhibitory properties. When males in that group were tested with jacket on, they displayed significantly longer ejaculation latencies and fewer ejaculations overall compared to males with the jacket off. This indicates that the jacket was a neutral stimulus that came to predict sexual excitation and arousal if paired with successful copulation, or sexual inhibition and de-arousal if paired with thwarted copulation.



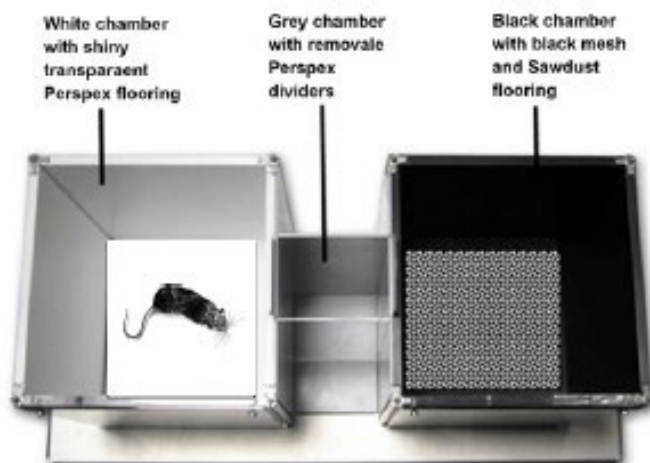
**Figure 3:** Male rats wearing a rodent-tethering jackets. Left: male in the conditioning paradigm of Pfaus, Erickson, and Talianakis (2013). Right: males wearing tethering jackets in an open field where a female rat is being tested for conditioned partner preference (see below).

*Conditioned place preference (CPP).* All animals display a conditioned preference for places associated with reward states. This is assessed in the laboratory using a CPP apparatus with two distinctive compartments that a rat can easily differentiate (**Figure 4**). This apparatus is used typically to examine the rewarding properties of drugs, or appetitive or consummatory



events that are either rewarding, negatively reinforced (e.g., after removal of an aversive stimulus), or aversive (Tzschentke, 1998).

**Figure 4: CPP apparatus.** Rats normally display an unconditioned preference for the dark compartment during a pre-test, though they will explore the light compartment. Rats are then given repeated sequential exposure to an event (e.g., ejaculation, paced copulation) or a drug effect while placed into the light compartment, and a control condition while placed into the dark compartment. On a final test, rats are placed into the center compartment, and the doors are opened on both sides so that the rat can roam freely between the compartments. Preference is assessed statistically by comparing the amount of time spent in the three compartments (light, dark, middle start compartment).



If the rat spends significantly more time in the light compartment, or shows a significant shift of time spent in the dark compartment on the pre-test to the light compartment on the final test, then it is assumed that the event or drug had rewarding or negatively reinforcing properties. If the rat avoids the light compartment, then it is assumed that the event or drug was aversive.

In male rats, CPPs have been established with sexual reward using two different conditioning paradigms. In one paradigm, copulation to ejaculation occurs within one distinctive compartment of the CPP box, and this compartment is subsequently preferred over the other compartment in which copulation was not allowed (e.g., Everitt, 1990); a CPP developed by this procedure is referred to as copulatory CPP. Copulation to ejaculation is required to establish a copulatory CPP (intromissions alone are not sufficient). However, once established, copulatory CPPs can be maintained by intromissions alone, whereas prevention of intromission extinguishes the CPP (Hughes, Everitt, & Herbert, 1990; Tenk et al., 2009). In a second more common paradigm, male rats are allowed to copulate to ejaculation in a separate chamber and then transferred immediately after ejaculation to the previously non-preferred compartment of the CPP apparatus. The control condition for the other compartment is either no copulation or copulation to a small number of intromissions (e.g., 5). Following such training the compartment associated with the postejaculatory reward state becomes preferred over the other compartment (e.g., Ågmo & Berenfeld, 1988). A CPP produced this way is referred to as postejaculatory CPP. Demonstrations of postejaculatory CPP might appear puzzling at first

glance because the place CS is presented following the UCS or reward (copulation to ejaculation). This appears to involve a “backward” pairing of the stimuli that is not supposed to yield conditioned responding to the CS. However, if the brain state induced by ejaculation is considered to be the rewarding UCS, then the pairing of CS and UCS is simultaneous and postejaculatory CPPs follow the rules of Pavlovian conditioning. In both paradigms, ejaculation is necessary and sufficient to induce a reward state that is interpreted as a pleasurable UCS. Copulation to ejaculation also induced CPP in male prairie voles (Ulloa et al., 2018)

As mentioned above, during copulation female rats typically control the rate of genital stimulation (CLS and VCS) that they receive from the male. This includes mounts with pelvic thrusting that induce external CLS and penile intromission/ejaculation that induce internal CLS and VCS. Paredes and colleagues were the first to show that paced copulation in unilevel pacing chambers (with the center barrier in place that only females can cross) induced a significant CPP in females relative to unpaced copulation in the same chamber with the center barrier removed (Paredes & Alonso, 1997; Paredes & Vazquez, 1999). This effect was dependent on full steroid priming with estradiol and progesterone. A subsequent study by Meerts and Clark (2007) suggested that unpaced copulation in a large chamber could also induce CPP. However, in that study, the chamber was large enough for the female to enforce a preferred interval between intromissions, something also observed by Jenkins and Becker (2003) using nose-pokes by the female as the operant that allowed entry to the male’s side.

Of the different types of stimulation received by the female, which results in CPP? Meerts and Clark (2009) found that artificial VCS applied every 30 sec (to mimic the normal inter-intromission interval) using a 1 ml syringe plunger with 200 g of force held on the cervix for 2 sec, induced significant CPP in sexually naïve females relative to a control condition of no VCS. Social interaction of the female with a castrated male did not result in CPP in that study. Parada et al. (2010) found that artificial CLS, using a soft natural fiber camel-hair paintbrush that was applied in 3 upward deflections against the external clitoral glans every 5 sec, also induced significant CPP in sexually naïve females relative to a control condition in which the tail was raised but no CLS was applied. CLS administered every sec did not induce a significant CPP, indicating that its distribution in time, like that of VCS, is part of the reward that females seek

by pacing the rate of copulation. Unlike copulation, however, distributed CLS induced significant CPP regardless of the hormonal priming of the female (oil vehicle, estradiol alone, or estradiol and progesterone; Parada et al., 2012). The effect of hormonal priming was also examined on the retention of pacing-induced CPP during extinction in that study. Fully primed females were given 6 alternating, counterbalanced copulatory conditioning trials that lasted 30 min each, 3 with the divider, and 3 without. Immediately after paced trials, females were placed into their initially non-preferred side of the CPP box for an additional 30 min. After unpaced trials, females were placed into their initially preferred side of the CPP box for an additional 30 min. CPP was tested 4 days after the final conditioning trial to be able to manipulate the female's hormonal state prior to the final preference test. Females were randomly assigned to one of three hormone groups and received estradiol and progesterone, estradiol alone, or the oil vehicle. Females were then placed into the middle compartment of the CPP chamber, and allowed free access to both distinctive compartments. After the test, females were maintained on their respective hormone treatments and put on a partial extinction schedule, in which the maintenance of CPP was tested again 4 days after the first CPP test but without prior copulation. All females, regardless of hormone condition, displayed CPP on the first CPP test. However, by the second CPP test, only the fully primed females showed significant extinction of CPP. Those data indicate that the memory of sexual reward can be retained in females despite being in a hormonal state that no longer supports copulation. In contrast, when females are primed to desire sexual interaction but do not receive it, then they begin to show extinction of CPP.

Finally, anesthesia of the clitoris (Parada et al., 2014) or clitoris and vagina (Meerts, Strnad, & Schairer, 2015) with 5% lidocaine altered the structure of paced copulation in unilevel pacing chambers, with females showing less time spent with males. It also blocked the development of CPP. Prior experience with paced copulation also blocked the ability of artificial distributed CLS to induce a CPP (Parada, Jafari, & Pfaus, 2013). This is similar to a “UCS preexposure effect” (sensory preconditioning; e.g., Hinson, 1982) typically found in studies of Pavlovian conditioning in which prior experience with a whole UCS (in this case, the OLR induced by the combination of CLS and VCS during paced copulation with a male) devalues the

reward associated with each separate component (Pfaus et al., 2016). Thus, to a sexually naïve female, external CLS induces a complete reward state; but to a sexually experienced female, external CLS is one part of a larger reward state that includes VCS, control of the rate of copulation through pacing behavior, cues from the gonadally-intact male, flank stimulation, etc.

In summary, the reward value of copulation for both male and female rats comes from genitosensory stimulation and OLRs experienced in contexts of increased sexual arousal.

### Conditioned partner preference (CPaP) and mate choice.

For many years, social and sexual partner preferences were studied in rats, voles, mice, and other rodents and lagomorphs using an apparatus in which the test subject could approach two potential conspecifics in a T- or Y-maze that were contained in a closed goal compartment with wire mesh. Although this allowed the animals to smell, hear, and see one another, they could not interact physically (**Figure 5, left and middle**) which made it unclear whether the preference was social or sexual, or based on novel vs. familiar olfactory or auditory cues. Other chambers were constructed that allowed physical interaction as a dyad (**Figure 5, right**), but not with the type of partner competition observed in the wild or group mating contexts, as reported by McClintock (1984). McClintock used a large open field to examine unobstructed



**Figure 5: Different partner preference chambers to test olfactory and sociosexual preferences in rats, mice, and voles.** In the chambers on the left and middle, rats or mice are allowed to roam freely between two isolated compartments, and the time spent near the two conspecifics is recorded. If the test subject spends significantly more time with one over the other, then a preference is inferred. In the schematic chamber on the right (Aragona & Wang, 2009), vole subjects could choose to physically interact with either the familiar or novel partner. An identical chamber was used to assess same- versus opposite-sex partner preferences in rats (Ramírez-Rodríguez et al., 2021).

group mating and partner preference between 2 males and 3 females (**Figure 1, bottom right**).

Taking McClintock's lead, my laboratory has used an open field (123 x 123 x 46 cm) in which the test subject (either male or female) can interact freely with two potential partners based on prior associations of olfactory or somatosensory stimuli with paced copulation (**Figure 1, top right**). For studies of CPaP in males, we use an "MFF (one male, two females) open field choice paradigm." Males are placed into the open field for 5 min, after which two sexually receptive females are introduced on opposite sides of the male and the animals allowed to interact for 30 min. One female bears the conditioned cue while the other does not. All tests are recorded on video and scored subsequently using a computerized event scoring program that provides data on which female received the first mount, intromission, and ejaculation, and the distribution of mounts, intromissions, and ejaculations per female throughout the 30-min test. For studies of CPaP in females, we use an "FMM (one female, two males) open field choice paradigm." Males wearing a rodent tethering jacket are attached to a thin wire spring at diagonal ends of the open field. The female is then introduced in the middle of the open field and allowed to interact with the two males for 30 min. One male bears the conditioned cue while the other does not. The video recording is then scored for the choice of male for first solicitation, first mount, first intromission, and first ejaculation, the number of mounts, intromissions, and ejaculations received from each male during the 30-min test, and the total time spent near each male during the test (quadrant analysis). During preliminary runs of the FMM paradigm, it was discovered that males were very cooperative, taking turns chasing and mounting the female, and crawling all over one another during the period between copulations with the female. This created a confound for our initial studies of odor conditioning because the scented male would essentially spread his scent onto the unscented male. Thus, it was necessary to tether them<sup>1</sup>. However, this gave the female far more control over which

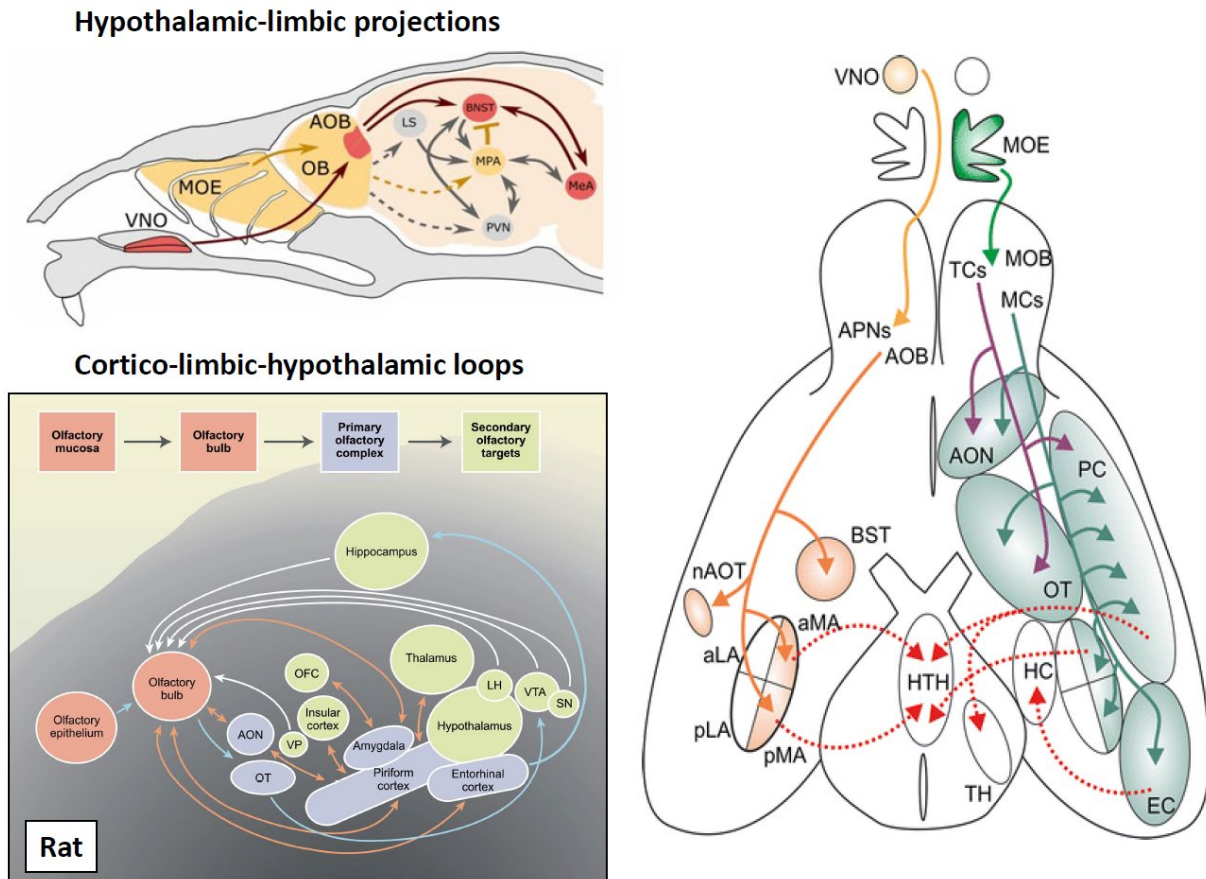
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<sup>1</sup> Serendipitously, when one of my graduate students attempted to use formerly tethered males as untethered stimuli for females in unilevel pacing chambers, those males displayed sluggish copulation. Testing them again with fully primed females in unilevel pacing chambers or the open field did not change their sluggish copulation. However, when tested with the tethering jacket on, those males displayed vigorous copulation. This led to the formal study by Pfaus, Erickson, and Talianakis (2013) reviewed above.

male she chose to be near. Routinely, the females would run away from the preferred male after receiving an intromission (similar to the behavior they display in both unilevel and bilevel pacing chambers) and run around the open field, passing the nonpreferred male, who would attempt to mount, but typically not be quick enough to catch the female before she ran out of his zone (his zone being defined as the extent of his tether). In cases where the nonpreferred male did catch the female, it was not uncommon to see her receive a mount or intromission and then rear up in a boxing posture and push the male away. She never displayed this behavior toward the preferred male.

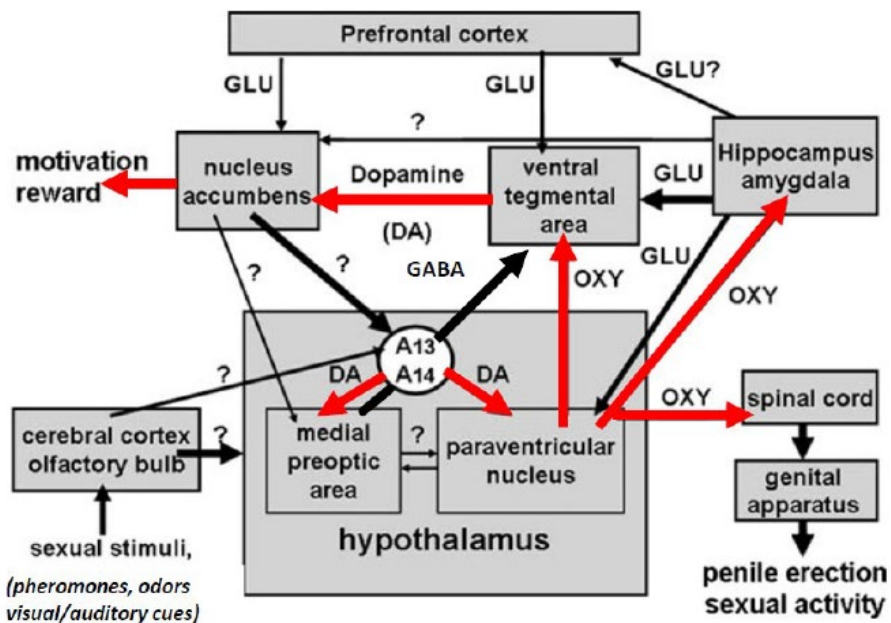
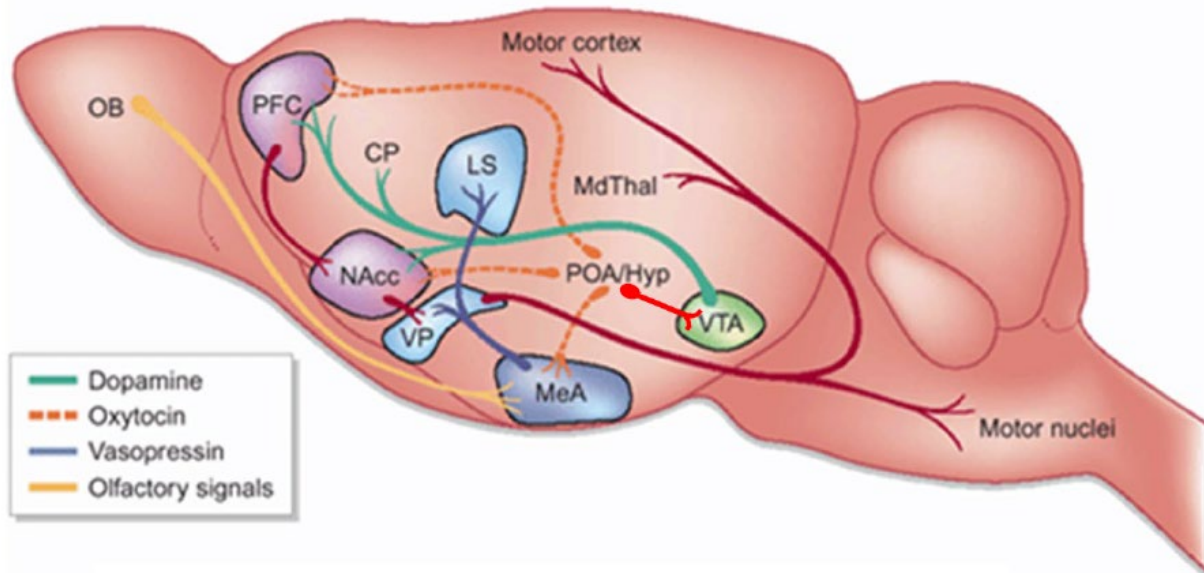
*Olfactory-based CPaP.* To understand fully how an olfactory-based CPaP might work, it is necessary to first provide an overview of the olfactory system of rats and how it is stimulated by pheromones and odors that result in the activation of attentional, bonding, and reward mechanisms. Olfaction is the dominant sense in rats (Barnett, 1963; Slotnick, 2001). Two olfactory systems exist that transmit pheromonal and odor stimulation: 1) the vomeronasal system, where pheromones (typically steroid hormone conjugates) activate the vomeronasal organ (VNO) which then activates the accessory olfactory bulbs; and 2) the main olfactory system, where odorants binding to receptors in the main olfactory epithelium (MOE) activate the main olfactory bulbs (**Figure 6**). The VNO system projects from the accessory olfactory bulbs to hypothalamic structures like the mPOA, bed nucleus of the stria terminalis (BNST), lateral septum (LS), and paraventricular nucleus (PVN), along with projections to limbic structures like the medial amygdala and hippocampus (**Figure 6, top left**). The MOE system projects from the main olfactory bulbs to cortical regions (e.g., olfactory tubercle, orbital frontal cortex, piriform cortex, entorhinal cortex, insula) that serve as olfactory and emotional interfaces with limbic structures like the amygdala and regulatory structures like the hypothalamus, along with the mesolimbic and nigrostriatal dopamine systems in the midbrain (**Figure 6, bottom left**). The two systems converge in the amygdala and thalamus (**Figure 6, right**), where they feed back to other cortical, limbic, and hypothalamic structures (Shipley, Ennis, & Puche, 2004). In particular, outputs from the olfactory tubercle to the VTA disinhibit mesolimbic dopamine neurons and activate mesolimbic (A10) dopamine release in regions like the NAc and medial

amygdala, and orbital and infralimbic frontal cortex. Outputs from both the main and accessory olfactory bulb activate incertohypothalamic (A13) dopamine release in the mPOA, which coordinates elements of sexual motivation with the activation of parasympathetic outflow necessary for genital arousal (reviewed in Pfaus, 2009). Outputs to the PVN also activate oxytocin neurons that are implicated in social, sexual, and maternal bonding (Carter, 2022; Coria-Avila et al., 2014). Thus, the bonding system overlaps with both the incertohypothalamic and mesolimbic dopamine systems, to activate attention to reward-related cues, and especially attention to conspecifics (or persons) that are predictive of reward (**Figure 7**).



**Figure 6: Main and accessory olfactory pathways in the rat.** Top left: Afferent pathways to the hypothalamus and amygdala. Bottom left: Primary and secondary afferent and efferent pathways to the cortex, limbic regions, hypothalamus, and midbrain. Right: Main and accessory pathways and their convergence in amygdala and thalamus. VNO: vomeronasal organ. MOE: main olfactory epithelium. MOB: main olfactory bulb. AOB: accessory olfactory bulb. AON: accessory olfactory nucleus. OT: olfactory tubercle. VP: ventral pallidum. OFC: orbital frontal cortex. PC: piriform cortex. LS: lateral septum. MPA: medial preoptic area. BST/BNST: bed nucleus of the stria terminalis. LH: lateral hypothalamus. MeA: medial amygdala. HC: hippocampus. EC: entorhinal cortex. TH: thalamus. VTA: ventral tegmental area. SN: substantia nigra.





**Figure 7: Interaction of attention and bonding pathways.** Attentional mechanisms involve the activation of mesolimbic dopamine pathways from the VTA to limbic structures like the NAc. Bonding mechanisms that utilize oxytocin and/or vasopressin also coordinate outputs that modify both motivation and behavior toward conspecifics through the interaction of oxytocin or vasopressin with both the VTA and NAc. A critical feature is the GABA output from the mPOA to the VTA that disinhibits dopamine neurons. Top: modified from Young and Wang, 2004. Bottom: modified from Melis and Argiolas, 2011. OXY: oxytocin. DA: dopamine. Glu: glutamate.

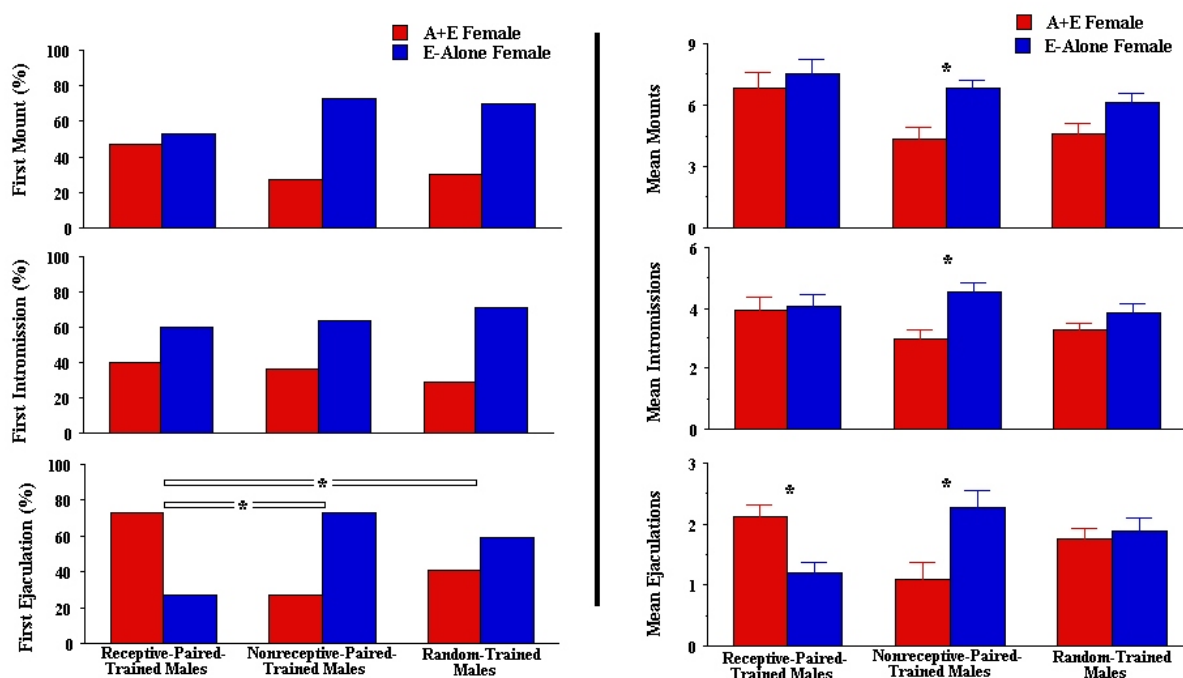
*Olfactory CPaP in males.* The first experiment to examine an olfactory-conditioned CPaP in male rats was by Pfaus, Jacobs, and Wong (1986) and utilized another neutral odor (almond extract) painted on the neck and anogenital regions of the receptive female, identical to the body regions scented with wintergreen in the hormonal experiment by Graham and Desjardins



(1980). Males were given their first and subsequent 9 multi-ejaculatory conditioning trials conducted at 4-day intervals in bilevel pacing chambers with either scented or unscented females. This formed the Odor-Paired and Odor-Unpaired groups, respectively. On the 10<sup>th</sup> trial, males were shifted to a large open field and given access to two receptive females, one scented and the other unscented, and their choice of female for first mount, intromission, and ejaculation, and the distribution of mounts, intromissions, and ejaculations on each female was recorded. First, males in the Paired group showed a faster acquisition of baseline rates of sexual responding in terms of number of intromissions and ejaculations during conditioning by trial 5 compared to males in the Unpaired group that had achieved baseline by trial 8. Second, during the open field CPaP test, males in the Paired group mounted and intromitted whichever female was closest, but ejaculated preferentially first and significantly more times with the scented female. Males in the Unpaired group also mounted and intromitted whichever female was closest, but showed a trend toward a significant preference to ejaculate with the unscented female.

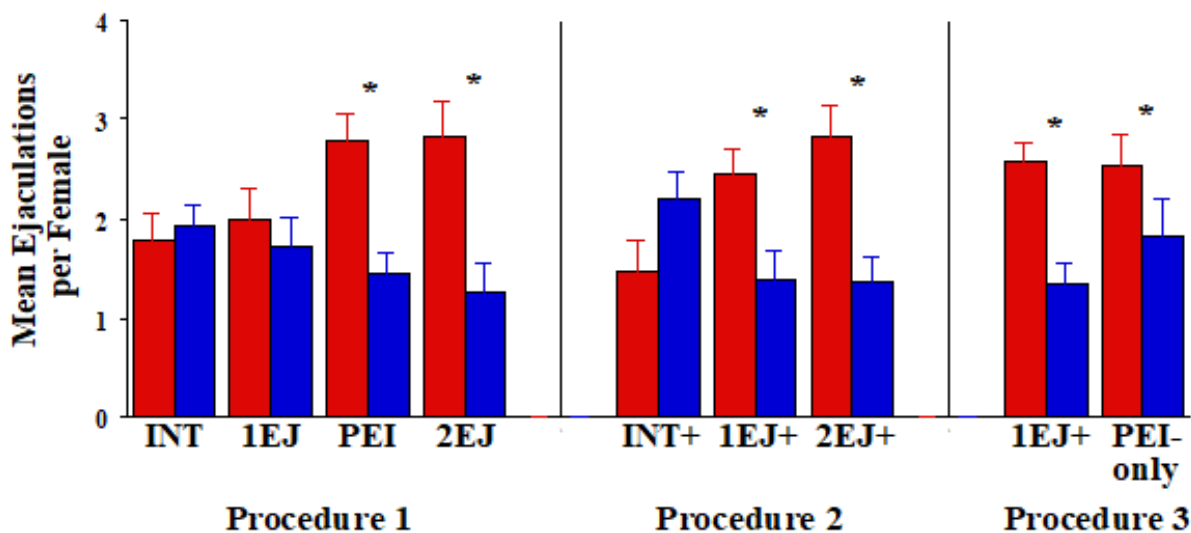
Although published as an extended abstract, this study lay dormant until my first PhD student, Tod Kippin, took it on for his dissertation research. In the first study, Kippin et al. (1998) replicated the original finding using two different paradigms, one with Paired and Unpaired groups of males (as above), and the other with explicitly Paired and Unpaired groups of males. In the explicitly paired condition, males received access to scented sexually receptive females at 4-day intervals in bilevel pacing chambers, but between the intervals (48 hr after each paired trial) they were given access to unscented, sexually nonreceptive females. Males in the explicitly unpaired condition had the opposite order of association, access to unscented sexually receptive females at 4-day intervals, but between the intervals they had access to scented, sexually nonreceptive females. Thus, in the explicitly Paired group, the odor meant “yes,” and in the explicitly Unpaired group, it meant “no.” In a previous study (Pfaus & Pinel, 1989), it had already been found that male rats would learn to inhibit their copulatory advances toward sexually nonreceptive females, so it was expected that the inhibitory state learned during the nonreceptive trials would make the explicitly unpaired conditioning more robust. Finally, a random-paired control was added in which the order of odor and no-odor association

was mixed between receptive and nonreceptive females (making the odor and no odor not predictive of reward or inhibition). All males received 9 sequential conditioning trials before being placed into the open field for the CPaP test. Results (**Figure 8**) showed that males in the explicitly Paired group chose the scented female for their first ejaculation, and had significantly more ejaculations with those females. Conversely, males in the explicitly Unpaired group chose the unscented female for their first ejaculation and had significantly more ejaculations with those females. Additionally, they also had significantly more mounts and intromissions with the unscented females, demonstrating that the inhibitory association of the odor with sexually nonreceptive females had produced more robust conditioning than no exposure to the odor in the first paradigm. The random-paired control did not show any preference. In summary, this study showed that the odor could be made excitatory or inhibitory by its association with copulation to ejaculation with sexually receptive females, or thwarted copulation with sexually nonreceptive females, and that it followed Pavlovian rules regarding the predictive nature of the odor. Kippin et al. termed this a “conditioned ejaculatory preference” or CEP.



**Figure 8:** Data from the explicitly paired and unpaired paradigm used by Kippin et al (1998). A+E females in red are scented. E-alone females in blue are unscented. Left: percentage of males that chose the scented or unscented females for first mount, intromission, and ejaculation. Right: distribution of mounts, intromissions, and ejaculations per each female during the 30-min test.

A second study into the nature of the unconditioned stimulus (Kippin & Pfaus, 2001a) showed that intromissions alone during the 9 conditioning trials did not result in CEP, nor did ejaculation with immediate removal of the female. Instead, the male had to be in the vicinity of the female during his first postejaculatory refractory phase of each trial for the effect to consolidate (**Figure 9, Procedure 1**). Indeed, allowing males to copulate to ejaculation with an unscented receptive female, but placing a scented receptive female into the chamber immediately after ejaculation (PEI only), also resulted in significant CEP (**Figure 9, Procedure 3**). Thus, although ejaculation was necessary, it was not sufficient. Males had to spend the postejaculatory period with the female bearing the odor for conditioning to occur.



**Figure 9: Nature of the unconditioned stimulus for CEP.** In Procedure 1, males were allowed 5 intromissions (INT), 1 ejaculation (1EJ), ejaculation plus the postejaculatory interval or refractory period (PEI), or 2 ejaculations (2EJ) with the scented female during conditioning. Procedure 2 contrasted ejaculation +PEI with a scented female being placed into the chamber immediately after males ejaculated with an unscented female, thus spending his PEI with the scented female.

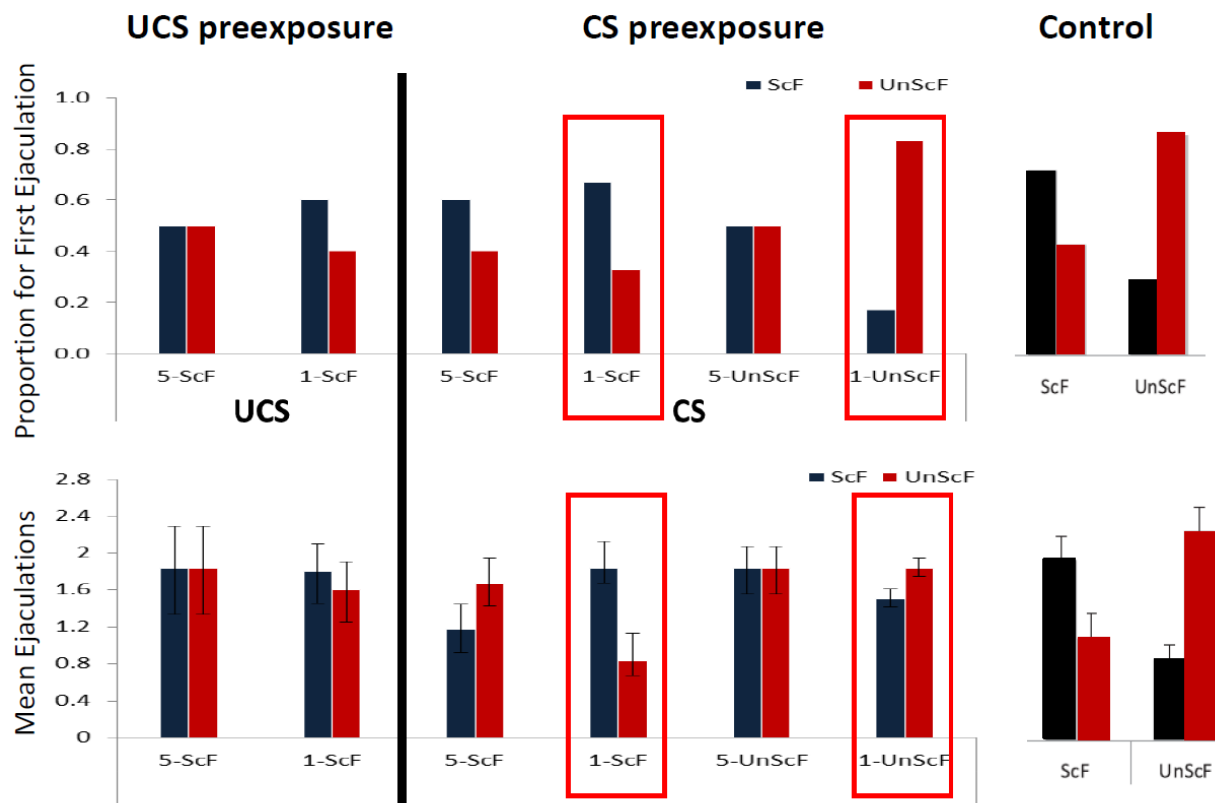
Subsequent studies established that males in the Paired groups would show more attention during the first ejaculatory series toward the unscented females, but would turn their attention toward the scented females approximately 3 intromissions prior to ejaculation (Kippin & Pfaus, 2001b). It was also found that males needed at least 5 multiejaculatory conditioning trials to begin to show CEP, suggesting that the conditioning required sensitization of different neural systems (e.g., dopamine), much like the acquisition of responding for drugs of abuse

(Kippin et al., 2001). Kippin, Cain, and Pfaus (2003) contrasted the difference in brain activation of Paired versus Unpaired males exposed to either estrous vaginal secretions, the conditioned odor, or a water control, in bedding. Immunohistochemical detection of Fos protein, an immediate-early gene product used routinely in my laboratory as a marker of neuronal activation (e.g., Pfaus & Heeb, 1997), was examined following exposure to the bedding. Following exposure to estrous vaginal secretions in both groups, Fos immunoreactivity (IR) increased in the AOB, MeA, medial BNST, mPOA, ventromedial hypothalamus (VMH), VTA, and both the NAc core and shell. Conversely, although Fos-IR increased in the MOB of males in both groups, it increased in the PC, basolateral amygdala, NAc core, and the anterior portion of the lateral hypothalamic area only in Paired males. The control condition did not induce significant activation of Fos-IR in any brain region. These data were the first to show conditioned activation of the main olfactory system by a neutral odor paired with the postejaculatory reward state. Although estrous and sexually conditioned odors are processed by distinct neural pathways, they appeared in this study to converge in the NAc, suggesting that this structure has a unique role in processing both pheromonal and olfactory sexual stimuli.

The effect of UCS or CS preexposure was examined on olfactory-based CPaP in two studies by Quintana et al. (2018a;b). The first of these (Quintana et al., 2018a) examined UCS preexposure. Males were given 1 or 5 copulatory trials with almond scented or unscented receptive females in unilevel pacing chambers with a one-hole divider at 4-day intervals. Males were allowed to ejaculate only once and spend the postejaculatory interval with the female. This was followed by 9 similar conditioning trials with scented or unscented females in the same chamber prior to an open field CPaP test. A control group that received conditioning exclusively with scented or unscented females showed significant CEP for the familiar females. However, males that had 1 or 5 trials with the scented or unscented females prior to conditioning with unscented or scented females, respectively, no longer showed any conditioning. This indicates that even one copulatory trial to ejaculation with an unscented female can inhibit conditioning with the scented female.

The second study examined CS preexposure. This was done with 1 or 5 presentations of the almond odor or water on a cotton gauze pad followed by 9 conditioning trials with scented

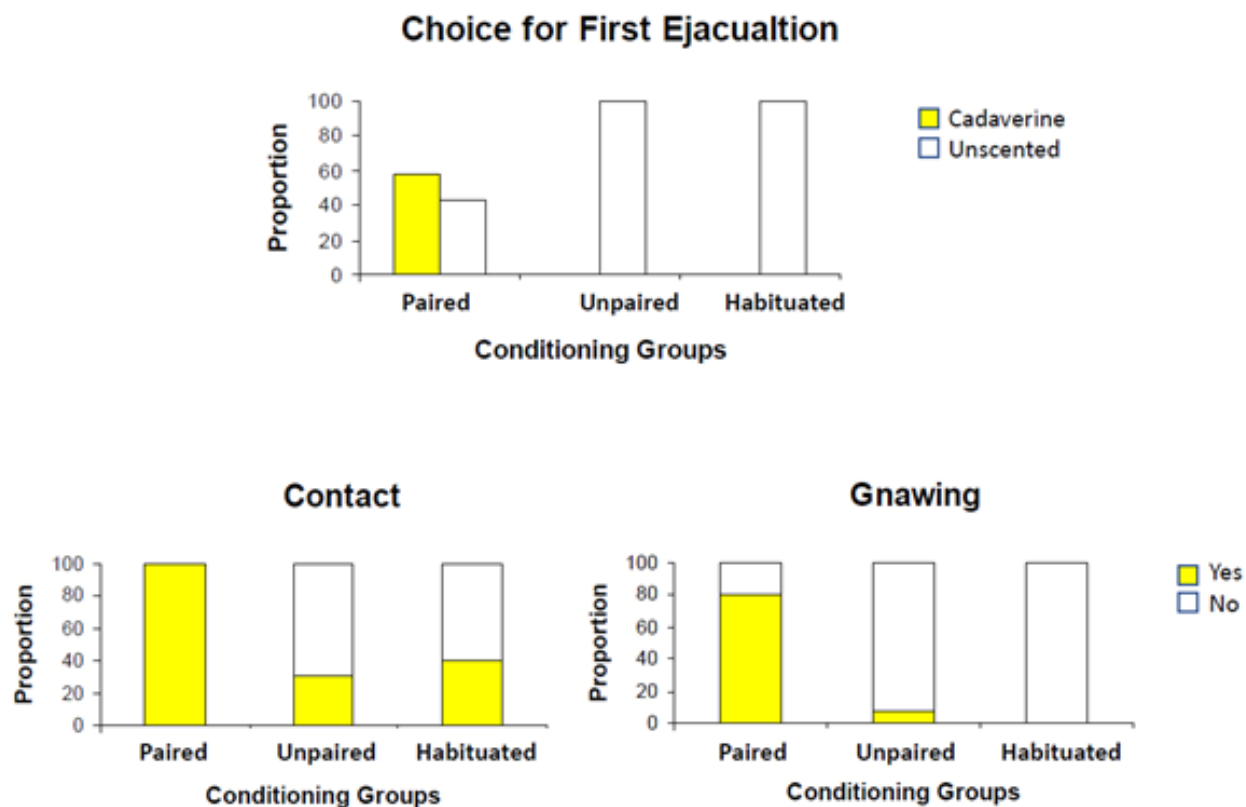
or unscented females. Although 1 presentation of the CS odor alone did not alter subsequent conditioning, 5 presentations induced latent inhibition. Data are shown in **Figure 10**.



**Figure 10: Effects of UCS or CS preexposure on the development of CEP.** In the UCS preexposure experiment (left side), 1 or 5 prior copulations to 1 ejaculation and the PEI with unscented females abolished subsequent conditioning with the scented female (ScF) or unscented female (UnScF). In the CS preexposure exposure experiment (middle), 1 prior exposure to the almond scent or water on gauze did not disrupt subsequent conditioning for the scented or unscented females (red boxes), whereas 5 prior exposures produced latent inhibition (learned irrelevance) that abolished conditioning. In the control condition (right side), males that received 9 trials with scented or unscented females showed significant CEP.

We have examined whether an unconditionally aversive odor (cadaverine) associated with sexual reward could make it conditionally appetitive in male rats (Pfaus, Kippin, & Centeno, 2001). Cadaverine is the smell of death, a polyamine that is given off by decaying flesh. This odor is aversive to rats and many other species. Rats, in particular, bury their dead, and will bury pellets soaked in cadaverine (Montoya, Sutherland, & Whishaw, 1981; Pinel, Gorzalka, & Ladak, 1981). In this study, run identically to those in Kippin et al. (1998), Paired males were given 9 copulatory trials with receptive females scented with 10% cadaverine,

whereas Unpaired males were given 9 copulatory trials with unscented receptive females. A Habituation control was run identically to the Unpaired group, but had the cadaverine odor placed into their home cages after each copulatory training trial. Four days after the last conditioning trials, males were placed into the open field with two sexually receptive females, one scented with cadaverine, and one unscented. Results (**Figure 11**) showed that half of the



**Figure 11:** Cadaverine data from Pfaus, Kippin, and Centeno (2001).

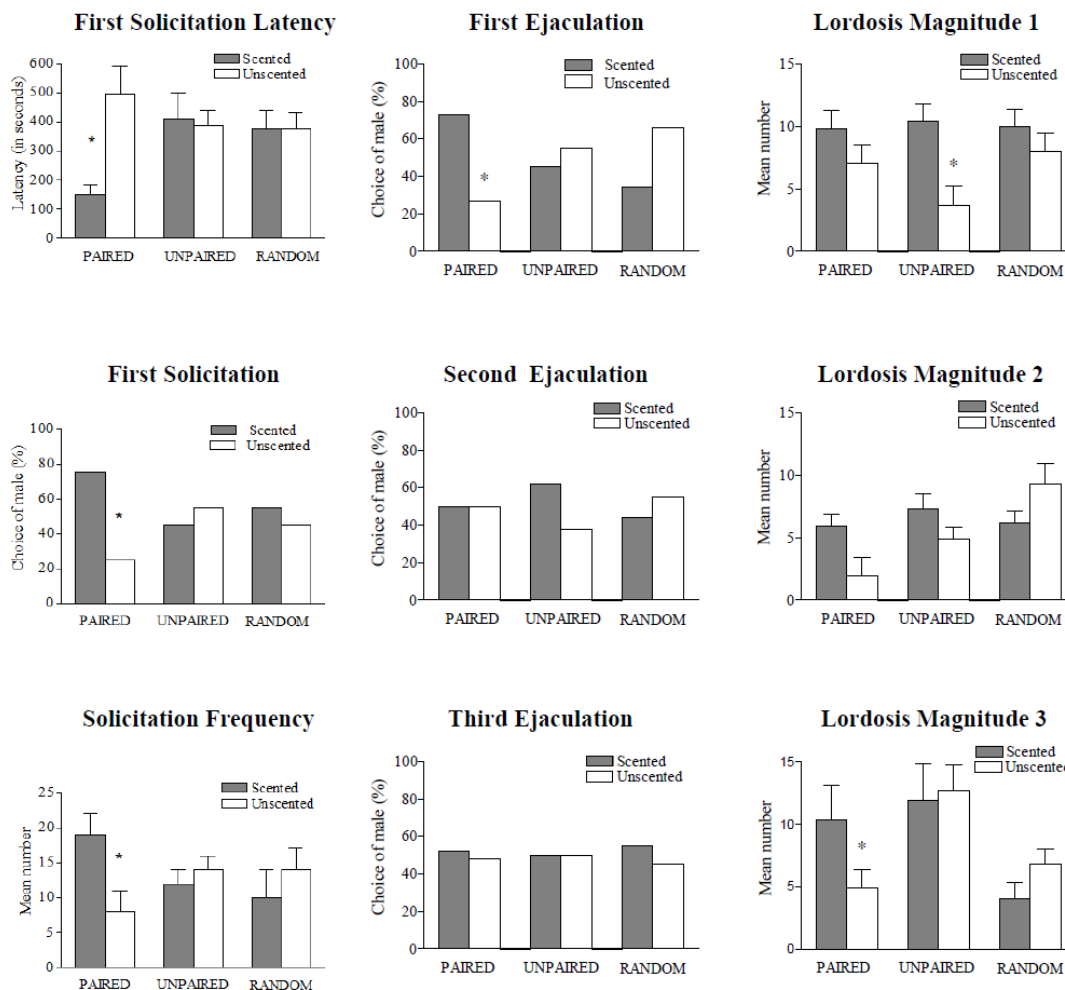
males in the Paired group ejaculated preferentially with the cadaverine-scented females, whereas all of the males in the Unpaired and Habituated groups ejaculated preferentially with the unscented females. Four days after the open field CPaP test, males were placed into a shoebox cage with 5 cm of corncob bedding. After a 5-min habituation period, a wooden dowel soaked in 10% cadaverine was placed in the chamber and the behavior of the rats scored. All of the males in the Paired group contacted the dowel and 80% picked it up and gnawed on it repeatedly, as they would if the dowel had a naturally rewarding odor like chocolate smeared

on it. Significantly fewer males in the Unpaired and Habituated groups contacted the dowel, and only 1 male in the Unpaired group gnawed on the dowel once before retreating from it. This study shows that males will actually copulate with dead smelling females and that the postejaculatory reward state supports conditioning that makes an aversive odor become conditionally appetitive. Although this was a new finding for sexual behavior, it was not new in the literature. For example, rats normally avoid bitter tastes. However, when forced to drink water containing morphine, over time they learn to like the bitter flavor of the water associated with the morphine reward state (Zellner et al., 1985).

Finally, an odor associated with a non-sexual reward state in an infant male rat can translate into a CEP when the rat is an adult. Fillion and Blass (1986) reported that neonatal male rats exposed to a neutral lemon odor (citral) painted on their mother's ventrum while nursing ejaculated faster as adults with sexually receptive, citral-scented females compared to unscented receptive females. We decided to take the mother out of the conditioning entirely by examining whether a citral odor paired with tactile reward in neonatal male rats would alter the subsequent expression of a CEP. In Ménard et al. (2020), newborn males were separated from their mothers each day from Postnatal Day 1 to 10 and placed into a Plexiglas cage that contained either citral-scented (Paired group) or unscented (Unpaired group) bedding. During each trial, males were stroked from their heads to the base of their tails with a soft, camel-hair paintbrush for 15 min, after which they were returned to their mothers. This conditioning mixed both positive and negative reinforcement with the odor. Starting in post-adolescence (between 70 and 80 days of age), males were habituated to the open field followed by their first sexual experience in the open field. Males were presented with two females, one scented with citral, and the other unscented, for a 30-min CPaP test. Males in the Paired group copulated and ejaculated preferentially with the scented female whereas males in the Unpaired group showed no preference. Pairing a neutral odor with a reward state in infancy generates a preference in male rats to ejaculate with sexually receptive females bearing the same reward-related odor in adulthood.

*Olfactory CPaP in females.* Similar conditioning phenomena have been found in female rats. Coria-Avila et al. (2005) used an explicitly Paired, Unpaired, and Random control paradigm.

OVX females were primed fully with estradiol benzoate (EB) and progesterone (P). In the Paired group, females associated paced copulation in a unilevel pacing chamber bisected by a four-hole divider with an almond scented stud male, and nonpaced copulation (with the divider removed) with an unscented stud male. Tests were 30 min in duration and 4 tests were conducted sequentially in each condition at 4-day intervals for a total of 8 tests. The Unpaired group had the opposite order of association, whereas the random paired group had males with or without the odor paired randomly with paced or nonpaced copulation. On the final 30-min open field CPaP test, each female was allowed access to two tethered males in the open field and their choice of male for solicitations, mounts, intromissions, and ejaculations were scored. Results (**Figure 12**) showed that females in the explicitly Paired group solicited the scented male



**Figure 12:** Data from the explicitly Paired and Unpaired paradigm used by Coria-Avila et al (2005) in female rats. Grey bars are scented males. White bars are unscented males.

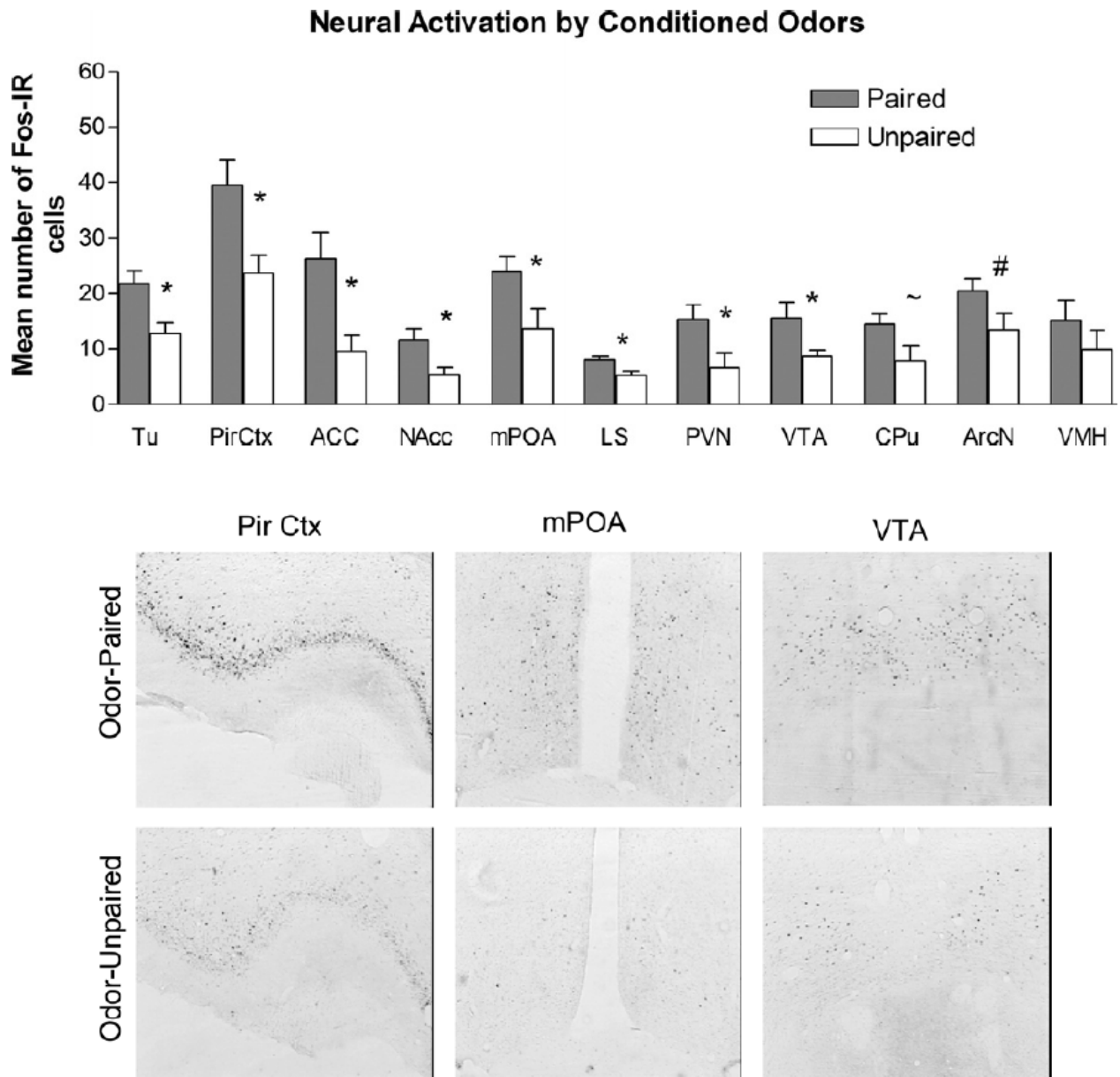


first, faster, and more times throughout the test relative to the two other groups. They showed more full lordosis postures (Lordosis Magnitude 3s) with the scented male compared to the unscented male, and they also took the scented male's first ejaculation preferentially compared to the other groups. Thus, in contrast to male CEP, females in the paired group solicit their preferred male, display high intensity lordosis with that male, and choose that male for their first ejaculation. The odor likely makes it easier to discriminate their preferred male in the open field.

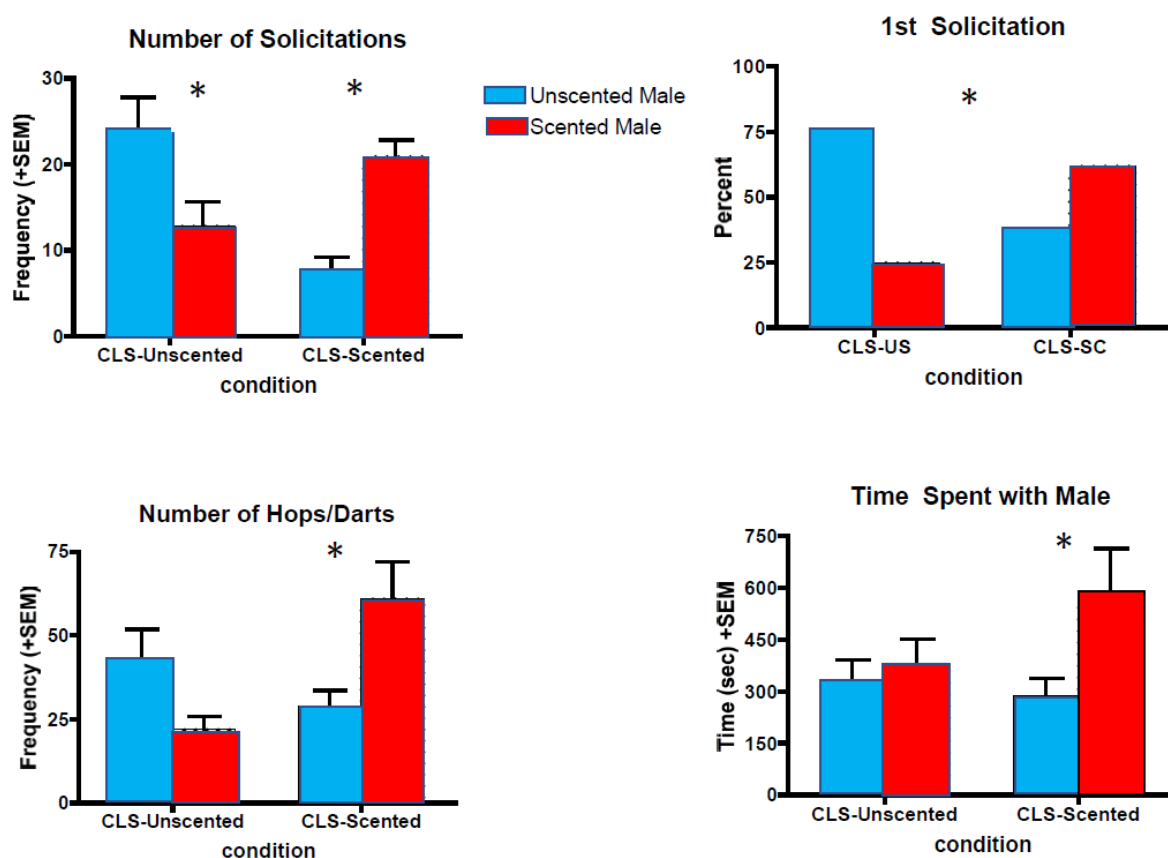
A subsequent experiment replicated these data and also examined brain activation by the odor alone using Fos protein as a marker (Coria-Avila & Pfaus, 2007). A similar pattern of neuronal activation to that observed in Paired males by the odor alone was found (**Figure 13**). This suggests that the primary and secondary regions stimulated by the main olfactory system underlie the conditioned responses made to odors paired with sexual reward in both males and females.

As with CPP, distributed CLS was also able to induce a reward state that supported an olfactory CPaP. In the first study (Parada et al., 2011), sexually naïve OVX females were primed with EB and P and received either 10 alternating trials of CLS and no CLS. Females in the Paired group had CLS associated with a cotton gauze pad soaked in almond extract and no CLS associated with water applied to the cotton gauze pad. Females in the Unpaired group had the opposite order of association. CLS applications were made as in Parada et al. (2010) for CPP induction. Four days after the last conditioning trial, females were placed into an open field with two tethered males, one scented and the other unscented for a 30-min test of CPaP. Results (**Figure 14**) showed that females in the Paired group solicited first and more the scented male than the unscented male, showed more hops and darts with him, and spent more time with him compared to the unscented male. Females in the Unpaired group showed the opposite preference, soliciting first and more the unscented male, but not showing more hops and darts or time spent with either male. Notably, the females did not display a preference to receive ejaculations from either male. It is possible that CLS needs to be paired with VCS and/or unconditioned male odors/pheromones to induce the ejaculatory preference. In another experiment of that study, Paired females were given CLS in the presence of an almond scented

male behind a wire-mesh screen and sham CLS in the presence of an unscented male behind the screen. The Unpaired group received the opposite order of association. Four days after the last conditioning trial, females were placed into an open field with two tethered males, one



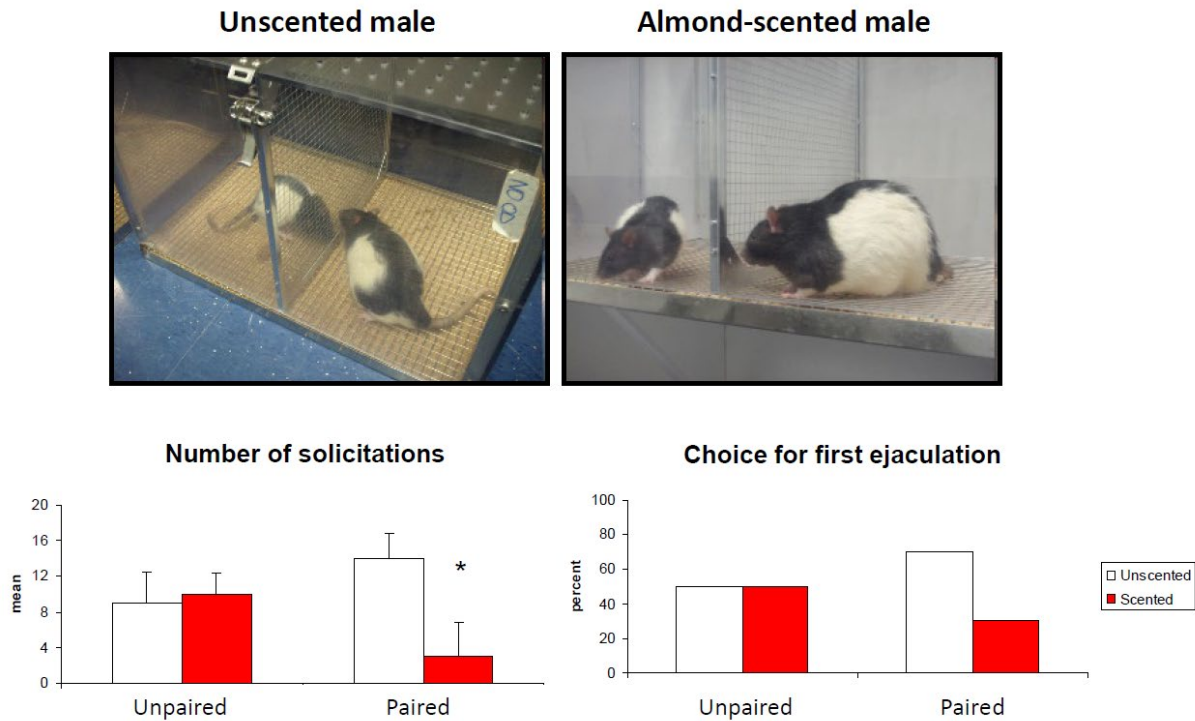
**Figure 13: Neuronal activation by the almond odor in Paired versus Unpaired females.** Top: means and SEMs of Fos counts in different regions. Tu: olfactory tubercle. PirCtx: piriform cortex. ACC: anterior cingulate cortex. ArcN: arcuate nucleus. Bottom: representative photomicrographs of Fos activation in PirCtx, mPOA, and VTA. Black dots show the nuclear Fos stain and the number of those are counted in each region. Note the Fos staining in the center of the VTA in the Paired example relative to the lack of Fos in the same region of the Unpaired example. This is the region where dopamine cell bodies are found, suggesting that they are selectively activated in the Paired group.



**Figure 14:** Data from Parada et al. (2011) showing selective solicitations toward scented males in the paired group and unscented males in the Unpaired group. Scented males are in red. Unscented males are in blue.

scented and the other unscented, for a 30-min test of CPaP. Contrary to our expectations, results showed that Paired females displayed an *avoidance* of the scented male for solicitations and ejaculations, whereas the Unpaired females showed no preference (**Figure 15**). Upon reviewing the videos of the conditioning trials, it was found that females were hopping, darting, and showing intense ear-wiggling toward the male following each distributed CLS. All showed nose pokes through the wire mesh and some even tried to climb the screen. It is likely that the females were frustrated receiving CLS in the presence of an inaccessible male, and this state of frustration became an aversive UCS associated with the almond odor, resulting in conditioned inhibition. It is unclear which male-related cues might have altered the females' behavior. In the study by Parada, Jafari, and Pfaus (2013), prior sexual experience altered the ability of CLS to induce a CPP, similar to the way in which prior sexual experience to ejaculation with an unscented female altered the development of CPP in male rats. It is possible that other

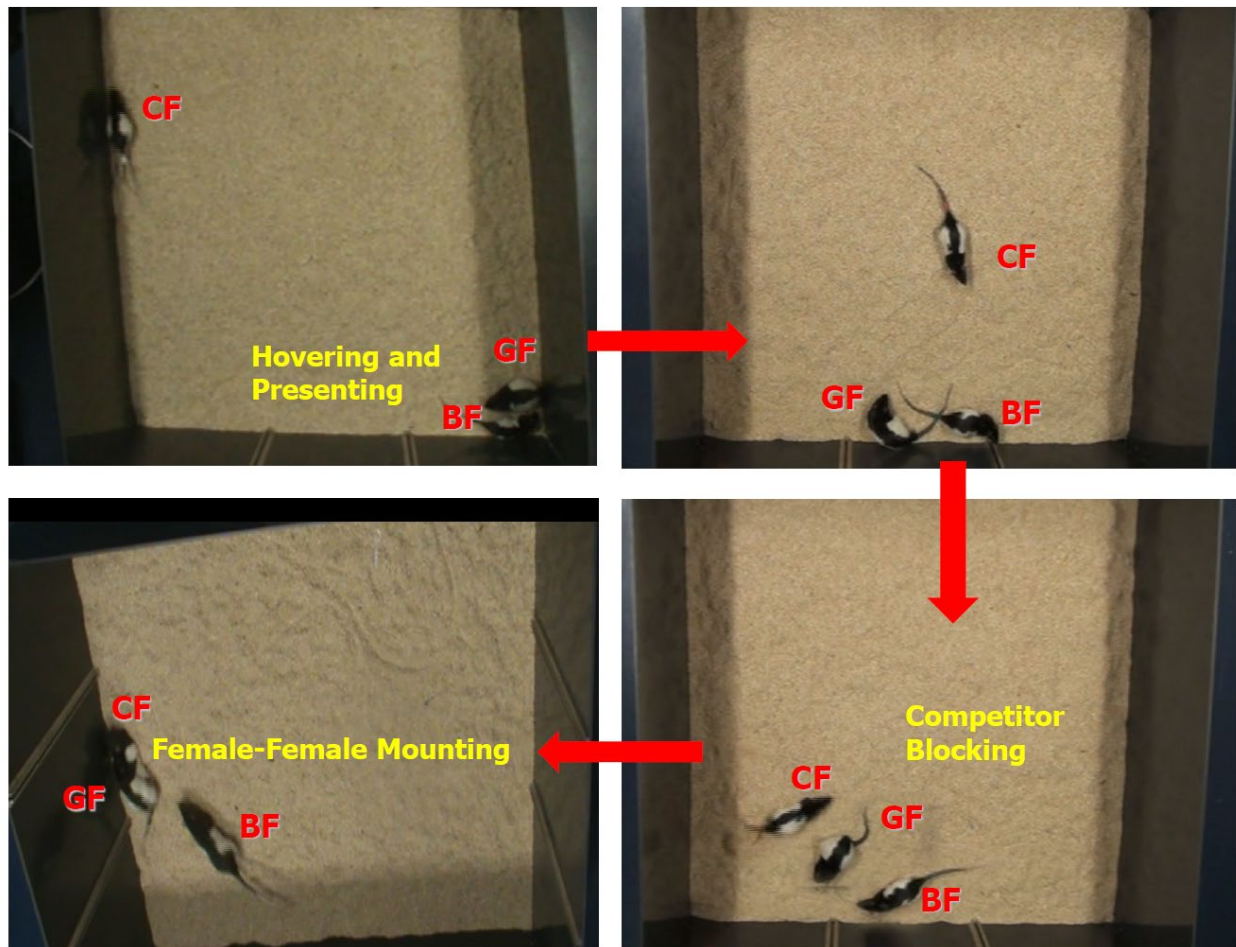
olfactory cues (e.g., pheromones, MHCs), and/or auditory stimuli provided by the male, altered the context in which CLS was received, turning it from a consummatory reward in its own right into an appetitive prelude for copulation.



**Figure 15:** Data from Parada et al. (2011) showing the potential effect of sexual frustration induced by CLS in the presence of an inaccessible male. Scented males are in red. Unscented males in white.

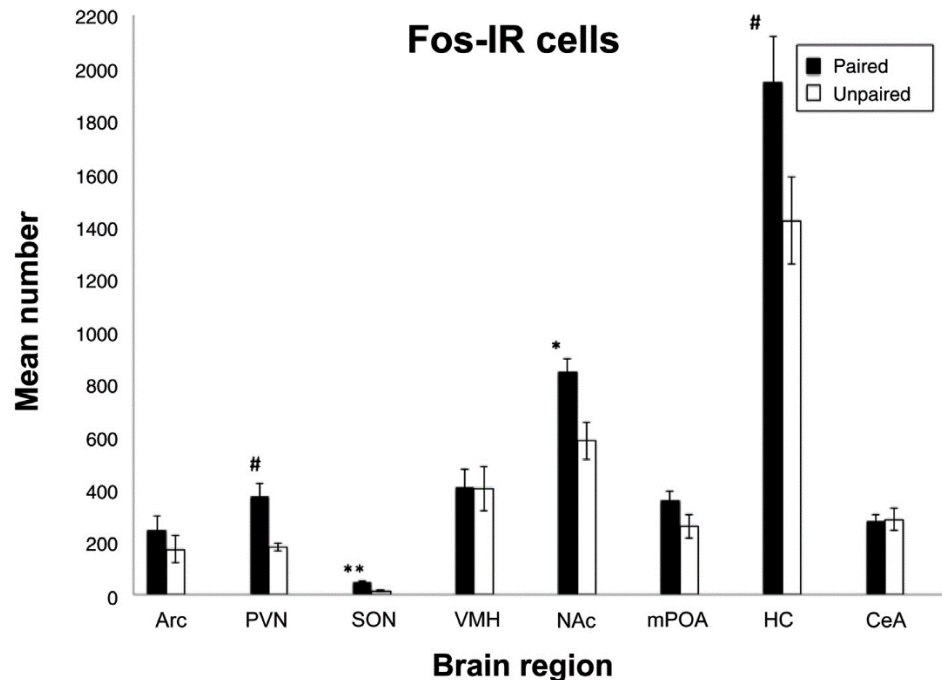
The experiment above raised the possibility that females could learn associations based on natural male cues alone. Preliminary studies showed that they could, but only if paired with the same male on each conditioning trial of paced copulation in unilevel chambers with the 1-hole divider. The 4-hole divider did not support conditioning without the odor. Holley et al. (2014) extended this to see if females might show mate-guarding behavior based on the reward state induced by 1-hole paced copulation with the same male every time. Females received 10 trials of either paced copulation in the unilevel chamber (bisected by the 1-hole divider), or nonpaced copulation with the divider removed. A final F-M/F test was conducted in the open field. The male was placed into the open field for a 5-min habituation period, after which the receptive female partner he had always copulated with during conditioning and a receptive

competitor female were placed into the open field. The paired female did indeed show mate guarding behaviors similar to those observed in pair bonded prairie vole females (**Figure 16**). These behaviors included hovering and presenting (H&P) around the paired male, competitor blocking (CB) behavior where the paired female would intercept the competitor female if she came close to the male, and female-female mounting behavior (FFM) observed when one female exerts dominance over another (Fang & Clemens, 1999). In contrast, females paired with males in the nonpaced condition did not show these behaviors, but instead competed with



**Figure 16: A sequence of conditioned mate guarding in the female rat.** Upper left: the paired female (GF) is hovering and presenting toward the paired male (BF), with the competitor female (CF) alone. Upper right: the CF coming toward the pair. Bottom left: the GF getting between the CF and the BF to block her access. Lower left: After the CF runs around the GF and solicits the BF, the GF runs after her and mounts her several times, sometimes pushing her head against the wall of the open field or displaying other forms of agonistic behavior (e.g., boxing, ventral attacks).

the other female as if they were with a novel male, displaying the same type of competitive behaviors that McClintock (1984) described. Indeed, even GF females given access to novel males when sexually receptive in an FFM open field paradigm also do not show any evidence of mate guarding, but do if the male is the BF. After the F-M/F open field test, females were sacrificed and their brains processed for Fos-IR (**Figure 17**).

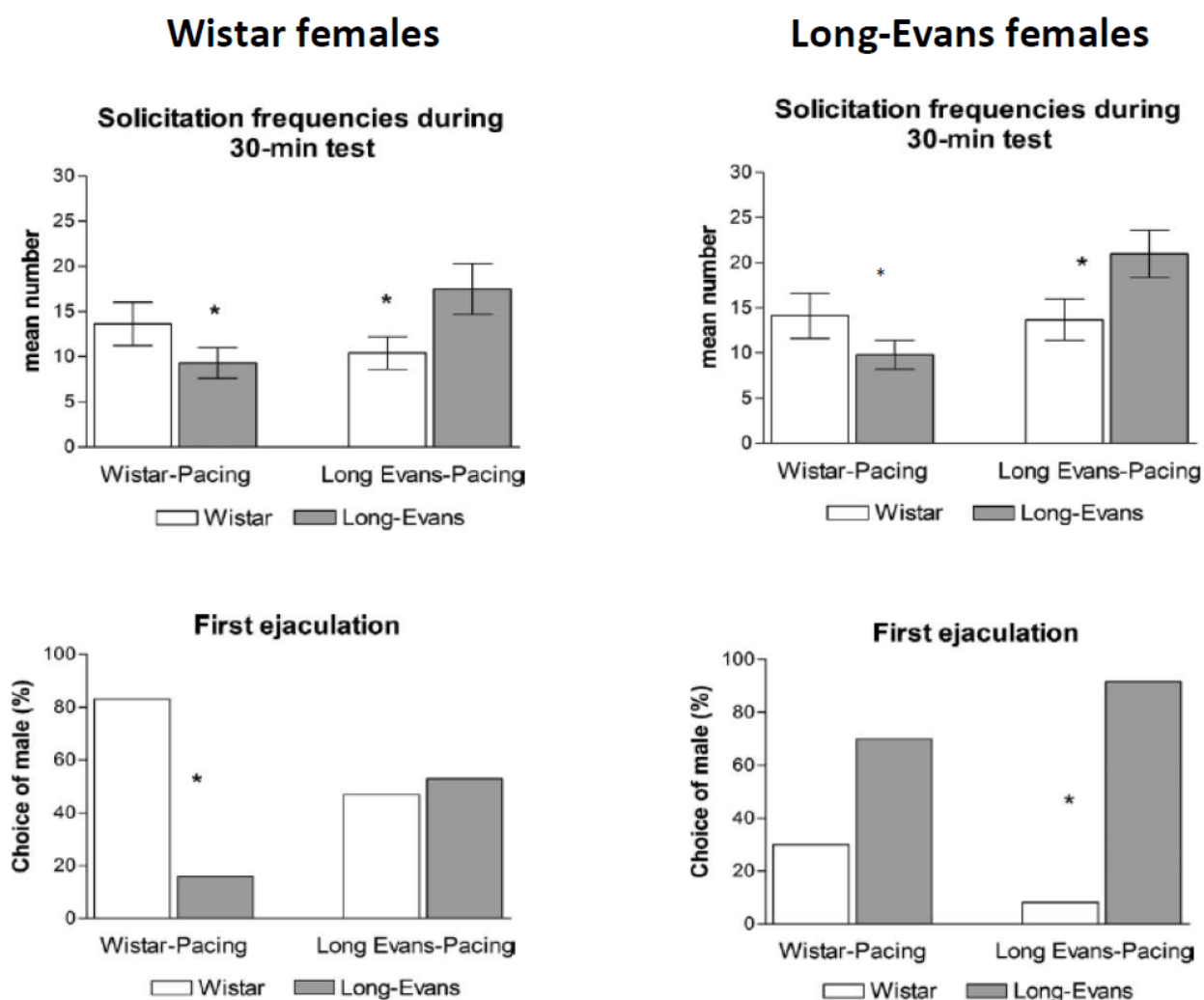


**Figure 17:** Fos expression in different brain regions in paired versus unpaired females following the open field test. #  $p < 0.1$ . \*  $p < 0.05$ . \*\*  $p < 0.01$ . Arc: arcuate nucleus. CeA: central nucleus of the amygdala.

These data show that both primary and secondary regions of the main and accessory olfactory system are activated when female rats show mate-guarding behaviors. Interestingly, taking together the data reported so far in both males and females, common areas of activation by the conditioned cues include the NAc, PVN, SON, VTA, and in most cases the mPOA. The HC likely also plays a role in differentiating copulatory contexts. Thus, there may be an integration of limbic and hypothalamic systems that modulate the expression of olfactory-based CPaP in rats.

*Strain-based CPaP.* Two studies showed that the strain of rat could be used as a CS for sexual reward. Coria-Avila et al. (2006) tested whether Wistar albino females given paced

copulation with either Wistar males or pigmented Long-Evans males, or Long-Evans females given paced copulation with either Long-Evans or Wistar males would show a conditioned preference for the pacing-related male. Females were given 10 sequential multi-ejaculatory experiences with one strain of male in the 4-hole unilevel pacing condition and the other strain in the nonpacing condition with the divider removed (5 paced and nonpaced experiences each). Conditioning trials were conducted at 4-day intervals. On the test day, females were placed into an open field with two tethered males, one Long-Evans and one Wistar. Results showed that, regardless of strain, females solicited significantly more the male associated with paced copulation relative to the male associated with nonpaced copulation (**Figure 18**).

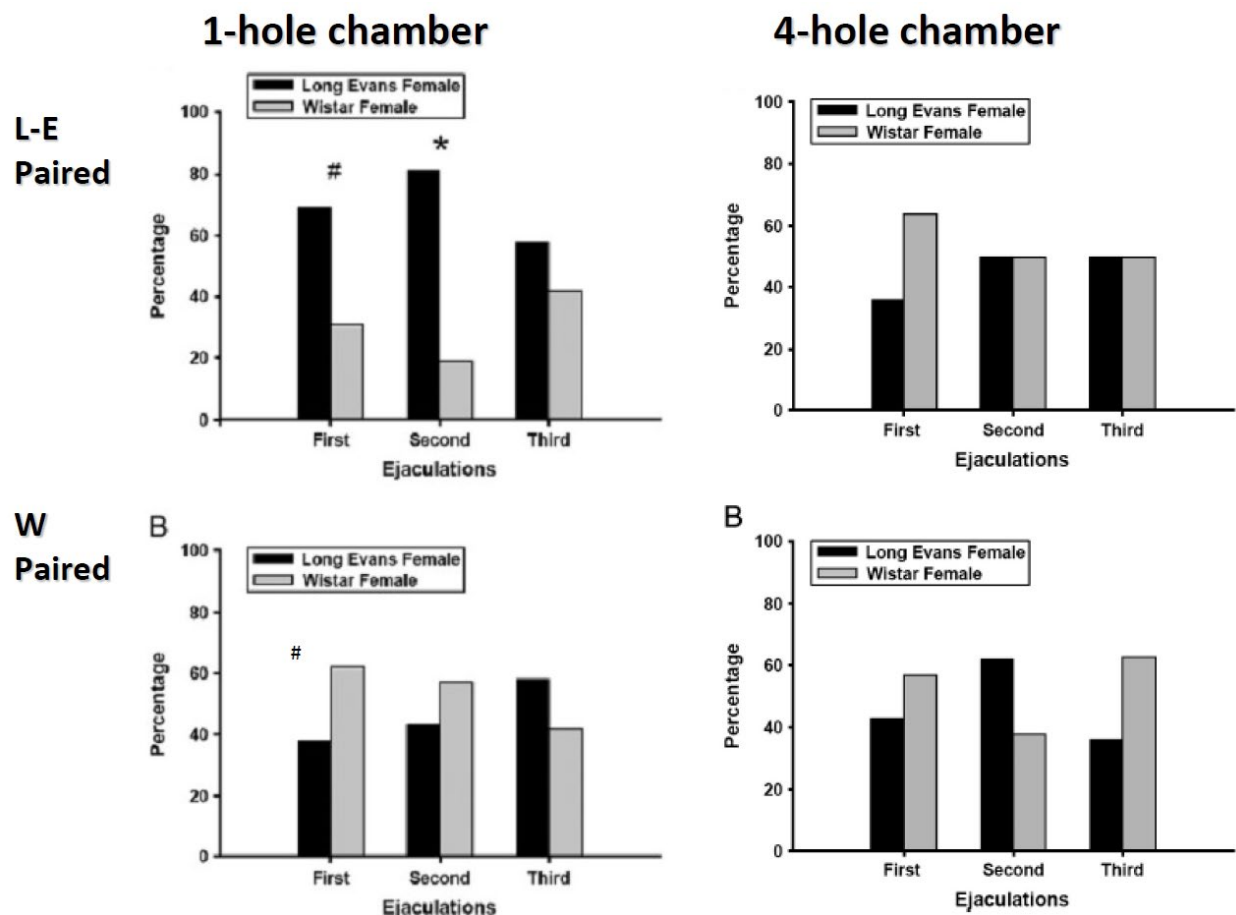


**Figure 18: Strain conditioning in Wistar (albino) and Long-Evans (pigmented) females.** Females solicited more the pacing-related male, and chose that male for their first ejaculation.



However, the females chose the pacing related male for their first ejaculation, but only if that male was of their own strain. This suggests an assortative mate choice based on pacing-related reward with males of similar phenotype.

Ismail et al. (2011) examined a similar phenomenon in Long-Evans males. Males received their first 10 multiejaculatory copulatory trials at 4-day intervals in the unilevel pacing chamber with either Long-Evans or Wistar females. Given that males develop different rates of copulatory behavior in the two pacing conditions (1-hole vs. 4-hole; Ismail, Zhao, & Pfaus, 2008) half of the males in each condition were trained with either the 1-hole or 4-hole divider. A final open field CPaP test was conducted 4 days after the final conditioning trial. Results showed that Long-Evans males developed a significant CEP for strain of female, but only in the 1-hole condition (**Figure 19**). Although this effect was shown for the first ejaculation, the males



**Figure 19: Strain conditioning in Long-Evans male rats as a function of unilevel pacing context.** Males showed a significant first ejaculation CEP for the strain of female they copulated with in the 1-hole, but not the 4-hole context.

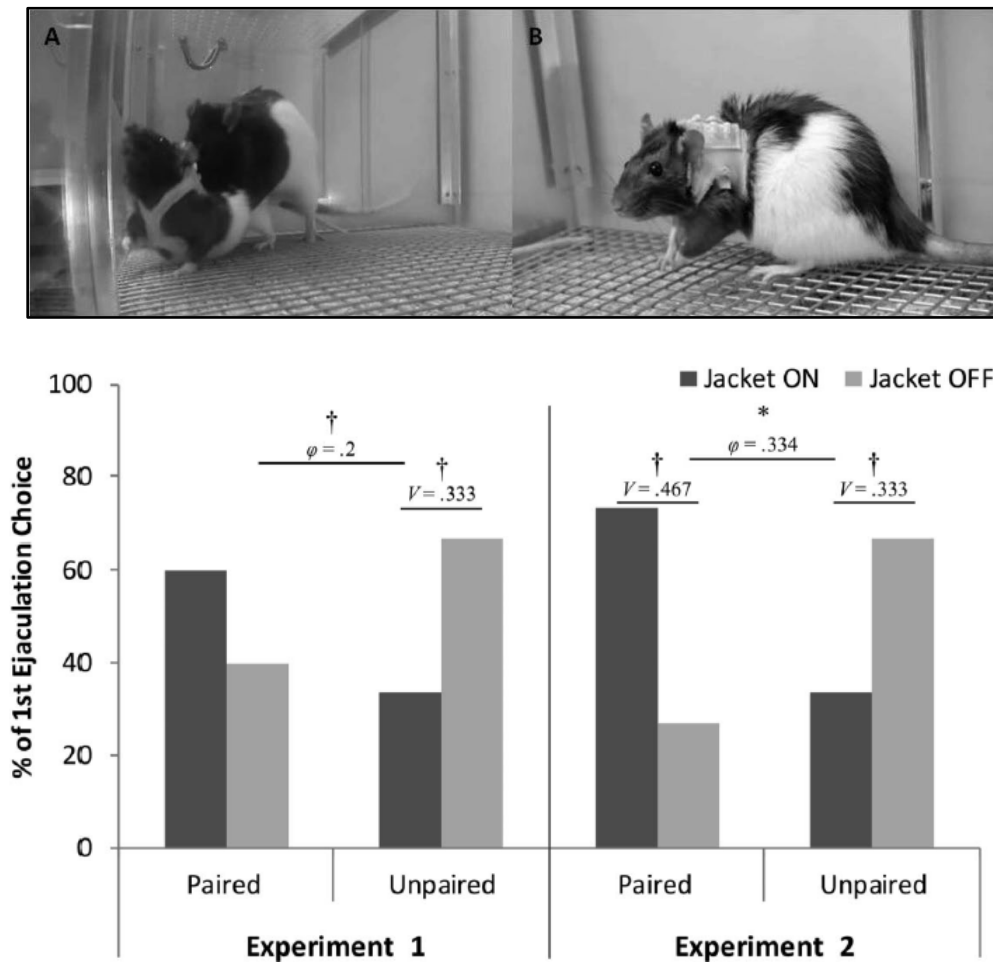


maintained a significant CEP with their own strain for the second ejaculation. As with the females, this finding suggests a stronger assortative mate choice based on some aspect of pacing-related reward in the 1-hole context with females of similar phenotype. Given that males must wait longer for the female to return in this context, it is possible that this increased their arousal. Just as food reward is greater to a food-deprived rat than to a free-feeding rat, it is possible that ejaculation riding on higher arousal is more rewarding.

Strain cues are clearly multisensorial. It is likely that the strains possess different MHC complexes that alter olfactory receptor genes, as is found in mice (Amadou et al., 2003). Thus, in addition to having the potential of different pheromonal or odorous secretions in urine (e.g., Brown, Singh, & Roser, 1987), their odor detection senses may be different. They also differ in their appearance (white fur vs. patchy white and dark fur). Pigmented rats have better visual acuity and are more aggressive than albino strains (Barnett, Dickson, & Hocking, 1979). It is not clear which cue(s) might be salient for the strain-based conditioning, but it is clear that rats can recognize their own and other strains and make partner preference judgements based on the association of strain cues with sexual reward.

*Somatosensory-based CPaP.* The finding that males could associate the rodent tethering jacket with their own sexual arousal (Pfaus, Erickson, & Talianakis, 2013) led Quintana et al (2019a) to ask whether the rodent tethering jacket on the partner could condition a CPaP in male and female rats. Two experiments were run with males, one Paired vs. Unpaired, and one explicitly Paired vs, explicitly Unpaired. In the first, males received 14 conditioning trials at 4-day intervals in unilevel pacing chambers bisected with the 4-hole divider. Males copulated with sexually receptive females that either wore the jacket (Paired) or were without the jacket (Unpaired). In the second experiment, males received sequential pairings of jacketed and unjacketed females in unilevel pacing chambers bisected by the 4-hole divider. In the explicitly Paired condition, jacketed females were sexually receptive and unjacketed females were sexually nonreceptive. In the explicitly Unpaired condition, unjacketed females were sexually receptive and jacketed females were nonreceptive. In this experiment, males received 7 alternating trials with each female at 4-day intervals. Four days after the final conditioning trial in both experiments, males were placed into the open field for 5 min, after which two receptive

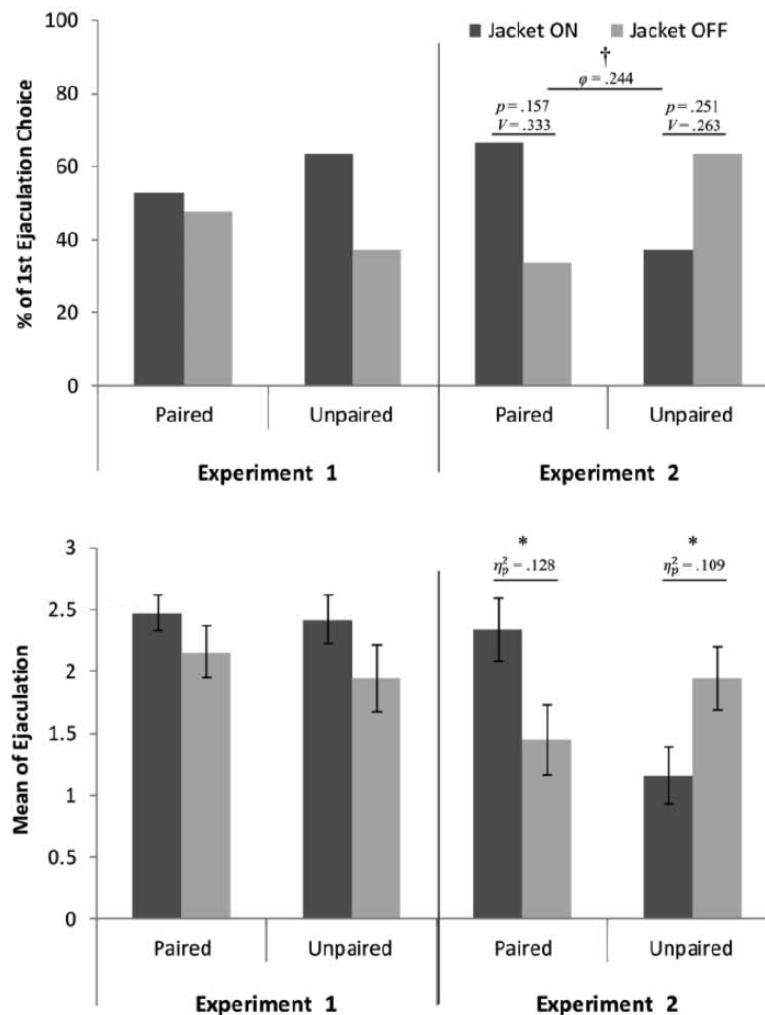
females were placed into the open field, one jacketed and the other unjacketed, for a 30-min CPaP test. In both experiments, Paired males showed a significant CEP for the jacketed female, though this preference was stronger in the explicitly Paired condition (**Figure 20**). Males in the Unpaired groups showed a significant CEP for the unjacketed female that was no different between the Paired and explicitly Paired conditions.



**Figure 20: Somatosensory-based CPaP in males.** Top left: Male copulating with a jacketed female. Top right: Jacketed female. Bottom: CEP for the jacketed vs. unjacketed female in the Paired vs. Unpaired condition in Experiment 1 (right) and the explicitly Paired vs. explicitly Unpaired condition in Experiment 2 (left).

Female conditioning occurred with the same experimental paradigms of Paired and Unpaired in Experiment 1, and explicitly Paired and explicitly Unpaired in Experiment 2 that had been used for males, except that the males had the jacket on or off. On the final open field CPaP test, females were paired with two males, one with the jacket and one without the jacket.

The males were not restrained because there was no possibility of mixing the cues, as had occurred during our preliminary trials with the odor conditioning. Results showed that females in Experiment 1 did not develop CPaP for either the jacketed or unjacketed male. There was no effect on solicitations or choice for ejaculation. In contrast, females in Experiment 2 developed a significant CPaP for the pacing-related male for first and overall ejaculations. No significant effect was observed for solicitations in either paradigm.



**Figure 21: Somatosensory-based CPaP in females.** Females did not display significant numbers of solicitations for either male in the unilevel chambers, nor did they display a significant choice of male for first ejaculation or overall numbers of ejaculations in the Paired vs. Unpaired condition of Experiment 1. In contrast, females displayed a significant choice for first and overall ejaculations in the explicitly Paired vs. explicitly Unpaired conditions of Experiment 2.

These results indicate that both males and females develop significant CPaP for a jacketed or unjacketed partner when those cues are explicitly paired with sexual receptivity (giving rise to a significant CEP in males) or paced copulation (giving rise to a significant choice for ejaculations in females). The lack of significant choice for solicitations in females in this study may stem from either the lack of a significant distal olfactory cue that attracts females to the males, or the fact that males were not tethered during the final open field test. Indeed, those males displayed cooperative pursuit of the female during the open field test which did not give her much of a chance to display full solicitations. It is remarkable that, despite the relative lack of a distal olfactory cue and less control over the copulatory contact in this study, females still displayed significant CPaP in terms of an explicit choice to receive ejaculations from the pacing-related male.

### **Neuropharmacology of sexual CPP and CPaP**

Testing drug effects on sexual CPP and CPaP have centered around compounds that either inhibit or facilitate their development, or their expression after they have been conditioned. Most of the studies have been conducted in males and have revolved around the notion that mesolimbic dopamine activates attention to cues associated with reward, whereas opioid systems actually carry the reward signal (Paredes, 2014; Pfaus, 2009).

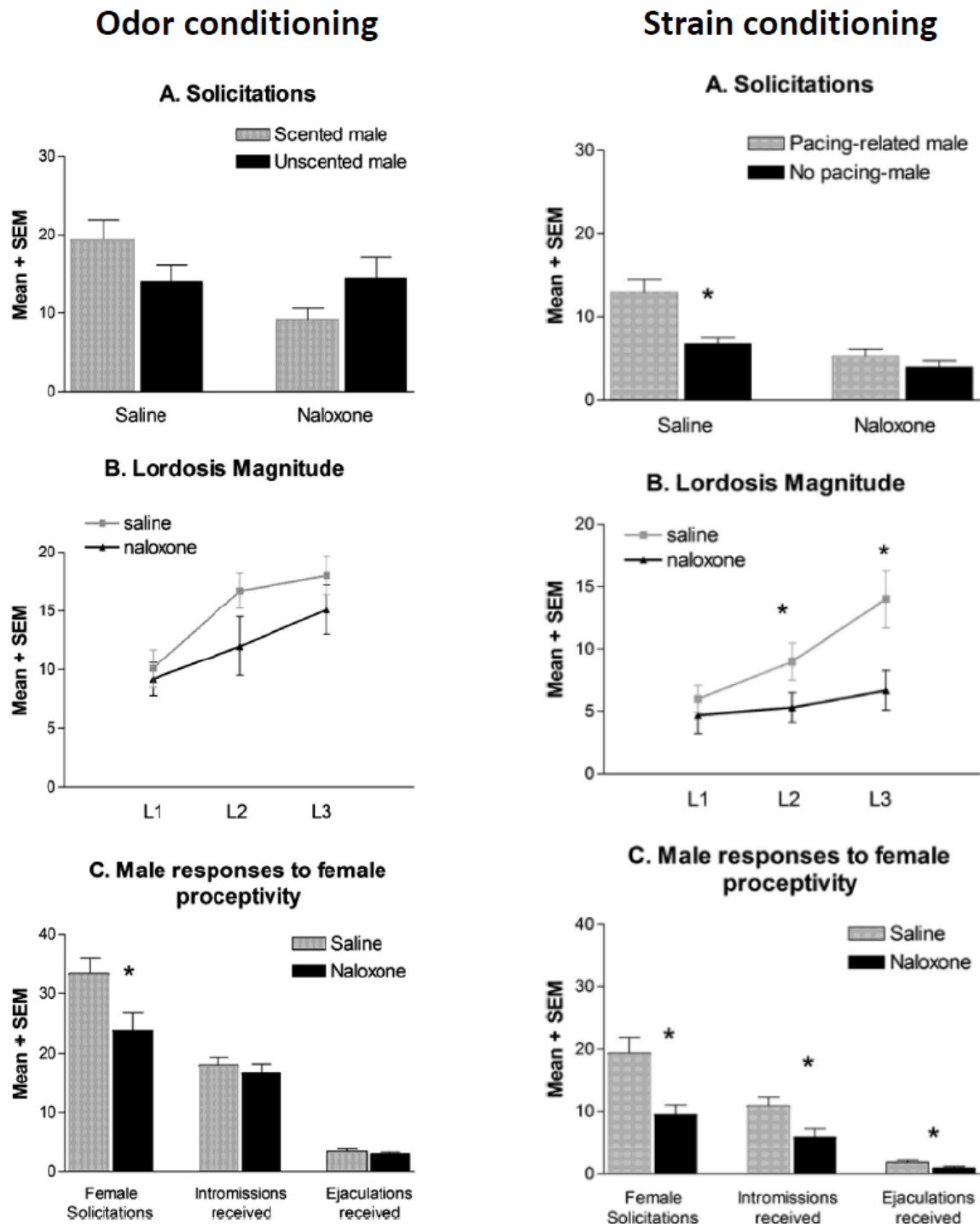
*Opioids.* Naloxone and naltrexone are direct acting broad spectrum opioid receptor antagonists that bind to all three opioid receptors, but with their highest affinity to MORs relative to  $\delta$  (DORs) or  $\kappa$  (KORs) opioid receptors (Paterson, Robson, & Kosterlitz, 1983). They are used commonly to treat opiate and opioid overdose, and as part of a regimen of treatments for drug addiction and problem paraphilias. Naloxone is short acting, with a half-life of 30-18 min in plasma, whereas naltrexone is longer acting with a half-life of 4-13 hrs in plasma.

In male rats, systemic injections of naloxone that did not alter sexual behavior disrupt the development of postejaculatory CPPs but not copulatory CPPs (Ågmo & Berenfeld, 1988; Meharra & Baum, 1990). However, once a copulatory CPP was developed, its expression was blocked by a systemic injection of naloxone prior to the final CPP test session (Meharra & Baum, 1990; Everitt, & Herbert, 1990). There is also evidence that the site of action of naloxone

is different for these effects. Naloxone infused into the mPOA prior to conditioning trials disrupted the development of postejaculatory CPP (Ågmo & Gomez, 1993) but not the expression of a copulatory CPP (Hughes, Everitt, & Herbert, 1990). Interestingly, lesions of this area caused a time-dependent disruption of a copulatory CPP (Hughes, Everitt, & Herbert, 1990). In female rats, systemic naloxone prior to paced copulation trials in a 1-hole unilevel pacing chamber blocked the development of CPP (Parades & Martínez, 2001). Infusions of naloxone to the mPOA, VMH, and MeA, but not the NAc, prior to paced copulation trials in the 1-hole unilevel pacing chamber also blocked the development of CPP (García-Horsman, Ågmo, & Paredes, 2008).

As with CPP, naloxone and naltrexone block the establishment of CPaP in rats and voles. Coria-Avila et al. (2008) examined the effects of naloxone in fully primed Long-Evans females in two paradigms: 1) an explicitly Paired odor-conditioning paradigm (almond odor paired with pacing in the 4-hole condition and no odor paired with nonpaced copulation with the divider removed), and 2) a strain conditioning paradigm in which either Long-Evans or Wistar males were paired explicitly with Pacing, and the other strain with nonpaced copulation. Females received either saline or naloxone prior to each of 10 conditioning trials at 4-day intervals (5 paced and 5 nonpaced). Saline was administered to all females prior to the final open field CPaP test. For the odor conditioning, females injected with saline prior to conditioning displayed a trend toward a significant preference to solicit the almond-scented male and take his ejaculations. In contrast, females injected previously with naloxone showed more solicitations toward the unscented male, and no significant preference of male for ejaculation. Females treated previously with naloxone also showed fewer solicitations overall, and fewer high intensity lordosis postures. For the strain conditioning, females injected with saline showed a significant preference to solicit the male associated with pacing and take his ejaculations preferentially. In contrast, females injected previously with naloxone did not show this preference. As with the odor conditioning experiment, females treated previously with naloxone displayed fewer high intensity lordosis postures (**Figure 22**). Given that all females were injected with saline prior to the final open field CPaP test, these data indicate that an expectancy of sexual nonreward induced by naloxone blocked CPaP and reduced the intensity

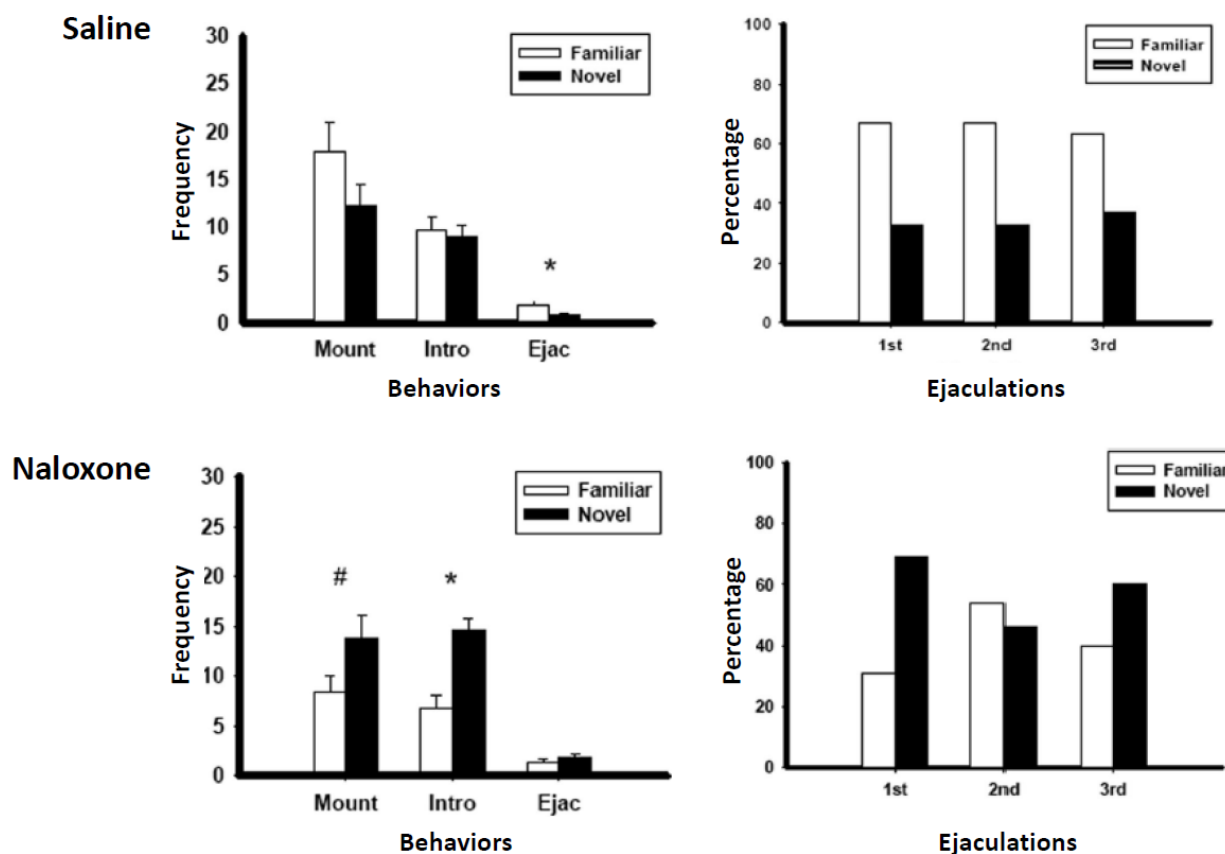
of lordosis. Lordosis is a reflex dependent on the actions of estradiol and progesterone in the brain. It is remarkable that the expectancy of sexual nonreward could modify this reflex in a



**Figure 22:** Effect of saline or naloxone on the development of an odor-based (left) or strain-based (right) CPaP in female rats.

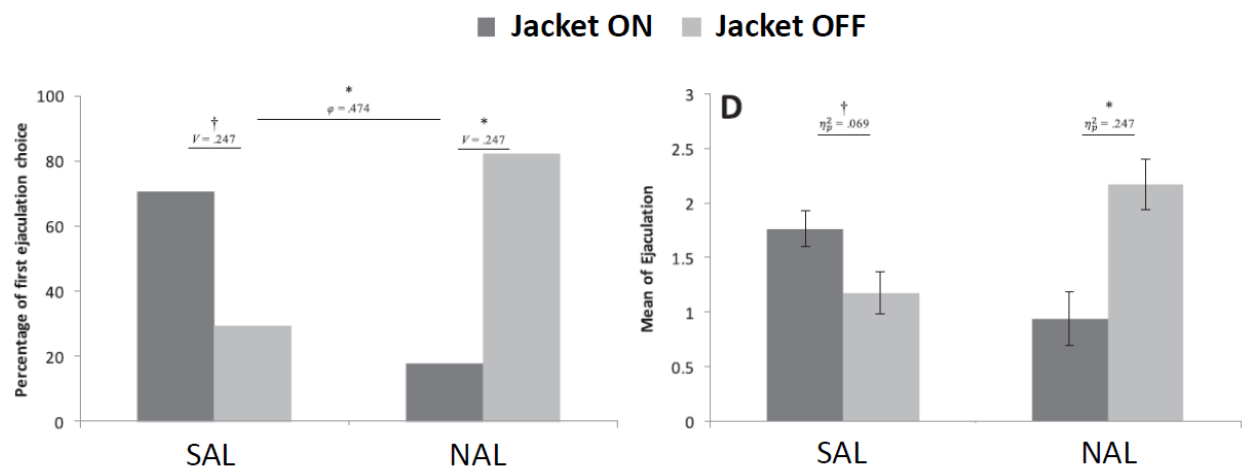
top-down manner.

Ismail et al. (2009) examined the effect of naloxone on the development of a CEP in male rats. Males were injected with either saline or naloxone immediately prior to ten 30-min multi-ejaculatory conditioning trials with almond scented receptive females conducted at 4-day intervals. The open field CEP test occurred 4 days after the last conditioning trial. All males were injected with saline immediately before being placed into the open field for 5 min before two receptive females were introduced, one scented with almond and the other unscented. The test lasted 30 min. Males trained with saline prior to each conditioning trial showed CEP for the almond scented female (**Figure 23, top left and right**). Males injected with naloxone prior to each conditioning trial changed their preference and mounted and intromitted more, and had their first ejaculations with, the novel (unscented) female during the CEP test (**Figure 23, bottom left and right**).



**Figure 23:** Effect of saline (top) or naloxone (bottom) on the development of an odor-based CEP in male rats.

Naloxone administration before conditioning of CEP for the tethering jacket also inhibits its acquisition. Quintana et al. (2019b) used an explicitly Paired procedure similar to that used in Experiment 2 to of Quintana et al. (2019a) above, but with a 1-hole divider instead of a 4-hole divider. Male rats received sequential pairings of jacketed receptive and unjacketed nonreceptive females. Half of the males received systemic injections of naloxone immediately before each pairing with receptive females and saline injections before each pairing with nonreceptive females. The other half received saline injections prior to each pairing with receptive and nonreceptive females. On the final CEP test, all males were injected with saline and placed into the open field for 5 min before two receptive females were introduced, one with the jacket on and one without the jacket, for the 30-min test. As in Ismail et al. (2009), males injected with saline showed a preference to ejaculate first and more with the jacketed female, whereas males injected with naloxone during training shifted their preference for the female without the jacket (**Figure 24**). Unlike Ismail et al. (2009), the males did not show a significant preference to mount or intromit one female over the other. This could be due to the use of the 1-hole divider or the fact that the jacket might be less of a distal cue.

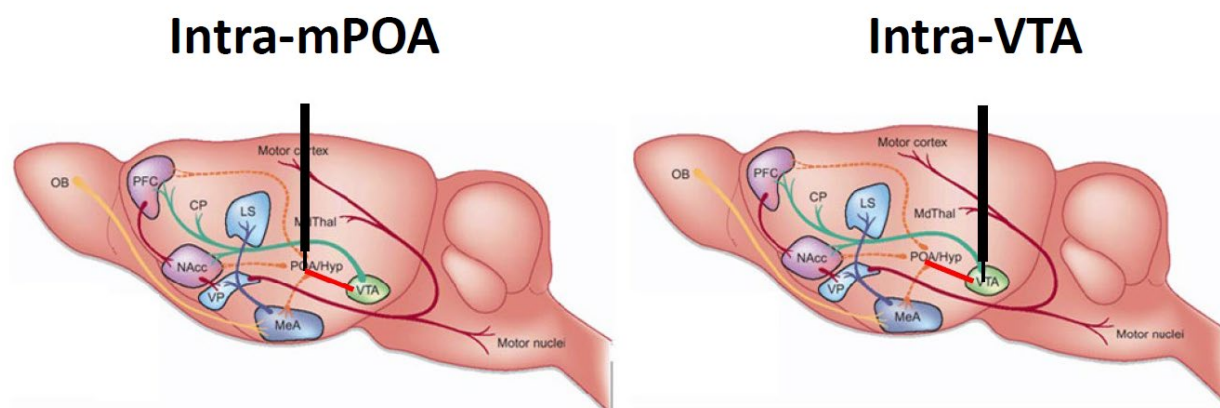


**Figure 24:** Effect of saline (SAL) or naloxone (NAL) on the development of a somatosensory-based CEP in male rats.

Taken together, these data suggest that copulation without the opioid effect at ejaculation induces an aversive state that becomes associated with the odor CS. This makes males shift their preference toward a female that does not bear the CS.

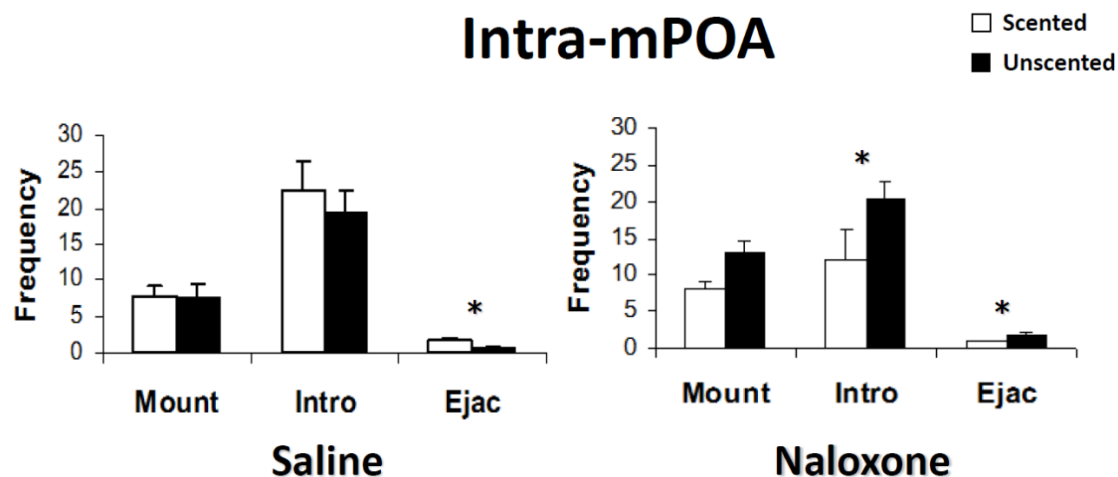


In another set of experiments, Quintana et al. (2019c) examined the effect of naloxone or saline infusions to the mPOA or VTA (**Figure 25**) of male rats given explicitly Paired training as above with the odor or jacket. Males received 10 alternating trials with the almond odor (as in Ismail et al., 2009) or 14 alternating trials with the jacket (as in Quintana et al, 2019a;b). Males



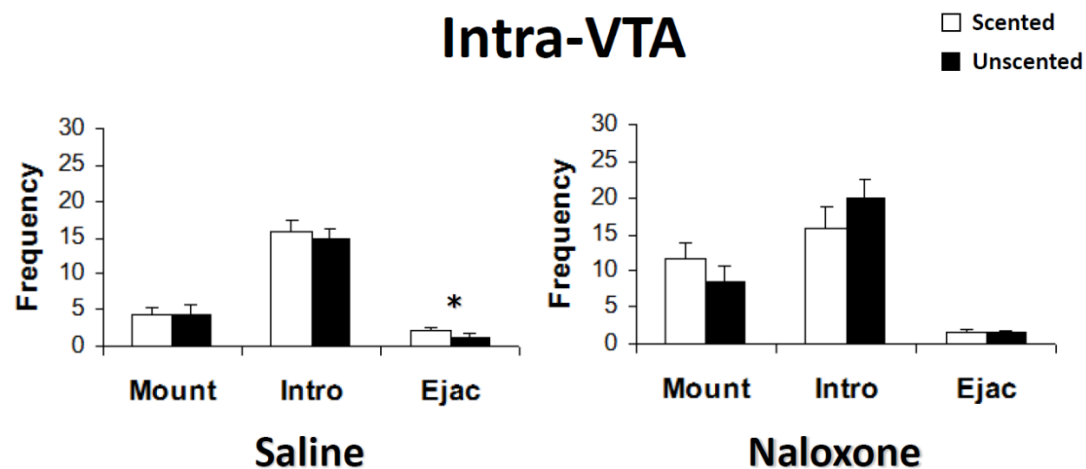
**Figure 25:** Bilateral cannula placements into the mPOA or VTA of male rats. Rats were infused with saline or naloxone in 1 $\mu$ l per side for 1 min, with the infusion cannula left in for another min to assure dispersal from the infusion site.

infused with saline to the mPOA displayed a significant preference to ejaculate first and more often with the scented female, whereas males infused with naloxone to this region showed a preference intromit and ejaculate first and more often with the unscented female (**Figure 26a**).

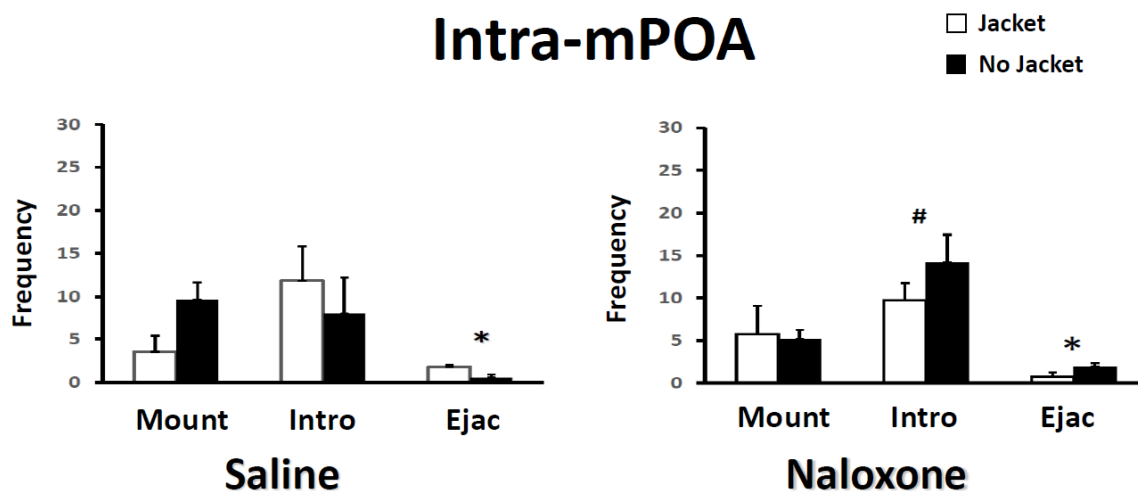


**Figure 26a:** Effect of intra-mPOA infusions of saline or naloxone on the development of an odor-based CEP.

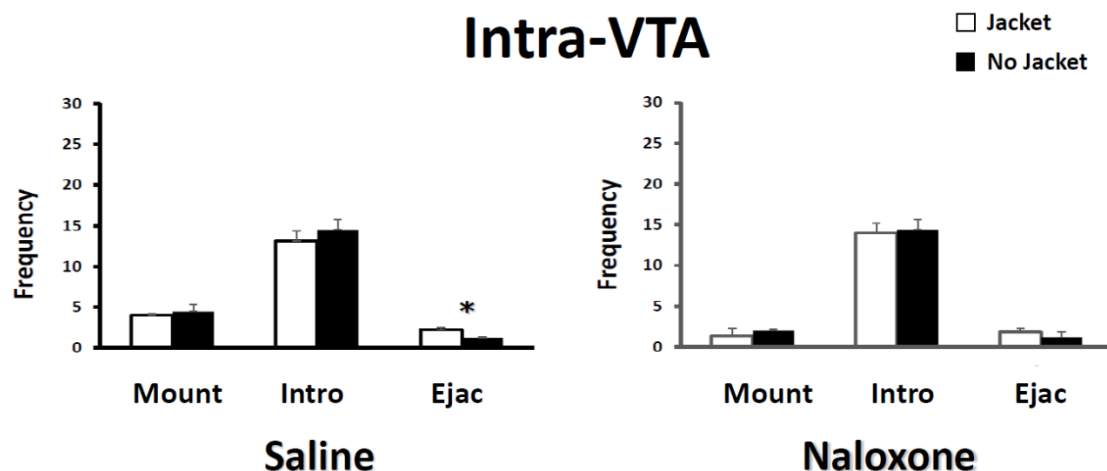
Males infused with saline to the VTA also showed a significant CEP for the scented female, whereas males infused with naloxone did not display a preference for either female (**Figure 26b**). Similar to the odor conditioning, males infused with saline to the mPOA displayed a significant preference to ejaculate first and more often with the jacketed female, whereas males infused with naloxone to this region showed a preference to intromit more and ejaculate first and more often with the female without the jacket (**Figure 26c**). Finally, males infused with saline to the VTA displayed a significant preference to ejaculate first and more often with the jacketed female, whereas males infused with naloxone did not display a preference for either female (**Figure 26d**).



**Figure 26b:** Effect of intra-VTA infusions of saline or naloxone on the development of an odor-based CEP.



**Figure 26c:** Effect of intra-mPOA infusions of saline or naloxone on the development of a somatosensory-based CEP.



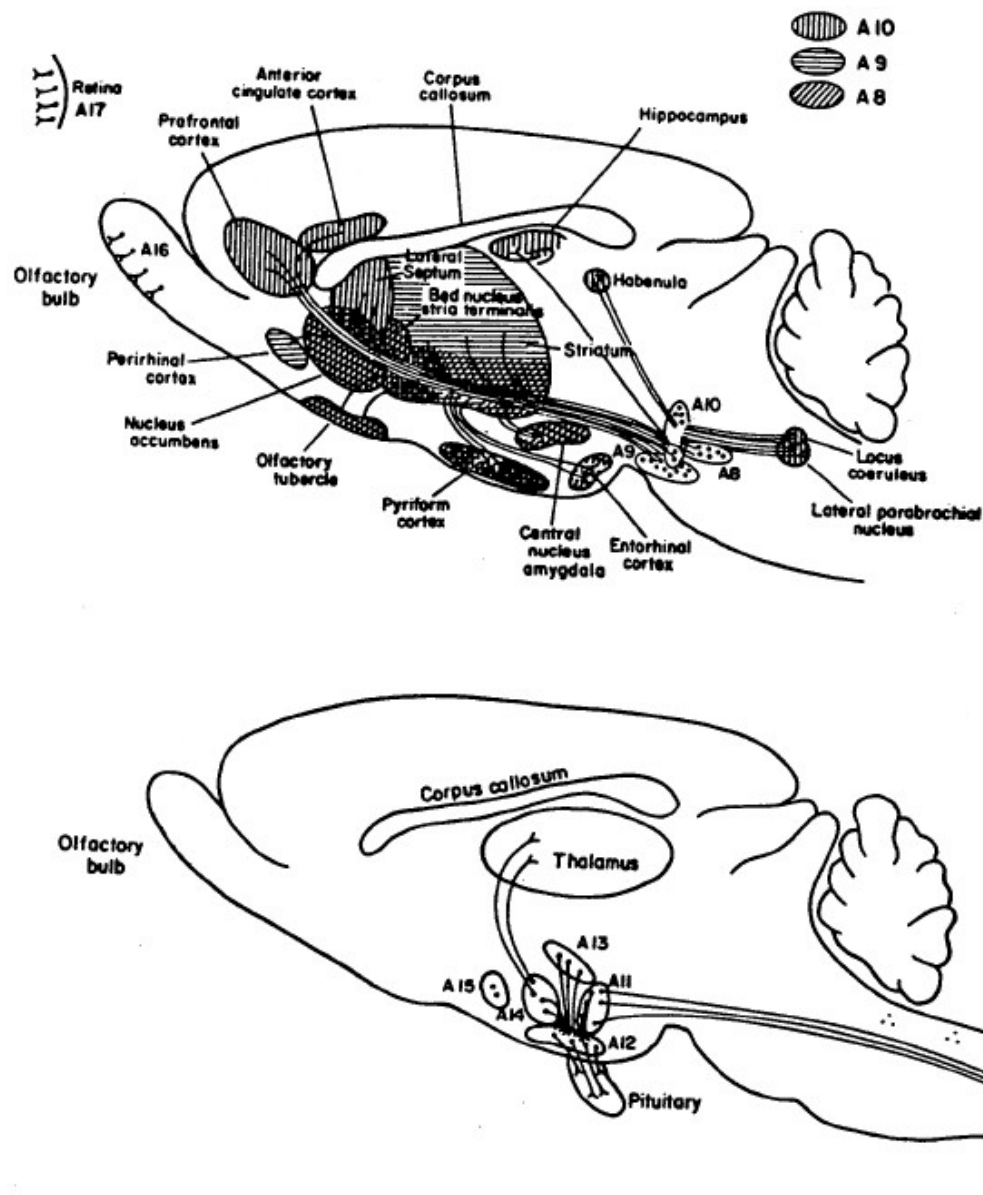
**Figure 26d:** Effect of intra-VTA infusions of saline or naloxone on the development of a somatosensory-based CEP.

As with CPP, intra-mPOA infusions of naloxone blocked the development of CEP for both the odor and jacket, and seems to have created an aversive state that mimicked the effect of systemic naloxone. Although naloxone infusions to the VTA also blocked the development of CEP, they did not shift the male's preference for a female not bearing the CS. This suggests that opioid actions in the mPOA may valence the reward value of sexual stimuli, whereas opioid actions in the VTA (presumably through the activation of mesolimbic dopamine transmission) modulate incentive salience.

Similar findings were reported in male prairie voles. Burkett et al. (2011) administered saline or naltrexone to males during their first sexual experiences and cohabitation with females. Males that received a single systemic injection of naltrexone did not display a partner preference. Males that received three injections throughout the cohabitation period displayed a preference for a novel female. In contrast, males injected with saline showed a typical partner preference for the familiar female. The MOR selective antagonist CTAP also blocked partner preference in male voles when infused into the caudate-putamen. Along the same lines, systemic injections of naloxone blocked CPP induced by copulation and cohabitation in male, but not female, prairie voles (Ulloa et al., 2018). Thus, sexual partner preference in male prairie voles, as in rats, is also dependent on opioid reward mechanisms acting through MORs during the male's first copulations with a female. As outlined above, opioids like  $\beta$ -endorphin are

released in different brain regions during at ejaculation (Szechtman, Hershkowitz, & Simantov, 1981). Binding to MORs induces receptor internalization that is critical for the activation of *Gi* proteins that disinhibit certain second messenger cascades, such as MAP kinases and CaM kinase II, both of which figure in the long-term sensitization of dopamine and glutamate transmission (see below). This occurs in the mPOA after ejaculation (Coolen et al., 2004). Thus, the postejaculatory state plays a critical role in the crystallization of opioid-dependent sexually conditioned place and partner preferences. Paced copulation and distributed CLS and VCS do the same thing in females given that those effects are also blocked by opioid antagonists. This gives rise to the notion that paced copulation, distributed CLS/VCS, and ejaculation, induce OLRs in rats that are homologous to orgasm in humans (Pfaus et al., 2016).

*Dopamine.* As mentioned above, two major ascending dopamine pathways exist in the vertebrate brain that are involved critically in motivation and movement (**Figure 27**): the A10 mesolimbic/mesocortical system (with cell bodies in the VTA that project axons to many limbic structures, notably the NAc, amygdala, lateral septum, hippocampus, and frontal cortex), and the A9 nigrostriatal system (with cell bodies in the substantia nigra that project axons to the dorsal striatum). These systems interact at the level of the ventral striatum (NAc), which integrates motivation and action, bringing animals from distal to proximal to interactive with all incentive stimuli (O'Connell & Hofmann, 2011). Intrinsic dopamine systems exist in the diencephalon, notably the A11 periventricular system that has diffuse dopamine cells around the third ventricle that project from the hypothalamus to the brainstem and spinal cord, the A12 tuberoinfundibular system that projects from the arcuate nucleus to the pituitary (where among other roles, it acts as the prolactin inhibiting factor), and the A13 incertohypothalamic system that projects from the zona incerta (ZI) under the thalamus to the mPOA, the VMH, with ancillary projections to the central nucleus of the amygdala (CeA) and the periaqueductal grey (PAG) in the midbrain. As shown in Figure 7, neurons in the mPOA send projections to the PVN and VTA (and also the VMH) where they facilitate both sexual arousal and sexual motivation to prepare animals to interact with incentive sexual stimuli (Pfaus, 2009). Both the mesolimbic and incertohypothalamic systems are activated by sensory stimulation, and in both systems, appetitive sexual behaviors cause a phasic release of dopamine, whereas copulation results in



**Figure 27: Major dopamine pathways in the rat brain.** Top: Major ascending pathways include the mesolimbic/mesocortical (A10) and nigrostriatal (A9) systems that pass through the medial forebrain bundle. Bottom: Major diencephalic pathways include the periventricular (A11), tuberoinfundibular (A12), and incertohypothalamic (A13) systems. After Moore (1987) and Roth, Wulf, and Deutsch (1987).

tonic dopamine release. Ejaculation in male rats results in an immediate inhibition of dopamine release in both the mPOA and NAc during the absolute refractory phase, and a drift upwards during the relative refractory phase (Blackburn, Pfaus, & Phillips, 1992; Pfaus et al., 1990). The increase in dopamine transmission in the mPOA must reach a critical threshold for copulation

to occur or resume after ejaculation (Dominguez & Hull, 2005; Putnam et al., 2001). A major source of excitation of dopamine transmission in the mPOA comes from the MeA which sends olfactory-mediated excitatory glutamate projections through the stria terminalis (Dominguez and Hull, 2001). A major source of inhibition of dopamine transmission in the mPOA and NAc comes from the actions of serotonin in the lateral hypothalamus (Lorrain et al., 1999). Serotonin release there activates local GABA interneurons in both regions that inhibit dopamine release presynaptically.

There are two families of dopamine receptors, the D1 and D2 families. D1 (D1 and D5) receptors, are linked to *G<sub>s</sub>* proteins that promote cAMP activity, whereas D2 (D2, D3, and D4) receptors are linked either to phosphatidylinositol hydrolysis that activates inositol triphosphate or diacylglycerol as second messengers, and/or *G<sub>i</sub>* proteins that inhibit cAMP activity (Strange, 1993). Receptors are found in the terminal regions of the pathways shown in **Figure 27**.

Conditioned approach and other appetitive sexual behaviors displayed by male rats (e.g., conditioned level changes in bilevel chambers) are reduced by systemic administration of dopamine receptor antagonists irrespective of receptor type (Pfaus & Phillips, 1991). However, in that study, nonselective antagonists like haloperidol and the D1-selective antagonist SCH 23390 also increased the mount and intromission latencies, and reduced the number of intromissions and ejaculations, whereas D2-selective antagonists like clozapine and sulpiride decreased conditioned level changes only. Bilateral infusions of haloperidol to the mPOA produced all the effects observed with systemic administration whereas infusions to the NAc decreased level changes but did not alter copulatory measures.

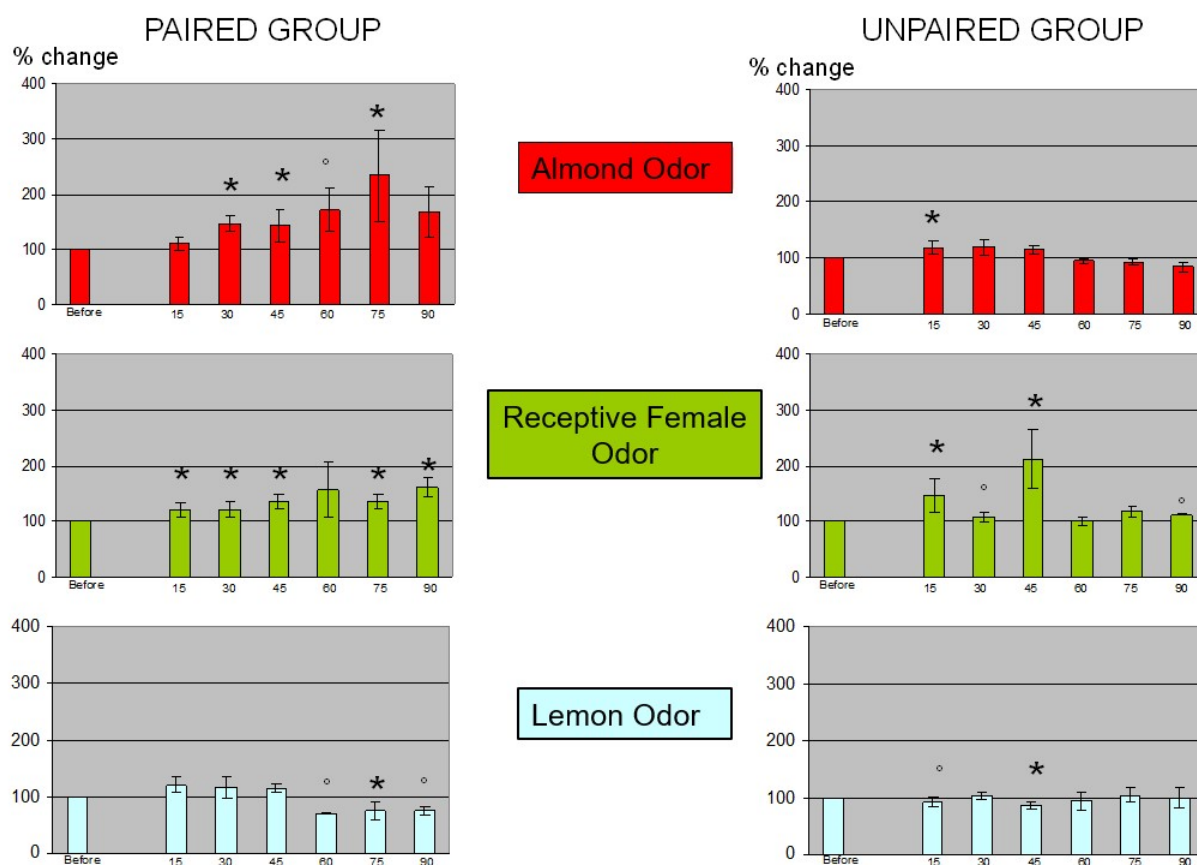
Ågmo and Berenfeld (1988) found that the development of postejaculatory CPP in male rats was not blocked by injections of pimozide (a D2/D3 receptor antagonist) prior to each training session. Conversely, Meisel, Joppa, and Rowe (1996) found that the development of copulatory CPP in female hamsters was blocked by injections of the D2 receptor antagonists sulpiride or raclopride prior to each training session. No studies have reported on the effects of dopaminergic drugs on the expression of CPP. However, other results suggest involvement of the mesolimbic dopamine systems in responding to conditioned stimuli. For example, West,

Clancy, and Michael (1992) examined the responsiveness of the NAc in male rats to several neutral odors (isoamyl acetate (banana), cedar wood oil, cineole (camphor), acrylic solvent (methyl methacrylate; a sharp, fruity odor), or geraniol (rose)) on filter paper paired with copulation to ejaculation, copulation but no ejaculation, presentation of a nonreceptive female, or no conspecific. After 10 training trials, they anesthetized the males and implanted electrodes aimed at the NAc to record single unit activity from medium spiny neurons that are activated by dopamine there in response to the presentation of the odors. Odors paired previously with access to a receptive female produced more single-unit activity than odors paired with either a nonreceptive female or with no conspecific. Further, the responsiveness was higher for males that ejaculated during training than in those who copulated but did not ejaculate.

Consistent with the findings of West, Clancy, and Michael (1992), Pfaus et al. (2012) found that the presentation of the almond odor that had been paired previously with copulation to ejaculation increased dopamine concentrations in microdialysates taken from the NAc of freely-moving Paired males (**Figure 28**). These concentrations peaked at approximately 230% of baseline, similar to the values obtained from a low dose of cocaine. Dopamine concentrations did not increase in freely-moving Unpaired males. In both groups, however, smaller increases were observed in response to estrous vaginal secretions, and no increases were observed in response to a novel lemon (citral) odor. It is also the case that the almond odor when presented to Paired males or females activates Fos-IR in the central region of the VTA, where dopamine cell bodies of the A10 system are found (e.g., Coria-Avila & Pfaus, 2007; **Figure 13**).

The effects of the nonselective dopamine receptor antagonist flupenthixol was examined on CPaP in female and male rats. Flupenthixol administered systemically before each conditioning trial in female rats abolished CPaP for the almond odor, but not for strain (Coria-Avila et al., 2008). In contrast, systemic flupenthixol administered before each conditioning trial in male rats did not alter the development of CEP (Ismail et al., 2009). Taken together with the results of Meisel, Joppa, and Rowe (1996), it would appear that females are more sensitive to the effect of dopamine antagonists than males, suggesting that dopamine transmission is necessary for conditioning to neutral odors or place cues in females. However, dopamine

antagonists compete with dopamine for receptor occupancy, and male rats release on average approximately 25% more dopamine in the NAc during copulation than female rats (Damsma et al., 1992; Pfaus et al., 1995). This makes it possible that the dose of flupenthixol used was not sufficient to antagonize dopamine receptors efficiently in males.



**Figure 28: Percent shifts in dopamine concentration from dialysates in the NAc.** Samples were taken during baseline (Before), and at 15 min intervals during the presentation of a Paired or Unpaired almond odor, estrous vaginal secretions from sexually receptive females, or a novel lemon odor.

Aragona et al. (2006) stumbled upon a very important finding regarding the role of D1 and D2 receptors in the NAc on pair-bond formation in prairie voles. Pair bonded males had an increase in D1 receptors and a decreased in D2 receptors in the caudal part of the NAc shell compared to non-pair bonded males. Sexually naïve males showed the opposite receptor density profile. Blockade of D2, but not D1, receptors in the NAc prevented copulation induced partner preferences, whereas the activation of D2 receptors with the selective agonist

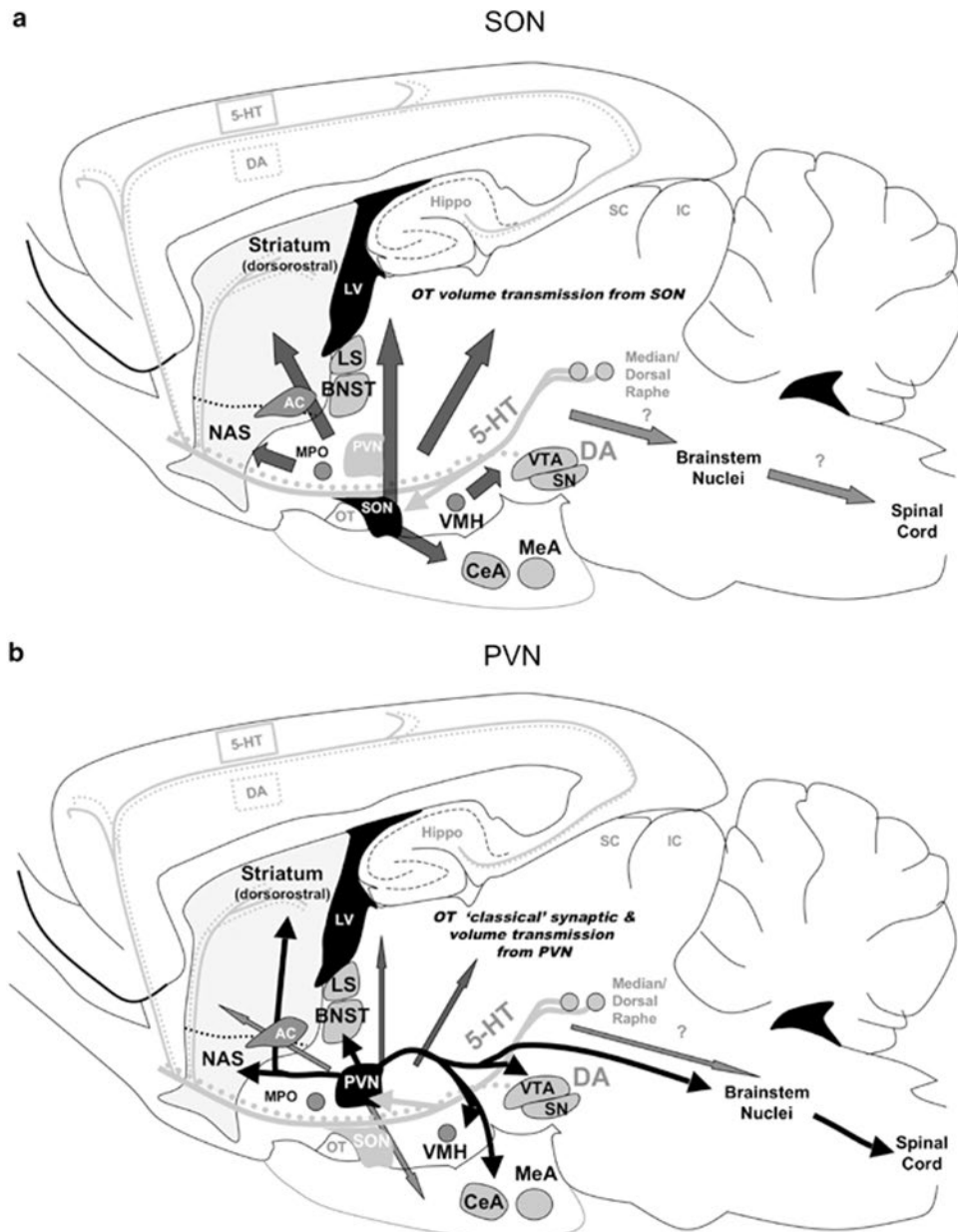


quinpirole induced partner preferences in the absence of copulation, when males were simply paired with a particular female while under the influence of quinpirole. In contrast, the D1 selective agonist SKF 38393 prevented pair bond formation in naïve males copulating with their first sex partner. This suggested that the caudal NAc shell displays plasticity during first sexual experiences that induce pair bond formation such that the increase in D1 receptors maintains the pair bond by preventing sociosexual interest in novel partners.

Triana-Del Rio et al. (2011) extended this finding to male rats. Males cohabitated with either almond-scented males or unscented stimulus males under the influence of systemic injections of quinpirole or saline. On a final nondrug test using tethered males in two separate compartments, one familiar and one novel, the males that cohabitated under the influence of quinpirole displayed a social preference toward the familiar male, regardless of the presence of the conditioned odor. They also displayed a preference for the familiar male over a novel receptive female. Interestingly, when subject males cohabitated with sexually active stimulus males under quinpirole, they also displayed noncontact erections during their final test in the presence of the familiar male. Cohabitation under quinpirole did not induce a social or sociosexual partner preference in females. A subsequent study showed that sexually naïve male rats cohabitating with juvenile male rats under the influence of systemic quinpirole also developed a sociosexual preference for juvenile versus novel adult males on the final test, along with noncontact erections (Ramírez-Rodríguez et al., 2021). It is not known if the density for D1 receptors increased after conditioning, but it was clear that the social partner preference lasted at least a month after the pairing and without further conditioning. Given that D1 antagonists reduce the number of ejaculations in sexually active male rats, it is possible that a greater D1/D2 ratio in the NAc maintains attention toward, and intromissions directed at, the preferred partner, especially when ejaculation is imminent (Kippin & Pfaus, 2001b).

*Oxytocin and vasopressin.* In addition to dopamine, the neuropeptides oxytocin and vasopressin play an important role in social and sexual bonding in voles and other mammalian species (Insel, Young, & Wang, 1997; Williams et al., 1994; Young & Wang, 2004). Like dopamine, oxytocin also plays an excitatory role in sexual arousal (Argiolas & Melis, 2013). Cell bodies for oxytocin and vasopressin are found in the supraoptic nucleus (SON) and

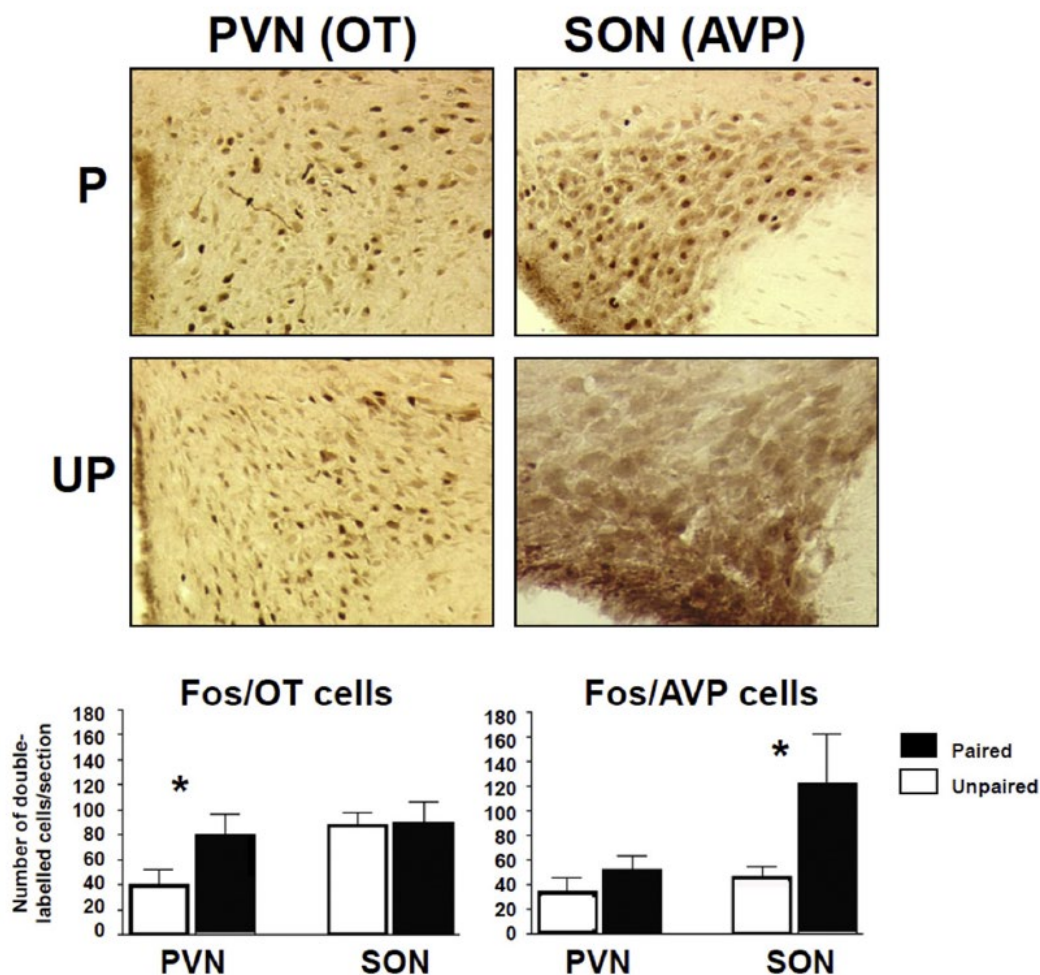
paraventricular nucleus (PVN) of the hypothalamus, and project to limbic structures like the NAc, CeA, lateral septum, and to the VTA. In addition to their actions in the brain as neurotransmitters (**Figure 29**), oxytocin and vasopressin are released from the posterior pituitary into the bloodstream, where they can act as peptide hormones at peripheral targets including the penis, clitoris, and cervix. In an important series of studies, central infusions of



**Figure 29: Oxytocin projections from the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus.** Note the overlap with dopamine terminal regions like the NAc, and projections to the VTA. After Froemke & Young (2021).

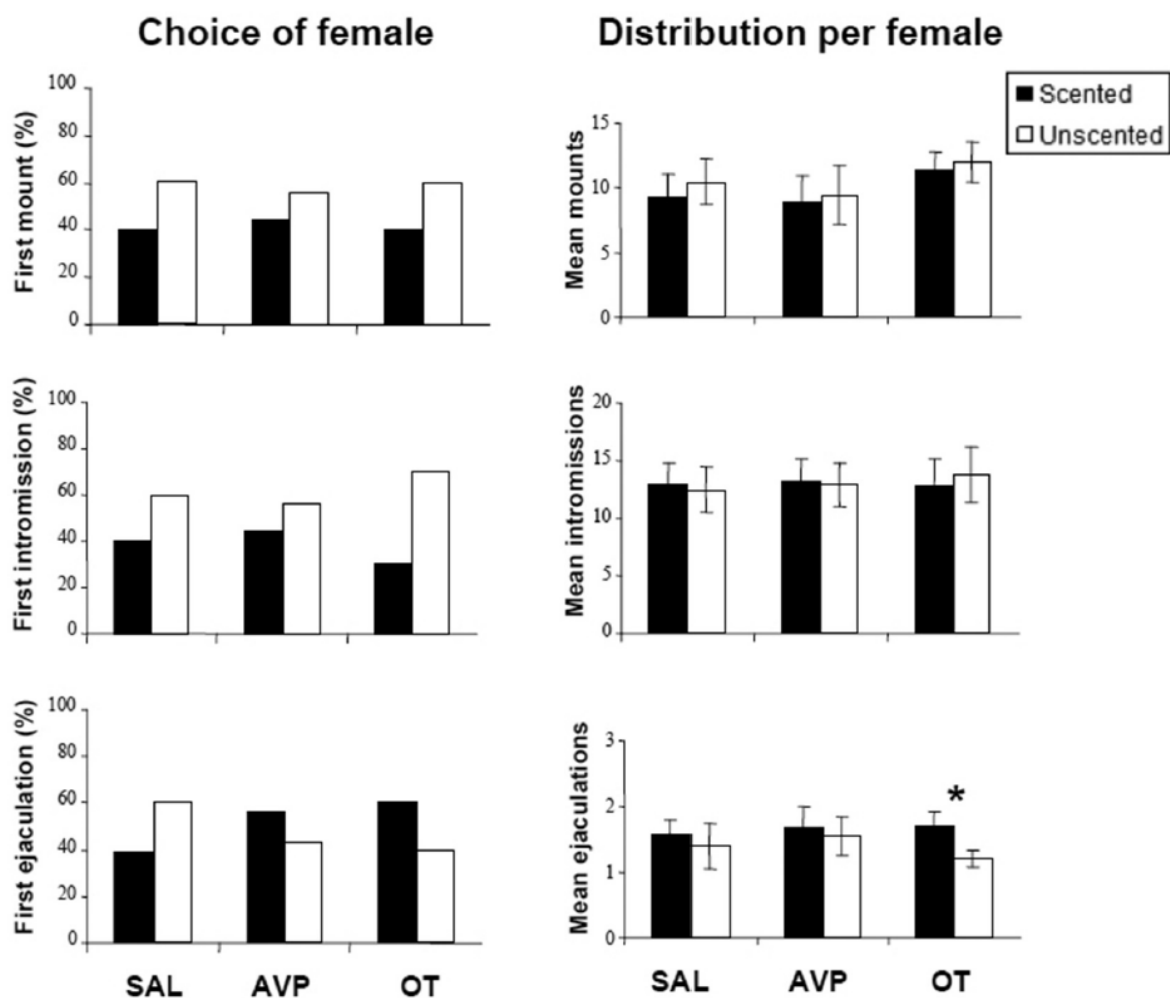
oxytocin enhanced the development of pair bonding in female prairie voles (Williams et al., 1994). In another study, overexpression of the vasopressin 1a receptor through viral transfection into the ventral pallidum facilitated pair-bonding in non-monogamous montane voles (Lim et al., 2004). The V1a receptor population is far greater in this region of the male prairie vole brain compared to the montane vole brain.

Ménard et al. (2019) gave male rats 9 multiejaculatory trials in bilevel pacing chambers with almond scented or unscented females. After confirming CEP for males in the Paired vs. Unpaired groups, the males were given 2 reconditioning trials and then presented with the odor alone on gauze for 1 hr before their brains were processed for double labeling of nuclear Fos and cytoplasmic oxytocin or vasopressin in neurons of the PVN and SON (Figure 30).



**Figure 30:** Activation of oxytocin (OT) and vasopressin (AVP) neurons in the PVN and SON of Paired (P) versus Unpaired (UP) males in response to the almond odor.

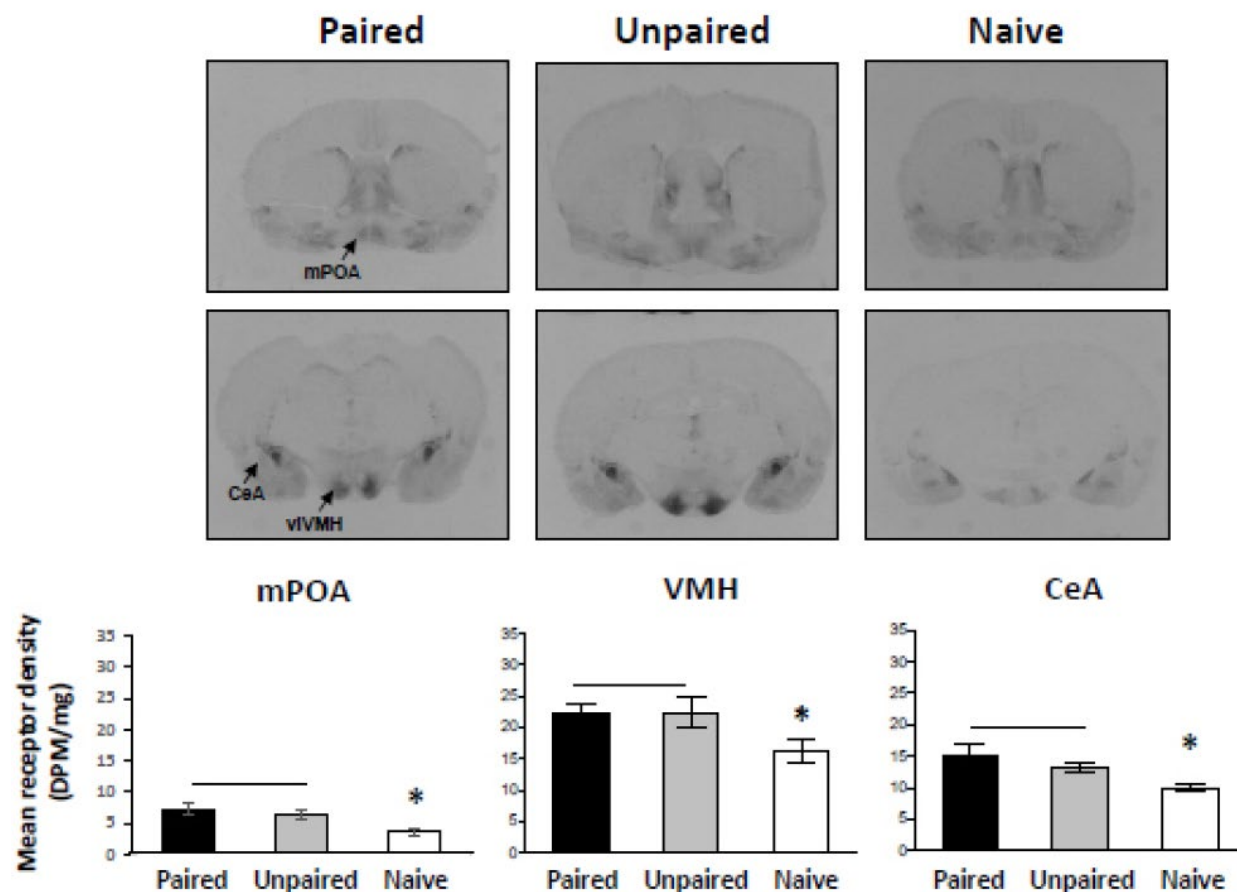
Activation of oxytocin neurons was observed in the PVN, but not SON, of Paired vs. Unpaired males. In contrast, activation of vasopressin neurons was observed in the SON, but not PVN, of Paired vs. Unpaired males. In a second experiment, Ménard et al. administered oxytocin, vasopressin, or saline systemically during the first Paired conditioning trial in a new group of males, and then tested CEP 4 days later. Males injected with oxytocin, but not vasopressin or saline, showed enhanced CEP (**Figure 31**), suggesting that oxytocin release from projections originating in the PVN are part of the circuit that enhances CEP.



**Figure 31:** Effect of saline (SAL), vasopressin (AVP), or oxytocin (OT) during the first multiejaculatory conditioning trial with almond-scented females. Data are from the second test 4 days after the conditioning trial.

In another study, Ménard et al. (2021) examined whether oxytocin or vasopressin receptor densities would be altered in Paired vs. Unpaired males, relative to a group of sexually

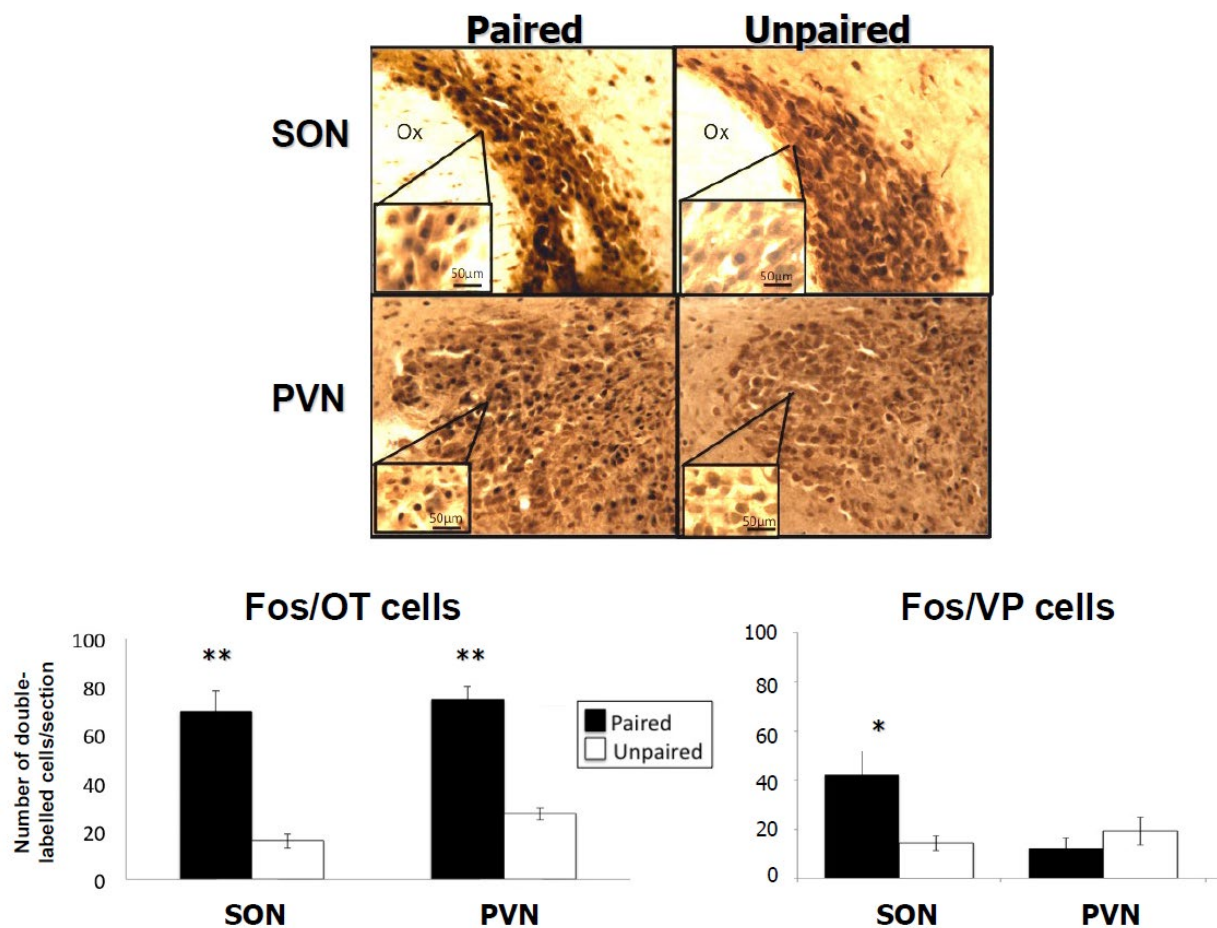
naïve males. As before, males were given 9 conditioning trials in bilevel pacing chambers with either almond-scented or unscented females. Brains were then processed for autoradiography using  $^{125}\text{I}$ -ornithine vasotocin as the ligand. Oxytocin receptor densities were increased by sexual experience in the mPOA, VMH, and CeA in both the Paired and Unpaired groups relative to the sexually naïve group. There was no effect on vasopressin receptor expression (Ménard et al., 2021; **Figure 32**).



**Figure 32: Oxytocin receptor densities in the mPOA, VMH, and CeA of Paired, Unpaired, and sexually naïve males.** Top shows representative autoradiograms. Bottom shows average counts. Sexual experience (9 multiejaculatory conditioning trials) increased oxytocin receptor binding relative to the sexually naïve group. No differences were found between Paired and Unpaired males.

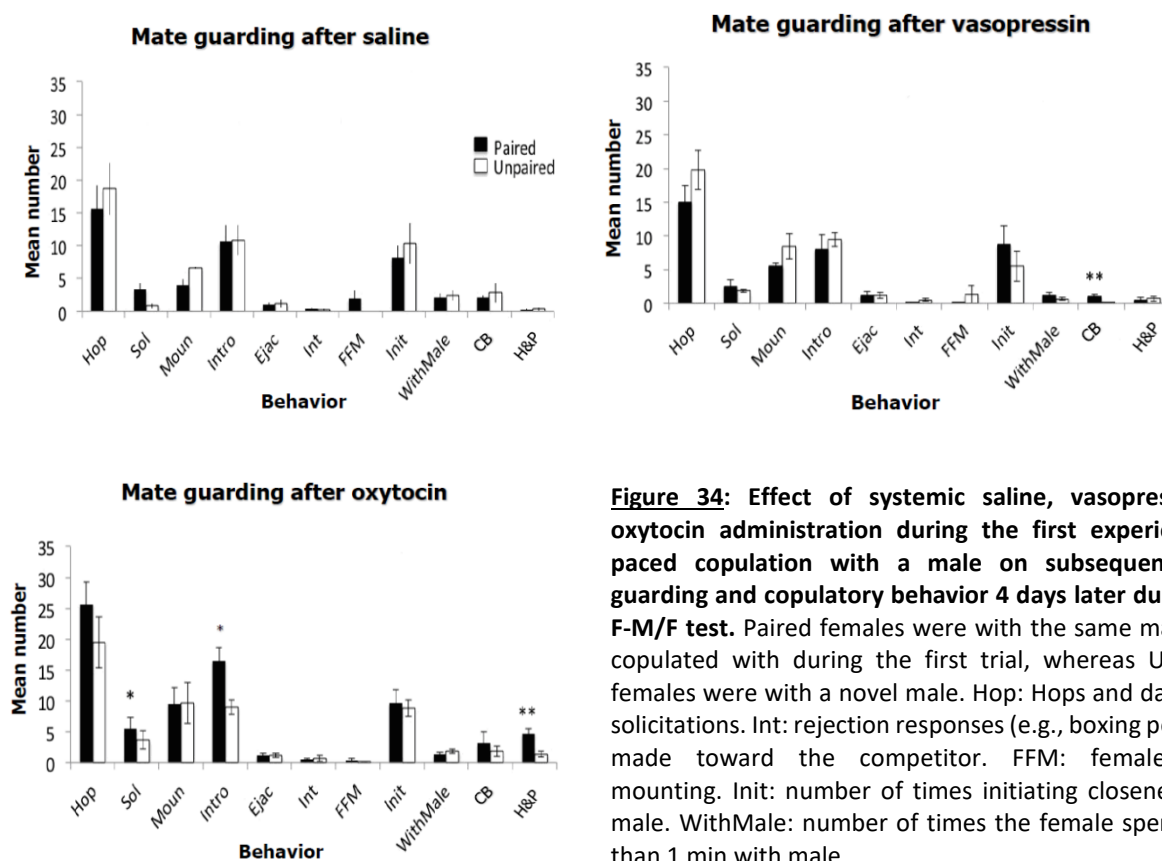
Finally, Triana-Del Rio (2015) found that systemic injections of quinpirole and oxytocin together greatly enhanced both the time spent with the cohabitated male and the display of noncontact erections, relative to either drug alone. This suggests a synergy between dopamine and oxytocin systems, as outlined in **Figure 7**.

Holley et al. (2015) replicated the female rat mate-guarding paradigm, but then tested the activation of oxytocin and vasopressin neurons following the final 1-hr mate-guarding test using double-labeling to examine Fos-IR in the nucleus of cells stained for oxytocin or vasopressin. As with Paired males, Paired females that displayed mate-guarding behavior had significantly increased numbers of double-labelled Fos/oxytocin neurons in the PVN, and more Fos/vasopressin neurons in the SON compared to Unpaired females. However, unlike Paired males, Paired females also had significantly more Fos/oxytocin neurons in the SON as well (Figure 33).



**Figure 33:** Activation of oxytocin (OT) and vasopressin (VP) neurons in the SON and PVN of Paired versus Unpaired females in response to the final mate-guarding test. Top, representative photomicrographs of double-labeled Fos/oxytocin or Fos/vasopressin neurons in the SON and PVN. Bottom: mean counts of double-labeled neurons in each region.

A second experiment tested whether a single systemic injection of oxytocin, vasopressin, or saline, during the first conditioning trial could potentiate mate guarding behaviors 4 days later in the open field with the same male (Paired condition), relative to the same injections but where the male was replaced with a novel male (Unpaired condition). No differences in mate-guarding or copulatory behaviors were observed after saline treatment on the first trial. In contrast, treatment with vasopressin potentiated competitor blocking (CB) behaviors displayed by Paired females during the test, whereas treatment with oxytocin potentiated the hovering and presenting (H&B) behavior and solicitations (Sol) displayed by Paired females during the test, such that they received significantly more intromissions from the Paired male relative to the number received by females in the Unpaired condition (**Figure 34**).



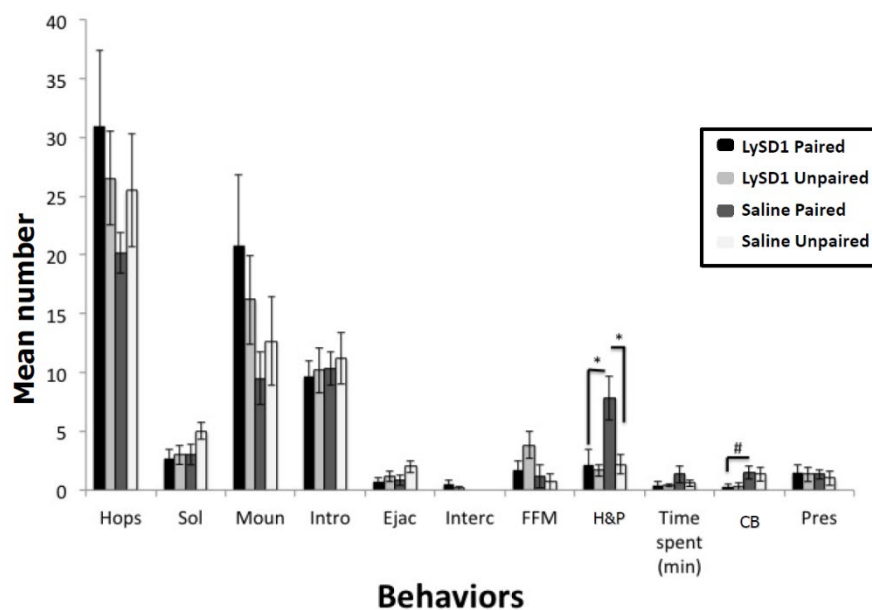
**Figure 34:** Effect of systemic saline, vasopressin, or oxytocin administration during the first experience of paced copulation with a male on subsequent mate guarding and copulatory behavior 4 days later during the F-M/F test. Paired females were with the same male they copulated with during the first trial, whereas Unpaired females were with a novel male. Hop: Hops and darts. Sol: solicitations. Int: rejection responses (e.g., boxing postures) made toward the competitor. FFM: female-female mounting. Init: number of times initiating closeness with male. WithMale: number of times the female spent more than 1 min with male.

These data show that oxytocin and vasopressin potentiate the acquisition of different mate-guarding and copulatory behavior in females. Oxytocin potentiates male-directed affiliative behaviors like H&P along with appetitive sexual behaviors like solicitations. Vasopressin



potentiates defensive behaviors like CB that indicate awareness of the competitor female. The activation of oxytocin neurons in the PVN and SON after 10 trials with the same male may be contributing differentially to these behaviors, whereas the activation of vasopressin neurons in the SON is likely contributing to the female's awareness of the competitor.

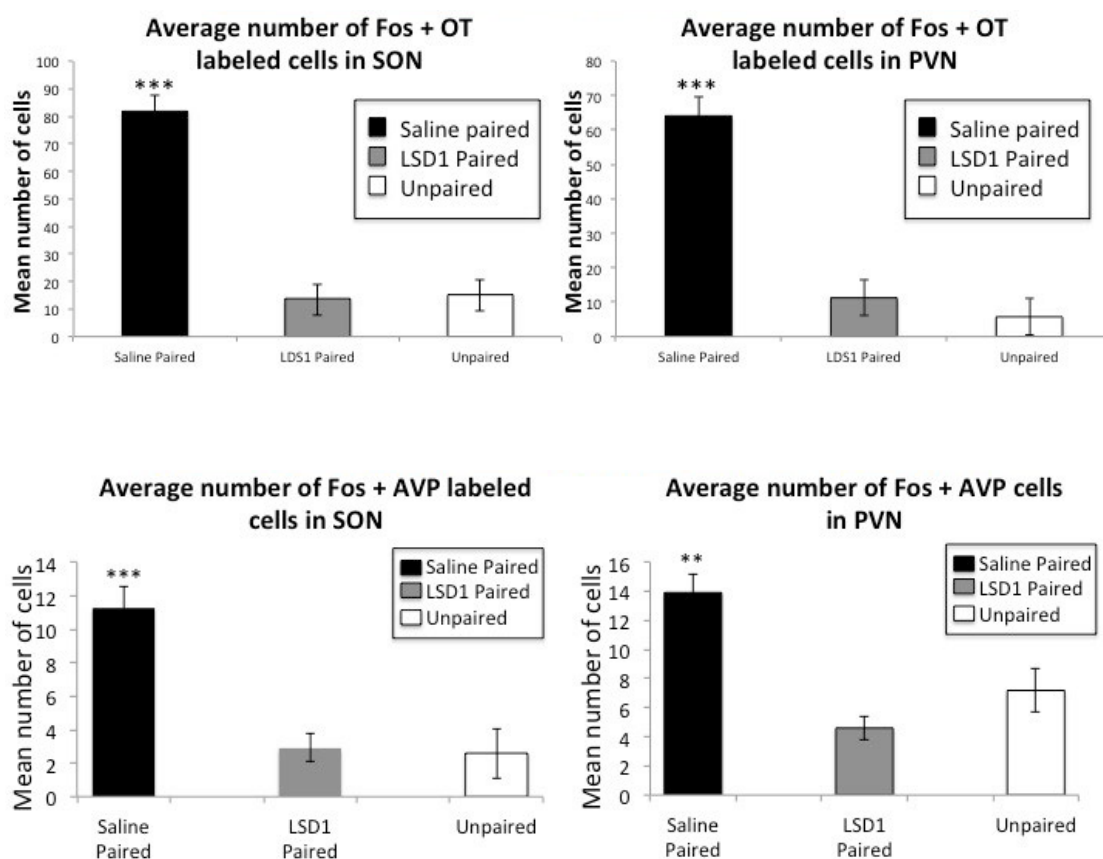
*Inhibition of lysine-specific demethylase.* In a final study of mate-guarding behavior in the female rat, Holley et al (2018) administered saline or oryzon, a lysine (K)-specific demethylase 1a (LSD-1a) inhibitor, prior to each of 10 Paired or Unpaired conditioning trials at 4-day intervals in unilevel pacing chambers with the 4-hole divider (Paired females copulated with the same male each trial, whereas Unpaired females copulated with a different male each trial). Relative to saline, oryzon inhibited the development of H&P and CB in the Paired group (**Figure 35**), but had no significant effect on behavior in the Unpaired group.



**Figure 35:** Effects of the LSD-1a inhibitor oryzon or saline on mate-guarding and copulatory behaviors of female rats.

Following the final F-M/F test, brains were removed and examined for double labelling of Fos with oxytocin or vasopressin in the Paired females receiving either saline or oryzon, and the Unpaired females receiving saline. Results showed that oryzon decreased the number of double-labelled cells for oxytocin or vasopressin in the SON and PVN of Paired females relative to saline and females in the Unpaired group (**Figure 36**).



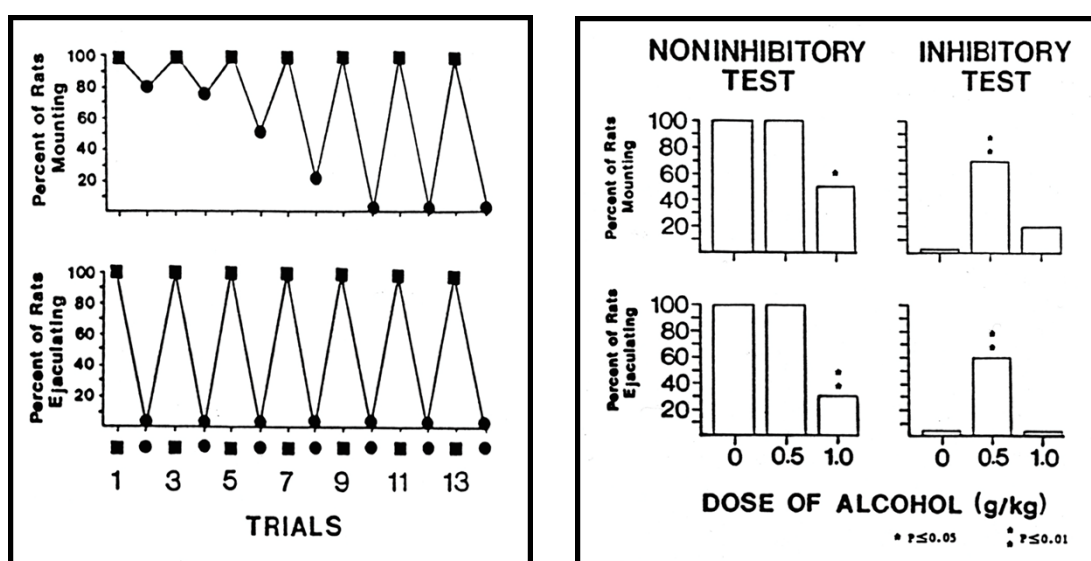


**Figure 36:** Effect of saline or oryzon (LSD1) in Paired females on the average number of double labelled cells for Fos/oxytocin (OT) or Fos/vasopressin (AVP) in the SON or PVN. Comparisons were made also with saline-treated females in the Unpaired group.

LSD1 codes for a flavin-dependent monoamine oxidase which demethylates mono- and di-methylated lysines. This unravels certain DNA strands that are twisted around histones, exposing (and sometimes also linearizing) the 5' to 3' regions around a gene that opens it up to transcription and subsequent translation into mRNA and protein with the right activators. LSD1 regulates gene expression epigenetically, and figures in various neuronal processes such as neuronal differentiation, activation of the circadian clock, and in presynaptic plasticity that underlies hippocampal learning and memory (Kim, Kim, & Baek, 2021; Lim et al., 2017). Oryzon inhibits the removal of methyl groups on lysines, keeping genes wound around histones and far less likely to be open to transcription. In the experiment above by Holley et al. (2018), oryzon inhibited the ability of sexual reward in females to become associated with a particular male, leading to inhibited mate-guarding behavior and a lack of activation of oxytocin and vasopressin

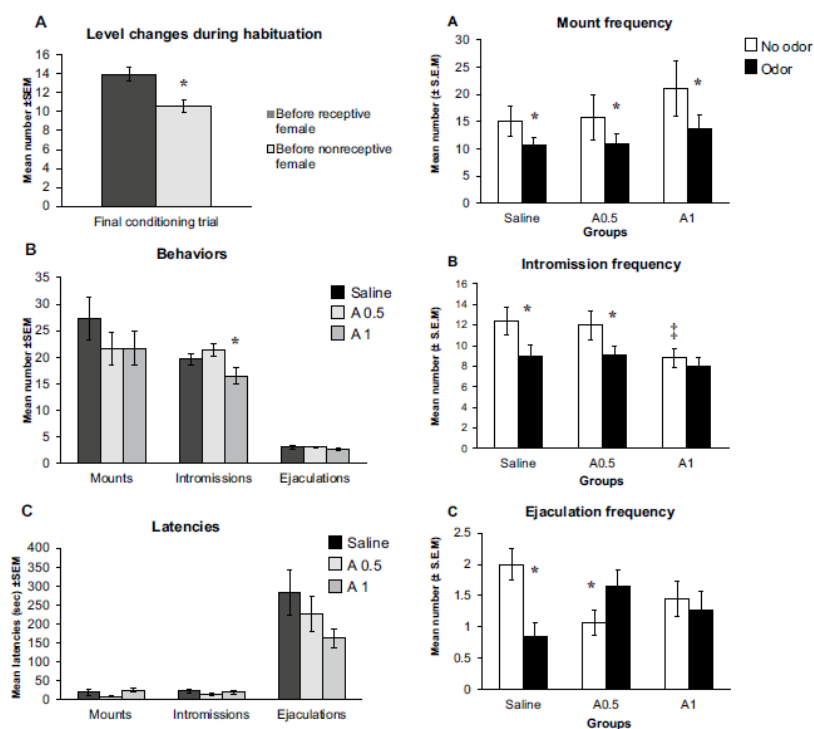
neurons in the SON and PVN that are an integral part of the neurochemical mechanism underlying CPaP and mate-guarding. Although not yet tested directly on mate guarding, this effect is similar to that of blocking opioid reward at MORs.

*Conditioned inhibition and disinhibition.* In the study by Kippin et al. (1998), copulation with unscented receptive females and almond scented nonreceptive females (i.e., in the explicitly unpaired group) led males to avoid copulation with scented receptive females on the final open field CEP test. This was an example of conditioned sexual inhibition (CSI) signaled by the odor, and likely accrued from the experience of nonreward through thwarted copulation (along with defensive behaviors on the part of the females). In a previous study (Pfaus & Pinel, 1989) virtually all male rats that had their first 10 multiejaculatory experiences in bilevel pacing chambers with unscented sexually receptive females attempted to mount sexually nonreceptive females the first time they were placed into the chamber. In that study, it took 5 alternating trials of access to sexually receptive and nonreceptive females, spaced 48 h apart, to get all males to learn not to mount nonreceptive females (**Figure 37**). Once learned (after trial 14), males were randomly assigned to two groups that received access to either receptive



**Figure 37:** Effect of sexual nonreward on male copulatory attempts (left) and alcohol disinhibition of the inhibitory state (right). Left: squares are trials with sexually receptive females; circles are trials with sexually nonreceptive females. Right: Noninhibitory test is with sexually receptive females. Inhibitory test is with sexually nonreceptive females.

females (Noninhibitory test) or nonreceptive females (Inhibitory test). Within each group, 3 subgroups were injected with saline, a low dose of alcohol, or a high dose of alcohol. In the Noninhibitory test, the low dose of alcohol had no effect on the percentage of males that mounted or ejaculated, whereas the high dose decreased these proportions significantly. In contrast, during the Inhibitory test, the low dose of alcohol disinhibited mounts and ejaculations (which occurred ex copula, often in mid-air) despite the females' defensive behaviors. Germé and Pfaus (2024) replicated this using the explicitly unpaired procedure of Kippin et al. (1998). Males were given a total of 20 conditioning trials at 2-day intervals, ten with an unscented receptive female and ten with an almond scented nonreceptive female. Immediately prior to the open field CSI test with two receptive females, one scented and the other unscented, males were injected with saline, or the same two doses of alcohol as in Pfaus and Pinel (1989). Males injected with saline displayed CSI and showed a CEP for the unscented female (**Figure 38**). In contrast, males injected with the low dose of alcohol had disinhibited CSI, with more males ejaculating with the scented female. A second study (Germé et al., 2025) found a similar effect of *d*-amphetamine.



**Figure 38: Conditioned sexual inhibition and disinhibition of ejaculation by alcohol.** Left: individual behaviors displayed by males in the drug groups on the final test. Right: percentages of males that mounted, intromitted and ejaculated with the scented or unscented females on the final test.

### **Cupid's epigenetics: Basic rules and mechanisms**

Findings from the experiments reported here give rise to some basic rules about the way the experience of sexual pleasure molds sexual motivation, arousal, and partner preference in the rat, and potentially other species, including humans.

Rule #1: The primacy of first experience(s) with sexual pleasure. An analogy from feeding: We cannot know the taste of chocolate until we taste it the first time. And, assuming it is sweet with the right texture and consistency, the brain tags it as a pleasurable experience which creates an expectancy of pleasure for subsequent experiences (Cabanac, 1992; Toates, 1986). This cannot be unlearned, although if all chocolate from then on was unsweetened, it would either be enjoyed differently as Mexican *molé* or be avoided due to extinction of pleasure and/or learned aversion to the bitter taste.

Sexual pleasure works the same way (Pfaus et al., 2012; 2020; Quintana et al., 2018a). First experiences of genital stimulation are generally pleasurable, though not truly *sexual* until puberty. Obviously, first sexual experiences are not always pleasurable in humans, but first experiences of sexual pleasure, by definition, are. Sexual pleasure comes in appetitive and consummatory forms, both of which are associated with genital and central arousal during solo and partnered sexual stimulation. Orgasms, or OLRs in other animals, are the zenith of both arousal and sexual pleasure. That phase of the sexual response cycle is a UCS that reinforces both the behaviors that led to it and salient features of the partner(s) associated with it. First experiences of the postorgasmic state lay the cornerstones of features in others (and in ourselves) that are found sexually attractive.

Rule #2: Opioids are the substrate of sexual pleasure. It is clear from the studies reported here that blocking opioid actions at opioid receptors blocks the ability of first experiences of sexual reward to condition sexual motivation, arousal, and partner preferences. Opioids likely control this by acting on MORs to create a state that reinforces both operant (response-reinforcer) and Pavlovian (stimulus-stimulus) contingencies in sexual circumstances. This state of the brain crystallizes partner related features that become attractive, a kind of “type” that is different for different individuals given different experiences. Component features congeal into a Gestalt, an organized whole that is beyond the sum of its parts, and that

comes to the foreground of attention amidst an array of possible partners. Opioid actions also result in an automation of sexual behaviors that lead to the opioid reward state. If this appears circular, it is. It is a basic principle of brain organization that allows all beings to identify and predict external causes for their internal states. In Pavlovian terms, the CS is a *representation* of the UCS. In cognitive parlance, the CS *primes* neural systems for the appropriate response. In our Fos studies, it is clear that the CS alone (odor, somatosensory stimulus) activates brain regions typically activated by CLS and VCS provided by paced copulation in females, or genital stimulation to ejaculation in males. Arousal also plays a key role in amplifying the reward state.

**Rule #3:** Stimuli associated with pleasure activate and sensitize mesolimbic and incertohypothalamic dopamine in the short-term and long-term, respectively. Both systems are critical substrates of focused attention, social recognition, and sexual desire or motivation. Opioids acting on MORs in both cell body and terminal regions sensitize dopamine systems through a molecular cascade that makes those neurons more sensitive to sexual incentive cues (see below). In turn, those cues activate attention, arousal, and desire with dopamine as the neural substrate.

**Rule #4:** Pleasure sensitizes oxytocin and vasopressin systems, which act as substrates for genital arousal, bonding, and partner recognition. Oxytocin, in particular, enhances genital arousal (Argiolas & Melis, 2013), and the conditioned activation of oxytocin neurons in the presence of a preferred partner or other sexual CS aligns partner preference with arousal. In this sense, the combination of oxytocin and dopamine shown in **Figure 7** focuses the activation of dopamine neurons in the VTA on partner- and sexual-related cues. The release of oxytocin at orgasm may also stimulate intimate closeness and cuddling behavior, keeping a bonded pair in relatively close proximity so that preferences can be continually reinforced. Vasopressin may have similar actions in monogamous males like prairie voles, but also in rats to act as a fast vasopressor to increase blood volume, cardiac output, and arterial pressure in the presence of a preferred sex partner. In humans, this could very well underlie the feeling of one's heart "skipping a beat" in the presence of a desired partner.

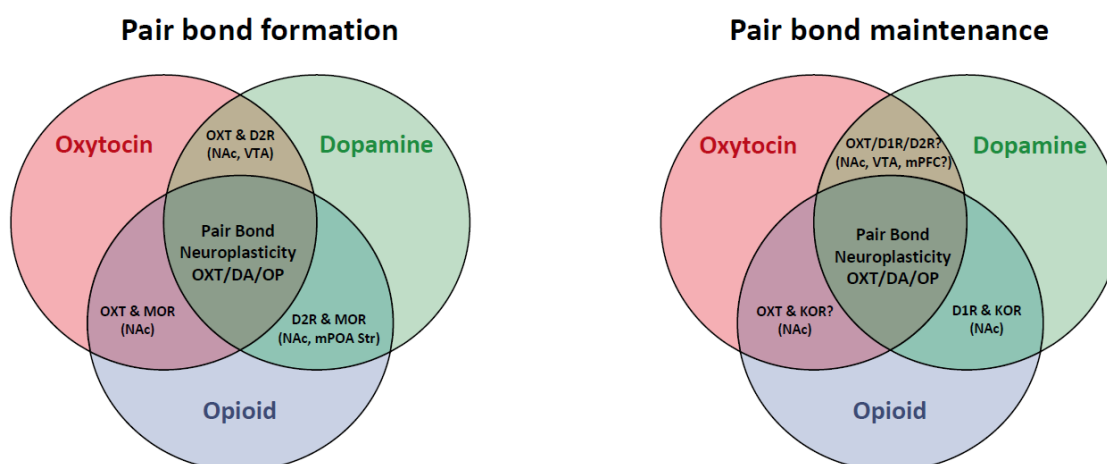
**Rule #5:** Blocking epigenetic mechanisms like demethylation acts like blockade of reward. It is well established that brain plasticity involves a complex interplay of genetic,

epigenetic, and environmental influences that show organisms how the world works. This information is processed and learned; essentially crystallized into sensory habits that focus attention on cues that predict positive and negative reinforcement or punishment, and motor habits that move us from distal to proximal to interactive with reinforcers, or away from punishers. Some of the information is “prewired” as instinct (e.g., Tinbergen, 1951) and hardwired as fixed or variable action patterns (Schleidt, 1971; Wise, 1987). Examples include the interest displayed by sexually naïve male rats in estrous vaginal secretions and the lordosis posture displayed by sexually naïve female rats activated by the first male palpation of the flanks when they are in heat. But it is equally instinctual to learn and to imprint even incorrect stimuli that happen to be in the right place at the right critical time (e.g., Konrad Lorenz and his goslings). These “mistakes” are merely variations on a theme: enough goslings will hatch and follow their real mothers that the few following Lorenz simply did not matter to the species.

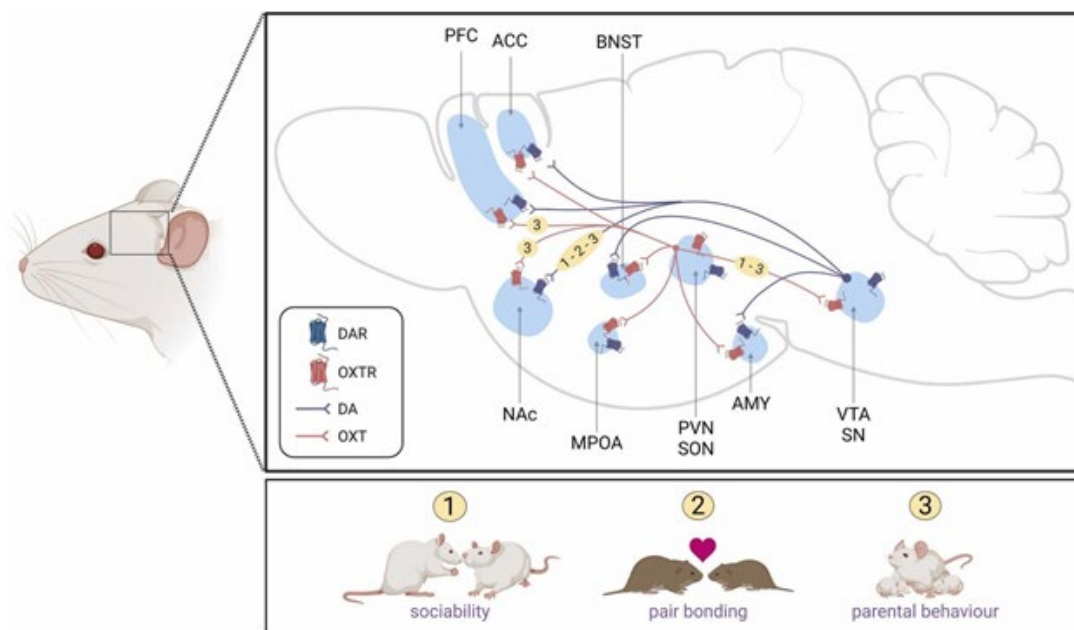
Reward coming from endogenous opioid actions that produce pleasure and euphoria, and the actions of serotonin that produce satiety and refractoriness, are critical components of the epigenetic changes in the brain that sculpt learning and memory in each individual. While the cytoarchitecture of the brain in each species is relatively fixed (Brodmann, 1909/2006; Butler & Hodos, 2005; Paxinos & Watson, 2013), the functional connections, neurochemical and intracellular signaling cascades, and molecular events that underlie them are shaped by experience. Blocking the experience of reward during first experiences of genital stimulation or copulation leads to conditioned inhibition of sexual behavior. This occurs by exposure to a condition of sexual nonreward (e.g., males exposed to nonreceptive females; females receiving clitoral stimulation in the presence of an inaccessible male behind a screen), by the blockade of MORs with naloxone or naltrexone, or by a more general blockade of the demethylase enzyme that blocks the ability of genes involved in sexual reward to be unraveled from histones during first sexual experiences.

*Mechanisms.* The activation of mesolimbic and incertohypothalamic dopamine systems, and oxytocin and vasopressin systems, occurs in the presence of stimuli with innate, prewired incentive salience. Salience itself is affected by sensory awareness in distal and proximal (interactive) space. For dopamine systems, stimuli are hierarchical based on the strength of

innate and dominant sensory systems: Humans have dominant visual and auditory sensory systems, whereas rats have dominant olfactory and auditory systems. This puts distal cues of those systems at the top of an inverted pyramid-shaped hierarchy. Olfactory cues in humans come next, with proprioceptive, tactile, and gustatory systems requiring proximal interaction with their respective cues. For rats, the requirement of proximal interaction would apply to visual, proprioceptive, tactile, and gustatory cues, since the rodent visual system is not as complex at the level of the lateral geniculate or primary visual cortex as that of humans (e.g., Meier & Reinagel, 2013; Priebe & McGee, 2014). It is a useful heuristic to think of stimuli or cues as we think of nouns: Person, place, or thing. Although mesolimbic and incertohypothalamic dopamine systems are activated by all three, the addition of oxytocin and vasopressin to the mix weights attention toward person cues, especially within interpersonal and social circumstances (Loth & Donaldson, 2020; Rappeneau & Castillo Díaz, 2024; **Figures 39 and 40**), and helps prepare the body for interaction. There is bidirectional feedback between brain dopamine and oxytocin systems at the level of the mPOA and PVN, and integration of them in the VTA and NAc (**Figure 7**). Both systems play somewhat redundant roles in controlling the autonomic nervous system, and relaying autonomic feedback to limbic and cortical structures (Festante et al., 2020; Thorner, 1975). Integration of these two systems underlies the formation and maintenance of pair bonds in monogamous prairie voles and is activated conditionally by stimuli associated with sexual reward in the rat (Quintana, Mac Cionnaith, & Pfaus, 2022).



**Figure 39:** Integration of oxytocin (OXT), dopamine receptor activation (D1, D2), and opioid receptor activation (MOR, KOR) in the formation and maintenance of pair bonds. After Loth & Donaldson (2020).



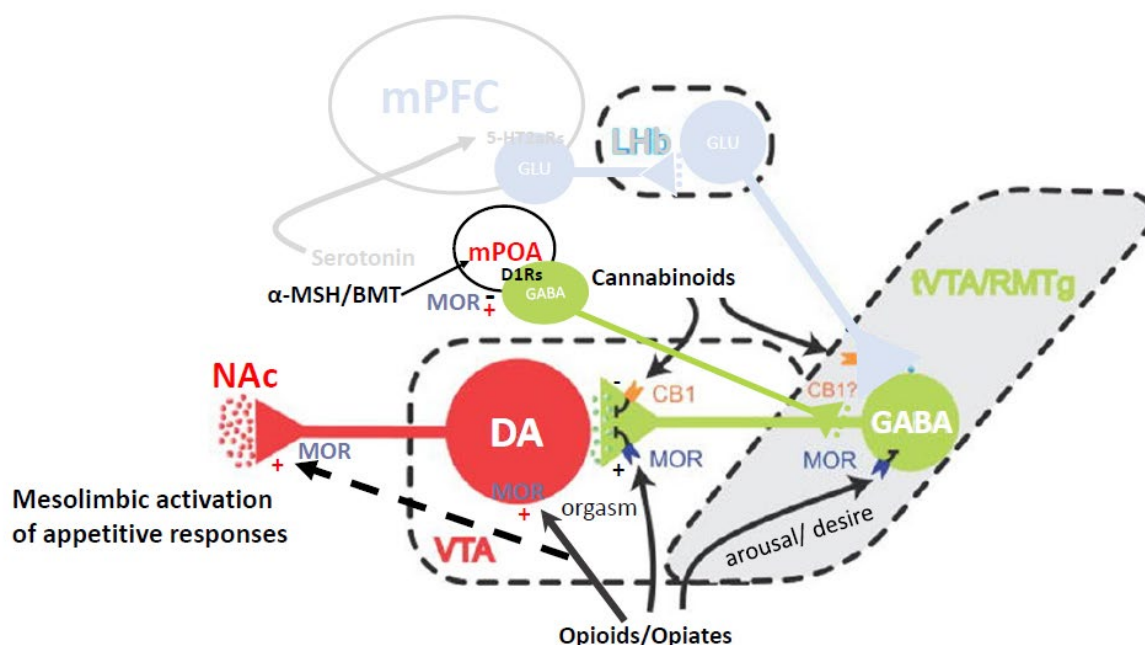
**Figure 40: Critical regions of integration of oxytocin (OXT) and dopamine (DA) receptor-mediated control of social bonding.** After Rappeneau & Castillo Díaz (2024).

Dopamine and oxytocin neurons are activated, inhibited, but also sensitized by opioids acting on MORs (Herz, 1998; Nisbett, 2024; Valentino & Volkow, 2018; Vanderschuren & Kalivas, 2000). For mesolimbic dopamine, activation by opioids occurs through disinhibition of dopamine neurons in the VTA. Dopamine neurons there are under tonic inhibitory control by GABA interneurons (**Figure 41**), which are activated by glutamate at NMDA receptors. Presynaptic MOR binding on presynaptic glutamate terminals inhibits cAMP as a second messenger and opens K<sup>+</sup> channels to hyperpolarize the membranes. This reduces the activation of GABA interneurons and leads to phasic firing of dopamine neurons. MORs are also found on the cell bodies of GABA interneurons, and opioid actions there further reduce their activation, leading to tonic firing of dopamine neurons. The net result is a phasic, then tonic, increase in dopamine release in mesolimbic and mesostriatal terminal regions (Jalabert et al., 2011; Margolis et al., 2014; McGovern et al., 2023), as was observed in the NAc of male rats during appetitive and consummatory phases of copulation, respectively (Blackburn, Pfaus, & Phillips, 1992; Pfaus et al., 1990). An identical phase-related release of dopamine was also observed in the mPOA, suggesting that dopamine neurons in the Zona Incerta are similarly organized.

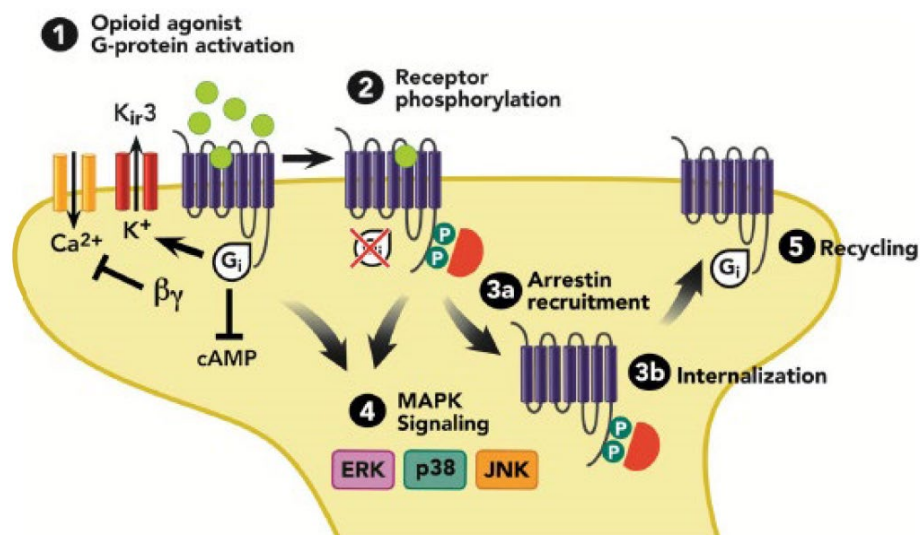


Activation of MORs on dopamine neurons leads to their inhibition, and sums with inhibitory inputs driven by serotonin during and after ejaculation in male rats. The result, as mentioned above, is a precipitous drop in extracellular dopamine concentrations in the NAc and mPOA, but not the dorsal striatum, immediately after ejaculation (detected with both microdialysis and voltammetry).

It is at this point that the “magic” begins during first experiences with sexual reward. Opioids like  $\beta$ -endorphin and enkephalins released at ejaculation or during OLRs bind to MORs on dopamine neurons. MORs activate Gi proteins that inhibit cAMP cascades and  $\text{Ca}^{2+}$  channels, and open  $\text{K}^{+}$  channels which hyperpolarizes the cell membrane (Figure 42). But agonist-bound MOR internalization also activates MAP kinases like ERK, JNK, and p38. Antagonists of ERK or p38 inhibit the development of CPP for amphetamine or cocaine and



**Figure 41: MOR actions in the VTA on sexual behavior.** Glutamate (GLU) inputs from diverse regions of the brain drive inhibitory GABA interneurons that tonically inhibit dopamine (DA) cell bodies. Inhibition of GLU and then GABA neurons drives phasic and tonic dopamine activity in dopamine neurons, leading to phasic or tonic dopamine release in terminal regions. Massive opioid release at ejaculation/orgasm spills over to MORs on dopamine cell bodies, leading to a precipitous decline in dopamine release. Activation of MORs in the NAc also contributes to the decreased release, as do serotonin-driven inputs to the VTA that underlie the continued inhibition during the refractory period. A similar set of receptor-mediated events appears to occur in the incertohypothalamic dopamine system.



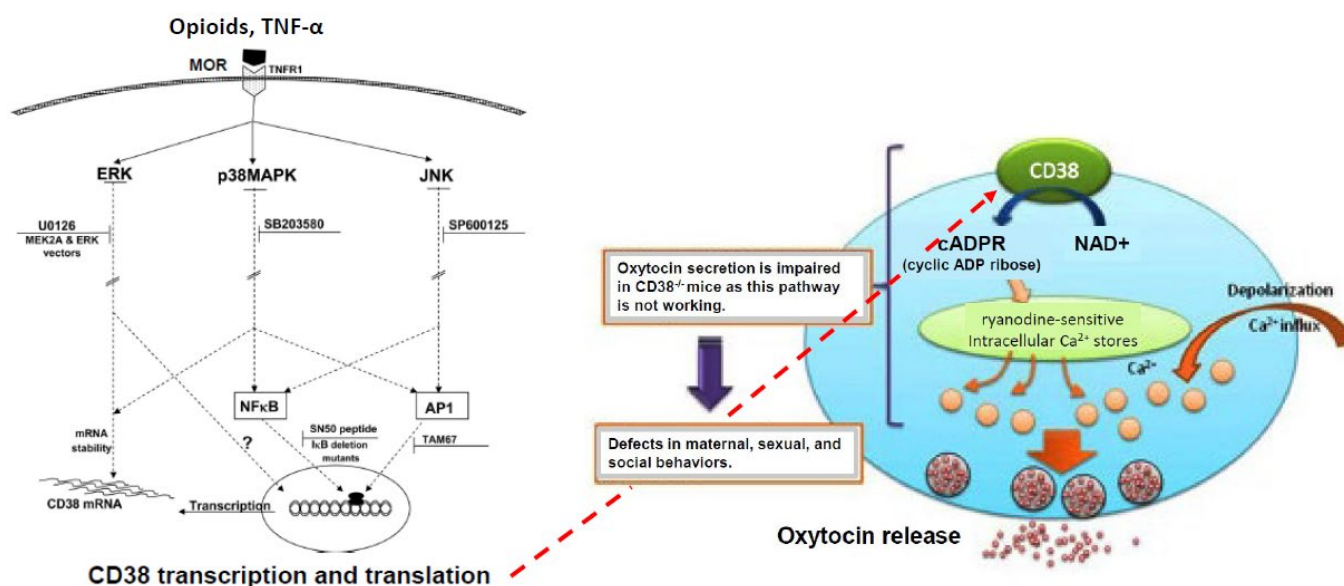
**Figure 42: Second messenger events related to agonist activation of MORs.** Figure describes the process of receptor binding, internalization, and recycling. 1) Agonist binding to MOR. 2) Receptor phosphorylation. 3)  $\beta$ -arrestin recruitment and receptor internalization. 4) Activation of MAP kinase signaling. 5) Receptor recycling.

prevent synaptic reorganization and long-term potentiation (LTP) that regulates sensitized Ca<sup>2+</sup> influx (Dudek & Fields, 2001; Gerdjikov, Ross, & Beninger, 2004; Thomas & Huganir, 2004; Valjent et al., 2000).

Thus, the process of sensitization in dopamine neurons occurs while they are inhibited. Upon subsequent stimulation, dopamine release will occur faster in terminal regions, with more and more potentiation of Ca<sup>2+</sup> influx. This translates into a faster focusing of attention on incentive cues associated with reward. Increased dopamine transmission in terminal regions like the NAc also induces changes in dendritic morphology and molecular actions in medium spiny neurons that have both types of dopamine receptors and that form a necessary interface between motivation and motor function. The transcription factor  $\Delta$ FosB is upregulated by sexual experience in medium spiny neurons of the NAc (Hedges et al., 2009; Nestler, 2014), which upregulates a number of genes related to second messenger function, including Ca<sup>2+</sup>-calmodulin kinases like CaMKII that are important for neurotransmitter release.

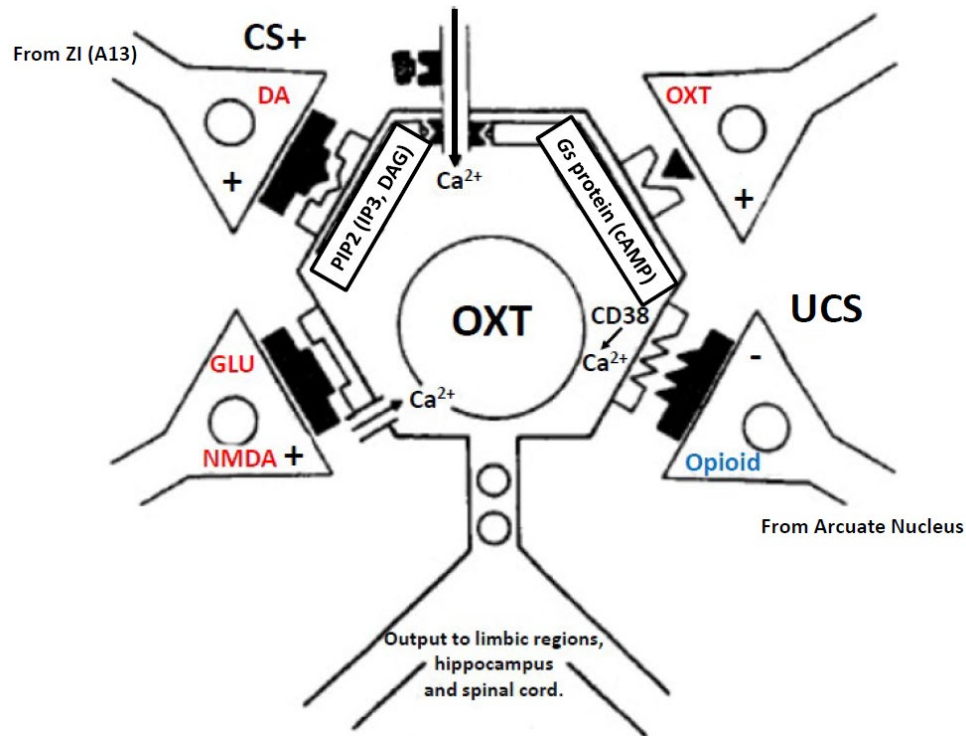
Sensitization also occurs as a function of MOR binding and internalization in oxytocin neurons. MAPKs activate the transcription of genes, one of which is the Cluster of Differentiation 38 (CD38) gene, a cyclic ADP ribose hydrolase that forms cyclic ADP ribose and nicotinic acid adenine dinucleotide phosphate (NAADP) which, similar to inositol triphosphate

(IP3), causes the release of  $\text{Ca}^{2+}$  from intracellular stores (**Figure 43**). This action is critical for oxytocin release (Higashida, 2016; Kim et al., 2016). Diminished CD38 gene transcription figures in autism-spectrum disorders (Peñagarikano, 2017; Zhang et al., 2017) and CD38 knockout mice show impaired object- and social-recognition memory (Kim et al., 2016). Thus, sexual reward



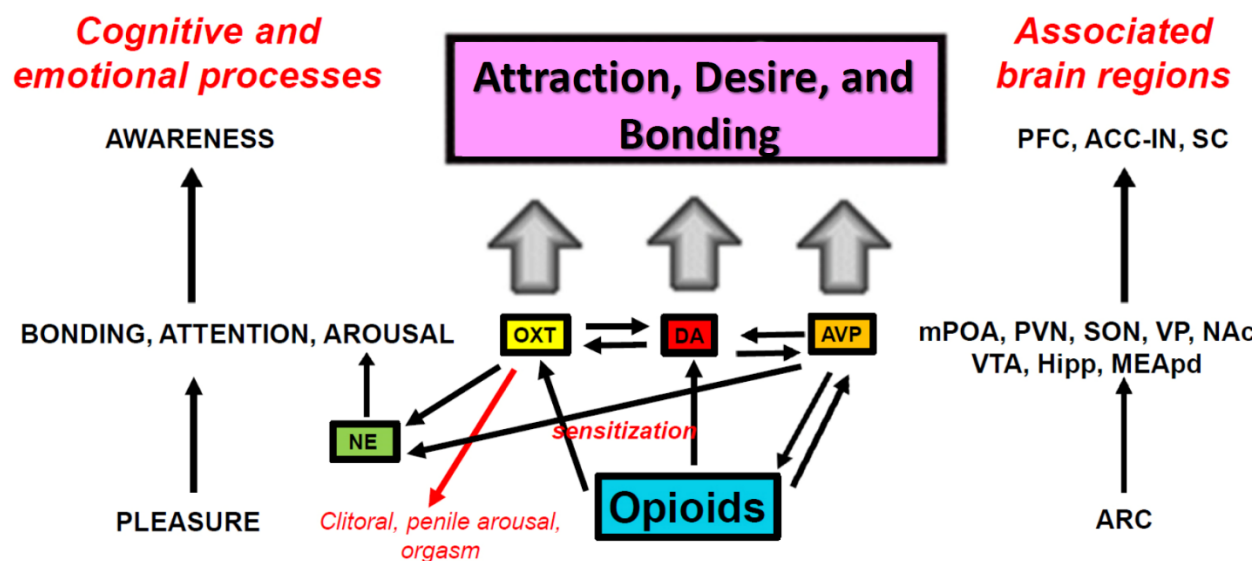
**Figure 43:** CD38 gene transcription by MOR activation of MAPKs in oxytocin neurons. After Higashida (2016).

and pleasure driven by MOR actions in oxytocin neurons augments the synthesis of CD38, which in turn sensitizes oxytocin release. And what activates oxytocin neurons? In addition to direct input to the PVN from the main olfactory pathway (Figure 6), the Incerthypothalamic dopamine pathway also sends efferents to the PVN that activate oxytocin neurons, as do outputs from the mPOA (**Figure 7**). This creates a molecular Hebbian circuit in oxytocin neurons in which opioids (UCS) sensitize oxytocin release through CD38 activation, and dopamine activated by conditioned cues and binding to D2 receptors (CS) stimulates release through the activation of phosphatidylinositol second messengers. (**Figure 44**).



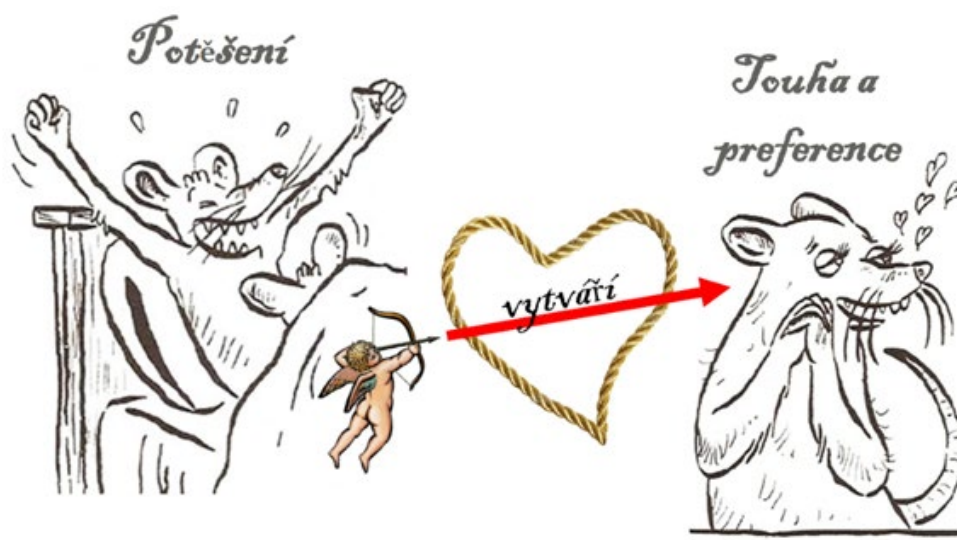
**Figure 44: Proposed molecular Hebbian circuit within oxytocin (OXT) neurons by which opioid activation of MORs and dopamine activation of D2 receptors augments oxytocin release.** Glutamate stimulated by the main olfactory system acting through NMDA receptors is also capable of activating this system, as is oxytocin itself. From Quintana, Mac Cionnaith, and Pfaus (2022).

Put into the perspective of functional pathways (**Figure 45**), opioid activation by sexual reward is the foundation of both monogamous pair bonding and conditioned partner preference. It rewards ongoing copulatory behavior and sensitizes dopamine and oxytocin mechanisms necessary for appetitive sexual motivation directed at preferred partners. Pleasure sensitizes mechanisms of bonding, attention, and arousal by actions on oxytocin, dopamine, and possibly vasopressin neurons that also activate peripheral genital arousal through autonomic control of sympathetic and parasympathetic outflow. This comes into conscious awareness by actions in the prefrontal cortex, anterior cingulate-insula complex, and somatosensory cortex. Thus, pleasure creates desire and preference, whether it is through an action on innately upregulated oxytocin or vasopressin systems (as in monogamous prairie voles) or through Pavlovian conditioning (in rats). The primacy of first experiences of pleasure



**Figure 45:** Neural mechanisms by which sexual pleasure and reward activate bonding, attention, and arousal to create motivation (desire), attraction, and bonding. Left: cognitive and emotional processes. Right: associated brain regions. ACC-IN = anterior cingulate-insula complex; ARC: arcuate nucleus. Hipp: hippocampus. NAc: nucleus accumbens (ventral striatum). MEApd: medial amygdala, posterior-dorsal region. mPOA: medial preoptic area. PFC: prefrontal cortex. PVN: paraventricular nucleus of the hypothalamus. SC: somatosensory cortex. SON: supraoptic nucleus of the hypothalamus. VP: ventral pallidum. VTA: ventral tegmental area.

tag salient incentive stimuli associated with those experiences as important and preferred. In turn, those stimuli grab attention and induce arousal because, in the words of Krafft-Ebing (1886/1929), “they awaken mighty associations with sexual pleasure.”



The path of Cupid's arrow

### Relationship of sexual reward to reproduction

Partner preference and mate choice are often thought to be guided by innately desired characteristics of fitness, like health, strength, resource acquisition, and reproductive capacity (fecundity, fertility). These are inferred from external stimuli like ornaments, nests (e.g., bowerbirds), calls, courtship displays, etc. (e.g., Halliday, 1983; Henshaw, Fromhage, & Jones, 2022), which have been selected for. But reproductive success is not always guaranteed, and it is not the case that alpha males inseminate all females (e.g., for Japanese macaques (*Macaca fuscata*); Takahata et al., 1999). Even in monogamous pair-bonded species like prairie voles, extra-pair paternity is common (Keane, Stevens, & Solomon, 2024). In many animals, internal physiological processes along with appropriate sexual behaviors contribute to successful fertilization. For example, in females that copulate with many males, sperm competition becomes a competitive variable (Coria-Avila et al., 2004). For males that copulate with many females, lower sperm content in each successive ejaculation becomes a competitive variable. And dominant female rats seem to “know” this: They take early ejaculations from males before removing themselves from the copulation game. Early ejaculations produce more male offspring in the litter, which increases the chances that females have been partially androgenized by sharing the fetal blood supply. In turn, this increases the likelihood of those female pups being dominant adults in the next generation (McClintock, 1984).

In contrast, Darwin (1871) clearly regarded mate preferences as being about aesthetics and beauty (p. 254), composites that may or may not reflect fitness and reproductive success for each individual (Prum, 2012). This raises the possibility that other factors are at work to generate partner preferences and reproductive success. In addition to epigamic characteristics, selection has conferred on many, if not all, species a reproductive system that requires proper stimulation. In rats, males require accessory olfactory stimulation from estrous secretions, tactile stimulation of the penis from intromissions, and the act of mounting with proprioceptive stimulation from pelvic thrusting, to ejaculate. But, as sexual behavior becomes more automated with practice, only pelvic thrusting seems to matter, as complete penile anesthesia or olfactory occlusion does not prevent it (Pfaus, Kippin, & Centeno, 2001). What also matters is that mounts with pelvic thrusting occur at an optimal rate. If females impose long inter-

intromission intervals, especially with defensive responses, male sexual arousal wanes. Likewise, for female rats, the initiation and rate of copulatory stimulation (CLS and VCS) need to be optimal. Females also need olfactory stimulation from the male, as olfactory occlusion dramatically reduces the display of solicitations in females primed fully with estradiol and progesterone (Afonso, Woehring, & Pfaus, 2006). When females in heat solicit and run away, they need to be chased by the solicited male. Lack of chasing (induced by haloperidol injections of the male) result in lower rates of solicitation and more agonistic confrontations with the male (e.g., boxing and kicking) prior to a runaway, perhaps as a supersollicitational behavior to get him to chase her (Pfaus, Smith, & Coopersmith, 1999).

Optimal rates of copulatory stimulation create sexual reward in both male and female rats (Martínez & Paredes, 2001) and lead to greater reproductive success in females. For example, Erskine (1985) had gonadally intact females in heat copulate with males in a unilevel pacing chamber with or without a 4-hole divider. Females received either 5 or 10 intromissions before ejaculation. In both cases, rates of pregnancy were between 15% and 20% in the chambers without the divider, but between 70% and 80% in the chambers with the divider. Likewise, in another study (Cibrian-Llenderal et al., 2010), females were given vibratory CLS at either massed or distributed intervals, or no CLS, prior to copulation with sexually vigorous males in small cylindrical chambers that do not allow proper pacing. Females in the distributed CLS group that received more than 9 intromissions before ejaculation showed an almost 70% pregnancy rate, relative to females that received massed CLS or no CLS (with pregnancy rates of less than 30% in those other groups). In contrast, none of the females that received less than 9 intromissions before ejaculation got pregnant regardless of the CLS priming.

In the study above by Coria-Avila et al. (2004), sperm competition was tested by allowing gonadally intact albino Wistar females in heat to mate with either albino Wistar or pigmented Long-Evans males, with the strain of male counterbalanced between first and second pairing. An experimental delay of 0 min, 5 min, and 10 min was imposed between the first ejaculation and pairing with the second male. Pigmentation of the offspring was used as the measure of paternity (either albino or partial pigmentation). Females that had the second male introduced immediately (0 min) after the first male's ejaculation sired offspring exclusively



by the second male. Females that had the second male introduced 5 or 10 min after the first male's ejaculation sired more offspring from the first male. Thus, after a delay of 5 min or more, first ejaculations are more likely to succeed in impregnation. Interestingly, in the strain conditioning study by Coria-Avila et al. (2006), it was noted that females regardless of strain stayed in the vicinity of the preferred male after receiving their first ejaculation from him for his entire postejaculatory refractory period (which can last 5 min or more).

The conditional activation of oxytocin neurons by cues associated with sexual reward is a potential mechanism that helps to assure reproductive success with preferred partners. This would occur by potentiating the coordination of sexual arousal, ejaculation, and the cervico-uterine reflex contractions that figure in sperm transport, in the presence of one's preferred partner or in the presence of cues associated with sexual reward. Indeed, oxytocin receptors have been found in the penis (Zhang et al., 2005) and in the glans and corpora of the clitoris and in the cervix (Mac Cionnaith et al., in preparation). In addition to potentiating sexual partner preference, peripheral administration of OXT abbreviates the duration of female rat sexual behavior by facilitating estrous termination (Mac Cionnaith et al., in preparation). Females that take themselves out of the mating context for over 5 min assure paternity for the last male's ejaculation (Coria-Avila et al., 2004). Doing this following the selective acceptance of ejaculations by the preferred familiar male sets up a behavioral system to assure paternity. Likewise, CEP with the same female would also be expected to facilitate paternity, especially if the male was also the female's preferred choice. This phenomenon of oxytocin coordination links ultimate causality with proximate causality, making successful reproduction more likely based on a prior history of sexual reward with the preferred partner

Thus, optimal rates of copulation lead to opioid-induced sexual reward, which in turn focuses partner preferences on cues associated with the reward state. In male rats, this creates a conditioned preference to ejaculate first and more times with familiar females bearing those cues. In female rats, this creates a conditioned preference to solicit copulation from, and receive first and sometimes several ejaculations from, their preferred familiar males. When reward is thwarted and the state of nonreward is associated with the same cue, males choose novel females without the cue whereas females either prefer the novel male without the cue



(e.g., Parada et al. 2011) or they show no preference, but display fewer solicitations and high intensity lordosis postures overall, and receive fewer intromissions and fewer ejaculations from either male, despite being fully primed with EB and P.

## Limitations

A reasonable question to ask is whether rats experience any of this type of learning in the wild. And the answer is that no one really knows. Female rats in seminatural laboratory environments show preferences for certain males, but it is unclear how those preferences form (Chu, Guarraci, & Ågmo, 2015; McClintock, 1984). The largest studies of the behavior of wild Brown rats (*Rattus norvegicus*) ever undertaken were led by American ethologist John B. Calhoun, first in the 1940s in Towson, MD, a suburb of Baltimore, in a 930 m<sup>2</sup> enclosure, and again in the 1950s in a large barn outside Rockville, MD. The now-classic publication from those studies revealed much about group mating behaviors and strategies, but did not discuss individual preferences except in the case of some homosexual behaviors shown by female rats and a few outcast male rats (Calhoun, 1962). But in all heterosexual mating cases, rats copulated in large groups, typically with one female pursued by several males that took turns. Likewise, a large study on conditioning and sexual behavior in the male albino lab rat (Larsson, 1956) described the automation of appetitive and consummatory sexual behaviors by different types of sexual experience, but nothing about the formation of partner preferences.

The laboratory apparatus in the studies reviewed here (unilevel and bilevel pacing chambers) were designed to provide rats with varying types of optimal sexual reward in the presence of partners bearing particular cues. The open field was used to examine the effect of those reward states on conditioned partner preferences in a proximal free choice situation where the animals could actually interact sexually. As with most studies in behavioral neuroscience, these are contrived situations. But what they reveal is an enormous flexibility in behavior (and therefore brain plasticity). Rats are able to adjust to many environmental circumstances and to optimize their behavior within those circumstances to achieve desired endpoints. For example, females in small testing chambers that do not allow them to pace in any optimal way use defensive responses to enforce desired intercopulatory intervals with

males. Likewise, McClintock's open field had large rocks and small plastic tubes which females would also use to delay copulating with males that were pursuing them. In bilevel chambers, females learn quickly to stay on the landing between levels if they do not want to be pursued (e.g., during estrus termination), as males cannot mount them on the landing. And in unilevel pacing chambers, females can simply stay on the side opposite the male's side and not re-enter. Thus, although it is impossible to know for sure if rats in the wild copulate under conditions of positive or negative reinforcement, the fact that they can adapt to entirely contrived situations means that plasticity has evolved to allow complex learning through experience, even about sexual behavior. This also means that "mistakes" will be made from a reproductive standpoint: animals will imprint sexually on nonreproductive stimuli if they are present during first experiences of sexual reward. But perhaps that is exactly what happens when animals imprint on ornaments through the same relationship with sexual reward.

Another potential limitation is our use of conditioning trials run at 4-day intervals. Although this interval approximates the 4-day estrous cycle of female rats, in the real world gonadally-intact females are likely to become pregnant after group mating, or at least pseudopregnant by the VCS they receive from multiple intromissions and ejaculations. Pseudopregnancy lasts approximately 12 days (the span of time it takes for the corpora lutea to regress and the placenta to be fully formed). When this does not happen, estradiol levels rise and the females go into heat 48 hr later. If pregnant, the female delivers pups 21 days after insemination, and spends another 21 days in the nest engaging in parental behaviors. Once the pups are weaned, the female's estrous cycle will begin again. This suggests that female rats may not spend more than a few cycles not pregnant or pseudopregnant. In fact, if females do not become pregnant or pseudopregnant, they become sensitized to their own estradiol, which activates more progesterone receptors in key regions of the mPOA and ventromedial hypothalamus the next time they come into heat (Jones, Gardner Gregory, & Pfaus, 2015). This makes them solicit more and show high intensity lordosis, making them of higher incentive value to males, which in turn increases the likelihood that they will be impregnated the next time they are in heat. Thus, gonadally-intact female rats are unlikely to copulate at 4-day intervals in the wild. Females in our laboratory situations are displaying an adaptive adjustment

to sex without pregnancy, which may alter the way that Pavlovian associations are made between sexual reward and incentive cues.

### **Implications for human sexual behavior**

Although human sexual behavior is best studied in humans, it is often impossible to do so directly for ethical, sociocultural, and scientific reasons. Understanding the impact of first experiences with sexual reward in humans therefore requires accurate recollection, something that can never be guaranteed. And, of course, such experiences cannot be intervened to advance science. At best, one is lucky to find populations that had different experiences, and use them in quasi-experimental designs.

The study of animal sexual behavior has helped fill many gaps in knowledge, for example, contributing to our understanding of neural, neurochemical, and hormonal systems that have been conserved in evolutionary history to control appetitive and consummatory sexual behaviors (Georgiadis, Kringelbach, & Pfaus, 2012; Pfaus et al., 2012). Common mechanisms of sexual arousal, desire, and reward exist in rodents and humans (Pfaus 2009), along with the ability to condition preferences for both reproductive and nonreproductive stimuli associated with sexual reward (Balon, 2016; Ford & Beach, 1951; Krafft-Ebing, 1886/1929). There is great diversity in human sexual preferences for positions, behaviors, and types of genital and erotic stimulation (Masters, Johnson, & Kolodny, 1986). There is even greater diversity in individual preferences for facial features, hair and eye color, and body types, that people find attractive (e.g., Havlíček et al., 2015), despite “norms” of beauty that differ by culture and even by generation within the same culture (Gabor, 1973). Thus, both “typical” and “atypical” sexual interests, paraphilias, fetishes, and even preferences for stimuli that might normally engender disgust, are modified by the same epigenetic mechanisms linked to first experiences of sexual reward. Base rates of paraphilic interests vary widely depending on the culture and the particular interest or activity (Joyal & Carpentier, 2017; Powls & Davies, 2012), but are generally higher in men than women, (although the percentages for women have been growing since the original studies by Kinsey and colleagues; Kinsey, Pomeroy, & Martin, 1948;

Kinsey et al., 1953). In the Czech population, base rates for at least one paraphilic interest are reported at 31% of men and 14% of women (Bártová et al., 2021).

Buss and Schmidt (1993) posited an “evolved psychological mechanism” to account for interests in non-fitness related stimuli, or, in another example, when “exotic becomes erotic” (Bem, 1996). It is likely that this mechanism is learning; experience with Pavlovian and operant contingencies that predict sexual reward, and crystallizing those experiences into individual patterns of sexual interest and behavior. This flexibility derives from brain plasticity, along with the generally chaotic choices that adolescents coming into their adult sexuality face. But this gives humans, and perhaps other species, an enormous amount of chance to recombine in different ways, yielding a higher degree of genetic diversity than if features and ornaments were preselected and stable. Such stimuli and behavior are likely to differ between individuals based on each person’s sexual experience. How excited an individual gets in anticipation of sex; how willing the individual is to work for sexual reinforcement; where an individual goes to find a sex partner; how vigorous the courtship, solicitation, and copulation; and which potential sex partner one actually mates with, are all determined by the counterbalancing of experience and conditioning on one hand and instinctual responses to unconditionally arousing stimuli on the other. But again, it also means that “mistakes will be made” from a reproductive standpoint. As with the limitations noted above for rats, the advent of birth control (from polished stones used as intrauterine devices (IUDs) by the ancient Bedouins, to the Roman ring pessary, to condoms, to the current incarnations of IUDs and hormonal birth control) has allowed women and men to delve into sexual behavior and reward without reproductive consequences. That sex for pleasure also augments reproductive processes underscores the kind of optimality and utility that natural rewards are believed to produce (Cabanac, 1992; Kahneman, 2000).

McKenna (2022) differentiated climax, a spinal sensory motor reflex, from orgasm, a brain phenomenon of pleasure and satiety. Climax is induced by sympathetic outflow that engages an epileptic-like motor response in the perineum and pelvic floor. Lateral spinothalamic neurons in the lower lumbar region of the cord induce this reflex, but also send information through the spinal cord up to the thalamus, which activates the orgasm circuit, resulting in opioid and serotonin release. Although it is possible to have orgasm without climax,

and climax without orgasm, the two typically co-occur. In men, pelvic floor contractions around the prostate induce ejaculation, which has a clear reproductive function. And, far from orgasm in women being some “vestige” of a male orgasm (Pfaus et al., 2016), the release of oxytocin at orgasm in women helps to generate cervical and uterine contractions that can aid in sperm transport (Shafik et al., 2005) and the doubling of daily prolactin secretion that is required for mammary gland development (Reuquen et al., 2017). There is also evidence that, during the ovulatory period in women, orgasms can augment luteinizing hormone release (Pfaus & Tsarski, 2022). This would aid in proper luteal function such as the maintenance of progesterone secretion from the corpora lutea during the first trimester. Thus, conditioned secretion of peripheral oxytocin could increase the likelihood of successful fertilization in pair-bonded heterosexual couples.

Naltrexone is currently used in the treatment of problematic paraphilias (particularly pedophilia), compulsive sexual behavior disorders, and other sexual offenses (Landgren et al., 2022; Ryback, 2004). Its efficacy likely stems from its ability to block orgasm-induced reward. However, it is not always effective given the treatment regimens (e.g., taken once per day in the morning; Raymond, Grant, & Coleman, 2010). Although longer acting than naloxone, the half-life of naltrexone in plasma is 3.9 to 10.3 hr depending on the route of administration (Crabtree, 1984). Even assuming a maximal time course, taking the drug in the morning means that it is essentially gone in the afternoon and evening, thus allowing orgasm during masturbation or actual sexual interactions to take place during that time. Our studies show that for the inhibitory conditioning to work, antagonism of MORs must occur during the sexual interaction, not long before or after.

### **Future directions**

Although much has been revealed from animal studies regarding the role of opioids in sexual reward states, the human literature has been speculative. Just as positron emission tomography (PET) can be used with the radioligand  $^{11}\text{C}$ -raclopride to examine dopamine transmission in humans, the radioligand  $^{11}\text{C}$ -carfentanil, which has high affinity for MORs, can be used with PET to quantify endogenous opioid transmission (Jern et al., 2023). Its use during

sexual stimulation and orgasm represents an important step forward in linking the animal and human literatures. However, this technique does not reveal MOR receptor internalization, which, as explained above, is a critical step in the activation of long-term changes to second messenger systems and epigenetic activation in neurons that possess MORs. To date, infusions of opioid antagonists to discrete brain regions have been used to determine whether opioid activation in a particular region contributes to the reward state. As reported above, microinfusions of naloxone to the mPOA and VTA have resulted in different effects on ejaculation-induced reward in male rats, with the mPOA mediating the reward value and the VTA mediating the sensitization of attention to reward related cues. Potential use of optogenetic techniques in Cre-mutant rats could stimulate opioid release by light in those regions to examine whether such release conditions place or partner preferences without copulation.

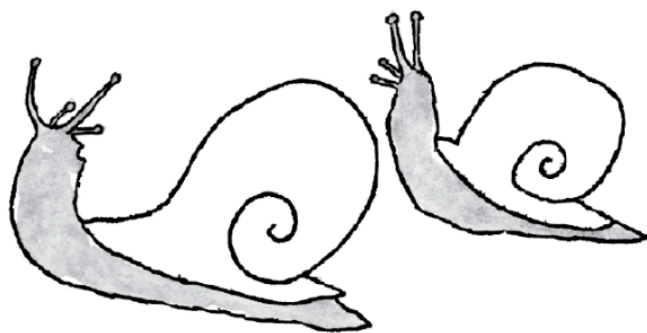
Whole-brain mapping of the sexual reward circuit in rats could be done using statistical analyses of Fos-IR, as has been done recently for pair-bonding in the prairie vole (Gustison, et al., 2024). Given the dependence of opioid reward on epigenetic changes that allow it to be linked by Pavlovian and operant contingencies to stimuli and particular sexual responses, respectively, it would also be of interest to examine whether genes associated with excitatory or inhibitory neurochemical systems are in methylated or demethylated states. Ideally this would be examined within terminal regions of the sexual and bonding brain circuit (e.g., PFC, NAc, CPu, mPOA, PVN, amygdala, hippocampus, and VTA) following conditioning. Genes of interest would include those related to dopamine synthesis (tyrosine hydroxylase), release (RIM1, ELKS2 and Bassoon proteins), reuptake (dopamine transporters 1, 2, and 3), dopamine DA1 and DA2 receptor families, oxytocin, oxytocin receptor, vasopressin, vasopressin receptors (V1AR, V1BR, and V2R),  $\alpha$ -melanocyte stimulating hormone and melanocortin receptors 3 and 4,  $\beta$ -endorphin, the 4 opioid receptors (MOR, DOR, KOR, and ORL-1), noradrenaline transporter, serotonin synthesis (tryptophan hydroxylase), transporters, NMDA glutamate receptor subunits, GABA A receptor subunits, and serotonin 1a, 2a, 2b, and 2c receptors.

Finally, we still know very little about the mechanisms of sexual refractoriness after orgasm(s) in humans (Levin, 2009), and especially if sex differences in orgasm quality and

number result in differences in opioid activation and level of sexual pleasure and how these might be related to refractoriness. However, because naloxone blocks the reinforcing effect of OLRs on place and partner preference in both female and male rats, similar mechanisms are likely activated. In addition, the neurochemical mechanisms of refractoriness in male rats have been studied in detail using the sexual exhaustion paradigm (Beach & Jordan, 1956), and the relative contribution of opioids, serotonin, dopamine, and endocannabinoids established (Fernández-Guasti & Rodríguez-Manzo, 1997; Garduño-Gutiérrez, León-Olea, & Rodríguez-Manzo, 2013; Guadarrama-Bazante & Rodríguez-Manzo, 2019; Rodríguez-Manzo & Fernández-Guasti, 1994; Rodríguez-Manzo & González-Morales, 2020). Understanding the mechanisms of sexual inhibition during refractory periods could help inform the treatment of problematic paraphilias and compulsive sexual behaviors, especially in men who engage in chronic masturbation and complain of “porn-induced erectile dysfunction.” Those same mechanisms may also be responsible for organic or drug-induced sexual arousal and desire disorders, delayed or absent orgasm, or anhedonic orgasm.

## Conclusions

“Cupid’s Epigenetics” refers to the neurochemical and molecular mechanisms by which opioid-mediated sexual reward or pleasure sensitizes cues and behaviors that predict it. Such sensitization requires the activation and augmentation of dopamine and oxytocin systems that focus attention on sexual incentives, increase sexual arousal and desire (motivation), and that reinforce appetitive behaviors that bring potential partners together and automate copulatory behaviors during consummatory sexual interaction. The net outcome is sexual imprinting; a preference for certain types of individuals, features, and even objects, along with certain behaviors, that are associated with sexual pleasure. Although this can lead in some individuals to a preference for nonreproductive sexual stimuli and behaviors, the more powerful effect is to promote reproduction in bonded individuals and maintain genetic and behavioral diversity overall in all species (e.g., Bagemihl, 1999; Lents, 2025). It is hoped that the experiments from my laboratory reviewed in this thesis help shed light on the phenomenon of sexual reward and the many ways it can shape sexual motivation and preference.



S. GROSS

*"I don't care if she is a tape dispenser. I love her!"*

*„Nezajímá mě, jestli je to dávkovač lepicí pásky. Miluju ji!“*



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**A.**

**Original review papers on conditioning and  
sexual behavior**



# Who, What, Where, When (and Maybe Even Why)? How the Experience of Sexual Reward Connects Sexual Desire, Preference, and Performance

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**Abstract** Although sexual behavior is controlled by hormonal and neurochemical actions in the brain, sexual experience induces a degree of plasticity that allows animals to form instrumental and Pavlovian associations that predict sexual outcomes, thereby directing the strength of sexual responding. This review describes how experience with sexual reward strengthens the development of sexual behavior and induces sexually-conditioned place and partner preferences in rats. In both male and female rats, early sexual experience with partners scented with a neutral or even noxious odor induces a preference for scented partners in subsequent choice tests. Those preferences can also be induced by injections of morphine or oxytocin paired with a male rat's first exposure to scented females, indicating that pharmacological activation of opioid or oxytocin receptors can “stand in” for the

sexual reward-related neurochemical processes normally activated by sexual stimulation. Conversely, conditioned place or partner preferences can be blocked by the opioid receptor antagonist naloxone. A somatosensory cue (a rodent jacket) paired with sexual reward comes to elicit sexual arousal in male rats, such that paired rats with the jacket off show dramatic copulatory deficits. We propose that endogenous opioid activation forms the basis of sexual reward, which also sensitizes hypothalamic and mesolimbic dopamine systems in the presence of cues that predict sexual reward. Those systems act to focus attention on, and activate goal-directed behavior toward, reward-related stimuli. Thus, a critical period exists during an individual's early sexual experience that creates a “love map” or Gestalt of features, movements, feelings, and interpersonal interactions associated with sexual reward.

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We have evolved a nervous system that acts in the interest of our gonads, and one attuned to the demands of reproductive competition.

—M. T. Ghiselin (1973)

Erotic fetishism makes an idol of physical or mental qualities of a person or even merely of objects...because they awaken mighty associations with...sexual pleasure.

—R. v. Krafft-Ebing (1886/1929)

The first cut is the deepest...

—Cat Stevens (1967)

## Introduction

Imagine yourself walking along a busy street when suddenly you smell something that reminds you of your very first love.



The odor is familiar, comforting, and despite the terrible adolescent angst that accompanied the breakup, it elicits a complete and happy memory of exciting experiences you shared. To another person, that smell may well rekindle horrible memories of abuse and pain. And to a third person, the smell has no meaning whatsoever. Now consider another scenario. You are at a bar with a good friend watching others and selecting those who are the sexiest. Although at first you both agree on some rudimentary symmetries and body types, the discussion quickly degenerates around particular features: you like brunettes while your friend likes blondes, you like almond-shaped eyes, while your friend likes round eyes, you like long faces while your friend likes round faces, you like a particular set of dimples while your friend cannot stand dimples, you like a particular size of breasts or pectorals, while your friend likes skinny. You like the one dressed in leather, while your friend likes the one in the business suit. It is even worse when the talk turns to personality characteristics. You like them loud, unbridled, and maybe a bit dangerous, while your friend likes them demure, soft-spoken, reliable, and boring. Eventually, you agree to disagree on who is hot and who is not.

If the ultimate drive for reproductive success causes us to select potential sex partners with epigamic characteristics that denote good genetic material, health, wealth, higher social status, and good potential parental investment (e.g., Buss, 1994), why would we ever have a difference of opinion? Why would we have “a billion wicked thoughts” (Ogas & Gaddam, 2011) instead of just a few that denote reproductive success? Why would we ever need to learn anything about, or from, sex? We should simply be drawn to men that are big and strong enough to tear the flesh off bears, and/or younger women with ample breasts and optimal waist-to-hip ratios, and then copulate as much as possible, preferably in the position(s) that provide the most effective stimulation for impregnation, and, of course, only when his sperm count is high and she is ovulating. But who are we to pick? Do we all possess the *same* degree of desired physical characteristics as those we want to mate with? Of course, the answer to that rhetorical question is no. Moreover, *mating* is clearly not the only reason we have sex and, in fact, may be second to a more proximate master: pleasure (with Tantric liberation coming a distant third). Indeed, sexual behavior in some species facilitates sociosexual goals; for example, brief female–female mounting in bonobos that promotes peaceful interactions among males (Furuichi, 2011; Hohmann & Fruth, 2000).

A nervous system that is prepared to copulate also needs to be flexible and learn strategies that maximize copulatory success in an ever-changing world. But what defines success? In order to have sex, we must first be able to respond to hormonal and neurochemical changes that signal our own sexual desire and arousal and distinguish it from other sympathetic activation, such as anxiety. This ability underlies our moment-to-moment level of sexual arousability (as conceived by Whalen, 1966) and defines a large part of the internal state that is commonly referred to as “sex drive.” Second, we must be able to make

sense of external cues that signal sexual attraction and receptivity in others (e.g., Salu, 2011). This ability requires a complex mix of instinct, learning, and feedback: a neural organization that allows for incentive-based motivation and expectancy (Bindra, 1968, 1974; Bolles, 1972; Toates, 1986). We must be able to identify external stimuli that predict where potential sex partners can be found, to seek out, solicit, court, or otherwise work to obtain sex partners, distinguish external cues and behavioral patterns of potential sex partners from those that are not sexually receptive, and to pursue sex partners once sexual contact has been made (Pfaus, Kippin, & Coria-Avila, 2003). In this way, we move in time from distal to proximal to interactive, with an ever-increasing load on sensory processing and motor sophistication as we interact more and more with a moving and sentient sensory stimulus.

Neural mechanisms also exist that allow sexual responding to become habitual or automated with practice and such processes may underlie the ability of desired features in others to be identified and responded to quickly (e.g., Ortigue & Bianchi-Demicheli, 2008) and of sexually experienced individuals to be less affected by treatments that disrupt sexual responding in sexually naïve individuals (e.g., Pfaus & Wilkins, 1995). Similarly, neural mechanisms exist that allow the stimulation received during sexual contact to be perceived as rewarding. Such reward alters subsequent behavior, for example, by contributing to the formation of preferences for salient stimuli associated with positive sexual reinforcement (Pfaus, Kippin, & Centeno, 2001), just as individuals develop preferences for stimuli associated with drug or food reward (Berridge, 2009; Robinson & Berridge, 2001). Many of these aspects of sexual responding go well beyond the traditional focus on copulation or genital reflexes/blood flow. Although some appetitive responses that individuals make prior to copulation are not specific to sexual behavior, they can be considered “sexual” if they are conditioned using sexual reward as the positive reinforcer (Pfaus, 1999). This is as true for appetitive bar pressing in male rats (e.g., Everitt, Fray, Kostarczyk, Taylor, & Stacey, 1987) as it is for giving flowers and remembering birthdays in men. Thus, we learn about *who* makes us feel desire, about *what* we like, and *where* we like it, and we learn what to do to get it.

It is becoming increasingly clear that there is a critical period of sexual behavior development that forms around an individual’s first experiences with sexual arousal and desire, masturbation, orgasm, and sexual intercourse itself. During this period, the sensory and motor mechanics of the behavior become integrated and crystallized along with the development of preferences for ideal activities and physical features of a partner. Such preferences often violate societal “norms” (e.g., as in the development of fetishes or paraphilias) and so-called “evolutionary laws” regarding features that represent genetic and reproductive strength, appearing more to be based on an egocentric evaluation of salient reward- or pleasure-related characteristics that differ from one individual to another. The formation of such experience-based

preferences can be found in historical texts, such as Stendhal's "Principle" in his work *De L'Amour* (1822), described explicitly in case histories, such as those in Krafft-Ebing's *Psychopathia Sexualis* (1886/1929), and more theoretically in the "love maps" proposed by Money (1986). This critical period may well form on the foundation laid by previous critical periods, especially those associated with attraction to other- or same-sex individuals, which itself may form on the foundation laid by a critical period for gender typical versus atypical behavior, and the sense of self as "female" or "male."

In this article, we propose a neurodevelopmental trajectory of sexuality that considers how innate predispositions are forged into successful sexual behaviors, expectations, and desires, based on an individual's experience with reward and capacity to link reward to cues that predict it. The integration of genetic and experiential factors in determining behavior is consistent with older ideas of the complementary nature of ultimate and proximate causality (Tinbergen, 1963) and new ideas concerning the role of epigenetic factors (e.g., Bale, 2011). A popular reading of genetic causality seems to infer that behaviors are fixed or hardwired in the brain, whereas learning infers that such brain mechanisms and the behaviors they underlie are flexible. We want to emphasize at the outset that even so-called "fixed action patterns" are subject to experience and that subsequent epigenetic changes in gene transcription and protein synthesis in neurons can alter the expression of behaviors differently in different individuals. Evolutionary pressures alter the costs and benefits of any behavior, and experience with reward (and possibly punishment) maintains the cost-benefit ratio. We note that this ratio may change in different environmental conditions, sometimes quickly and radically. Those who can learn to respond in the wake of sudden changes to a niche (e.g., a decrease in population density) will likely out-reproduce those who do not learn. Thus, ultimate and proximate causes of behavior are complementary. We posit that reward mechanisms sit in the middle of these two domains.

### Genetic and Hormonal Conditions and Predispositions

Sexual activity or partner preferences that stray from so-called "normal" are often attributed to genetic or hormonal differences. Such differences are believed to create different brains that process the world differently, or to generate physiological factors that predispose individuals to certain sexual dysfunctions, such as lifelong premature ejaculation (Waldinger, 2008) or erectile dysfunction (Bancroft & Janssen, 2000).

#### Biomarkers

A number of genetic and neural correlates of male homosexuality have been reported as "female" phenotypes (assumed to be due to either perinatal feminization and/or demasculinization). For example, relative to heterosexual men, gay men possess a smaller

female-like interstitial nucleus III of the anterior hypothalamus (LeVay, 1991), a larger female-like suprachiasmatic nucleus (Swaab, Gooren, & Hoffman, 1992), and in male-to-female transsexuals a smaller female-like central region of the bed nucleus of the stria terminalis (Zhou, Hofman, Gooren, & Swaab, 1995). Of course, it is not known whether those neuroanatomical differences actually contribute to the sexual orientation of the individual or whether they simply reflect an unrelated endpoint of some differentiated developmental process. Biomarkers have also been suggested for a distinct (and not putatively feminized or demasculinized) "gay" genetic makeup in the expression of the X-linked marker Xq28 (Hamer, Hu, Magnuson, Hu, & Patatucci, 1993), in the 2D:4D ratio of gay men relative to heterosexual men (e.g., Robinson & Manning, 2000), in a leftward directional handedness asymmetry (Martin, Puts, & Breedlove, 2008), hair-whorl direction (Rahman, Clarke, & Morera, 2009), visuospatial performance tasks (Collaer, Reimers, & Manning, 2007), and in the fraternal birth order effect (Blanchard, 2007; Blanchard & Lippa, 2007; Bogaert & Skorska, 2011; Rahman, 2005; Vasey & VanderLaan, 2007). Handedness and general IQ also appear as correlates in incarcerated male pedophiles (Cantor, Blanchard, Bobichaud, & Christensen, 2005; Cantor, Klassen et al., 2005), and cerebral white matter deficiencies in the superior fronto-occipital and right arcuate fasciculi have been found in incarcerated male pedophiles relative to an incarcerated non-pedophilic male population (Cantor et al., 2008), suggesting that frontal output (responsible for executive function and behavioral inhibition) of pedophilic individuals is compromised.

#### Sex Differences and Steroid Hormones

Another source of innate differences in sexual function involves the pattern of circulating steroid hormone levels that differ between the sexes. This occurs in two phases in mammals: (1) an "organizational" phase in which the male phenotype differentiates from the female "default" through the combined action of androgens and estrogens during a critical perinatal period that "sculpt" a male brain; and (2) during a subsequent "activational" phase in which gonadal steroid secretion acts on those differentiated brain regions to activate the neuroendocrine systems necessary for reproduction and the behaviors that will bring it about (Bonthuis et al., 2010; Lenz & McCarthy, 2010; Nelson, 2005). Thus, the brains of male mammals are differentiated from the female default by early hormone actions, which lead to different patterns of sexual responding in adulthood driven by two distinct steroid hormone secretion patterns, one continuous (male) and one cyclic (female).

The cyclic nature of estradiol, testosterone, and progesterone action in females leads to changes in sexual responding and increases in sexual arousal and desire around the time of ovulation in all vertebrate species, including humans (Slob, Ernste, & van der Werff ten Bosch, 1991; Stanislaw & Rice, 1988), although a smaller increase has been reported around the time of menstruation

(Singer & Singer, 1972). The hormonal milieu during the periovulatory follicular phase alters the way in which visual sexual stimuli are processed in women (Gizewski et al., 2006; Krug, Pihl, Fehm, & Born, 2000; Mass, Hölldorfer, Moll, Bauer, & Wolf, 2009; Rupp et al., 2009), which presumably leads to a shift in the incentive value of the stimuli. Analogous findings have been reported in our primate cousins, for example, in approaches and solicitations made around the time of the mid-cycle estradiol peak in rhesus macaques (Wallen, Winston, Gaventa, Davis-DaSilva, & Collins, 1984) and in the appetitive and consummatory sexual behaviors that characterize the periovulatory period of female rats (e.g., Beach, 1976; Erskine, 1989; McClintock, 1984; Pfaff, 1980, 1999; Pfaus et al., 2003). This contrasts with the relatively stable and continuous testicular androgen secretion in mammalian males (and its reduction to neural androgens or aromatization to neural estradiol in different regions of the brain) that maintains sexual arousability and responsiveness in a relatively continuous manner (Hull & Dominguez, 2007).

### Biological Differences in Mating Strategy

Although a number of innate differences in mating strategies between species have been described, perhaps the most studied is the partner preference displayed by monogamous prairie voles relative to promiscuous meadow or montane voles (Lim, Hammock, & Young, 2004; Lim & Young, 2004; Winslow, Hastings, Carter, Harbaugh, & Insel, 1993; Witt, Carter, & Walton, 1990; Young & Wang, 2004). Differences in functional vasopressin and oxytocin receptor densities have been described between these two species of vole (Insel, Winslow, Wang, Young, & Hulihan, 1995) and, in particular, greater vasopressin 1A receptor density in the ventral pallidum (a motor output region that contributes to goal-directed behavior) of monogamous prairie voles compared to promiscuous voles (Lim, Hammock et al., 2004). Indeed, overexpression of this receptor in meadow voles by injection of an adenoviral vector that contains the whole receptor gene resulted in male meadow voles developing a significant partner preference for the first female they mate with (Lim, Wang et al., 2004). These data suggest strongly that a genetic difference in receptor density for those two neuropeptides results in a predisposition toward social recognition, affiliation, and pair-bonding, which, in turn, underlies the display of particular reproductive strategies in a species.

### Temperament and Response Style

Finally, what is called “temperament” is believed to reflect innate differences in brain function that start in childhood and lead to individual differences in sensory processing and motor response styles. This results in stable personality traits such as extroversion, introversion, impulsivity, and inhibition (Kagan, 1994). One of the first classification schemes was that of the ancient “humoral

doctrine” of Hippocrates (460–370 BCE). This doctrine described four human personality traits (sanguine, choleric, melancholic, and phlegmatic) that allegedly explained both individual and group differences in responding. Sexually speaking, someone who is sanguine and prone to humor and passion would likely process sexual stimulation more readily than the calm phlegmatic person who is prone to sluggishness. That latter individual may require more sensory stimulation to achieve a requisite level of arousal for sexual responding and may gravitate to highly arousing, naughty, even violent sex play. Likewise, an introverted individual may have less sexual experience than an extroverted individual based simply on exposure to social situations and concomitant availability of potential sex partners. The two may possess different sexual attitudes and different levels of sexual arousal and desire (Meston & Buss, 2007). Indeed, it is possible that whatever the genetic bases of temperament are also produce differences in the activation patterns of neurochemical systems that excite or inhibit sexual behavior (Pfaus, 2009). However, introversion in women is correlated with a history of childhood sexual abuse (Talbot et al., 2000), making it difficult to know what comes first, response style or particular sexual experiences that may reinforce response style. Differences in temperament can also change how drugs affect sexual behavior (Pfaus, Wilkins, et al., 2010), with one person’s “aphrodisiac” producing a loss of sexual function in another.

In summary, innate genetic predispositions are assumed to underlie different sexual responses and partner preferences. Those differences may form generally around different hormone actions that sexually differentiate the brain and induce a differential activation of excitatory or inhibitory neurochemical systems, which leads to specific types of sensory processing and/or motor function. Together, they create individual brains with different sexual phenotypes and proneness to sexual excitation and inhibition, either as a spontaneous “drive” or in the presence of incentive sexual stimuli. Such differences are believed to underlie sexual orientation in humans and different species-specific mating strategies, such as monogamy or polygamy.

### Associative Conditions

Another innate characteristic of brains is that they learn and remember. Brain function (and, therefore, behavior) is modified to a large extent by experience. The ability to form operant associations (Skinner, 1938) between motor actions (responses) and their consequences (reinforcers) and Pavlovian associations (Pavlov, 1927) between a neutral external cue that serves as a conditioned stimulus (CS) and different emotional states like pleasure, reward, or aversion, induced by an unconditioned stimulus (UCS), require a brain capable of experience-based synaptic plasticity, as in the formation of Hebbian circuits and neural networks that sensitize neural responses to stimuli (Hebb, 1949; Johnson,

LeDoux, & Doyère, 2009). There are a number of molecular events that shape such plasticity. These include: (1) the activation of growth factors that direct the formation of new synapses; (2) augmentation of intracellular events, such as transmitter receptor proliferation and the availability of second messengers linked to receptors; and (3) epigenetic factors, including DNA methylation or histone acetylation of genes to suppress or enhance the generation of proteins they code for that ultimately facilitate or inhibit individual responses that comprise the behavior. All three processes are stimulated and sensitized during phases of neuronal activation following the first encounter with a new sensory stimulus (see, e.g., McEwen, 2010). The emotional state induced by the consequences of that encounter feed back to strengthen or inhibit the likelihood of future encounters. Because the molecular protein synthetic events that subserve those phenomena take time, along with a degree of repeated experience with the conditions, the final form of the response accrues across a “learning curve” that can be defined individually or for a group of individuals exposed to the same stimuli and consequences. Regardless of the species, all neurons store the memory of salient events in this way (Kandel, 2001). Thus, activation of the network itself by a formerly “neutral” CS associated with it generates, as Pavlov said, a “representation of the UCS” which serves to predict, activate, and prime the emotional state. An incentive motivational account of Pavlovian conditioning (e.g., Bindra, 1968) has the CS activating a “central motivational state,” which moves the body in the direction of the goal (e.g., activates appetitive responses) and prepares it viscerally to act on the goal (e.g., salivation in response to a CS that predicts food; erection in response to a CS that predicts sexual gratification).

As with Pavlovian conditioning, a motor response that results in the arrival of a needed or preferred stimulus becomes strengthened, such that doing it on its own after it is learned can be reinforcing to a certain extent. Motor patterns also become automated with repeated activation, as in the development of “habits” or “routines” that require very little cognitive loading to be engaged once they are formed (Hull, 1943; Tolman, 1932). This also makes them extremely difficult to break without an extensive program of extinction and/or alternate reinforcement and, even then, spontaneous recovery can occur, showing the primacy of early relative to subsequent experiences. Thus, associative conditioning creates a brain in which the ends “justify” the means and in which the cues that predict the ends become conditioned incentives. To a brain that generates some degree of self-awareness (as in humans), conditioning results in circular reasoning and tautologies. When do we ever thank our bodies and ourselves for our pleasurable responses to salient erotic stimulation? Instead, we ascribe their valence and intensity to our sex partner(s), as in the phrase “you make me feel so good.” Of course, when such stimulation loses its arousing potential, as often happens when it is repeated over and over again the same way (and at intervals that produce tolerance, rather than sensitization), we tend to blame that loss on our sex partners no longer arousing us as they once did.

## Setting the Stage

If we were creating a sexually reproductive universe, would we make sexual responses as “innate” as possible to assure that everyone would do the same thing at the right time with presumably the most optimal mate(s)? After all, like a male black widow spider or praying mantis, we may get only one crack at it. Yet, anyone who has had the opportunity to watch utterly inept sexually naïve male rats try to copulate no doubt wondered why there are so many rats. Like language, sexual desire, preference, and performance must be learned and crystallized before they appear as a relatively “automatic” set of species-specific responses. Essentially, genetic factors and early hormonal differentiation help to set the stage upon which experience creates the play. But the actors have to rehearse before the play can be performed properly. The sexual brain must have feedback systems that coordinate, facilitate, and crystallize the learning of appropriate and competent sexual responses. Those systems must link together the activation of both sympathetic and parasympathetic divisions of the autonomic nervous system by desired sexual stimuli with appetitive sexual behaviors directed at those stimuli and drive them into a coordinated set of consummatory (e.g., copulatory) responses. What kind of feedback is required and how can this be studied empirically?

Obviously, human sexual behavior is best studied in humans, but it is impossible to control the conditions under which humans have their first sexual experiences. Far too much occurs randomly and chaotically despite the best-laid plans. Therefore, we depend on animal models in which different aspects of sexual experience can be controlled methodically (for comprehensive reviews, see Ågmo & Ellingsen, 2003; Pfaus et al., 2003). Sexual behavior in animals, like that of humans, can be divided along appetitive and consummatory lines (Pfaus, 1999; Pfaus, Damsma, et al., 1990) and can be studied with respect to the stimuli animals are given to respond to and the motor patterns that become strengthened and automated with repeated sexual experience. Although the behavioral structure of sexual responding may be the same for all vertebrates, it is true that laboratory rats are not humans. Drawing conclusions from one species and applying it to another must be done with caution and an eye toward predictions made from one species about the functions of another, e.g., mechanisms underlying erection in males or sexual solicitations in females (Beach, 1950; Pfaus et al., 2003). We do not know if rats experience “orgasm” as we do, although at another level of analysis they clearly experience a sexual reward state that augments their responsiveness to cues associated with it.

## Learning What to Do

In his seminal analysis of conditioning and copulatory behavior in male rats, Larsson (1956) demonstrated learning curves for baseline rates of mounts, intromissions, and ejaculations in male rats. Baseline rates were typically achieved in 3–5 trials to ejaculation



and were faster if males achieved more ejaculations in their initial trials. Notably, sexually naïve males typically took a long time to sniff and lick the female's anogenital region before mounting whereas some males never copulated at all despite repeated exposure to females and their progressively intense attempts to solicit copulation. Some sexually sluggish or non-copulating males (referred to colloquially as “duds” relative to the “studs” that copulate) could be stimulated to mount if unavoidable, low-level footshock, short-term pain (e.g., tail-pinch), or neutral stimuli paired with them were applied (Barfield & Sachs, 1968; Caggiula, 1972; Crowley, Poplaw, & Ward, 1973). Such stimuli also reduced the number of intromissions required for ejaculation in sexually active males (Beach & Fowler, 1959; Sachs, Macaione, & Fegy, 1974).

In other experiments, repeated exposure of the males to the copulation testing chambers for several days prior to their first exposure to the female increased the proportion that mounted to 100%, suggesting that the stress of a novel environment may preclude copulation in sensitive males (Pfaus & Wilkins, 1995). Endogenous opioids are activated in response to novelty stress (Izquierdo & McGaugh, 1987) and may disrupt copulation in sexually naïve males by  $\mu$  receptor activation in an important hypothalamic region known as the medial preoptic area (mPOA) (Hughes, Everitt, & Herbert, 1990; Matuszewich & Dornan, 1992). Administration of the opioid receptor antagonist naloxone to sexually naïve males increased the proportion of males that copulated on their first test in a manner identical to that of preexposure to the testing chamber (Pfaus & Wilkins, 1995). In fact, naloxone had no effect in that study if males had been preexposed. In addition to genetic reactivity to novelty stress, sexually sluggish males were found to have decreased nuclear estrogen receptor in the mPOA relative to sexually active males (Clark, Davis, & Roy, 1985), suggesting a genetic factor that would either fail to activate excitatory pathways, or fail to disinhibit them in the presence of stressful inhibitory external cues.

### Crystallization

Learning the appropriate set of appetitive responses is crucial to gaining access to sex partners and learning how to copulate properly is crucial to successful reproduction. Both types of responses become “crystallized” in an animal's behavioral repertoire. Many examples of this exist in the literature. In rats, those behaviors include performance in obstruction boxes (Jenkins, 1928; Moss, 1924; Stone, Barker, & Tomlin, 1935; Warner, 1927), straight-alley running (Beach & Jordan, 1956; Sheffield, Wulff, & Backer, 1951; Ware, 1968), maze learning (Drewett, 1973; Eliasson & Meyerson, 1975; Hetta & Meyerson, 1978; Kagan, 1955; Meyerson & Lindstrom, 1973; Warner et al., 1991; Whalen, 1961), crossing of electrified grids (Moss, 1924), nose-pokes and other attempts to “get to” a potential sex partner behind a wire-mesh screen (Damsma, Pfaus, Wenkster, Phillips, & Fibiger, 1992; Pfaus, Damsma, Wenkster, & Fibiger,

1995; Pfaus, Mendelson, & Phillips, 1990), digging through sand (Anderson, 1938), bar-pressing for a sex partner (Beck, 1971, 1974, 1978; Beck & Chmielewska, 1976; French, Fitzpatrick, & Law, 1972; Jowaisas, Taylor, Dewsbury, & Malagodi, 1971; Larsson, 1956; Sachs et al., 1974; Schwartz, 1956) or for a cue light associated with the arrival of a sex partner (Everitt, 1990; Everitt & Stacey, 1987; Everitt et al., 1987), and psychomotor stimulation in anticipation of the arrival of a sex partner (Mendelson & Pfaus, 1989). To gain access to receptive females, male guinea pigs learn to run an alley (Seward & Seward, 1940), male pigeons learn to peck keys (Gilbertson, 1975), and male stickleback fish learn to swim through rings (Sevenster, 1973). Male songbirds learn to sing particular songs that attract females (MacDougall-Shackleton, 2009; Mooney, 2009).

Larsson (1956) also found that, once established, individual patterns of copulatory behavior appear stable and relatively resistant to environmental change. Similarly, Pattij et al. (2005) demonstrated stable individual differences in ejaculatory latency in male rats that resembled those found in humans using the stopwatch method to determine intravaginal ejaculatory latency (e.g., Walddinger, McIntosh, & Schweitzer, 2009), and could be assigned statistically to groups of rapid, normal, and delayed ejaculators. In fact, such assignment predicted certain drug effects, such as the facilitation of ejaculation by the presynaptic 5-HT<sub>1a</sub> agonist drug 8-OH-DPAT (which occurred in normal and delayed ejaculators, but not in rapid ejaculators). However, the early conditions under which copulatory patterns are established can alter the way that they appear in subsequent tests. For example, Larsson discovered an “enforced interval effect” (EIE) in which males that had the receptive female pulled out of the test arena for durations of up to 1 min would ejaculate with progressively fewer intromissions. Once established, this effect would last despite the fact that males had free access to females. However, although males given early experience with free copulation showed an EIE within a copulatory session, they would resume their “normal” pattern under conditions of free copulation.

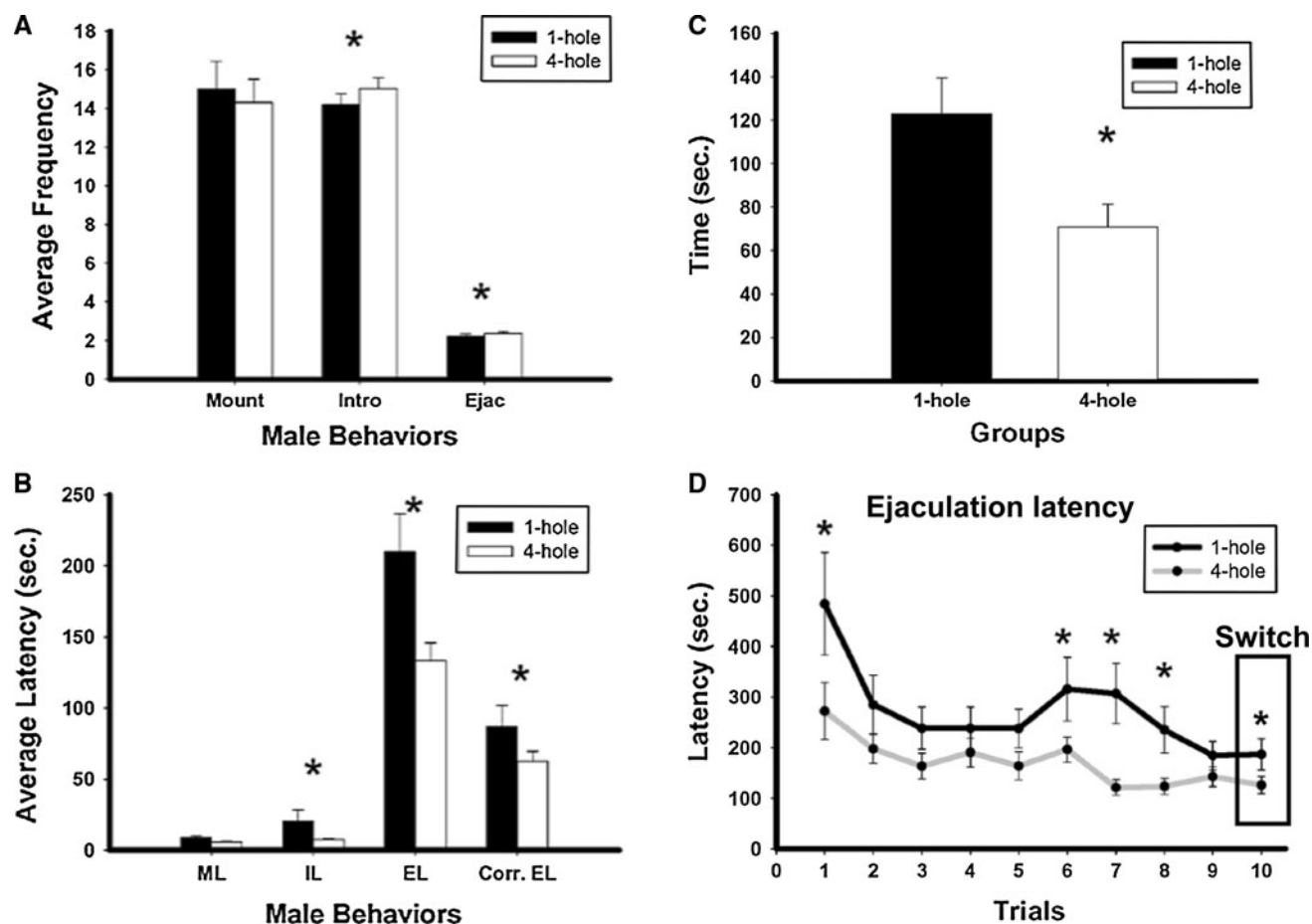
In an elegant and well-controlled extension of the EIE, Silberberg and Adler (1974) trained sexually naïve male rats to copulate in three different conditions. Control males were allowed to copulate freely with sexually receptive females for 30 min on each of 20 tests of sexual behavior. Experimental males were allowed to achieve seven intromissions before the receptive female was removed during each test. A third group was yoked in time to the experimental group, but not in terms of a particular number of intromissions prior to the female being removed. A large and significant proportion of males in the experimental group consistently achieved ejaculation prior to the 7th intromission relative to males in the other groups. Intromissions thus could be interpreted as “operants” that brought about ejaculation and the intromission pattern (along with the ejaculation latency) could come under external control.

To examine the development of particular patterns of sexual responding, we have used unilevel pacing chambers with two

types of Plexiglas divider, one with four holes and another with one hole, that separate the chamber into two equal sides. The holes are small so that only the female can cross from one side to the other. This sequesters the male to one side and allows the female to control or “pace” the initiation and rate of copulation. However, this creates two distinctive pacing conditions, one in which the female has free access to the male (4-hole) and one in which she has relatively restricted access (1-hole) given that the male typically puts his head in the a hole (presumably trying to get to the other side). Of course, from the male’s perspective, the female is always available in the 4-hole condition whereas he has to wait longer in the 1-hole condition. Male rats given their first nine trials of sexual activity in one of the two pacing conditions developed significantly different patterns of copulatory activity (Ismail, Zhao, & Pfaus, 2008). Males trained in the 4-hole condition developed faster ejaculation latencies and fewer intromissions before ejaculation relative to males trained in the 1-hole condition (Fig. 1). Notably, when males in

each group were switched to the other condition on the 10th trial, they maintained their previously-established ejaculatory pattern, despite the difference in condition.

Beach (1956) proposed that two separate but interactive sexual mechanisms existed in male rats, an arousal mechanism (AM) and a copulatory mechanism (CM). The AM integrated distal olfactory, auditory, and visual cues from receptive females. When the cue strength became sufficiently intense, the AM activated the CM to initiate copulatory responding (mounts and intromissions). The CM then integrated tactile stimulation from the penis with each vaginal intromission, leading eventually to ejaculation and the highly stereotyped post-ejaculatory refractory period. Given that sexually naïve males can show preferences for receptive over non-receptive females and that they are responsive to sex odors from receptive females, the first unconditioned reinforcer in the cascade of sexual reinforcers would be sex odors. The goal would be getting to them. In Beach’s terms, this would be the primary function of the AM.



**Fig. 1** Development of male rat sexual behavior in 1-hole vs. 4-hole unilevel pacing conditions. **a** Average frequencies of mounts, intromissions, and ejaculations during the nine conditioning trials in the two pacing conditions. **b** Average mount, intromission, and ejaculation latencies during the nine conditioning trials in the two pacing conditions.

**c** Average time (s) the females spent away from males during the nine conditioning trials in the two pacing conditions. **d** Ejaculation latencies in the two pacing conditions across the nine conditioning trials and during the 10th test when the males were switched into the other pacing condition

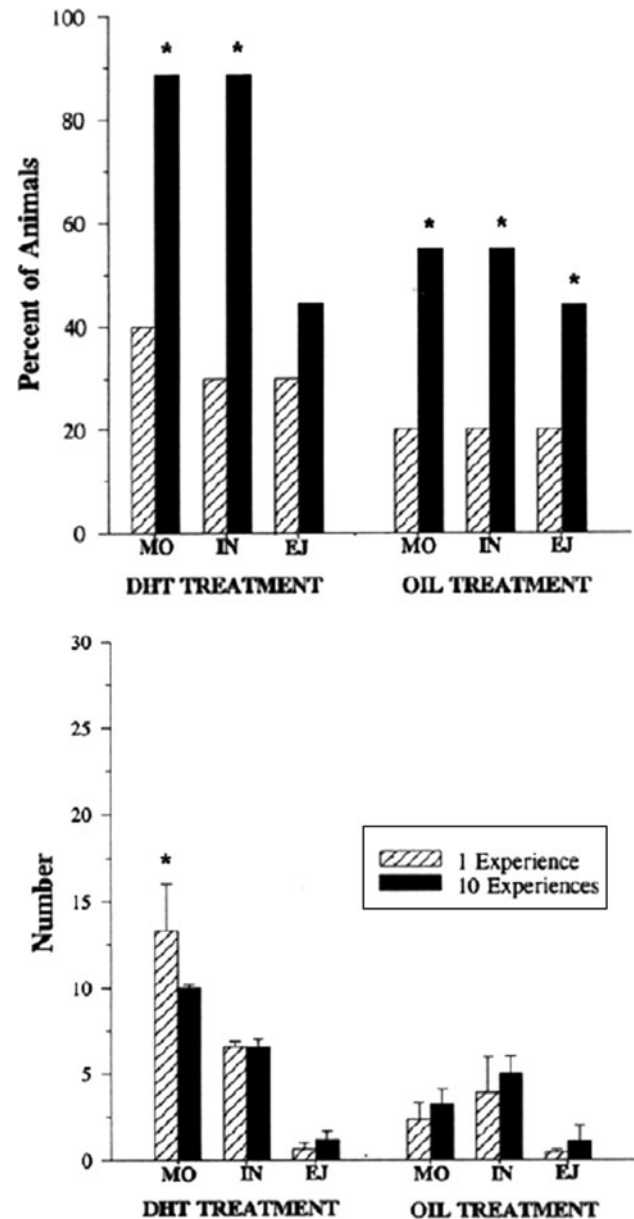
Whalen (1961) asked what the necessary stimulation must be for the development of copulatory behavior. The answer was penile stimulation. Whalen varied whether males achieved mounts without intromission, intromissions without ejaculation, or intromissions with ejaculation with sexually receptive females. On a final test, males were allowed to copulate to ejaculation with receptive females. Many rats that achieved only mounts during their sexual experience trials did not copulate whereas rats that achieved intromissions with or without ejaculation were able to copulate to ejaculation normally. Thus, exposure to sex odors alone was not sufficient to crystallize patterns of copulation; sensory feedback from penile stimulation was necessary. This makes penile stimulation a second goal or reinforcer in the cascade (and the first of the CM).

Whalen also asked whether mounts with or without intromission could augment the ability of male rats to learn to run a T maze and find a sexually receptive female. Males with 1 or 4 mounts alone, or 1 or 4 intromissions, both scored higher than chance (choosing the side with the receptive female more often than the non-receptive female). However, experience with intromissions produced a significantly higher percentage of “correct” responses, indicating that tactile stimulation of the penis summed with mounts (and other close interaction with the female during training) to augment the learning. López, Olster, and Ettenberg (1999) asked whether sexually naïve rats would run faster in a straight-arm runway if their prior copulatory experience was intromissions with or without ejaculation. Only rats that achieved ejaculation showed faster running times. Thus, it would seem that a cascade of reinforcing events, from perception of sex odors to chasing receptive females to penile stimulation during mounting to ejaculation, is necessary for the normal display of appetitive and consummatory sexual responses.

### Resistance to Disruption

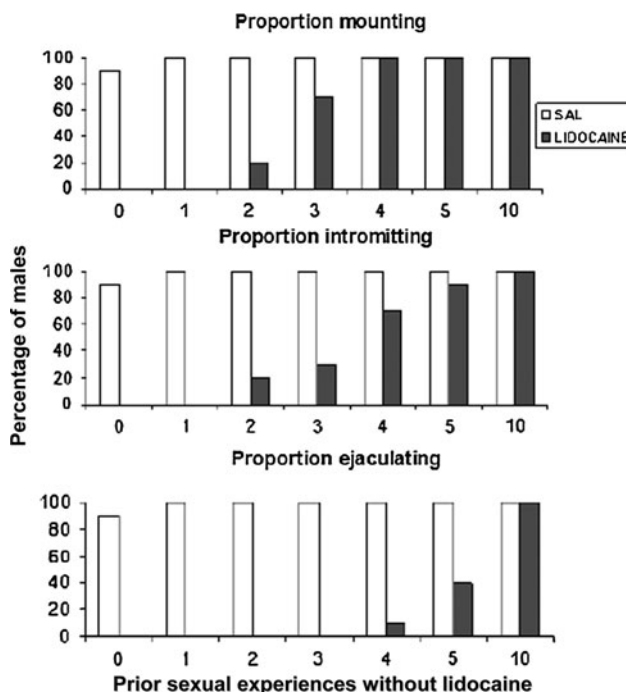
Sexual experience has profound effects on physiology and makes animals relatively resistant to treatments that disrupt sexual behavior. For example, compared with sexually inexperienced males, sexually experienced males have larger testes (Drori & Folman, 1964), heavier penises (Herz, Folman, & Drori, 1969), lighter body weights (Siegel, Nunez, & Wade, 1981), and increased secretions from accessory sex glands (Drori & Folman, 1964). Sexual experience has also been shown to ameliorate the disruptive effects of anosmia (Thor & Flannelly, 1977), castration (Lisk & Heiman, 1980; Fig. 2), penile deafferentation (Lodder, 1975), penile anesthesia with lidocaine (Fig. 3), and age (Gray, Smith, Dorsa, & Davidson, 1981). Gonadally intact, sexually experienced males prefer the odors of receptive females over those of non-receptive females whereas sexually naïve or castrated males do not display a significant preference (Carr, Loeb, & Dissinger, 1965; Carr, Loeb, & Wyllie, 1966).

We have shown that sexually naïve males are susceptible to the disruptive effects of novelty stress on copulation whereas



**Fig. 2** Effects of castration with or without dihydrotestosterone treatment on consummatory sexual behaviors of male rats. *Top* Proportion of males displaying mounts, intromissions, and ejaculations a month after castration. *Bottom* Frequencies of mounts, intromissions, and ejaculations a month after castration. Males were given either 1 or 10 trials of sexual behavior to one ejaculation each prior to castration. \* $p < .05$  compared to oil-treated controls

males with 10 prior multi-ejaculatory sexual experiences are not (Pfaus & Wilkins, 1995). Although both sexually naïve and experienced males that were placed into a novel testing apparatus displayed behaviors consistent with a fear state (e.g., freezing followed by exploration near the walls of the chamber), the sexually experienced males responded immediately to the introduction of a sexually receptive female with the initiation of copulation whereas most of the sexually naïve males ignored the receptive female for a long period of time, and, as noted above,



**Fig. 3** Effect of the topical anesthetic lidocaine (5% topical cream) or saline applied to the penis of male rats on the proportion of males that mount, intromit, and ejaculate as a function of prior sexual experience. Males received either 1, 2, 3, 4, 5, or 10 prior experiences to one ejaculation before the application of lidocaine or saline. For percentages of 60 or lower,  $p < .05$

some sexually naïve males did not initiate copulation, although novelty-induced opioid transmission in certain regions of the brain leads to an endogenous state in which copulatory behavior is suppressed, the induction of this state in males with sexual experience was not sufficient to suppress copulation once the female was placed into the chamber.

### Learned Inhibition

Sometimes animal have to learn what *not* to do. Sexually experienced male rats learn not to attempt copulation with sexually non-receptive females. This was shown when males that had 10 prior multiejaculatory trials at 4-day intervals with females were presented with a sexually non-receptive female (Pfaus & Pinel, 1989). All males attempted to mount those females despite their attempts being thwarted aggressively by the females' defensive behaviors. When males were presented sequentially with sexually non-receptive and receptive females in alternating trials, the males learned not to mount the non-receptive females over the course of approximately five trials while maintaining high rates of sexual responding on trials with sexually receptive females. Interestingly, a low dose of alcohol increased the proportion of males that attempted to mount the non-receptive females in a subsequent test, showing that alcohol possesses disinhibitory properties only if animals were inhibited in the first place.

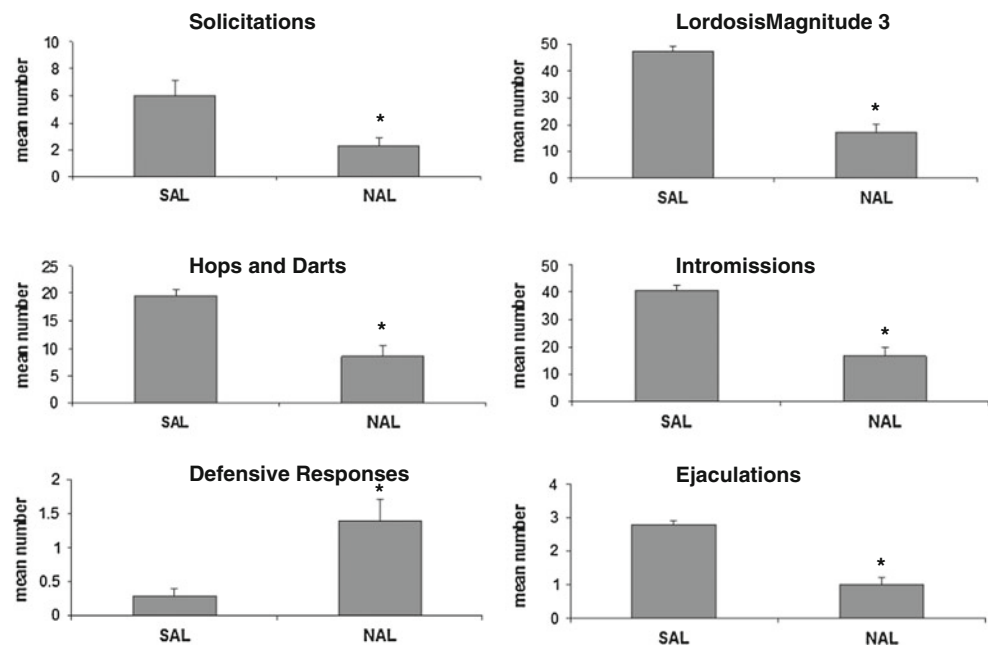
When presented with a sexually inactive male rat, sexually receptive and experienced females will solicit the males with increasing vigor before mounting them repeatedly (Afonso & Pfaus, 2006; Beach, 1968). This “female–male mounting” (FMM) behavior was displayed only when the gonadally-intact females were sexually receptive, when ovariectomized (OVX) females were primed with estrogen, and was terminated abruptly if the males finally “got it” and mounted back. Thus, it is regarded as a “super-solicitation” behavior. Importantly, FMM is displayed by sexually naïve females presented with a castrated, sexually inactive male on their first exposure to males. However, if the males never mounted back (as the long-term castrates do not), the FMM behavior diminished over five successive trials (Afonso, Bablekis, & Pfaus, 2006). It is as if the females learned that this behavior does not successfully stimulate the males to mount them, although it could be restored if the males mounted intermittently. Fully receptive female rats also learned to suppress both appetitive and consummatory aspects of sexual behavior if they are paired with an explicitly nonrewarding state. We have created such a state with injections of the opioid receptor antagonist drug naloxone. Females were given their first six experiences of copulation with sexually vigorous males at 4-day intervals in unilevel pacing chambers either under the influence of naloxone or saline. On the 7th test, all rats were injected with saline. Females that had experienced sex without opioid reward showed dramatic reductions in solicitations, hops and darts, lordosis, and increased defensive responses compared to control rats and received fewer intromissions and ejaculations from the males (Fig. 4). As the half-life of naloxone is approximately 1–2 h in plasma (Fishman, Roffwarg, & Hellman, 1973), it is highly improbable that the reduction was due to a long-term or sustained action of naloxone itself. It is more likely that it stemmed from an expectancy of nonreward during sex.

### Neural Correlates

Whereas critical periods of perinatal and pubertal steroid hormone action alter brain structure and function within hypothalamic, limbic, and cortical regions associated with sexual arousal and desire, experience with salient sexual stimulation (and reward) also changes or refines brain structure and neurochemical function (Pfaus, 2009). For example, whole-brain endorphin content increases significantly in male rats following repeated ejaculations (Szechtman, Hershkowitz, & Simantov, 1981). Copulation of sexually naïve rats to one ejaculation results in sensitized dopamine release and greater induction of the immediate-early gene product Fos (a marker of neuronal activation) in the nucleus accumbens (NAc) in response to a female behind a screen, relative to males allowed to intromit only during their first experience or that remain sexually naïve (Biały & Kaczmarek, 1996; Bradley & Meisel, 2001; López & Ettenberg, 2002). Males allowed 10 multiejaculatory experiences with sexually receptive females have increased numbers of dendritic spines (and therefore synapses) in the NAc relative to males allowed one multiejaculatory experience. Males



**Fig. 4** Effects of acquiring sexual experience under the influence of saline or naloxone (5 mg/kg, ip) on appetitive and consummatory sexual behaviors in OVX female rats primed fully with estradiol and progesterone. Females received six multiejaculatory experiences at 4-day intervals prior to the final test in which all rats received an injection of saline



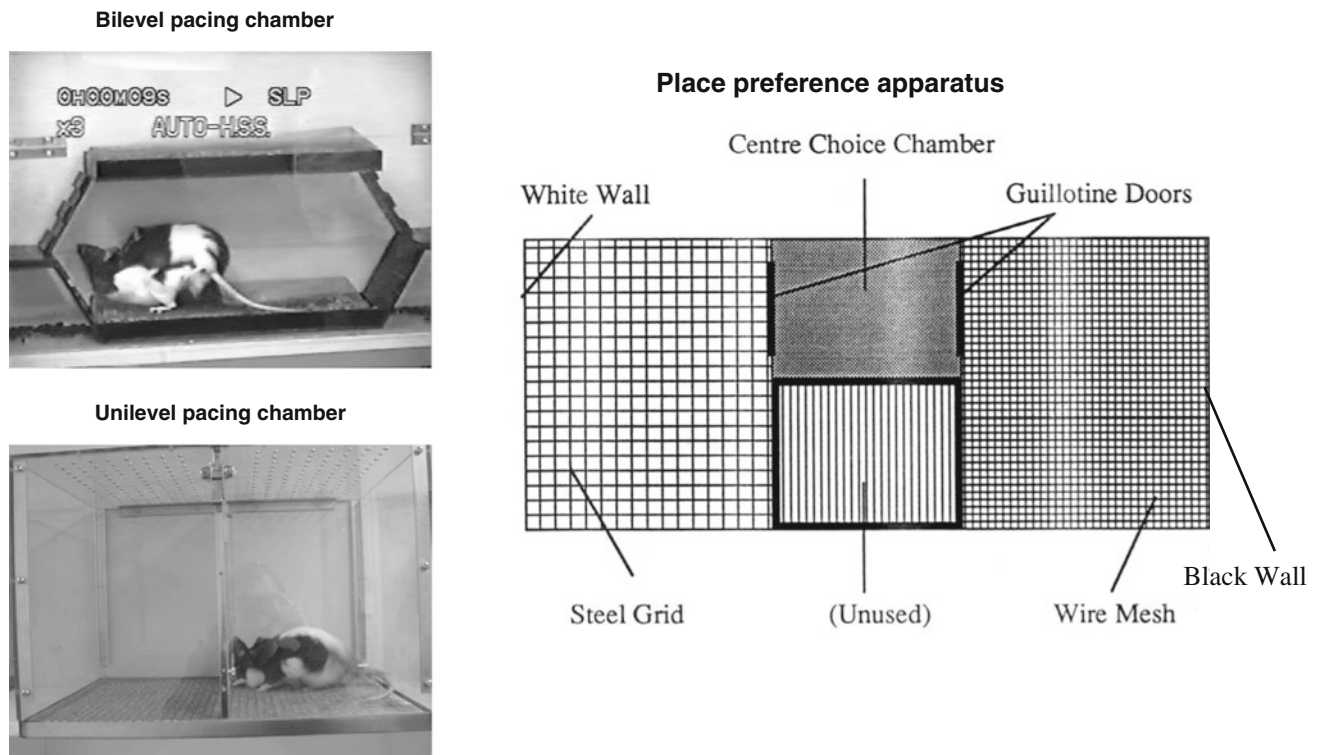
allowed 16 multiejaculatory experiences have increased glucose metabolism in limbic structures relative to sexually naïve males or those allowed three multiejaculatory experiences (Sakata, Gonzalez-Lima, Gupta, & Crews, 2002). Those data suggest that experience with ejaculation in males activates endogenous opioid reward systems and sensitizes mesolimbic systems associated with incentive motivation in male rats (Robinson & Berridge, 1993). Experience with paced copulation in females appears to activate the same reward and incentive systems. Females that mount sexually inactive males show increased Fos induction in the mPOA, ventromedial hypothalamus (VMH), and medial amygdala, but females that either do not mount or that have learned not to mount over five trials with castrated, sexually inactive males do not show Fos induction in those regions (Afonso, Lehmann, Tse, Woehrling, & Pfaus, 2009).

Sexual experience also activates the cerebellum. Sexually naïve male rats allowed to copulate to one ejaculation at 4-day intervals showed activation of the cerebellar vermis (notably lobule 7) whereas males given non-copulatory exposure to females had relatively little activation (Manzo et al., 2008). Using an identical paradigm, dynamic increases were found in GAD-65 (assessed by RT-PCR), the enzyme that converts glutamate into the inhibitory neurotransmitter GABA, during the first three ejaculations experienced by males that were sexually naïve at the beginning of the experiment (Bolivar-Duarte, Silva, Manzo, & Pfaus, 2012); however, this increase had dropped back to baseline levels by the fourth ejaculation. Thus, the acquisition of baseline rates of sexual behavior in male rats appears to correspond to dynamic reorganization of the cerebellar vermis, consistent with the critical role of the cerebellum

in motor habit learning (Salmon & Butters, 1995). It is tantalizing to consider that the particular pattern of sexual responding that crystallizes in animals is mediated by dynamic changes in cerebellar function during their initial sexual experiences.

### Learning Where to Do It

Whereas the crystallization of responses to unconditioned sexual incentives and copulatory behavior itself appears to be conditioned by genital stimulation, learning where to find sex partners, or optimal places to experience copulation, are conditioned by sexual pleasure or reward. Contextual factors, such as settings, are important components of positive sexual experiences for both men and women (e.g., Basson, 2001; Hoon, 1984; Kinsey, Pomeroy, & Martin, 1948; Kinsey, Pomeroy, Martin, & Gebhard, 1953; McCarthy, 1977). Salient cues in the environment may be associated with sexual reward in such a way that they increase arousal or desire directly in their presence. Such is the case with places that are associated with sexual gratification. Animals often display a preference to remain in a context that has been paired consistently with access to a reward (e.g., drugs of abuse, highly palatable foods, a mate) over a context that has not. This conditioned place preference (CPP) is typically demonstrated in an apparatus with two distinctive compartments that are connected to either side of a third neutral compartment (Fig. 5). During training, the compartments are paired differentially with unconditional stimuli (e.g., one side is paired with a sex partner, food, or a rewarding drug, and the other side is paired with either



**Fig. 5** Apparatus used to test CPP. Rats are allowed different types of copulatory experience in either bilevel or unilevel pacing chambers after which they are transferred to one side of a CPP box. On alternating trials, rats receive 15 min of no copulation in the pacing chamber, after which they are transferred to the other side of the CPP box. On the final test, rats are placed

into the central start compartment, the guillotine doors raised, and rats are allowed to roam freely from one side to the other over a specified amount of time. The amount of time spent in each compartment is marked by photo-beam breaks between the compartments, and tabulated automatically

nothing or a control manipulation). On the final test, the animal is placed into the neutral compartment with the two doors on either side opened to allow free access to either compartment. CPP is said to have developed if the subject spends significantly more time in the reward-paired compartment than the other compartment. Stimuli or events that are capable of supporting CPP are referred to as “rewards” rather than “reinforcers,” because the subject has never been required to move into the paired compartment to experience them. Thus, CPP is not reinforced, per se, because it is displayed spontaneously on the final test. However, the increased time spent in the side paired with reward is clearly conditional upon the Pavlovian association of those contextual cues with the reward state.

In male rats, sexual CPPs have been established using two different conditioning procedures. In one, copulation to ejaculation is allowed to occur within one of the distinctive environments and this environment is subsequently preferred over one in which no copulation occurred (Everitt, 1990). CPPs developed by this procedure are referred to as “copulatory CPPs.” Copulatory CPPs can be maintained by intromissions alone whereas prevention of intromission disrupts a previously established CPP (Hughes et al., 1990). In a second procedure, male rats are allowed to copulate to ejaculation in a separate testing chamber and are then transferred

immediately to one distinctive compartment of the CPP apparatus. As with the first testing procedure, the other distinctive compartment is paired on intermediate days with a control condition (usually no copulation). Following such training, the compartment paired with copulation is preferred over the other compartment (e.g., Ågmo & Berenfeld, 1990). A CPP induced by this procedure is referred to as a “post-ejaculatory CPP.” Demonstrations of post-ejaculatory CPPs might appear puzzling at first glance because the CS (i.e., the distinctive environment) is presented after the UCS (copulation to ejaculation), in what learning theorists call “backward conditioning” (that would not be expected to yield conditional responding to the environment). However, if the neural reward state induced by ejaculation is the unconditional stimulus, then the pairing of environmental cues with it is simultaneous. Thus, post-ejaculatory CPP can be accounted for by the rules of Pavlovian conditioning.

CPPs have also been demonstrated in female rats and hamsters. Oldenburger, Everitt, and de Jonge (1992) found that when copulation occurred within one of the distinctive compartments of a CPP apparatus, female rats showed a weak CPP. Subsequently, Paredes and Alonso (1997) and Paredes and Vazquez (1999) demonstrated a robust CPP in female rats that depended on whether the females were able to pace the rate of copulation with-

out having to employ defensive behaviors. This was accomplished using unilevel pacing chambers bisected by a Plexiglas divider with one or more small holes that only the female can pass through (Erskine, 1985, 1989; Paredes & Alonso 1997; Paredes & Vazquez, 1999). The male was sequestered on one side of the chamber and the female was then free to pace the copulatory contact by running from side to side whenever she wanted. Like males, females acquired a strong preference for a distinctive environment only if they were placed into the CPP box immediately after paced copulation. No preference was found if the copulation was unpaced prior to placement in the CPP box (meaning that it had occurred in the same pacing chamber but without the divider). Thus, for a female rat, CPP develops only if she has been able to control the initiation and rate of copulation freely without having to use defensive behaviors.

Although a sexually vigorous male rat is a clear UCS for approach and solicitation in female rats (e.g., Ågmo, 1999), contextual cues associated with pacing elicit a conditioned sexual reward state in those females. However, these results may also indicate the presence of an unconditional aversive state during unpaced copulation. To examine this possibility, Afonso, Woehrling, and Pfau (2006) allowed female rats to copulate in two unilevel pacing conditions using Plexiglas dividers that had either 4 holes or 1 hole. This was done to eliminate the possibility of an “aversive” state resulting from unpaced copulation. Trials were conducted sequentially at 4-day intervals and each pacing condition was paired with one of the distinctive sides of a CPP apparatus, in a counterbalanced fashion. Control groups contrasted the 4-hole or 1-hole condition with a no-divider condition (as was done by Paredes & Alonso, 1997).

Control females developed significant CPP for either the 1-hole or 4-hole condition, relative to unpaced copulation with no divider. Those control data replicate the findings of Paredes and Alonso (1997) and indicate that both the 4-hole and 1-hole condition were rewarding relative to the unpaced (no divider) condition. However, they do not rule out the possibility that the real distinction being made was between an aversive condition (unpaced copulation) and a rewarding condition (paced copulation). This was addressed in the group allowed to contrast the 4-hole vs. 1-hole condition. In this group, females developed significant CPP for the 4-hole condition relative to the 1-hole condition, suggesting strongly that copulatory CPP reflects a true sexual reward state in females. Similarly, Jenkins and Becker (2003) found that female rats developed significant CPP for paced relative to unpaced mating, but also for unpaced mating in which the experimenter removed the male for a period that approximated the female's imposed interintromission interval, relative to unpaced mating in which male removal did not occur. Thus, female rats develop CPP for sex at their own preferred intervals. Taken together with the results of Matthews et al. (1997), these data suggest that reward comes from the sexual stimulation that females receive, namely mounts with intromission, so long as that stimulation occurs at the desired time intervals.

What about paced copulation leads to CPP in females? Meerts and Clark (2009) reported that artificial vaginocervical stimulation (VCS) applied with a 1 ml syringe plunger at 200 g of pressure for 2 s at 30-s intervals, for a total of 15 stimulations, induced a reliable CPP in OVX females primed with estradiol and progesterone. Given that VCS stimulates both internal clitoris and cervix, we asked whether clitoral stimulation (CLS) alone could induce CPP (Cibrian-Llenderal et al., 2010; Parada, Chamas, Censi, Coria-Avila, & Pfau, 2010). In these studies, CLS was administered either with a lubricated paintbrush or a small cotton-tipped vibrator at preferred intervals for 10–15 min over 5–6 reinforced sessions. Both types of stimulation induced robust CPP. Importantly, clitoral reward can be induced in OVX females with or without hormone priming (Parada, Abdul-Ahad, Censi, Sparks, & Pfau, 2011), indicating that sexual reward is independent of steroid priming, although such priming would normally be required for females to experience CLS from mounts with pelvic thrusting (Pfaff, Montgomery, & Lewis, 1977).

Domjan and Hall (1986) demonstrated that male Japanese quail will stay in the vicinity of a window in their home cage through which they could see a sexually receptive female during a pre-copulatory period. However, this behavior developed only if the males had the opportunity to copulate with the female after the pre-copulatory period. A variant of this procedure, similar to that used to study anticipatory motor responding in rats, was used by Balthazart, Reid, Absil, Foidart, & Ball (1995) to study the role of hormones and brain dopamine systems in conditioned sexual behavior in quail. Male quail were placed into a chamber that contained a window and sliding door at one end through which the male could see a sexually receptive female. After a 10-min period, the sliding door opened and the animals could interact freely. As in Domjan and Hall (1986) and Mendelson and Pfau (1989), only males that copulated with the females during this period developed the behavior, in this case, a preference to stay close to the window in the pre-copulatory period of subsequent tests. Castrated males did not develop this conditioned proximity behavior nor did males that did not copulate. Castration also reduced the time spent near the window males trained prior to castration, and subsequent replacement with testosterone or estradiol restored the behavior. Subsequently, Castagna, Ball, and Balthazart (1997) reported that nomifensine, a dopamine re-uptake inhibitor, decreased the appetitive social proximity response, but increased the frequency of mount attempts. In contrast, amfonelic acid, a compound that enhances dopaminergic tone, increased aspects of both appetitive and consummatory sexual behaviors. Thus, brain dopamine systems in birds and mammals seem to have analogous functions in the control of appetitive or conditioned sexual approach behaviors.

Although both copulatory and post-ejaculatory CPP procedures produce effects of similar magnitude in male rats, there are differences in the underlying neurobiology. The opioid receptor antagonist naloxone disrupts both copulatory and post-ejaculatory CPPs, but in different ways. Ågmo and Berenfeld (1990) found that the development of post-ejaculatory CPP was blocked

by injections of naloxone prior to each training session. Conversely, the development of a copulatory CPP was unaffected by naloxone prior to each training session (Meharrra & Baum, 1990). However, once a copulatory CPP had developed, its expression was blocked by naloxone injections prior to the final test (Hughes et al., 1990; Meharrra & Baum, 1990).

There is also evidence that the site of action of naloxone is different for these effects. Ågmo and Gomez (1993) found that naloxone's disruption of the development of post-ejaculatory CPP occurred following infusions into the mPOA whereas infusions of naloxone into this brain region did not disrupt the expression of a copulatory CPP (Hughes et al., 1990). In females, pacing-related CPP was blocked by systemic injections of naloxone (Paredes & Martinez, 2001), or following infusions of naloxone to the mPOA, VMH, or medial amygdala, but not the NAc (Garcia-Horsman, Ågmo, & Paredes, 2008). These data suggest that common opioid systems in the brains of male and female rats are activated by sex-related cues (Paredes & Martinez, 2001) and constitute a primary reward signal. Indeed, as mentioned above, repeated ejaculations increase whole-brain endorphin content in male rats (Szechtman et al., 1981). As with approach behaviors toward sex-related odors, castration disrupts the expression of a copulatory CPP on the first postoperative test in male rats (Hughes et al., 1990; Miller & Baum, 1987), and acquisition of a copulatory CPP was blocked by naloxone in castrated, but not gonadally-intact, male rats (Meharrra & Baum, 1990). Endocrine responses have also been examined in male rats following exposure to contextual stimuli associated with copulation. Kamel, Mock, Wright, & Frankel (1975) reported that serum testosterone, luteinizing hormone, and prolactin levels were elevated after 45 min of exposure to an arena in which prior copulation occurred. Although dopamine antagonists have not been reported to alter the development or expression of copulatory CPPs in either male or female rats, or postejaculatory CPPs in male rats (Ågmo & Berenfeld, 1990; Garcia-Horsman & Paredes, 2004), Meisel, Joppa, and Rowe (1996) found that the development of a copulatory CPP in female hamsters was blocked by injections of the D2-receptor antagonists sulpiride or raclopride prior to each training session.

In summary, sexual reward in the form of ejaculation in male rats, or paced copulation in female rats, involves the activation of brain opioid systems. A similar effect may underlie the copulatory reward in male and female Japanese quail. This is reminiscent of the generalization made by some opiate addicts that the drug effect reminds them of the pleasure experienced at orgasm (Pfaus & Gorzalka, 1987), an effect referred to explicitly as a “pharmacogenic orgasm” by Chessick (1960). As with the reward state induced by heroin or cocaine, both male and female rats spend more time in contexts associated with state induced by sexual reward. Moreover, conditioned contextual stimuli associated with those reward states activate pituitary and gonadal hormone release in male rats, suggesting a priming or “occasion-setting” response that gets male rats ready to copulate.

## Learning When to Do It

When to have sex seems straightforward in terms of arousal, desire, and opportunity. If you have all three, you are all set. But that is a big “if”! Sexual arousal is almost always conditional upon the provoking stimuli being adequate to activate the necessary sympathetic (e.g., increased heart rate) and parasympathetic (e.g., genital blood flow) divisions of the autonomic nervous system. Desire often depends on whether the stimuli are competent as incentives and what else might be going on at the time. And opportunity is often fleeting.

Sexual arousal and desire are constrained further by experience. In addition to habituation that occurs when one does the same thing over and over, or the inhibition that occurs during a refractory period, the reward-related occasion setting mentioned above may well underlie the ability of external cues (and in their most obvious manifestation, fetish objects) to control sexual arousal and thereby control when to have sex. Inanimate objects, such as articles of clothing, particular forms and textures, and, in fact, any antecedent tactile, olfactory, auditory or visual stimulation, can during an animal's initial experiences with sexual reward, come to control both arousal and desire. Thus, the development of paraphilias may well follow the same pattern of Pavlovian and operant sexual learning discussed above.

Some opportunity is constrained by hormonal activation. Obviously, having a sexual “heat” makes things simple, constraining sexual arousal, desire, and behavior to the periovulatory period when estrogens and progestins can stimulate it (Pfaff, 1980, 1999). However, despite the fact that women's desire peaks around the time of ovulation (Stanislaw & Rice, 1988), we and some of our primate cousins (e.g., rhesus macaques, Goy, 1978) can have sex anytime throughout the cycle. And males *can* learn when it is appropriate and not appropriate to attempt copulation depending on the receptive versus defensive behaviors of the female (Pfaus & Pinel, 1989). Male rats also display “psycho-genic” or noncontact erections in response to unconditioned sex odors of sexually receptive females (Sachs, Akasofu, Citron, Daniels, & Natoli, 1994), an effect that is facilitated mildly by brief olfactory, visual, and auditory contact with inaccessible receptive females, moderately by experience with mounting alone, and dramatically by full copulatory experience to ejaculation (Sachs & Liu, 1997). Thus, although erections can be induced by presumably “prepotent” unconditional olfactory stimuli, the stimuli become far more effective—perhaps even sensitized—by pairing them with the state induced by sexual reward.

What about other sensory cues? In his classic work *Psychopathia Sexualis*, Krafft-Ebing (1886/1929) presented numerous case histories (almost all male) of fetish development in which particular inanimate objects, actions or states of arousal induced by painful stimulation (e.g., spanking, piercing the skin, etc.) or other tactile sensations (e.g., the feeling of feathers) became associated with an individual's first sexual erection or first set



of sexual experiences to orgasm. In some of those cases, the initial experience occurred prior to the normative age of puberty, suggesting that the individual may well have been in precocious puberty or that at least his genital responses were in a state of relative sexual awakening. Often, the individual would masturbate to ejaculation while reminiscing about the early event and/or fantasizing about experiencing it again with others. Of course, the fetish became problematic when it was attempted with an unwilling partner or when the individual was caught engaging the fetish in public (e.g., hiding in bushes and spilling water from a pitcher on an unsuspecting woman's bustier). The case histories were often accompanied by a psychological profile of the individual, noting a history of odd behavior and any potential eugenic abnormalities (e.g., low level cognitive functioning) that ran in the family. Given that such behavior was not "normal" in its day, and that the individual appeared to suffer from some genetic basis for abnormal behavior in general, it was easy to conclude that the sexual behavior was a "psychopathic" symptom of a more general genetic-based syndrome. And, after all, how could it serve reproduction if an individual can't get an erection unless he or his partner wears leather or lace? Surely, such objects did not play a central role in our species' evolutionary sexual history.

An alternate hypothesis from an incentive-based analysis is that *anything* that predicts sexual reward becomes an occasion setter for sexual arousal (and maybe desire too). But here is where the situation gets dicey. Whereas sexual reward may be studied objectively in animals, in humans the notion is muddled in peculiar definitions, along with particular phases of the human sexual response (Pfaus, 1999). It is also the case that human subjects in laboratory settings show relatively weaker effects than are typically observed in animals. This may be due to the fact that the typical human subject has already acquired sexual experience prior to the study, an effect that reduces the possibility of robust conditioning. Thus, the sexual UCSs used may not create a "strong enough" sexual reward state to condition the particular CSs, especially as they are not occurring during an early critical period of sexual behavior development.

There have been several notable attempts to condition sexual arousal in humans (see Hoffmann, 2012). Rachman (1966) and Rachman and Hodgson (1968) found that following pairing with erotic visual slides, a pair of women's boots was able to elicit erections in men. Similarly, McConaghy (1970, 1974) demonstrated conditioned erection elicited by colored circles or squares paired previously with erotic videos or still pictures in heterosexual and homosexual men. A particularly informative study by Kantorowitz (1978) examined the nature of association between the UCS and conditioned arousal induced by still pictures. For each subject, three different slides were paired with the plateau, absolute refractory, and later resolution stages during and after masturbation to orgasm. During subsequent testing, stimuli paired with the plateau phase produced an increase in penile erection whereas stimuli paired with the refractory phase produced a decrease in erection. Stimuli paired with the later resolution phase had no

effect. Remarkably, those responses were still present after 3 months. Lalumière and Quinsey (1998) reported significant conditioned genital arousal in heterosexual men to a picture of a moderately attractive, partially nude woman that was paired with a video depicting highly arousing sexual interaction. A control group that received access to the picture alone (without the video) showed habituation.

Regarding women, Letourneau and O'Donohue (1997) failed to find significant effects of conditioning on sexual arousal. However, it was noted that the UCSs (erotic films) produced only moderate levels of arousal whereas in studies with male subjects such stimuli produced high levels of arousal. Thus, this failure to demonstrate conditioned arousal in women may have been due to an ineffective UCS. Indeed, Both, Laan et al. (2008) found that neutral pictures of male headshots paired with 2 s of intensely pleasurable vibrotactile CLS produced greater vaginal pulse amplitude (VPA) during extinction in the paired versus unpaired groups. In another study, stimuli were presented briefly (30 ms), followed by a masking stimulus (Both, Spiering, et al., 2008). Although only the paired group showed increased VPA to the CS during the first extinction trial, there was no increase in the conscious affective value of the stimulus. Finally, Hoffmann, Janssen, and Turner (2004) varied the duration and relevance of a CS (abdominal area vs. a gun) that was paired with short erotic film clips in both men and women. Interestingly, when the stimuli were presented subliminally for brief durations, the relevant abdominal stimulus increased arousal in both men and women. However, when the stimuli were presented for longer periods prior to the erotic film clips, a sex difference emerged in which the relevant CS alone (abdominal area) induced genital arousal in men, but the presumably irrelevant stimulus alone (gun) induced genital arousal in women. This latter effect may indicate that women require CSs that increase autonomic arousal to a higher extent than men, a potential corollary of the "discordance" experienced by women, but not men, between genital and subjective sexual arousal (Chivers & Rosen, 2010).

Several studies have attempted to demonstrate instrumental control of sexual arousal in men and women. Rosen, Shapiro, and Schwartz (1975) found that given feedback and contingent monetary reinforcement, men learned to become sexually aroused in the absence of erotic stimuli. Other studies have found that men can suppress (Rosen, 1973; Rosen & Kopel, 1977) or increase (Reynolds, 1980) penile erection with instructional feedback; however, those studies failed to demonstrate learning effects across trials. Given similar instructions, women can increase their vaginal pulse in the absence of erotic stimulation (Zingheim & Sandman, 1978) or decrease vaginal pulse in the presence of erotic stimulation (Cerny, 1978), but, again, no learning effects were reported. In summary, the evidence regarding instrumental control of sexual arousal is limited to the one report in which monetary reinforcement and feedback were provided.

Can animals show sexual arousal in response to cues associated with sexual arousal or reward? Graham and Desjardins

(1980) placed sexually naïve male rats in a chamber scented with a methyl salicylate (wintergreen oil) CS for 7 min before transferring them to an adjacent chamber with an unscented receptive female for 15 min of copulation (UCS). Control males received CS alone, UCS alone or handling. Training occurred once a day for 14 days. On the 15th day, males were placed in either the CS or UCS chamber and trunk blood was taken within 15 min to assay for luteinizing hormone and testosterone. Exposure to females increased concentrations of both hormones in plasma whereas the CS was effective only in males in the paired group. Sachs and Garinello (1978) reported that placing male rats into a chamber in which copulation had previously occurred dramatically reduced the latency for males to display penile erections. Male rats trained to associate an almond odor with copulation to ejaculation (see below) initiated copulation faster with an almond-scented receptive female than with an unscented receptive female (Kippin & Pfaus, 2001b). Similarly, male marmosets trained to copulate to ejaculation with lemon-scented receptive females display erections and increased place preference in response to the odor alone relative to unpaired males (Snowdon, Tannenbaum, Schultz-Darken, Ziegler, & Ferris, 2011).

Placement of male rats into a holding cage has been used as a conditioned contextual stimulus to signal non-copulatory exposure to a receptive female behind a wire-mesh screen (Zamble, Hadad, Mitchell, & Cutmore, 1985; Zamble, Mitchell, & Findlay, 1986). Placing males into the holding cage prior to a final test of unrestricted copulation resulted in significantly shorter latencies to intromit and ejaculate relative to males placed directly from their home cages into the copulatory condition. Subsequent studies found that second-order conditioned stimuli (e.g., a plastic toy fish found in the holding cage) were effective in enhancing the same measures of arousal (Zamble et al., 1985) and such conditioning in sexually naïve or sluggish males could increase the proportion of males that copulated on a subsequent test (Cutmore & Zamble, 1988). Hollis, Cadieux, and Colbert (1989) demonstrated that repeatedly pairing a light with non-contact exposure to a receptive female resulted in conditioning of sexual behavior in male gouramies, a type of Labyrinth fish. They found that males receiving the conditioning treatment displayed significantly lower latencies to initiate copulation and lower levels of aggression towards females when the CS was presented before access to a female. Similar results have been demonstrated in Japanese quail. Male quail that received repeated exposure to females following the presentation of a CS light displayed significantly shorter latencies to initiate copulation when the stimulus was present compared to when it was absent (e.g., Domjan, O'Vary, & Greene, 1988).

Finally, if male rats are given their first copulatory trials wearing a Velcro tethering jacket, the jacket will become a CS+ for sexual arousal. Sexually naïve males in the paired group were given nine multi-ejaculatory trials at 4-day intervals with the jacket on, whereas males in the unpaired group received the same number of trials without the jacket. On the 10th trial, males in both groups

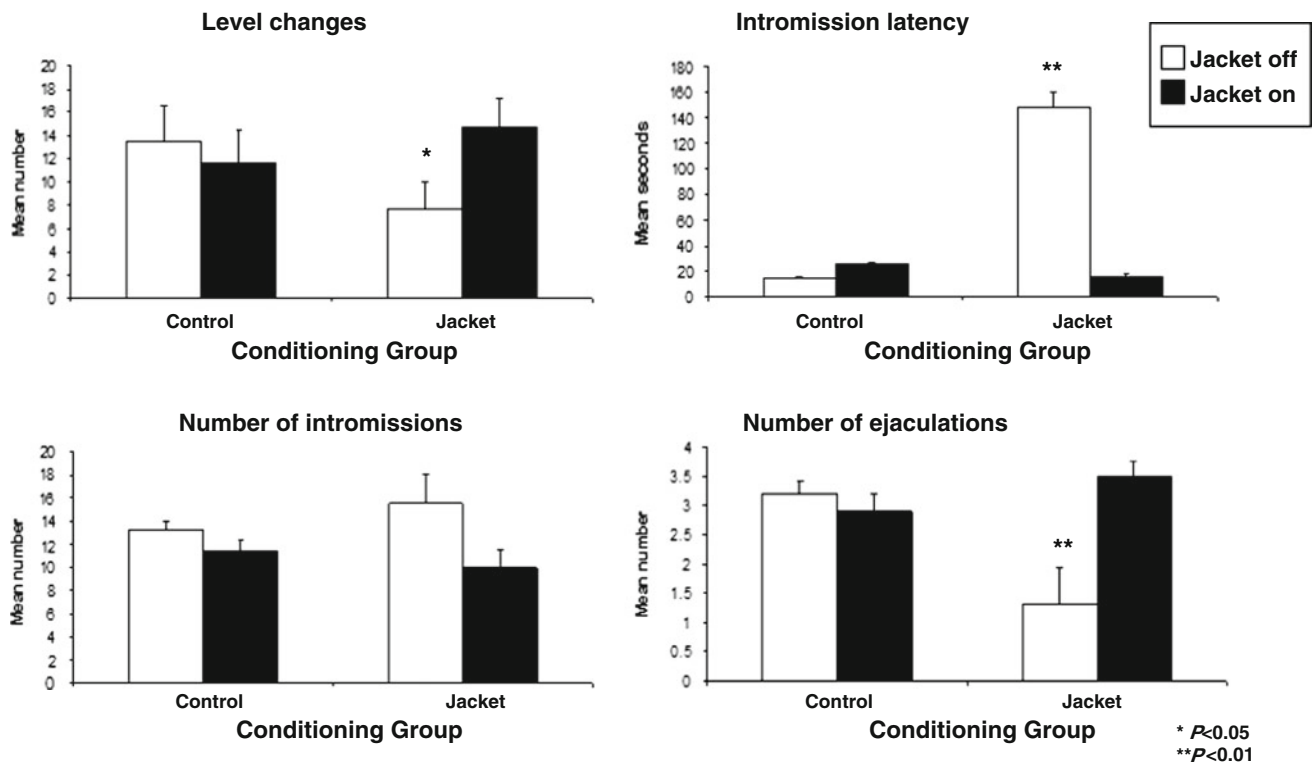
were assigned randomly to two subgroups, one with the jacket on and one with the jacket off, and given a final 30-min test of copulation. Males that had been trained without the jacket displayed normal baseline rates of copulatory behavior with or without the jacket, as did males trained with the jacket that had the jacket on during the final test (Fig. 6). In contrast, males trained with the jacket that had the jacket off during the final test displayed a severe copulatory deficit, including significantly increased mount, intromission, and ejaculation latencies, a decreased number of conditioned level changes, and a reduction in the proportion of males that achieved ejaculation during the test. The pattern suggests significantly diminished sexual arousal and desire and a failure to engage sympathetic tone for ejaculation (Pfaus, 2009). Thus, the jacket had become a CS+ for sexual arousal only in the paired group that had their early formative sexual experiences with it.

The ability of neutral olfactory and tactile stimuli associated with sexual reward to direct sexual arousal in males of a variety of species indicates a high degree of plasticity in the neural systems that control sexual behavior in general, rather than a specific genetic difference that makes some males susceptible to conditioning. Could this plasticity extend to the development of preferences for individual partner-related cues?

### Learning *Who* to Do It With

Although it is clear that animals learn what to do sexually and where and when to do it, the question of *who* they prefer to do it with seems like it should be less affected by experience and more by innate, genetic, and species-specific causes geared toward reproductive success. And in the case of homosexuality, it would seem that innate causes play a large role in determining sexual orientation long before sexual activity and sexual reward is initiated. Indeed, sexual partner preferences that are not geared toward reproduction (as in homosexuality) or that are highly constrained by the presence or absence of inanimate objects (as in many paraphilias) should either not exist or should confer some other reproductive benefit. One such benefit could be related to kin selection (e.g., Wilson, 1975), as in the “gay uncle hypothesis” that has received much attention but little empirical support in Western culture (Bobrow & Bailey, 2001), although it appears to be supported among the Samoan *fa'afafine* (Vasey & VanderLaan, 2010). Yet, from a reward-based incentive view, there is absolutely no reason why partner-related cues that can be detected at a distance (such as sex, gender, race, hair color or length, facial features, body type) or more proximally (such as a multitude of personality characteristics) cannot also become conditioned by early experience with sexual reward, although there are important developmental constraints that make the preference for gender far less amenable to conditioning (see below).

Evidence of this in animals would provide strong support for the alternative hypothesis put forth here that the “genetic” basis of sexual preference rests in reward-based learning, as do



**Fig. 6** Appetitive and consummatory sexual behaviors by male rats given their first nine trials of sexual behavior with or without a rodent jacket. On the final test, rats in each group either had the jacket on or off. \* $p < .05$  between jacket on and jacket off conditions

virtually all other motivational and regulatory functions performed by organisms on this planet. This does not make it a “choice” except at an epiphenomenological level of interpretation. Once conditioned, sexual preferences should feel as natural and “hardwired” as one’s food preferences. And, in humans, the conditioning does not occur overnight, but rather through a progressive and iterative process that begins well before the periadolescent awakening of sexual arousal, and becomes relatively refined and fixed during formative experiences with masturbation and orgasm prior to actual sexual activity with other people, when further refinement and fixing occur. Interpersonal sexual reward would thus reinforce the already-established template.

#### Features Conditioned by Sexual Reward

To study the effects of Pavlovian conditioning on sexual preferences, we took as our lead the findings of Graham and Desjardins (1980) above. Could a male rat’s primary sexual experiences with scented females come to elicit a preference for scented partners in a choice paradigm?

Kippin, Talianakis, Schattmann, Bartholomew, and Pfaus (1998) trained male rats to associate an almond odor smeared on the back of the neck and anogenital area of females that were either sexually receptive or non-receptive. Males in the Paired group received sequential access to scented receptive and unscented non-receptive females at 4-day intervals for nine trials each. Males in

the Unpaired group received the opposite order of association, such that the scent was on the non-receptive females and no scent was on the receptive females. Another group of males received Random pairing of almond odor with the receptive and non-receptive females. On the final test, males were placed into a large open field for a 5-min acclimation period, after which two receptive females were placed equidistant from the male, one scented and the other unscented. The choice of female for first mount, intromission, and ejaculation and the distribution of mounts, intromissions and ejaculations throughout the 30-min open field test were recorded.

Males in all groups copulated with the females closest to them, displaying no significant preference of female to receive their mounts and intromissions. However, males in the Paired group ejaculated preferentially with the *scented* females, choosing them significantly more than the unscented females to receive their first ejaculations, and more often to receive their ejaculations throughout the test. Males in the Unpaired had the opposite preference, choosing to ejaculate first, and more times overall, with the unscented females. Males in the Random group did not show any ejaculatory preference. Interestingly, males in the Unpaired group displayed a preference to mount and intromit with the unscented females on the final test, suggesting that the almond odor had acquired conditioned inhibitory properties that dampened their interest in now-receptive females bearing the odor. In fact, those males showed robust avoidance behavior toward the scented

females during the final test (an effect that was responded to with increased solicitations on the part of those females).

Subsequent studies established that the conditioned ejaculatory preference (CEP) developed only if males were in the vicinity of the females during their post-ejaculatory refractory period (Kippin & Pfau, 2001a) and was more robust if males had more ejaculations (Kippin, Samaha, Sotiropoulos, & Pfau, 2001). It did not develop if males were allowed only to intromit or if the female was removed immediately after ejaculation. An analysis of the conditioned response showed that males became “choosy” during the last three mounts prior to ejaculation and directed those mounts at the female bearing the familiar cue (Kippin & Pfau, 2001b). This is a period in which males are sensitive to their impending ejaculation and emit a 50 kHz call that attracts females immediately prior to ejaculation (McGinnis & Vakulenko, 2003). Using unilevel pacing chambers bisected by a Plexiglas partition that contained either 1 or 4 holes, males were shown to develop significant CEP only when trained in the 1-hole condition, in which they had to wait longer between intromissions, had more intromissions before ejaculation, and longer ejaculation latencies compared to males in the 4-hole condition (Ismail, Gelez, Lachapelle, & Pfau, 2009), suggesting that the ejaculatory reward state may rest on increased parasympathetic arousal during copulation, something that occurs naturally in bilevel pacing chambers as the males must chase the females in order to copulate with them (Pfau, Mendelson et al., 1990). Finally, Erskine (2005) raised the issue of whether such partner preferences might be displayed in more natural contexts (e.g., between different strains of partner). So, instead of neutral olfactory cues on the same strain of partner, we varied the strain of partner (pigmented Long-Evans rats vs. albino Wistar rats) associated with the ejaculatory reward state (Ismail, Jones, Graham, Sylvester, & Pfau, 2011). Pigmented Long-Evans males received nine multi-ejaculatory copulatory training trials with either pigmented or albino females in a 1-hole pacing chamber. In a final open field test with two females, one pigmented and one albino, the males chose the strain of female associated with the 1-hole condition significantly for their first ejaculation. However, the males chose pigmented females significantly for their second ejaculation only if they were associated with the 1-hole condition. This suggests that a degree of “assortative” choice (in which animals have a natural preference for less related members of their own strain, or at least that look a little like them) may strengthen the development of CEP (for a discussion of optimal in- and out-breeding as it relates to assortative mating, see Bateson, 1983).

Association with reward can also reduce the impact of aversive stimuli. For example, although rats initially reject a bitter morphine solution that replaces their drinking water, they learn to like the bitter taste as the morphine reward state sensitizes over time (Zellner, Berridge, Grill, & Temes, 1985). The sexual reward state in males is clearly powerful enough to condition a preference for a neutral odor, so we asked whether it could also blunt the impact of an aversive odor like cadaverine, a polyamine that is produced in decaying flesh. Rats are well known to bury aversive

smelling objects such as noxious food, dead conspecifics, and anesthetized conspecifics speared with cadaverine (Wilkie, MacLennan, & Pinel, 1979), so cadaverine is considered unconditionally aversive.

We gave male rats their first nine sexual experiences with either cadaverine-scented females (unanesthetized, of course), or unscented control females. A third group of males was given habituation to the cadaverine odor in their home cages and copulation trials with unscented females (Pfau et al., 2001). On the 10th trial, males from the three groups were placed into an open field and allowed to copulate freely with two receptive females, one scented with cadaverine and one unscented. Males in the control group pursued the unscented females selectively and ejaculated exclusively with them. Males that had been habituated in their home cages did the same. In contrast, males that had copulated previously with cadaverine-scented females pursued, copulated, and ejaculated with both females, indicating that the aversive properties of cadaverine had been diminished after pairing with sexual reward. Four days later, males received a wooden dowel that had been saturated in cadaverine into their home cages. Males in the control and habituated groups avoided the dowel, and some attempted to bury it. In contrast, all males in the cadaverine-paired group made contact with the dowel and more than half picked it up and gnawed on it, as they would if the dowel had been laced with something hedonically positive like estrous vaginal secretions or chocolate.

First and foremost, these findings demonstrated that a simple Pavlovian conditioning paradigm can modify male rat sexual strategies away from an assumed innate polygamous preference for novel females toward a preference for familiar females. Second, they showed that male rats can distinguish copulation from mating, as the conditioned preference was not displayed in the Paired group for choice of female for mounts and intromissions but rather for ejaculations. Third, the association of the odor with sexually non-receptive females induced a conditioned inhibitory state that compelled males to avoid sexually receptive females bearing the odor on the final test. Fourth, the state induced by ejaculation was the critical UCS, especially if it occurred on a background of high arousal. Finally, an unconditionally aversive odor (cadaverine) was made less aversive and possibly conditionally appetitive by pairing with sexual reward.

Female rats also show olfactory conditioned partner preference for males associated with a pacing-induced reward state (Coria-Avila, Ouimet, Pacheco, Manzo, & Pfau, 2005). This was accomplished easily in unilevel pacing chambers in which the paced condition involved the placement of either a 1-hole or 4-hole Plexiglas divider through which the female could regulate the initiation and rate of copulation. The non-paced condition involved copulation in the same chamber but without the divider. Females in the Paired group were given paced copulation with males that have had the almond odor smeared on their necks and anogenital area versus non-paced copulation with males that have had distilled water smeared on the same areas. After four paced versus non-paced trials, females were placed into a large



open field with two tethered males, one scented and the other unscented, and choice of male to solicit, copulate with, and receive ejaculations from, was recorded. Females for which the odor was paired with the paced condition selectively solicited, copulated with, and received ejaculations from the scented male. Females that had the odor explicitly unpaired or paired randomly with pacing did not display a preference.

As with males, females showed a similar preference for strain cues associated with paced copulation (Coria-Avila et al., 2006), although it was stronger if the strain associated with paced copulation was their own. Interestingly, in that study, pigmented or albino females solicited whichever strain of male was associated with paced copulation, but received ejaculations preferentially from males of their own strain and only if that male had been associated with paced copulation. This also revealed a degree of assortative choice, especially for mating, and showed that females, like males, can differentiate copulation from mating. Finally, female rats that experienced manual CLS in the presence of a cotton gauze pad soaked in almond extract chose to copulate selectively with almond-scented males over unscented males during their first sexual experience in a large open field with both males (Parada et al., 2011). Interestingly, they did not show a preference to receive the scented male's ejaculations, suggesting that the VCS received from males during paced copulation induced a further reproductive or mate choice. It is not yet known whether this stems from specific stimulation of the cervix (and pelvic nerve) or from full stimulation of internal and external aspects of the clitoris. Experience with paced, relative to non-paced, copulation in unilevel chambers induces significant neurogenesis in the granular layer of the accessory olfactory bulbs (Corona, Larriva-Sahd, & Paredes, 2011), a region known to contain intrinsic memory systems related to pheromonal stimulation and recognition of conspecifics (Brennan, Kaba, & Keverne, 1990; Brennan & Keverne, 1997). Thus, as Darwin (1859) suggested, females are indeed more “choosy” than males in terms of choosing a male to copulate and mate with. However, like males, they base this choice on the external (epigamic) characteristics of the male associated with their own experience of a copulatory reward state.

Females also learn inhibitory associations. In addition to the effect of nonreward induced by training under the influence of the opioid receptor antagonist naloxone, sexual frustration in the presence of an inaccessible male can also induce an inhibitory state. Parada et al. (2011) gave sexually naïve female rats five trials of CLS in the presence of a sexually active male scented with almond behind a screen. On alternating days, the females received sham CLS in the presence of an unscented male behind the screen. During the final open field test with two males, one scented and the other unscented, females solicited selectively the *unscented male* and showed a trend to receive that male's ejaculations preferentially. At first glance, these data seem at odds with the fact that CLS induces a reward state. However, it was noted that females attempted to solicit the males behind the screen following CLS during the training trials, which, of course, were not successful because the

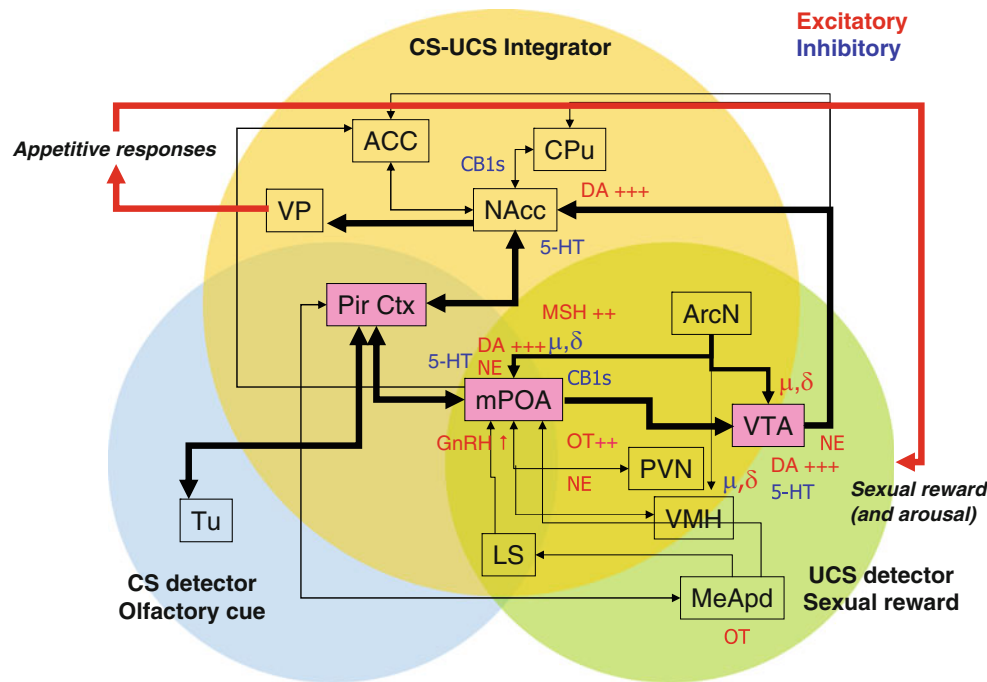
male was behind a screen. Thus, it is likely that the female was in a state of frustrated non-reward that she associated with the odor and generalized to the choice of male for her first sexual experience.

## Neural Correlates

One of the strengths of the olfactory conditioned partner preference paradigm is that the odor can be presented alone in the animal's bedding or on a cotton gauze pad and neuroanatomical and neurochemical activation in response to the odor examined. This takes the mess of copulation (and interpretation based on different types or amounts of sensory stimulation) out of the analysis and makes the study far more cognitive in terms of examining responses made to a second-order conditioned cue. In fact, it is now possible using this paradigm to relate areas of the rat brain activated by the conditioned odor with areas of the human brain activated by visual erotic cues.

We have found significant activation of the immediate-early gene product Fos following presentation of the odor in paired versus unpaired groups in a number of brain regions that constitute a sexual circuit similar to that found activated in fMRI studies of sexual arousal and desire in men and women (e.g., Karama et al., 2002; Ortigue & Bianchi-Demicheli, 2008; Paul et al., 2008). These regions included cortical structures such as the medial prefrontal cortex, piriform cortex, insula, and anterior cingulate gyrus; limbic/motor regions such as the olfactory tubercle, nucleus accumbens, dorsal striatum, lateral septum, basolateral and medial amygdala; hypothalamic regions such as the mPOA, bed nuclei of the stria terminalis, anterior and lateral hypothalamus, and the midbrain anterior ventral tegmentum (a region that gives rise to the mesolimbic dopamine (DA) system) of males (Kippin, Cain, & Pfaus, 2003) and females (Coria-Avila & Pfaus, 2007) after presentation of the paired odor alone relative to the odor unpaired or random. Previous studies showed these regions to be activated by unconditioned olfactory or genitosensory stimulation during copulation (reviewed in Pfaus & Heeb, 1997); thus, their conditional activation by the odor suggests that they are activating a representation of the UCS, as Pavlov (1927) originally suggested for all CS+s. Notably, a nearly identical set of regions is activated by stimuli that predict cocaine or heroin reward (Childress et al., 2008; Garavan et al., 2000), suggesting that these regions function together as a general system for appetitive arousal and desire related to reward.

We suggest that the conditioned cue acts as a “priming stimulus” to activate cortical, limbic, and hypothalamic circuits involved in the facilitation of sexual arousal and desire and/or in the suppression of inhibitory systems (Fig. 7). Some of those circuits involve selective processing of the sexual stimulation that generates the reward state (UCS), the olfactory stimulus (CS), and a system that integrates the CS and UCS so that animals can focus their attention and engage appropriate forward-directed locomotion toward the CS when it is present (Robinson & Berridge, 1993). What is immediately obvious from the figure is that systems for sexual



**Fig. 7** Neural systems which are critical for the display of conditioned olfactory preferences in the rat. Appetitive behaviors made toward conditioned stimuli lead to sexual reward that is processed by three interactive systems. Two systems process olfactory stimuli and sexual reward relatively independently, whereas a third, mesolimbic DA system, acts to integrate both the conditioned olfactory cue and its rewarding sexual outcome. Three common regions, the piriform cortex, mPOA, and VTA, are activated in male and female rats by conditioned olfactory stimuli. Opioid actions in the VTA potentiate mesolimbic DA activation, whereas opioid actions in the mPOA inhibit sexual arousal and desire. Neurotransmitter systems or their receptors in red/lighter are excitatory for sexual motivation whereas those in blue/darker are inhibitory. Note that opioids can be excitatory in the VTA, inhibitory in the mPOA, or either in the VMH (depending on the receptor type). Dopamine, gonadotropin releasing hormone, melanocyte stimulating hormone, noradrenaline, and oxytocin are excitatory whereas serotonin, opioids, and the endocannabinoids are inhibitory. ACC anterior cingulate cortex; ArcN arcuate nucleus of the hypothalamus; CB1 cannabinoid type 1 receptor; CPU caudate-putamen (striatum); DA dopamine;  $\delta$  delta opioid receptors; GnRH gonadotropin releasing hormone; LS lateral septum; MeApd posterior-dorsal nucleus of the medial amygdala; mPOA medial preoptic area; MSH melanocyte stimulating hormone;  $\mu$  mu opioid receptors; NAcc nucleus accumbens; NE noradrenaline; OT oxytocin; PirCtx piriform cortex; PVN paraventricular nucleus of the hypothalamus; Tu olfactory tubercle; VMH ventromedial nucleus of the hypothalamus; VP ventral pallidum; VTA ventral tegmental area; 5-HT serotonin. Adapted from Pfaus, Ismail, and Coria-Avila (2010) (Color figure online)

reward and incentive responding overlap with systems proposed for sexual (and maternal) bonding (e.g., Young & Wang, 2004). These involve the interaction of at least three neurochemical systems, including DA, hypothalamic oxytocin (OT), and opioids that inhibit hypothalamic structures like the mPOA, but sensitize mesolimbic DA systems through a process of disinhibition (Kalivas & Stewart, 1991). This is strikingly similar Fisher's (2000) proposal of three primary emotional systems for mating, reproduction, and parenting.

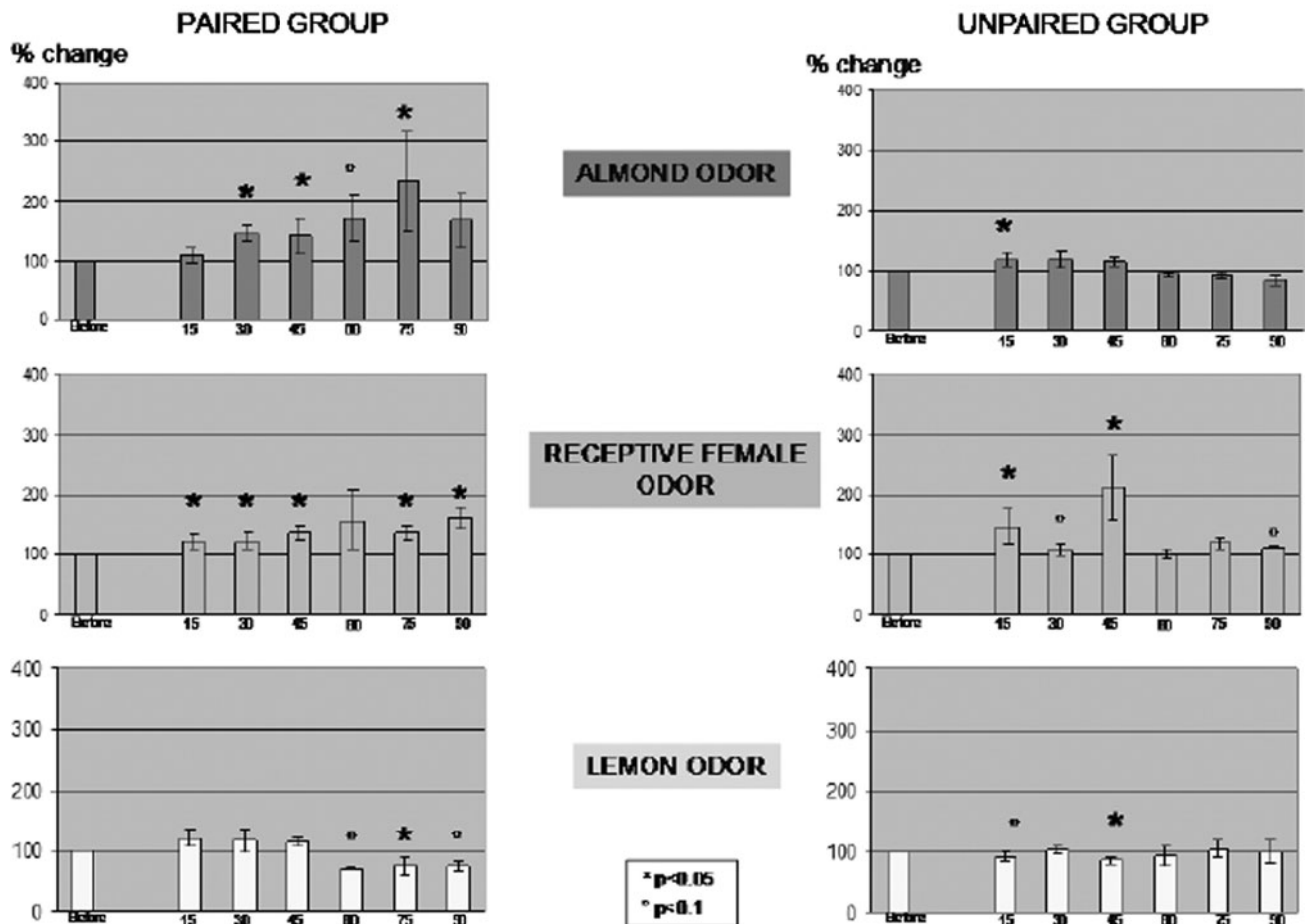
As reviewed above, inhibition of opioid receptors with naloxone inhibits the development of sexual CPPs in both male and female rats and produces an expectancy of non-reward that inhibits sexual desire and behavior in female rats that have their first sexual experiences with the drug. Naloxone administration during training also inhibits the development of sexually-conditioned ejaculatory and partner preferences in male and female rats (Coria-Avila et al., 2008; Ismail, Girard-Bérault, Nakanishi, & Pfaus, 2009). In these studies, naloxone or saline was administered immediately before a male rat's first 10 multiejaculatory sexual experiences

with a scented partner or during a female's first five experiences of paced copulation with a scented partner (relative to five trials of non-paced copulation with unscented males that were interspersed). Rats were then given a final test in a large open field with two sexually receptive and vigorous partners, one scented and the other unscented, following saline injections. Rats trained with saline injections showed the partner preference reviewed above whereas rats trained with naloxone did not display the preference for the scented partner. In fact, males chose the *unscented* female significantly more frequently for their first ejaculation and females solicited the *unscented* male significantly more frequently and received more mounts, intromissions, and ejaculations from that male.

We have also found conditioned DA release in the NAc of Paired-trained male rats in response to the almond odor, but not in Unpaired-trained males (Fig. 8) and conditioned activation of Fos within parvocellular OT neurons of the paraventricular nucleus (PVN) that likely project to other forebrain regions. Fos was also found activated by the conditioned odor in magnocellular

mine, gonadotropin releasing hormone, melanocyte stimulating hormone, noradrenaline, and oxytocin are excitatory whereas serotonin, opioids, and the endocannabinoids are inhibitory. ACC anterior cingulate cortex; ArcN arcuate nucleus of the hypothalamus; CB1 cannabinoid type 1 receptor; CPU caudate-putamen (striatum); DA dopamine;  $\delta$  delta opioid receptors; GnRH gonadotropin releasing hormone; LS lateral septum; MeApd posterior-dorsal nucleus of the medial amygdala; mPOA medial preoptic area; MSH melanocyte stimulating hormone;  $\mu$  mu opioid receptors; NAcc nucleus accumbens; NE noradrenaline; OT oxytocin; PirCtx piriform cortex; PVN paraventricular nucleus of the hypothalamus; Tu olfactory tubercle; VMH ventromedial nucleus of the hypothalamus; VP ventral pallidum; VTA ventral tegmental area; 5-HT serotonin. Adapted from Pfaus, Ismail, and Coria-Avila (2010) (Color figure online)

## Dopamine release in the NAc



**Fig. 8** Dopamine release in the nucleus accumbens of male rats in response to an almond odor paired or unpaired with sexual reward, estrous vaginal secretions, or a lemon odor unpaired in both groups. Unilateral dialysates were taken

every 15 min and analyzed via high performance liquid chromatography. Almond and lemon odors were presented on gauze. Estrous vaginal secretions were presented on Q-tips after vaginal lavage

vasopressin (VP) neurons of the supraoptic nucleus that likely project into the posterior pituitary. Systemic injections of OT (5  $\mu\text{g/kg}$ ), but not vasopressin, to sexually naïve male rats during their first exposure to almond-scented receptive females resulted in one-trial CEP.

These data suggest that conditioning in the putatively polygamous rat sensitizes a circuit similar to that activated in monogamous Prairie voles during their formative sexual experiences (Aragona et al., 2006; Young, Murphy Young, & Hammock, 2005; Young & Wang, 2004) and following parturition (Wang, Liu, Young, & Insel, 2000). The results of Aragona et al. (2006) are particularly instructive, as the sexual bond formation was inhibited by activation of D1 receptors, but facilitated by the activation of D2 receptors. This suggests a neural reorganization in mesolimbic terminals after formative sexual experiences that “seals the bond,” making it less likely for other stimuli to acquire associative strength.

Such an effect is consistent with modern theories of learning (e.g., Harris, 2011; Rescorla & Wagner, 1972) and has been implicated in the susceptibility to drug addiction, especially in terms of responding to cues that predict drug reward (Flagel et al., 2010), and more generally in response to food-related cues (Berridge, 2009; Blackburn, Pfaus, & Phillips, 1992). The interaction of OT and DA in the PVN, mPOA, VTA, and NAc of male rats induces penile erections and links them to appropriate appetitive sexual behaviors (Succu et al., 2007). Thus, opioid reward states may form the rudimentary mechanism of bonding because they sensitize DA release in the presence of reward-related cues compelling animals to focus their attention and goal-directed behavior toward those cues. Activation of brain OT systems (by DA or other means) adds a reduced social distance and bonding to this neurochemical reward state. Given that pharmacological activation of opioid receptors induces a direct suppression of both hypothalamic and pituitary OT secretion (Vuong, Van Uum, O'Dell,

Lufty, & Friedman, 2010), sensitized and potentially reorganized mesolimbic and hypothalamic DA systems must be a necessary intermediary. This is consistent with a multifaceted role of mesolimbic DA in incentive salience and response initiation (e.g., Blackburn et al., 1992; Robinson & Berridge, 1993).

### Assortativeness

In social systems, assortativeness refers to a bias in favor of similar characteristics. This bias could be toward any outwardly definable (or distal) trait, including gender, race, size, age, dress, and more proximal traits, including socioeconomic status, political belief or religious orientation. In mating systems, assortativeness refers to choosing characteristics that are similar to oneself, as in species or strain (race/size) whereas disassortativeness refers to choosing characteristics that are dissimilar, such as the sex of the partner in heterosexual circumstances. Such choice is believed to reflect sexual imprinting, in which early postnatal (PN) conditions require the newborn to recognize and stick close to its mother or caregiver. Bateson (1983) defined heterosexual assortative mate choice for different species along an optimality curve between inbreeding and outbreeding, with a number of external and genetic weighting coefficients that define the costs and benefits of both. The choices are then made on the basis of dominant distal sensory cue(s), such as coloration in the Japanese quail studied by Bateson.

But all is not quite as it should be. Many different external cues can be imprinted during the early PN period depending on who (or what) happens to be in the right place at the right time (as in the famous picture of Konrad Lorenz with Greylag goslings following him). Several important studies have demonstrated that adult males mate preferentially with females that have attributes similar to those of the female(s) that nursed them early in life. Yamazaki et al. (1988) found that male mice nursed by foster mothers chose to mate with females that resembled their foster mother rather than females that resembled their biological mother. Similarly, adult male Lesser Snow Geese prefer the coloration of the female that nursed them over a different coloration (Cooke & McNally, 1975) and field studies (Cooke, Finney, & Rockwell, 1976; Cooke, Mirsky, & Seiger, 1972). One of the most provocative reports of sexual imprinting was that of Kendrick, Hinton, Atkins, Haupt, and Skinner (1998), who demonstrated that sexual partner preferences can be achieved between goats and sheep using cross-fostering to manipulate the imprinting process. In males and females of both species, sexual partner preferences were toward members of the opposite sex of the species of the foster, rather than biological, mother.

Is assortative mate choice dependent on mom per se or on a state of reward that her features are associated with? We have found that an olfactory cue associated with early PN reward in rats directs mate choice in adulthood (Ménard et al., in preparation). Newborn rats separated from their mothers display distress vocalizations that are reduced or eliminated by stroking their heads and backs with a soft fine paintbrush. Thus, stroking is both

negatively reinforcing (in the reduction of acute distress) and positively reinforcing as a reward (Sullivan & Hall, 1988). In the Ménard et al. study, we separated rat pups from their mothers for 15 min a day on PN days 1–10 and placed them on bedding scented with lemon (Paired) or left unscented (Unpaired). Pups were then stroked gently for the 15 min of maternal separation, after which they were returned to their mothers. On PN 60, after adolescence had been reached, the males were given their first sexual experience in an open field with two sexually receptive females, one scented with lemon and one unscented. All males in the Paired group ejaculated preferentially with the lemon-scented females whereas males in the Unpaired group did not display a preference. Imprinting alters neurogenesis and synapse formation in a variety of limbic and cortical brain structures associated with sensory coding, reward, emotion, and memory, especially the hippocampus and related cortical structures (Bolhuis, 1999; Moriceau & Sullivan, 2004). It is likely, therefore, that those changes essentially sculpt the bias in brain.

### Experience and Expectancy

Experience leads to expectancy. Reward to desire. In a Pavlovian scheme, the CS comes to elicit a representation of the UCS and conditioned responses that lead to reinforcement strengthen the association. It is circular, but that is a fundamental property of the experience-driven brain plasticity that underlies Hebbian synapses. To the extent that “first cuts are the deepest,” and that first experiences with arousal and reward form a critical period of behavioral development and crystallization, the onset of sexuality then becomes a fluid critical period during which distal cues, proximal cues, and interpersonal cues that signal sexual reward are assembled into “wholes” or sexual Gestalts made up of desired features in potential sex partners (assembled perhaps hand-in-hand with desired activities with those partners). Individual differences in arousability, attention, temperament, reward intensity, and inhibition would then determine the success and timing of early sexual experiences. And if the process is iterative and additive, which it must be by definition, then new and unexpected experiences of greater sexual arousal and reward could refine the features further (e.g., as suggested by Morin, 1995) and lead to a conscious awareness that one has bonded sexually, emotionally, and romantically with another individual. Conversely, experiences with sexual non-reward could loosen or extinguish these associations, leading the features that were once in the “foreground” into the “background” (to paraphrase Köhler, 1929). However, if the process of reward learning is iterative and additive, it may never be completely possible to extinguish salient features entirely. Former drug addicts always want or crave some aspect of the early drug effect, even if the rewarding aspects of the drug had diminished prior to abstinence (Robinson & Berridge, 1993). This can be stimulated by cues that predict drug reward, and also by stress or other unexpected factors, and can lead to spontaneous recovery of drug taking



(Shaham, Erb, & Stewart, 2000), recovery of fears and phobias (Jacobs & Nadel, 1985), and perhaps also to the sexual gestalts and activities that we initially preferred.

Experience plays a vital role in some theories of sexual orientation. In his “exotic becomes erotic” theory, Bem (1996, 2000) provides support for the argument that gender-atypical behavior in childhood roots individuals into a biological framework in which same-sex activities become stronger predictors of sexual reward. This occurs in a social context in which gender role is fixed into “male” and “female” modes and imposed, and always confounded with sexual behavior. A number of studies have shown a strong correlation between gender nonconformity in childhood and subsequent homosexual orientation (e.g., Bailey & Zucker, 1995) and Bem’s own path analysis from a large sample of twins (Bem, 2000) reinforces this notion. So a first set of pieces in the puzzle of sexual orientation (and preference) comes from early interactions of genetic and sociocultural factors.

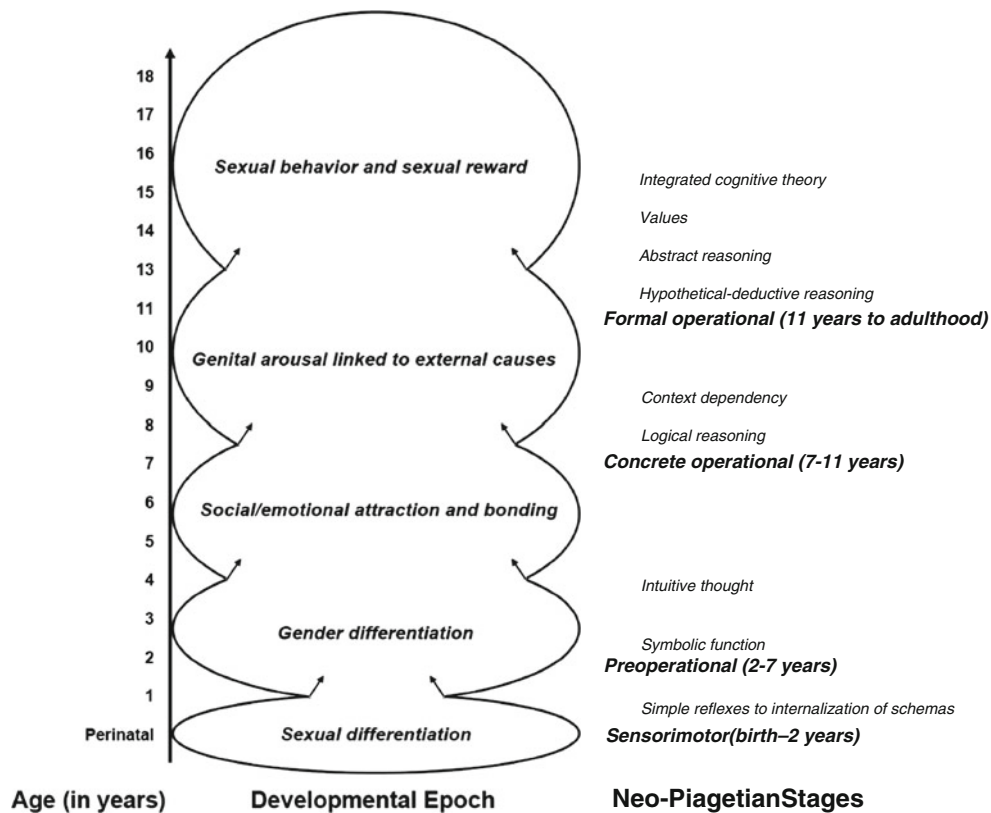
A second set of pieces comes from sexual experience itself. Storms (1981) argued that sex drive development occurs in concert with erotic development during a peri-adolescent period when individuals are moving from predominantly homosocial to heterosocial peer groupings. The content of an individual’s early sexual fantasies and desires is then consciously assessed in the context of actual sexual behavior and the dominant sociocultural definition of the appropriate gender and sexual orientation for that behavior, so that a convenient (and sometimes devastating) cognitive “label” ends up incorporated in one’s self-definition. And given the magnitude of sexual awakening in most people’s adolescent experiences, this addition to the self-definition is usually large and profound. One prediction in Storms’ theory is that sexual experiences that occur in the early phase of adolescence may be more likely to involve salient individuals in one’s same-sex social group. Interestingly, the modal retrospective age of puberty onset reported by the sample of gay men in Hamer et al. (1993) was between 11 and 12 years, somewhat earlier than the “normative” 12–13 years typically reported for boys in Western countries (Slyper, 2006). Of course, puberty does not occur suddenly but rather progresses in a cascade of events related to brain maturation, pituitary peptide hormone release, gonadal steroid hormone secretion, growth, and metabolism. Nevertheless, this leads us to entertain a tantalizing possibility that the X-linked genetic locus discovered by Hamer et al. could be related to a “female”-like timing of puberty onset. The Hamer et al. study can be mined for other gems of support for some of Bem’s and Storms’ ideas. The retrospective age of onset of first same-sex attraction had three phases, one from ages 5 to 8, a second from ages 9 to 13, and a third from 13 years on. The retrospective age of self-acknowledgement of homosexuality began just before the modal peak onset of puberty (approximately between 10 and 11 years), and rose steadily to age 17, then leveled off. Trailing this was the retrospective age of acknowledgement of homosexuality to others, which had a dynamic rise from ages 16 to 22, then leveled off in two phases, one from ages 22 to 32, and then less steeply after age 32. One

might imagine that exotic was becoming erotic through iterations of sexual reward achieved from formative same-sex sexual activities (starting, for example, with arousal, then rehearsal during masturbation, then actual sex play to genitally-based intercourse to orgasm) roughly between the ages of 11 and 16. It is not known from the data reported whether the men in that sample had experienced gender atypical behavior in that first period of attraction to other boys, but it is likely given the preponderance of supportive data.

### Critical Periods and Bias: Disentangling Orientation from Preference

The data of Hamer et al. (1993) suggest the existence of at least three critical periods during the trajectory toward full sexual responsiveness: attraction, then arousal, and finally the emergence of a sexual reward system linked predominantly to genital pleasure and orgasm during and after puberty. The sexual nature of this last system is explicit: by the time people have incorporated sexual activity into their daily routines, they have already imbued it with names and given it personal and social definitions and values that are specific to it. Although the animal work reviewed above indicates a high degree of plasticity regarding the development of preferences for certain types of stimuli associated with sexual arousal and reward during an animal’s first experiences with genital stimulation and copulation, it does *not* reveal how sexual orientation toward members of the opposite or same sex develops. The development of sexual orientation then must form a “constraint” on learning (Gallistel, 1980; Lashley, 1951), biasing the subsequent development of sexual preferences toward the preferred gender. In fact, we argue that each critical period biases the formation of the next, although the next critical period follows the rules of an emergent property as far as the brain is concerned and cannot be reduced solely to the organization and activation that occurred in the previous period. That these critical periods emerge on a self-defining continuum is consistent with the trajectory outlined in Bancroft’s (2009) seminal review of sexual development.

To begin to understand how an orientation bias might develop, we much consider the role of two earlier critical periods, one of perinatal hormone-induced brain differentiation (McCarthy & Arnold, 2011) followed by another within the first 2–3 years of life linked to gender differentiation and the display of gender typical versus atypical behaviors. A cascade of developmental epochs or critical periods is depicted in Fig. 9 (and is contrasted with a neo-Piagetian cascade of stages of cognitive development that occurs during the same time frame, e.g., Demetriou, 1998; Piaget, 1967). These first two critical periods form according to a relative binary code linked to “male” and “female” (Money & Ehrhardt, 1972). Thus, we refer to sexual differentiation of anatomy and brain as the first critical period out of which should emerge an anatomical “boy” or “girl” that we assume has a brain differentiated accordingly. The second critical period differentiates gender-



**Fig. 9** *Left* Developmental epochs during which critical periods mold the development of sexual behavior as a function of age. *Right* Stages of cognitive development according to neo-Piagetian models age-matched to the critical periods. Orientation, we argue, derives from the first two epochs, although it is not recognized by an individual as a sexual orientation until the 4th or 5th epochs. Social development ensues during the 3rd epoch becoming more and more sociosexual, giving rise to general sympathetic and parasympathetic genital arousal that is more and more recognized as being caused by external circumstances. This gives rise to the 5th epoch when masturbation and copulation are achieved. During early experience with sexual arousal and reward, critical features of partners and objects are reinforced in both Pavlovian and operant ways, as are the necessary movements that predict and facilitate

sexual reward states. These are built upon prior critical period outcomes that allow individuals to identify themselves as “female” or “male” in terms of sex and gender, which in turn lays important cornerstones for social development and reinforcement. The developmental epochs occur generally around the ages of the widest spread although they can develop earlier or later depending on genetic and hormonal factors. Arrows indicate that the lower epoch blends into the next one such that there are predominant periods but no absolute start or end. An exception to this is the last epoch, which likely ends with a definitive sense of sex and gender, sexual orientation and sexual preferences for desired features and activities. Of course, peak sexual experiences can occur throughout the subsequent lifespan and give rise to an iterative redefinition of reward and preference

specific behavior (and perhaps self-identity as female or male). Overlaid onto this differentiation is the emergence of operant reward-based learning as it relates to the development of gender identity. During the second critical period, the individual experiences *intrinsic* reward acting as a male or female and this expression may be further rewarded or punished externally depending on whether the expression is gender typical or atypical. Thus, dressing and acting in a gender “appropriate” versus “inappropriate” manner leads to positive reinforcement (reward), negative reinforcement, or outright punishment. Gender atypical behaviors in Western culture typically lead to punishment and/or partial reinforcement (e.g., when one parent “accepts” the gender atypical behavior while the other does not). The impact of the reward or punishment, in turn, is affected by an individual’s temperament. What must be stressed here is that the tendency toward gender typical or atypical expression is intrinsically rewarding when the child engages in it.

Whatever brain mechanism(s) generate this bias become vitally important in the subsequent emergence of sexual orientation. The second critical period also generates conscious awareness of strain features (e.g., race and size) that define the traits of one’s family and/or clan, which can then be used to assess future assortative traits.

The third critical period forms around social and emotional attraction and rudimentary social bonding to opposite- or same-sex individuals. As cognitive development continues, individuals become more and more aware of peer groups, social structure, and the rules of engagement. During this time (roughly between the ages of 4 and 8, social and emotional attraction to opposite- or same-sex peers crystallizes and defines friendships and identity within a peer group social structure. The development of social bonds and hierarchies is also intrinsically reinforced and children who develop friendships and feel accepted have a very different

psychological trajectory compared to children that feel alone and without friends or outright ostracized. Thus, bonding itself is intrinsically rewarding and is signaled in a Pavlovian way by the individual features (typically facial and gender-related) of the other person(s). Storms (1981) argued that the predominant organization through this pre-pubertal period is homosocial, with boys having more friends that are boys and girls having more friends that are girls. However, in contrast to Storms' prediction, by the "magical" age of 10, gender nonconforming children are more likely to be in a predominantly heterosocial organization and experience more acceptance from opposite-sex peers than gender conforming children (Bailey & Zucker, 1995; Wallien, Veenstra, Kreukels, & Cohen-Kettenis, 2010). And superimposed on this third critical period is the conscious awareness of "grey zones" between other dichotomies (e.g., good and bad). Thus, during this third critical period, children may learn how good or bad it is to be gender typical or atypical in terms of the development of emotional and social bonds with others, gravitating naturally to friends and peer groups that are more accepting.

The fourth critical period involves the linkage of genital arousal to a state of general arousal that is associated with particular individuals or actions. This period, between approximately 8 years old and puberty, is associated with greater episodic awareness and context-dependency of events. Of course, genital arousal can occur anytime after the genitalia have been formed and certainly occurs well before puberty in a semi-spontaneous manner (driven in boys, for example, by bladder distention). It is also clear that tactile self-stimulation of the genitals can be perceived as pleasurable and comforting in young children. The linking of genital arousal (parasympathetic) to general sympathetic arousal during specific events involving persons, places, and things sets the stage for the pubertal linkage to sexual reward. It is, in essence, a dress rehearsal, and although the events do not have to be sexual in nature (e.g., Ramsey, 1943), they *can* be. This is the golden thread weaving through Krafft-Ebing's (1886/1929) case histories and the sudden emergence of sexually arousing events creates a set of antecedent biases on the type of external stimuli that will eventually be linked to sexual reward. But in the case of Krafft-Ebing's data, those events biased individuals toward fetish objects or highly arousing "naughty" behavior. Precocious puberty (that occurs before the normative time for girls and boys) adds a dimension of daring to this. Girls and boys who undergo precocious puberty are more likely to be norm-breaking, take risks, and engage in sexual masturbation, sex play, and sexual intercourse at an earlier age than people with normative onset puberty (Bancroft, 2009; Johansson & Ritzén, 2005; Kinsey et al., 1948, 1953). It is clear that the emergence of sexual arousal that leads to even a small degree of genital and sexual pleasure during the early phase of puberty creates a bias for sexual attraction and preference later on (e.g., Herdt & McClintock, 2000). This biasing occurs earlier in people with precocious puberty. It may well be during this phase that the groundwork for "exotic becomes erotic" is laid. For example, the groundwork for fetish development may be laid

during this period if the object or action results in high general and sexual arousal that predict some degree of reward.

To the extent that peri-pubertal experience with genital arousal biases the types of stimuli one can be attracted to, first experiences of sexual reward with those stimuli through masturbation or sexual intercourse during the fifth and final critical period powerfully reinforce those "pre-potent" biases (marking the events through which exotic truly become erotic). In this way, the critical periods generate a reward-based trajectory that moves first through gender-specific antecedents of sexual orientation and preference, to emotional and social attraction and bonding to others (based on gender and perhaps also strain), to awareness of genital arousal as a reward itself, and finally to *sexual* arousal, attraction, bonding, and motor responses that are reinforced by sexual reward with others (allowing people to differentiate lovers from friends for the first time). The preferred features move along a continuum as well, from distal features that are more pre-potent, such as gender, to those that are more flexible, such as hair length or color. It is becoming clear that children in the first 2 years of life are also able to distinguish features that play a role in assortativeness, such as race and size, depending on familial makeup and availability in the immediate peer group (Lam, Guerrero, Damree, & Enesco, 2011). It may well be that the ability to detect gender and strain in oneself and others precedes everything. Age-related features likely come next, defining the peer group during the third and fourth critical periods, and the pool of potential sex partners during the fifth critical period. Gender, strain, and age represent three of the most obvious group-related distal visual features. Surely there are more at a proximal distance, and more still when interaction adds interpersonal qualities to the mix.

## Synthesis

We propose that the development of sexual "Gestalts" and sexual "scripts" (from the standpoint of both movements and language) are affected strongly by early formative experiences with sexual arousal and reward that feed forward to create desire for distal, proximal, and interactive features that predict the reward state. This occurs to some extent uniquely in the development of everyone's sexual preferences, although some commonalities may be easy to detect in terms of species-specific behavior or stimulation patterns, or as distal features of "attractiveness", such as the gender of the desired individual, race, age, body type, hair or eye color, facial features, and even the intergenerational styles of personal presentation (e.g., differences in facial structure, hair style, presence or absence of pubic, body, and facial hair of pin-ups from the first half of the twentieth century relative to the second half; see Gabor, 1973). It is likely, however, that some of those distal features, such as the gender of the desired partner, are relatively pre-potent, forming through mechanisms related to early critical periods of sexual differentiation and gender role

development, and reinforced naturally and intrinsically during periods of social and emotional attraction, and in the emergence of pleasurable genital arousal that is causally related to the gender of the individual that stimulates it. It is thus hard to escape the conclusion that there are as many sexual preferences as there are people, on a background of a gender orientation that has already been laid down. Every desired feature is to some extent a “fetish” that is sought after in the people we find attractive, in the styles they wear, and in the things they do (and we do with them). Although it is difficult to have rewarding sex with someone we are not attracted to, or worse, disgusted by, it is not impossible. As our rats have shown us, the unconditionally aversive odor of cadaverine can become less aversive and even conditionally desired when paired with a sexual-reward state during their early sexual experiences. There may be some features that are more “hardwired” at the outset, including those that define orientation and perhaps also those that contribute to assortativeness or optimal in- and out-breeding, and experience with sexual reward reinforces them considerably. One prediction of this is that people who experience sexual reward with someone of a non-preferred gender may well come to experience less aversion toward that type of sexual expression under certain circumstances (e.g., Pathela et al., 2006). This does not mean that sexual orientation and preferences can be altered once they are established. Reward gives us the power to know what we like, and directs our attentional mechanisms to focus on cues that predict the reward—to the exclusion of cues that do not, even if they *could*.

The fact that no one dies from lack of sex does not mean we don’t die trying. One of the important repercussions of making sex rewarding is that many individuals will try to do it despite the potential of predation or murder. Another repercussion is that a major source biological “determinism” for partner preference will be based on one’s own egocentric experience with cues that predict sexual reward. But this might make the most evolutionary sense of all, especially in a species in which individuals are, well, *individual* from a genetic and experiential perspective. If it is all about finding the “best” features of a mate that promote genetic survival, what then maintains diversity in a species, especially in those that are inbred or that live in unchanged environments? Shouldn’t the strong just eliminate the weak? Diversity in a species makes hybrid vigor possible and thus enhances survival across generations, especially when environmental changes occur in an unpredictable manner. Bonding to epigamic characteristics that are not associated with reproductive “strength” or “fitness” in one environment or epoch, may ensure survival in another. Moreover, reproduction may well be a fringe-benefit of sex that serves reward. As such, just having sex, especially good sex, makes an adult in any species “successful” from a more proximate sexual reward standpoint.

To the extent that mechanisms of sexual reward sensitize mechanisms of sexual bonding, it is likely that sexual reward and bonding merge in conscious awareness under the right circumstances

as romantic love (Aron, Fisher, Mashek, Li, & Brown, 2005; Fisher, Aron, Mashek, Li, & Brown, 2002), and bonded individuals can then begin to create the conditions for parental and even alloparental care. But they don’t have to. And that is as true for heterosexuals as it is for homosexuals and paraphilic individuals who favor inanimate objects. There is not one hormonal or gamete-related reason why gays and lesbians “can’t” reproduce, except that most do not desire heterosexual contact. Indeed, the number of gay men who provide sperm, and lesbians who become pregnant through artificial insemination, is growing in Western culture, as are the number of gays and lesbians who commit themselves to raising children. Does this reflect some emergent evolutionary change in homosexual brains or reproductive status? Probably not. More likely it reflects the fact that sexual reward and reproduction are separate and highly flexible processes that can interact under the right circumstances, and that our current cultural and legal climate has finally made it available.

The study of sexual learning in animals has given us a fresh perspective into the biological and social mechanisms of sexual reward and desire, and refined our knowledge of how patterns of sexual behavior crystallize during critical periods of early sexual experience. That allegedly polygamous rats show elements of monogamy suggests an extraordinary level of flexibility in brain function that can alter preferences dramatically as a function of early experience. Thus, Bagemihl’s (1999) concept of “biological exuberance” applies not just to homosexual behavior as a form of natural diversity, but to *all* forms of sexual expression and preference, no matter how constrained or socially sanctioned. To understand desire and preference we must understand reward and how it connects the external world to our internal representation of it.

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# Sex for fun: a synthesis of human and animal neurobiology

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**Abstract** | Sex is a fundamental pleasure, and crucial to the survival of our species. Though not many people would disagree with the proposition that sexual behaviour depends on the brain, the neuroscientific study of human sex is still relatively taboo and much remains to be discovered. On the contrary, excellent experimental animal models (mostly rat) are available that have uncovered major behavioural, neurochemical, and neuroanatomical characteristics of sexual behaviour. Restructuring sexual behaviour into broader terms reflecting behavioural states (wanting, liking, and inhibition) facilitates species comparison, revealing many similarities between animal and human sexual pleasure cycles, some of which can serve as potential avenues of new human sex research. In particular, behavioural and brain evidence clearly shows that motivational and consummatory phases are fundamentally distinct, and that genitally-induced sexual reward is a major factor in sexual learning mechanisms.

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## Introduction

Sex has the potential to be both pleasurable and frustrating. Attitudes vary such that, perhaps surprisingly, sexual dysfunctions including sexual desire disorder, anorgasmia, and premature ejaculation are not always perceived as problematic.<sup>1,2</sup> On the other hand, even when their sexual function is well within the normal range, individuals might deem their sexual experiences unsatisfying or their performance inadequate.<sup>3–5</sup> Although crucial to the survival of our species, human sex is clearly more complex than mere reproduction.

Neuroscience can provide a better understanding of the behavioural mechanisms involved in human sex.<sup>6–8</sup> In recent years, increasing emphasis has been put on understanding sexual reward-based learning and sexual incentive motivation. Similar to other forms of learning, sexual behaviour develops over time as people learn to associate stimuli such as bodily features, personality, and contextual cues with genitally-induced sexual pleasure.<sup>7</sup> Adolescence is arguably the most critical phase in sexual development.

The spectacular progress of human functional brain imaging in recent years has not completely bypassed the sexual domain, but the resulting data are often descriptive rather than mechanistic.<sup>6</sup> By contrast, experimental animal (mostly rat) studies have provided invaluable insights into the way the central nervous system organizes sexual reward and sexual incentive motivation,<sup>7–9</sup> although the question of whether animal data can be translated to human sexual behaviour remains unanswered (Box 1).

In this Review we provide a comprehensive description of the available animal and human literature on sexual

reward and sexual incentive motivation. We structure our Review around the sexual pleasure cycle, which has phases of wanting, liking and inhibition that map onto the more classical concepts of sexual excitement, plateau, orgasm and refraction. Following this sexual pleasure template, we highlight the major points of convergence and divergence across species, discuss a neural concept of human sexual behavioural control and suggest novel testable hypotheses for future sex research.

## The sexual pleasure cycle

Sexual response is at the core of sexual behaviour and is perhaps best conceptualized as the recurring cycle of events and behaviours that, in a heterosexual couple, can potentially lead to reproduction. Excitement (emotional desire, genital arousal), plateau (physical sexual activity and bodily and genital arousal), orgasm, and refraction are the phases of sexual pleasure that are traditionally distinguished.<sup>10</sup> The sexual pleasure cycle adheres to the basic structure of pleasure cycles related to other rewards (such as food), and can therefore also be expressed in terms of motivation–consummation–satiety or wanting–liking–inhibition (Figure 1; Box 2).<sup>6,11,12</sup>

Sexual behaviour is orchestrated by the brain through integration of incoming sensory information with the internal state. This might seem at odds with the fact that erection and ejaculation are organized as spinal reflexes,<sup>13,14</sup> and the fact that these reflexes can be functional in people with complete spinal cord transections;<sup>15</sup> however, sexual behaviour and sexual pleasure require integration of many different types of information, many of which are not genital. For example, the optimal use of sildenafil requires sexual desire, which can be triggered by the presence of a sexual partner or visual erotic stimuli.

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## Competing interests

The authors declare no competing interests.



In the following sections, we discuss what is known about the major brain mechanisms of the sexual pleasure cycle spanning sexual wanting, liking, and inhibition.

## Wanting sex

Sexual desire can be induced by virtually any external stimuli, encompassing touch, vision, scent, sound, and also mental representations. Unconditioned sexual stimuli (that is, those for which the pleasurable effect requires no learning) include proximal genital tactile stimulation in humans and distal stimuli such as pheromones, odours, and certain auditory vocalizations in rats.<sup>7,16</sup> Popular belief holds that humans also respond to some distal sexual incentive stimuli (breasts, pheromones) in an unconditioned manner, but this has been difficult to evaluate empirically (Box 1). In the following sections, we discuss the animal and human studies that have improved our knowledge of sexual incentive stimuli.

## Animal studies

Most sexual incentive stimuli are conditioned by genitally-induced sexual reward states, such as high sexual arousal and orgasm, such that they become predictors of sexual reward. However, nongenital contextual cues can also become sexual reward predictors, and hence preferred. This means a vast array of stimuli can become sexual in the right circumstances, and can even lead to the development of fetishes, or sexual wanting induced by inanimate objects.<sup>7,17</sup> This, in turn, can have major repercussions for copulatory behaviour. A striking example of this is the use of a rodent jacket as a somatosensory cue. Repeatedly pairing the jacket with each exposure to, and copulation with, sexually receptive females (made receptive at 4-day intervals to approximate the normal ovulatory cycle), leads to the jacket eliciting conditioned sexual reward in male rats, such that paired rats show dramatic copulatory deficits when not wearing the jacket.<sup>7</sup>

Reviewing the animal literature reveals that sexual wanting is stimulated by excitatory neurochemical mechanisms in the brain, including mesolimbic and incertohypothalamic dopamine critical for sexual incentive motivation, oxytocin and melanocortins acting in the hypothalamus and limbic system to stimulate sexual attraction, and ascending noradrenaline acting in hypothalamic, limbic, and cortical regions to augment sexual arousal.<sup>8</sup> Although these systems are activated by sexually relevant stimuli, they are primed for activation by gonadal steroids acting at both genomic and nongenomic levels to create a state of sexual arousability and responsivity.<sup>7,18</sup> This can occur via direct activation of excitatory substrates,<sup>18</sup> or through a process of disinhibition, in which systems that mediate stress, sexual refractoriness, or competing motivational states, are themselves inhibited by steroid hormone priming.<sup>19</sup> Thus, male animals that are not seasonal breeders and do not undergo a seasonal phase of testicular regression have an ambient state of sexual arousability<sup>20</sup> and responsivity, whereas female animals experience cyclic peaks in desire and arousability around the time of ovulation.<sup>21</sup> A similar situation applies to humans.

## Key points

- Restructuring sexual behaviour into broader terms reflecting behavioural states (wanting, liking, and inhibition) facilitates species comparison; similarities between animal and human sexual pleasure cycles can serve as potential avenues of new human sex research
- Sexual wanting in both rats and humans involves interaction between gonadal hormones and external stimuli that become sexual incentives through association with genitally-induced sexual reward; pleasurable genital stimulation is thus a major factor in sexual learning
- In terms of underlying brain networks and neurochemistry identified in both rat and human, wanting sex is something completely different to liking sex
- Sexual inhibition involves similar brain mechanisms in rats and humans
- Rats show a similar pattern of brain activation to humans in response to cues related to sexual reward
- Cortical, limbic, hypothalamic, and cerebellar regions are activated by sex-related stimuli in both humans and rats

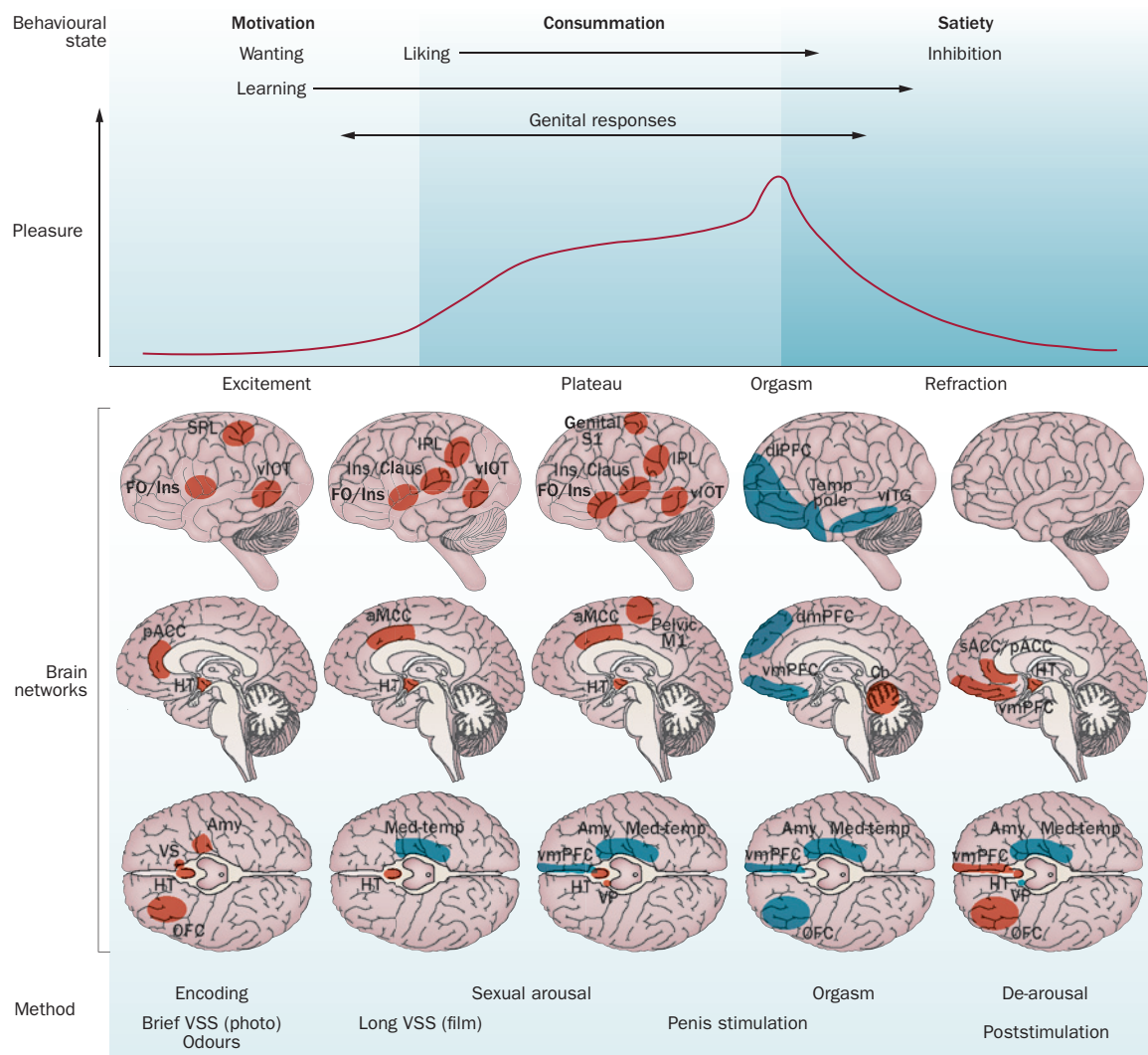
## Box 1 | Species differences in sexual behaviour

In his intriguing behavioural analysis of sex, Anders Ågmo<sup>16</sup> makes a strong argument that the human is not simply just another mammal. In his view, there are two major reasons for this: social learning (that is, learning from observation) and language (which might be associated with vivid mental representations). Introspection and moral thinking might be additional factors to take into consideration. These functions certainly contribute to any human behaviour and there is no reason to believe that they are not involved in sex.<sup>125</sup> This might explain the divergence in, for example, recruited brain networks during sex between animals and humans. In addition to these factors, Ågmo proposed that in humans all sexual incentive stimuli are learned, whereas animals can at least depend on a fixed set of unconditioned sexual stimuli (pheromones, vocalizations). Some human studies have suggested pheromones are involved in sexual attraction,<sup>126</sup> but the fact remains that humans lack a functional vomeronasal organ (the main pheromone detection organ in rats).<sup>127</sup> The full extent of sexual divergence between species remains to be elucidated, but human sexual behaviours and preferences are certainly extremely versatile, ranging from paedophilia to gerophilia, and from tantric sex to sadomasochistic practices. Under natural conditions, the same sexual variety is not observed in any other species (though some primates show extraordinary versatility).<sup>128</sup> Notably, social perceptions can critically drive sexual preferences in humans (for example, in terms of body shape), and these perceptions vary greatly over time and between populations.<sup>129</sup>

## Human studies

Human brain activity can be measured with neuroimaging techniques (Box 3). Neuroimaging studies of sexual incentive stimuli have mainly employed passive viewing paradigms in healthy men.<sup>6</sup> Women are somewhat under-represented in imaging studies, which could reflect researchers' hesitance to deal with 'female' methodological challenges related to their menstrual cycle, genital responses, attitude towards visual sexual stimulation (VSS), or relative discordance between reported and measured sexual arousal.<sup>6,11,12</sup>

Humans are extremely sensitive to their preferred sexual stimuli; when presented subliminally, VSS can induce both an attention bias<sup>22</sup> and measurable brain responses,<sup>23,24</sup> which are largely observed in the ventral striatum (which includes the nucleus accumbens), the amygdala, the anterior cingulate cortex (ACC), and the orbitofrontal cortex (OFC).<sup>23,24</sup> Responses to sexual incentive stimuli in the area of the nucleus accumbens and in the ACC are sensitive to pharmacological manipulation of dopamine levels,<sup>24</sup> which suggests an important parallel with rodent physiology.



**Figure 1** | The sexual pleasure cycle. The sexual response cycle involves phases related to excitement, plateau, orgasm, and refraction, which can also be expressed in terms of wanting, liking and inhibition. Sexual liking typically peaks at orgasm, but is by no means restricted to it. Orgasm (especially in men) tends to signal transition to the satiety phase, which can temporally prevent the individual from entering the cycle again. The sexual response cycle typically occurs over a relatively short time span (from minutes to hours) and may or may not include all of the phases and elements described. Neuroimaging studies have elucidated the key nodes and hubs that are responsible for sustaining and switching between the different phases of the pleasure cycle and can lead to changes in behaviour. VSS paradigms are likely to elicit sexual motivation and wanting. Genital stimulation is usually required to enter the consummatory plateau. The brain regions involved in mediating changes in the sexual response cycle are shown on only one side of the brain but most regions are involved bilaterally. Some regions have multiple roles at different points in the sexual response cycle, for example activity in the amygdala is involved in encoding sexual salience, sexual arousal, orgasm, and poststimulation. Permission obtained from Elsevier Ltd © Georgiadis, J. R. & Kringelbach, M. L. *Prog. Neurobiol.* **98**, 49–81 (2012). Abbreviations: aMCC, middle cingulate cortex (anterior part); Amy, amygdala; Cb, cerebellum; dlPFC, dorsolateral prefrontal cortex; dmPFC, dorsomedial prefrontal cortex; FO/Ins, frontal operculum/anterior insula; Genital S1, primary somatosensory cortex of external genitalia; HT, hypothalamus; Ins/Claus, posterior insula/claustrum; IPL, intraparietal lobule; Med-temp, medial temporal lobe; OFC, orbitofrontal cortex; pACC, pregenual anterior cingulate cortex; Pelvic M1, primary motor cortex of pelvic floor; sACC, subgenual anterior cingulate cortex; SPL, superior parietal lobule; Temp pole, temporal pole; vITG, ventral aspect of inferior temporal gyrus; vmPFC, ventromedial prefrontal cortex; vIOT, ventrolateral occipitotemporal cortex; VP, ventral pallidum; VS, ventral striatum/nucleus accumbens; VSS, visual sexual stimulation.

Further evidence for the involvement of meso-limbic dopamine in sexual wanting comes from studies on natural interindividual variations in expression of dopamine-related genes, which can be assessed in either peripheral venous blood or buccal cells from the mouth. Individuals with genetically predisposed high synaptic

dopamine not only had the strongest nucleus accumbens activity (measured with functional MRI [fMRI]) in response to monetary rewards,<sup>25</sup> but also the highest number of sexual partners<sup>26</sup> and the lowest age of first sexual intercourse.<sup>27</sup> Moreover, sublingual apomorphine (a dopamine agonist) could elicit penile erection in men

with psychogenic erectile dysfunction while they watched a long erotic video, although this did not coincide with measurable mesolimbic activity.<sup>28,29</sup> Indeed, VSS videos >30 s long very rarely yield measurable activity in the nucleus accumbens, suggesting that mesolimbic dopamine is critical for the onset of sexual wanting but not for sustained genital responses.<sup>6</sup>

Natural variations in gonadal steroid levels, between and within individuals, affect both reported sexual desire and the strength of overall VSS-induced brain activity.<sup>30–32</sup> Although these observations support the critical role of sex steroids in sexual wanting, the association is tentative at best until controlled studies are performed, including direct statistical group comparisons or placebo treatment. Better controlled studies have been performed outside the field of sexual medicine. In women, nucleus accumbens activity induced by anticipation of a monetary reward varies over the course of the menstrual cycle,<sup>33</sup> and can be manipulated by exogenous testosterone.<sup>34</sup> Similar interactions between gonadal hormones and mesolimbic dopamine might fuel sexual wanting, as suggested by animal research.<sup>7,18</sup>

Emotional stimuli are better remembered than neutral stimuli and this function depends heavily on the amygdala.<sup>35</sup> The same holds true for VSS; individual differences in VSS-induced amygdala activity can predict differences in recall of those stimuli 4 weeks later.<sup>36</sup> The ability of VSS to make a lasting impression is probably due to its incentive value, which, in turn, might be associated with the magnitude of sexual pleasure induced by such stimuli. Such associations might be instrumental in classical conditioning of sexual responses. In a series of studies performed in women, pleasurable clitoral stimulation (the unconditioned stimulus [UCS]) was paired with neutral visual stimuli (the conditioned stimulus [CS]). After 8–10 trials the neutral stimuli acquired reinforcing properties, meaning that presentation of the CS alone elicited enhanced vaginal pulse amplitude (a genital measure of sexual desire and arousal).<sup>37–39</sup>

Gluck and co-workers<sup>40</sup> revealed a central signature of sexual conditioning in healthy men and women. After pairing two different geometric shapes (CS) with either VSS (sexual reward, UCS) or nonrewarding (neutral) stimuli (non-UCS), volunteers were invited to participate in an fMRI study. While the VSS-predicting geometric shape (CS+) came to produce subjective sexual desire in some of the volunteers, the non-UCS predicting shape (CS–) did not do so in any of the volunteers. Accordingly, watching the CS+, compared to CS–, resulted in increased activity in the OFC, ACC, anterior insula, nucleus accumbens and midbrain. Of note, volunteers who reported that they had inferred the contingency between CS+ and UCS ('aware') showed stronger activity in this network than those who remained oblivious to it ('unaware'). Most aware volunteers were men, and most unaware volunteers were women.<sup>40</sup>

In another study,<sup>41</sup> men were shown visual cues that indicated the likelihood (25% or 75%) of being presented with pictures of attractive young women. The women in the pictures were either naked or wearing a bathing suit, the occurrence of which was also predicted in the cue.

## Box 2 | Terminology of sexual behaviour research

Sexual response has been conceived both in terms of dichotomies that reflect the processes of excitement and inhibition and as a cycle that moves through sequences of sexual or copulatory behaviour. Dichotomies typically reflect initiation and termination of sexual response along a general appetitive (behaviours that increase the probability of achieving a goal) versus consummatory (behaviours engaged with the goal) continuum. Included here are distinctions between courtship and copulation, motivation and performance, among others. The response cycle concept was made popular by Masters and Johnson in their 'EPOR' model,<sup>10</sup> which involves the transition from excitement (a mix of genital arousal and emotional desire for sex) to a plateau of arousal as sexual activity ensues, to an apex of excitement (orgasm), and then to an immediate resolution or refractory phase. In men, the refractory phase is generally longer than in women, especially those who are capable of having a series of orgasms. Superimposed on this are clinical dichotomies of conscious awareness, as reflected in objective versus subjective measures of arousal, desire, orgasm, and resolution. The sexual response cycle has more recently been compared to other well-established pleasure cycles that consist of phases of wanting, liking, and inhibition.<sup>6</sup> In this way, sexual responses follow a pattern similar to other motivations, such as thirst, hunger, and drug addiction, in which an individual wants or craves, likes (or dislikes), and experiences feedback in the form of satisfaction of the need state. These conceptualizations are essentially heuristics that allow researchers to separate sexual responses into the activation of different brain regions or neurochemical systems. Wanting and liking are distinguished by different systems in the brain. Wanting is driven by mesolimbic dopamine<sup>130</sup> that focuses attention and behaviour toward acquiring sexual incentives,<sup>8</sup> whereas liking is synonymous with reward or pleasure, and is driven by opioid and other reward systems.<sup>8</sup> Inhibition as either direct or indirect feedback regulation, is driven by opioid, serotonin, and endocannabinoid systems in the brain.<sup>8</sup> In this way, direct comparisons can be made between men and women, and across species, both in terms of the behaviours that are expressed in each phase and the neuroanatomical and neurochemical systems that underlie them.

Thus, expectancies ranged from a low chance of getting a minor sexual reward to a high chance of obtaining a reasonable sexual reward. When volunteers watched the stimuli in the fMRI scanner, the positive subjective value assigned to the stimuli (which was generally higher for the nude woman reward) correlated with activity in a number of brain regions, most notably the OFC, ACC, anterior insula, nucleus accumbens and midbrain. Importantly, the same set of brain regions were involved in processing the reward prediction error, that is, the degree of incongruence between the reward expected and obtained.<sup>41</sup>

These findings reveal a network of brain regions that are particularly sensitive to sexual incentive stimuli, reward contingencies and expectancy. By extrapolation, therefore, these areas might underlie sexual wanting. However, basically the same network is involved in processing the desire for other incentives, such as food.<sup>6</sup> Moreover, the regions comprising this putative 'sexual interest network' are not—or less—involved when high levels of sexual arousal are involved, suggesting that their role is primarily restricted to recognizing sexual opportunity and directing motivated behaviour accordingly (Figure 1).<sup>6</sup>

## Liking sex

Physical sexual activity can lead to enhanced genital and cardiovascular arousal, and genital stimulation can lead to orgasm. Opiates, such as heroin, produce a rush of euphoria followed by a prolonged period of relaxation,<sup>42</sup> a state that has been referred to as a 'pharmacogenic orgasm'.<sup>43</sup>



**Box 3** | Methods used to study the brain control of sex in animals and humans

Knowledge of brain activation by sexual stimulation or sex-related cues in rats comes from a variety of techniques, including lesions, electrical recording, and the activation of immediate-early gene products, such as Fos, that are typically expressed in the nuclei of cells that are activated by the stimuli. Additional neuropharmacological manipulations that activate or block specific neurotransmitter receptors in specific brain regions, or that use microdialysis or voltammetry to assess neurochemical turnover in extracellular fluid, have also been used extensively. Molecular studies in brain tissue have revealed hormone-induced transcription factor activation that has had a profound impact on our understanding of how hormones activate genes to make proteins that, when expressed, alter the functionality of brain pathways, making animals attend more to sex-related cues than other external incentives. Human functional neuroimaging works through the mechanism of neurovascular coupling, whereby local neural activity is followed by measurable blood flow, volume and oxygenation changes.<sup>131</sup> PET and functional MRI (fMRI) are the most widely used techniques. Though fMRI clearly outperforms PET in terms of the spatial and temporal (continuous versus intermittent scanning) resolution, and hence popularity, it is important to realize that both techniques measure indirect traces of pooled neuronal activity, often over many seconds. An inevitable consequence of sexual functional neuroimaging experiments is that sex happens at a preset time, in a preset way, and in a preset place. Though natural human sexual activity may also be less spontaneous than we sometimes would like to admit, the current state of sexual functional neuroimaging experiments reflects approximations of real sexual confrontations at best.

This opioid reward state induces a dramatic decline in sexual wanting in both men and women, and inhibits the ability to achieve orgasm during subsequent sexual intercourse.<sup>42</sup> It has been argued that a 'natural' version of this state is induced by human orgasm<sup>44</sup> and by certain copulatory behaviours in rats; for example, ejaculation in male rats induces the release of endogenous opioids.<sup>45</sup> Early phases of the sexual pleasure cycle might induce considerable positive affect and carry positive incentive value, although genital stimulation and orgasm in humans, and genital stimulation and ejaculation in male animals, are considered the chief phases that provide positive incentive value.

**Genital source of sexual reward**

There are various nongenital methods of achieving orgasm,<sup>46</sup> but the primary source of sexual pleasure is the brain's response to genital stimulation.<sup>8,47</sup> Spinal cord injury does not always completely eliminate genital sensation or functionality because sensory fibres from external and internal genitalia run through four main nerves—one somatosensory (pudendal) and three autonomic (hypogastric, pelvic, vagal)—that enter the central nervous system at different levels.<sup>15</sup>

Sensations that arise from stimulation of the glans of the penis or clitoris are thought to be the most important contributors to genitally-induced sexual reward (Figure 2). Indeed, anaesthetic lidocaine spray applied topically to the glans penis significantly prolongs ejaculation latency time.<sup>48</sup> Likewise, clitoral stimulation is the easiest way for women to achieve orgasm.<sup>49</sup> These similarities are not surprising given that, in mammals, the clitoris and penis follow the same developmental path prior to differentiation,<sup>50</sup> resulting in virtually identical innervation with similar distribution of central nervous system afferents and efferents.<sup>51–53</sup>

The role of the penis glans in sexual liking has been studied much more than that of the clitoris. Millions of years of copulation seem to have resulted in evolutionary pressures that have tailored glans penis morphology and neurophysiology so that it is suited to the stimulatory elements that comprise an aroused vagina: warmth, lubrication, and friction. For example, for bulls to penetrate a rubber tube for semen collection, the tube must be sufficiently warm (>42 °C),<sup>54</sup> suggesting that glans receptors might activate genital reward only within a narrow stimulatory range. Notably, many other types of stimulation, besides vaginal, might fulfill these conditions to provide genital reward. However, our current understanding of the specific receptive properties of the glans is incomplete. Although the sensory threshold of the penis (including glans) to high frequency vibratory stimulation has been assessed,<sup>55</sup> psychophysical studies in humans using more naturalistic stimulation are lacking.

Morphological studies conducted on human and rat penises have shown that the receptor constellation of the glans penis is remarkably similar across species and that it is unlike that of other skin.<sup>56,57</sup> Specifically, the glans penis is remarkable for an abundance of free nerve endings and thin C and Aδ fibres typical of protopathic somatosensation.<sup>56,57</sup> Moreover, it contains coiled receptors—genital end bulbs—that are not found anywhere else in the body.<sup>56,57</sup> These receptors seem to change their orientation during erection,<sup>56</sup> which might conceivably underlie the different sensitivity of the penis under engorged versus flaccid circumstances. Encapsulated mechanoreceptors and thick Aβ fibres, structures classically associated with touch, are virtually absent in the glans,<sup>56,57</sup> leading to the intriguing possibility that friction of the glans, a mechanical stimulus, is processed through the thin fibre protopathic route. Affective touch, that is the speed and pressure associated with a gentle caress, is known to be signalled through C-fibres in the arms and legs.<sup>58,59</sup> Similarly, friction of the glans is a hallmark of affective touch and a critical component of genital reward.

Thus, there is evidence to suggest that sexual liking is deeply encoded in mammalian genitalia, most notably in the glans, which might be considered a specialized sensor for somatosensory stimuli that result in genital reward. Stimulation of other skin areas, such as nipples,<sup>60</sup> is known to incite and augment sexual pleasure, but whether this is unconditioned or learned is an open question. Experimental partial lesions of the spinal cord in rats have demonstrated that sensory information important for ejaculation runs in the ventrolateral (spinothalamic) columns.<sup>61</sup> Indeed, men with injury to the ventral part of the spinal cord are likely to develop anorgasmia.<sup>62</sup> The spinothalamic tract is the main afferent pathway for protopathic information (such as pain), which fits with the concept of penis glans innervation outlined above. Similar studies on the clitoris do not exist. However, animal studies using anatomical tracers injected into penis and clitoris glans seem to indicate that the way clitoral and penile stimulation is conveyed to the brain is likely to be similar.<sup>51,52</sup> Pelvis viscera, including the prostate, the vaginal wall and vaginocervix, probably express different

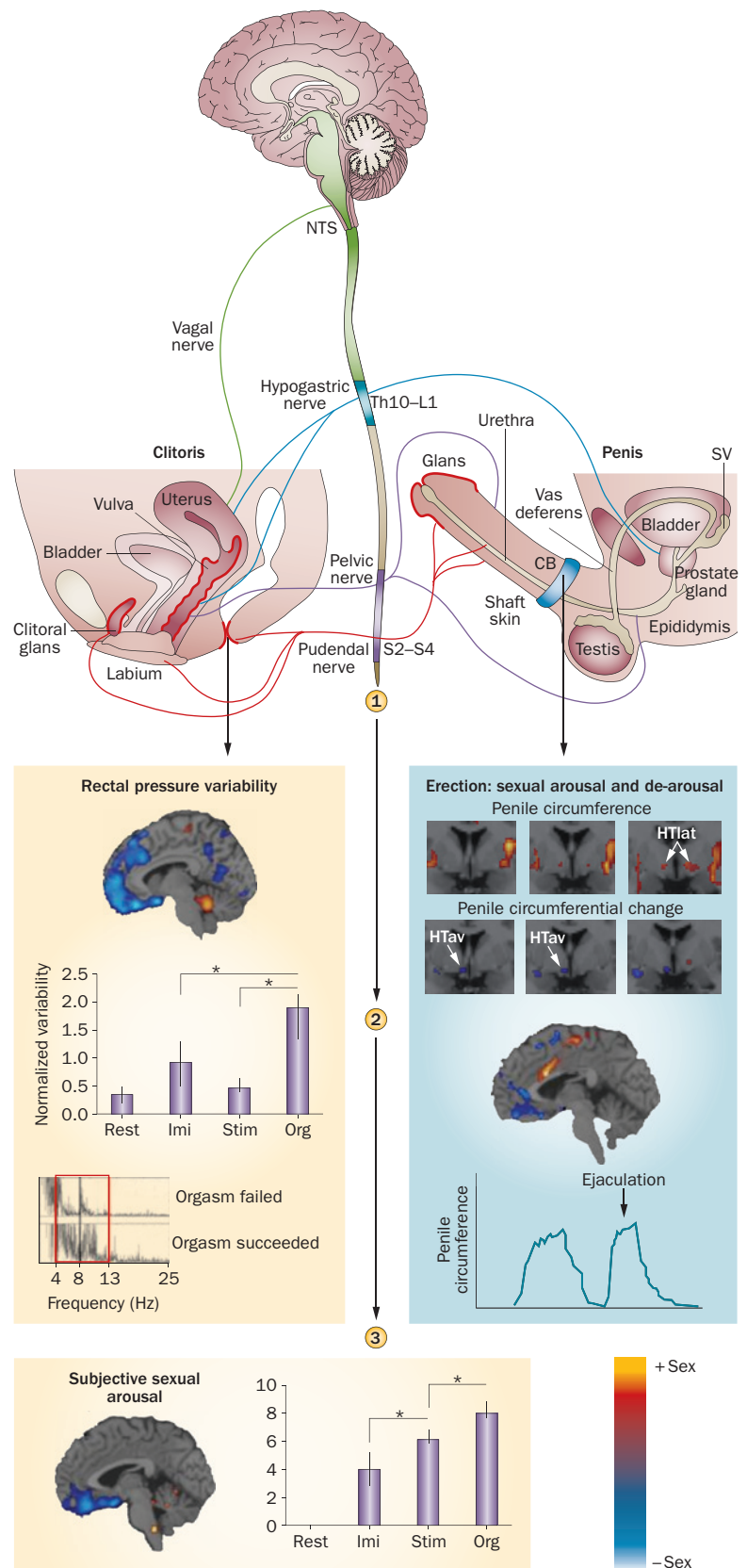
**Figure 2** | Partnered stimulation protocols (PSPs) to study the central mechanisms of human sexual consummation. (1) Partnered stimulation of the clitoris or penis was performed with simultaneous measurement of rectal pressure in women or penile circumference in men. The four major genital nerves are also shown, with the pudendal nerve (which innervates penis and clitoris) marked in red. (2) In women, rectal pressure patterns during orgasm had clearly distinguishable fast frequency characteristics, which correlated positively with cerebellar blood flow (orange). Negative correlations were observed in ventral and dorsal parts of the prefrontal cortex (blue). In men, positive correlations with penis stimulation, penile circumference, and the rate of circumferential change, were seen in a number of areas including the middle cingulate cortex (sagittal section, orange) and lateral hypothalamus (top panel, orange). After stimulation ceased, and arousal decreased, a completely different network became active, including ventral prefrontal cortex, rostral cingulate cortex (sagittal section, blue), and the anterior hypothalamus (top panel, blue). Differences between men and women with respect to cingulate and hypothalamic effects may reflect the superior sensitivity of the continuous measurements in the male PSP relative to the intermittent nature of the female PSP. (3) Subjective reports of sexual pleasure in women obtained after each scan correlated negatively with ventral prefrontal (3, blue) and medial temporal blood flow (not visible in section). \*Highlights significant differences. Abbreviations: HTav, anteroventral hypothalamus; HTlat, lateral hypothalamus.

receptors, but their role in sexual liking is undisputed. At present, however, this notion is primarily based on popular belief, anecdotal reports and behavioural studies in animals.<sup>63</sup>

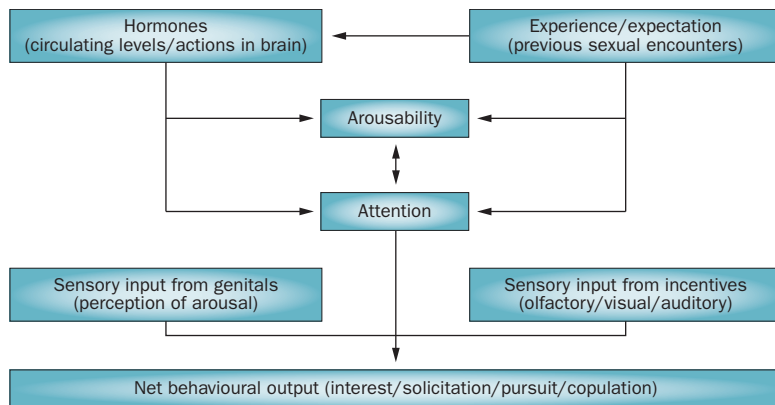
### The neural basis of genital reward

#### Animal studies

The neural basis of sexual liking has primarily been elucidated in animal studies. In rats, preferences can be instated through coupling with genital reward, leading, for example, to conditioned preferences for place or partner.<sup>7</sup> In male rats, ejaculation induces the reward state necessary for the induction of both conditioned place preference (CPP) and preferences for partner cues that are associated with the reward states.<sup>7,64,65</sup> In female rats, the ability to control or 'pace' the initiation and rate of copulation induces the reward state. This conditioning is achieved by repeated association of the reward state with a particular set of place cues in the CPP box, or with a particular partner cue (for example, almond odour on the partner). Animals are trained to contrast these cues with a different set of place or partner cues associated with sexual nonreward, such that on the final test day animals are given a choice between place or partner cues of high reward versus cues of lower reward or no reward. Not surprisingly, on the final test animals prefer the high reward cues. The administration of an opioid receptor antagonist, such as naloxone, during training blocks the development of such preferences suggesting that the reward state is dependent on opioid transmission.<sup>65</sup> Furthermore, injections of opioid antagonists can reverse the sexual inhibition displayed by male rats after



reaching sexual exhaustion.<sup>66</sup> Similarly, in female rats, CPP is blocked if naloxone is administered during training.<sup>67-71</sup> Manual clitoral or vaginocervical stimulation associated with place or partner cues can induce CPP



**Figure 3** | Scheme representing the most important neurobiological and behavioural elements of sexual responsiveness, as well as the interaction between them.

in sexually naïve rats,<sup>9,72,73</sup> and females selectively solicit sex from males bearing an odour associated with manual clitoral stimulation.<sup>74</sup>

Interestingly, in male rats, the reward state also requires a critical level of sexual arousal prior to ejaculation to support the conditioned preferences,<sup>75</sup> suggesting an interaction between the intensity of sexual activity prior to ejaculation or orgasm and the degree of reward experienced from it. Indeed, in sexually naïve, but not experienced, male rats, sexual intercourse without ejaculation supports the development of CPP.<sup>76</sup>

Studies examining *Fos* induction (a marker of neuronal activity) in the brains of male and female rats have revealed a common set of regions activated by copulatory stimulation.<sup>77</sup> Additionally, microdialysis has been performed to assess dopamine and glutamate transmission in the nucleus accumbens and hypothalamus during copulation. In both male and female rats, a number of relatively common neural structures are activated by copulatory stimulation, including sensory nerves that innervate the penis or vagina or cervix, by unconditioned olfactory or pheromonal stimuli, or by conditioned sexual incentives. This network includes specific subdivisions of the hypothalamic area, basal forebrain, thalamus, amygdala, and hippocampus, all of which contain classic nuclear hormone receptors.<sup>18</sup> Regions that do not contain classic intracellular steroid receptors, such as the ventral and dorsal striatum, and areas of cortex, are also activated.<sup>77</sup>

*Fos* has also been found colocalized with the cytoplasmic proteins gonadotropin releasing hormone, glutamate, dopamine, and oxytocin.<sup>77</sup> There is significant overlap in brain regions activated by ejaculation and vaginocervical stimulation in male and female rats, respectively, including the medial amygdala (posterior dorsal part), the subparafascicular (parvocellular) and intralaminar thalamus, the lateral putamen and claustrum, and the ventromedial hypothalamus.<sup>77</sup>

Several cortical regions are activated in both male and female rats by copulatory stimulation, including cingulate, piriform, somatosensory, and entorhinal cortex.<sup>77</sup> The insula is also activated by copulation with a preferred scented partner, and to a lesser extent by the conditioned odour alone. This is of particular interest because those

regions are also activated in humans during sexual activity<sup>78</sup> and while observing erotic pictures or films.<sup>79,80</sup> The ability of neutral odours to activate many of these regions following pairing with sexual reward states (induced by ejaculation in male rats and paced copulation in female rats) suggests that they are acting as conditional priming stimuli that generate anticipatory sexual responses in animals.<sup>7</sup>

Dopamine transmission in the nucleus accumbens is a critical substrate for sexual incentive motivation.<sup>81</sup> In both male and female rats, extracellular concentrations of dopamine increase during the presentation of a sexually receptive partner and during copulation. However, in male rats ejaculation produces a dramatic decrease in nucleus accumbens dopamine release that remains low during the absolute refractory phase, but moves back upward during the relative refractory period.<sup>82</sup> A neutral odour paired with the postejaculatory reward state in male rats selectively increases dopamine release in the nucleus accumbens, whereas the same odour unpaired does not.<sup>7</sup> These observations were recently confirmed using single unit recordings from nucleus accumbens shell neurons, as different functional classes of neurons were found to exhibit different firing patterns across the sexual pleasure cycle.<sup>83</sup>

Taken together with data from lesion, physiological, and neuropharmacological studies, the activation of mesolimbic dopamine systems by unconditioned and conditioned sex cues forms the core of an incentive motivational system that critically links sexual wanting to sexual reward. This system is activated in the presence of priming cues, and also by actual sex partners, especially those that bear stimuli predictive of reward (Figure 3). The brain regions discussed are involved in many different behaviours, but they act together as a 'system' for sexual behaviour.

#### Human studies

Human neuroimaging research of genital stimulation is much sparser than that of visual sexual incentives.<sup>6</sup> Furthermore, about half of these studies have been performed using PET (Box 3), rather than fMRI. Genital stimulation can be investigated in sexually aroused or nonaroused volunteers, and can be partnered or involve self-stimulation. In a partnered stimulation protocol with sexual arousal, volunteers lying inside the scanner receive manual genital stimulation by their real-life sexual partners.<sup>6,84</sup> Self-stimulatory protocols with sexual arousal have been performed in the context of vaginal self-stimulation, which required the use of custom-made phallus-like device.<sup>85–87</sup> Partnered protocols have a number of important advantages over genital self-stimulation, including less strong head movements, less interference from the experimenters, and sexual interaction with a real-life sexual partner.

Partnered genital stimulation studies (both aroused and nonaroused) have demonstrated that stimulation of the penis and clitoris leads to activity in the full somatosensory matrix. Most of the evidence shows that the penis and clitoris are represented in deep layers of the dorsal

primary somatosensory cortex.<sup>78,88–92</sup> Furthermore, the penis and clitoris also map to the secondary somatosensory cortex, in the parietal operculum.<sup>78,87,88,90,91,93,94</sup> The third area implicated in genital sensory processing is the insula (middle and posterior).<sup>78,88,91,93,94</sup> Of these areas, the posterior insula is most relevant in processing penile tumescence.<sup>77,79,89,95,96</sup>

The most advanced partnered stimulation protocol reported was an fMRI experiment performed in men (Figure 2),<sup>78</sup> which revealed involvement of the lateral hypothalamus, ventral pallidum, middle cingulate cortex, anterior insula, frontal operculum, inferior parietal lobule, and occipitotemporal cortex in both penile tumescence and sexual penis stimulation. The ventral pallidum, known to mediate food liking in rats,<sup>97,98</sup> signalled the onset of penis stimulation and, through decreased activity possibly reflecting inhibitory processes, the absence of penis stimulation.<sup>78</sup> These areas seem to be part of a 'sexual consummation network' that is fundamentally distinct from the earlier mentioned 'sexual interest network'.<sup>6</sup>

Georgiadis and Kringelbach<sup>6</sup> recently identified the substantial neuroanatomical overlap between sexual consummation and sympathetic arousal states induced by music, cocaine, or pain.<sup>6</sup> As mentioned previously, in male rats the intensity of sexual activity (prior to ejaculation) is predictive of the reward experienced,<sup>75</sup> which is supported by recent ideas that sympathetic arousal plays a crucial role in mammalian sexual activity.<sup>99,100</sup> This might explain how people and animals can transfer from a non-sexual high arousal state to sexual consummation, or vice versa if the arousal is not correctly identified as sexual.<sup>8</sup>

Activity in some brain regions decreases as sexual pleasure increases. This is true for the amygdala,<sup>78,88,89</sup> even when the amygdala readily responds to distant visual erotica.<sup>41,101,102</sup> However, prolonged amygdala activity corresponds to heightened vigilance,<sup>103</sup> which promotes avoidant responses and precludes physical interaction.<sup>104</sup> Conversely, people are less sensitive to fearful stimuli, such as white noise, during sexual arousal.<sup>105</sup> Downregulated neural activity associated with sexual liking is observed not only in the amygdala, but also in parts of the medial temporal lobe and ventromedial prefrontal cortex.<sup>78,88,93,106</sup> Intriguingly, all of these areas have been shown to be important for interpersonal judgements.<sup>107</sup>

Human orgasms are difficult to study, primarily because of their unpredictable and uncontrolled nature.<sup>84</sup> The most insightful orgasm data come from a partnered clitoral stimulation protocol in women (Figure 2). Orgasm in women was verified by measuring rectal pressure fluctuations; fast pressure fluctuations probably reflect involuntary pelvic muscular activity,<sup>108</sup> and are a defining feature of orgasm in both men and women.<sup>109,110</sup> In the imaging study, fast pressure fluctuations were indeed most prominent during orgasm.<sup>88,108</sup> The level of activity in ventromedial temporal and prefrontal cortices was lowest during orgasm, when sexual arousal was reported to be highest.<sup>88</sup> During orgasm, activity in prefrontal, but not temporal, areas correlated

with the frequency of rectal pressure fluctuations—fast fluctuations were associated with low prefrontal cortex activity. This indicates a relative loss of behavioural control,<sup>111</sup> which is supported by the lack of voluntary pelvic floor motor cortex activity during orgasm.<sup>88</sup>

The brain effect most specific to orgasm was observed in mid-anterior and medial subregions of the OFC.<sup>88</sup> Activity changes in these regions could be used to distinguish between sexual clitoral stimulation and orgasm, which fits with data from other neuroimaging studies demonstrating that similar OFC activity changes represent the subjective pleasure of food intake.<sup>12</sup>

Taking into consideration the temporal resolution of PET, which is about 1 min, the results described here might also be related to postorgasmic sexual inhibition or pre-orgasmic sexual arousal. For the same reason, it is possible that areas active specifically during orgasm might no longer be identified when data are pooled over a 1 min period.

## Sexual inhibition

Sexual inhibition can be induced by stressful events or high sexual rewards, such as during the refractory period after ejaculation in male rats, during which reproductive capacity must be regenerated prior to a resumption of copulation,<sup>8,112</sup> and after multiple paced intromissions and ejaculations in female rats that bring about oestrus termination.<sup>113</sup> In both cases, the activation of inhibitory pathways for sexual arousal and desire generates a state of reduced sexual wanting.

## Animal studies

In rats, activation of opioid, serotonin and endocannabinoid release during sexual reward is associated with an inhibition of ongoing sexual behaviour. Male rats allowed to copulate (with multiple ejaculations) to sexual exhaustion do not respond to female solicitations for a period of 24–72 h. This inhibition can be reversed by the serotonin 1A agonist 8-OH-DPAT (an autoreceptor agonist that inhibits serotonin release), by the  $\alpha_2$  adrenoceptor blocker yohimbine, and by naloxone.<sup>114</sup> Thus, blockade of opioid or serotonin transmission, or activation of parasympathetic pathways involved in erection, can overcome the state of inhibition induced by sexual exhaustion.

Yet, somewhat paradoxically, activation of opioid transmission by stress might also play a role in sexual inhibition. Male rats find new environments stressful. In fact, males that are not desensitized to the environment in which they have their first sexual experiences often do not copulate.<sup>115</sup> Pre-exposure to the environment, or treatment with naloxone, increased the proportion of males that copulated on their first trial.<sup>115</sup> Interestingly, sexually naïve males sensitized to amphetamine do not show inhibition during their first exposure to females in a novel environment, despite exposure to the drug being weeks before.<sup>116</sup> Although sexually experienced male rats show signs of fear in novel environments, they do not show subsequent sexual inhibition if a receptive female is placed into the environment.



Together, these data suggest that stress-induced inhibition of sexual response in male rats can be overcome by sensitization of dopamine systems by either sexual experience or exposure to amphetamines, or the blockade of opioid signalling. Indeed, in male rats trained not to copulate with sexually nonreceptive females, a low dose of alcohol or cocaine can disinhibit sexual advances.<sup>117,118</sup>

Interestingly, it has been shown that pairing exposure to a neutral odour with access to sexually nonreceptive females (which is a nonreward) induces *Fos* expression within regions of the medial prefrontal cortex and central nucleus of the amygdala in male rats.<sup>119</sup> In humans, these regions are associated with behavioural inhibition (as a form of executive function) and stress response, and activity in these regions has been shown to correlate negatively with sexual arousal.<sup>78,88,106</sup>

In female rats, a state of sexual nonreward can be induced by sexual frustration (achieved in an experimental setting by application of clitoral stimulation in the presence of an inaccessible male)<sup>7</sup> or by injections of naloxone (capable of eliminating both sexual place and partner preferences<sup>67,67,70,120</sup>) during the females' first sexual experience with a male. Female rats in such a state of sexual nonreward develop an inhibited sexual response.<sup>7</sup> Despite full hormone priming and an injection of saline on the test day, females do not solicit, show low-level lordosis reflexes (the arching of the back and raising of the rump that allows for clitoral and vaginocervical stimulation by male mounts and intromissions), and engage in intense fighting with males that attempt to mount.<sup>7,68</sup>

### Human studies

Few human neuroimaging studies have addressed sexual inhibition, and most of those that have, did so by presenting VSS to subjects with low sexual desire. Some of these studies seem to indicate that sexual inhibition correlates with prefrontal hyperactivity in hypogonadal individuals,<sup>31,80</sup> and that this hyperactivity can be downregulated by testosterone treatment.<sup>32</sup> Studies of psychogenic hyposexual patients suggest that increased activity in ventral prefrontal<sup>80,121</sup> and dorsal parietal areas<sup>121,122</sup> and decreased activity in the middle cingulate cortex<sup>121,122</sup> are indicative of differences in sexual arousability relative to healthy controls. Interestingly, male hyposexual patients showed sustained superior parietal activity during an erotic video (and a failure to achieve erection), whereas control patients demonstrated a drop in superior parietal activity and a rise in middle cingulate and posterior insula activity in association with penile erection.<sup>121</sup> Indeed, volitional inhibition of sexual arousal in healthy men elevated neural activity in the superior parietal and ventrolateral prefrontal cortex.<sup>102</sup> These observations seem to suggest that sexual inhibition, be it intended or unintended, is related to exaggerated activity of components of the sexual interest network (including the amygdala, superior parietal lobule, and ventral prefrontal cortex). For some reason this prevents a shift to activation of areas belonging to the sexual consummation network (including the ventral pallidum, posterior insula,

and middle cingulate cortex). Importantly, this implies that hyposexual patients might still be able to identify sexual incentives, but these incentives fail to produce a switch to the sexual consummation network.

Poststimulatory de-arousal (including that which occurs during the postejaculatory refractory period) also involves sexual inhibition. A partnered stimulation protocol in healthy men has shown that the amygdala, ventromedial prefrontal cortex, rostral cingulate cortex, and anterior hypothalamus were most active when the rate of penile detumescence was fastest.<sup>78</sup> This is similar to the areas activated in hyposexual individuals presented with VSS. In fact, the anterior hypothalamus is inhibitory to sexual arousal in male rats,<sup>71</sup> while functional connectivity between the anterior hypothalamus and the rostral cingulate cortex might be especially critical in arousal downregulation.<sup>123</sup>

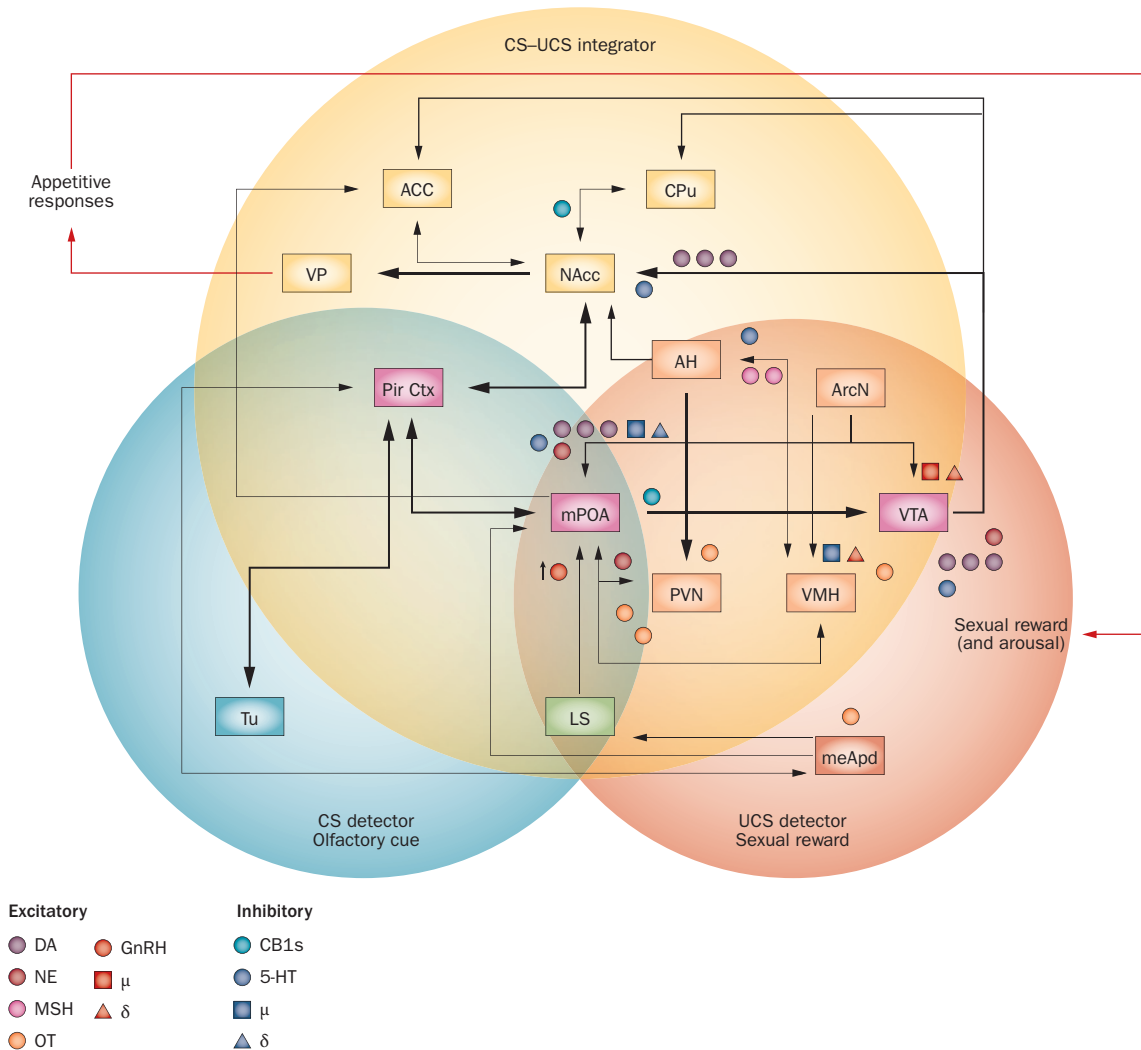
### Conclusions

We have finally started to get a better handle on the functional neurobiology of sexual behaviour. In broad terms, the brain integrates sensory sexual stimuli with the internal state, which in turn gives rise to unconditioned or learned expressions of sexual wanting, liking and inhibition. The restructuring of sexual behaviour—and other survival behaviours<sup>124</sup>—into these three phases is critical in order to achieve a meaningful species comparison; although introspection and subjective feelings are major topics in human research, it is impossible to know how animals perceive the world. The structure of the sexual pleasure cycle proposed here and elsewhere<sup>6</sup> renders such differences irrelevant and allows very different incentives and behavioural expressions displayed by animals and humans to be grouped under the same category. Human and animal studies have thus clearly elucidated the different phases and behaviours of the sexual pleasure cycle (Figure 4).

The evidence shows that sexual wanting in both rats and humans involves an interaction between gonadal hormone levels and external stimuli that become sexual incentives through association with sexual reward. The principal source of this reward is genital; in particular, the glans might be considered an extraordinary sensor for somatosensory stimuli that provide sexual reward. Human sexual behaviour involves balancing activity of distinct brain networks over distinct phases of the sexual response cycle, as well as sexual inhibition. As elucidated primarily by animal models, these phases are supported by quite distinct neurotransmitter systems.

Mesolimbic dopamine release is important throughout the pleasure cycle, but perhaps chiefly in sexual incentive motivation and sexual wanting. Human and rat studies seem to largely agree on this point, though the absence of nucleus accumbens activity during sexual consummation in humans is not in line with persistent nucleus accumbens activity in rats during elements of copulation. It is possible that the complex activity pattern of the nucleus accumbens across the pleasure cycle cannot yet be captured by functional neuroimaging methods, or perhaps the fact that rats constantly chase one another during





**Figure 4** | Neural systems critical for the display of unconditioned and conditioned sexual behaviour in the rat. Appetitive behaviours made toward CS lead to sexual reward that is processed by three interactive systems. Two systems process olfactory stimuli and sexual reward relatively independently, whereas a third, mesolimbic DA system, acts to integrate both the conditioned olfactory cue and its rewarding sexual outcome (UCS). Three common regions, the Pir Ctx, mPOA, and VTA, are activated in male and female rats by conditioned olfactory stimuli. Opioid actions in the VTA potentiate mesolimbic DA activation, whereas opioid actions in the mPOA inhibit sexual arousal and motivation. Opioids can be excitatory in the VTA, inhibitory in the mPOA, or either in the VMH. DA, GnRH, MSH, NE, and OT are excitatory whereas 5-HT and endocannabinoids are inhibitory. Neurotransmitter systems or their receptors in red are excitatory for sexual motivation whereas those in blue are inhibitory. Abbreviations: 5-HT, serotonin; ACC, anterior cingulate cortex; AH, anterior hypothalamus; ArcN, arcuate nucleus of the hypothalamus; CB1, cannabinoid Type 1 receptor; CPU, caudate-putamen (striatum); CS, conditioned stimuli; DA, dopamine;  $\delta$ , delta opioid receptors; GnRH, gonadotropin releasing hormone; LS, lateral septum; MeApd, posterior-dorsal nucleus of the medial amygdala; mPOA, medial preoptic area; MSH, melanocyte stimulating hormone;  $\mu$ , mu opioid receptors; NAcc, nucleus accumbens; NE, noradrenaline; OT, oxytocin; Pir Ctx, piriform cortex; PVN, paraventricular nucleus of the hypothalamus; Tu, olfactory tubercle; UCS, unconditioned stimuli; VMH, ventromedial nucleus of the hypothalamus; VP, ventral pallidum; VTA, ventral tegmental area. Adapted with permission from © Pfaus, J. G., Ismail, N. & Coria-Avila, G. A. in *Encyclopedia of behavioral neuroscience* (vol. 3) (eds Koob, G., Thompson, D. & Le Moal, M.) 201–209 (Elsevier, New York, 2010).

copulation requires a continuously activated mesolimbic dopamine system.

Endogenous opioids are released primarily upon ejaculation in rats, which not only temporarily shuts down sexual wanting through manipulation of mesolimbic dopamine, but also creates the sexual reward state that is most efficient at driving future sexual behaviour through learning (Figure 4). Although this learning mechanism is supported by solid empirical proof in rats, in humans

virtually nothing is known. Nevertheless, conditioning of sexual responses with pleasurable genital stimulation (without orgasm) is feasible in humans. Sexual stimulation without orgasm induces sexual liking as shown in both rats and humans, and it might be that high sympathetic arousal has an important role in both species. Serotonin is generally inhibitory to sexual responses in both species, which might explain the exaggerated prefrontal activity observed in people with low sexual desire.

Many of the same brain regions are active during human and rat sexual response cycles, including the hypothalamus, amygdala, nucleus accumbens, insula, cingulate cortex, and somatosensory cortex. However, in both species the collection of areas that is active at a given point depends on the phase of the sexual pleasure cycle. At least in humans, the shift from sexual wanting to liking induces a major shift in the balance between brain networks. Very similar network shifts can be found in other pleasure cycles, such as food-related behaviours, suggesting that dominant involvement of these networks reflects general behavioural phenomena.<sup>6</sup> Though the evidence is still limited, it seems that people are able to prevent this shift if responding to a sexual incentive stimulus is inappropriate. Conversely, perhaps sexual desire disorder could be explained by the failure to shift the balance between these networks when faced with sexual incentive stimuli, although obviously other explanations cannot be ruled out, such as negative associations with particular sexual incentive stimuli.

The reasons for the divergent changes in activity in brain areas, as well as brain network shifts, between animals and humans are potentially numerous. The most likely explanation is that humans use mental representations, introspective thinking, and empathy during their sexual pleasure cycle, whereas it is unknown whether this is the case for other animals. The human neuroimaging data discussed here have neither the spatial nor the temporal resolution to make comparisons with the neuroanatomical findings of animal studies.

Currently, animal models of the central control of sexual behaviour are much more advanced than human models. However, human neuroimaging techniques are developing very fast, and advances in both scanner hardware and analytic approaches promise to deliver, in the not-so-distant future, the tools necessary to understand the human central sexual system in a mechanistic way. Examples of such techniques include near-infrared spectroscopy and magnetic encephalography, which not only provide excellent (millisecond) temporal resolution but also allow for behavioural 'freedom' and natural interaction between sexual partners. Given the clear similarities between rat and human sexual response cycles outlined in this Review, we propose that paradigms

conducted in, and results obtained from, animal research should guide future efforts in humans. In particular, human neuroimaging paradigms have virtually ignored the role of experience-based sexual learning, the role of contextual cues, and the putative role of genitally-induced sexual reward. Yet, it might be that identifying the mechanisms behind formation of sexual associations will be instrumental to understanding sexual phenotypes such as psychogenic sexual dysfunction and paraphilias.

So, what does the future hold? We hope that the theory of sexual incentive motivation (built around genital reward) will lead to new avenues of human sexual (brain) research, including the investigation of novel paradigms that investigate how the brain mediates sexual learning. Paradigms should be developed to study how the human brain deals with sexual novelty, habituation and expectancy. The human propensity to learn through observation seems particularly accessible with functional neuroimaging techniques. Finally, human sexual inhibition is currently underexplored. In particular, it would be of interest to understand the role of the prefrontal cortex in promoting sexual inhibition. Natural variations in sexual phenotype could be of major importance in all of the above suggested lines of research.

A deeper understanding of human sexual behaviour will only emerge when we get a grip on how the brain, at multiple neurobiological levels, deals with sexual stimuli, and how it interacts with its most important sensors. In time, we hope that this knowledge will help to develop new and better treatments for sexual dysfunction.

#### Review criteria

We searched the Thompson ISI Web of Knowledge and PubMed databases. We used the key words "sexual desire", "penis", "clitoris", "vagina", "brain", "stimulation", "genital", "spinal cord", "fMRI", "neuroimaging", "dopamine", "opioids", "sexual inhibition", "learning", "conditioning". We selected publications on the basis of high reputation and quality, which means that we also referenced highly regarded older publications. We also searched the reference lists of articles identified by this search strategy. All papers referenced were published in English.

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# Author contributions

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CLINICAL RESEARCH ARTICLE

## Do rats have orgasms?

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**Background:** Although humans experience orgasms with a degree of statistical regularity, they remain among the most enigmatic of sexual responses; difficult to define and even more difficult to study empirically. The question of whether animals experience orgasms is hampered by similar lack of definition and the additional necessity of making inferences from behavioral responses.

**Method:** Here we define three behavioral criteria, based on dimensions of the subjective experience of human orgasms described by Mah and Binik, to infer orgasm-like responses (OLRs) in other species: 1) physiological criteria that include pelvic floor and anal muscle contractions that stimulate seminal emission and/or ejaculation in the male, or that stimulate uterine and cervical contractions in the female; 2) short-term behavioral changes that reflect immediate awareness of a pleasurable hedonic reward state during copulation; and 3) long-term behavioral changes that depend on the reward state induced by the OLR, including sexual satiety, the strengthening of patterns of sexual arousal and desire in subsequent copulations, and the generation of conditioned place and partner preferences for contextual and partner-related cues associated with the reward state. We then examine whether physiological and behavioral data from observations of male and female rats during copulation, and in sexually-conditioned place- and partner-preference paradigms, are consistent with these criteria.

**Results:** Both male and female rats display behavioral patterns consistent with OLRs.

**Conclusions:** The ability to infer OLRs in rats offers new possibilities to study the phenomenon in neurobiological and molecular detail, and to provide both comparative and translational perspectives that would be useful for both basic and clinical research.

**Keywords:** *sex; pleasure; animals; male; female; genitosensory; ultrasonic vocalizations; conditioned preference; opioids; dopamine*

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*Well you tried it just for once found it all right for kicks  
But now you found out that it's a habit that  
sticks 'Orgasm Addict' by the Buzzcocks (1977)*

Orgasms are among the most wonderful and pleasurable events we experience as human beings. The term comes from the Greek term *orgasmos* meaning 'excitement' or 'swelling' and is synonymous with sexual 'climax' or the autonomic apex of copulation or masturbation (Coolen, Allard, Truitt, & McKenna, 2004; McKenna, 1999a).

The Roman poet Ovid, in *Ars Amatoria* (2CE/1855), describes orgasm as a phase which 'relieves' or 'completes' the lovemaking of both partners (Vol. 2, p. 663). Albert Moll (1908/1912), one of the early German sexologists, described four phases of human sexual response resulting from a 'tumescence and detumescence drive' (referring to blood flowing into and out of erectile tissues), positioning orgasms as a 'voluptuous acme' or high point in tumescence that yields to a 'sudden cessation of the voluptuous sensation' and detumescence (pp. 22–23). Wilhelm Reich

(1927/1945) described orgasms as a ‘bioelectric discharge’ of purely sexual tension, breaking away from Freud’s (1920/1922) notion of orgasm as a death instinct or ‘Thanatos’ that the life instinct ‘Eros’ always moved its ‘libido’ toward. Kinsey, Pomeroy, and Martin (1948) and Kinsey, Pomeroy, Martin, and Gebhard (1953) noted that orgasms come largely from penile stimulation in men and clitoral stimulation (CLS) in women, although stimulation of other erogenous zones on the body could also sum up to, or in some cases generate, orgasms. Masters and Johnson (1966) reiterated Moll’s four stages of human sexual response and identified orgasm at its own phase, the ecstatic period when some or all of the built-up sexual tension is released, followed by a longer-term ‘resolution’ or refractory phase where pelvic muscles and blood flow are relaxed. Despite a plethora of poetry and songs devoted to orgasms and their repercussions, the phenomenology of orgasm in humans is fraught with a multitude of definitions and interpretations, different subjective experiences across individuals, and even different experiences in the same individual across the lifespan. Another problem is that orgasms have occupied the ‘highest’ place in the human sexual response cycle. This drives many people to ‘achieve’ them or somehow ‘give’ them to others, which can diminish the value of sexual sensations experienced during other phases of the response cycle. That said, people’s experiences with orgasms, especially first experiences, can be profound and life-changing.

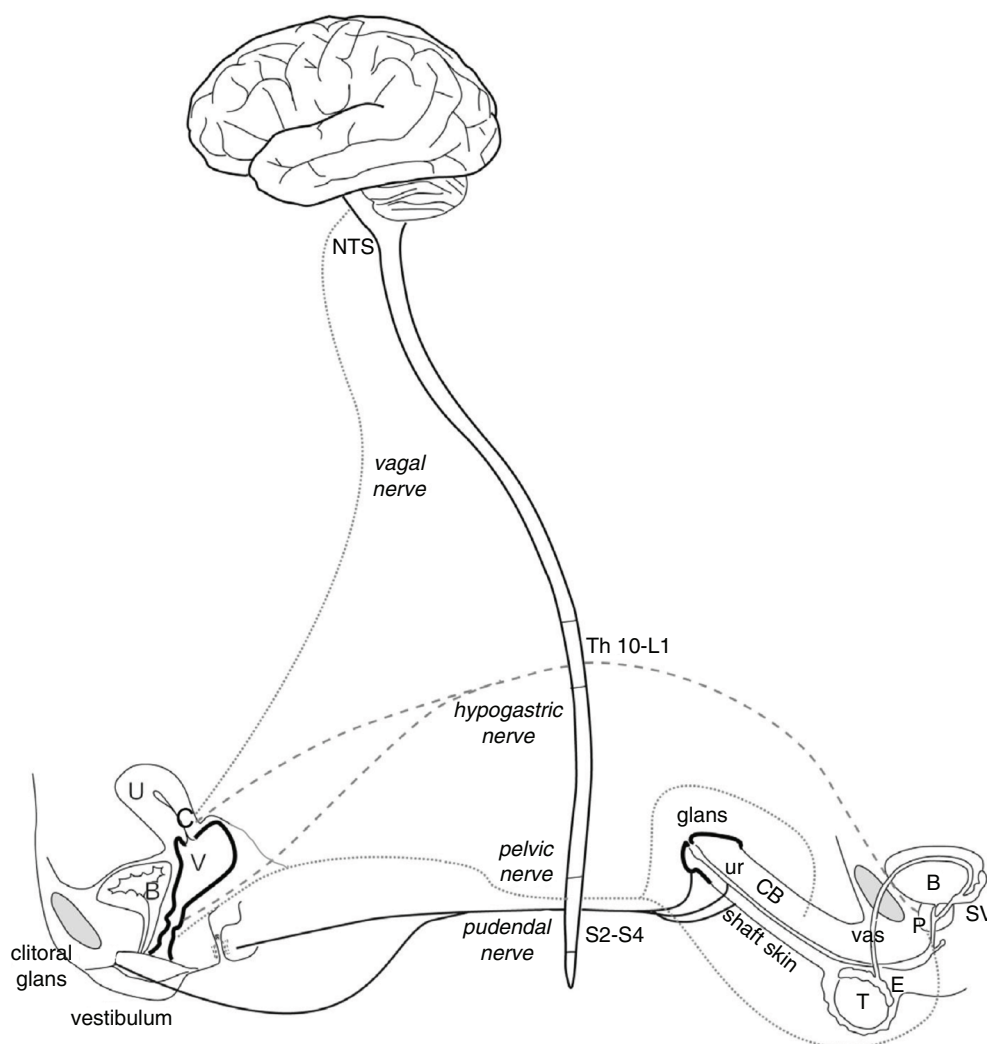
### Genitosensory, motor, and autonomic control of orgasm

Orgasms are controlled by the autonomic nervous system (ANS) and spinal cord, and ultimately processed in the brain (Komisaruk, Beyer-Flores, & Whipple, 2006). They can be defined and observed objectively as a spinal reflex that results in rhythmic muscle contractions of the pelvic floor and anus, and a urethrogenital reflex often coincident with seminal emission (controlled by the hypogastric nerve) and ejaculation (controlled by the pudendal nerve) in men (Giuliano, 2011; McKenna, 1999b; McKenna, Chung, & McVary, 1991) and contractions of the uterus and cervix in women (Meston, Levin, Sipski, Hull, & Heiman, 2004). Stimulation of the glans and shaft of the erect penis also give rise to pelvic floor muscle contractions as orgasm nears. Stimulation of the vagina and erect clitoris induces pelvic floor muscle contractions (Shafik, 1995; Shafik, El-Sibai, Mostafa, Shafik, & Ahmed, 2005) that appear to increase in duration and intensity as orgasm approaches. The glans penis and external clitoris are homologous structures, and tactile stimulation activates similar sensory nerves (e.g. dorsal clitoral and penile nerves) that course into the pudendal nerve, which enters the spinal cord at the sacral divisions S2–S4 (Fig. 1). The pudendal nerve is a mixed sensory and motor nerve that serves the entire sacral

plexus. In addition to the clitoris and penis, the pudendal nerve receives sensory inputs from labia, scrotum, and anus. Its motor division controls contractions of the external urethral and anal sphincters along with the rest of the pelvic floor muscles. The pelvic nerve controls contractions of the internal urethral and anal sphincters and is responsible for erection of the clitoris and penis. Stimulation of the hypogastric nerve activates the sympathetic outflow responsible for orgasm. In turn, the pelvic nerve carries sensory information from the cervix and pelvic floor muscle contractions during orgasm. Input from the cervix is likely also carried by the vagus nerve, which bypasses the spinal cord and sends sensory input directly to the medulla in the brainstem (Komisaruk, Beyer-Flores, & Whipple, 2006; Komisaruk & Whipple, 2005). These nerves thus subserve the genitosensory input to the spinal cord and brain that are processed ultimately as genital swelling, sexual stimulation, and orgasm (Fig. 2). In male rats, a set of spinothalamic neurons in the lower lumbar spinal cord (LSt neurons) has been identified that act as an ejaculation generator, coordinating inhibitory and excitatory outflow from brainstem and hypothalamic nuclei (notably nucleus paragigantocellularis, paraventricular (PVN) nucleus, and medial preoptic area (mPOA)) to control sympathetic, parasympathetic, and motor actions (Coolen et al., 2004; Truitt & Coolen, 2002). It is not known to what extent these neurons play a role in orgasm relative to the control of ejaculation, as ejaculation can occur without corresponding orgasm in men (McKenna, 1999b).

### Neuroanatomical and neurochemical correlates of orgasm

Orgasms produce a similar signature of brain activation in women and men undergoing functional magnetic resonance imaging (fMRI) scans, including deactivations of left ventromedial and orbitofrontal cortices, and activations of anterior cingulate, insula, parietal lobe, hippocampus, amygdala, basal ganglia (especially the putamen), nucleus accumbens (NAc), bed nucleus of the stria terminalis-preoptic area, hypothalamic PVN nucleus, cerebellum (including the anterior lobe of the cerebellar vermis and deep cerebellar nuclei), and lower brainstem (central gray, mesencephalic reticular formation, and nucleus of the solitary tract), with a slight bias for more activation of the central gray in men (Bianchi-Demicheli & Ortigue, 2007; Georgiadis, Kringelbach, & Pfaus, 2012; Georgiadis, Reinders, Paans, Renken, & Kortekaas, 2009; Komisaruk & Whipple, 2005; Stoléru, Fonteille, Cornélis, Joyal, & Moulier, 2012). Using positron emission tomography to analyze regional cerebral blood flow, Huynh, Willemsen, Holstege et al. (2013) found a greater activation of the pituitary following masturbation to orgasm in women relative to men. In another study, similar blood flow to the right ventrolateral



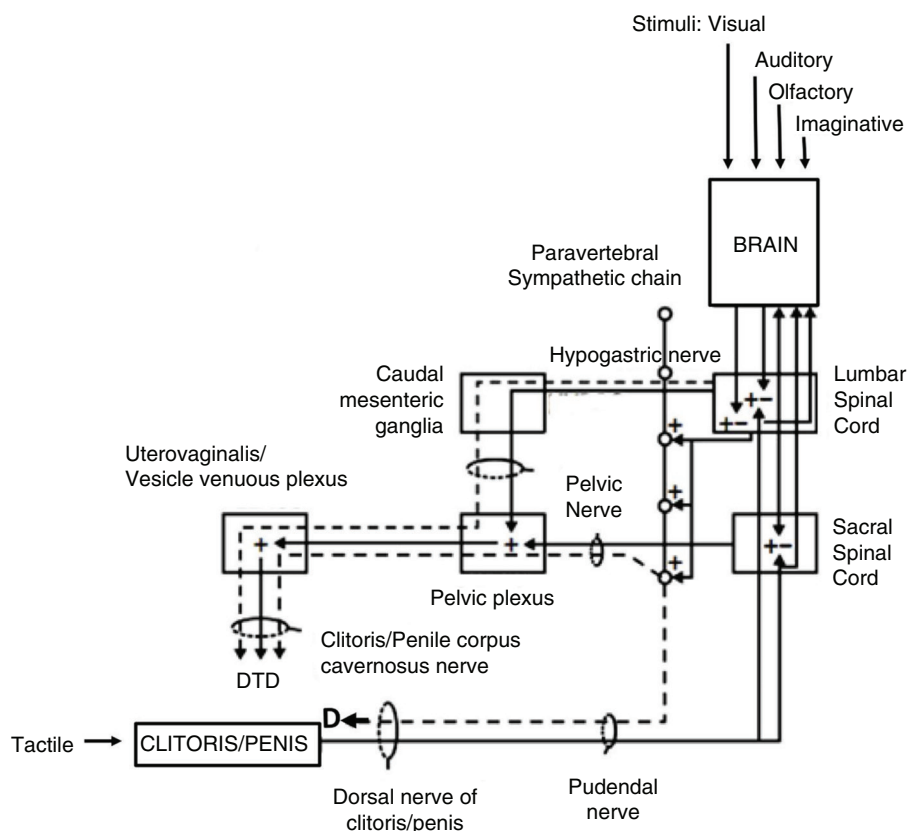
**Fig. 1.** Nerves (pudendal, pelvic, hypogastric, and vagus) that subserve sexual arousal and orgasm in women and men. B: bladder. C: cervix. CB: corpora spongiosum of the bulbocavernosus. E: epididymis. NTS: nucleus of the solitary tract (brainstem). P: prostate. SV: seminal vesicle. T: testis. U: uterus. ur: urethra. V: vagina. vas: vas deferens. Adapted from Georgiadis, et al. (2012).

pedunculopontine nucleus of the brainstem accompanied ejaculation in men and CLS-induced orgasm in women, with activation patterns corrected for head and facial movements made during ‘faked’ orgasm (Huynh, Willemsen, Lovick, & Holstege, 2013).

Multiple neurotransmitter systems, including endogenous opioids, serotonin, and endocannabinoids, are involved in the subjective feelings of pleasure, satiety, and sedation common to orgasms and their aftermath (Pfaus, 2009). In addition, plasma oxytocin and prolactin levels rise in both men and women during and following orgasm (Krüger et al., 2003, 2006; Murphy, Checkley, Seckl, & Lightman, 1990). The euphoric pleasure of orgasm is likely induced by opioids acting at  $\mu$ -opioid receptors in different limbic and hypothalamic structures (Ågmo & Paredes, 1988; Pfaus & Gorzalka, 1987; Parada, Sparks, Censi, & Pfaus, 2014). Indeed, heroin addicts describe the rush of euphoria they experience upon injecting heroin in

sexual terms, often equating it with orgasm (Chessick, 1960; Pfaus & Gorzalka, 1987). Accordingly, administration of the opioid receptor antagonist naloxone to men blunts the pleasure of orgasm and eliminates the oxytocin release associated with it (Murphy et al., 1990). In addition to the opioid contribution, orgasm depends critically on sympathetic activation, and there appear to be individually defined optimality curves for orgasm across increasing levels of arousal. For example, plasma levels of adrenaline and noradrenaline peak at orgasm and then fall precipitously after, perhaps giving rise to the subjective experience of a ‘release’ of tension and arousal that built up during sexual interaction. Furthermore, drugs or situations that reduce sympathetic tone can delay or abolish orgasm, whereas drugs or situations that increase sympathetic tone can either increase the magnitude or number of orgasms, although such increases must be specific to, or associated with, sexual interaction





**Fig. 2.** Schematic diagram of the spinal and supraspinal regulation of tumescence (T) and detumescence (D) of the clitoris and penis. Dashed lines are excitatory and solid lines inhibitory for genital blood flow. After De Groat and Steers (1998). LSt neurons that comprise the ejaculation generator in males are located in the lower lumbar cord (see Coolen et al., 2004).

(Pfaus et al., 2010). However, if stressors are of sufficient magnitude, then the likelihood of becoming sexually aroused is reduced, making sexual behavior and orgasm unlikely to impossible (Barlow, 1986; Both, Everaerd, & Laan, 2003; Magariños & Pfaff, 2016).

### Are orgasms unique to humans?

The study of sexual behavior in animals, mostly rodents and non-human primates, has provided analogies and homologies of human sexual behavior and revealed much about underlying physiological processes that subserve sexual arousal and desire, and to a lesser extent sexual reward and inhibition, that simply could not be obtained from humans (Ågmo, Turi, Ellingsen, & Kaspersen, 2004; Aragona & Wang, 2004; Frohmader, Pitchers, Balfour, & Coolen, 2010; Pfaus, Kippin, & Coria-Avila, 2003; Pfaus et al., 2010, 2012; Wallen, 1995). Accordingly, these models are used widely in sexual and reproductive medicine (Chianese et al., 2011; Giuliano et al., 2010; McMurray, Casey, & Naylor, 2006; Olivier et al., 2006; Pattij, Olivier, & Waldinger, 2005). Mechanisms of erection, ejaculation, and the post-ejaculatory refractory period have been studied in detail in rodents and other species (e.g. Chen, Chan, & Chang, 1999; Giuliano & Rampin, 2004; Levin,

2009; Newman, Reiss, & Northup, 1982). Ejaculation in a variety of species has been viewed as synonymous with sexual ‘climax’ (Fox & Fox, 1971), and indeed the autonomic and neurochemical mechanisms that control erection, seminal emission, and ejaculation are virtually identical in males of a variety of mammalian species, including humans. This begs a question: Can we assume that a male rat or rhesus macaque experiences the pleasure of ejaculation as we experience an orgasm? It may be more accurate to refer to this as an ‘orgasm-like response’ (OLR). Likewise, during copulation, female macaques can display intense tonic/clonic uterine contractions and sudden increases in heart rate coincident with a male’s ejaculation (Goldfoot, Westerborg-van Loon, Groeneveld, & Slob, 1980; Slob, Groeneveld, & van der Werff ten Bosch, 1986; Troisi & Carosi, 1998; Zumpe & Michael, 1968), although this response is not always observed. During this time, some female macaques also open their mouths and tense their facial muscles in what Slob et al. referred to as ‘climax face’. Do female macaques or females of other species experience OLRs in response to sufficient copulatory stimulation? And if so, how could we know?

One advantage of studying orgasms in humans is that people can provide a subjective verbal assessment of their

feelings that can be contrasted with physiological correlates. However, this comes with a caution: at best, humans do not have a common way to scale orgasm quality or intensity (despite available validated rating scales, like that of Mah and Binik (2002), which researchers doing objective assessments have not utilized); at worst, they lie. Moreover, it is virtually impossible to study human orgasms in any kind of natural environment without necessarily altering the context. And of course, we cannot study first experiences of orgasm except as Kinsey and colleagues did in the subjective retrospective of their participants.

### Inferring subjective states from objective behaviors

Obviously, we do not know what it feels like to be another animal (Nagel, 1974). In fact, our empathy and intuition take us only so far in inferring the feelings of other people. For example, to someone who is sexually active but has never experienced an orgasm, the tales of those that have multiple orgasms must seem outlandish if not also depressing. However, consistent with Dennett (1991), if an orgasm could be distilled into observable (and testable) components, it would be easier to make quantitative determinations of differences in expression that might reflect lawful differences in qualitative experience. What might those components be? A hint is given from the work of Mah and Binik (2002).

In developing their adjective-based rating scale, Mah and Binik (2002) examined factor loadings and structure among a large set of words used by women and men to describe their subjective experience of orgasm from masturbation and copulation. These terms were then rated by several hundred university-age women and men, and then organized by principal components analysis into 12 factors. Further refinement was done by collapsing factors that reflected essentially the same phenomena into a two-factor model that encompassed sensory and cognitive/affective dimensions of the experience of orgasm. This model accounted for 73–84% of the intersubject variance across all measures. The sensory dimension consisted of terms that described sensations of building, flooding, flushing, shooting, throbbing, and muscle spasms. The cognitive–affective dimension described ecstasy, pleasurable and satisfying sensations, relaxation, and emotional intimacy. These dimensions appear to correspond to three components: (1) physical sensations generated by muscle contractions and sympathetic activation, (2) a cognitive/affective sense of ecstasy and pleasure, and (3) the relaxation/intimacy that corresponds to a longer-term refractory period. If we distill these components further into what might be observed in humans or other animals, we can derive at least three phenomena that could characterize an OLR:

1. Physiological changes, including pelvic floor and anal muscle contractions that stimulate seminal emission and/or ejaculation in the male, or that stimulate uterine and cervical contractions in the female. These contractions should occur in temporal synchrony with sympathetic activation and/or bloodflow from erectile tissues;
2. Short-term behavioral changes that reflect immediate awareness of a pleasurable hedonic reward state. These include measurable vocalization patterns, rejection of further genitosensory stimulation, and/or behavioral relaxation or quiescence, during and/or immediately after the OLR; and
3. Long-term behavioral changes that depend on the reward state induced by the OLR. These include an identifiable state of sexual satiety, the strengthening of patterns of sexual arousal and desire in subsequent copulations, and the generation of conditioned place and partner preferences for contextual and partner-related cues associated with the reward state.

How well these criteria might be applied to rats?

### Copulatory responses in rats

To understand the potential expression of OLRs in a rat model, it is necessary to consider the form and patterning of rat copulatory behavior. Copulation in rats is initiated and controlled by females, who enforce or ‘pace’ the multiple mounts, vaginal intromissions, and ejaculations they receive from males (Madlafousek & Hlinák 1978; McClintock, 1984; Pearce & Nuttall, 1961; Pfaus, Jones, Flanagan-Cato, & Blaustein, 2015). Sexually receptive females actively solicit sexual interaction with males by making a headwise orientation to the male followed by an abrupt runaway. This often entices the male to chase them, after which the female holds a pre-lordosis crouch. Males mount with flank palpation, further increasing the lordosis posture of the female, allowing vaginal penetration as the male intromits with brief pelvic thrusts that typically last less than a second. If vaginal penetration has occurred, the male dismounts abruptly and grooms his erect penis into detumescence. The female typically runs away a short distance, and either hops and darts around the male or makes another full solicitation. Hopping and darting typically entices the male to mount again and is used as a proximal solicitation, especially if the male is not chasing or if the female does not have enough room in a testing environment to make a full solicitation with a longer runaway. Finally, stimulation of the cervix results in an inhibitory state. Indeed, in pacing chambers, females typically begin to fight with males after they receive approximately four ejaculatory series and display decreased sexual solicitations and hops and darts (Coopersmith, Candurra, & Erskine, 1996; Pfaus,

Smith, & Coopersmith, 1999). This fighting behavior enforces longer and longer intervals between intromissions. Similarly, in large open fields with attached burrow systems, females typically take themselves out of the copulations after a smaller number of ejaculations compared with small chambers (McClintock, 1984). Collectively, these patterns of behavior reflect an 'estrous termination' that is dependent on the amount of vaginocervical stimulation (VCS) received by the female (Pfaus, Smith, Byrne, & Stephens, 2000) and corresponding activation of the pelvic nerve (Lodder & Zeilmaier, 1976; Pfaus, Manitt, & Coopersmith, 2006), which in turn activates a glutamate system in the ventromedial hypothalamus (VMH) that inhibits appetitive sexual behavior (Georgescu, Cyr, & Pfaus, 2012; Georgescu, Sabongui, Del Corpo, Marsan, & Pfaus, 2009). Ejaculation by males results in a post-ejaculatory refractory period, after which males resume copulating with the female toward another ejaculation. This can repeat through approximately 6–10 successive ejaculatory series until the male is 'sexually exhausted' (Beach & Jordan, 1956) and cannot engage in full copulatory responses for approximately 3–4 days (Rodríguez-Manzo, 1999; Rodríguez-Manzo & Fernández-Guasti, 1994, 1995).

#### Male responses to copulatory stimulation

In response to female solicitations, male rats mount, intromit, and ejaculate, and males have relatively stable individual patterns of these behaviors (Pattij et al., 2005). In terms of sensory stimulation, males receive both olfactory stimulation from the odors/pheromones of the female along with penile stimulation from intromissions and ejaculations (Fig. 3). Deprivation of sensory stimulation from the penis by topical anesthesia or deafferentation results in males that mount at a higher rate than

males given a control treatment but cannot intromit or ejaculate (Gray, Davis, & Dewsbury, 1976; Lodder, 1976). Deprivation of olfactory information, either from destruction of the olfactory epithelium by zinc sulfate, or bulbectomy, abolishes copulatory behavior in males (Carr, Loeb, & Dissinger, 1965; Thor & Flannelly, 1977). However, these disruptions of copulatory behavior do not occur if male rats have sufficient sexual experience (Pfaus et al., 2012), suggesting that sexual experience crystallizes and automates copulatory patterns in males. Males are also aware of their sexual state during copulation. For example, prior to each mount with intromission, males emit a short ultrasonic call in the 50 kHz range (McIntosh, Barfield, & Thomas, 1984; White, Cagiano, Moises, & Barfield, 1990). These precontact vocalizations increase in frequency as males learn to associate the ejaculatory reward state with their pre-ejaculatory copulatory behavior (Bialy, Rydz, & Kaczmarek, 2000; McGinnis & Vakulenko, 2003). These vocalizations are eliminated after castration and restored with exogenous androgen (Harding & Velotta, 2011). Immediately after ejaculation, males emit a long 22 kHz vocalization which corresponds to their period of behavioral quiescence (Burgdorf et al., 2008; McIntosh, Barfield, & Thomas, 1984; White et al., 1990).

#### Female responses to copulatory stimulation

Pacing is facilitated for females in large open fields, bilevel chambers (where she can run from level to level), or in unilevel chambers bisected by a Plexiglas partition with holes at the bottom that allow the female – but not the male – to cross from side to side. Females show regular patterns of pacing in these three testing environments. For example, in unilevel chambers with a partition, females display different latencies to return to the

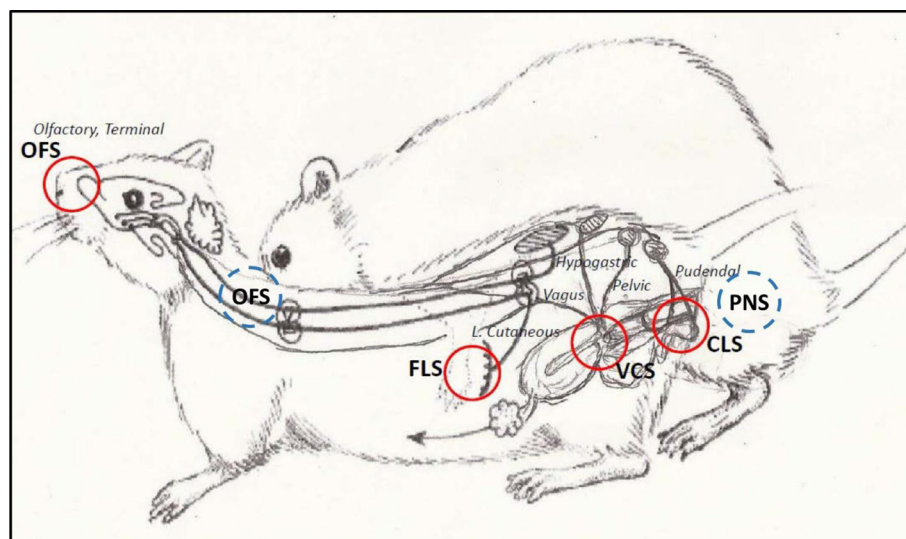


Fig. 3. Sensory stimulation of female (red) and male (blue) rats during sexual interaction. OFS: olfactory stimulation. FLS: flank (tactile) stimulation. VCS: vaginocervical stimulation. CLS: clitoral stimulation. PNS: penile stimulation.

male (called 'contact return latencies') depending on the preceding copulatory stimulation (short if mounted, longer if intromitted, and longest if she received an ejaculation; Erskine, 1985). These observations bolstered the notion that females enforce an optimal timing between intromissions that leads to optimal timing for the induction of a progestational state (Adler, 1978). If females are tested in small chambers that do not allow efficient pacing, they are more likely to display defensive responses such as rearing or boxing to enforce intervals between mounts, intromissions, and ejaculations. They are also less likely to get pregnant (Frye & Erskine, 1990), suggesting that female rats have a similar 'vaginal code' to that first observed in female mice by Diamond (1970).

During copulation, females receive olfactory stimulation from male odors and pheromones, somatosensory stimulation from the flanks (FLS) where males palpate, CLS from pelvic thrusts, and VCS from penile intromissions, which results in the seminal plug after ejaculation (Fig. 3), in addition to the cognitive-affective sense of control given the pacing conditions. Interestingly, VCS alone does not activate LSt neurons in the lower lumbar spinal cord that act in males as the spinal ejaculation generator (Truitt, Shipley, Veening, & Coolen, 2003). However, deprivation of sensory input from the clitoris and cervix through topical anesthesia resulted in shorter contact return latencies in unilevel pacing chambers following intromissions and ejaculations (Meerts, Strnad, & Schairer, 2015). This effect was also observed after transection of the pelvic nerve alone (Meerts & Clark, 2009). In contrast, chronic anesthesia of the external gland of the clitoris alone had the opposite effect in which females had a greater number of exits from and entrances to the male compartment but spent less time with the male (Parada et al., 2013). Females also emit ultrasonic vocalizations in the 'appetitive' 50 kHz range during copulation, especially as they approach males (White, Colona, & Barfield, 1991). These calls may be in response to male odor (Matochik, Barfield, & Nyby, 1992) or to the anticipation of rewarding copulatory contact. The calls are displayed reliably in females during proestrus/estrus, are eliminated by ovariectomy, and restored by hormone treatment that restores sexual receptivity (Matochik et al., 1992).

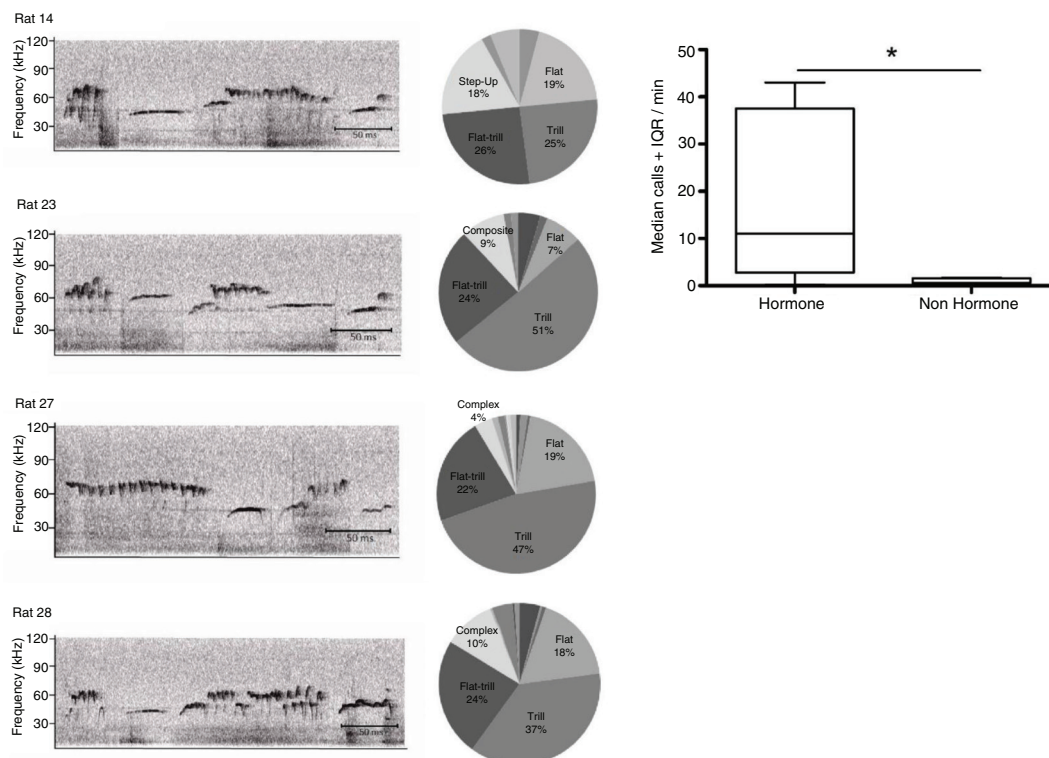
Paced copulation induces more vigorous mounts and intromissions by the male (Erskine, Kornberg, & Cherry, 1989), which result in stronger CLS and VCS from the male's pelvis and penis, respectively, during each mount with intromission and pelvic thrusting.

Together, these data suggest that genitosensory stimulation of the penis or clitoris induce a pleasurable state that males and females are aware of in terms of appetitive calls, and which they attempt to compensate for behaviorally if the stimulation is reduced by pharmacological agents or lesions to specific nerves.

## Effects of repeated pleasurable copulatory stimulation

Copulation in both male and female rats also induces a reward state that can be associated by Pavlovian processes with contextual- or partner-related cues to form long-term conditioned place preferences (CPPs) or conditioned partner preferences. CPP is often demonstrated in an apparatus with two distinctive compartments that are connected to either side of a third neutral compartment. During training, the compartments are paired sequentially with different unconditional stimuli, (e.g. one side is paired with the reward state induced by copulation with a sex partner, eating food, or a drug with abuse potential, whereas the other side is paired with either nothing or a control manipulation). In the final test, the animal is placed into the neutral compartment between the two distinctive compartments, with the two doors on either side opened to allow free access to either compartment. CPP is said to have developed if the rat spends significantly more time in the reward-paired compartment than the other compartment. Sexual CPP is observed when male rats are placed into a distinctive compartment of the CPP box after ejaculation, in contrast to being placed into the other compartment after mounts or intromissions alone (Ågmo & Berenfeld, 1990; Everitt, 1990; Hughes, Everitt, & Herbert, 1990; Mehara & Baum, 1990). However, intromissions alone can maintain copulatory CPPs in males that have not yet experienced ejaculation (Hughes et al., 1990; Tenk, Wilson, Zhang, Pitchers, & Coolen, 2009). For females, sexual CPP is achieved by pairing either paced copulation or non-paced copulation with one of two distinct environments (Afonso, Woehrling, & Pfau, 2006; Paredes & Alonso, 1997; Paredes & Vazquez, 1999). CPP can also be induced by CLS alone (Parada, Chamas, Censi, Coria-Avila, & Pfau, 2010). We have mimicked the CLS females receive from males by applying it to the clitoris directly with a #4 camelhair paintbrush (Parada et al., 2010; Parada, Abdul-Ahad, Censi, Sparks, & Pfau, 2011). CLS applied in intervals to mimic its distribution during paced copulation induces a strong CPP relative to continuous CLS or a control condition in which the female's tail is raised but no CLS is applied. Interestingly, the CPP induced by CLS is independent of hormone administration to ovariectomized females (Parada, Vargas, Kyres, Burnside, & Pfau, 2012). CLS induces immediate 50 kHz vocalizations that follow individual trill-flat patterns (Fig. 4). These patterns are emitted in response to distributed but not continuous stimulation and are maximal in females that are fully sexually motivated and receptive. Thus, like male 50 kHz vocalizations, females appear to be aware of timed CLS and respond to it in a way consistent with the induction of a reward state.





**Fig. 4.** Ultrasonic vocalizations made by female rats in response to distributed CLS. Left side depicts raw calls. Middle depicts the proportion of total calling during CLS taken by flats, trills, flat-trills, step-ups, and compound calls. Right side depicts median calls in females that receive full hormone priming with estradiol benzoate and progesterone versus no hormone (oil vehicle). \* $P < 0.01$ .

In another group of studies, we have shown that placement of a neutral odor, such as almond or lemon, on a vigorous and receptive sex partner can be paired with the reward state induced by first experiences of ejaculation in males or paced copulation in females. Accordingly, these odors become discrete, partner-related, conditioned stimuli that direct a Pavlovian conditioned partner and mate preference in females (Coria-Avila, Ouimet, Pacheco, Manzo, & Pfaus, 2005; Coria-Avila & Pfaus, 2007) and conditioned ejaculatory preferences in males (Ismail, Gelez, Lachapelle, & Pfaus, 2009; Kippin, Talianakis, Schattmann, Bartholomew, & Pfaus, 1998; Kippin & Pfaus, 2001a, 2001b). Typically, these preferences are formed by paired copulation in bilevel or unilevel pacing chambers and then tested in a large open field with two potential sex partners, one scented and the other unscented. Unpaired controls who received repeated pairing with an unscented partner show a slightly weaker conditioned preference for the unscented partner on the final open-field test, and random-paired controls do not show any conditioning. Strain cues from pigmented versus non-pigmented partners also induce significant partner preferences in both females (Coria-Avila et al., 2006) and males (Ismail, Jones, Graham, Sylvester, & Pfaus, 2011). Interestingly, a female rat given her first paced copulatory experience with one particular male displays conditioned mate-guarding behavior in the

presence of that male and a competitor female (Holley et al., 2015; Holley, Shalev, Bellevue, & Pfaus, 2014), a behavior that she does not display if the male is novel or there is no competition for him. These behaviors do not depend on an olfactory cue per se, instead are associated with more ‘pheromonal’ cues (e.g. major histocompatibility complexes) from the familiar male.

The reward state induced by ejaculation or paced copulation is dependent on the release of endogenous opioids in key limbic and hypothalamic brain regions (Paredes, 2014; Pfaus et al., 2012; van Furth, van Emst, & van Ree, 1995; van Furth, Wolterink, & van Ree, 1995). Repeated ejaculations increase whole-brain endorphin content in male rats (Szechtman, Hershkowitz, & Simantov, 1981). Indeed, opioid activation may also mediate the ability of orgasms in humans, and ejaculation in rats, to reduce anxiety and dysphoria (Fernández-Guasti, Roldán-Roldán, & Saldívar, 1989; McCarthy, 1977; Pfaus & Wilkins, 1995; Rodríguez-Manzo, López-Rubalcava, & Fernández-Guasti, 1999). Systemic treatment with the opioid antagonist naloxone, but not dopamine antagonists, during training abolishes the development of CPP and conditioned partner/ejaculatory preferences in both female and male rats (Ágmo & Berenfeld, 1990; Coria-Avila et al., 2008; Ismail, Girard-Bériault, Nakanishi, & Pfaus, 2009; Mehara & Baum, 1990; Paredes & Martinez, 2001). In fact, female rats given their first few sexual

experiences under the influence of systemic naloxone lose their desire for sex, as indicated by the loss of solicitations and lordosis, and the induction of defensive responses when injected subsequently with saline (Pfaus et al., 2012). In males, the conditioned ejaculatory preference for the odor is shifted from the familiar scented female to unfamiliar unscented female if naloxone is infused to the mPOA during training, whereas it is abolished altogether if naloxone is infused to the ventral tegmental area (VTA) or NAc during conditioning (Quintana et al., in preparation). Similarly, infusions of naloxone into the mPOA abolish conditioning of CPP in both male (Ågmo & Gomez, 1993) and female (García-Horsman, Ågmo, & Paredes, 2008) rats. Naloxone was also effective in eliminating pacing-related CPP following infusions to the VMH or medial amygdala but not the NAc (García-Horsman et al., 2008). This suggests that the mPOA (and related hypothalamic structures such as the PVN and supraoptic (SON) nuclei) links the reward to particular partner-related cues, whereas the VTA and NAc are involved in the general attending to reward-related cues in males but not necessarily in females. Opioids are known to sensitize dopamine cell activity in the VTA, which results in sensitized release of dopamine in the NAc and elsewhere during reward-seeking behavior (Koob et al., 2014; Kornetsky, 2004; Spanagel, 1995). Opioid actions are also likely responsible for the sensitized activation of oxytocin and vasopressin neurons in the PVN and SON when conditioned rats are in the presence of partner-related cues (Holley et al., 2015).

### The case for OLRs in rats

Both male and female rats display physiological and behavioral changes consistent with the induction of OLRs during sexual interaction. They are clearly aware of the sexual stimuli they receive and behave in ways to maximize the reward received by them. The urethrogenital ejaculatory reflexes of male rats, and the cervicouterine reflexes of female rats, depend on pelvic floor muscle contractions that are experienced at discrete intervals during sex. Responses made to those physiological events include both the induction of short- and long-term inhibitory states for further sexual stimulation, such as refractory periods in males, pacing behaviors by females, along with sexual exhaustion and estrous termination. Longer-term sensitization of contextual- and partner-related cues also occurs that is associated with the induction of a hedonically positive, opioid-induced sexual reward state. This reward state augments the activation of excitatory neurochemical systems, such as dopamine, noradrenaline, oxytocin, and vasopressin (Pfaus, 2009), that drive sexual arousal, CPP, and partner/mate preference in the appropriate circumstances. Thus, female and male rats meet the criteria set forth for OLRs, and they do so with a rich repertoire of behaviors to

study beyond copulatory responses alone. Although we can never know if rats experience OLRs the same way we experience orgasms, it is notable that these cognitively mediated behaviors reflect both evaluation and expectation of sexual pleasure or reward. This places them squarely into the realm of responses studied by human sex researchers as a function of sexual pleasure (Georgiadis et al., 2012). In turn, the ability to define OLRs opens new possibilities to study the effects of sexual reward and non-reward, and the manner in which arousal, sexual disinhibition, and other developmental, situational, or partner-related variables might enhance the experience of sexual pleasure from a comparative and translational perspective.

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Review

# Behavioral, Neural, and Molecular Mechanisms of Conditioned Mate Preference: The Role of Opioids and First Experiences of Sexual Reward

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**Abstract:** Although mechanisms of mate preference are thought to be relatively hard-wired, experience with appetitive and consummatory sexual reward has been shown to condition preferences for partner related cues and even objects that predict sexual reward. Here, we reviewed evidence from laboratory species and humans on sexually conditioned place, partner, and ejaculatory preferences in males and females, as well as the neurochemical, molecular, and epigenetic mechanisms putatively responsible. From a comprehensive review of the available data, we concluded that opioid transmission at  $\mu$  opioid receptors forms the basis of sexual pleasure and reward, which then sensitizes dopamine, oxytocin, and vasopressin systems responsible for attention, arousal, and bonding, leading to cortical activation that creates awareness of attraction and desire. First experiences with sexual reward states follow a pattern of sexual imprinting, during which partner- and/or object-related cues become crystallized by conditioning into idiosyncratic “types” that are found sexually attractive and arousing. These mechanisms tie reward and reproduction together, blending proximate and ultimate causality in the maintenance of variability within a species.

**Keywords:** opioids; dopamine; oxytocin; vasopressin; reward; first sexual experiences; mate preference; conditioned partner preference; conditioned place preference; paraphilias

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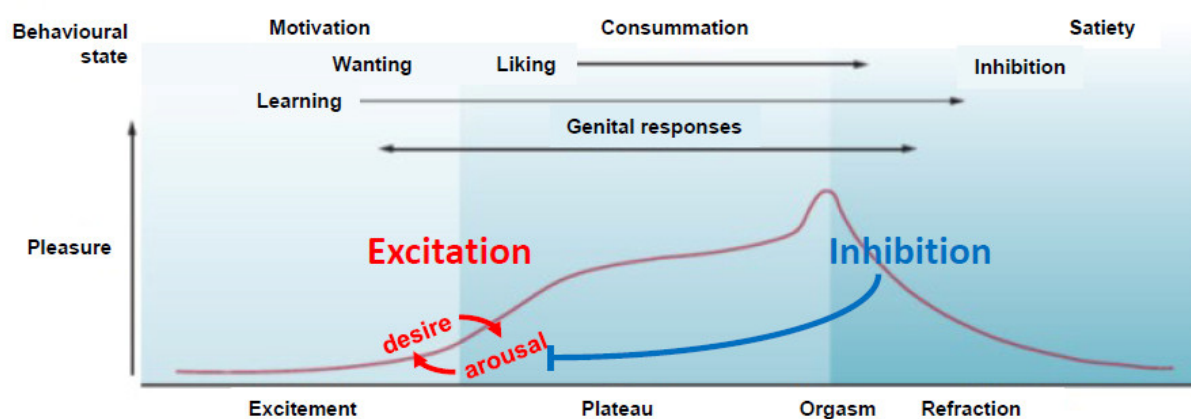


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## 1. Sexual Excitation and Inhibition

Most theoretical models of sexual behavior in humans and animals describe dichotomous phases of behavior (e.g., appetitive vs. consummatory), or internal influences on behavior (e.g., excitation vs. inhibition) that flow in time and within different types of brain, spinal, and/or peripheral systems [1–4]. Orthogonal to this are linear, or multi-stage, models of sexual response. Physiological sexual responses, at least in humans, are thought to follow a four-stage model which was first conceived of by Moll [5] and made popular by Masters and Johnson [6] as their “EPOR” model of sexual Excitement (arousal and desire), genital and extragenital stimulation during the Plateau, the peak of excitement and pleasure at Orgasm, and the inhibition of excitement that occurs during Resolution (refractoriness, see Figure 1). All phases of the sexual response have both excitatory and inhibitory influences, giving behavior a substantial flexibility. Indeed, although sexual behavior is controlled by hormonal and neurochemical actions in the brain, sexual experience induces an even greater degree of plasticity that allows animals to form instrumental and Pavlovian associations that predict sexual outcomes, thereby directing the

strength of sexual response. First, humans and animals must be able to respond to hormonal and neurochemical changes that signal sexual desire and arousal, and distinguish it from other sympathetic activation, such as anxiety. This ability underlies the moment-to-moment level of sexual arousability (as conceived by Whalen [7]) and defines a large part of the internal state that is commonly referred to as “sex drive.” Second, humans and animals must be able to make sense of external cues that signal sexual attraction and receptivity in potential sex partners (e.g., [8]). This ability requires a complex mix of instinct, learning, and feedback that follows a neural organization for incentive-based motivation and expectancy [9–12]. Humans and animals must be able to identify external stimuli that predict where potential sex partners can be found, to seek out, solicit, court, or otherwise work to obtain sex partners, distinguish external cues and behavioral patterns of potential sex partners from those that are not sexually receptive, and to pursue sex partners once sexual contact has been made [13]. In this way, humans and animals move in time from distal to proximal to interactive, with an ever-increasing load in sensory processing and motor sophistication as we interact more closely with either a stationary or moving and sentient array of sexual stimuli. Additionally, superimposed on this is the determination of attractivity, represented by hierarchies of stimuli from each sensory domain that are attractive to each individual.

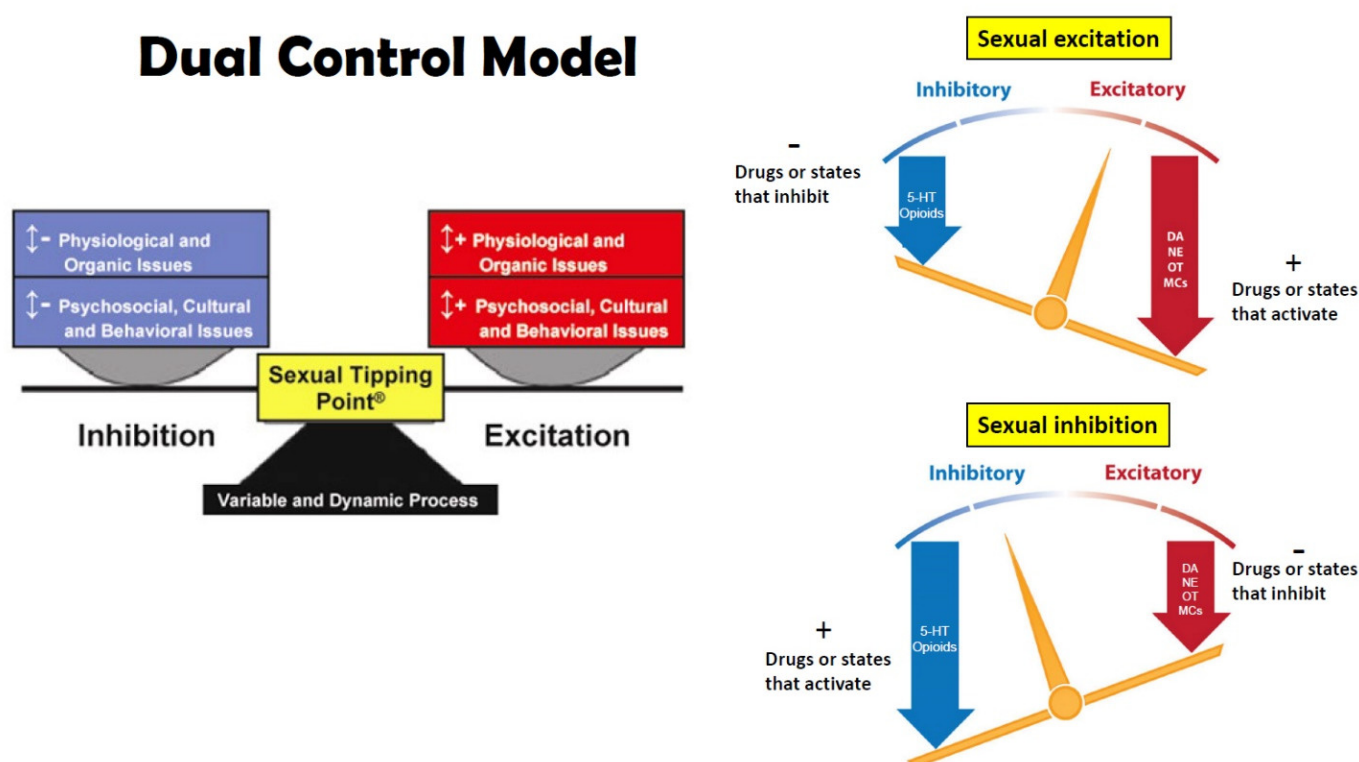


**Figure 1.** Depiction of Masters’ and Johnson’s “EPOR” model of human sexual response and related the activation of corresponding excitatory (red) and inhibitory (blue) brain regions. Opioid and serotonergic transmissions at orgasm activate inhibitory feedback on mechanisms of sexual arousal and desire. Adapted with permission from Georgiadis, Kringelbach, and Pfau [14] (2012. Nature Publishing Group).

It is becoming increasingly clear that there is a critical period of sexual behavior development that forms around an individual’s first pleasurable experiences with sexual arousal and desire, masturbation, sexual intercourse, and especially orgasm. During this period, the sensory and motor mechanics of the behavior become integrated and crystalized along with the development of preferences for ideal activities and physical features of a partner, and even objects associated with the sexual reward state. Such preferences often violate societal “norms” (e.g., as in the development of fetishes or paraphilias) and so-called “evolutionary laws” regarding features that represent genetic and reproductive strength, appearing more to be based on an egocentric evaluation of salient reward- or pleasure-related characteristics that differ from one individual to another, and create a “type” that each individual finds attractive and arousing. The formation of such experience-based preferences can be found in historical texts, such as Stendhal’s “Principle” in his work *De L’Amour* [15], in the case histories of Krafft-Ebing’s *Psychopathia Sexualis* [16], and more theoretically in the “love maps” or sexual “gestalts” proposed by Money [17]. This experiential critical period may well build upon the foundation laid by previous critical periods, especially those associated with attraction to other- or same-sex individuals,

which themselves may build on the foundation laid by a critical period for gender typical vs. atypical behavior, and the sense of self as “female” or “male” [18,19].

Beyond the priming role of steroid hormones to allow animals to respond to sexual incentives (for a review see [20,21]), the orchestration of sexual behavior and partner or mate preference for all animals is a fine-tuning process between mechanisms of sexual excitation and inhibition in the central and peripheral nervous system (see Figure 2). The main neurotransmitters involved in sexual excitation are dopamine (DA), norepinephrine (NE), melanocortin (MC), and oxytocin (OXT) acting mainly in hypothalamic, limbic, and cortical regions that integrate sexual arousal, attention, and motivated behavior. Neurotransmitters involved in sexual inhibition are mainly serotonin (5-HT), and the endogenous opioids (e.g.,  $\beta$ -endorphin) that regulate reward, satiety, sedation, and refractoriness [3,22]. For obvious ethical reasons, much of our knowledge regarding the effects of first experiences with sexual reward, the neuropharmacological and molecular mechanisms that underlie them comes from animal models (e.g., rats, quail, voles). Despite species’ differences in copulatory behavior, it is very likely that similar, if not identical, mechanisms underlie those experiences in humans [19,23].



**Figure 2.** Left: dual control model of Perelman [1] (Reprinted with permission from Perelman, 2006, Wiley) that depicts sexual excitation and inhibition forming around a labile sexual tipping point. The dual-control model is based on work by Bancroft and Janssen [4], who based their model on the work of Gray [24] for fear conditioning. Gray, in turn, based his ideas on those of Pavlov [25] for the role of excitation and inhibition of cortical function in conditioned reflexes. Right: neurochemical mechanisms and processes associated with sexual excitation and inhibition. DA = dopamine, NE = norepinephrine, OT = oxytocin, MC = melanocortins, 5-HT = serotonin, reprinted with permission from Pfau [3] (2009, Elsevier).

Mesolimbic, nigrostriatal, and hypothalamic DA facilitate general attention to incentive stimuli, copulatory proficiency, and genital reflexes [3,26]. Systemic administration of DA agonists facilitates male sexual behavior [27], induces copulation in sexually sluggish [28] or sexually exhausted males [29], whereas DA antagonists impair sexual behavior in male rats [30], and eliminate sexual solicitations in females [31]. The NE system has been shown to play a role in general arousal and the control of autonomic outflow, shown to

facilitate as well as inhibit male sexual behavior depending on which receptor they may bind [3,32]. For instance, yohimbine, an adrenergic receptor antagonist, enhances mounting in male rats [33], whereas lesions of noradrenergic cell bodies in the locus ceruleus increases the post-ejaculatory refractory period [34]. Finally, OXT has been implicated in sexual arousal, orgasm, satiety, and partner preference [35]. OXT cell bodies are located mainly in the paraventricular and supraoptic nuclei of the hypothalamus. Infusions of OXT into the paraventricular nucleus of male rats stimulated penile erection, while systemic administration facilitated ejaculation in male rats treated with the selective serotonin reuptake inhibitor (SSRI) fluoxetine [36,37].

The inhibitory neurotransmitters serotonin and the endogenous opioids subserve different aspects of behavioral inhibition. Serotonin neurons originate in the Raphé nuclei and send projections toward brain areas located in the brainstem, midbrain, and forebrain, as well as several structures in the periphery [3,32]. Serotonin turnover in the brain regulates satiety through a complex mechanism at many levels of processing, including the hypothalamus and prefrontal cortex, where serotonin mediates the behavioral inhibition indicative of “executive function”. Blocking serotonin reuptake with SSRIs such as fluoxetine or paroxetine inhibits sexual behavior in both female and male rats [36], whereas inhibiting its synthesis, release, or receptor binding, facilitates male sexual behavior [3,38]. Moreover, systemic injections of a serotonin synthesis inhibitor restored sexual behaviors in sexually sluggish, as well as in gonadally intact or castrated male rats [39].

Like the effects of exogenous opiates, endogenous opioids are well known for being involved in the rewarding aspects of a variety of motivated behaviors including sex [40], but also as a fundamental substrate of sexual refractoriness in humans and rats [41]. Accordingly, copulation to ejaculation increased whole brain  $\beta$ -endorphin content [42], and  $\mu$ -opioid receptor activation in the medial preoptic area (mPOA; [43]) and ventral tegmental area (VTA; [44]), regions implicated in the control of reward in general and sexual behavior in particular [32]. Moreover, opioid use dramatically reduced sexual arousal, increased the ejaculation latency, and/or inhibited sexual responding altogether in humans and animals [22,45]. Bilateral infusions of  $\beta$ -endorphin to the mPOA mimicked the post-ejaculatory state behaviorally [46]. Interestingly, Chessick [47] likened the injection of synthetic opiates such as heroin to a “pharmacogenic orgasm”.

## 2. Learning and Sexual Experience

Almost every behavior is the product of the interaction between the animal’s central nervous system and its learned experiences. Yet, in order to determine which prevails over the other when it comes to partner preference, or the classical question “nature vs. nurture”, studies have shown that an animal’s own experience (proximate causality) appears to override biological predetermination (ultimate causality). Previously, it was shown that inbred mice prefer to copulate with partners of a different haplotype (or genetic parents; [48]). To evaluate whether this is a natural or learned preference, Yamazaki and collaborators took a litter of newborn mice and transferred them to foster parents whose litters were removed approximately at the same time after birth. At day 21, mice were separated into different cages with other mice of the same genotype and fostering conditions until they reached sexual maturity. At the preference test, males were given the choice to copulate with two females, one from their fostering genetic profile and another of the same H-2 haplotype. Contrary to what was previously shown [48], male mice nursed by fostered mothers chose to copulate preferentially with sexual partners that would resemble their foster mother rather than their biological mother, thus showing that H-2 selective preference is acquired by family imprinting. However, a crucial experiment showed that sexually naïve animals would choose a mate that better resembles an adoptive mother rather than the genetic mother [49]. In their experiment, Kendrick and colleagues separated male and female sheep and goats and cross-fostered them. These animals were also allowed to engage in social contact with members of their genetic species during development. When animals reached adulthood, animals were tested for social

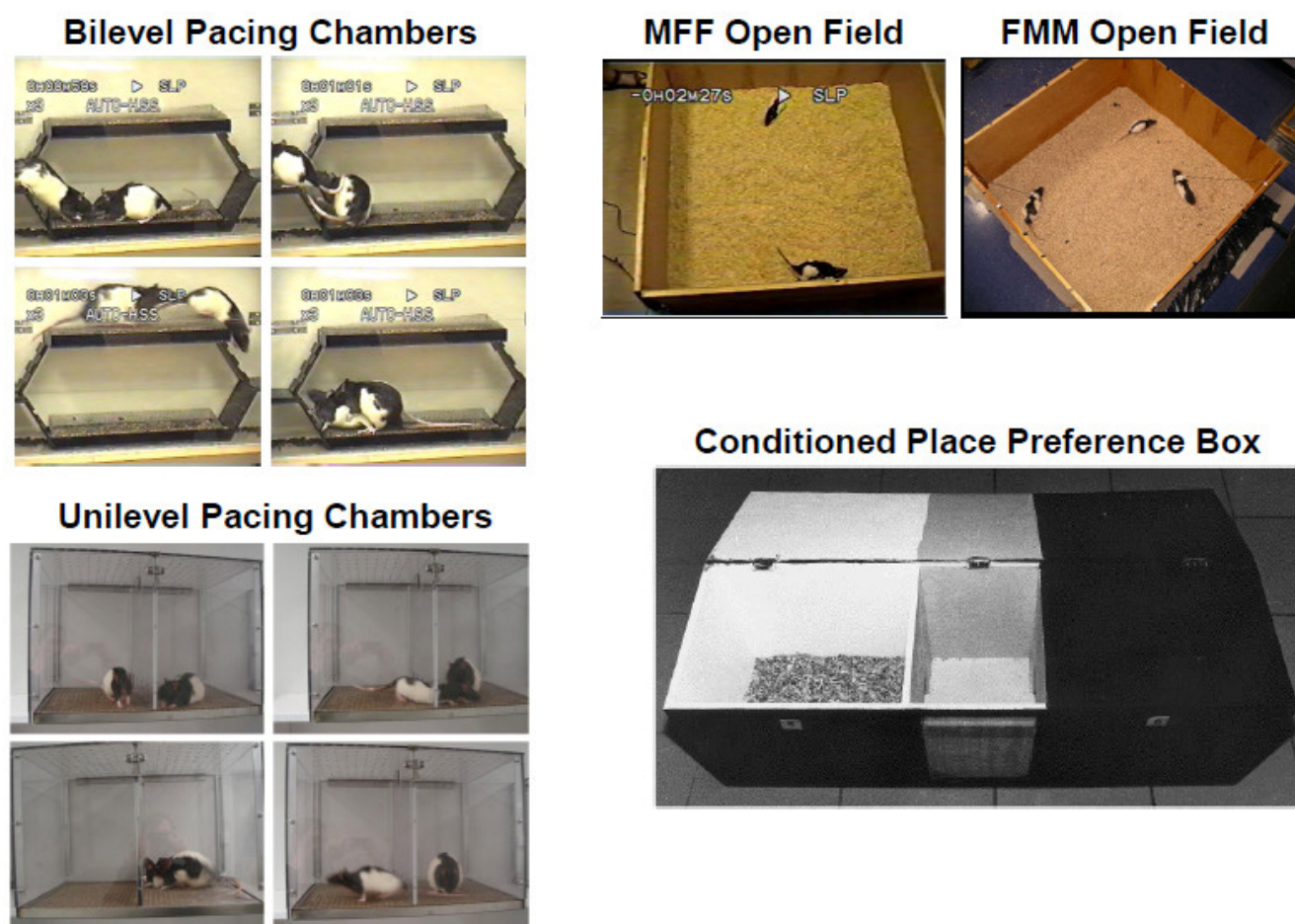


and mate preference between members of their own and foster species. Results showed that both cross-fostered males and females significantly chose more frequently to socialize and selectively mate with partners of their maternal species. These effects were more pronounced and long-lasting in males than in females. In contrast, all control animals preferred to socialize and mate exclusively with members of their own genetic species [49]. These findings provided insightful evidence on how the environment and some learning experiences shape our partner preference choices even beyond what is believed to be pre-set biologically, and suggests that epigenetic changes in attention and bonding mechanisms are altered by the experience of reward.

### *2.1. Classical Conditioning of Sexual Behavior*

One of the fundamental forms of learning by which animals can acquire references from their environments and experiences was described by Pavlov [25], often referred to as Pavlovian or classical conditioning. This form of learning states that neutral cues, or conditioned stimuli (CSs), acquire meaning by their predictive association with biologically relevant cues, or unconditional stimuli (USs). This would lead ultimately to a CS becoming a priming cue that elicits a conditioned response (CR) similar to that elicited by the US, or unconditioned response (UR; e.g., [50]).

One of the first demonstrations that copulation induced a sexual reward state came from Domjan and Hall [51] who showed that male quail would remain around the vicinity of a test box window where they had previously seen a female quail. This behavior developed only if the male quail had copulated previously with that female. Subsequently, Ågmo and Berenfeld [52] found a similar phenomenon in male rats using the conditioned place preference (CPP) paradigm. In their study, male rats were placed in a CPP box with three compartments (see Figure 3). Initially, males were placed in the middle compartment and allowed to roam freely among the three compartments, each of them with different characteristics (i.e., floor texture, background color, and illumination as either dark or light). The time spent in each determined the rat's natural preference, where males expressed a typical preference for the dark side over the light side. Male rats were then given several experiential training trials. One group of males was allowed to copulate to ejaculation with receptive females in a different chamber and immediately afterward put in the non-preferred (typically light) side of the box and left for 30 min. Two more groups were given the same sexual experience, but injected with either the opioid receptor antagonist naloxone (NAL), or the dopamine receptor antagonist pimozide (PIM) five min before copulation. Experiential control groups were injected with either NAL, PIM, or the  $\mu$ -opioid agonist morphine without subsequent copulation. Finally, male rats were tested for CPP by measuring the time spent in either compartment of the box after being put in the middle compartment. Males who were allowed to copulate until ejaculation, identically to those injected with morphine, shifted their preference and spent more time in the previously non-preferred side, whereas NAL-treated males that copulated did not prefer either of the sides. Furthermore, PIM-treated males did not show a preference for either side of the box, whereas PIM-treated males who were also allowed to copulate until ejaculation did show a preference for their non-preferred side. Control groups that did not copulate or were only injected with vehicle before copulation did not show any preference for either of the sides of the box [52]. Together, these results show that the aftermath of ejaculation, while the male is in a post-ejaculatory refractory state, has rewarding properties capable of establishing positive conditioned associations with cues of the immediate context or environment, and that these associations are founded in the action of opioid, but not dopamine, neurotransmission.



**Figure 3.** Left: Bilevel and unilevel pacing chambers used for conditioning of partner preferences (adapted from Pfau et al., [53] with permission. 2015, Elsevier). Pacing chambers allow the female to regulate copulatory contact with the male by either running from level to level (**top**) or by darting through small holes in a divider that are big enough to allow the female to pass through, but too small to allow the male to pass through (**bottom**). In the bilevel pacing chambers, females make a head-wise orientation to the male (**upper left panel**), then hop over him exposing their anogenital area (**top right panel**). Females then run away, forcing the male to chase them (**bottom left panel**), after which they run to the original level and assume a lordosis crouch, allowing the male to mount with intromission (**bottom right panel**). Similarly, in unilevel pacing chambers females can pass through the hole into the male's side (**top left and right panels**) and solicit an intromission (**bottom left panel**), then exit the male's side (**bottom right panel**). Each of these sequences depict the initiation and termination of a copulatory bout by the female. **Right top:** Open fields used for testing conditioned partner preferences and CEP (adapted from Pfau et al., [53] with permission. 2015, Elsevier). To test CEP, a male is placed into the open field with two females, one scented or jacketed and the other unscented or unjacketed. The male can then copulate freely with either one. To test partner preferences in females, a female is placed with two tethered males, one scented or jacketed, and the other unscented or unjacketed. Males are tethered to avoid their tendency to huddle together. Females compete, so they can remain untethered. **Right bottom:** Apparatus used to condition and test place preferences after sexual reward training (Reprinted with permission from Paredes and Vasques [54]. 1999. Elsevier). After a requisite amount of copulatory stimulation, a female or male is placed on one side of the box (usually the light side as this is typically not their naturally preferred side). This is contrasted without stimulation, after which the female or male is placed into the naturally preferred (e.g., dark) side. If the copulatory stimulation induces a reward state, then on a final test, after the animal is placed into the middle compartment, it will move to the side with cues that predict the reward state and spend more time there compared to the other side.

As with CPP, male rats develop a conditioned ejaculatory preference (CEP) toward females bearing an odor that has been previously associated with sexual reward [55]. Working on the findings of a previous study by Graham and Desjardin [56] showing that plasma luteinizing hormone and testosterone were elevated significantly in male rats by a neutral wintergreen odor paired with copulation to ejaculation, Kippin and colleagues trained male rats to associate a neutral almond odor or no odor with copulation to ejaculation. The following three groups were tested: a paired group trained to differentiate almond-scented receptive females from unscented non-receptive females; an unpaired group trained to differentiate unscented receptive females from scented non-receptive females; and a random-paired group randomly paired with receptive and non-receptive scented females (making the odor irrelevant). In a final preference test, males were allowed to copulate freely with two receptive females, one scented and the other unscented (see Figure 3). Males in the paired group displayed a CEP for the females bearing the odor whereas males in the unpaired group displayed a CEP for the unscented females. Males in the random-paired group did not display a preference for one female over the other [55]. Subsequently, Kippin and Pfau [57] found that the CEP depended on the male being in the presence of the scented female during the post-ejaculatory reward state. Male rats even learned to develop a preference for an unconditionally aversive odor (cadaverine) if it was associated with the postejaculatory reward state [19]. Finally, a generalized reward state can alter CEP. Ménard et al. [58] found that pairing a lemon odor in the bedding while applying gentle strokes on rat pups after separation from their mother would imprint a preference towards this odor, leading the male pups as adults to ejaculate preferentially with a lemon-scented female partner in their first sexual experience, compared to pups who did not have the lemon odor while stoked. This not only suggests that early experiences play a role in partner choice, but raises the question of whether first experiences with sexual reward in particular may shape the individual's future partner choice by similar mechanisms.

Although rats rely heavily on their sense of smell, similar findings were reported for somatosensory and visual CSs. Domjan, Huber-McDonald and Holloway [59] used Japanese quail males and assigned them to two groups. Both were presented with an inanimate taxidermic female quail with which they could copulate for 30 sec followed by access to a sexually receptive quail hen. In the fading group, the taxidermic object was gradually covered with terrycloth over successive trials, until fully covered leaving no quail features in the last trial of training. The non-fading group was always presented with the fully covered inanimate object. After the training, each subject was tested in the training boxes for five min. with the fully covered inanimate object, except that no live female quail was introduced. Overall, males trained in the fading group spent more time around the object and displayed more copulatory behaviors (i.e., grabs, mounts, and cloacal contacts) towards the fully covered inanimate object than the males trained in the non-fading group. These data demonstrated that sexual behavior is able to be conditioned towards an inanimate stimulus object that has no natural connection with sexual reward [59]. Similarly, Köksal et al. [60] demonstrated persistence in copulation with an inanimate object after an extinction procedure only when the trained CS was the same terrycloth used by Domjan et al. [59], but not when it was a light. Male rats were also trained to show CEP for the strain of female (e.g., pigmented vs. albino) that was paired with the post-ejaculatory reward state [61]. Although the two strains clearly differ in terms of visual cues, differences in terms of accessory or main olfactory cue strength cannot be ruled out. However, male rats have also been conditioned to show a CEP for females wearing a rodent tethering jacket versus no jacket [62], suggesting a high degree of flexibility in the sensory modality of the CS that is paired with the ejaculatory reward state US. As with olfactory conditioning, the development of this CEP was blocked by systemic naloxone administration [63]. Interestingly, the same rodent jacket somatosensory CS can also be conditioned to modulate sexual arousal. Pfau, Erikson and Talianakis [64] trained males to have their first 10 sexual experiences to ejaculation with or without wearing a rodent tethering jacket. On

the final test, rats in both groups were randomly assigned to have the jacket on or jacket off. Males trained and tested with the jacket on copulated normally, as did males trained without the jacket and tested with it. However, significantly fewer males trained with the jacket copulated to ejaculation with the jacket off; and those that did displayed significantly fewer anticipatory behavior, ejaculations, and longer intromission latencies than males in the other groups. A second experiment showed that the jacket could acquire inhibitory properties if it was on the male when he was paired with a sexually non-receptive female. These data show that a somatosensory CS that the male comes into physical contact with, much like the terrycloth inanimate object used by Köksal et al. [60], can come to acquire sexually arousing properties if paired with the post-ejaculatory reward state.

Both CPP and conditioned partner preference were also demonstrated in female rats. Previous studies demonstrated that females develop a preference for the non-preferred side of the CPP box if the contextual cues are paired with paced, versus non-paced copulation in unilevel pacing chambers [54,65]. Bilevel or unilevel pacing chambers allow females to control the initiation and rate of copulation, either by running from level to level [66] or to exit from and return to the side with the male through a divider with holes that accommodate the female to pass through, but that are too small for the male to pass through [67], respectively (see Figure 3). Importantly, pacing conditions in the unilevel chamber can be modulated simply by removing the divider. With the divider, the female controls the initiation and rate of copulation. Without the divider, the male is more likely to control the initiation and rate. Females also developed a conditioned partner preference based on a neutral almond odor placed on a male rat in either bilevel chambers or unilevel chambers with the divider [68]. Using a similar conditioning training procedure as Kippin et al. [55], paired females developed a preference for scented versus unscented males which they displayed on a final open-field test with two tethered males, one scented and the other unscented: Females showed more solicitations of the scented males, more high-magnitude lordosis, and chose the scented male more often to receive his ejaculations. Systemic naloxone administration blocked the development of this conditioning [69]. Similar results were obtained when the strain of male (pigmented vs. albino) was used as a composite CS [70] and when the rodent jacket was used as a CS on the male [62].

Although pacing allows the female to control the initiation and rate of copulation, in fact it allows her to receive clitoral stimulation (CLS) and vaginocervical stimulation (VCS) from male mounts with intromissions and ejaculations at a preferred interval. Artificial CLS that mimics the rate of paced copulation not only produces CPP in sexually naïve females [71,72], but it also supports the conditioning of part of the partner preference observed by Coria-Avila and colleagues. Parada et al. [73] gave sexually naïve females repeated pairings of distributed CLS in the presence of an almond-scented gauze pad versus no CLS in the presence of an unscented gauze pad. On a subsequent open field test with two tethered males, one scented and the other unscented, the females solicited more frequently and showed higher magnitude lordosis with the scented male compared to the unscented male. However, they did not choose the scented male to receive his ejaculations selectively, suggesting that VCS may contribute to the mate choice aspects of conditioning in females.

Sexual reward states that ride on high arousal are also more potent and differentiate contextual cues from partner-related cues. For example, using CPP, male rats appeared to prefer a four-hole divider over a one-hole divider in a unilevel pacing context (see Figure 3). However, for CEP to be conditioned, rats must be trained with the one-hole divider [74]. Similar results were obtained in female rats [75], especially as it concerned the female's choice of male from whom to take ejaculations. This typically imposes a greater inter-intromission interval for the male and longer return latency of the female after mounts, intromissions, and ejaculations, especially if the male puts his head into the hole to try to reach the female. Females typically impose a longer inter-intromission interval prior to a male's ejaculation in bilevel chambers [66]. They do this by running from level to level more times, which forces the male to chase them for longer periods. It appears

that extending the time the animals have to wait to get genital stimulation increases their arousal, which, in turn, increases the potency of the postejaculatory or CLS reward state.

## 2.2. Instrumental Learning and Sexual Behavior

Instrumental learning originates with Thorndike's law of effect [76] that states that if a behavior in the presence of a stimulus is followed by a satisfying consequence, the established association between the behavior and the stimulus becomes strengthened. Similarly, when the behavior is followed by an aversive consequence, the association weakens. This type of conditioning defines a contingency between responses and their reinforcers, and the term "operant" was coined by Skinner [77]; for behaviors that "operate" on the environment), who advanced the study of this form of learning by allowing animals to freely perform the behavior, as opposed to discrete-trial procedures. That way, animals were free to perform and repeat the operant response over and over. Thus, the behavior could be shaped based on different schedules of reinforcement or punishment [78].

Several demonstrations of male rats performing lever-pressing operants to gain access to sexually receptive females were documented [79,80], along with overcoming obstacles [81], or crossing shock grids or other aversive tasks [38,82–84]. Other preparations that manipulated brain neurochemistry and anatomy were also explored. For example, axon-sparing neurotoxic lesions of the basolateral amygdala disrupted lever pressing for a secondary sexual reinforcer (a stimulus light paired with access to a sexually receptive female), whereas it did not affect copulation in males [85]. In contrast, the same lesions of the mPOA disrupted copulation, leaving conditioned lever pressing virtually unaffected at the time of testing [86].

## 2.3. First Experiences of Sexual Reward

In every case, the studies reviewed above controlled an animal's first sexual experience and provided the context for sexual reward or non-reward during that and subsequent sexual experiences. In humans, first sexual experiences may or may not coincide with sexual reward, and indeed sexual reward from orgasm or other sensory stimulation may not occur every time a person has sex. However, more controlled manipulations of first experiences have been tested experimentally. In order to elucidate what first experiences with neutral or biologically relevant stimuli actually do, researchers exposed animals to either the CS or US before training for conditioned preference, resulting in different outcomes depending on what is being pre-exposed and for how long.

*US pre-exposure (blocking)*: US pre-exposure is the phenomenon in which there is retardation or outright blocking of the establishment of a CR if its CS is paired with the US in a context in which the animal was previously exposed to the US alone (e.g., [87]). For example, Taylor [88] conditioned the blinking response of human participants using an air puff signaled by a light. Before training, one group received several presentations of the air puff in three different intensities to the cornea of their eyes without light. The number of eye-blink responses was greater in the group that was not pre-exposed to the air puff, whereas in the pre-exposed group the number of eye-blink responses was in an indirect correlation with the intensity of the air puff during the pre-exposure phase. Two different explanations were proposed, one associative and the other non-associative. The former proposes that the US pre-exposure effect is due primarily to an association between the US and cues in the context in which the initial US pre-exposure takes place prior to training (e.g., [50,87]). The latter claims that by pre-exposing the US, there is a reduction in initial emotional reactivity of the animal's response due to general habituation that reduces the salience of the US, and thereby attenuates subsequent excitatory conditioning (e.g., [89]). In a study of blocking [90], male rats were given one or five copulatory experiences to one ejaculation each with unscented receptive females. Subsequently, males were given another 10 experiences with almond-scented receptive females, and later on tested in an open field on their 11th trial with two receptive females, one scented and the other

unscented. Regardless of the number of US pre-exposures to the unscented female, males did not display any CEP to the scented or unscented females, indicating that the first experience of the post-ejaculatory reward state was sufficient to block the ability of the odor to control the associative strength. Likewise, CLS is no longer capable of inducing CPP in female rats that have had experience with paced copulation in a bilevel chamber [91].

*CS pre-exposure (latent inhibition):* As with blocking, pre-exposing an animal to the CS produces a disruption or retardation of a subsequent trained association with the same CS (e.g., [92]). For instance, animals pre-exposed to a saline solution used later on to train conditioned taste aversion showed retardation of the association in comparison to a control group that was not exposed to it previously [93]. Most theories coincide in that latent inhibition is the result of a reduction in associability or attention to CS during pre-exposure [94]. This learning phenomenon and its properties highlights the ability of animals to form new associations through passive, non-reinforced pre-exposure of CSs, demonstrating that previous experiences influence them when being trained to learn new associations with neutral cues. Zamble, Mitchell and Findlay [95] demonstrated that single CS or contextual cues can facilitate copulation in Japanese quail (i.e., reduced ejaculation latency) if they predicted copulation with a receptive female. However, when animals were pre-exposed enough times to the mating context, the background cues were shown to be subjected to latent inhibition. Similarly, Quintana et al. [96] gave male rats one or five pre-exposures to the neutral almond odor on gauze prior to giving males 10 copulatory trials to ejaculation with scented receptive females. On the final test, males were placed into an open field with two receptive females, one scented and the other unscented. The group provided with one odor pre-exposure displayed significant CEP, whereas the group that received five pre-exposures did not.

#### 2.4. Potentiating or Inhibiting Conditioning during First Experiences with Reward

In addition to high arousal, the ability of certain drugs to potentiate conditioning during first experiences with sexual reward was examined. The results of these studies begin to frame the neurochemical systems that sensitize during first experiences with sexual reward. Ménard et al. [97] found that parvocellular OXT neurons in the paraventricular nucleus of the hypothalamus (PVN) that project to other brain regions and magnocellular vasopressin (AVP) neurons in the supraoptic nucleus of the hypothalamus (SON) that project to the posterior pituitary were conditionally activated by the almond odor CS in male rats. A subsequent experiment injected either OXT, AVP, or saline, subcutaneously to males prior to their first sexual experience to ejaculation with receptive females. All males were injected with saline prior to their second sexual experience in an open field with two receptive females, one scented and the other unscented. Only males injected with OXT during their first sexual experience to ejaculation showed a weak but statistically significant CEP during the open field trial.

Likewise, female rats given repeated paced copulation with an unscented male rat displayed classic mate-guarding behavior when placed into an open field with their familiar male and a competitor female [98]. This behavior consists of hovering and presenting postures close to the male, attempts to block access of the competitor female to the male by getting between them, and the aggressive mounting of, or outright fighting with the competitor female if she solicits the male. In contrast, if the conditioned female is receptive and with a novel male and a receptive competitor female, she displays a species' typical pattern of competitive solicitations and interceptions of the male, similar to the observations of McClintock [99]. Holley et al. [100] found that conditioned females exposed to their familiar males also had significant activation of parvocellular OXT neurons in the PVN and magnocellular OXT and AVP neurons in the SON. Holley et al. [100] also examined the effect of subcutaneous OXT, AVP, or saline injections during the females' first paced experiences with a male on subsequent mate guarding during their second experience. All females were injected with saline prior to their second sexual experience,

which took place in an open field with their familiar male and a competitor female. Females injected with OXT showed significantly more incidents of hovering and presenting relative to saline controls, made significantly more solicitations, and received significantly more intromissions and ejaculations. In contrast, females injected with AVP displayed significantly more incidents of conspecific blocking of the competitor female relative to females previously treated with OXT or saline. Thus, in both male and female rats, the hyper-OXT state augmented the experience of sexual reward and produced significant learning of a partner preference. A hyper-AVP state did not do this in males, but potentiated the vigilance of the female toward the competitor. Finally, female rats that received systemic injections of naloxone during their first paced sexual experiences demonstrated a lack of sexual interest, solicitations, and lordosis on a final saline test despite being fully hormonally primed [19]. Similarly, females that received subcutaneous injections of the lysine-specific demethylase inhibitor oryzon (to prevent epigenetic alterations resulting from sexual experience) during paced conditioning trials with the same male did not display any mate-guarding behaviors with that male, nor did they display an increased activation of OXT or AVP neurons in either the PVN or SON [101]. Male rats that received systemic naloxone injections during their first sexual experiences with almond-scented females displayed a preference for the unscented female on the final test [74]. These data indicate an important interaction of the sexual reward state with mechanisms of arousal, attention, and bonding that were induced by both contextual and partner related cues that predict the reward state. How might this occur?

### 3. Brain Activation by Sexual Experience and Cues Associated with Sexual Reward

As with other motivational systems and rewards, experience with sexual reward changes the brain and sensitizes behaviors aimed at acquiring sexual reward [102–105]. These changes occur within neural excitatory systems, including up-regulation of nitric oxide synthase in the mPOA (NOS; [106,107]), OXT receptor in the VMH and central amygdala [108], immediate early gene proteins such as  $\Delta$ FosB in the VTA, NAc, medial prefrontal cortex and infralimbic cortex [104], enhanced synthesis of brain-derived neurotrophic factor (BDNF) and its tyrosine kinase receptor B (trkB; [104]), sensitized DA release in the NAc and medial prefrontal cortex [109–112], and altered DA cell morphology and enhanced function in the VTA [113]. In addition to the activation of OXT and VP neurons in the PVN and SON reviewed above, cues associated with sexual reward activate overlapping neural circuits for sexual excitation, reward, and bonding [114,115]; similar to the regions activated by direct copulatory stimulation [72,116] and stimulate DA release conditionally in the NAc [19]. Pitchers et al. [113] found that the activation of  $\Delta$ FosB in the NAc was necessary for the reinforcing properties of sexual reward in male rats, presumably on the role played by mesolimbic DA transmission in this critical terminal region.

### 4. Dual Role of Endogenous Opioids in Sexual Behavior

There are three main types of endogenous opioids: endorphins, enkephalins, and dynorphins. They are the result of an enzymatic process of three different precursor molecules, pro-opiomelanocortin (from which melanocortins such as  $\alpha$ -MSH and corticotropins such as ACTH are derived), pro-enkephalin, and pro-dynorphin [117–119]. There are also three types of opioid receptors,  $\mu$ ,  $\kappa$ , and  $\delta$ . These receptors are located predominantly in hypothalamic, limbic, and cortical areas [120,121] and have different roles in the control of sexual behavior. Although enkephalins and dynorphins are the natural ligands of  $\delta$  and  $\kappa$  receptors, respectively, the  $\mu$  receptor has a number of endogenous ligands that bind to it, including endomorphins, endorphins, enkephalins, and morphiceptin (derived from the milk protein  $\beta$ -casein). Upon agonist stimulation, opioid receptors are internalized into clathrin-coated endosomes and are no longer expressed on the cell surface [43,122]. Opioid receptor internalization, then, can be used as a measure of agonist binding.



#### 4.1. Inhibition and Refractoriness or Disinhibition

During copulation, and after several vaginal intromissions, male rats ejaculate and fall into a period of sexual quiescence in which another erection cannot be achieved in the span of a few minutes, a period known as the post-ejaculatory interval (PEI) or refractory phase (for a review see [6,32]). Likewise, female rats have relatively short refractory periods (30–60 s) after ejaculation or distributed CLS [67], and much longer refractory periods after multiple VCSs that define estrous termination [123].

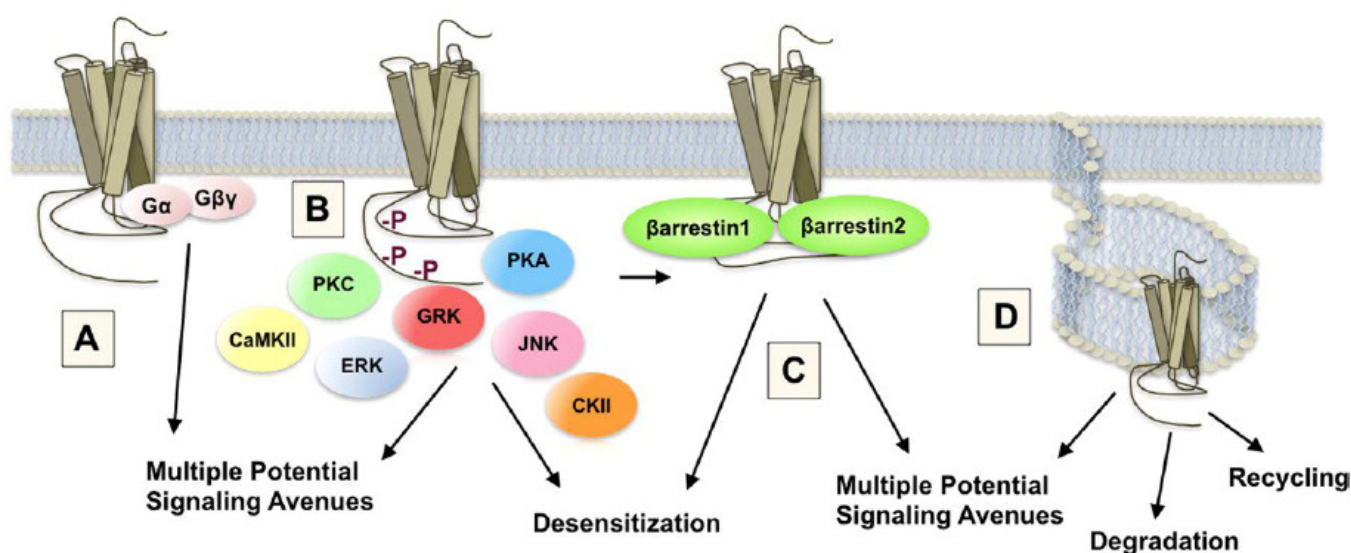
Many of these inhibitory behavioral responses can be mimicked by the systemic or central administration of opiate drugs or by the central administration of endogenous opioids [22,40]. For example,  $\beta$ -endorphin infused into the mPOA of sexually experienced male rats inhibited their copulatory behaviors in a dose-dependent fashion [46,124]. Furthermore, compared to sexually active males, sexually inactive males demonstrated an increment of endogenous opioid octapeptide Met-Arg<sup>6</sup>-Gly<sup>7</sup>-Leu<sup>8</sup> in the hypothalamus [125], as well as an increment of pro-enkephalin and pro-dynorphin mRNA expression in the paraventricular nucleus [126]. Infusions of morphiceptin into the mPOA of male rats produced a delay in their initiation of copulation compared to the control animals infused with a vehicle solution [127]. Moreover, inhibition of copulatory behaviors (e.g., copulation latency and ejaculation) was found when infusing a  $\kappa$  opioid receptor agonist (U-50488H), an effect that disappeared differentially when a  $\kappa$  opioid receptor antagonist (nor-binaltorphamine) was infused into the VTA, mPOA, or NAc [128]. Facilitation of male sexual behavior was reported when the longer-acting opioid receptor antagonist, naltrexone, was administered in sexually inactive males [129], sexually naïve males [130], and sexually satiated males [131], and also reduced the ejaculation latency and increased ejaculation frequency in sexually active male rats [132]. However, some of these effects may be disinhibitory and counteract the effect of stress-induced opioid release (e.g., [130]) or a progressive buildup of opioid binding after multiple ejaculations [131]. These data suggest that the NAc, mPOA, and VTA are important sites for the opioid mediation of sexual inhibition.

Much of the work on opioids in females has been limited to lordosis, with selective  $\mu$  receptor activation being inhibitory and selective  $\delta$  or  $\kappa$  activation having a facilitative effect [22,133]. NAL in all cases reversed these effects. In many cases, however, endogenous  $\beta$ -endorphin or morphiceptin had a dose-dependent dual effect, with low doses facilitating and higher doses inhibiting lordosis. Different opioids have inhibitory effects in different brain regions. For example, endomorphins inhibit lordosis after intraventricular infusions or site-specific infusions to the lateral septum and diagonal band of Broca, whereas the synthetic  $\mu$  agonist D-Ala<sup>2</sup>-Met<sup>5</sup>-enkephalin (DALA) inhibited lordosis following bilateral infusions to the mPOA and VMH [134]. Facilitation of proceptive behaviors was observed following infusions of a  $\delta$  agonist to the third ventricle of female rats primed with estradiol and progesterone, but not estradiol alone [133]. Recently, Johnson, Hong and Micevych [135] inhibited lordosis using the optogenetic activation of  $\beta$ -endorphin terminals in the medial nucleus of the mPOA of female POMC-cre mice primed with estradiol and progesterone. The inhibition was accompanied by the internalization of  $\mu$  opioid receptors.

#### 4.2. Reward-Related Sensitization of Incentive Cues

Opiates and opioids are well known for their rewarding or positively reinforcing properties. Morphine and  $\beta$ -endorphin both induce CPP and support both peripheral and intracranial self-administration [136–140]. Likewise, copulation and ejaculation in males, or paced copulation/distributed CLS in females, are crucial rewarding events in the establishment of CPP [141], CEP [57,74], and conditioned partner preference [69]. As with all reward-related learning, the administration of the opioid receptor antagonist NAL or naltrexone block the acquisition of these conditioned responses indicating that endogenous

opioid transmission is critical. Indeed, whole-brain  $\beta$ -endorphin content increases dramatically after ejaculation in male rats [42], with specific increases in enkephalin content observed in the midbrain and hypothalamus [93]. Copulation to ejaculation also induces  $\mu$ -receptor internalization (a marker of ligand-induced receptor activity) in the mPOA [43] and VTA [44]. More specifically, it has been shown that different numbers of ejaculations render different  $\mu$ - and  $\delta$ -receptor internalization in the VTA, and although the internalization of both receptors increased as a result of ejaculation, only  $\mu$ -receptor internalization was correlated with the number of times a male ejaculated [41,142], suggesting that this receptor carries the reward or pleasure signal. Indeed, bilateral infusions of DALA to the mPOA, induce significant CPP [141]. These data are consistent with the overall notion of  $\mu$  receptors in many reward-related regions of the brain carrying the reward signal (e.g., [143]), with  $\delta$  receptors in the NAc shell further enhancing reward [144]. The reward signal impinges on neural systems for motivation, or more specifically, desire and attention (see Figure 4).



**Figure 4.** Structure and function of the  $\mu$  opioid receptor and interaction with second messenger systems. The schematic demonstrates key points in opioid receptor signaling and regulation that was shown to be influenced by differential agonist occupation. (A) heterotrimeric G proteins represent 16 individual gene products for  $G\alpha$ , 5 individual gene products for  $G\beta$  and 11 for  $G\gamma$  proteins. Together, the diversity arising from heterotrimeric G protein subunit composition presents a gateway to potentially high diversification of agonist-directed coupling between  $\mu$  opioid receptor and G proteins. These interactions can determine access to secondary cascade activation. (B) the  $\mu$  opioid receptor can be phosphorylated in response to agonist occupation by multiple kinases, each of which has multiple isoforms. Phosphorylation by a particular kinase may dictate secondary cascade interactions or subsequent receptor fate. CKII, casein kinase II. (C) receptor interaction with scaffolding partners such as  $\beta$ -arrestins can be dependent or independent of receptor phosphorylation. Agonist occupancy may determine these interactions with potential binding partners. Such interactions can prevent (desensitization) or promote subsequent signaling. (D) the  $\mu$  opioid receptor can be internalized in response to agonist occupancy. Endocytosis may involve clathrin- or caveolin-dependent processes and may result in the activation of subsequent signaling pathways, receptor recycling or degradation. Reprinted with permission from Raehal et al. [145] (2011, The American Society for Pharmacology and Experimental Therapeutics).

#### 4.3. Antagonism of Opioid Reward

As mentioned previously, copulatory-induced CPP in both females and males, CEP in males and conditioned partner preference in females can be disrupted by the administration of NAL during training. Appetitive aspects of sexual behavior that denote desire

are strongly affected by the blunting of opioid reward. Male rats in bilevel chambers naturally develop a characteristic level searching behavior prior to the introduction of a sexually receptive female [146]. This behavior was blocked by the systemic administration of NAL [147]. In females, repeated training trials with NAL resulted in a dramatic decline in solicitations and lordosis overall, and an increase in the display of rejection responses made toward both males in the open field choice test, despite females being fully primed with estradiol and progesterone [69]. However, regarding CEP in males, if the training with NAL is paired exclusively with the scented female, the disruption of CEP for the scented female is typically a shift in choice to the unscented female during the final open field choice test, despite those males copulating to multiple ejaculations during the training trials. A similar phenomenon was reported in male prairie voles. Typically, males display a partner preference for a familiar female with whom they cohabitated and copulated relative to an unfamiliar female. However, if that cohabitation occurred under the influence of naltrexone, the males shift their preference to the unfamiliar female [148]. This suggests that the antagonism of opioid receptors during copulation induces a state of non-reward that is associated with the familiar female, and shifts the male's preference to the unfamiliar or novel female. A state of non-reward induced by frustration also shifts the preference of female rats toward the male associated with it. Parada et al. [91] gave females distributed CLS in the presence of a scented but inaccessible male using a wire-mesh screen to divide the unilevel pacing chamber. On the final open field choice test, females solicited and received ejaculations selectively from the unscented male relative to the scented male.

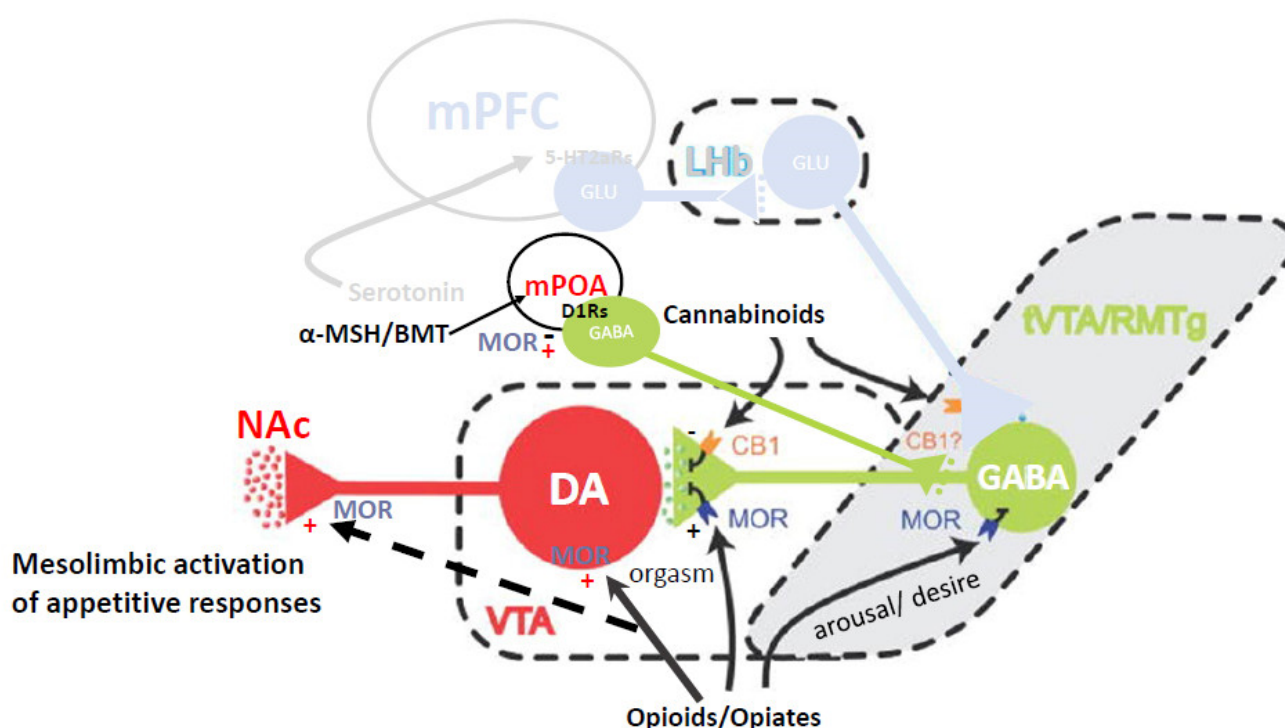
Brain regions involved in this behaviour have begun to be examined. Burkett et al. [148] infused the selective  $\mu$  receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH<sub>2</sub> (CTOP) to either the NAc or caudate-putamen (CP) of male prairie voles (identical to the group above that received naltrexone). Infusions to the CP, but not NAc, disrupted partner preference and the time spent huddling, relative to saline infusions. Quintana et al. [149] examined the role of NAL infusions to the mPOA or VTA on CEP. Relative to males infused with saline, infusions of NAL to the mPOA shifted the preference towards the unfamiliar female (similar to peripheral injections of NAL), whereas infusions to the VTA abolished CEP completely. Taken together with the data of Ågmo and Gomez [141], the mPOA would appear to be a region where the reward value of sexual behavior (and perhaps other motivated behaviors) is determined by opioid action. In contrast, regions of the mesolimbic pathway (e.g., VTA and NAc) appear to be involved in the opioid sensitization of attention. This raises a paradox: opioids inhibit sexual behavior in these regions but, with time, sensitize the systems responsible for sexual incentive motivation, including reward-related attention and partner preference. How might this work at a neuropharmacological and molecular level?

## 5. Neuropharmacological, Signal Transduction, and Molecular Mechanisms of Opioid Sensitization

### 5.1. Mesolimbic DA Sensitization

The ability of sexual reward to induce CPP, CEP, partner preference, and to enhance appetitive sexual behaviors, must involve a process of sensitized attention to environmental and partner-related cues. One of the classic mechanisms of attention to reward-related cues is the sensitized activation of mesolimbic/mesocortical DA [150–154] (see Figure 5). The A10 DA neurons that make up this pathway originate in the ventromedial portion of the VTA and project to a number of cortical and limbic sites, including the prefrontal cortex, anterior cingulate, septum, amygdala, and NAc [155–157]. In prairie voles, DA release in the NAc is particularly important for pair-bond formation [158], with DA actions on the D2 receptor family being important for formation and actions on the D1 receptor family important for retention of the pair bond [159]. Sensitized DA cell body activation in the VTA, sensitized DA release in the septum, and/or sensitized DA release in the NAc, can

be induced by opioids acting at the  $\mu$  or  $\kappa$  receptor, respectively, and plays a major role in Pavlovian learning. This has been shown for stimulus–drug associations [160,161] and other forms of incentive learning in which an external stimulus predicts a central reward state. When conditioned, such stimuli become particularly resistant to extinction. We note that DA release in response to the familiar odor associated with CEP caused an increase in NAc DA release to an average of 180% to 200% in paired males, but produced only a small and non-significant increase in unpaired males [19]. Similarly, paired males also showed a slight but nonsignificant increase to a novel odor not associated with CEP. This indicates that the familiar odor as a predictor of sexual reward (and CEP) had sensitized DA release in the NAc. Although not tested, it is likely that NAL blocked this sensitization given that it blocked the acquisition of CEP. Indeed, NAL prevents the ability of morphine infusions to the VTA to induce CPP, and naltrexone treatment blocks the reinstatement of both heroin and cocaine self-administration in rats [162].



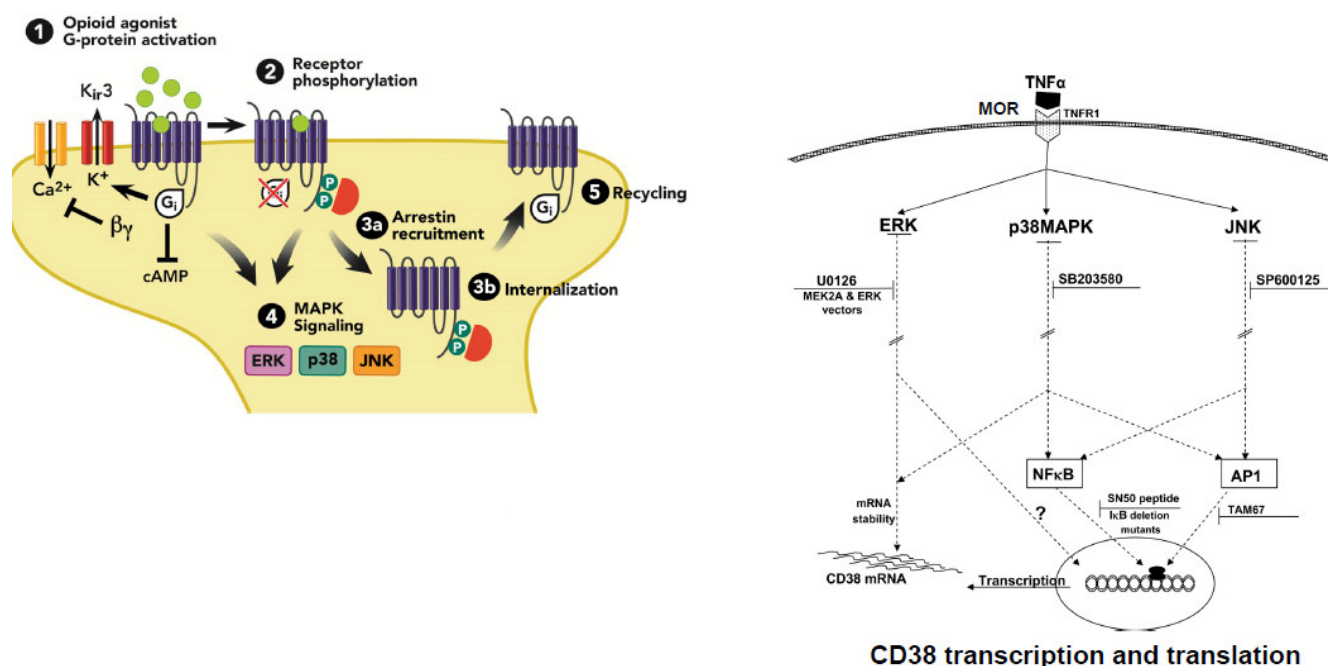
**Figure 5.** Neuroanatomical mechanisms of opioid inhibition and sensitization of mesolimbic and incerto-hypothalamic DA transmission. Opioids such as  $\beta$ -endorphin disinhibit dopamine release during sexual arousal and desire, but inhibit it at orgasm. This inhibition, however, results in sensitization of dopamine in subsequent trials [163,164]. Adapted from Barrot et al. [165] (2012, Barrot et al.)

The mechanisms responsible for DA sensitization by  $\mu$  opioid receptor agonists have been studied in the VTA and NAc. In the VTA, DA neurons are typically under tonic inhibition by GABA neurons from the ventral pallidum or rostromedial tegmental nucleus, and by local interneurons that are activated by glutamate outputs from regions such as the cortex. These interneurons are inhibited by agonist action at  $\mu$  receptors, thus disinhibiting DA neuronal activity [44,166]. However, DA neurons can be activated by glutamate projections directly, and presynaptic  $\mu$  receptors on glutamate terminals can inhibit this activation. This provides opioid, GABA, and glutamate projections to the VTA with the ability to inhibit or facilitate DA cell body activation. In the NAc, DA release occurs in the following two forms: transient or phasic DA release caused by the depolarization of DA cell bodies in the VTA, and sustained or tonic release regulated by afferents from the PFC and amygdala to the NAc [167]. In male rats, the appetitive period of sexual

behavior is characterized by phasic DA release in the NAc, whereas copulation is characterized by tonic release until ejaculation, when DA levels plummet and remain low for the absolute refractory period, then slowly rise up during the relative refractory period [109,168]. The decrease in DA is caused in part by opioid and serotonin actions that inhibit DA cell bodies and presynaptic terminals in the NAc. Infusions of morphine or dynorphin into the VTA increases DA release in the NAc, which ultimately facilitates male sexual behavior [169]; however,  $\mu$  receptor agonists increase the length of the PEI (e.g., [170]), again showing that opioids acting on  $\mu$  opioid receptors can facilitate or inhibit different aspects of copulation in males. Importantly, Kippin and Pfaus [57] determined that being in the presence of the scented female during the post-ejaculatory refractory period is when the incubation of learning occurs that results in the CEP. This is a period when DA activity is inhibited, but opioid activity is enhanced. In particular, this enhancement comes from the following two opioids working simultaneously:  $\beta$ -endorphin activation of  $\mu$  receptors on GABA interneurons in the VTA and dynorphin activation of  $\kappa$  receptors on presynaptic DA terminals in the NAc.

### 5.2. DA Related Signal Transduction and Genomic Mechanisms

The signal transduction and molecular mechanisms that underlie opioid sensitization of DA transmission occur during this period of DA suppression. All opioid receptors are coupled to G-proteins, making opioid receptors members of the GPCR “family”. Agonist binding is diminished by guanine nucleotides and agonist-stimulated GTPase activity. In addition, all opioid receptors inhibit adenylate cyclase [171]. Many GPCRs stimulate mitogen-activated protein kinase (MAPK or ERK) activity, although MAPK activation is not dependent on  $\mu$  receptor internalization [172]. Although opioid receptors inhibit voltage-gated N-type  $\text{Ca}^{2+}$  channels, they activate inwardly rectifying  $\text{K}^{+}$  channels and phospholipase C- $\beta$  (PLC- $\beta$ ), responsible for the splitting of membrane bound phosphatidylinositol into its constituent second messengers, inositol trisphosphate (IP3) which liberates intracellular  $\text{Ca}^{2+}$  from the endoplasmic reticulum, and diacylglycerol (DAG) which can activate  $\text{Ca}^{2+}$  binding proteins and enzymes [173]. However, in many cases, this requires the additional stimulation of Gq-coupled receptors, which also stimulate  $\text{Ca}^{2+}$  release from intracellular stores via the IP3 pathway. Ultimately, these multiplicative actions stimulate more and more intracellular  $\text{Ca}^{2+}$  that can activate binding proteins in the cell membrane (e.g., PKC and CAM kinase) and stimulate calcium response element binding (CREB) proteins to regulate gene expression. This allows opioids to regulate the transcription of proteins linked to  $\mu$  receptor trafficking, such as G-protein coupled receptor kinase 2 (GRK2) and  $\beta$ -arrestin 2, as well as receptors for other neurotransmitters, such as DA receptors, NMDA receptors, GABA-A receptors, and alpha adrenergic 2A receptors [174]. Interestingly,  $\beta$ -arrestin 2 may regulate opioid sensitized DA release. Transgenic  $\beta$ -arrestin 2 knockout mice exhibit a greater sensitization of DA release and greater reward in response to alcohol [175], which is known to activate opioid-mediated DA sensitization (see Figures 4 and 6).



**Figure 6.** Stimulation of MAP kinase signaling by opioid agonist actions on  $\mu$  opioid receptors leads to a cascade of second messenger related events (**left**), ultimately amplifying the transcription and translation of CD38 (**right**), a protein critical for  $\text{Ca}^{2+}$  mobilization within OXT neurons and augmentation of OXT neurotransmission when stimulated.

### 5.3. Downstream Activation of OXT and AVP Neurons

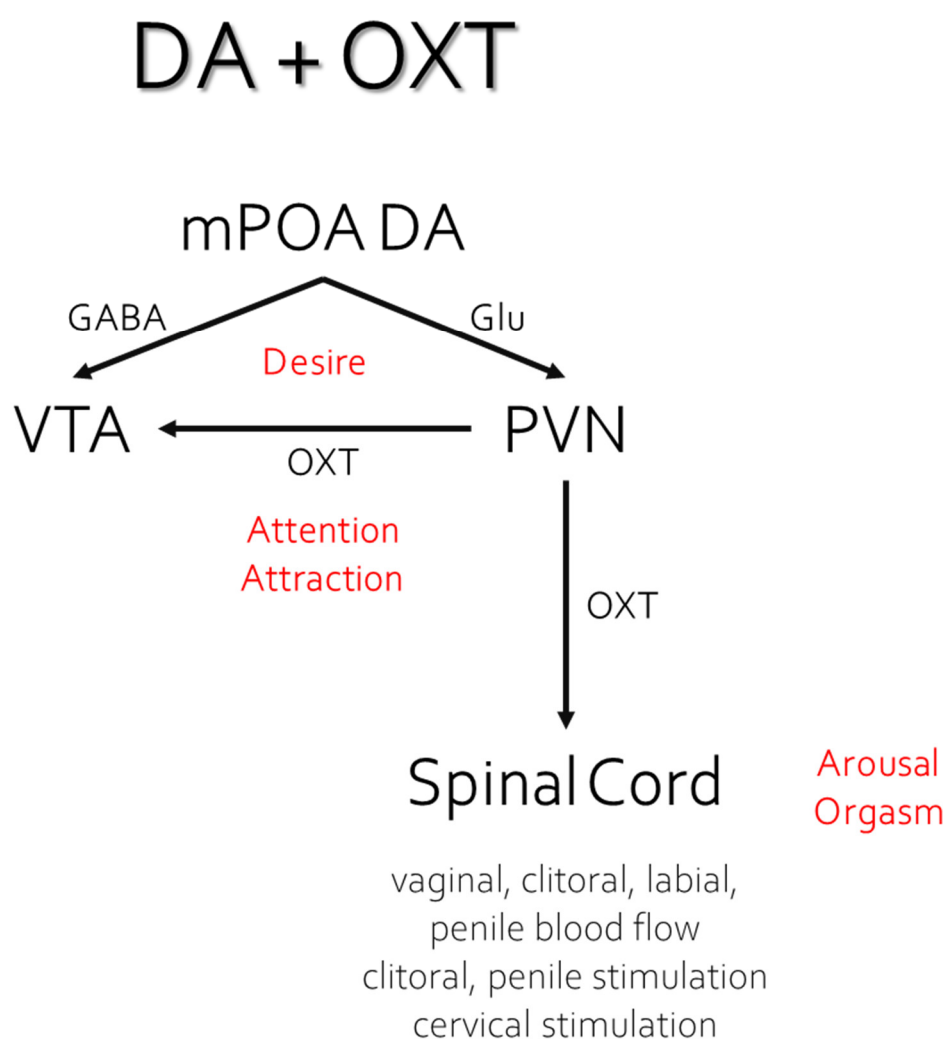
Of the many neuropeptides that modulate sexual behavior, OXT and AVP figure prominently in sexual arousal [176–179] and the establishment of monogamous partner preferences in prairie voles [180–186]. As mentioned above, the mating-induced preference by male prairie voles for a familiar female can be inhibited by naltrexone, suggesting that opioids play an important role in their development. However, so does DA. Opioids and DA both participate in the sensitization and activation of OXT and AVP neurons. The following two populations of OXT neurons exist in rats and other rodents that comprise two systems within the PVN and SON of the hypothalamus [187]: small parvocellular neurons that project largely to midbrain (periaqueductal grey), brainstem, and spinal cord, and larger magnocellular neurons that project diffusely to limbic and forebrain sites, and to the posterior pituitary [188–190]. Parvocellular OXT neurons also make direct dendritic contact with magnocellular OXT neurons in the SON [191] and regulate OXT release from those neurons into the posterior pituitary vasculature. Thus, OXT can coordinate sexual arousal with partner preference by both central and peripheral actions.

As an antidiuretic hormone, AVP increases water retention by the kidneys. As a vasopressor, AVP causes vasoconstriction and increases blood pressure. Central activation of V1a receptors in the ventral pallidum is also critical for the display of partner preference in prairie voles, and the overexpression of V1a receptors in a promiscuous vole species can induce monogamous-like partner preferences [192]. Despite its clear role in monogamous partner preferences, injections of AVP during early experience with sexual reward did not augment CEP in males or conditioned partner preferences in females. This leaves conditional activation of OXT as a potential integrator of sexual arousal and partner preference.

The conditional activation of OXT neurons in particular by cues associated with sexual reward likely helps to assure reproductive success with preferred partners. This would occur by potentiating the coordination of sexual arousal, ejaculation, and the cervico–uterine reflex contractions that figure in sperm transport, in the presence of one's preferred partner or perhaps even just in cues associated with sexual reward. Indeed, OXT



receptors were found in the penis [193], in the glans and corpora of the clitoris and in the cervix [194]. In addition to potentiating sexual partner preference, the peripheral administration of OXT shortens the duration of female rat sexual behavior by facilitating estrous termination [194]. Females that take themselves out of the mating context for over 10 min assure paternity for the last male's ejaculation [195]. This behaviour following the selective acceptance of ejaculations by the preferred familiar male sets up a behavioral system to assure paternity. Likewise, CEP with the same female would also be expected to facilitate paternity, especially if the male was also the female's preferred choice. This phenomenon of OXT coordination links ultimate causality with proximate causality, making successful reproduction more likely based on a prior history of orgasm-like sexual reward with the preferred partner (see Figure 7).



**Figure 7.** Coordination of genital blood flow, sexual desire, attraction, and orgasm by the interaction of DA and OXT in response to conditioned sensory cues; based on Melis and Argiolas [196]. Details are found in the text.

#### 5.4. Epigenetic Mechanisms

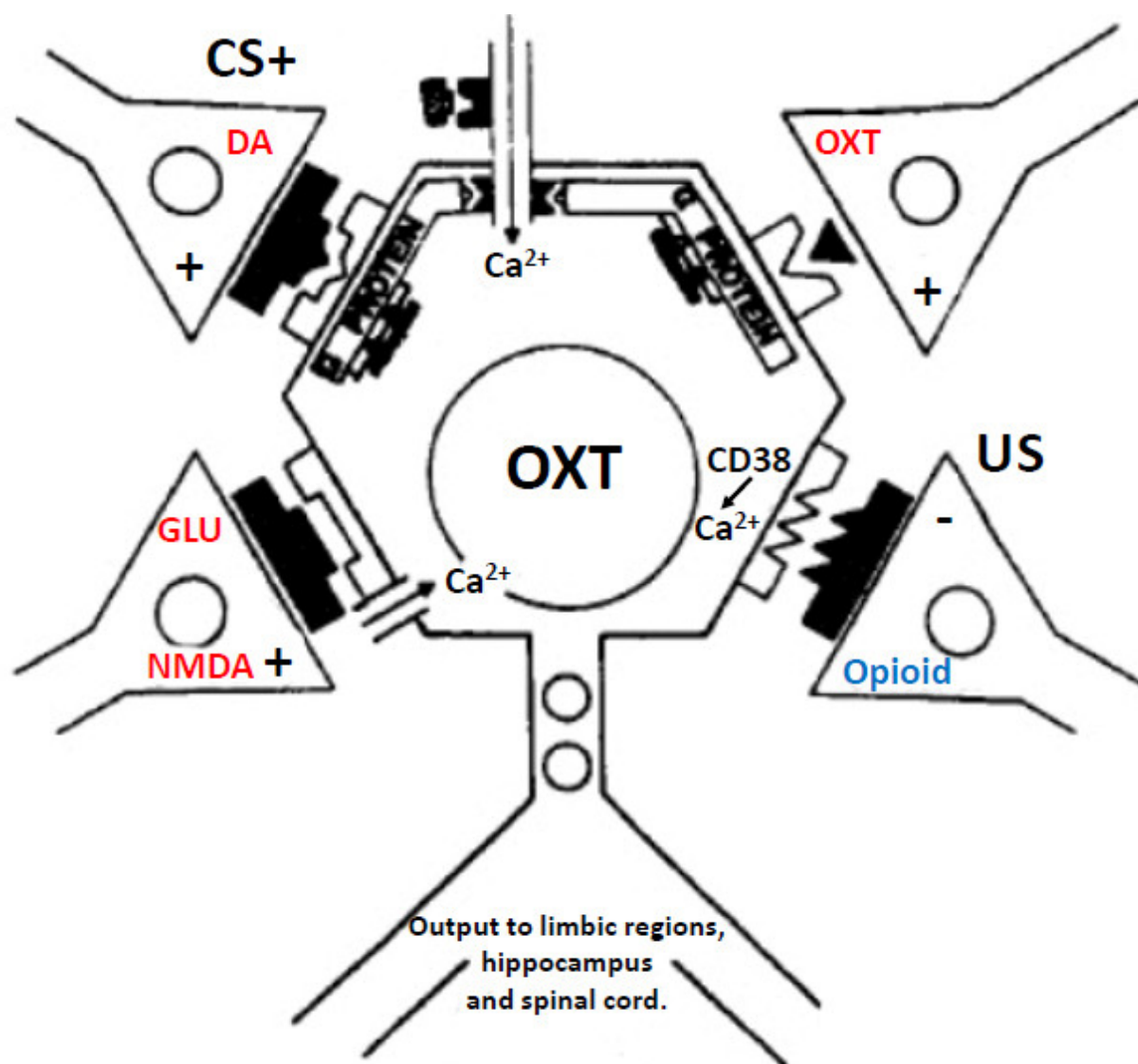
The interaction between the environment, behavior, genes, and the underlying mechanisms by which they influence each other is an important focus for research on sexual behavior and partner preference [197,198]. By manipulating the way DNA is unfolded from histones and expressed, either by silencing or expressing certain parts of the gene,



scientists observed behavioral changes in different animal models. Epigenetics refers to the study of how environmental changes modify the way the genome is expressed through the manipulation of the enzyme responsible for the addition or removal of epigenetic tags in histone proteins, without altering the DNA sequence [199]. For instance, through the administration of a histone deacetylase inhibitor (HDACi), a drug that promotes DNA acetylation and transcriptional activation, sexually naïve female voles developed a partner preference by simply being exposed to a male vole partner in the absence of mating [200]. Conversely, demethylation also promotes the unpacking of genes [201], and the administration of oryzon, a specific inhibitor of lysine specific demethylase-1 (LSD-1), blocked the development of conditioned mate guarding behavior in the female rat in a manner similar to NAL [101]. Females in the group that received oryzon prior to each training trial copulated normally with males during the training trials indicating that sexual arousal and opioid reward were processed normally despite demethylase inhibition. However, those females failed to display the mate guarding behaviors shown by females that received saline before each trial. Moreover, double-labeled cell counts for Fos within OXT and AVP neurons were significantly lower in the oryzon-treated group compared to the saline-treated group in both the PVN and SON. Histone modifications are a key element in gene regulation through chromatin remodeling. LSD-1 demethylases have been shown to demethylate repressive histone markers thus leading to transcriptional activation [201], and blocking this keeps those genes repressed. The results of Holley et al. [101] suggest that genes involved in the activation of OXT and AVP neurons, but not the rewarding effects of opioids, are especially affected by LSD-1 demethylase inhibition. Interestingly, D2Rs and D3Rs were found on both OXT and AVP neurons in the PVN that also express BDNF [202,203]. Demethylase inhibition reduces the viable expression of a number of genes related to DA receptor function, including an attenuation of D2R-activation of a G protein-coupled inwardly rectifying potassium (GIRK) channel [204], which, in turn, would be expected to diminish the conditional activation of OXT and AVP neurons. These data also suggest that the activation of OXT and AVP neurons occurs downstream of the DA neuronal populations sensitized by opioids. Indeed, neuroanatomical and microinjection studies show that A13 incerto-hypothalamic DA neurons project axons to the PVN and are themselves activated by efferents stimulated by DA release in the NAC [196].

#### *5.5. Molecular Mechanisms of Opioid Sensitization of OXT Neurons and the Role of DA*

Opioids also sensitize OXT neurons directly by their actions on  $\mu$  opioid receptors. This occurs by a complex interaction with the activation of  $G_i$  proteins that inhibit cAMP, but disinhibit MAP kinase signaling (via ERK, p38MAPK, and JNK pathways). In turn, MAPK pathways activate the transcription of genes, one of which is the Cluster of Differentiation 38 (CD38) gene, a cyclic ADP ribose hydrolase that acts as a marker of cell types in addition to being an activator of B cells and T cells [205]. However, its enzymatic activity also forms cyclic ADP ribose and nicotinic acid adenine dinucleotide phosphate which, similar to IP<sub>3</sub>, release Ca<sup>2+</sup> from intracellular stores. This action is important for OXT neurotransmission [206–211]. Diminished CD38 action figures in autism-spectrum disorders [212,213] and CD38 knockout mice show impaired object- and social-recognition memory [208]. Thus, sexual reward and pleasure driven by  $\mu$  opioid actions on OXT neuronal membranes may augment the synthesis of CD38, which in turn could sensitize OXT release in the presence of sexual reward-related cues. If true, then when DA release activates OXT neurons (via opioid sensitized mesolimbic DA transmission that activates efferents to A13 DA neurons projecting to the PVN and SON), the augmented DA activation would stimulate more OXT release, in addition to the DA-sensitized and focused appetitive responses made toward preferred sex partners or merely stimuli associated with sexual reward (see Figure 8).



**Figure 8.** Synaptic inputs to OXT neurons in the PVN and possibly the SON. In this scheme, the CS + signal is carried by augmented incerto-hypothalamic DA transmission driven by sensitized meso-limbic DA. The US signal is carried by opioid transmission acting on  $\mu$  opioid receptors. Although this signal inhibits OXT cell membranes, it leads to an augmentation of CD38 gene transcription, mobilization of  $\text{Ca}^{2+}$ , and sensitization of OXT transmission when neurons are stimulated (in this case by DA, but potentially also by OXT and N-methyl D-aspartate [NMDA] glutamate [GLU] inputs).

## 6. First Experiences of Sexual Reward in Humans; or the Path of Cupid's Arrow

First sexual experiences in humans typically involve masturbation to orgasm with the use of sexual content material (e.g., pornography) before having first copulatory contact with another person [214,215]. Although first sexual experiences likely involve arousal and desire, they do not necessarily end in orgasm. This is especially true for women [216–219]. It is also the case that not all orgasms for men or women are alike, and will depend, as with our rats and their orgasm-like responses [220], on the context. For example, orgasms experienced in the context of coercive sex, compliant sex, and/or pressure to have an orgasm, not only dampen the pleasure of orgasm, but make it feel “bad” (e.g., [221]). Likewise, orgasms can be diminished in quality when sex is frustrating, inhibited, habitual, or otherwise during low arousal [19,222–226] or when heteronormative scripts that emphasize male pleasure from vaginal penetration alone are driving the sexual interaction are followed [227,228] (see also [229]). Even ejaculation quality in terms of



augment sexual motivation and desire [248] and likely extends to person-related cues (e.g., shape of face, eyes, hair, body type, ethnic characteristics, interpersonal attributes), places, or even objects, conditioned by Pavlovian associations, and/or particular appetitive and consummatory sexual behaviors (e.g., body movements, use of toys, etc.) conditioned by response-reinforcer (operant) associations with arousal and pleasure [249]. Together, Pavlovian and operant associations with sexual arousal and pleasure partially define a preference for a familiar “type” and the movements that become one’s typical pattern of sexual response, including particular paraphilias [19,23].

## 7. Conclusions

It is clear that sexual experience generates plasticity in the brain that allows sexual reward to modulate the way predictive cues come to focus attention, augment sexual arousal and desire, and facilitate bonding. These are critical elements for the creation of partner preference and mate choice, especially for familiar partner-related features. The action of opioids as substrates of sexual reward facilitates the cascade of molecular events that lead to these enhanced responses, essentially the “personification of reward” that allows animals to differentiate the features they find attractive and prefer in potential sex partners. In Figure 8, we propose a process in which opioids such as  $\beta$ -endorphin derived from cell bodies in the arcuate nucleus bind to  $\mu$  opioid receptors and produce the sensations of reward and pleasure that grow during sexual interaction, reaching their peak at orgasm. Through second-messenger and molecular actions, opioids sensitize DA, AVP, and OXT systems in hypothalamic and limbic structures to promote attention, arousal, and bonding with cues that predict sexual reward. These processes are then summed in the cortex and translate to a conscious awareness of attraction and desire.

Although partner features that depict health and the propensity for protection and survival of offspring may well be laid down genetically [250,251] and induce a preference for optimal inbreeding and outbreeding (e.g., [252,253]), such preferences rely on sexual imprinting, a phenomenon that is supposed to enable adults to recognize members of their own species for reproduction and parenting [254]. However, cross-fostering studies between goats and sheep indicate that experience with maternal care determines the species chosen for first sexual experiences, regardless of whether the species is one’s own [49]. Additionally, presumably the animals still have to copulate to seal the deal. Indeed, Fillion and Blass [255] found that male rats reared with dams that had their teats painted daily with citral odor investigated, copulated, and ejaculated more readily with citral-scented receptive females compared to unscented females, suggesting that the odor was paired with reward through suckling and feeding. Subsequently, Ménard et al. [58] examined whether a similar lemon odor paired with tactile reward in neonatal male rats would alter the subsequent expression of CEP. Newborn Long-Evans male rats were separated from their mothers each day beginning on Postnatal Day 1 and placed into a Plexiglas cage that contained either unscented or citral-scented bedding. During each trial, rats were stroked from head to toe with a soft, narrow paintbrush, after which they were returned to their mothers. When the males were adults, they were given their first sexual experience in an open field with two females, one scented with lemon and the other unscented. Males in the paired group copulated and ejaculated preferentially with the lemon-scented female whereas males in the unpaired group showed no preference. Pairing a neutral odor with a reward state in infancy generates a preference in male rats to ejaculate with sexually receptive females bearing the same odor in adulthood. As Lorenz noted in his seminal paper, the first experience of a greylag goose seeing a large object immediately after hatching induced a process of imprinting that compelled the gosling to follow Lorenz as if he was the gosling’s mother.

It is clear that first experiences with sexual arousal and reward induce sexual imprinting through a process of plasticity in the brain that allows sexual reward to modulate the way predictive cues come to focus attention, augment sexual arousal and desire, and facilitate bonding. These are critical elements for the creation of partner preference and

mate choice. The actions of opioids as substrates of sexual reward facilitate the cascade of molecular events that lead to these enhanced responses, essentially the “personification of reward” that allows animals to discriminate and attend to the familiar features they find attractive and prefer in potential sex partners or objects associated with sexual reward. Obvious ethical constraints make it virtually impossible to manipulate, much less test, conditioning during critical first experiences with sexual reward that can occur any time across the lifespan in humans. We note that sexual interests span a continuum of preferences, from particular features and specific behavioral patterns to fetishes and paraphilias, as well as sexual orientation based on anatomical sex and perceived gender [19,23]. The wide variety of idiosyncratic features that can be conditioned in our species means that everyone will be found desirable by at least another, assuming that they possess the ability for competent sexual stimulation. Additionally, once conditioned, the type is not easy to extinguish, although it is likely iterative with new sexual “firsts” that alter the perception and definition of sexual reward into the realm of peak experiences.

Perhaps all species on this planet have sex for pleasure most of the time. Mating can occur—or not—with a bit of luck in a mating context, but mating is not critical for the individual so long as enough individuals in each generation of a species are successful at it. Attraction to particular individuals works hand-in-hand with the desire for sex. Such sexual bonding, even for a short time, requires conditioning and expectation of reward to produce effective appetitive responses toward preferred partners. First experiences with sexual reward matter in this, as they set the stage for the maintenance of variability that is the hallmark of hybrid vigor and continued survival within a species.

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**B.**

**Original studies on the conditioning of sexual  
behavior in male rats**



## Olfactory Conditioning of Sexual Behavior in the Male Rat (*Rattus norvegicus*)

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The role of classical conditioning in the copulatory preferences of male Long-Evans rats (*Rattus norvegicus*) was examined by pairing a neutral olfactory stimulus (almond odor) with female reproductive status. During training trials, the males were given access to scented or unscented females that were either sexually receptive or unreceptive. Subsequently, copulatory preferences were tested in males given simultaneous access to 2 receptive females, 1 scented and 1 not. Males trained with scented-receptive females displayed an ejaculatory preference for the scented female. Males trained with scented-unreceptive females or with unscented-receptive females displayed an ejaculatory preference for the unscented female. Males displayed no preference when scent and reproductive status were paired randomly. These results demonstrate that classical conditioning produces an ejaculatory preference.

Although associational factors have long been hypothesized to be important in the development and expression of sexual behavior (Beach, 1956; Larsson, 1956; McConaghy, 1987), it is only recently that progress has been made in the development of animal models to study such processes. The success of these models has been aided in part by the conceptual framework within which different sexual behaviors are cast (Pfaus, 1996; Toates, 1992). Accordingly, the influence of conditioning in both appetitive and consummatory phases of sexual behavior has been examined using a variety of species (for a review, see Domjan & Hollis, 1988).

The role of conditioning in appetitive sexual behaviors has been studied for both preparatory and anticipatory behaviors. Conditioning of preparatory behaviors has been studied most extensively by imposing an operant on the acquisition of a mate. Instrumental lever pressing for sexual reinforcement has been demonstrated in both male rats (Beck, 1971, 1978; Beck & Chmielewska, 1976; Jowaisas, Taylor, Dewsbury, & Malagodi, 1971; Schwartz, 1956) and female rats (Beck, 1971, 1974, 1978; Bermant, 1961; Bermant & Westbrook, 1966; French, Fitzpatrick, & Law, 1972). Everitt, Fray, Kostarczyk, Taylor, and Stacey (1987) demonstrated that male rats will press a bar for the presentation of a second-order stimulus (e.g., a light or a tone) that has been paired with copulation. In addition, male rats learn to navigate T and X mazes correctly to locate a receptive

mate (Hetta & Meyerson, 1978; Hull et al., 1991; Moses, Loucks, Watson, Matuszewich, & Hull, 1995; Warner et al., 1991; Whalen, 1961). Mendelson and Pfaus (1989) developed a method to study anticipatory sexual excitement in male rats using bilevel chambers. They found that the development of anticipatory locomotion, as measured by level changes before the introduction of the female into the chamber, was conditional on Long-Evans male rats being allowed access to receptive rather than nonreceptive females (see also Pfaus, Mendelson, & Phillips, 1990). In contrast, Wistar male rats developed appetitive level changing that was conditional on olfactory cues from previous copulations being present in the bilevel chambers, regardless of whether the males were presented subsequently with receptive or nonreceptive females (Van Furth & Van Ree, 1996).

Studies examining the influence of conditioning on consummatory sexual behaviors have focused on measures of sexual arousal and ejaculation during copulation. Zamble and colleagues (Zamble, Hadad, Mitchell, & Cutmore, 1985; Zamble, Mitchell, & Findlay, 1986) used the placement of male rats in a holding cage as a conditioned stimulus to signal noncopulatory exposure to a receptive female on several training trials. On subsequent copulatory tests, males placed into the holding cage before copulation had significantly shorter latencies to intromit and ejaculate than males that were placed into the test arena directly from their home cages. Similarly, Domjan and colleagues (Domjan, Greene, & North, 1989; Domjan, Lyons, North, & Bruell, 1986; Köksal, Domjan, & Weisman, 1994) demonstrated that the presentation of a stimulus previously paired with access to a receptive female will reduce the latency to initiate copulation in Japanese quail. Hollis and colleagues (Hollis, Cadieux, & Colbert, 1989; Hollis, Pharr, Dumas, Britton, & Field, 1997) have found that a cue that consistently precedes the presentation of a female becomes able to inhibit aggression, speed copulation, and provide an advantage in terms of number of offspring sired in male blue Gouramis. Classical conditioning has also been used to produce inhibition of sexual behavior. Johnston and Zahorik found that a single

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pairing of vaginal secretions with lithium chloride injections caused male hamsters to develop aversions to this initially attractive substance (Johnston & Zahorik, 1975; Zahorik & Johnston, 1976) and to suppress their sexual behavior toward a receptive female as indicated by a decreased number of mounts, decreased time spent mounting, increased latency to mount, and an increased latency to sniff or lick the female's vagina (Johnston, Zahorik, Immler, & Zakon, 1978). Moreover, one study reported that when lithium chloride injections were paired repeatedly with access to a receptive female, copulation was suppressed in rats (Peters, 1983). Lawrence and Kiefer (1987) found that such inhibitory conditioning was facilitated by the addition of a neutral odor (almond extract). Finally, Silberberg and Adler (1974) demonstrated that consummatory sexual behavior was influenced by imposing an operant on ejaculation. In that study, male rats learned to decrease the number of intromissions required to achieve ejaculation if the stimulus females were removed after the seventh intromission of the first ejaculatory series.

The role of conditioning in sexual partner preference has also been examined. Domjan, O'Vary, and Greene (1988) found that male Japanese quail exhibited shorter latencies to engage and complete copulation with a female bearing a conditioned stimulus (CS; coloured feathers) paired previously with copulation compared with the latencies of males that copulated with a female not bearing the CS. Fillion and Blass (1986) found that adult male rats displayed shorter ejaculation latencies with receptive female rats bearing an odor paired with the males' experience of nursing during infancy compared with receptive females not bearing the odor (see also Marr & Gardner, 1965). In these studies, the conditioned effect was demonstrated by differences in the males' responses to females either bearing or not bearing the CS. Although such differential responding is indicative of conditioned increases in sexual arousal, it should not be taken as evidence of a conditioned preference for a particular mate for one important reason: There was no opportunity for the male to select between potential mates. Investigation of the relation between conditioned stimuli and mate preferences would require that (1) the conditioned stimulus be attached to a potential mate (as in the Domjan et al., 1988, and Fillion & Blass, 1986, studies) and that (2) males would have unrestricted access to at least 2 potential mates.

In a preliminary study (Pfaus, Jacobs, & Wong, 1986), a neutral odor (almond extract) used as an olfactory CS paired with copulation produced an ejaculatory preference. Male rats were allowed access to sexually receptive females with either the CS or distilled water applied to their neck and anogenital regions during copulatory training trials. The influence of this conditioning procedure on copulatory preference was subsequently tested by allowing males to copulate to ejaculation with simultaneous, unrestricted access to 2 receptive females, 1 bearing the CS and 1 not. More males in each group ejaculated with females similar to those they had previously copulated with. The present study replicates the results of this pilot study and provides the explicitly unpaired and randomly paired control conditions

necessary to establish classical conditioning as the mechanism underlying the development of the ejaculatory preference.

## Experiment 1

In Experiment 1, we examined the effect of repeated pairings of an olfactory stimulus (almond extract) with access to a receptive female rat on the acquisition of sexual behavior and on the subsequent copulatory preferences between potential mates in the male rat. The explicitly unpaired and the randomly paired control conditions necessary to establish classical conditioning were also examined (Rescorla, 1967). This task was accomplished by giving three groups of male rats alternating training trials with both receptive and nonreceptive female rats. One group always had exposure to the almond odor paired with access to receptive females (paired-training condition) and trials with nonreceptive females that were unscented. A second group always received exposure to the almond odor paired with nonreceptive females (unpaired-training condition) and trials with receptive females that were unscented. A third group had exposure to the almond odor paired with receptive or nonreceptive females in a random fashion (random-training condition).

## Method

### Subjects

**Males.** The 54 Long-Evans rats (*Rattus norvegicus*) that served as subjects in this experiment were obtained from Charles River, Canada (St. Constant, Québec). The males weighed approximately 300 g and were sexually naive at the start of the experiment. They were housed in pairs in Plexiglas cages (36 cm × 26 cm × 19 cm) with ad lib access to food (Purina Rat Chow) and water. All rats were kept in a 12-hr reversed light-dark cycle colony room maintained at 21 °C.

**Females.** Female Long-Evans rats (*Rattus norvegicus*) from the same supplier as above were ovariectomized via bilateral lumbar incisions under ketamine-xylazine anesthesia at least 2 months before the start of the experiment; they were sexually experienced. Sexual receptivity was induced by subcutaneous administration of estradiol (10 g) 48 hr before and progesterone (500 g) 4–6 hr before each test trial. Females were housed under the same conditions as males. Stimulus females were selected at random for use during conditioning trials and copulatory preference tests. Female rats were scented with approximately 1 ml of either almond extract (Blue Ribbon, Etobicoke, Ontario, Canada) or distilled water applied to both the back of the neck and the anogenital area using a cotton swab.

### Apparatus

Conditioning trials took place in bilevel chambers constructed of Plexiglas (outside dimensions: 18 cm × 25 cm × 65 cm) with a platform (40 cm in length) elevated by a set of ramps at each end dividing the chamber into two levels (see Pfaus et al., 1990, for further details). The bilevel chambers were cleaned with water and Coverage 256 (Conva Tec, St. Louis, MO) and soiled bedding was replaced with clean bedding before each conditioning trial. All conditioning trials were recorded on videotape and later scored using a PC-based program (Ogawa, 1991). Copulatory preference

tests took place in a large open field (123 cm  $\times$  123 cm  $\times$  46 cm) and were scored at the time of testing.

### Procedure

**Conditioning phase.** Male rats were preexposed to the bilevel chambers once a day for 15 min each day to habituate them to the training environment. This habituation procedure lasted 7 days and has been shown previously to increase the proportion of males that become vigorous copulators (Pfaus & Wilkins, 1995). Then all males received a total of 18 conditioning trials at 2-day intervals during the middle third of the dark phase of the light-dark cycle. Access to sexually receptive and sexually nonreceptive females occurred on alternating trials; the first trial was counterbalanced with respect to female status for each group. For males in the paired-training condition, all trials with sexually receptive females were with females that had almond extract applied to each area (A + E females), and all trials with sexually nonreceptive females were with females that had distilled water applied to each area (N-alone females). For males in the unpaired-training condition, all trials with sexually receptive females were with females that had distilled water applied to each area (E-alone females), and all trials with sexually nonreceptive females were with females that had almond extract applied to each area (A + N females). And for the males in the random-training condition, half the trials with sexually receptive females were with A + E females and half with E-alone females, and half the trials with sexually nonreceptive females were with A + N females and half with N-alone females. The scent of the females on all these trials was counterbalanced and followed a pseudorandom schedule determined before the experiment.

For all conditioning trials, males were placed individually into a bilevel chamber for 5 min, after which a female of the appropriate sexual status and appropriate scent was placed into the chamber for a 30 min test of copulation. Latency and frequency data for all mounts, intromissions, and ejaculations were recorded during each conditioning trial. Criteria for sexual behaviors were those described by Sachs and Barfield (1976) and Meisel and Sachs (1994).

**Copulatory Preference Test.** Four days after the final conditioning trial, each male was placed in the large open field and allowed to habituate for 5 min. At the end of this period, one A + E female and one E-alone female were placed simultaneously into two diagonal corners of the open field at approximately equal distances from the male. All copulatory behaviors and the females to which they were directed were recorded during each male's test. Tests were terminated 30 min after the females were introduced.

### Statistical Analysis

Mixed-design between-groups and within-groups analyses of variance (ANOVAs) were used to analyze the level-changing data from the receptive and nonreceptive conditioning trials, and significant values were followed by post hoc analysis of individual means using the Tukey method. Chi-square analysis was used for the analysis of proportions of female selected for first mounts, first intromissions, and first ejaculations on the copulatory preference test. Mixed ANOVAs were used for analysis of the distributions of mounts, intromissions, and ejaculations throughout the 30 min copulatory preference test. In addition, *t* tests were used for the analysis of proportions of mounts and intromissions directed toward the female selected for ejaculation in a series. The level of significance for all comparisons was .05.

## Results

### Conditioning Phase

No substantial between-group differences were detected during the conditioning phase. Mean level change latency decreased and mean level change frequency increased across trials for all groups (data not shown); levels were similar to those found in previous studies using the bilevel chambers (see Pfaus et al., 1990). The proportion of males that ejaculated with receptive females increased during the conditioning phase from 67% on the first trial to 95% on the final trial; there were no between-group differences. The proportion of males that mounted nonreceptive females decreased during the conditioning phase from 30% on the first trial to 5% on the final trial; again there were no between-group differences.

### Copulatory Preference Test

Of the 54 males, all but 11 copulated to ejaculation on the copulatory preference test. Of the males that failed to ejaculate, more were in the unpaired-trained group ( $n = 7$ ) than in the paired-trained group ( $n = 3$ ) and the random-trained group ( $n = 1$ ). The proportion of unpaired-trained males that failed to copulate on the copulatory preference test was significantly greater than on Conditioning Trial 9,  $\chi^2(35) = 8.69$ ,  $p < .05$ , and was significantly greater than the proportion of random-trained males,  $\chi^2(35) = 5.79$ ,  $p < .05$ .

Figure 1 displays the selection of females for the first mount, first intromission, and first ejaculation for each group. More paired-trained males ejaculated first with A + E females than did males in the other two groups, and more unpaired-trained males ejaculated first with E-alone females than did males in the paired group. Chi-square analyses confirmed the statistical significance of these observations: paired-trained versus unpaired-trained,  $\chi^2(25) = 5.42$ ,  $p < .05$ ; paired-trained versus random-trained,  $\chi^2(32) = 3.35$ ,  $p < .05$ ; unpaired-trained versus random-trained,  $\chi^2(28) = 0.56$ ,  $p > .05$ . No significant differences were found between the groups for the selection of females for the first mount or first intromission.

The mean mounts per female per series, intromissions per female per series, and mean ejaculations per female over the 30 min test are displayed in Figure 2. Paired-trained males mounted more frequently than did the males in the other two groups, and the E-alone females received more mounts than did the A + E females; there was not a significant interaction between group and female type. The significance of these observations were confirmed by a  $3 \times 2$  mixed ANOVA, for group:  $F(2, 80) = 6.86$ ,  $p < .05$ . Post hoc comparisons revealed that paired-trained males differed significantly from unpaired-trained and random-trained males, but that unpaired-trained and random-trained did not differ significantly; for female type:  $F(1, 80) = 10.56$ ,  $p < .05$ ; for Group  $\times$  Female Type:  $F(2, 80) = 0.37$ ,  $p > .05$ . Also, E-alone females received more intromissions than did A + E females, but there were no other significant differences in the distribution of intromissions; the significance of these obser-

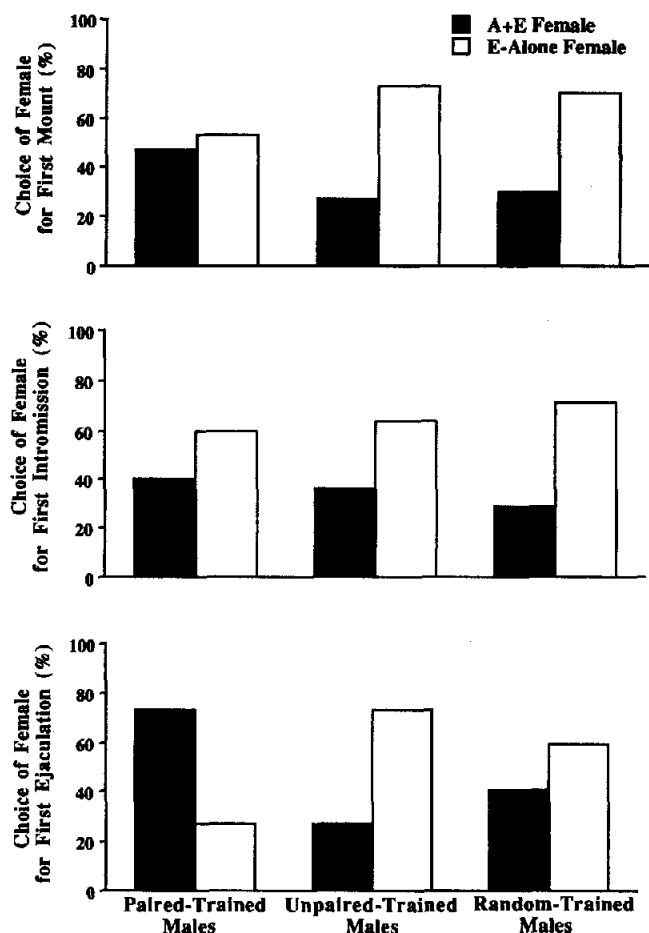


Figure 1. Proportional distribution by female type of first mount (top panel), first intromission (middle panel), and first ejaculation (bottom panel) during the copulatory preference test in Experiment 1. A + E Female = Sexually receptive females that had almond extract applied to the back of the neck and to the anogenital area; E-Alone Female = Sexually receptive females that had distilled water applied to the back of the neck and to the anogenital area.

variations was confirmed by a  $3 \times 2$  mixed ANOVA, for group:  $F(2, 80) = 1.03, p < .05$ ; for female type:  $F(1, 80) = 6.10, p < .05$ ; for Group  $\times$  Female Type:  $F(2, 80) = 1.45, p < .05$ . Moreover, for ejaculation distribution, paired-trained males ejaculated more frequently with A + E females than with E-alone females, unpaired-trained males ejaculated more frequently with E-alone females than with A + E females, and random-trained males ejaculated with both females with equal frequency. A  $3 \times 2$  mixed ANOVA revealed no significant main effects, for group:  $F(2, 80) = 0.40, p > .05$ ; or for female type:  $F(1, 80) = 0.50, p > .05$ . However, there was a significant interaction between group and female type,  $F(2, 80) = 11.01, p < .05$ . Post hoc comparisons revealed that paired-trained males ejaculated more times with A + E females than they did with E-alone females; that paired-trained males ejaculated with A + E females more times than the males in the other two groups; that paired-trained males ejaculated with E-alone females

less times than did the males in the other two groups; that unpaired-trained males ejaculated more times with E-alone females than they did with A + E females; that unpaired-trained males ejaculated more times with E-alone females than did random-trained males; and that unpaired-trained males ejaculated with A + E females less times than did random-trained males.

The proportion of males that switched females for consecutive ejaculations is displayed in Figure 3. Analysis of choice of females for consecutive ejaculations revealed that there were differences between groups. On the second series, more than half of the random-trained males (59%) switched females, whereas approximately half of the paired-trained males (47%) and less than half of the unpaired-trained males (27%) switched females. A chi-square analysis revealed the statistical significance of the difference between

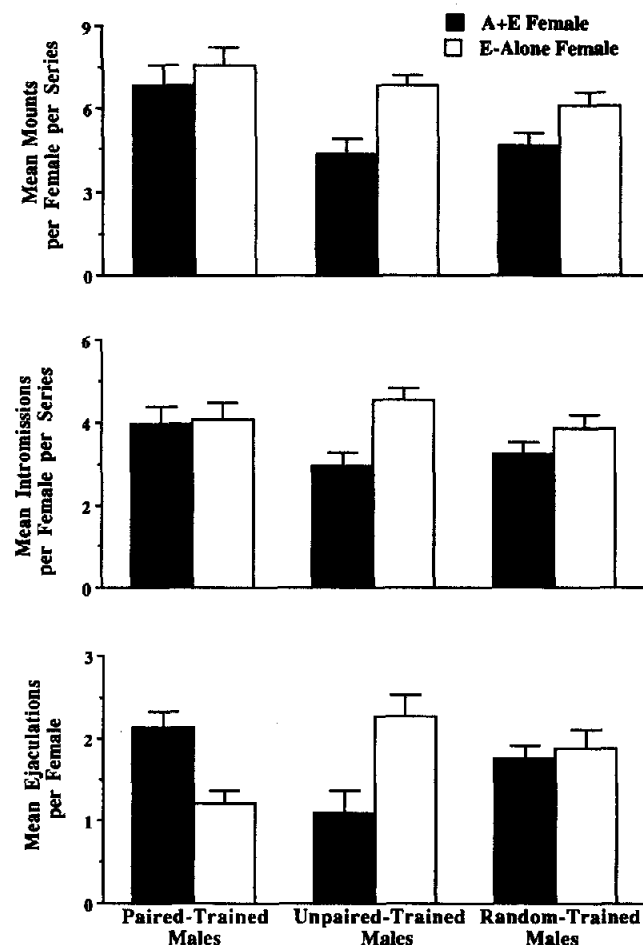


Figure 2. Distribution of mean (+SE) mounts per female per series (top panel), mean (+SE) intromissions per female per series (middle panel), and mean (+SE) ejaculations per female (bottom panel) during the copulatory preference test in Experiment 1. A + E Female = Sexually receptive females that had almond extract applied to the back of the neck and to the anogenital area; E-Alone Female = Sexually receptive females that had distilled water applied to the back of the neck and to the anogenital area.

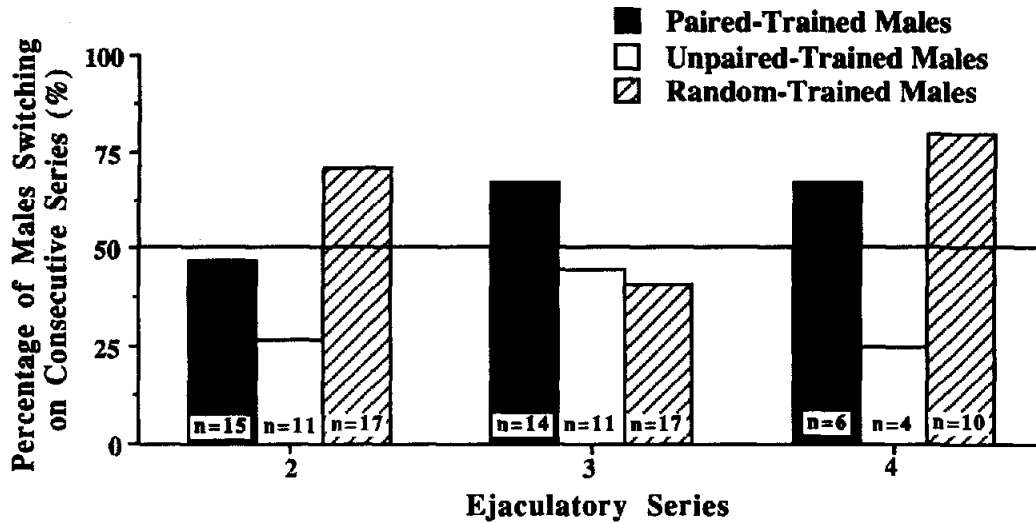


Figure 3. Proportion of males in each group switching females for ejaculation on consecutive ejaculatory series during the copulatory preference test in Experiment 1.

the random-trained and unpaired-trained groups,  $\chi^2(28) = 5.04$ ,  $p < .05$ , but no other differences were significant. Analysis of subsequent ejaculatory series failed to reveal significant differences in the proportions of males switching on the third or fourth series.

No other measures of copulatory behavior differed significantly between the two groups. However, it is noteworthy that the selection of females for ejaculation was not related to either the distribution of mounts or the distribution of intromissions. The same female was selected for first ejaculation and for first mount by approximately half of the paired-trained (60%), unpaired-trained (43%), and the random-trained (53%) males, and for first intromission by approximately half of the paired-trained (53%), unpaired-trained (36%), and random-trained (59%) males. For all three groups of males, the mean proportion of mounts across all ejaculatory series directed toward the female selected for ejaculation on the same series was not substantially different from chance (50%) probability, for paired-trained males:  $t(14) = 0.33$ ,  $p > .05$ ; for unpaired-trained males:  $t(10) = 1.9$ ,  $p > .05$ ; for random-trained males:  $t(16) = 0.21$ ,  $p > .05$ . Similarly, the mean proportion of intromissions across all ejaculatory series directed toward the female selected for ejaculation on the same series was not substantially different from chance probability in either group, for paired-trained males:  $t(14) = 0.87$ ,  $p > .05$ ; for unpaired-trained males:  $t(10) = 0.41$ ,  $p > .05$ ; for random-trained males:  $t(16) = 0.31$ ,  $p > .05$ .

### Discussion

The finding that the paired- and unpaired-trained males ejaculated preferentially with females during the copulatory preference test refines the generally accepted notion that male rats mate indiscriminately with groups of females (see Dewsbury, 1982; McClintock, 1984; Symons, 1979) or that

they have a preference for novel females (Dewsbury, 1981). Males were allowed to copulate with 2 females that had been rendered highly distinguishable by the addition of the CS (almond) odor to 1 of the females. Under these conditions, males displayed discriminative mating. On the copulatory preference test, more males in paired- and unpaired-trained groups ejaculated with the female that was similar to females with which they had previously copulated than ejaculated with the other female; the paired-trained males displayed a preference for A + E females and unpaired-trained males displayed a preference for E-alone females. These findings support previous work demonstrating that under certain conditions a male can discriminate between and prefer to mate with certain females (Dewsbury, 1982; McClintock, Anisko, & Adler, 1982; see also McClintock, 1984) and further suggest that when there are distinguishable differences between females, males will use cues that have previously been paired with female reproductive status in directing their ejaculations.

It is important to note that in the present experiment, the expression of the male preference occurred in the presence of interactions between the 2 females. McClintock et al., (1982) and Tiefer (1969) have shown that during group mating female rats compete actively while mating with a male and that such competition is highest when a male is about to ejaculate, especially for early ejaculations. This competition between females (e.g., interception of the male as he chases another female) was observed during the copulatory preference test. Thus, the current demonstration of male sexual preference for a particular female for his first ejaculation in the presence of 2 females suggests that conditioned ejaculatory preference in the male rat is a particularly robust phenomenon.

An unexpected and potentially interesting finding in this experiment was the significant reduction in the proportion of



unpaired-trained males copulating to ejaculation on the copulatory preference test. The reason for this reduction is not clear. One possible explanation is that the almond odor may have produced inhibition of sexual behavior on the test. During the conditioning trials, the unpaired-trained males received repeated trials with A + N females. Male rats in all of the groups learned to inhibit their sexual behavior toward the nonreceptive females, but for the unpaired-trained males, the almond odor was always a reliable indicator of the necessity of this inhibition. Thus, through classical conditioning, the almond odor alone may have become able to inhibit sexual behavior. During the copulatory preference test, the almond odor was likely present from two sources: first, from the A + E female and second, from the open field (because it was not cleaned between tests, it is likely there was a residual almond odor). This intriguing pattern of results is of potential importance because this effect may provide a useful model for studying the effects of inhibitory conditioning on sexual behavior. Previous studies of conditioned inhibition of sexual behavior used nausea produced by lithium chloride injections paired with copulation to reduce sexual arousal (e.g. Johnston & Zahorik, 1975). The present findings suggest that conditioned sexual inhibition may be produced by pairing a CS (a neutral odor) with a naturally inhibiting unconditioned stimulus (UCS; a nonreceptive female).

Another unexpected finding was that significantly more random-trained males switched their choice of female for the second ejaculation ("switching") compared with the other groups, suggesting an alteration in the general mating strategies of male rats in the presence of an arbitrary odor that has been previously paired with female sexual receptivity. The significance of switching lies in its resemblance to the Coolidge effect (Beach & Jordan, 1956; see Dewsbury, 1981). As is the case with the Coolidge effect, switching involves an increase in the incentive value of a second (or novel) female following copulation with a first (or familiar) female. In the original study by Beach and Jordan (1956), the increased incentive value of a novel female was apparent by her ability to stimulate copulatory behavior from a male rat that had become sexually exhausted through repeated ejaculations with a familiar female. In the present study, the increased incentive value of a female that has not received an ejaculation over a female that has received an ejaculation was apparent in random-trained males, most of which switched their choice of females for the second ejaculation. The fact that the other groups of males were less likely to display switching suggests that in addition to a conditioned ejaculation preference, the conditioning procedure used here can influence the general mating strategy used by male rats. The nature and consequences of these differences in copulatory strategy are unclear, and to our knowledge similar conditioning effects have not been reported previously. However, the finding that random-trained and unpaired-trained males differed in terms of their selection of females for the second ejaculation under equivalent conditions further suggests that male selection controlled the distribution of ejaculations during the copulatory preference test.

Finally, it is of interest to note that the addition of training

trials with nonreceptive females does not appear to influence the development of conditioned level changing. Mendelson and Pfaus (1989) found that sexually naive males receiving access to receptive females developed conditioned level changing, whereas those receiving access to nonreceptive females did not. They further found that after males had developed conditioning level changing, substitution of a nonreceptive female for the receptive one did not disrupt the behavior. Thus, the present results are consistent with the notion that the development of conditioned level changing is dependent on receiving access to receptive females in the bilevel chamber. Moreover, because the chambers were cleaned before each trial, these findings also support the notion that sex odors from previous copulations are not necessary for the development of conditioned level changing in Long-Evans rats.

## Experiment 2

The purpose of Experiment 2 was to replicate and extend the results of Experiment 1. In Experiment 1, we found that when males were given training trials with both receptive and unreceptive females that always involved a consistent odor pairing, males would develop a preference for the receptive females of the odor type that they had previously encountered as sexually receptive. The purpose of Experiment 2 was to determine whether mate preferences could be generated with only the trials with receptive females. Accordingly, paired-trained males received nine trials with A + E females and nonpaired-trained males received nine trials with E-alone females. Then all males received a copulatory preference test with one A + E and one E-alone female.

## Method

### Subjects

Thirty male rats ( $n = 15$  per group) of the same strain from the same supplier were housed in the same conditions as those of Experiment 1 and served as subjects. Female rats were housed and receptivity was induced in the same way as in the first experiment.

### Apparatus

All conditioning trials were conducted in the same bilevel chambers (cleaned before each trial) and the copulatory preference tests were conducted in the same open field as in Experiment 2. All conditioning trials were recorded on videotape and later scored using a PC-based program (Ogawa, 1991).

### Procedure

**Conditioning phase.** As in Experiment 1, male rats received seven daily 15-min preexposure sessions to the bilevel chambers. The subsequent nine conditioning trials were identical to the trials with receptive females in the first experiment. Both paired- and nonpaired-trained males were placed individually into a bilevel chamber for 5 min, after which a receptive female bearing the appropriate scent (almond extract or distilled water) was placed into the chamber for a 30 min test of copulation.

**Copulatory Preference Test.** Four days following the final conditioning trial, each of the males was placed in the open field and allowed to habituate for a period of 5 min. As in Experiment 1, 1 A + E female and 1 E-alone female were placed simultaneously into two diagonal corners of the open field at approximately equal distances from the male. All copulatory activity and the female to which they were directed was recorded during each male's test. The test was terminated after 30 min.

### Statistical Analysis

Mixed-design between-groups and within-groups ANOVAs were used to analyze all data from the conditioning trials with significant values being followed by post hoc analysis of individual means using the Tukey method. Chi-square analysis was used for analysis of proportions of females selected for first mount, first intromission, and first ejaculation on the copulatory preference test. Mixed ANOVAs were used for analysis of the distributions of mounts, intromissions, and ejaculations throughout the 30-min test period. In addition, *t* tests were used for analysis of proportions of mounts and intromissions directed toward the females selected for ejaculation in a series. The level of significance for all comparisons was .05.

### Results

#### Conditioning Phase

Of the 30 males used as subjects in this experiment, 4 failed to ejaculate on the first conditioning trial, but all males mounted, intromitted, and ejaculated on subsequent trials. No significant differences between groups were observed during the conditioning phase. The mean latency to level change decreased, and the mean number of level changes increased across conditioning trials; however, there were no between-group differences on these measures (data not shown). Similarly, the mean latencies of rats in each group to intromit and ejaculate decreased across trials during the conditioning phase; again, no between-group differences were detected (data not shown). Data was consistent with other studies of copulation in bilevel chambers (see Pfaus et al., 1990).

#### Copulatory Preference Test

All 30 males mounted, intromitted, and ejaculated at least twice during the copulatory preference test. Figure 4 displays the choice of females for first mount, first intromission, and first ejaculation. Consistent with the findings of Experiment 1, more paired-trained males ejaculated with A + E females, whereas approximately equal numbers of nonpaired-trained males ejaculated with E-alone and A + E females; a chi-square confirmed the statistical significance of this observation,  $\chi^2(29) = 3.60, p < .05$ . No significant differences were found between the groups for choice of female for first mount,  $\chi^2(29) = 0.56, p > .05$ , or first intromission,  $\chi^2(29) = 1.2, p > .05$ .

The mean mounts per female per series, intromissions per female per series, and mean ejaculations per female over the 30 min test are displayed in Figure 5. For mount distribution, there were no significant effects; this was confirmed by a

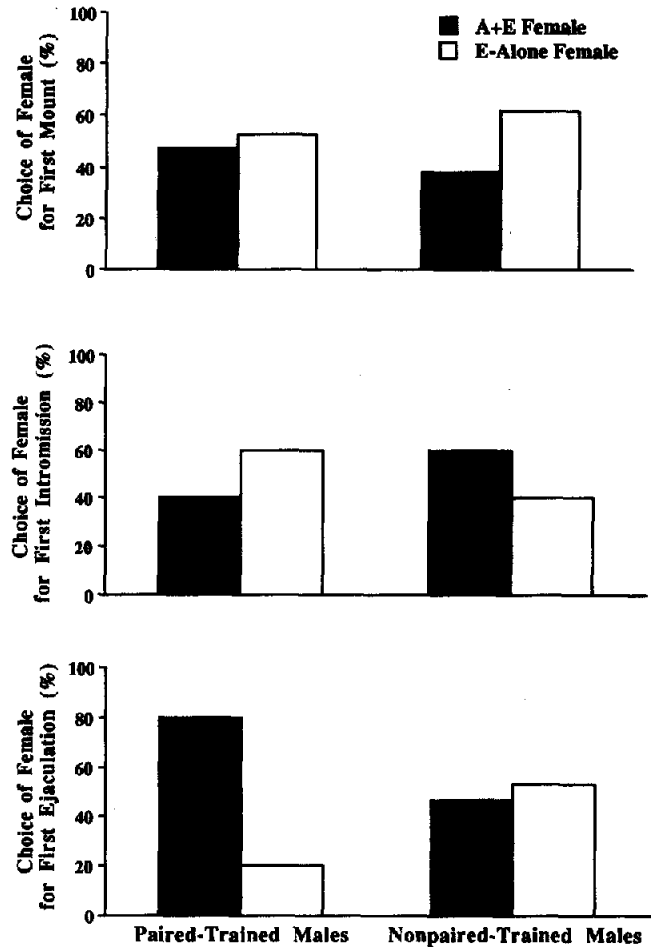


Figure 4. Proportional distribution by female type of first mount (top panel), first intromission (middle panel), and first ejaculation (bottom panel) during the copulatory preference test in Experiment 2. A + E Female = Sexually receptive females that had almond extract applied to the back of the neck and to the anogenital area; E-Alone Female = Sexually receptive females that had distilled water applied to the back of the neck and to the anogenital area.

2 × 2 mixed ANOVA, for group:  $F(1, 28) = 1.59, p > .05$ ; for female type:  $F(1, 28) = 0.091, p < .05$ ; for Group × Female Type:  $F(1, 28) = 1.003, p > .05$ . For intromission distribution, there were also no significant effects, for group:  $F(1, 28) = 0.123, p > .05$ ; for female type:  $F(1, 28) = 0.704, p < .05$ ; for Group × Female Type:  $F(1, 28) = 0.574, p > .05$ . Moreover, for ejaculation distribution, a 2 × 2 mixed ANOVA revealed no significant main effects, for group:  $F(1, 28) = 1.92, p > .05$ ; for female type:  $F(1, 28) = 0.13, p > .05$ . There was, however, a significant interaction between group and female type:  $F(1, 28) = 7.36, p < .05$ . Post hoc comparisons revealed that paired-trained males ejaculated significantly more times with A + E females than E-alone females and that nonpaired-trained males ejaculated significantly more times with E-alone females than A + E females.

Figure 6 displays the proportion of males in each group that switched female for choice of ejaculation on consecu-

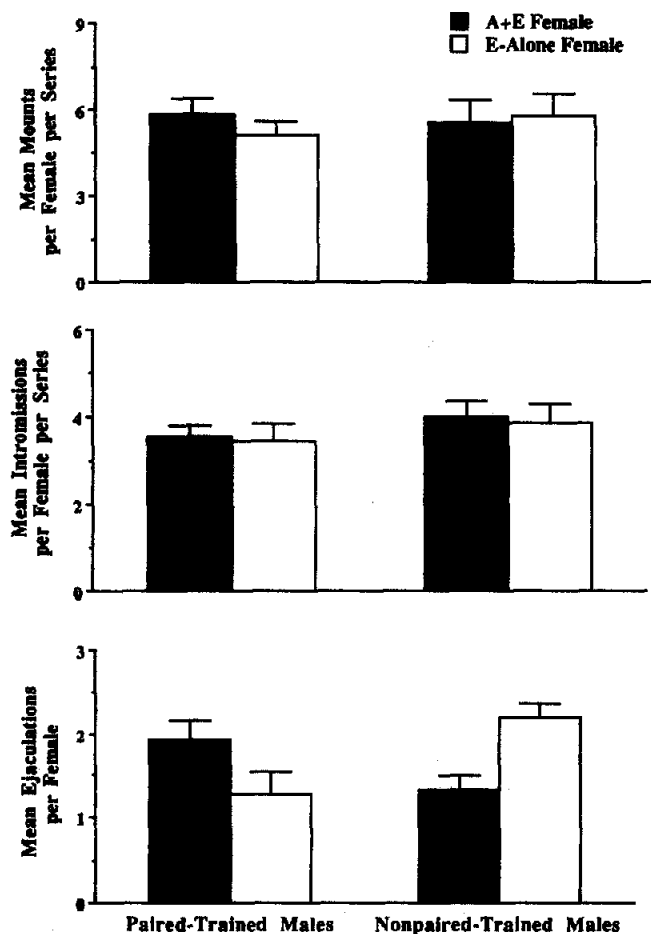


Figure 5. Distribution of mean ( $\pm SE$ ) mounts per female per series (top panel), mean ( $\pm SE$ ) intromissions per female per series (middle panel), and mean ( $\pm SE$ ) ejaculations per female (bottom panel) during the copulatory preference test in Experiment 2. A + E Female = Sexually receptive females that had almond extract applied to the back of the neck and to the anogenital area; E-Alone Female = Sexually receptive females that had distilled water applied to the back of the neck and to the anogenital area.

tive series. Analysis of the second ejaculatory series revealed that the two groups differed in the choice of female for ejaculation with respect to the female with which they ejaculated on the first series. A large proportion (87%) of the nonpaired-trained males switched their choice of female for ejaculation from the first to the second series, whereas less than half of the paired-trained males switched (47%). A chi-square analysis confirmed the statistical significance of this observation,  $\chi^2(29) = 5.40$ ,  $p < .05$ . Analysis of subsequent ejaculatory series failed to reveal significant differences in the proportion of males switching females on consecutive ejaculatory series, for the third ejaculatory series:  $\chi^2(26) = 1.78$ ,  $p > .05$ ; for the fourth ejaculatory series:  $\chi^2(13) = 0.03$ ,  $p > .05$ . Furthermore, just over half (60%) of the paired-trained males ejaculated with both females during the 30-min test compared with all (100%) of the nonpaired-trained males. A chi-square analysis con-

firmed the statistical significance of this observation,  $\chi^2(29) = 7.50$ ,  $p < .05$ .

No other measures of copulatory behavior differed significantly between the two groups. Again, it is noteworthy that the selection of females for ejaculation was not related to either the distribution of mounts or the distribution of intromissions. The same females were selected for first ejaculation and for first mount by approximately half of the paired-trained (53%) and nonpaired-trained (60%) males, and for first intromission by approximately half of the paired-trained (40%) and nonpaired-trained (53%) males. For both groups of males, the mean proportion of mounts across all ejaculatory series directed toward the females selected for ejaculation on the same series was not substantially different from chance (50%) probability, for paired-trained males:  $t(14) = 0.63$ ,  $p > .05$ ; for nonpaired-trained males:  $t(14) = 0.68$ ,  $p > .05$ . Similarly, the mean proportion of intromissions across all ejaculatory series directed toward the females selected for ejaculation on the same series was not substantially different from chance probability in either group, for paired-trained males:  $t(14) = 1.67$ ,  $p > .05$ ; for nonpaired-trained males:  $t(14) = 0.28$ ,  $p > .05$ .

### Discussion

The results of Experiment 2 confirmed the general findings of Experiment 1 on the copulatory preference test. Paired-trained males directed their ejaculations preferentially toward A + E females as indicated by the selection of females for their first ejaculation and the distribution of ejaculations, whereas nonpaired-trained males directed their ejaculations preferentially toward E-alone females as indicated by the distribution of ejaculations; however, their selection of females for their first ejaculation did not indicate a preference (Figures 5 and 6). Together the results of these experiments demonstrate that the pairing of a neutral odor with access to a receptive female produces a preference for a female bearing that odor over a female that does not. The fact that differential distribution of copulatory behaviors between the two female types was only observed with ejaculatory measures suggests that such conditioned preferences influence only the distribution of ejaculations and not the distribution of mounts or intromissions.

### General Discussion

The present series of experiments demonstrate that the pairing of a neutral odor with a receptive female alters consummatory measures of sexual behavior and mate selection in the male rat. Males trained with A + E females ejaculated with A + E females significantly more than with E-alone females. Males trained with E-alone females showed the opposite, albeit weaker, pattern; they tended to ejaculate more often with E-alone females. However, this effect was strengthened with the addition of training with A + N females. Males trained with random pairings of almond odor and female receptivity showed no preference for one female over the other. Furthermore, for the second ejaculation, paired-trained and unpaired-trained males displayed a sig-

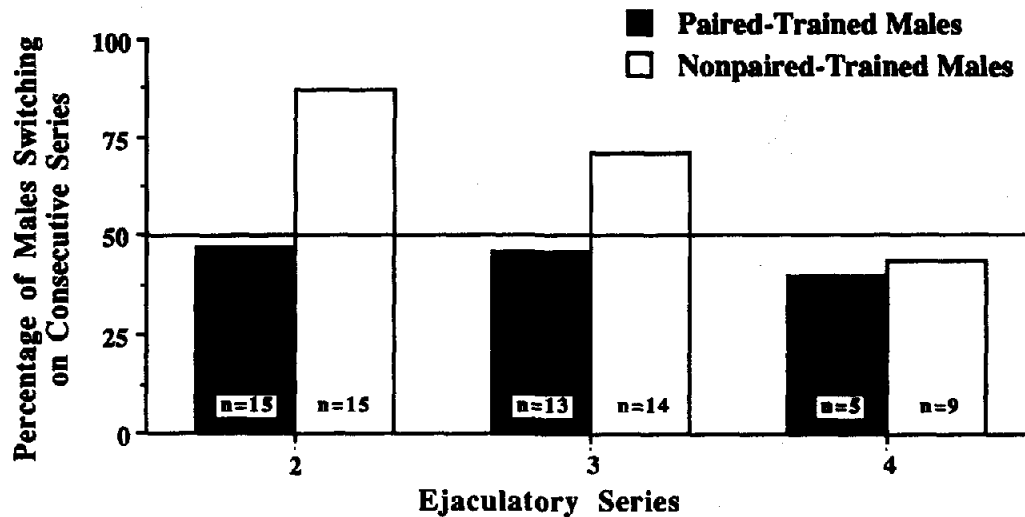


Figure 6. Proportion of males in each group switching females for ejaculation on consecutive ejaculatory series during the copulatory preference test in Experiment 2.

nificant reduction in switching behavior, whereas almost all of the nonpaired-trained and most of the random-trained males switched females. Together, these results suggest that olfactory conditioning produces an ejaculatory preference in male rats by making them more likely to direct their ejaculation toward cues previously paired with sexually receptive females.

The present findings that male rat sexual behavior can be conditioned by pairing a CS with a copulatory UCS also stand in contrast to those of Zamble, Hadad, and Mitchell (1985), who failed to find any evidence of conditioning using a copulatory UCS. Probably the most important reason for these differences in results is the manner in which each study assessed conditioning. The present study assessed the influence of conditioning on a qualitative measure, that is, the selection of a mate in a triad mating situation; however, Zamble, Hadad, and Mitchell assessed the influence of conditioning on a quantitative measure, that is, the ejaculation latency in a dyad mating situation. Thus, the type of conditioned effects reported here could not have been detected in the study by Zamble, Hadad, and Mitchell. We do not know whether the present conditioning procedure might have produced differential response latencies similar to those reported by Zamble, Hadad, Mitchell, and Cutmore (1985) because we did not use dyad testing conditions, although we note that there were no differences between the groups on copulatory latencies during the conditioning phase.

Second, the CS-UCS interval was qualitatively different between studies. In our experiments, the CS was present throughout the period of exposure to the receptive female, whereas in the Zamble, Hadad, and Mitchell (1985) study, it was presented only before access to the receptive female on training trials. Akins, Domjan, and Gutierrez (1994) have demonstrated that in Japanese quail, the topography of the conditioned responses is dependent on the CS-UCS interval: With a long interval, males displayed a general increase

in locomotion, whereas with a short interval, males displayed behavior directed toward the CS (i.e., conditioned approach). If the same principles apply to the rat, then it seems likely that the relatively long interval used by Zamble, Hadad, Mitchell, and Cutmore (1985) facilitated sexual behavior by increasing arousal in general, whereas the shorter CS-UCS interval in our experiments produced conditioned effects on sexual behavior directed at the CS (e.g., the selection of females with the CS odor).

The present findings demonstrate not only the ability of a conditioned odor to produce an ejaculatory preference in the male rat but also the utility of testing male sexual behavior in the presence of multiple receptive females. The conditioned effects observed on the pattern of copulatory preferences could not have been detected if a more standard dyad test had been used simply because there would be no opportunity for the males to display a preference. An alternative test strategy could have been one similar to those used in studies of conspecific preference (e.g., Gilman & Westbrook, 1978; de Jonge, Burger, Van Haaren, Overdijk, & Van de Poll, 1987). In those studies, subjects are allowed to investigate confined conspecifics or bedding from conspecifics. Although such experiments would be of interest, they do not allow direct assessment of copulatory preferences per se but rather are useful in assessing preferential approach behavior. Furthermore, rats mate naturally in groups (see McClintock, 1984), and the triad mating test might therefore represent a more ethologically relevant method of assessing mate preferences in this species.

Comparison of the results from distribution of mounts and intromissions with the distribution of ejaculations during the copulatory preference test reveals another unexpected finding. Both groups of males preferentially distributed their ejaculations, but the same was not true for other measures of copulatory activity. Paired-trained males displayed preferences for A + E females for first ejaculation, and both groups of males displayed a preference for one type of

female over the other for total number of ejaculations. They did not show any consistent preference for either female for first mount, for first intromission, or for the mean number of mounts or intromissions in any ejaculatory series or across series. A male's choice of female for the first mount or intromission on a given ejaculatory series thus could not be used to predict which female that male would ejaculate with. This finding suggests that in group mating situations there are differences in how males distribute mounts and intromissions compared with how they distribute ejaculations. Although our results are consistent with McClintock's view (1984) that "usually, during group mating, a male rat simply mates with the female that is closest and soliciting him" (p. 26), this does not appear to be true for ejaculation. At the point of ejaculation or immediately before it, males may become discriminating and choose between potential mates. Thus, in the male rat, as in the male human, selection of a partner for copulation per se and selection of a partner for mating may be differentiated (Buss & Schmitt, 1993; Kenrick, Sadalla, Groth, & Trost, 1990; Townsend & Levy, 1990; Townsend & Roberts, 1993).

In conclusion, the present results extend the findings of several previous studies that reported the conditioning of different aspects of sexual behavior in the male rat. These studies demonstrated that conditioned stimuli can increase sexual excitement (e.g., Pfaus et al., 1990), arousal (e.g., Zamble, Hadad, Mitchell, & Cutmore, 1985), and neuroendocrine function (Graham & Desjardins, 1980), and that subjects can be trained to perform arbitrary operants to obtain access to a mate or a second-order stimulus associated with a mate (e.g., Everitt et al., 1987). The present study demonstrates that conditioned stimuli can also direct ejaculation in a situation in which a male rat has the opportunity to copulate with multiple partners.

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## Original Articles

# The development of olfactory conditioned ejaculatory preferences in the male rat

## I. Nature of the unconditioned stimulus

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**Abstract**

We have demonstrated previously that repeated pairing of a neutral odor with copulation produces a subsequent conditioned ejaculatory preference (CEP) for a female bearing that odor. Here we examine the copulatory components that comprise the unconditioned stimulus (UCS). In Experiment 1, male Long–Evans rats were allowed to copulate with scented females for nine sessions in which they achieved two ejaculations, one ejaculation plus the first intromission following the postejaculatory interval (PEI), one ejaculation without a PEI, or five intromissions without ejaculation. Only the males that achieved two ejaculations or one ejaculation plus the PEI displayed significant CEP. In Experiment 2, males were allowed to remain in the presence of the scented female without access to her after different amounts of copulatory stimulation. Under these conditions, both one and two ejaculations, but not five intromissions, supported the development of CEPs. In Experiment 3, males were allowed to copulate to ejaculation with an unscented female followed by exposure without access to a scented female. This treatment also supported the development of CEP. These results indicate that ejaculation plus a PEI are necessary for the development of CEPs and that the female must be present during the PEI for this to occur. These findings indicate that events during the PEI are the critical components of the UCS for CEP development. © 2001 Elsevier Science Inc. All rights reserved.

**Keywords:** Sexual behavior; Copulation; Mating; Ejaculation; Postejaculatory interval; Rats; Males; Learning; Pavlovian conditioning; Preferences

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**1. Introduction**

The expression of many sexual behaviors is dependent upon, or modified by, prior experience. Learning has an influence on the performance of operants, mate location, sexual excitement, mate recognition, sexual arousal, courtship behaviors, mate preferences, and copulatory efficiency (for reviews see Refs. [8,9,37]). Many effects of learning on sexual behavior involve the development of associations between a natural reinforcer, unconditional stimulus (UCS), and an initially neutral stimulus, conditioned stimulus (CS). The nature of the learning for several sexual behaviors has been probed by examining the types of UCSs that support learning. Conditioned sexual excitement, as measured by

increased anticipatory level changing in bilevel chambers [30], can be produced either by repeated exposure to estrous odors without copulation in bilevel chambers [50] or by repeated copulatory experience in cleaned bilevel chambers [21]. The performance of operants is supported by intromissions without ejaculation [23,48,52], however, ejaculations increase the level of operant performance. And, the development of conditioned place preferences requires ejaculation, but can develop when a contextual CS is paired only with postejaculatory events [1,33]. Thus, different conditioned sexual behaviors require the pairing of CSs with different unconditioned components of copulation.

The present study examines the UCS for the influence of conditioning on copulatory preferences. In previous studies [20,21], a neutral odor (almond extract) used as an olfactory CS paired with copulation produced a conditioned ejaculatory preference (CEP). In these studies, male rats were allowed access to sexually receptive females with a neutral odor applied to their neck and anogenital region during copulatory training sessions. The influence of this condi-

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tioning procedure on copulatory preferences was tested subsequently by allowing males to copulate simultaneously with unrestricted access to two receptive females, one bearing the CS and one not. On the copulatory preference test, most males trained with the almond-scented females ejaculated first and more often with the almond-scented female than the unscented one. Males with no experience with almond odor, or trained with almond odor in an unpaired or randomly paired manner, did not display a preference for almond-scented female. Thus demonstrating that the learning was of a Pavlovian nature. Further, the effect of the conditioning appeared to be specific to ejaculation as no difference were found for the distribution of mounts or intromissions between the almond-scented and unscented female. Additional studies [19] have found that the olfactory CS can elicit copulatory behavior when it is applied to a nonreceptive female and that conditioned males show a bias to mount the almond-scented female only immediately prior to ejaculation, suggesting that CEP is mediated by a preference for the scented female at or near the point of ejaculation.

The UCS for CEP was determined by examining the effect of different amounts of copulation with scented females on the development of CEP. This was accomplished by allowing males either multiple intromissions without ejaculation, ejaculation, or multiple ejaculations with almond-scented females, and then assessing their copulatory preferences for an almond-scented female or an unscented female.

## 2. Experiment 1

Our previous demonstrations of CEPs have involved training schedules of nine sessions, each 30 min in duration. In Experiment 1, Long–Evans male rats were given nine sessions with almond-scented receptive females which were terminated following either two ejaculations, one ejaculation plus the first intromission after the postejaculatory interval (PEI), one ejaculation without a PEI, or five intromissions without ejaculation. Then subsequent copulatory preferences were assessed during a 30-min test with two females, one almond-scented and one not.

### 2.1. Methods

#### 2.1.1. Subjects

**2.1.1.1. Males.** The 66 Long–Evans rats that served as subjects in Experiment 1 were obtained from Charles River Canada, (St. Constant, Québec, Canada). The males weighed approximately 300 g and were sexually naive at the start of the experiment. They were housed in pairs in Plexiglas cages (36 × 26 × 19 cm) with ad lib access to food (Purina Rat Chow) and water. All rats were kept in a 12:12 h reversed light/dark cycle colony room maintained at 21°C.

**2.1.1.2. Females.** Female Long–Evans rats from the same supplier as above were ovariectomized via bilateral lumbar incisions under ketamine/xylazine anesthesia at least 2 months prior to the start of the experiment and were sexually experienced. Sexual receptivity was induced by subcutaneous administration of estradiol (10 µg) 48 h prior and progesterone (500 µg) 4–6 h prior to each training or test session. Females were housed under the same conditions as males. Stimulus females were selected at random for use during conditioning sessions and copulatory preference tests. Female rats were scented with either approximately 1 ml of almond extract (Blue Ribbon, Etobicoke, Ontario, Canada) or distilled water applied to both the back of the neck and the anogenital area using a cotton swab.

#### 2.1.2. Apparatus

Conditioning sessions took place in bilevel chambers constructed of Plexiglas (outside dimensions of 18 × 25 × 65 cm) with a platform (40 cm in length) elevated by a set of ramps at each end dividing the chamber into two levels (for further details see Ref. [35]). The bilevel chambers were cleaned with water and Coverage 256 (Conva Tec, St. Louis, MO) and the soiled bedding was replaced with clean bedding prior to each conditioning session. All conditioning sessions were recorded on video and scored subsequently using a PC-based program. Copulatory preference tests took place in a large open field (123 × 123 × 46 cm) with a thin layer of bedding covering the floor. Copulatory preference tests were scored at the time of testing.

#### 2.1.3. Procedure

**2.1.3.1. Conditioning phase.** Conditioning sessions were conducted in the same manner as in Kippin et al. [21] with the exception that trials were terminated once a criterion of copulation was achieved. Male rats were preexposed to the chambers once a day for 15 min each day in order to habituate them to the training environment. This habituation procedure lasted 7 days and has been shown previously to increase the proportion of males that become vigorous copulators [38]. Then males were allowed to achieve either two ejaculations (2EJ;  $n = 14$ ), one ejaculation plus the first intromission after the PEI (PEI;  $n = 14$ ), one ejaculation (1EJ;  $n = 14$ ), or five intromissions (INT;  $n = 24$ ); conditioning sessions were conducted at 4-day intervals during the middle third of the dark phase of the light/dark cycle. In each of nine conditioning sessions, males were placed in the chamber for 5 min then a sexually receptive scented female (A + E female) was placed into the chamber. Rats were then allowed to copulate until they reached their copulatory criterion then they were returned to their home cages; conditioning sessions were terminated by removing the female followed by removal of the male approximately 1 min later. Latency and frequency data for all mounts,

intromissions, and ejaculations were recorded during each conditioning session. Criteria for sexual behaviors were those described by Sachs and Barfield [44] and Meisel and Sachs [29].

**2.1.3.2. Copulatory preference test.** Four days after the final conditioning session, each male was placed in the large open field and allowed to habituate for 5 min. At the end of this period, one A+E female and one female scented with distilled water (E-Alone female) were placed simultaneously into two diagonal corners of the open field at approximately equal distances from the male. All copulatory behaviors and the females to which they were directed were recorded during each male's test. Tests were terminated 30 min after the females were introduced.

A number of procedures were used to ensure that the two females used during the copulatory preference tests differed only in the applied odor. Females were selected at random from a separate pool of females than those used during training. Female identification was verified by small bands on the tail made with two different colored markers of the same make that were applied at least 4 days in advance of the test. The two markers were used in a counterbalanced fashion, with the type of female for each color marker alternating on consecutive tests.

#### 2.1.4. Statistical analysis

Mixed-design between–within ANOVAs were used to analyze the level changing data from the conditioning sessions with significant values being followed by post hoc analysis of individual means using the Tukey method. Chi-square analyses for multiple independent samples [47] were used to analyze group differences in the proportion of female selected for first mount, first intromission, and first ejaculation on the copulatory preference test; significant values were followed up by chi-square analyses for two samples. Previous experiments (e.g., Ref. [22]) have consistently found that olfactory conditioning procedures similar to those used in the present experiment produce preferences on ejaculation, but not on mount or intromission, measures. Accordingly, the distribution of ejaculations between females on the copulatory preference tests were analyzed using planned orthogonal comparisons [13]; comparisons were made only between the A+E and E-Alone female for each group of males. Effect size estimates [13] were calculated from the distribution of ejaculations between females for each group. The distribution of mounts and intromissions between the two females during the copulatory preference tests were analyzed using mixed ANOVAs with significant values being followed by post hoc analysis of individual means using the Tukey method. Chi-square analyses were used to compare the proportions of males that switched choice of female for ejaculation on consecutive series. The level of significance for all comparisons was .05.

## 2.2. Results

### 2.2.1. Conditioning phase

Of the 66 males used as subjects in this experiment, 7 failed to achieve the copulatory criterion on the first conditioning session and were excluded from the experiment; 1 in the 2EJ group, 3 in the PEI group, and 3 in the INT group. An additional four males in the INT group were excluded from analysis because they ejaculated on one or more conditioning sessions.

The mean latency to level change decreased and the mean number of level changes increased across conditioning sessions for all groups. Interestingly, males in the INT group reached an asymptotic latency to level change higher than that of the other three groups and an asymptotic level change frequency lower than that of the 2EJ and 1EJ groups (see Fig. 1). The statistical significance of these differences were confirmed by mixed-design between–within ANOVAs. For level change latency, there was a significant effect of group [ $F(3,48)=4.04$ ,  $P<.05$ ; INT group differed significantly from the other three groups and no other groups differed significantly], a significant effect of session [ $F(8,384)=35.47$ ,  $P<.05$ ; Session 1 differed from all other sessions, but no other sessions differed significantly], but the interaction between group and sessions failed to reach significance [ $F(8,384)=0.92$ ,  $P>.05$ ]. For level change frequency, there was a significant effect of group [ $F(8,48)=5.90$ ,  $P<.05$ ; INT group differed significantly from the 2EJ and 1EJ groups, no other groups differed significantly], a significant effect of sessions [ $F(8,384)=35.47$ ,  $P<.05$ ; Session 1 differed from all other sessions, Session 2 differed from Sessions 4 to 9, and Session 3 differed from Session 7, no other sessions differed significantly], and a significant interaction between group and session [ $F(24,384)=1.60$ ,  $P<.05$ ; for Session 1 2EJ group differed from the 1EJ and PEI groups, for Session 3 2EJ group differed from the other three groups and 1EJ group differed from INT group, for Sessions 4, 5, and 7 INT group differed from the other three groups, for Sessions 6, 8, and 9 INT group differed from 2EJ and 1EJ groups].

Conversely, males in all groups displayed similar copulatory behaviors during training; latency to mount, latency to intromit, and interintromission intervals decreased to asymptotic levels that did not differ between groups, intromission ratio (number of successful intromission to intromission attempts, i.e., mounts) increased to asymptotic levels that did not differ between groups, and pursuit of female (number of level changes per mount) increased to asymptotic levels that did not differ between the four groups. Similarly, ejaculation latency decreased to asymptotic levels that did not differ between the 2EJ, PEI, and 1EJ groups, and postejaculatory refractory period decreased to asymptotic levels that did not differ between the 2EJ and PEI groups (data not shown).

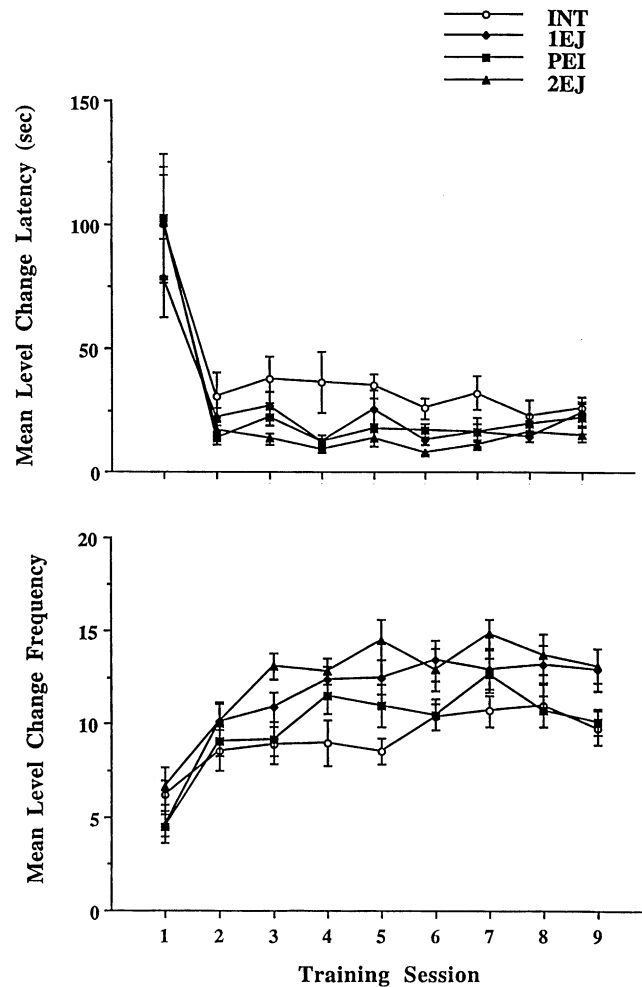


Fig. 1. The mean level change latency (top panel) and mean level change frequency (bottom panel) for each group of males during each conditioning session prior to the female being placed into the chamber. For mean level change latency the INT group differed significantly from the other three groups and for mean level change frequency the INT differed significantly from the 1EJ and 2EJ groups; see text for details.

### 2.2.2. Copulatory preference test

Of the 55 males tested, 6 failed to copulate to ejaculation on the copulatory preference test (1 in the 2EJ group, 2 in the PEI group, and 3 in the INT group). All copulatory parameters were similar between all groups; no significant differences were detected between groups, between female types, or for interactions between groups and female types for intromission latency, intromission frequency, interintromission interval, ejaculation latency, and PEI.

The choice of female for first mount, first intromission, and first ejaculation for each group in Experiment 1 are displayed in Fig. 2, left panel. More males in the 2EJ and PEI groups ejaculated first with A+E females than did males in the 1EJ or INT groups. The significance of this observation was confirmed by chi-square analysis:  $\chi^2(df=3, n=48)=6.52, P<.05$ . The differences between the 2EJ and the INT groups and between the PEI and the INT groups, but no other between group comparisons, were statistically significant as confirmed by chi-square analyses:

2EJ versus PEI  $\chi^2(n=21)=0, P>.05$ ; 2EJ versus 1EJ  $\chi^2(n=26)=1.47, P>.05$ ; 2EJ versus INT  $\chi^2(n=25)=4.81, P<.05$ ; PEI versus 1EJ  $\chi^2(n=23)=1.24, P>.05$ ; PEI versus INT  $\chi^2(n=22)=4.18, P<.05$ ; 1EJ versus INT  $\chi^2(n=27)=1.19, P>.05$ . More males in the 2EJ, PEI, and 1EJ group mounted and intromitted first with the almond-scented female than males in the INT group; none of the males in the INT group mounted or intromitted first with the almond-scented female. Chi square analyses confirmed the statistical significance of these observations. For mounts:  $\chi^2(df=3, n=48)=10.91, P<.05$ ; follow-up comparisons: 2EJ versus PEI  $\chi^2(n=21)=0.06, P>.05$ ; 2EJ versus 1EJ  $\chi^2(n=26)=0, P>.05$ ; 2EJ versus INT  $\chi^2(n=25)=8.55, P<.05$ ; PEI versus 1EJ  $\chi^2(n=23)=0.07, P>.05$ ; PEI versus INT  $\chi^2(n=22)=9.35, P<.05$ ; 1EJ versus INT  $\chi^2(n=27)=8.77, P<.05$ . For intromissions:  $\chi^2(df=3, n=48)=9.00, P<.05$ ; follow-up comparisons: 2EJ versus PEI  $\chi^2(n=21)=0.06, P>.05$ ; 2EJ versus 1EJ  $\chi^2(n=26)=0.13, P>.05$ ; 2EJ versus INT  $\chi^2(n=25)=8.55, P<.05$ ; PEI versus 1EJ  $\chi^2(n=23)=0.01, P>.05$ ;

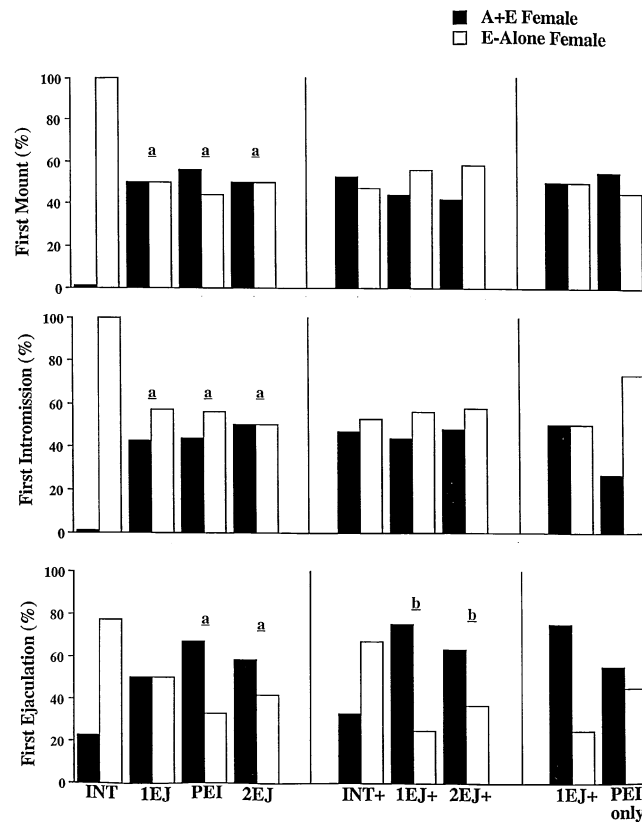


Fig. 2. Proportional distribution by female type of first mount (top panel), first intromission (middle panel), and first ejaculation (bottom panel) during the copulatory preference test for each group in Experiment 1 (left panel), Experiment 2 (center panel), and Experiment 3 (right panel). For statistical analysis, the nonparametric chi-square test was used;  $P < .05$ : **a** for comparison between INT group versus 1EJ, PEI, and 2EJ groups, **b** for comparison between INT+ group versus 1EJ+ and 2EJ+ groups.

PEI versus INT  $\chi^2(n=22)=7.06$ ,  $P < .05$ ; 1EJ versus INT  $\chi^2(n=27)=7.16$ ,  $P > .05$ .

The mean mounts per female per series, mean intromissions per female per series, and mean ejaculations per female over the 30 min test for each group in Experiment 1 are displayed in Fig. 3, left panel. Males in the 2EJ and PEI groups ejaculated more frequently with the A + E female than the E-Alone female, whereas the males in the 1EJ and INT groups ejaculated with both females with approximately the same frequency. Planned orthogonal contrasts confirmed the statistical significance of these observations: 2EJ group  $t(88)=3.88$ ,  $P < .005$ ; PEI group  $t(88)=2.83$ ,  $P < .05$ ; 1EJ group  $t(88)=0.80$ ,  $P > .05$ ; INT group  $t(88)=-0.39$ ,  $P > .05$ . Effect size estimates for the differences in distribution of ejaculations were for the 2EJ group 1.31, for the PEI group 1.58, for the 1EJ group 0.30, for the INT group  $-0.17$ . No significant differences were found for distribution of mounts (results of  $3 \times 2$  mixed ANOVA: for Group:  $F(3,98)=0.09$ ,  $P > .05$ ; for Female Type:  $F(1,98)=0.12$ ,  $P > .05$ ; for Group  $\times$  Female Type:  $F(3,98)=0.91$ ,  $P > .05$ ). Similarly, no significant differences were found for distribution of intromissions (results of  $3 \times 2$  mixed ANOVA: for Group:  $F(3,98)=1.01$ ,  $P > .05$ ; for Female Type:  $F(1,98)=1.11$ ,  $P > .05$ ; for Group  $\times$  Female Type:  $F(3,98)=0.21$ ,  $P > .05$ ).

The proportion of males that switched females for consecutive ejaculations are displayed in Table 1. There did not appear to be any consistent pattern in the proportion of males that switched females for choice of ejaculation between consecutive series. The between group analyses failed to reach statistical significance for all series: for second ejaculation  $\chi^2(df=3, n=53)=0.98$ ,  $P > .05$ ; for third ejaculation  $\chi^2(df=3, n=49)=2.84$ ,  $P > .05$ ; for fourth ejaculation  $\chi^2(df=3, n=40)=0.55$ ,  $P > .05$ . Nor did the proportions of males switching across groups differ from chance (50%) on any series: for second ejaculation  $\chi^2(df=1, n=53)=0.17$ ,  $P > .05$ ; for third ejaculation  $\chi^2(df=1, n=49)=0.02$ ,  $P > .05$ ; for fourth ejaculation  $\chi^2(df=1, n=40)=0.55$ ,  $P > .05$ . These patterns of choice of female for ejaculation indicate that an ejaculatory preference is not merely a function of choice of female for first ejaculation followed by alternation of choice of female for ejaculation on subsequent series.

### 2.3. Discussion

The findings of the present experiment demonstrate that the development of CEPs is dependent upon ejaculation with a scented female during the conditioning sessions. Males not allowed to ejaculate during training, as in the INT group, failed to demonstrate a preference.

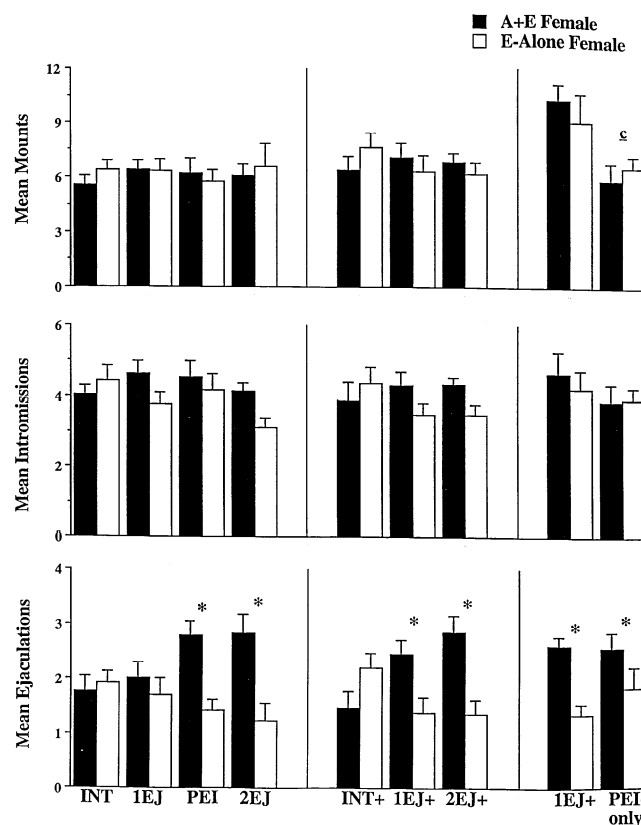


Fig. 3. Distribution of mean ( $\pm$ S.E.) mounts per female per series (top panel), mean ( $\pm$ S.E.) intrusions per female per series (middle panel), and mean ( $\pm$ S.E.) ejaculations per female (bottom panel) during the copulatory preference test in Experiment 1 (left panel), Experiment 2 (center panel), and Experiment 3 (right panel). For statistical analysis of the distributions of mounts and intrusions, between-within ANOVAs;  $P < .05$ : c for comparison between 1EJ+ group versus PEI-only group. For statistical analysis of the distribution of ejaculations, planned orthogonal contrasts;  $P < .05$ : \* for comparisons between A + E female and E-Alone female.

Moreover, it appears that the male must be exposed to the scented female following ejaculation for the preference to develop. When the female was removed immediately following ejaculation, as in the 1EJ group, no preference was displayed. These results suggest that the UCS for CEP is an event produced by ejaculation, not the ejaculation per se. However, the present experiment failed to control for duration of exposure to the almond-scented female, thus the differences between groups may simply be due to the length of exposure resulting from copulatory criteria that require more time to achieve. This confound was addressed subsequently in Experiment 2. Alternatively, the removal of the males following ejaculation in the 1EJ group may have disrupted the development of conditioning. However, the male was only removed following a 1-min interval and no distress to the male was apparent upon removal from the chamber.

Interestingly, in the INT group, which did not display CEP for the scented female, it appears that the males in this group displayed an initial copulatory preference for the unscented female. Remarkably, all males in this group choose the unscented female for first mount and first intrusion and the majority of them choose the unscented female for first ejaculation. This finding is interesting for

two reasons. First, it is the first demonstration that conditioned copulatory preferences can be displayed in choice of

Table 1

Males switching choice of female for ejaculations on consecutive series

|                     | Series 2 | Series 3 | Series 4             |
|---------------------|----------|----------|----------------------|
| <i>Experiment 1</i> |          |          |                      |
| Overall             | 47 (53)  | 51 (49)  | 40 (40)              |
| INT                 | 56 (18)  | 59 (17)  | 33 (12)              |
| 1EJ                 | 50 (14)  | 43 (14)  | 40 (10)              |
| PEI                 | 44 (9)   | 67 (9)   | 50 (8)               |
| 2EJ                 | 33 (12)  | 40 (10)  | 33 (9)               |
| <i>Experiment 2</i> |          |          |                      |
| Overall             | 34 (50)* | 38 (48)  | 57 (37)              |
| INT+                | 40 (15)  | 29 (14)  | 45 (8)               |
| 1EJ+                | 44 (16)  | 25 (16)  | 73 (11)              |
| 2EJ+                | 21 (19)  | 56 (18)  | 50 (18)              |
| <i>Experiment 3</i> |          |          |                      |
| Overall             | 60 (20)  | 53 (19)  | 50 (16)              |
| 1EJ+                | 56 (9)   | 63 (8)   | 17 (6)               |
| PEI-only            | 55 (11)  | 5 (11)   | 70 (10) <sup>†</sup> |

Percentage of males switching choice of female for ejaculation in: (number of subjects per group ejaculating in series).

\* Across groups proportion different from chance (50%),  $P < .05$ .

<sup>†</sup> Between groups differences,  $P < .05$ .

female for mounts or intromissions (in this case, choice of female for first mount and first intromission). Our previous findings found differences only for distribution of ejaculations. Second, the finding that the direction of the preference displayed by the INT group was toward the unscented female, whereas all their previous copulatory experience was with scented females, suggests that interruption of copulation prior to ejaculation may produce an aversive state sufficient to act as a UCS for a CEP in the opposite direction of the CEP observed when males are allowed to copulate to ejaculation.

The difference between groups in the development of conditioned level changing in anticipation of the arrival of the female also reveals a number of interesting points regarding the nature of sexual conditioning. First, consistent with the findings of Kippin et al. [21], all groups in the present experiment displayed levels of anticipatory level changing that increased with copulatory experience in chambers that were cleaned, suggesting that conditioned level changing is not elicited solely by sex odors in Long–Evans males. Nonolfactory stimuli or perhaps even the scent of the clean chambers must have served as the CS to facilitate anticipatory level changing. This finding clarifies conclusions of Van Furth and Van Ree [50] that conditioned level changing is driven by sex odors based on their finding that olfactory bulb lesions disrupt the development of this conditioned behavior. Our results do not stand in contrast to theirs because the chambers in our experiment, although cleaned, undoubtedly contained distinctive odors (e.g., that of Coverage 256 detergent). However, it is not known whether conditioned level changing could be elicited by strictly nonolfactory cues. This idea could be tested by changing the odors in the chambers between training and testing sessions. Second, although all groups displayed a decrease in latency to level change and an increase in frequency of level changes, the INT group reached asymptotic levels with a longer latency and lower latency than the other groups. This suggests that the type of copulation obtained can affect the magnitude of the conditioned response. Cues associated with ejaculation appear to produce more sexual excitement than cues associated with interrupted copulation. Third, the finding that INT group displayed an increase in an appetitive conditioned behavior (level changing) during training, but that the scented female appeared to be devalued during the copulatory preference test demonstrates not only that there are different UCS for different conditioned behaviors but also that a single UCS can have various influences and in apparently opposing directions depending on what behavior is measured (i.e., increase sexual excitement and decrease in ejaculatory preference).

The present study also reveals novel information regarding the acquisition of copulatory behavior. Although previous studies (e.g., Ref. [52]) have looked at the maintenance of copulation following intromissive experience alone, the subjects in these experiments were all

sexually experienced. In the present experiment, all subjects were initially sexually naive and received various amounts of copulation during training. Consistent with findings in sexually experienced animals, intromissive experience alone was sufficient to maintain copulatory behavior in sexually naive males. Moreover, parameters of copulatory behavior did not differ between groups either during training or on the copulatory preference test, demonstrating that intromissive experience alone is sufficient for the acquisition of the copulatory efficiency typical of sexually experienced males.

### 3. Experiment 2

The results of Experiment 1 demonstrate that either 2EJ or PEI with a scented female during conditioning sessions is sufficient for the development of CEP. However, it is not clear why males in the 1EJ and the INT conditions failed to develop CEP. It may be argued that the critical reason that PEI group, but not the 1EJ group, displayed CEP was due to the length of exposure to the scented female and not due to achieving a specified amount of copulation (i.e., exposure to female for PEI group was approximately 15–20 min on Session 1 and 8–10 min on Session 9 versus for 1EJ group was approximately 10–15 min on Session 1 and 3–5 min on Session 9). Accordingly, the goal of Experiment 2 was to determine whether length of exposure to the scented female, or copulation during exposure, is the critical factor in the UCS for CEP. This was achieved by allowing males to copulate to different criterion followed by exposure without access to the scented female for a 30-min period.

#### 3.1. Methods

##### 3.1.1. Subjects

Male rats of the same strain from the same supplier and housed in the same conditions as those of Experiment 1 served as subjects in Experiment 2 ( $n = 78$ ). Female rats were housed and treated in the same way as in the first experiment.

##### 3.1.2. Apparatus

All conditioning sessions were conducted in semicircular chambers fitted with dividers. The chambers were 65 cm long at the front and 40 cm at the widest point and were 40 cm high. The chambers had a Plexiglas front, a wood floor covered with a thin layer of bedding, a rounded metal rear portion, and a metal mesh lid (grid of 1.2 cm). The front and back had Plexiglas grooves so that a divider of plastic coated metal mesh (grid 2.5 cm) could be easily inserted to form two separate compartments. Subjects on either side of the divider could see, hear, smell, and have limited contact with each other, but could not copulate. Chambers were cleaned according to the same procedures used in Experiment 1 and soiled bedding was replaced with clean bedding prior to each conditioning session. Copulatory preference tests were conducted in the same open field as

used in Experiment 1. All conditioning sessions were recorded on video and scored subsequently using a PC-based program and copulatory preference tests were scored at the time of testing.

### 3.1.3. Procedure

**3.1.3.1. Conditioning phase.** As in Experiment 1, male rats received seven daily 15-min preexposure sessions to the training chambers. Each male received nine conditioning sessions consisting of two phases: First, male subjects and stimulus females were allowed to copulate to criterion and then the male was exposed without access to the female. Males were allowed to copulate with an A+E female to either two ejaculations (2EJ+;  $n=26$ ), one ejaculation (1EJ+;  $n=26$ ), or five intromissions (INT+;  $n=26$ ) before the divider was inserted. On each conditioning session, males in each group were placed individually into a chamber for 5 min, after which an A+E female was placed into the chamber and the pair was allowed to copulate until the criterion was achieved. Then the divider was quickly inserted and, if necessary, the female was placed on the opposite side of the divider of the male. Then, the pair was left undisturbed until 30 min had elapsed from the time that the female was first placed into the chamber. After insertion of the divider, both of the animals spent most of the time in close proximity of the divider and engaged in substantial climbing of the divider. Accordingly, the animals were generally within a few centimeters of each other throughout the exposure periods. Thus, this procedure ensured that all males in different groups received the same duration of exposure to the A+E females.

**3.1.3.2. Copulatory preference test.** Four days following the final conditioning session, each male was placed in the open field and allowed to habituate for a period of 5 min. As in Experiment 1, one A+E female and one E-Alone female were placed simultaneously into two diagonal corners of the open field at approximately equal distances from the male. All copulatory behaviors, and the female to which they were directed, were recorded during each male's test. The test was terminated after 30 min.

### 3.1.4. Statistical analysis

The statistics used in Experiment 1 were used in Experiment 2. Chi-square tests were used to analyze between group differences in the proportion of female selected for first mount, first intromission, and first ejaculation on the copulatory preference test. The distribution of ejaculations between females on the copulatory preference tests was analyzed using planned orthogonal comparisons of the ejaculations received by the A+E and E-Alone females for each group of males. Effect size estimates were calculated from the distribution of ejaculations between females for each group. The distribution of mounts and intromissions between the two females during the copulatory pref-

erence tests was analyzed using mixed ANOVAs. Chi-square analyses were used to compare the proportions of males that switched choice of female for ejaculation on consecutive series. The level of significance for all comparisons was .05.

## 3.2. Results

### 3.2.1. Conditioning sessions

Of the 78 males used in Experiment 2, 23 males failed to ejaculate during the first conditioning session and were not included in any analyses; 7 in the 2EJ+ group, 8 in the 1EJ+ group, and 8 in the INT+ group. Males in all groups displayed similar copulatory behaviors during training; latency to mount, latency to intromit, and interintromission intervals decreased to asymptotic levels that did not differ between groups and intromission ratio increased to asymptotic levels that did not differ between groups. Similarly, ejaculation latency decreased to asymptotic levels that did not differ between the 2EJ+ and 1EJ+ groups (data not shown).

### 3.2.2. Copulatory preference test

Of the 55 males tested in Experiment 2, 5 failed to copulate to ejaculation during the copulatory preference test; 2 in the 1EJ+ group, and 3 in the INT+ group. Copulatory behaviors were similar between all groups. Most copulatory parameters were similar between all groups; no significant differences were detected between groups, between female types, or for interactions between groups and female types for intromission latency, intromission frequency, interintromission interval, and ejaculation latency. A significant difference was found between groups for the PEI [ $F(2,47)=3.98$ ,  $P<.05$ ; follow-up analysis revealed the INT+ group had longer PEIs than the other two groups]; however, no significant effects of female type or groups  $\times$  female type interaction were detected.

The choice of female for first mount, first intromission, and first ejaculation for each group of males in Experiment 2 are displayed in Fig. 2, center panel. More males in the 2EJ+ and 1EJ+ groups ejaculated first with the A+E female than males in the INT+ group. The statistical significance of these observations were confirmed with chi-square analyses:  $\chi^2(df=2, n=50)=6.21$ ,  $P<.05$ ; follow-up comparisons: 2EJ+ versus 1EJ+  $\chi^2(n=35)=0.57$ ,  $P>.05$ ; 2EJ+ versus INT+  $\chi^2(n=34)=2.98$ ,  $P<.05$ ; 1EJ+ versus INT+  $\chi^2(n=31)=5.43$ ,  $P<.05$ . No significant differences were observed between groups for first mount [ $\chi^2(df=2, n=50)=0.48$ ,  $P>.05$ ] or first intromission [ $\chi^2(df=2, n=50)=0.08$ ,  $P>.05$ ].

The mean mounts per female per series, mean intromissions per female per series, and mean ejaculations per female during the copulatory preference test for each group in Experiment 2 are displayed in Fig. 3, center panel. Males in the 2EJ+ and 1EJ+ groups ejaculated more frequently with the A+E female, whereas the males in the INT+ group



ejaculated more frequently with the unscented female. Planned orthogonal contrasts confirmed the statistical significance of these observations: 2EJ+ group  $t(94)=4.01$ ,  $P<.05$ ; 1EJ+ group  $t(94)=2.65$ ,  $P<.05$ ; INT+ group  $t(94)=-1.77$ ,  $P>.05$ . Effect size estimates for the differences in distribution of ejaculations were for the two ejaculations group 1.24; for the 1EJ+ group 1.10; for the INT+ group  $-0.66$ . No significant differences were found for distribution of mounts (results of  $3 \times 2$  mixed ANOVA: for Group:  $F(2,94)=0.54$ ,  $P>.05$ ; for Female Type:  $F(1,94)=0.06$ ,  $P>.05$ ; for Group  $\times$  Female Type:  $F(2,94)=1.16$ ,  $P>.05$ ). Similarly, no significant differences were found for distribution of intromissions (results of  $3 \times 2$  mixed ANOVA: for Group:  $F(2,94)=0.29$ ,  $P>.05$ ; for Female Type:  $F(1,94)=1.37$ ,  $P>.05$ ; for Group  $\times$  Female Type:  $F(2,94)=1.86$ ,  $P>.05$ ).

The proportion of males that switched females for consecutive ejaculations are displayed in Table 1. There did not appear to be any consistent pattern in the proportion of males that switched females for choice of ejaculation between consecutive series. The between group analyses failed to reach statistical significance on all series: for second ejaculation  $\chi^2(df=2, n=50)=2.41$ ,  $P>.05$ ; for third ejaculation  $\chi^2(df=2, n=48)=3.06$ ,  $P>.05$ ; for fourth ejaculation  $\chi^2(df=2, n=37)=1.61$ ,  $P>.05$ . The proportion of males switching across groups was significantly lower than chance (50%) for second ejaculation [ $\chi^2(df=1, n=50)=5.12$ ,  $P<.05$ ], but not for third ejaculation [ $\chi^2(df=1, n=48)=3.00$ ,  $P>.05$ ] or fourth ejaculation [ $\chi^2(df=1, n=37)=0.68$ ,  $P>.05$ ]. These patterns of choice of female for ejaculation indicate that an ejaculatory preference is not merely a function of choice of female for first ejaculation followed by alternation of choice of female for ejaculation on subsequent series.

### 3.3. Discussion

The results of Experiment 2 confirm and extend the findings of Experiment 1. The finding that the INT+ group did not display a preference for the scented female even when time of exposure during training sessions was 30 min demonstrates that length of exposure to the scented female is not in itself sufficient for the development of CEP. Additionally, the finding that the 2EJ+ and 1EJ+ groups displayed a preference for the scented female demonstrates that ejaculation is critical for the development of CEP. Moreover, these results show that the presence of the scented female following ejaculation is critical to the development of CEP. When the female was removed following ejaculation (1EJ group in Experiment 1) no CEP developed; however, when the female was present even without access (1EJ+ group in Experiment 2) a significant CEP developed.

The males in the INT+ group in the present experiment displayed an ejaculatory preference for the unscented female as demonstrated by choice of female for first ejaculation and distribution of ejaculations. This finding is in partial agree-

ment with the finding for the INT group in Experiment 1. In Experiment 1, the INT group displayed only an initial preference for the unscented female, as demonstrated by choice for first mount, first intromission, and first ejaculation, but did not show a preference for distribution of ejaculations for the rest of the test session. The significance of these findings is unclear for two reasons. First, given the discrepancies between the nature of the preference displayed by each group, it is not clear if the incomplete copulation treatment results in a preference that is altered by removal or presence of the female. Second, in other studies, males trained with unscented females displayed an ejaculatory preference for unscented females when given the opportunity to copulate with a scented and unscented female. It is not clear whether this represents a default condition or if there is a conditioned preference displayed by males trained with unscented females. In such a case the addition of an odor would produce a deviation from the conditioned preference. Thus, the preference for unscented females displayed by males trained with unscented females and males trained with incomplete copulation with scented females may be interpreted as an absence of any conditioned preferences (i.e., a default) or that one or both of these treatments resulted in conditioning that is the same in expression but not in underlying nature. The resolution of these issues will require further investigation.

## 4. Experiment 3

The findings of Experiments 1 and 2 suggest that the critical aspect of copulation that comprises the UCS for development of CEP was the presence of the scented female following ejaculation. The purpose of Experiment 3 was to determine if exposure to an A+E female only following ejaculation is sufficient to support CEP. Accordingly, male rats were allowed to copulate to ejaculation with an unscented female immediately after which they were exposed (without access) to the same female bearing almond odor for a period of 30 min.

### 4.1. Methods

#### 4.1.1. Subjects

Male rats of the same strain from the same supplier and housed in the same conditions as those of Experiments 1 and 2 served as subjects ( $n=30$ ). Female rats were housed and treated in the same way as in the first two experiments.

#### 4.1.2. Apparatus

All conditioning sessions were conducted in the semi-circular chambers fitted with dividers that were used in Experiment 2. Copulatory preference tests were conducted in the same open field as used in Experiments 1 and 2. All conditioning sessions were recorded on video and scored

subsequently using a PC-based program and copulatory preferences tests were scored at the time of testing.

#### 4.1.3. Procedure

**4.1.3.1. Conditioning phase.** As in Experiments 1 and 2, male rats received seven daily 15-min preexposure sessions to the testing chambers. Each male then received nine conditioning sessions consisting of two phases: First, male subjects and stimulus females were allowed to copulate until the male ejaculated, after which the male was exposed to the female without access. Males were allowed to copulate to one ejaculation with either an A + E (1EJ+;  $n = 12$ ) or unscented female (PEI-only;  $n = 18$ ), after which both groups were exposed without access to an A + E female behind the divider. At the start of each conditioning session, males in each group were placed individually into a chamber for 5 min, after which a receptive female bearing the appropriate scent (almond extract or distilled water) was placed into the chamber until the male ejaculated. Then, the female was immediately removed and scented with almond odor, the divider was inserted, and the female placed on the opposite side of the divider as the male for 30 min of exposure.

Extreme care was taken to ensure that males in the PEI-only condition were not exposed to the almond odor prior to ejaculation during conditioning. Four rooms, each with its own ventilation system, were used for training, only one male was run in each room on a training day. Each chamber was cleaned as in Experiments 1 and 2 and the soiled bedding was replaced with clean bedding prior to each training session. All the males to be tested on a given day were brought to the testing area at the same time and placed in one of the rooms. Then, each male was run simultaneously by different experimenters or the experimenters washed their hands between running consecutive subjects — different procedures were used based on the number of experimenters available on a given day with the resultant variability serving to randomize experimenter effects across training sessions. Accordingly, it is unlikely that these males were exposed to the almond odor prior to ejaculation during each training session.

**4.1.3.2. Copulatory preference test.** Four days following the final conditioning session, each male was placed in the open field and allowed to habituate for a period of 5 min. As in Experiments 1 and 2, one A + E female and one E-Alone female were placed simultaneously into two diagonal corners of the open field at approximately equal distances from the male. All copulatory behaviors, and the female to which they were directed, were recorded during each male's test. The test was terminated after 30 min.

#### 4.1.4. Statistical analysis

The statistics used in Experiments 1 and 2 were used in experiment 3. Chi-square tests were used to analyze between group differences in the proportion of female selected for first

mount, first intromission, and first ejaculation on the copulatory preference test. The distribution of ejaculations between females on the copulatory preference tests were analyzed using planned orthogonal comparisons of the ejaculations received by the A + E and E-Alone females for each group of males. Effect size estimates were calculated from the distribution of ejaculations between females for each group. The distribution of mounts and intromissions between the two females during the copulatory preference tests were analyzed using mixed ANOVAs. Chi-square analyses were used to compare the proportions of males that switched choice of female for ejaculation on consecutive series. The level of significance for all comparisons was .05.

#### 4.2. Results

##### 4.2.1. Conditioning sessions

Of the 30 males used in Experiment 3, 9 failed to copulate to ejaculation on the first conditioning session and were not included in any analyses; 6 in the PEI-only group and 3 in the 1EJ+ group. No significant differences on any copulatory behaviors were detected between the groups.

##### 4.2.2. Copulatory preference test

All of the 21 the males that completed training copulated to ejaculation. All copulatory parameters were similar between all groups; no significant differences were detected between groups, between female types, or for interactions between groups and female types for intromission latency, intromission frequency, interintromission interval, ejaculation latency, and PEI.

The choice of female for first mount, first intromission, and first ejaculation for each group of males in Experiment 3 are displayed in Fig. 2, right panel. More males in the 1EJ+ group ejaculated first with the A + E female than males in the PEI-only group; however, this difference failed to reach statistical significance [ $\chi^2(n = 20) = 1.25, P > .05$ ]. No significant differences were found between groups for first mount or first intromission.

The mean mounts per female per series, mean intromissions per female per series, and mean ejaculations per female during the copulatory preference test for both groups in Experiment 3 are displayed in Fig. 3, right panel. Males in the both groups ejaculated more frequently with the A + E female than the E-Alone female. Planned orthogonal contrasts confirmed the significance of these observations: 1EJ+ group  $t(36) = 2.28, P < .05$ ; PEI-only group  $t(36) = 2.00, P < .05$ . Effect size estimates for the differences in distribution of ejaculations for the 1EJ+ group and the PEI-only group are 1.92 and 0.69, respectively. Additionally, males in the 1EJ+ group mounted more frequently than males in the PEI-only group [ $F(1,36) = 10.46, P < .05$ ], but there was no significant difference for distribution of mounts between females [ $F(1,36) = 0.04, P > .05$ ], nor was there a significant interaction between group and female type [ $F(2,50) = 0.44, P > .05$ ]. No significant differences were found for distribu-

tion of intromissions (results of  $3 \times 2$  mixed ANOVA: for Group:  $F(1,36)=2.75$ ,  $P>.05$ ; for Female Type:  $F(1,36)=0.01$ ,  $P>.05$ ; for Group  $\times$  Female Type:  $F(1,36)=0.10$ ,  $P>.05$ ).

The proportion of males that switched females for consecutive ejaculations are displayed in Table 1. There did not appear to be any consistent pattern in the proportion of males that switched females for choice of ejaculation between consecutive series. Significantly more males in the PEI-only group switched choice of female for ejaculation on the fourth series than males in the 1EJ+ group [ $\chi^2(df=1, n=16)=4.20$ ,  $P>.05$ ], but the groups did not differ significantly on the second series [ $\chi^2(df=1, n=20)=0.30$ ,  $P>.05$ ] or third series [ $\chi^2(df=1, n=19)=0.55$ ,  $P>.05$ ]. Nor did the proportions of males switching across groups differ from chance (50%) on any series: for second ejaculation  $\chi^2(df=1, n=20)=0.80$ ,  $P>.05$ ; for third ejaculation  $\chi^2(df=1, n=19)=0.05$ ,  $P>.05$ ; for fourth ejaculation  $\chi^2(df=1, n=16)=0.00$ ,  $P>.05$ . These patterns of choice of female for ejaculation indicate that an ejaculatory preference is not merely a function of choice of female for first ejaculation followed by alternation of choice of female for ejaculation on subsequent series.

#### 4.3. Discussion

The results of Experiment 3 confirm the findings of the first two experiments that one ejaculation per training session followed by exposure to the scented female is sufficient for the development of CEP. Moreover, the finding that the PEI-only group displayed CEP provides support for the notion that the critical component of the UCS for CEP is the presence of scented female during the PEI, and further, that it does not require continuous copulation with a scented female.

Interestingly, the CEP displayed by the PEI-only males was weaker than that displayed by the 1EJ+ males. This pattern of results is consistent with the interpretation that events during the PEI constitute the critical UCS. The difference in magnitude of the CEP between the two groups is also in agreement with the general principles of Pavlovian conditioning which is typically stronger when the CS (i.e., almond-scented female) is present prior to the UCS (i.e., postejaculatory events) than when the CS is present at the same time as the UCS [43]. This pattern of results may also be interpreted as suggesting that the CEP produced by pairing an odor with the UCSs present during the postejaculatory period is augmented by pairing the odor with other UCSs present during copulation. However, the finding in Experiment 1 that removal of the scented female immediately following ejaculation does not produce CEP demonstrates that pairing the CS with copulatory UCSs is, by itself, insufficient for the development of CEP. Further, it is unlikely that the disturbance of removing the female blocks CEP development because both groups in the present experiment experienced the removal of the female (and subsequent return) but still displayed CEP.

## 5. General discussion

The results of the present study reveal that the development of CEP is dependent upon ejaculation followed by the presence of a scented female. CEPs can be displayed when the male has access to a scented female throughout the PEI or when he is exposed without access to a scented female for an extended interval following ejaculation (presumably throughout the PEI). Perhaps the most remarkable finding is that males do not actually have to copulate with a scented female in order to subsequently display CEP; the female must only be scented following ejaculation. Together these findings implicate processes during the PEI as the critical components of sexual behavior which constitute the UCS that supports the development of CEP.

The findings of the present study that the UCS for CEP and the UCS for conditioned sexual excitement are different highlights the fact that not all conditioning effects on sexual behavior involve the same UCS. Conditioned sexual excitement [21,50], conditioned sexual arousal [25,28,41, 42,55,56], and instrumental behavior [23,48,52] all appear to be supported by UCSs which do not involve ejaculation. Conversely, CEP and conditioned place preferences [1,33] both require ejaculation. Further, they both are supported by pairing stimuli with the postejaculatory period. These findings suggest that highly complex interactions occur between various components of sexual behavior (i.e., UCSs) and environmental stimuli (i.e., CSs) which result in a multifaceted impact of learning on subsequent sexual behaviors.

The PEI is defined as the period from an ejaculation to the next mount with intromission [29,44]. Accordingly, there are at least two sequential processes that occur during the PEI. Immediately following ejaculation there is an inhibitory phase, often referred to as a refractory period, which is accompanied by a characteristic vocalization produced by the male [5]. The inhibition diminishes progressively until the male can be rearoused and copulates. From the present studies, it is unclear whether the inhibitory processes, the rearousal processes, or both, constitute the UCS for CEP. Given that the UCS for CEP are events following ejaculation, it would be useful to identify what these events are. However, it is inherently difficult to isolate the underlying neurobiological events of individual components of copulation. For example, the details regarding the processes of genital stimulation that trigger ejaculation and that produce and remove the transient inhibition of copulatory behavior following ejaculation are not completely understood at the neural level.

Several studies aimed at correlating neural activation with sexual behavior have elucidated certain neural events related to ejaculation. This has been done by measuring electrophysiological activity and using induction of the immediate-early gene, *c-fos*, as a marker of neuronal activation. In the hippocampus, electrophysiological recordings have revealed that theta rhythm predominates prior to intromissions followed by transient desynchronized, large-amplitude irregular

activity. This irregular activity is markedly prolonged following ejaculation [24]. Similarly, activity in the mPOA increases prior to intromission, and is followed by a sharp drop in firing rate. This drop in firing rate is also prolonged following ejaculation [15]. Fos protein appears to be induced selectively by ejaculation in a number of brain regions in several species (for a review see Ref. [36]). Ejaculation-related Fos has been reported in the posterodorsal preoptic nucleus [2,6,14], caudal portion of the medial bed nucleus of the stria terminalis in rats [6], but not in gerbils [14], posterodorsal portion of the medial amygdala (rat [2,6,51], hamsters [10,22,53,54], gerbils [14]), and lateral tegmentum (rats [2,51], gerbils [14]). There are also greater amounts of Fos found in the parvocellular regions of the hypothalamic paraventricular nuclei in rats allowed to ejaculate than in those not allowed to ejaculate [53]. These findings are highly consistent across species despite differences in the behaviors that induce them, suggesting that the individual sensory afferents or their convergence may be critical components for brain activation by sexual stimulation [36]. However, it must be noted that ejaculation versus intromission comparisons involve one group receiving more stimulation (e.g., more intromissions, longer exposure) than the other. Subtractive differences between groups may be qualitative, quantitative, or both in these studies. Accordingly, interpreting the relevance of such effects to the neural basis of the UCS for CEP must be viewed cautiously.

Other studies have examined the correlation between sexual behavior and neurochemistry. Microdialysis has shown that levels of dopamine (DA) and its metabolites rise with the initiation of mating in the nucleus accumbens [7,12,34,39] and the medial preoptic area [17,46]. These levels appear to remain elevated throughout copulation. In studies employing voltammetry, DA oxidation signals in the nucleus accumbens and medial preoptic area, levels rose and fell with the onset and termination of each ejaculatory series [3]. This drop in DA was confirmed subsequently with microdialysis [31]. Similarly, luteinizing hormone secretion increases during initiation of copulation [4,18,32]. Conversely, brain levels of serotonin (5-HT) in the lateral hypothalamic area [26], and 5-HT metabolite levels in brain homogenates [27], are elevated only following ejaculation. Similarly, ejaculation is associated with cerebrospinal fluid increases in oxytocin [16], prolactin [4,32], and gamma-aminobutyric acid (GABA) [40]. Further, endogenous opioids have been implicated in ejaculatory mechanisms because opioid antagonists disrupt ejaculation-induced analgesia [49], antianxiety [11,45], and conditioned place preferences [1]. Although oxytocin likely plays a role in triggering ejaculation, opioids, 5-HT, prolactin, and GABA, which are inhibitory to copulation, may be involved specifically in the inhibitory phase of the PEI. Determining the exact neurochemistry and neuroanatomy of CEP will require further investigation.

In summary, the present experiments demonstrate that the UCS for CEP are postejaculatory events that do not require

copulation with a scented female per se. This stands in sharp contrast to the development of copulation or conditioned sexual excitement which requires intromissions but not ejaculation as the necessary UCS. Understanding the specific components of sexual behavior that comprise UCSs for conditioned behaviors will guide subsequent investigations into the neural substrates of conditioned influences on sexual behavior.

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## ESTROUS ODORS AND SEXUALLY CONDITIONED NEUTRAL ODORS ACTIVATE SEPARATE NEURAL PATHWAYS IN THE MALE RAT

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**Abstract**—Olfactory stimuli play important roles in sexual behavior. Previous studies have demonstrated that both estrous odors and initially neutral odors paired with copulation influence the sexual behavior of male rats. The present study examines the pattern of neural activation as revealed by Fos immunoreactivity (Fos-IR) following exposure to bedding scented with either a neutral odor (almond) paired previously with copulation, estrous odors or no odor. Following exposure to estrous odors Fos-IR increased in the accessory olfactory bulb, medial amygdala, medial bed nucleus of the stria terminalis, medial preoptic area, ventromedial hypothalamus, ventral tegmental area, and both the nucleus accumbens core and shell. Conversely, following exposure to the sexually conditioned odor Fos-IR increased in the piriform cortex, basolateral amygdala, nucleus accumbens core, and the anterior portion of the lateral hypothalamic area. In addition, following exposure to almond odor Fos-IR increased in the main olfactory bulb independent of its pairing with copulation. These patterns of Fos-IR following exposure to estrous or sexually conditioned odors were not influenced by either the addition or omission of the other type of odor. These findings demonstrate that estrous and sexually conditioned odors are processed by distinct neural pathways and converge in the nucleus accumbens core, suggesting that this structure has a unique role in processing sexual stimuli of both pheromonal and olfactory natures. © 2003 IBRO. Published by Elsevier Science Ltd. All rights reserved.

**Key words:** Fos, sexual behavior, associative learning, odor, nucleus accumbens, hypothalamus.

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**Abbreviations:** AOB, accessory olfactory bulb; aLHA, anterior portion of the lateral hypothalamic area; A+E, almond-scented estrous females; BAOT, bed nucleus of the accessory olfactory tract; BLA, basolateral amygdala; CEA, central amygdala; DAB, 3,3'-diaminobenzidine; E-Alone, unscented estrous females; Fos-IR, Fos immunoreactivity; MEA, medial amygdala; mBNST, medial bed nucleus of the stria terminalis; MOB, main olfactory bulb; mPOA, medial preoptic area; NAc Core, nucleus accumbens core; NAc Shell, nucleus accumbens shell; NGS, normal goat serum; Pir Ctx, piriform cortex; TBS, Tris-buffered saline; VMH, ventromedial hypothalamus; VTA, ventral tegmental area.

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Olfactory stimuli play important roles in the modulation and expression of sexual behavior in many species (e.g. Cain and Paxinos, 1974; Edwards et al., 1990; Larsson, 1971; McClintock, 1978, 1984; McClintock and Adler, 1978; Schank and McClintock, 1997), including humans (McClintock, 1971; Stern and McClintock, 1998). In rats, naturally occurring pheromonal stimuli emitted from sexually receptive females (i.e. estrous odors) are attractive to males (Bressler and Baum, 1996), produce sexual arousal (Sachs, 1997), and increase testosterone and luteinizing hormone levels in blood (Graham and Desjardins, 1980). The neural structures mediating the effects of estrous odors have been investigated extensively using Fos immunoreactivity (IR) as a marker of neuronal activation. A common set of structures appears to be activated in rats, hamsters, gerbils, and ferrets by exposure to estrous odors (for reviews see Bialy and Kaczmarek, 1996; Newman et al., 1997; Pfaus and Heeb, 1997; Swann and Fiber, 1997; Veening and Coolen, 1998). Briefly, following exposure to estrous odors Fos-IR is increased in the accessory olfactory bulb (AOB)/vomeronasal organ, medial amygdala (MEA), medial bed nucleus of the stria terminalis (mBNST), nucleus accumbens core and shell (NAc Core and NAc Shell), medial preoptic area (mPOA), and the ventral tegmental area (VTA) in several mammalian species. Additionally, dopamine transmission in the nucleus accumbens is elevated during exposure to estrous odors in both sexually naïve and experienced male rats (Mitchell and Gratton, 1991, 1992; Wenkstern et al., 1993) indicating that this pathway is responsive to estrous odors regardless of prior sexual experience.

Neutral odors associated with sexual behavior can also modulate neuroendocrine function and sexual behavior. For example, Graham and Desjardins (1980) found that plasma levels of testosterone and luteinizing hormone increased in response to a neutral odor (methyl salicylate or wintergreen) that had been paired previously with copulation to ejaculation. The same odor not paired with copulation did not increase hormone levels. Previously, we (Kippin et al., 1998; Kippin and Pfaus, 2001a,b; Kippin et al., 2001) demonstrated that a neutral odor (almond or lemon) paired with the sexual reward state induced by ejaculation comes to direct the subsequent choice of sexual partners. During training, males received access to sexually receptive females that were either scented with almond (paired group) or unscented (unpaired group). A final choice test was conducted in an open field in which males received access to two receptive females, one scented and the other unscented. Although all males copulated indiscriminately with the two females, those in the paired group

ejaculated preferentially with the scented female. Little is known about the neural basis of these sexually conditioned effects. Exposure to a variety of neutral odors paired previously with access to a sexually receptive female increases the firing rates of NAc neurons (West et al., 1992); however, the afferent pathway for this effect is not known. Additionally, a somatosensory cue (an i.p. injection) paired previously with copulation increases glucose utilization in the basolateral amygdala (BLA), central amygdala (CEA), and bed nucleus of the accessory olfactory tract (BAOT) (de Jonge et al., 1992). In the present study, we compared the pattern of neural activation produced by estrous odors and sexually conditioned odors in male rats using Fos-IR as a marker of neuronal activation.

## EXPERIMENTAL PROCEDURES

Fos-IR was examined in the male rat brain following exposure to estrous odors, a novel neutral odor, or a sexually conditioned odor, individually or in combination. Experiment 1 examined the pattern of Fos-IR following exposure to estrous odors using procedures shown to be effective in previous studies (Bressler and Baum, 1996; Kelliher et al., 1999). Experiment 2 examined the pattern of Fos-IR following exposure to a neutral odor (almond extract) or the same odor after it was paired with copulation. Experiment 3 examined the patterns of Fos-IR following exposure to combinations of the estrous and sexually conditioned odors. In addition to replicating the conditions examined in experiments 1 and 2, experiment 3 compared the level of Fos-IR in structures activated by the combination of estrous and sexually conditioned odors, the combination of estrous and neutral odors, and estrous odors with the omission of the sexually conditioned odor.

## Subjects

**Males.** In all experiments, male Long–Evans rats obtained from Charles River Canada (St. Constant, Québec) served as subjects. The males weighed approximately 300–350 g and were sexually naive at the start of the experiment. They were housed in pairs in Plexiglas cages (36 cm×26 cm×19 cm) with *ad libitum* access to food (Purina Rat Chow, Woodstock, Ontario, Canada) and water. All rats were kept in a 12-h reversed light/dark cycle colony room (lights off at 0800 h) maintained at 21 °C. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and all efforts were made to minimize the number of rats used and to reduce their suffering.

**Stimulus females.** Female Long–Evans rats from the same supplier as above were ovariectomized via bilateral lumbar incisions under ketamine/xylazine anesthesia at least 2 months prior to the start of the experiment and were sexually experienced. Sexual receptivity was induced by subcutaneous administration of estradiol (10 µg) 48 h prior and progesterone (500 µg) 4–6 h prior to each test trial. Females were housed in separate rooms under the same conditions as males. Stimulus females were selected at random for use during training sessions and copulatory preference tests. Almond-scented estrous females (A+E) had approximately 1 ml of almond extract (Blue Ribbon, Etobicoke, Ontario, Canada) applied to both the back of the neck and the anogenital area using a cotton swab. Unscented estrous females (E-Alone) had approximately 1 ml of distilled water applied to both the back of the neck and the anogenital area using a cotton swab.

## Apparatus

Training sessions took place in pacing chambers constructed from a standard laboratory Plexiglas cages (36 cm×26 cm×19 cm)

with a Plexiglas divider-insert that allowed the female to pace the copulatory behavior of the male by moving around the divider forcing the male to pursue her. The insert was made by attaching a Plexiglas divider (30 cm×20 cm×0.5 cm) lengthwise to the center of a Plexiglas base (35 cm×18 cm×0.5 cm). The insert was then placed into the Plexiglas cage, the base was covered with bedding material, and a piece of wire mesh (0.25-cm grid, 35 cm×18 cm) with a groove cut into the center to fit around the divider was placed on top of the bedding. A cover constructed of wire mesh (0.5-cm grid, 36 cm×20 cm) was placed over the chamber. All training sessions were recorded on video and scored subsequently using a PC-based program. Olfactory stimulation tests took place in identical Plexiglas cages without the divider-insert and with uncovered bedding material.

## Procedure

**Training phase.** The training procedures used in each experiment were identical to those in Kippin et al. (1998). Male rats were exposed to the chambers once a day for 15 min each day for 7 days in order to habituate them to the training environment. This treatment has been shown to increase the proportion of males that copulate (Pfaus and Wilkins, 1995). Then all males received nine training sessions at 4-day intervals during the middle third of the dark phase of the light/dark cycle. For males in the A+E-trained condition, all training sessions were with A+E females. For males in the E-Alone-trained condition, all training sessions were with E-Alone females. For all training sessions, males were placed individually into a chamber for 5 min, after which a female of the appropriate sexual status and appropriate scent was placed into the chamber for a 30-min test of copulation. Latency and frequency data for all mounts, intromissions, and ejaculations were recorded during each training session according to the criteria of Sachs and Barfield (1976) and Meisel and Sachs (1994). As in previous studies (Kippin et al., 1998), the addition of the neutral almond odor had no significant effect on copulation at any point during the conditioning phase and each male copulated to ejaculation on at least the last five training sessions (data not shown). Males were randomly assigned to odor conditions in such a way that intromission latency, ejaculation latency, and ejaculation frequency on the final training session were approximately equal between groups.

**Olfactory stimulation test.** Four days after the final training session, each male was placed in a Plexiglas chamber which contained bedding from one of three odor conditions. In the no-odor condition, 5 ml of distilled water was mixed into the bedding immediately prior to the test. In the estrous-odors condition, bedding that had been soiled by three sexually receptive (estrous) females for 6 h following progesterone injection was used (as in Bressler and Baum, 1996; Kelliher et al., 1999). In the almond-odor condition, 5 ml of almond extract was mixed into the bedding immediately prior to the test. In all conditions, males were allowed to remain undisturbed on the bedding for 2 h. Males were then injected with a lethal dose of sodium pentobarbital (120 mg/kg, i.p.) and perfused intracardially with 400 ml of phosphate-buffered saline followed by 400 ml of 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde for 8 h and stored overnight in 30% sucrose solution.

**Fos-IR.** Coronal brain sections were incubated sequentially with 30% w/w hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in Tris-buffered saline (TBS) for 30 min at room temperature, 3% normal goat serum (NGS) in 0.05% Triton TBS for 90 min at 4 °C, rabbit polyclonal anti-Fos (Oncogene Science, Boston, MA, USA; diluted 1:75,000) in 0.05% Triton TBS with 3% NGS for 72 h at 4 °C, biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; 1:200) in 0.05% Triton TBS with 3% NGS for 1 h at 4 °C, and avidin–biotinylate–peroxidase complex (Vectastain Elite ABC Kit,



**Table 1A.** Fos induction by estrous female odors (mean±S.E.M.)

| Training/test odor | Pir Ctx  | NAC Cor   | NAC Shell | mBNST     | vBNST    | mPOA      | LHA      | VMH       | MeA       | BLA      | VTA       |
|--------------------|----------|-----------|-----------|-----------|----------|-----------|----------|-----------|-----------|----------|-----------|
| E-Alone/none       | 26.6±.4  | 21.5±6.5  | 25.7±7.0  | 17.7±2.8  | 17.0±3.3 | 15.2±2.9  | 17.6±5.0 | 11.3±2.3  | 16.5±2.1  | 22.1±2.8 | 15.3±2.3  |
| E-Alone/estrous    | 34.8±5.0 | 38.4±5.4* | 44.1±5.0* | 32.2±6.2* | 23.9±2.0 | 23.5±2.3* | 22.8±4.3 | 25.7±3.7* | 33.7±3.9* | 22.7±2.5 | 37.5±2.2* |

**Table 1B.** Fos induction by a sexually conditioned odor (mean±S.E.M.)

| Training/test odor | Pir Ctx   | NAC Cor   | NAC Sh   | mBNST    | vBNST    | mPOA     | LHA       | VNH      | MeA      | BLA       | VTA      |
|--------------------|-----------|-----------|----------|----------|----------|----------|-----------|----------|----------|-----------|----------|
| E-Alone/none       | 19.1±6.4  | 24.9±4.4  | 25.7±4.8 | 23.8±6.5 | 23.0±6.8 | 16.6±5.4 | 22.4±4.9  | 13.1±5.5 | 18.1±5.5 | 28.7±9.3  | 16.4±4.6 |
| A+E/none           | 18.6±5.5  | 27.4±5.5  | 18.6±5.5 | 19.9±5.8 | 18.6±4.5 | 15.4±3.4 | 19.7±5.0  | 10.4±4.3 | 21.9±4.1 | 19.6±5.8  | 19.9±5.6 |
| E-Alone/almond     | 23.6±6.1  | 20.7±3.5  | 29.7±5.3 | 21.4±4.9 | 20.4±6.1 | 18.0±5.2 | 24.7±6.7  | 11.0±3.6 | 16.3±6.1 | 25.7±5.3  | 21.0±5.6 |
| A+E/almond         | 45.6±7.4* | 64.3±9.1* | 22.8±5.1 | 16.1±5.1 | 18.0±4.8 | 12.6±4.3 | 45.7±7.1* | 9.57±2.5 | 19.1±4.4 | 50.4±8.7* | 18.6±5.2 |

<sup>a</sup> Abbreviations: Pir Ctx, piriform cortex; NAC Cor, core region of the nucleus accumbens; NAC Shell, shell region of the nucleus accumbens; mBNST, medial region of the bed nucleus stria terminalis; vBNST, ventral region of the bed nucleus stria terminalis; mPOA, medial preoptic area of the hypothalamus; LHA, anterior lateral hypothalamus; VMH, ventral medial hypothalamus; MeA, medial amygdala; BLA, basolateral amygdala; VTA, ventral tegmental area.

\*  $P < 0.05$  versus all other groups in experiment.

Vector Laboratories; diluted 1:55) for 2 h at 4 °C. Sections were washed in TBS (3×5 min) between each incubation. Immunoreactions were stained by sequential treatments at room temperature with 50-mM Tris for 10 min, 3,3'-diaminobenzidine (DAB) in 50-mM Tris (0.1 ml of DAB/Tris buffer, pH 7.8) for 10 min, DAB/3% H<sub>2</sub>O<sub>2</sub> in 50-mM Tris for 10 min, and 8% nickel chloride (400 µl per 100 ml of DAB/Tris buffer+H<sub>2</sub>O<sub>2</sub>). Sections were mounted on gel-coated slides and allowed to dry, then dehydrated, cleared in Hemo-D, coverslipped, and examined under a microscope.

### Histological and statistical analysis

Tissue sections were examined at 40× and the number of Fos-positive cells was counted bilaterally for each region from five sections per rat using a computerized image-analysis system (M1; Imaging Research Inc., St. Catharines, Ontario, Canada) linked to a Leitz Laborlux microscope. Only the brain regions in which Fos cells were observed clearly in at least one condition were included in this study. These regions were defined by using the borders in Paxinos and Watson (1986): AOB (plates 2 and 3), main olfactory bulb (MOB; plates 2 and 3), NAC Core (plates 13 and 14), NAC Shell (plates 13 and 14), piriform cortex (Pir Ctx; plates 13 and 14), mPOA (plates 20 and 21), mBNST (plates 21 and 22), anterior portion of the lateral hypothalamic area (aLHA; plates 23 and 24), ventromedial hypothalamus (VMH; plates 27 and 28), MEA (plates 28 and 29), BLA (plates 28 and 29), and VTA (plates 37 and 38). A mean was calculated for each area for each rat and statistical analyses were conducted for each area using either planned orthogonal comparisons (Glass and Hopkins, 1985) or two-factor ANOVA where appropriate. Significant F values were followed by post-hoc analysis using the Tukey method. The Bonferroni correction was employed when follow-up comparisons used a single mean repeatedly. The level of significance for all comparisons was 0.05.

## RESULTS

### Neuronal Fos induction by exposure to estrous odors

To determine the pattern of neuronal activation produced by estrous odors, males were trained with unscented females (E-Alone-trained males) and subsequently exposed to bedding with either no additional odor or estrous odors ( $n$ =five per test-odor condition). Following exposure to estrous odors Fos-IR was increased significantly in the AOB (mean±S.E.M. for E-Alone/no odor,  $4.3 \pm 1.7$ ; for E-Alone/estrous odors,  $12.3 \pm 3.2$ ), NAC Core, NAC Shell, mBNST, mPOA, VMH, MEA, and VTA (see Table 1A). The significance of these observations was confirmed by planned orthogonal contrasts. For AOB,  $t(8)=3.67$ ,  $P < 0.05$ . For the NAC Core,  $t(8)=3.41$ ,  $P < 0.05$ . For the NAC Shell,  $t(8)=3.59$ ,  $P < 0.05$ . For the mBNST,  $t(8)=3.89$ ,  $P < 0.05$ . For the mPOA,  $t(8)=2.54$ ,  $P < 0.05$ . For the VMH,  $t(8)=3.53$ ,  $P < 0.01$ . For the MEA,  $t(8)=5.53$ ,  $P < 0.05$ . For the VTA,  $t(8)=7.84$ ,  $P < 0.05$ . No significant effects were found in the Pir Ctx, aLHA, or BLA.

### Neuronal Fos induction by exposure to the conditioned odor

In order to determine the pattern of neuronal activation produced by a sexually conditioned odor, males were trained with either almond-scented females (A+E-trained males) or unscented females (E-Alone-trained males) and subsequently exposed either to no odor or almond odor in bedding ( $n$ =seven per each training group/test-odor con-

### Abbreviations used in the figure

3v third ventricle  
ac anterior commissure  
E ependyma and subependyma layer  
DEn dorsal endopiriform nucleus  
IGr internal granular layer  
lo lateral olfactory tract

lv lateral ventricle  
Mi mitral cell layer  
ox optic chiasm  
ot optic tract  
st stria terminalis

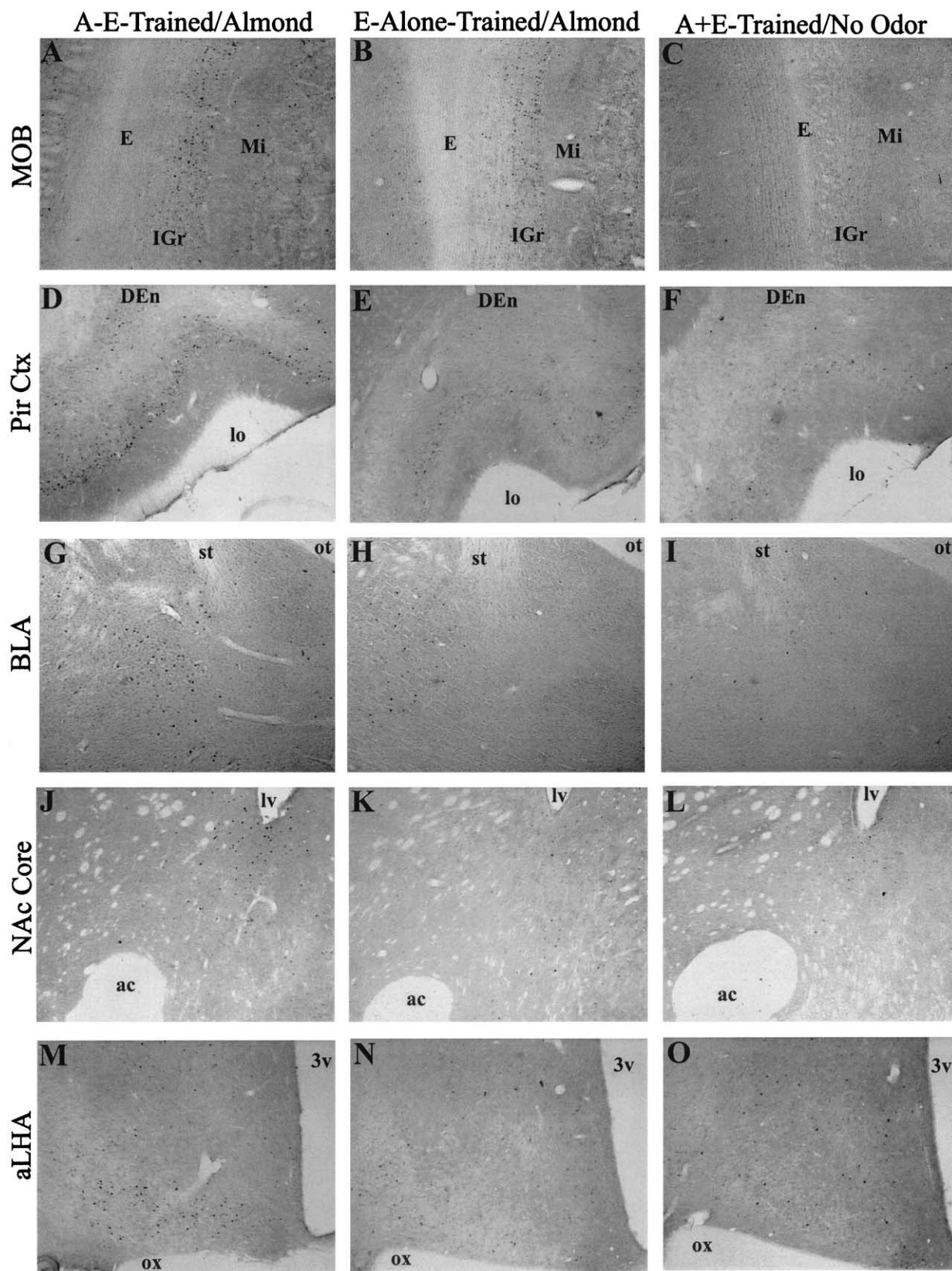


Fig. 1.

**Table 2.** Interactions in Fos induction between the estrous odors and sexually conditioned odor systems

| Training/test odor     | NAc Cor                 | NAc Shell               | mPOA                   | LHA                     |
|------------------------|-------------------------|-------------------------|------------------------|-------------------------|
| E-Alone/none           | 25.1±8.1                | 16.5±4.8                | 14.5±4.2               | 22.3±4.8                |
| E-Along/estrous        | 42.3±9.9* <sup>‡</sup>  | 42.5±10.9* <sup>‡</sup> | 32.8±5.9* <sup>‡</sup> | 27.3±6.3                |
| E-Alone/almond         | 26.0±11.4 <sup>†</sup>  | 14.0±4.5 <sup>†</sup>   | 11.3±3.4 <sup>†</sup>  | 20.0±8.0                |
| E-Alone/almond+estrous | 47.5±12.8* <sup>‡</sup> | 47.5±13.5* <sup>‡</sup> | 38.3±9.2* <sup>‡</sup> | 28.3±11.9               |
| A+E/none               | 27.5±5.4                | 14.0±4.5                | 14.8±3.6               | 19.0±5.0                |
| A+E/estrous            | 39.0±9.5*               | 50.3±12.6*              | 27.3±5.4*              | 21.5±7.0                |
| A+E/almond             | 56.5±11.4*              | 17.8±6.6                | 15.3±5.9               | 48.0±7.1* <sup>†</sup>  |
| A+E/almond+estrous     | 68.5±17.2* <sup>†</sup> | 43.8±9.1* <sup>‡</sup>  | 34.3±4.0* <sup>‡</sup> | 55.3±13.1* <sup>†</sup> |

<sup>a</sup> For abbreviations see Table 1.

\*  $P < 0.05$  for comparison to no-odor control.

<sup>†</sup>  $P < 0.05$  for comparison to estrous-odors condition.

<sup>‡</sup>  $P < 0.05$  for comparison to almond-odor condition.

dition). Following exposure to the novel neutral odor, which was used as the conditioned stimulus, Fos-IR was also increased in the MOB (mean±S.E.M. for A+E-trained/almond, 33.1±4.6; for E-Alone-trained/almond, 28.5±5.2; for A+E-trained/no odor, 8.2±1.2; for E-Alone-trained/no odor, 6.9±2.3). Moreover, following exposure to the sexually conditioned odor, Fos-IR was increased significantly in the NAc Core, Pir Ctx, BLA, and aLHA (see Table 1B and Fig. 1). The significance of these observations was confirmed by two-way (training group X test odor) ANOVAs. For MOB, there was a significant effect of test odor,  $F(1,24)=3.99$ ,  $P < 0.05$ , but no significant effect of training group,  $F(1,24)=2.01$ ,  $P > 0.05$ , or significant interaction,  $F(1,24)=1.02$ ,  $P > 0.05$ . For the Pir Ctx, there were significant effects of training group,  $F(1,24)=6.60$ ,  $P < 0.05$ , and test odor,  $F(1,24)=4.95$ ,  $P < 0.05$ , but no significant interaction,  $F(1,24)=3.09$ ,  $P > 0.05$ ; follow-up analyses revealed that the A+E-trained males exposed to almond odor had higher Fos-IR than all other groups (all  $P < 0.05$ ), but no other groups differed significantly. For the BLA, there was a significant interaction,  $F(1,24)=9.05$ ,  $P < 0.05$ , and a significant effect of training group,  $F(1,24)=6.13$ ,  $P < 0.05$ , but not test odor,  $F(1,24)=1.92$ ,  $P > 0.05$ ; follow-up analyses revealed that the A+E-trained males exposed to almond odor had higher Fos-IR than all other groups (all  $P < 0.05$ ), but no other groups differed significantly. For the NAc Core, there was a significant interaction,  $F(1,24)=12.34$ ,  $P < 0.05$  and significant effects of training group,  $F(1,24)=7.77$ ,  $P < 0.05$ , and test odor,  $F(1,24)=15.48$ ,  $P < 0.05$ ; post hoc analyses revealed that the A+E-trained males exposed to almond odor had higher Fos-IR than all other groups (all  $P < 0.05$ ), but no other groups differed significantly. For aLHA, there was a significant interaction,  $F(1,24)=6.83$ ,  $P < 0.05$ , and a significant effect of training group,  $F(1,24)=9.72$ ,  $P < 0.05$ , but

not test odor,  $F(1,24)=4.06$ ,  $P > 0.05$ ; post hoc analyses revealed that the A+E-trained males exposed to almond odor had higher Fos-IR than all other groups (all  $P < 0.05$ ), but no other groups differed significantly. No significant effects were found in the AOB, NAc Shell, mBNST, mPOA, VMH, MEA, or VTA.

#### Neuronal Fos induction by exposure to estrous and sexually conditioned odors

In order to study the interaction between the patterns of neuronal activation produced by exposure to estrous and sexually conditioned odors, males were trained with either almond-scented females (A+E-trained males) or unscented females (E-Alone-trained males) and subsequently exposed to either no odor, estrous odors, almond odor, or a combination of estrous and almond odors in bedding ( $n$ =four to five per each training group/test-odor condition) and a subgroup of brain regions (NAc Core, NAc Shell, mPOA, and aLHA) were analyzed. In addition to replicating the results from the preceding experiments, three specific comparisons were examined. First, the induction of Fos-IR was examined following exposure to the estrous and sexually conditioned odors in commonly activated structures (i.e. the NAc Core). Second, the induction of Fos-IR was examined in males trained previously with A+E females following exposure to estrous odors and the omission of the almond odor in the NAc Core, NAc Shell, and mPOA. Third, the induction of Fos-IR was examined following combined exposure to estrous and sexually conditioned odors in all structures examined.

Table 2 displays the mean Fos-IR (±S.E.M.) for each training/test group for each structure examined in this experiment. As in the previous experiments, following exposure to the sexually conditioned odor alone increased sig-

**Fig. 1.** Fos immunoreactivity (Fos-IR) following exposure to the sexually conditioned odor in the almond-scented estrous females (A+E)-trained males, the same odor in unscented estrous females (E-Alone)-trained males, or no odor in A+E-trained males in the main olfactory bulb (MOB) (A–C), piriform cortex (Pir Ctx) (D–F), the basolateral amygdala (BLA) (G–I), the nucleus accumbens core (NAc Core) (J–L), and the anterior portion of the lateral hypothalamic area (aLHA) (M–O). Fos-IR in the MOB was increased following exposure to the conditioned odor in both the A+E-trained and the E-Alone-trained males (A, B) compared with the A+E-trained males not exposed to the odor (C). Conversely, levels of Fos-IR were increased in the A+E-trained males exposed to the sexually conditioned odor in the Pir Ctx (D), BLA (G), NAc Core (J), and aLHA (M) compared with the E-Alone-trained males exposed to the same odor (E, H, K, and N) or the A+E-trained males exposed to no odor (F, I, L, and O). See Table 1 for mean±S.E.M. and text for statistical comparisons.

nificantly Fos-IR in the NAc Core and aLHA, whereas following exposure to the estrous odors alone increased significantly Fos-IR NAc Core, NAc Shell, and mPOA. Moreover, prior training with A+E females did not have a significant effect on Fos-IR in response to estrous odors alone in the NAc Core, NAc Shell, and mPOA, nor did the combination of estrous and sexually conditioned odors alter Fos-IR in the structures examined.

The significance of these observations was confirmed by two-way (training group X test odor) ANOVAs. For the NAc Core, there was a significant interaction,  $F(2,20)=3.89$ ,  $P<0.05$  and significant effects of training group,  $F(1,20)=10.50$ ,  $P<0.05$ , and test odor,  $F(2,20)=10.91$ ,  $P<0.05$ . Post hoc analyses revealed that the A+E-trained males exposed to almond odor had higher Fos-IR than either the no-odor groups or the E-Alone-trained males exposed to almond odor. The A+E-trained and E-Alone-trained males exposed to estrous odors did not differ significantly from each other ( $P>0.05$ ) but had higher Fos-IR than their respective no-odor group controls ( $P<0.05$ ). Fos-IR did not differ significantly between the almond and estrous odors in the A+E-trained males ( $P>0.05$ ). Finally, the combination of estrous and conditioned odors did not alter the response relative to either odor alone ( $P>0.05$ ).

In the NAc Shell, there was a significant effect of test odor  $F(2,20)=30.41$ ,  $P<0.05$ , but no significant effect of training group,  $F(1,20)=0.16$ ,  $P>0.05$  or significant interaction,  $F(2,20)=0.72$ ,  $P>0.05$ . Post hoc analyses revealed that males in both training groups exposed to estrous odors (alone or in combination with almond odor) had higher Fos-IR than either the no-odor or almond-odor alone groups (all  $P<0.05$ ). The combination of almond and estrous odors did not significantly alter Fos-IR in either training group from estrous odors alone ( $P>0.05$ ).

In the mPOA, there was a significant effect of test odor  $F(2,20)=34.70$ ,  $P<0.05$ , but no significant effect of training group,  $F(1,20)=0.46$ ,  $P>0.05$  or significant interaction,  $F(2,20)=1.20$ ,  $P>0.05$ . Post hoc analyses revealed that males in both training groups exposed to estrous odors (alone or in combination with almond odor) had higher Fos-IR than either the no-odor or almond-odor alone groups (all  $P<0.05$ ). The combination of almond and estrous odors did not significantly alter Fos-IR in either training group from estrous odors alone ( $P>0.05$ ).

In the aLHA, there was a significant interaction,  $F(2,20)=10.12$ ,  $P<0.05$  and significant effects of training group,  $F(1,20)=15.60$ ,  $P<0.05$ , and test odor,  $F(2,20)=10.80$ ,  $P<0.05$ . Post hoc analyses revealed that the A+E-trained males exposed to almond odor had higher Fos-IR than either the no-odor groups, the E-Alone-trained males exposed to almond odor, or the estrous-odor groups (all  $P<0.05$ ). The A+E-trained and E-alone-trained males exposed to estrous odors did not differ significantly from each other ( $P>0.05$ ) nor did they differ from their respective no-odor group controls ( $P>0.05$ ). Finally, the combination of both odors did not differ significantly from the effect of almond alone ( $P>0.05$ ).

## DISCUSSION

The present study demonstrates that distinct neural pathways are activated by estrous odors and a sexually conditioned odor. Consistent with previous studies, we found that male rats exposed to bedding soiled by estrous females had elevated Fos induction in the AOB, NAc Core, NAc Shell, mBNST, mPOA, VMH, MEA, and VTA. The functions and possible connections of these structures have been reviewed elsewhere (see Bialy and Kaczmarek, 1996; Newman et al. 1997; Petrusis and Johnston, 1999; Pfau and Heeb, 1997; Swann and Fiber, 1997; Veening and Coolen, 1998). In contrast, male rats exposed to bedding scented with an odor paired previously with copulation had increased Fos induction in the Pir Ctx, NAc Core, aLHA, and BLA. Although it is unclear how to relate the lack of Fos induction in a specific brain structure to the absolute amount of cellular activity of that region (for discussions, see Komisaruk et al., 2000; Pfau and Heeb, 1997), different patterns of Fos-IR indicate that processing within specific structures is of a different nature or is produced by different levels of sensory activation. Accordingly, the different pattern of Fos-IR following exposure to estrous and sexually conditioned odors in the present study indicates that these types of odors are either processed by distinct pathways or that the level of activity within specific structures is differentially induced by these odors.

The finding that a sexually conditioned odor activates the NAc Core and the BLA are consistent with the findings of West et al. (1992) and de Jonge et al. (1992), respectively. However, the findings of de Jonge et al. are inconsistent with the present findings and those of West et al. in that the former failed to reveal activation in the NAc. Conversely, the present findings failed to find activation of the CEA or BAOT, which were activated in the de Jonge et al. study. It is unclear whether these inconsistencies are due to differences in the nature of the CSs (olfactory versus somatosensory), differences in the dependent measures (Fos induction versus 2-DG incorporation), or other factors.

It is not known to what extent there is interaction between the pathways activated by estrous and sexually conditioned odors. The only site of common activation of these stimuli in the present experiment was the NAc Core. This suggests that these odors, both of which are important cues for different aspects of sexual behavior, activate largely independent pathways that may converge in the NAc Core. Males trained with scented females displayed the same pattern of Fos induction following exposure to estrous odors regardless of whether the sexually conditioned odor was present. Fos induction in the NAc Core, therefore, does not appear to be additive in response to the two types of odors. This suggests that a common set of neurons were activated within the NAc Core in response to the different types of sex-related odors.

Although a definitive serial pathway cannot be elucidated from the pattern of Fos induction alone, it is possible to construct probable pathways processing the conditioned stimuli based on known synaptic afferents and efferents. Such pathways can be verified using a combination of Fos-IR and lesions. Given that the stimuli of interest in the

present study were olfactory cues, the initial structure in the pathway must lie in the olfactory bulb. The almond odor used as a conditioned stimulus increased Fos induction in the MOB independent of conditioning. Whereas, consistent with previous reports (Bressler and Baum, 1997; Kelliher et al., 1999), the pheromonal cues from the estrous odors activated the AOB, but not the MOB.

The Pir Ctx receives direct input from the MOB (Scott, 1986; Shipley and Ennis, 1996; Haberly, 1998) and in the present study had significantly higher Fos induction in response to the odor only when it had been paired previously with sexual behavior. Although it is commonly regarded as primary olfactory cortex, the architectural organization of the Pir Ctx more closely resembles that of associative cortex rather than sensory cortex, and the MOB itself appears to serve as primary sensory cortex (Haberly and Price, 1978; Johnson et al., 2000; Luskin and Price, 1983). These findings suggest that the Pir Ctx is probably involved in higher-level processing of sensory information (i.e. perception and response generation; Haberly, 1998; 2001). Consistent with this notion, firing rates in neurons within the Pir Ctx are increased as a function of the associational contingencies between odors and reinforcement (Schoenbaum and Eichenbaum, 1995). Moreover, in the present study, the Pir Ctx responded significantly to the almond odor only when it was paired previously with sexual behavior, and thus appears to be important in the responding to olfactory stimuli that have acquired meaning through association with natural incentives or rewards. It would appear that such stimuli are processed via a MOB to Pir Ctx pathway, whereas pheromonal sexual stimuli are processed via an AOB to MEA pathway, consistent with the notion that cellular responses to pheromones are largely independent of sexual experience (e.g. Dulac and Axel, 1998). However, it is important to note that experience does alter responses to pheromones, for instance, repeated exposure to estrous odors, with or without sexual contact, sensitizes the ability of those odors to induce dopamine release in the NAc (Mitchell and Gratton, 1991).

The BLA receives direct input from the Pir Ctx (Haberly, 1998; Johnson et al., 2000) and, similar to the Pir Ctx, had significantly higher Fos induction in response to almond odor only when it had been paired previously with sexual behavior. These findings suggest that the MOB–Pir Ctx pathway is responsible for activation of the BLA during exposure to an odor paired with sexual behavior. However, BLA also receives input from other amygdala nuclei, such as the cortical nuclei (Pitkanen et al., 1997; Swanson and Petrovich, 1998), with direct MOB innervation (Shipley and Ennis, 1996; Haberly, 1998). Although it is possible that this latter pathway could bring the olfactory information to the BLA during exposure to the almond odor in parallel to processing in the Pir Ctx, increased activation following exposure to the sexually conditioned odor was not found in the cortical amygdala. Further, the BLA has been shown to be critical in the acquisition and expression of other forms of associative learning (for recent reviews see Davis et al., 1993; Everitt et al., 1999; Fanselow and LeDoux, 1999) and for the expression of operant responding to second-order sexual incentives (Everitt et al., 1989). Accordingly,

the BLA may be involved in similar functions in response to odors previously paired with sexual behavior.

The NAc Core receives direct input from the BLA, and this pathway has been implicated in responding for sexual incentives but not in copulatory performance (Everitt et al., 1989). Accordingly, it is likely that the activation of the NAc Core in the present study is mediated via a BLA-dependent projection. The NAc has been hypothesized to be involved in reward processes (e.g. Wise and Bozarth, 1981, 1987), instrumental learning (e.g. Hall et al., 2001; Ikemoto and Panksepp, 1999; Kalivas and Nakamura, 1999; Kelley, 1999), and the attribution of salience to incentive cues (Berridge and Robinson, 1998; Robinson and Berridge, 1993). Consistent with these processes, dopamine release in the NAc increases during copulation in male rats but decreases following ejaculation (Blackburn and Pfaus, 1992; Fiorino et al., 1997). Dopamine levels in the NAc also increase during exposure to estrous odors (Mitchell and Gratton, 1991, 1992) and cellular activity in the NAc increases during exposure to a sexually conditioned odor (West et al., 1992). Furthermore, the conditioning procedures used in the present study have been shown previously to condition male rats to ejaculate preferentially with estrous females that have the odor relative to another estrous female that does not have the odor. This conditioned ejaculatory preference is dependent on an increase attractiveness or incentive salience of the conditioned odor and female immediately prior to ejaculation (Kippin and Pfaus, 2001). Together these findings suggest that the NAc Core may play a role in the assessment of incentive value of conditioned stimuli. Moreover, the activation of the NAc Core by sexually conditioned and estrous odors suggests that this region is important in responding to both unconditioned and conditioned sexual stimuli.

The aLHA serves as an integrator for the limbic forebrain and autonomic nervous system and thus receives diverse and complex input from many brain regions via the medial forebrain bundle (for reviews see Bernardis and Bellinger, 1993; Geeraedts et al., 1990a,b; Nieuwenhuys et al., 1982; Veening et al., 1982). These inputs include projections from the Pir Ctx (Price et al., 1991; Scott and Leonard, 1971), the BLA (Johnson et al., 1996; Ono et al., 1985), and the NAc Core (Heimer et al., 1991; Zahm and Heimer, 1993). The aLHA has been implicated in a number of neural functions related to reward and regulation of body weight (see Bernardis and Bellinger, 1993, 1996), including roles in sensory processing during food selection (Carlisle and Stellar, 1969; Mufson and Wampler, 1972) and in the generation of conditioned autonomic responses (Iwata et al., 1986; Powell and Levine-Bryce, 1989). A number of recent findings have implicated the aLHA in sexual behavior. Serotonin levels in the aLHA are stable during copulation but increase following ejaculation (Lorrain et al., 1997). Axon-sparing lesions of the aLHA facilitate erection during noncontact exposure to receptive female, but impair ejaculation without interfering with mounts or intromissions (Kippin et al., 2000). Conversely, lesions of the posterior portions of the LHA disrupt all copulatory behaviors (Edwards and Malliard, 1990) and nocturnal erection (Schmidt

et al., 2000). Together these findings suggest that subdivisions of the LHA play different roles in the regulation of copulation with posterior regions controlling arousal or erection and anterior regions controlling ejaculation. The present Fos findings, along with those implicating the aLHA in ejaculatory function (Kippin et al., 2000; Lorrain et al., 1997) and those demonstrating that conditioned ejaculatory preferences are dependent upon the presence of the conditioned stimulus during the postejaculatory refractory period (Kippin and Pfau, 2001a), suggest that the aLHA may serve a critical role in the neural representation of the unconditioned stimulus during the acquisition of conditioned ejaculatory preferences.

The present study demonstrated a clear dissociation between the Fos induction produced by sexually conditioned and estrous odors. The sexually conditioned odor activated the MOB, Pir Ctx, BLA, aLHA, and NAc Core. Conversely, estrous odors activated the AOB, mBNST, mPOA, VMH, MEA, NAc Core, NAc Shell, and VTA. Thus, the neural pathways mediating the processing of these odors appear to be distinct, although they may converge in NAc Core.

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# Naloxone, but Not Flupenthixol, Disrupts the Development of Conditioned Ejaculatory Preference in the Male Rat

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Male rats display a conditioned preference to ejaculate with a female bearing an odor paired previously with copulation to ejaculation. The present study examined the role of endogenous opioid and dopamine systems in this preference. Male rats received saline, the opioid receptor antagonist naloxone, or the dopamine receptor antagonist flupenthixol prior to 10 conditioning trials in a pacing chamber with an almond-scented female. On the final test, all males were injected with saline and given access to 2 females, 1 scented and the other unscented, in an open field. Only males injected with naloxone during training failed to manifest a conditioned ejaculatory preference. These findings suggest that activation of opioid, but not dopamine, systems during sexual interaction are necessary for conditioned ejaculatory preference in male rats.

**Keywords:** opioids, dopamine, naloxone, flupenthixol, partner preference

It has been shown that polygamous male rats can develop a conditioned ejaculatory preference (CEP) for females bearing a familiar odor cue paired previously with the reward state induced by ejaculation (Kippin, Talianakis, Schattmann, Bartholomew, & Pfaus, 1998). In a subsequent study, Kippin and Pfaus (2001) demonstrated that ejaculation is necessary for conditioning to occur, and that the postejaculatory interval is the stage of copulation during which males associate a conditioned stimulus with sexual reward. These findings are consistent with those of conditioned place preference (CPP) in which ejaculation is also necessary for conditioning to occur (Miller & Baum, 1987; Mehrara & Baum, 1990). Recently, we showed that males can develop CEP for a familiar female after repeated copulation with this female in pacing chambers bisected by a partition containing one hole, but not four holes (Ismail, Gelez, Lachapelle, & Pfaus, 2008). Pacing chambers are often used to study female sexual behavior (Coria-Avila, Ouimet, Pacheco, Manzo, & Pfaus, 2005; Erskine, Kornberg, & Cherry, 1989; Paredes & Vazquez, 1999). Although males ejaculated in the one-hole and in the four-hole conditions, only males trained in the one-hole condition developed CEP. These findings suggest that ejaculation alone is not sufficient for conditioning to occur. This makes it possible that the arousal level experienced during copulation also plays a role in the development of conditioning.

The pharmacology underlying the development of CEP for a familiar female remains to be investigated. However, previous studies have identified both endogenous opioids and dopamine (DA) as possible candidates. Opioids are released in the brain during ejaculation (Szechtman, Hershkowitz, & Simantov, 1981), and  $\mu$  opioid receptors are activated in the medial preoptic area (mPOA) during this period (Coolen, Fitzgerald, Yu, & Lehman, 2004). Systemic administration of the general opioid receptor antagonist naloxone disrupts the display of a sexually conditioned place preference in male rats that develops when a place is paired with the postejaculatory refractory period (Ågmo & Berenfeld, 1990; Miller & Baum, 1987; Mehrara & Baum, 1990). Naloxone also disrupts the acquisition of a conditioned partner preference in female rats for males bearing odor or strain cues associated with paced copulation (Coria-Avila, Gavrilu, et al., 2008). DA has long been known to be important for male sexual motivation and behavior (Blackburn, Pfaus, & Phillips, 1992; Pfaus & Phillips, 1991; Sachs & Meisel, 1988) and for directing attention to a rewarding stimulus (Mirenovic & Schultz, 1994). DA release increases phasically in hypothalamic and mesolimbic DA terminals, such as the mPOA and nucleus accumbens (NAc), respectively, in response to cues associated with copulation, such as estrous vaginal secretions or neutral odors associated with the ejaculatory reward state (Blackburn, Pfaus, & Phillips, 1992; Damsma, Pfaus, Wenkstern, Phillips, & Fibiger, 1992; Gelez, Coria-Avila, Woehrling, Rajabi, & Pfaus, 2005; Mitchell & Gratton, 1991; Pfaus et al., 1990; Richardson & Gratton, 2008). DA is released in a relatively continuous or tonic manner during copulation in the mPOA, NAc, and striatum of male rats, but declines precipitously in the mPOA and NAc after ejaculation (Blackburn et al., 1992; Lorrain, Riolo, Matuszewich, & Hull, 1999). Given that DA plays an important role in the prediction of the occurrence of a rewarding event (Schultz, 1998), it is possible that activation of DA release is part of the mechanism that directs male rats toward odors or other cues associated with sexual reward. It is interesting that, in females, administration of the DA receptor

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antagonist flupenthixol disrupts the acquisition of sexually conditioned partner preference for an odor cue associated with paced copulation but not for strain cues associated with paced copulation (Coria-Avila, Gavrilu, et al., 2008). Moreover, administration of DA antagonists does not disrupt the expression of sexually conditioned CPP (Ågmo & Berenfeld, 1990; Garcia Horsman & Paredes, 2004; Paredes & Ågmo, 2004).

Given that opioid, but not DA, transmission is important for the expression of sexual CPP in male and female rats, but that both play a role in sexually conditioned partner preference in female rats, the present study examined whether endogenous opioid or DA transmission plays a role in the development of CEP in males.

### Experiment 1: Effect of the Opioid Receptor Antagonist Naloxone

#### Method

##### Subjects and Surgery

**Males.** Forty male Long-Evans rats, weighing approximately 300 g at the start of the experiment, were obtained from Charles River Laboratories (St. Constant, QC). Rats were housed in groups of four, in plastic solid floor cages, with ad libitum access to food (Purina Rat Chow) and water, and they were maintained on a 12-hr dark–light cycle (lights on at 8 p.m.) in a temperature-controlled room ( $21 \pm 2^\circ\text{C}$ ). Behavioral testing was conducted in the dark phase of the cycle. Males were sexually naïve at the start of the experiment.

**Females.** Forty female Long-Evans rats, weighing approximately 200 g at the start of the experiment, were obtained from the same supplier and were housed under the same conditions as the males. Females were anesthetized with a mixture of ketamine (50 mg/ml) and xylazine hydrochloride (4 mg/ml), at a ratio of 4:3, respectively, administered intraperitoneally in a volume of 1 ml/kg of body weight. Anesthetized females were then bilaterally ovariectomized via a lumbar incision. All females were given a week of postsurgical recovery prior to sexual training. Following recovery, all females were placed in a pacing chamber with intact and sexually vigorous males during four sexual training trials. For all behavioral tests, sexual receptivity was induced by priming the females with subcutaneous injections of 10  $\mu\text{g}$  (in 0.1 ml of sesame oil) of estradiol benzoate (EB) 48 hr and 500  $\mu\text{g}$  (in 0.1 ml of sesame oil) of progesterone (P) 4 hr prior to behavioral testing.

##### Drugs

Naloxone hydrochloride (Sigma; St. Louis, MO), a nonspecific opioid receptor antagonist, was dissolved in 0.9% physiological saline, and was injected into the males subcutaneously at a dose of 5 mg/kg in a volume of 1 ml/kg (as in Miller & Baum, 1987), 5 min before each trial. Males in the control group were injected subcutaneously with 0.9% physiological saline in a volume of 1 ml/kg 5 min before each trial.

##### Apparatus

Copulatory conditioning trials took place in rectangular pacing chambers (46 cm  $\times$  39 cm  $\times$  37 cm) covered with a grid floor and bedding and bisected with a clear Plexiglas divider with a hole cut

into the bottom of the divider. This hole was small enough to allow only the female to cross, but not the male. All conditioning sessions were recorded on a DVD and subsequently scored using a PC-based program (Cabilio, 1996).

#### Procedure

**Conditioning phase.** The 40 naïve males were randomly assigned to one of two groups. Both groups were subjected to 10 copulatory trials in a pacing chamber with the same almond-scented female; however, one group was injected subcutaneously with saline 5 min before each trial, and the other group was injected subcutaneously with naloxone, a nonspecific opioid receptor antagonist (5 mg/kg), 5 min before each trial. For all conditioning sessions, males were given a 5-min habituation period to a pacing chamber, after which a female was placed into the chamber for 20 min. Females were primed with EB and P as described above. Conditioning trials occurred at 4-day intervals during the middle third of the rat's dark cycle following hormone priming.

**Copulatory preference test.** Four days following the last conditioning trial, males were placed in an open field and allowed to habituate for 5 min. At the end of this period, the familiar scented female and a novel unscented female were placed simultaneously into two diagonal corners of the open field at approximately equal distance from the male. All animals were able to interact freely with one another for 30 min. All males were drug-free during this test.

#### Behavioral Measures and Statistical Analyses

Latency and frequency measures were recorded for all mounts, intromissions, and ejaculations. Criteria for sexual behaviors were those described by Sachs and Barfield (1970). Female solicitations (defined as a headwise orientation to the male followed by a runaway, forcing the male to chase the female), hops and darts, and lordosis magnitude (on a scale from 1 to 3, with 1 representing low magnitudes, 2 representing moderate magnitudes, and 3 representing high magnitudes, as in Hardy & DeBold, 1972) were recorded. Latency and frequency measures of mounts, intromissions, and ejaculation during conditioning trials were recorded to examine differences in the pattern of copulation between males treated with either naloxone or saline. To study copulatory preference, the frequency of ejaculation with each female, and the female chosen for the first three ejaculations, were also recorded. One-way analyses of variance (ANOVAs) were used to analyze all frequency and latency data. Chi-square analyses were used to determine the difference in the proportion of females selected for the first two ejaculations during the copulatory preference test. The criterion for statistical reliability for all comparisons was set to  $p < .05$ .

#### Results

Figure 1 illustrates the mean frequencies of copulatory behaviors with the familiar or novel females displayed by males treated previously with saline or naloxone. Saline-treated males ejaculated significantly more often with their familiar female as opposed to the novel one,  $F(1, 22) = 9.54$ ,  $p < .05$  (see Figure 1a). In contrast, males injected with naloxone during conditioning ejaculated as

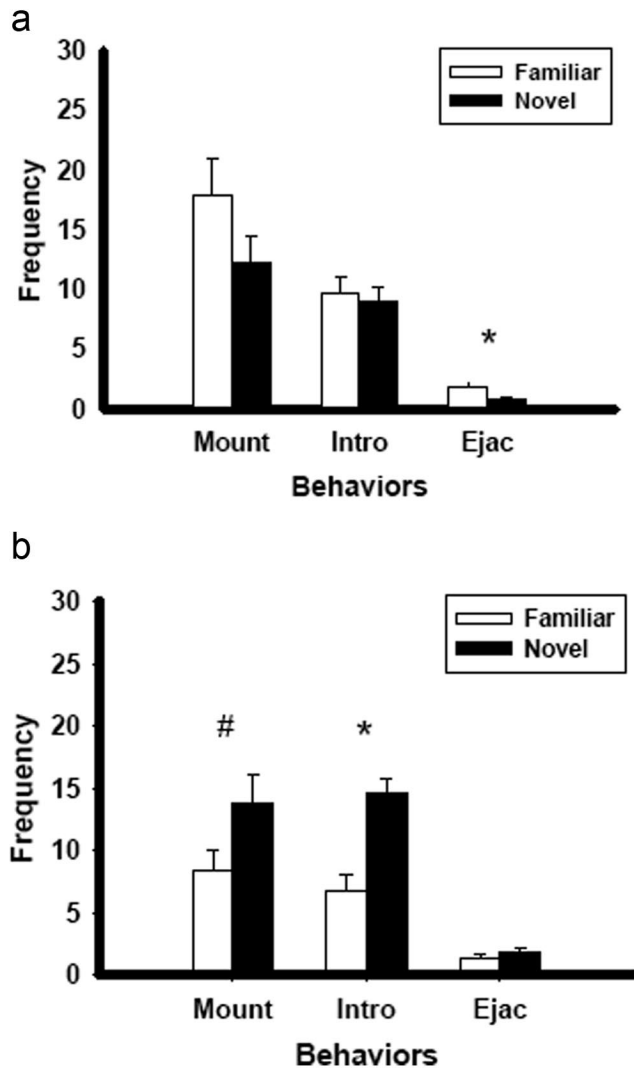


Figure 1. Mean frequency of mounts, intromissions, and ejaculations in (a) saline- and (b) naloxone-treated male rats with the familiar scented and novel unscented female rats. \* $p < .05$ . # $p < .1$ .

often with the familiar as with the novel females. It is interesting that these males tended to mount,  $F(1, 24) = 3.70$ ,  $p < .1$ , the novel female more frequently and to intromit significantly,  $F(1, 24) = 18.27$ ,  $p < .05$ , more frequently the novel female (see Figure 1b).

No differences in the latencies of male copulatory behavior were found between males previously treated with naloxone or saline (see Table 1).

Males previously injected with saline also seemed to choose their familiar female more often than the novel one for the first three ejaculations, but this difference did not reach statistical significance. Naloxone-treated males seemed to choose more often the novel female for the first and third ejaculation, but this did not reach statistical reliability (see Figure 2).

Tables 2 and 3 depict the frequencies and latencies of male copulatory behaviors during conditioning Trials 5, 7, 9, and 10. On Trial 5, naloxone-treated males mounted, intromitted, and ejacu-

Table 1

Average Latency (Mean  $\pm$  SEM) of Male Sexual Behavior During Partner Preference Test in Male Rats Treated Previously With Naloxone or Saline

| Treatment | Mount latency    | Intromission latency | Ejaculation latency |
|-----------|------------------|----------------------|---------------------|
| Saline    | 15.34 $\pm$ 2.18 | 29.66 $\pm$ 6.8      | 372.8 $\pm$ 67.7    |
| Naloxone  | 91.19 $\pm$ 51.0 | 112.6 $\pm$ 51.0     | 265 $\pm$ 33.3      |

lated significantly less than saline-treated ones:  $F(1, 24) = 7.05$ ,  $p < .05$ ;  $F(1, 24) = 6.84$ ,  $p < .05$ ;  $F(1, 24) = 4.11$ ,  $p < .05$ , respectively. Similarly, on Trial 7, naloxone-treated males mounted, intromitted, and ejaculated significantly less than saline-treated ones:  $F(1, 38) = 14.26$ ,  $p < .05$ ;  $F(1, 38) = 31.57$ ,  $p < .05$ ;  $F(1, 38) = 14.52$ ,  $p < .05$ , respectively (see Table 2). Naloxone-treated males also displayed significantly longer mount and intromission latencies than saline-treated females:  $F(1, 24) = 10.39$ ,

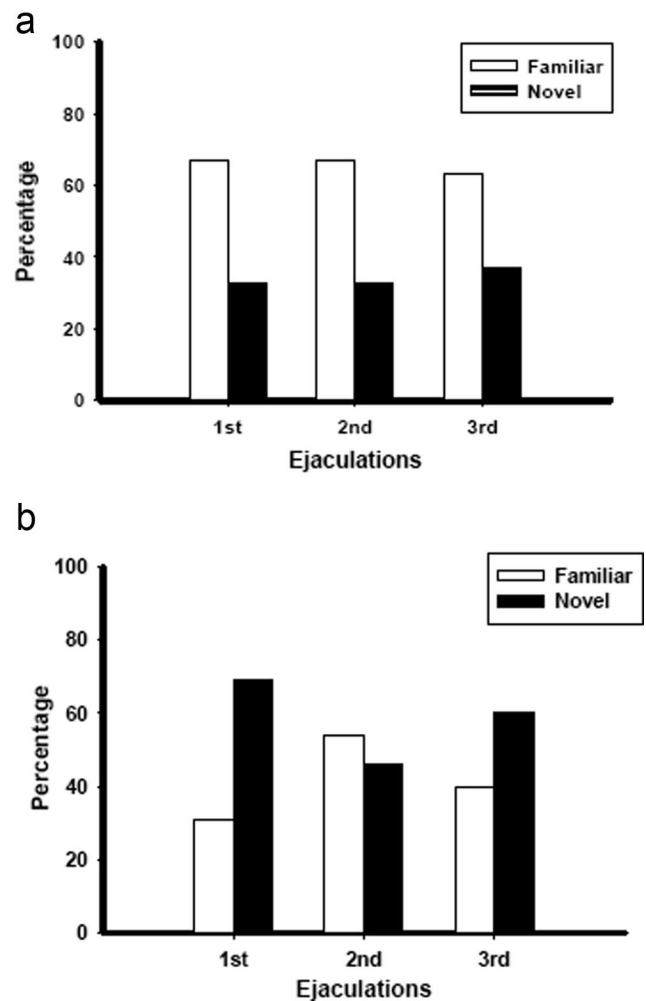


Figure 2. Distribution of first three ejaculations between familiar almond-scented and novel unscented female rats in (a) saline- and (b) naloxone-treated male rats.

Table 2

*Average Frequency (Mean  $\pm$  SEM) of Male Sexual Behavior During Conditioning Trials in Male Rats Treated With Naloxone or Saline*

| Behavior     | Treatment | Trial 5         | Trial 7          | Trial 9         | Trial 10         |
|--------------|-----------|-----------------|------------------|-----------------|------------------|
| Mount        | Saline    | 8.06 $\pm$ 1.42 | 10.2 $\pm$ 1.91  | 12.3 $\pm$ 2.05 | 4.4 $\pm$ 0.99   |
|              | Naloxone  | 2.8 $\pm$ 1.03* | 2.3 $\pm$ 0.85*  | 3.7 $\pm$ 1.02* | 2.44 $\pm$ 0.77  |
| Intromission | Saline    | 8.25 $\pm$ 1.18 | 9.4 $\pm$ 1.26   | 7.1 $\pm$ 1.06  | 16.4 $\pm$ 1.66  |
|              | Naloxone  | 3.3 $\pm$ 1.47* | 1.55 $\pm$ 0.61* | 1.8 $\pm$ 0.67* | 3.56 $\pm$ 1.66* |
| Ejaculation  | Saline    | 1.5 $\pm$ 0.20  | 1.65 $\pm$ 0.24  | 1.4 $\pm$ 0.21  | 1.3 $\pm$ 0.21   |
|              | Naloxone  | 0.8 $\pm$ 0.29* | 0.45 $\pm$ 0.20* | 0.45 $\pm$ 0.2* | 0.33 $\pm$ 0.24* |

\*  $p < .05$ .

$p < .05$ ;  $F(1, 24) = 13.2$ ,  $p < .05$ , respectively (see Table 3; Tables 4, 5, and 6). Moreover, on Trial 9, naloxone-treated males displayed significantly fewer mounts, intromissions, and ejaculations than saline-treated males:  $F(1, 38) = 14.12$ ,  $p < .05$ ;  $F(1, 38) = 17.94$ ,  $p < .05$ ;  $F(1, 38) = 10.80$ ,  $p < .05$ , respectively (see Table 2). Naloxone-treated males also displayed significantly longer mount and intromission latencies compared with saline-treated males:  $F(1, 26) = 5.86$ ,  $p < .05$ ;  $F(1, 26) = 17.43$ ,  $p < .05$ , respectively (see Table 3). On Trial 10, naloxone-treated males intromitted and ejaculated significantly fewer times than saline-treated males,  $F(1, 17) = 29.75$ ,  $p < .05$ ;  $F(1, 17) = 9.29$ ,  $p < .05$ , respectively; and they displayed significantly longer mount and intromission latencies,  $F(1, 17) = 10.78$ ,  $p < .05$ ;  $F(1, 14) = 7.96$ ,  $p < .05$ , respectively, than saline-treated males.

## Experiment 2: Effect of the Dopamine Receptor Antagonist Flupenthixol

### Method

#### Subjects

Forty male and 40 female Long-Evans rats served as subjects in this experiment. Subjects were acquired from the same supplier and housed under the same conditions as subjects in Experiment 1. For all conditioning trials and the copulatory preference test, females were primed with EB and P.

#### Drugs

Cis(Z)- Flupenthixol (Sigma; St. Louis, MO), a D1/D2 receptor antagonist was dissolved in 0.9% physiological saline and was injected intraperitoneally at a dose of 0.5 mg/kg in a volume of 1

ml/kg 30 min before every trial (as in Ågmo, 2003). Males in the control group were injected intraperitoneally with 0.9% physiological saline with a volume of 1 ml/kg 30 min before each trial.

#### Apparatus

All conditioning sessions and copulatory preference tests took place in the same pacing chambers and open fields used in Experiment 1.

#### Procedure

**Conditioning phase.** The 40 naïve males were randomly assigned to one of two groups. One group was given 10 copulatory trials in a pacing chamber with the same scented female and was injected with saline before each trial. The other group was given 10 copulatory trials in pacing chambers with the same scented female and was injected interperitoneally with flupenthixol (0.1 mg/kg), a nonspecific dopamine antagonist, before every trial. Conditioning trials occurred in the same way as in Experiment 1.

**Copulatory preference test.** Copulatory preference test also took place in the same way as in Experiment 1. The males were drug-free during this test.

#### Behavioral Measures and Statistical Analyses

Latency and frequency data of male and female sexual behaviors during the conditioning trials and the open field test were recorded and analyzed using one-way ANOVAs as in Experiment 1. Chi-square analyses were used to determine the differences in the proportion of females selected for the first two ejaculations. Copulatory preference was determined the same way as in Exper-

Table 3

*Average Latency (Mean  $\pm$  SEM) of Male Sexual Behavior During Conditioning Trials in Male Rats Treated With Naloxone or Saline*

| Behavior     | Treatment | Trial 5            | Trial 7              | Trial 9              | Trial 10            |
|--------------|-----------|--------------------|----------------------|----------------------|---------------------|
| Mount        | Saline    | 37.4 $\pm$ 11.9    | 17.64 $\pm$ 4.69     | 85.08 $\pm$ 67.4     | 43.04 $\pm$ 9.38    |
|              | Naloxone  | 63.86 $\pm$ 17.82  | 390.48 $\pm$ 147.95* | 404.62 $\pm$ 127.58* | 528.8 $\pm$ 157.6*  |
| Intromission | Saline    | 50.1 $\pm$ 20.6    | 21.34 $\pm$ 5.01     | 25.34 $\pm$ 3.94     | 43.04 $\pm$ 9.39    |
|              | Naloxone  | 149.075 $\pm$ 91.5 | 493.96 $\pm$ 202.7*  | 542.4 $\pm$ 156.4*   | 471.45 $\pm$ 200.2* |
| Ejaculation  | Saline    | 176.28 $\pm$ 25.05 | 137.38 $\pm$ 19.27   | 292.95 $\pm$ 73.08   | 595.5 $\pm$ 120.9   |
|              | Naloxone  | 162.65 $\pm$ 36.46 | 330.16 $\pm$ 195.15  | 168.78 $\pm$ 69.19   | 727.5 $\pm$ 118.65  |

\*  $p < .05$ .

Table 4  
Average Latency (Mean  $\pm$  SEM) of Male Sexual Behavior  
During Partner Preference Test in Male Rats Treated  
Previously With Flupenthixol or Saline

| Treatment    | Mount latency    | Intromission latency | Ejaculation latency |
|--------------|------------------|----------------------|---------------------|
| Saline       | 15.3 $\pm$ 2.45  | 22.51 $\pm$ 3.64     | 294.74 $\pm$ 40.5   |
| Flupenthixol | 26.52 $\pm$ 9.35 | 42.8 $\pm$ 11.7      | 347.9 $\pm$ 59.46   |

iment 1. The criterion for statistical reliability for all comparisons was set to  $p < .05$ .

### Results

Figure 3 illustrates the frequency of sexual behavior in males injected with saline or with flupenthixol during conditioning. It can be seen in this figure that saline-treated males displayed significantly more ejaculations with their familiar female compared with the novel one,  $F(1, 29) = 4.12$ ,  $p = .05$  (see Figure 3a). It is interesting that males injected with flupenthixol during conditioning also ejaculated significantly more with the familiar female as opposed to the novel one,  $F(1, 36) = 4.01$ ,  $p = .05$  (see Figure 3b).

Saline-treated males also seemed to choose more frequently their familiar female for the first and third ejaculations, but this did not reach statistical significance. Similarly, flupenthixol-treated males also seemed to choose more frequently their familiar female for the first two ejaculations, but this failed to reach statistical criterion for reliability (see Figure 4). No differences were found in the latencies of male copulatory behavior during the partner preference test between males treated with flupenthixol or with saline (see Table 6).

Tables 5 and 6 show no difference in frequencies and latencies of male copulatory behavior between flupenthixol- and saline-treated males during Trial 10 of the conditioning phase.

### General Discussion

The present study shows that the development of CEP is disrupted by the administration of naloxone, but not flupenthixol, during conditioning trials. This suggests that activation of opioids receptors, but not DA receptors, is necessary for the development of CEP for a familiar female following copulatory training in one-hole pacing chambers. These findings are similar to those of Ågmo and Berenfeld (1990) in which naloxone, but not the DA receptor antagonist pimozide, disrupted the expression of sexually conditioned CPP in male rats. Thus, similar neurochemical mechanisms appear to underlie the development of CPP and CEP.

Table 5  
Average Frequency (Mean  $\pm$  SEM) of Male Sexual Behavior  
During the 10th Conditioning Trial in Male Rats Treated With  
Flupenthixol or Saline

| Treatment    | Mount           | Intromission     | Ejaculation    | Anogen          |
|--------------|-----------------|------------------|----------------|-----------------|
| Saline       | 8.45 $\pm$ 1.36 | 11.95 $\pm$ 1.53 | 1.5 $\pm$ 0.17 | 1.05 $\pm$ 0.30 |
| Flupenthixol | 7.55 $\pm$ 0.92 | 10.55 $\pm$ 1.22 | 1.8 $\pm$ 0.17 | 1.9 $\pm$ 0.53  |

Table 6  
Average Latency (Mean  $\pm$  SEM) of Male Sexual Behavior  
During the 10th Conditioning Trial in Male Rats Treated With  
Flupenthixol or Saline

| Treatment    | Mount              | Intromission       | Ejaculation        |
|--------------|--------------------|--------------------|--------------------|
| Saline       | 74.89 $\pm$ 47.08  | 79.62 $\pm$ 47.80  | 342.94 $\pm$ 58.65 |
| Flupenthixol | 134.59 $\pm$ 34.64 | 170.01 $\pm$ 37.09 | 270.73 $\pm$ 50.27 |

Endogenous opioids figure prominently in the control of pleasure and reward. There are three major types of opioid receptors:  $\mu$ ,  $\delta$ , and  $\kappa$  (Patterson, Robson, & Kosterlitz, 1983). Those receptors are found predominantly in hypothalamic, limbic, and cortical areas such as the amygdala, the mPOA, the paraventricular nucleus (PVN), the nucleus of the solitary tract (NST), and the cingulate

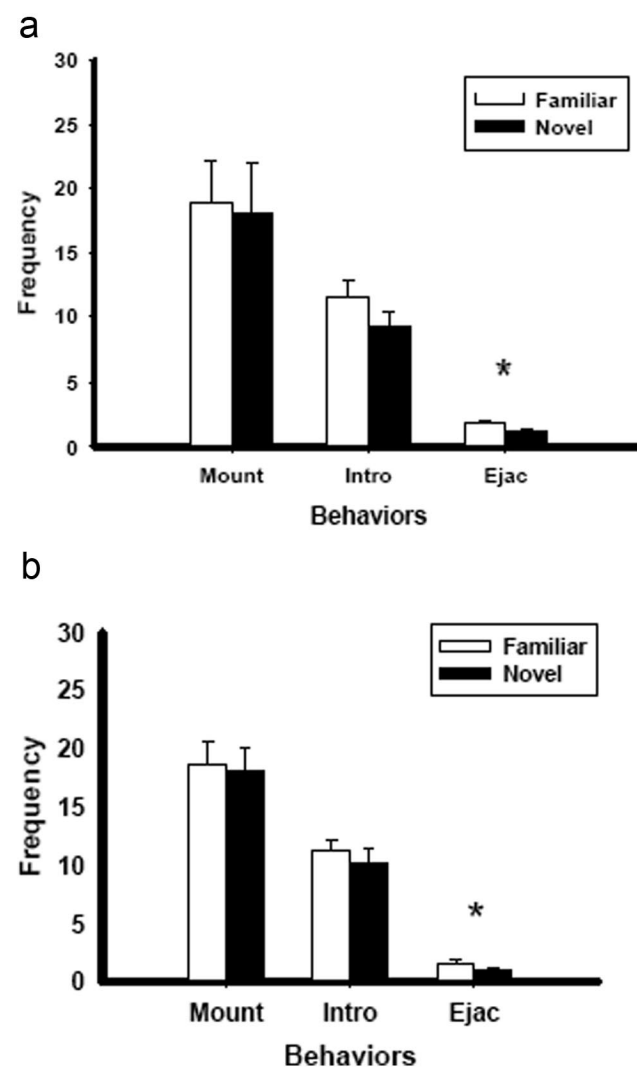


Figure 3. Average frequency of mounts, intromissions, and ejaculations in (a) saline- and (b) flupenthixol-treated male rats toward the familiar scented and novel unscented female rats on the partner preference test when no drugs were given. \*  $p < .05$ .



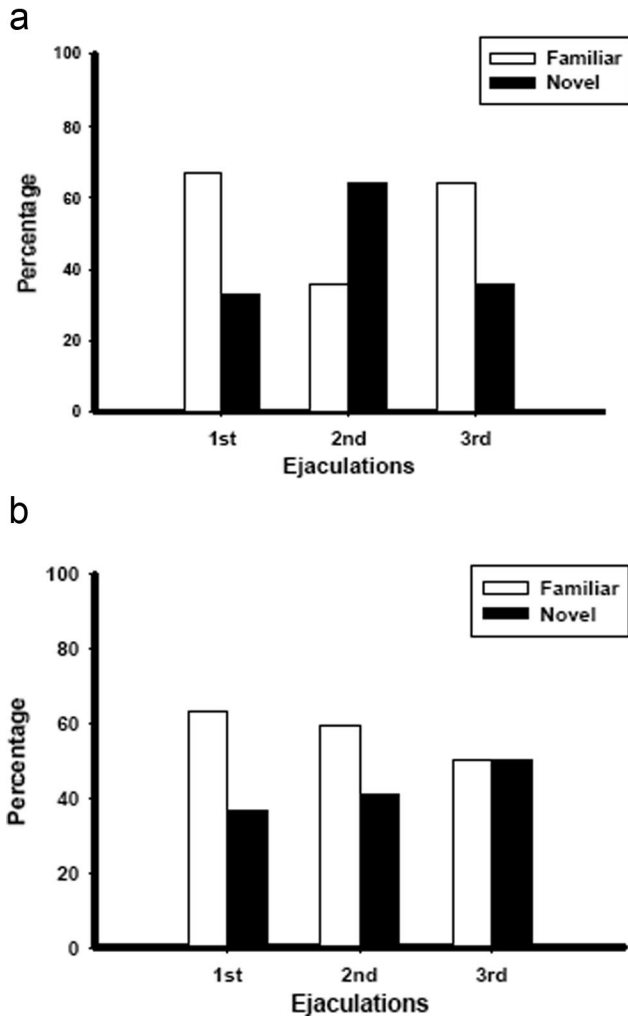


Figure 4. Distribution of first three ejaculations between familiar almond-scented and novel unscented female rats in (a) saline- and (b) naloxone-treated male rats.

cortex (Clarke, Zimmer, Zimmer, Hill, & Kitchen, 2003; Reinoso-Barbero & de Andres, 1995). The effect of opioids on sexual behavior was first reported in clinical accounts of long-term opioid users who experienced a deterioration of their sexual function. In both women and men, long-term opioid use results in anorgasmia, hypoactive sexual desire, decreased ejaculate volume (men), absence of menstrual periods (women), elimination of sexual dreams, and infertility (Pfaus & Gorzalka, 1987). Whipple and Komisaruk (1985) have shown that vaginal stimulation significantly increases pain tolerance and pain detection thresholds in a naloxone-reversible fashion. Similarly, González-Mariscal, Gómora, and Bayer (1994) have reported in male rats that during copulation, analgesic effects reach peak levels during ejaculation. On the basis of these findings, endogenous opioids appear to be released during genital stimulation and ejaculation, and may play an important role in the perception of the intensity of sexual reward by binding to opioid receptors in the amygdala, the mPOA, the PVN, the NST, or cingulate cortex, and by mediating the activation of other areas

that receive projections from these areas. Indeed, infusions of naloxone to the mPOA, ventromedial hypothalamus, or medial amygdala, but not NAc, disrupt the development of a sexual CPP in female rats (García-Horsman, Ágmo, & Paredes, 2008), suggesting that a distributed network of opioid actions in hypothalamic and limbic regions is critical for the association of sexual reward with conditional stimuli.

In the present study, naloxone-treated males displayed lower frequencies and longer latencies for mounts, intromissions, and ejaculations during the conditioning trials compared with saline-treated males. These results suggest a decrease in the intensity of sexual reward during conditioning trials, given that the state induced by ejaculation is necessary for the development of a CEP (Kippin & Pfaus, 2001; Kippin, Samaha, Sotiropoulos, & Pfaus, 2001). However, the overall copulatory pattern on the final test did not differ between males previously treated with naloxone or saline (see Table 1), indicating that the effects of naloxone on copulatory behavior were short-lived, and that the lack of partner preference of males treated previously with naloxone was not secondary to a general disruption of copulation on the final open-field test. Those results also suggest that chronic treatment with naloxone may induce a state of nonreward that alters the willingness of male rats to copulate with females, as it does to the willingness of female rats to initiate copulation with males (Coria-Avila, Solomon, et al., 2008). This stands in contrast to the ability of acute naloxone to facilitate copulatory behavior in sexually naïve males or males that are stressed (Pfaus & Wilkins, 1995), and suggests that the baseline state of the animals is a critical variable in the interpretation of drug effects on sexual behavior.

Although Coria-Avila and colleagues (Coria-Avila, Gavrila, et al., 2008; Coria-Avila, Solomon, et al., 2008) found that both naloxone and flupenthixol blocked the development of conditioned partner preference in female rats following paced copulation, the present studies found this effect only for naloxone. One obvious possible explanation is that slightly different neuronal mechanisms underlie the development of partner preference in males and in females. However, another potential explanation is that DA does play a role in the development of CEP in males but that the effect of flupenthixol was not sufficient to block it with our current experimental design. Although both DA and opioid antagonists bind competitively with their respective neurotransmitters, the binding kinetics and release patterns of opioids and DA during sexual behavior are not equivalent. Endogenous opioids are released as a "bolus" in different brain regions at the time of ejaculation (Szechtman et al., 1981), whereas DA transmission is increased continuously throughout copulation but decreases precipitously after ejaculation (Pfaus et al., 1990). As mentioned above, the postejaculatory interval is the critical period during which CEP develops (Kippin & Pfaus, 2001; Kippin et al., 2001), and opioid levels are high but DA levels are low during this stage of copulation. Taken together with the present data, it is likely that opioid release is necessary for conditioning to occur. Although microinjections of flupenthixol to the mPOA or ventral tegmental area disrupt male copulatory behavior and appetitive maze running to obtain a receptive female (Pehek et al., 1988; Warner et al., 1991), and although the dose of flupenthixol used in the present study blocked the acquisition of conditioned partner preference in female rats (Coria-Avila, Gavrila, et al., 2008), it is possible that this dose did not compete efficiently with the increased DA release

during copulation. Indeed, the increase in NAc DA in females during paced copulation is far lower than the increase observed in males (Jenkins & Becker, 2003), so it is possible that a higher dose of flupenthixol would be needed to block DA receptors sufficiently. In contrast, the dose of naloxone used in the present study has consistently prevented CPP in male and female rats and conditioned partner preference in female rats. This suggests that naloxone pretreatment competes effectively with opioids released as a bolus during the experience of sexual reward.

Paredes and Ågmo (2004) have argued against a role of DA in sexual behavior. This argument is predicated on the fact that DA antagonists do not alter sexual CPP or an unconditioned olfactory preference for an estrous versus nonreceptive female. However, DA release in mesolimbic or incertohypothalamic terminals during copulation may be a critical antecedent for conditioned sexual reinforcement. For example, data from several laboratories have indicated that extracellular DA in the NAc increases in the presence of sexually relevant stimuli (Pfaus et al., 1990; Mas, Gonzalez-Mora, Louilot, Sole, & Guadalupe, 1990; Damsma et al., 1992; Fiorino, Coury, & Phillips, 1997; Fiorino & Phillips, 1999), including conditioned odors associated with ejaculation (Gelez et al., in preparation). Moreover, subcutaneous injection of apomorphine, a DA agonist, elicited penile erection, whereas this effect was blocked by the subcutaneous injection of the DA antagonist haloperidol (Rampin, Jerome, & Suaudeau, 2003). López and Ettenberg (2000) demonstrated that males injected with haloperidol before their first copulatory experience exhibited diminished motivation to approach estrous females on subsequent drug-free trials, even though all males ejaculated on their first copulatory experience. Dopamine is also widely believed to be associated with the anticipatory phase of reward-seeking behaviors (Schultz & Dickinson, 2000). Moreover, studies comparing monogamous prairie voles to nonmonogamous meadow voles reported that blockade of DA D2 receptors in the NAc disrupts copulation-induced partner preference (Liu & Wang, 2003; Young, Lim, Gingrich, & Insel, 2001). Thus, although in the present study disruption of the dopaminergic system failed to block the development of CEP for a familiar female, DA may still play an important role in the pattern of male copulatory behavior.

Oxytocin may be an important intermediary of opioid and DA actions on CEP. Oxytocin treatment in male rats shortens both the ejaculation latency and postejaculatory interval (Arletti, Bazzani, Castelli, & Bertolini, 1985), and can restore normal ejaculatory behavior in male rats that have delayed ejaculation following chronic treatment with selective serotonin reuptake blockers such as fluoxetine (de Jong, Veening, Olivier, & Waldinger, 2007; Cantor, Binik, & Pfaus, 1999). Those data suggest that oxytocin release is a critical antecedent for ejaculation. Oxytocin also modulates the development of pair bonding in monogamous prairie voles (Young & Wang, 2004). For instance, female prairie voles injected with oxytocin develop pair bonds even in the absence of copulation (Young et al., 2001). Moreover, injection of an oxytocin antagonist directly in the NAc disrupts the development of pair bonding following mating. Conversely, systemic injection of oxytocin to male rats prior to their first sexual experience with an almond-scented female increases the number of males that display CEP (Gelez et al., 2005). Injections of naloxone block stress-induced increases in local oxytocin in the PVN, but not supraoptic nucleus, of virgin female rats (Wigger & Neumann, 2002). Hypo-

thalamic dopamine also appears to facilitate oxytocin release in the PVN in the control of penile erection (Baskerville & Douglas, 2008). Thus, oxytocin release is important for erection and ejaculation, may require the actions of DA and opioids, and may form an important pathway by which sexual reward generates the rudiment of pair bonding that CEP for a familiar female represents.

In summary, the results of the present study suggest that the release of endogenous opioids but not DA is necessary for the development of partner preference in males for a familiar female. However, the role of DA in the development of CEP requires further investigation.

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## Partner preference for strain of female in Long–Evans male rats

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### ABSTRACT

Although male rats are reported to show greater sexual arousal and mating preference for a novel female compared to a familiar one, we have shown that after repeated copulation to ejaculation with a female bearing a neutral odor in bilevel pacing chambers, or unilevel pacing chambers bisected by a 1-hole divider, male rats display a conditioned ejaculatory preference for a female bearing the odor relative to a female not bearing the odor. The aim of the present study was to examine whether males might also develop a conditioned ejaculatory preference for the strain characteristics of the female after repeated copulation with the same female in a pacing chamber bisected by either a 1-hole or 4-hole divider. In this experiment, male Long–Evans rats were given 10 copulatory trials with the same Long–Evans or Wistar female in either the 1-hole or 4-hole condition. Copulatory preferences were then examined in an open field where males had the choice to copulate with either the familiar female or a novel one of a different strain from the familiar female. Results indicated that Long–Evans males trained in the 1-hole condition with the same Long–Evans female displayed a conditioned ejaculatory preference for the familiar vs. novel female. However, males trained in the 1-hole condition with the same Wistar female at every trial copulated indiscriminately with the familiar and novel females. No preference was detected in males trained in the 4-hole condition. These findings suggest that, following training in a 1-hole pacing chamber, males displayed an ejaculatory preference only if the familiar female is of their own strain.

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### 1. Introduction

Rats are generally assumed to have polygamous mating strategies [1,2]. In the wild, males have been reported to chase estrous females as a pack and compete to mate with the female [3]. Male rats also display a “Coolidge effect” in which the inhibition of sexual arousal by repeated copulation with the same partner is delayed with the introduction of novel sex partners [4–6]. Similarly, in semi-natural settings, females have been found to shift partners throughout the mating sequence, although some females take ejaculations exclusively from a single male [7,8]. Moreover, female hamsters display a longer duration of sexual receptivity if their male partners are switched throughout the test [9]. However, the copulatory behavior of males and females during group mating differs considerably from those of dyadic mating, suggesting that sexual response patterns are variable and modifiable by experience.

We have shown previously that both male and female rats develop sexual partner preferences based on stimuli associated with sexual reward states. For example, male rats develop a conditioned ejaculatory preference for females bearing a neutral odor (e.g., almond) paired with the sexual reward state induced by ejaculation [10,11]. Similarly, female rats develop a conditioned sexual place preferences and partner preferences for males bearing an almond

odor associated with the reward state induced by copulation in pacing chambers, where the female can control the initiation and rate of sexual activity [12–14]. Thus, simple Pavlovian associations of external stimuli, such as neutral odors, with sexual reward states can direct the mate choice of male and female rats. Although compelling from a learning perspective, a more general question concerns the relevance of those data in terms of ecological validity: When are rats presented with scented partners in the wild?

Erskine [15] argued that the conditioned partner preference paradigm provides important tools to examine sexual motivation and mate choice in laboratory settings but it is unclear whether this information applies to rats in their natural environment. However, it is possible that rats can differentiate potential partners by other characteristics, including the hormonal state and strain of the animal. Different strains of rats (such as albino and pigmented) have been used to study sexual competition and females seem to interact more with partners of their own strain relative to those of different strains [16,17]. These findings suggest that females are able to identify individuals of their own strain. Although the exact cues the females attend to have not been identified (e.g., olfactory, visual, and/or auditory), these cues may ultimately influence their partner choice. Coria-Avila et al. [18] examined whether the development of partner preference in females for males associated with paced copulation was dependent on the strain of the male. Indeed, they showed that the strain of the partner plays an important role in the development of partner preference. In this study, Long–Evans and Wistar females

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experienced either paced copulation with a Long–Evans male and non-paced copulation with a Wistar male or paced copulation with a Wistar male and non-paced copulation with a Long–Evans male. Females solicited males associated with paced copulation selectively, regardless of the strain. However, females chose to receive their first ejaculation from males of their own strain, and to receive more ejaculations from those males if they were paired with paced copulation. This suggests that females have a natural preference for males of the same strain, although appetitive aspects of sexual behavior can be modulated away from this preference if the other strain is paired with a sexual reward.

The present study asked whether male rats develop a preference for the strain of female paired with the reward state induced by ejaculation. Based on the results reported by Coria-Avila et al. [18], we hypothesized that Long–Evans males given copulatory trials with Long–Evans females will be more likely to develop partner preference than Long–Evans males given copulatory trials with Wistar females.

## 2. Methods

All animal procedures conformed to the guidelines of the Canadian Council on Animal Care, and were approved by the Concordia University Animal Research Ethics Committee.

### 2.1. Subjects and surgery

Fifty-eight sexually naïve male Long–Evans rats, weighing approximately 300 g at the start of the experiment, were purchased from Charles River Laboratories (St-Constant, QC). Rats were housed in groups of four, in plastic solid floor cages lined with 2 cm of beta chip, with *ad lib* access to food (Purina Rat Chow) and water, and maintained on a 12-h dark/light cycle (lights on at 8 pm) in a temperature-controlled room ( $21 \pm 2^\circ\text{C}$ ). Behavioral testing was conducted in the dark phase of the cycle.

Twenty-nine Long–Evans and 29 Wistar female rats, weighing approximately 200 g at the start of the experiment, were purchased from the same supplier and were housed under the same conditions as the males. After a week of habituation, females were anaesthetized with a mixture of ketamine (50 mg/ml) and xylazine hydrochloride (4 mg/ml), at a ratio of 4:3, respectively, administered intraperitoneally in a volume of 1 ml/kg of body weight. Anaesthetized females were then bilaterally ovariectomized via a lumbar incision. All females were given one week of postsurgical recovery prior to sexual training. Following recovery, all females were placed in a pacing chamber with intact and sexually vigorous males during four sexual training trials. For all behavioral tests, sexual receptivity was induced by priming the females with an injection of 10  $\mu\text{g}$  (sc in 0.1 ml of sesame oil) of estradiol benzoate (EB) 48 h and 500  $\mu\text{g}$  (sc in 0.1 ml of sesame oil) of progesterone (P) 4 h prior to behavioral testing.

### 2.2. Apparatus

Copulatory conditioning trials took place in rectangular pacing chambers (46 cm  $\times$  39 cm  $\times$  37 cm) covered with a grid floor and bedding and bisected with a clear Plexiglas divider with either one hole or four holes cut into the bottom of the divider. These holes were small enough to only allow the female to cross and not the male. We have shown previously that copulation in these chambers induces CEP [19]. All conditioning sessions were recorded on a DVD and subsequently scored using a PC-based program [20].

### 2.3. Procedure

#### 2.3.1. Conditioning phase

The 58 sexually naïve Long–Evans males were assigned randomly to one of two groups. One group was given 10 copulatory trials in 1-hole

pacing chambers and the other group was given 10 copulatory trials in 4-hole pacing chambers. Half of the males assigned to the 1-hole condition were paired with the same Long–Evans female while the other half were paired with the same Wistar female during every conditioning trial. Similarly, the males assigned to the 4-hole condition were also given access to either the same Long–Evans or the same Wistar female during every conditioning trial. Prior to all conditioning trials, males were placed individually into a pacing chamber for 5 min of habituation. Then, either a Long–Evans or Wistar female primed with EB and P as described above was placed into the chamber for 20 min. Conditioning trials occurred at 4-day intervals during the middle third of the rat's dark cycle following hormone priming.

#### 2.3.2. Copulatory preference test

Four days following the last conditioning trial, males were placed in an open field and allowed to habituate for 5 min. At the end of this period, the familiar female and a novel female of a different strain (Long–Evans or Wistar) than the familiar one were placed simultaneously into two diagonal corners of the open field at approximately equal distance from the male. All animals were able to interact freely with one another for 30 min.

### 2.4. Behavioral measures and statistical analyses

Latency and frequency data were recorded for all mounts, intromissions, and ejaculations during the copulatory preference test. Criteria for male sexual behaviors were those described by Sachs and Barfield [21]. Female solicitations (defined as a headwise orientation to the male followed by a runaway, forcing the male to chase the female; [2,7]), hops and darts, lordosis magnitudes (on a scale from 1 to 3, with 1 representing low magnitude, 2 representing moderate magnitude, and 3 representing high magnitude, as in Hardy & DeBold [22]), and the time spent away from the male were also recorded. Mixed analyses of variance (ANOVAs) were used to determine differences in copulatory behaviors among the two conditioning groups. When appropriate, post hoc tests (using the Tukey Least Significant Difference test) were carried out to examine pairwise comparisons. Chi-square ( $\chi^2$ ) analyses were used to examine the choice of female for each ejaculation. The criterion for statistical reliability for all comparisons was set to  $p < 0.05$ .

## 3. Results

Fig. 1 illustrates the frequency of ejaculation in a) 1-hole-trained and b) 4-hole-trained males with Long–Evans and Wistar females during the copulatory preference test. Mixed ANOVA showed no

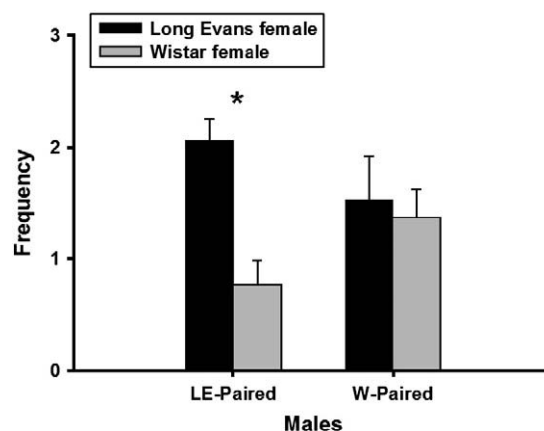


Fig. 1. Average ejaculation frequencies during the copulatory preference test in males trained in 1-hole pacing chambers with either a Long–Evans (L–E-paired) or a Wistar female (W-paired). \* $p < 0.05$ .

significant main effect but revealed a significant female  $\times$  familiarity interaction,  $F(1, 52) = 4.79$ ,  $p < 0.05$ . Pairwise comparisons of ejaculation frequencies between the familiar and novel female revealed that males trained in 1-hole pacing chambers and paired with a Long-Evans female ejaculated significantly more with their familiar Long-Evans female than with the novel Wistar one (Mean difference = 1.31 and SEM = 0.41,  $p < 0.005$ ). In contrast, males trained in 1-hole pacing chambers and paired with a Wistar female ejaculated indiscriminately with the novel Long-Evans and the familiar Wistar females (Mean difference = 0.2 and SEM = 0.43,  $p > 0.05$ ). Moreover, Fig. 2A shows that 1-hole-trained males paired with a Long-Evans female tended to choose more often their familiar Long-Evans as opposed to the novel Wistar female for their first ( $\chi^2 = 2.25$ ,  $p = 0.1$ ) and second ejaculations ( $\chi^2 = 6.25$ ,  $p < 0.05$ ). One-hole-trained males paired with a Wistar female did not display such preferences for their first three ejaculations (Fig. 2B). Contrary to 1-hole trained males, 4-hole-trained males ejaculated indiscriminately with both Long-Evans or Wistar females (Figs. 1b and 3).

Differences in female behaviors were also observed. Mixed ANOVA revealed a significant female  $\times$  familiarity interaction,  $F(1, 52) = 11.86$ ,  $p < 0.005$ . Pairwise comparison of solicitation frequency between the familiar and novel females revealed that 1-hole Long-Evans females solicited the Long-Evans-paired males significantly more frequently than did the Wistar females (Mean difference = 6.44 and SEM = 2.45,  $p < 0.05$ ) (Fig. 4). However, 4-hole Wistar females solicited the Wistar-paired males significantly more often than did the Long-Evans females (Mean difference = 6.90 and SEM = 2.95,  $p < 0.05$ ) (Fig. 5).

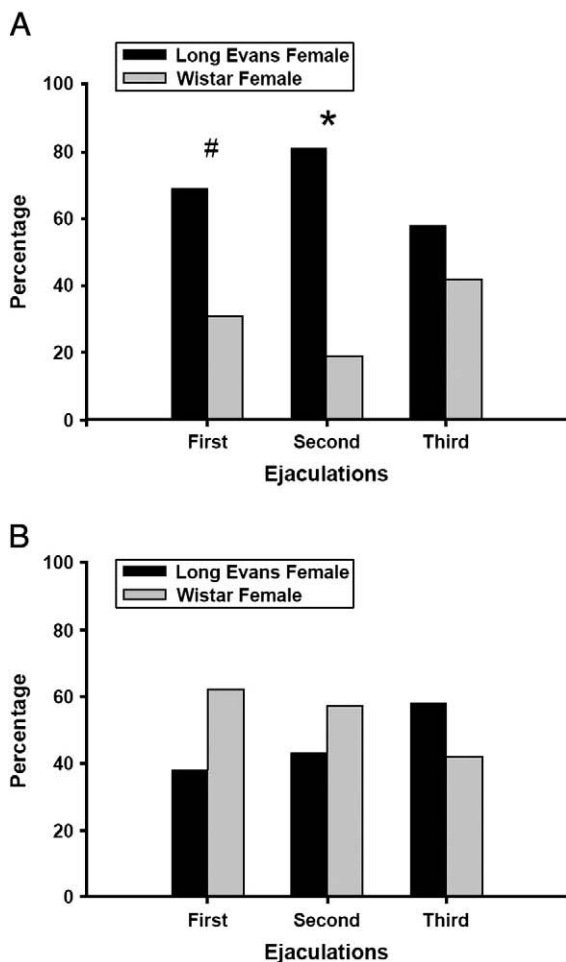


Fig. 2. Choice of female for the first three ejaculations in A) L-E-paired or B) W-paired 1-hole males. \* $p < 0.05$ , # $p < 0.1$ .

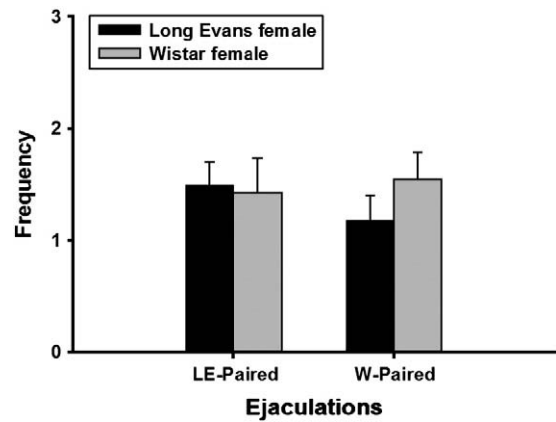


Fig. 3. Average ejaculation frequencies during the copulatory preference test in males trained in 4-hole pacing chambers with either a Long-Evans (L-E-paired) or a Wistar female (W-paired).

#### 4. Discussion

In the present study, Long-Evans male rats displayed a significant CEP for the strain of partner, but only if the familiar female is of the same strain as the male. This preference was demonstrated by a higher ejaculation frequency and the choice of the familiar Long-Evans female for the first two ejaculations in Long-Evans males trained in 1-hole pacing chambers.

Partner preference is a well-established paradigm to study sexual motivation [10–12,15,23–27]. It requires animals to identify,

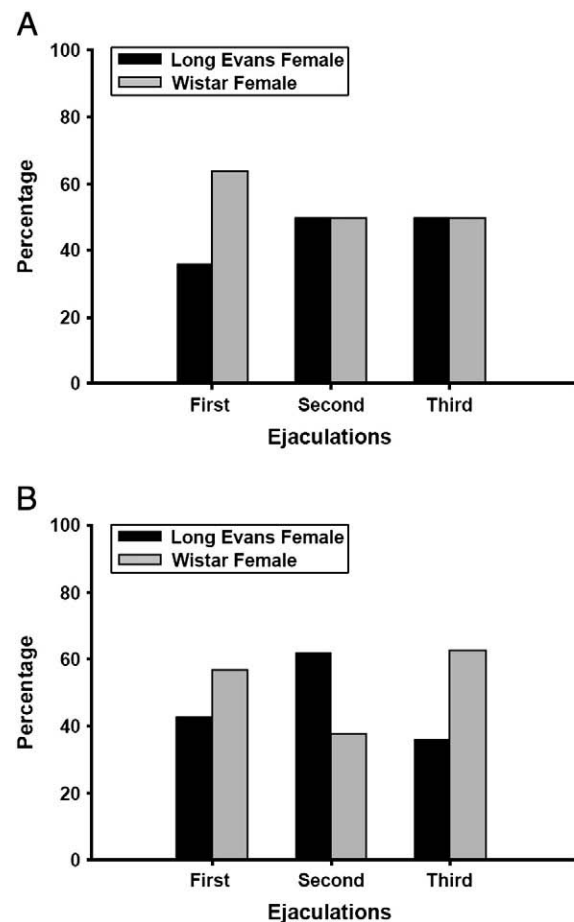


Fig. 4. Choice of female for the first three ejaculations in A) L-E-paired or B) W-paired 4-hole males.



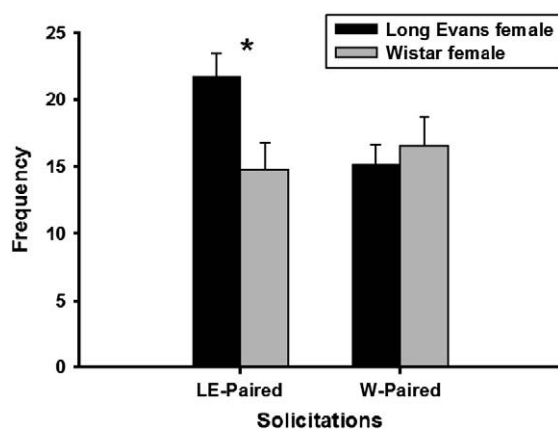


Fig. 5. Average solicitation frequency displayed by Long-Evans or Wistar females with L-E-paired or W-paired males in the 1-hole condition. \* $p < 0.05$ .

interpret, and respond to cues such as odors, appearance, and vocalizations from the stimulus partners [2–4]. In the present study, it was observed that 1-hole-trained Long-Evans males paired with Long-Evans females developed CEP for their familiar female whereas males paired with Wistar females did not. This suggests that conditioning is stronger when males are paired with females of their own strain during copulatory trials, perhaps because they have a natural preference for females of their own strain. Early life experiences may shape animals to recognize individuals of their own strain and selectively mate with them. D'Udine & Alleva [28] showed that early postnatal experiences with parents and siblings induce sexual preference in animals for their own strain. Early postnatal exposure of male rats to a neutral citral odor smeared on their mother's teats results in more ejaculations with scented females relative to unscented females during their first sexual experiences [29]. Moreover, mice raised by rat mothers attempt to copulate more with rats than with mice [30]. In agreement with the findings of Coria-Avila et al. [18], our results suggest that animals engage in assortative mating, a strategy that maintains homozygosity by preventing strains from outbreeding positive characteristics. However, this phenomenon appears more plastic in females than in males.

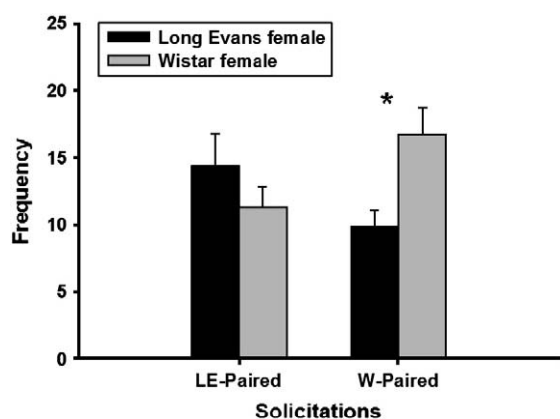
Coria-Avila et al. [18] showed that Long-Evans and Wistar females can develop a conditioned partner preference for males associated with paced copulation, regardless of the strain of males but the development of this preference is facilitated when females are given access to paced copulation with males of their own strain, indicating that females have a natural preference for males of the same strain. This suggests that females' initial sexual experiences can modify the effects of early postnatal experience on partner preference. However, this does not appear to be the case for males. In our study, 1-hole-trained males displayed CEP for their familiar female only if she was of their own strain. This suggests that in males, experience with sexual reward superimposes a mate preference only for traits (e.g. a neutral odor) associated with an already-established preference for a familiar strain. It is also possible that females are more sensitive to certain strain cues (e.g., pheromones or olfactory cues derived from major histocompatibility complexes) that males cannot detect. Thus the apparent sex differences in the development and display of conditioned partner preferences may be attributable to differences in sensory ability.

The distribution of ejaculations during the final test differed between males trained in the 1-hole and 4-hole conditions: Males trained in the 4-hole condition displayed an apparent Coolidge effect (a shift in the distribution of ejaculations) whereas males trained in the 1-hole condition consistently chose their familiar female to ejaculate with. We have reported similar findings using odor conditioning in partners of the same strain, such that significant CEP

for almond-scented females developed in males trained in the 1-hole, but not 4-hole, condition [31]. This preference may correspond to the male's preferred rate of copulation [32], an effect that also underlies the development of a sexually conditioned place preference [33]. Thus, we argue that in the 1-hole condition, the strength of the sexual reward may have overcome the males' "natural tendency" to prefer novel females. It could be argued that the CEP observed in 1-hole-trained Long-Evans-paired males for their familiar Long-Evans female in the present study was influenced by the increased solicitations from their familiar female during the copulatory preference test. However, this does not appear to be the case as the Wistar females also tended to solicit more often toward the 4-hole Wistar-paired males and this did not result in the display of CEP in these males for Wistar female. We have shown that female solicitation does not influence the display of CEP in male rats [32].

The results of the present study show that Long-Evans males trained in 4-hole pacing chambers failed to display a CEP for their familiar female regardless of the strain of the female. As we have shown previously [31,32], the development of conditioning for place or partner cues is not solely dependent on ejaculation, as suggested previously by Miller and Baum [34] and Kippin and Pfaus [11,35,36], but rather seems additionally modulated by the level of sexual arousal during copulation. Males trained to copulate in 1-hole and 4-hole pacing chambers display different patterns of copulation which result from different levels of arousal experienced during copulation in these chambers [31]. For instance, males trained in 1-hole pacing chambers display significantly longer ejaculation latencies, and more intromissions prior to ejaculation, than males trained in 4-hole pacing chambers. Moreover, females spend significantly more time away from the males in the 1-hole condition, compelling the males to wait for the females for longer periods of time than males in the 4-hole condition [32]. This is reminiscent of the "enforced interval effect" reported in 1956 by Larsson [37], in which shorter interintromission latencies between 30 s to 3 min imposed by the experimenter lead to increased sexual arousal and activity. It is possible that anticipated access to the female in 1-hole pacing chambers may result in higher levels of arousal in males and this may facilitate conditioning and the development of partner preference. This idea is also consistent with the findings of Lipp [38] that the arousal level of an anticipated event augments the development of conditioned responding. Indeed, Richardson and Gratton [39] recently showed that dopamine transmission in the nucleus accumbens was increased when a reward was presented at variable, rather than fixed, intervals. Those findings suggest that rats are more aroused and excited in situations in which the occurrence of a reward is anticipated and unpredictable than in situations in which the occurrence of the reward is expected and predictable. We speculate that males trained in the 1-hole paced condition may have experienced high levels of arousal during copulation because the contact with the female partner was unpredictable. In contrast, males trained in 4-hole pacing chambers may have experienced lower levels of sexual arousal because the contact with the female partner occurred at shorter intervals. Indeed, females in the 4-hole condition typically leave and return to the male following an intromission at faster intervals because they can re-enter the male's side more easily given the availability of more than one hole. In contrast, following an intromission and female exit in the 1-hole condition, the male often stays near the hole that the female exited, making it difficult for her to re-enter until moves away.

Finally, we note that Long-Evans females displayed significantly more solicitations toward Long-Evans-paired males in the 1-hole condition relative to Wistar-paired males (Fig. 5), whereas Wistar females displayed significantly more solicitations toward Wistar-paired males in the 4-hole condition relative to Long-Evans-paired males (Fig. 6). Given that the frequency of solicitations is dependent on the experience of pacing-induced sexual reward [12,18], an effect induced by endogenous opioid release [40], this finding suggests a



**Fig. 6.** Average solicitation frequency displayed by Long-Evans or Wistar females with L-E-paired or W-paired males in the 4-hole condition. \* $p < 0.05$ .

strain difference in the perception of sexual reward by female rats. Long-Evans females appear to derive more reward from the 1-hole condition whereas Wistar females derive more reward from the 4-hole condition. McClintock [7] reported differences in the pacing behavior of wild (pigmented) vs. domesticated (laboratory-raised, nonpigmented) females, such that wild females enforced longer interintromission intervals in a large open field compared to domesticated females. It is possible that this effect was also influenced by genetic factors inherent to pigmented vs. nonpigmented strains. Indeed, clitoral stimulation applied every second to Wistar rats increases solicitations over stimulation applied once every 5 s [41]. In contrast, clitoral stimulation applied every 5 s induces a conditioned place preference in Long-Evans females whereas stimulation applied every second does not [42]. Previous reports indicate that pigmented Long-Evans males display noncontact erections in response to inaccessible estrous females, whereas nonpigmented Wistar males are severely impaired in this ability [43]. However, systemic administration of the serotonin synthesis inhibitor p-chlorophenylalanine to Wistar males facilitates the display of noncontact erections [44], indicating that the relative impairment in this strain may be due to increased basal concentrations of serotonin that inhibit erection. To the extent that serotonin plays a critical role in sexual inhibition and satiety [45], it may be that the increased activity of serotonin in Wistar females requires them to receive intromissive stimulation at faster frequencies to support sexual reward.

In summary, the results of the present study suggest that males trained to copulate in 1-hole pacing chambers develop CEP for strain cues only if the familiar female is of their own strain. Thus, although cues associated with sexual reward come to direct appetitive sexual responses in both male and female rats, mate choice (in this case the choice to give or receive ejaculations to or from a particular partner) may be more “hardwired” by assortative pressures in which animals prefer their own strain over a different one.

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# Somatosensory conditioning of sexual arousal and copulatory behavior in the male rat: A model of fetish development



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## HIGHLIGHTS

- Rodent jackets can be conditioned to excite or inhibit sexual arousal in male rats.
- The jacket acts as a contextual cue.
- Somatosensory conditioning can be used as a model of sexual fetish development.

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## ABSTRACT

Previous studies have established that neutral olfactory cues associated with sexual reward or inhibition become conditioned stimuli that direct male or female rats toward or away from potential sex partners bearing the cue. Here we examined the ability of a somatosensory cue to exert stimulus control over sexual arousal and copulation in male rats. In the first experiment, two groups of sexually naïve male rats had their first copulatory experiences with receptive females in bilevel chambers with or without a rodent jacket. On a final copulatory test, half of the rats in each group were tested with the jacket on or off. Rats trained and tested without the jacket copulated normally, as did rats trained and tested with the jacket on, and rats trained without the jacket but tested with the jacket on. However, significantly fewer rats trained with the jacket on but tested with the jacket off copulated to ejaculation, and those that did made significantly fewer anticipatory level changes and had fewer significantly longer mount, intromission, and ejaculatory latencies, and fewer ejaculations. In the second experiment, one group of sexually naïve males was given differential conditioning trials in bilevel chambers to associate the jacket on with sexual reward (copulation to ejaculation with a sexually receptive female) and the jacket off with sexual inhibition (thwarted copulatory attempts with a sexually nonreceptive female). A second group had the opposite order of association. A final test with receptive females was made for all males with the jacket on. Males trained to associate the jacket with excitation displayed normal copulation whereas males trained to associate the jacket with inhibition displayed significantly fewer anticipatory level changes and ejaculations, and had significantly longer ejaculation latencies. Thus, somatosensory cues can signal sexual excitation or inhibition in male rats depending on the conditioning history.

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## 1. Introduction

Sexual behavior in all species depends on a complex interplay of innate and learned factors. All animals must be able to respond to hormonal and neurochemical changes that signal their own sexual desire and arousal, and innate factors create individual differences in this ability, contributing to a moment-to-moment level of sexual arousability [1,2]. However, experience plays a key role in automating and consolidating baseline rates of sexual responding [3–6], and forming conditioned responses to first- or second-order sexual reinforcers (reviewed in [1,7,8]). Animals use conditioned cues to become aroused and interact

with external sexual stimuli. Thus, in addition to recognizing their own state of sexual arousal and desire, animals must be able to identify external stimuli that predict where potential sex partners can be found, to seek out, solicit, court, or otherwise work to obtain sex partners, distinguish external cues and behavioral patterns of potential sex partners from those that are not sexually receptive, and to pursue sex partners once sexual contact has been made. In rats, these conditioned responses have been modeled using conditioned place and partner preferences, and depend on sexual reward achieved through copulatory contact that induces a preferred rate of intromissive penile stimulation leading to ejaculation in males, or that is paced by females to induce clitoral and vaginocervical stimulation at their preferred rates [1].

In our previous work, neutral olfactory stimuli (e.g., almond or lemon odors) were paired with states of sexual reward induced by interaction

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with sexually receptive partners, or inhibition induced by interaction with sexually non-receptive partners, to conditioned partner preference or avoidance, respectively. The odor was always placed on the partner (neck and anogenital area) so that the subject would learn to associate it as a partner-related cue and direct conditioned responding (solicitations in females, ejaculations in males) toward the partner bearing the odor associated with sexual reward [9,11]. In cases where the odor was paired with sexual nonreward, females would solicit and receive ejaculations more from an unscented partner relative to a scented one [12,13] and males would mount, intromit, and ejaculate preferentially with an unscented partner relative to a scented one [10,11]. Male rats trained to associate an odor with sexual reward displayed shorter intromission latencies with sexually receptive scented females relative to sexually receptive unscented females, but did not show any other enhancement of appetitive or consummatory sexual behaviors [14]. Similarly, male rats have greater activation of luteinizing hormone and higher plasma testosterone concentrations following exposure to an odor paired with sexual reward [15] relative to males that have not had the odor paired. Finally, male marmosets trained to copulate to ejaculation with lemon-scented receptive females display erections and increased place preference in response to the odor alone relative to unpaired males [16]. These data indicate that discrete odor cues on the partner that are paired with sexual reward come to direct partner and place preferences, and can enhance certain aspects of sexual arousal and preparatory neuroendocrine function in males.

Contextual cues can also control sexual responses conditionally. Placing male rats into a chamber in which copulation has occurred previously induced penile erections whereas placement into a novel chamber did not [17]. Placement of male rats into a holding cage associated with exposure to a receptive female behind a wire-mesh screen resulted in significantly shorter latencies to intromit and ejaculate in a final test of unrestricted copulation relative to males placed directly from their home cages into the copulation chamber [18,19]. Male rats trained to associate two distinctive environments with morphine reward or saline initiate copulation faster and display more vigorous approach and pursuit of receptive females in the morphine-paired environment [20]. Similar results have been demonstrated in Japanese quail and fish. Male quail that received repeated exposure to females following the presentation of a conditioned stimulus house light displayed significantly shorter latencies to initiate copulation when the stimulus was present compared to when it was absent [21]. Likewise, repeatedly pairing a house light with non-contact exposure to a receptive female reduced the latencies for male gouramies to initiate copulation when the house light was presented prior to unrestricted access to the female [22].

It appears that discrete cues on sex partners become important incentive stimuli for the development of conditioned partner and place preferences relative to more diffuse contextual cues that facilitate more general measures of sexual arousal and the initiation of copulation. This relationship echoes the difference between discrete and contextual cues that promote sensitization of motor responses to stimulant drugs like amphetamine [23], and may well reflect differences in what is associated and what can be predicted from the cues. Contextual cues are present at all times during interaction with rewarding unconditional stimuli (UCSs), and thus act as conditional stimuli (CSs) that predict the reward state. Discrete sexual CSs may well vary in stimulus intensity and proximity, such that they are less predictive of immediate reward states and more work has to be done to approach and consume them (e.g., a discrete odor on a sexually receptive female “runs away” as the female controls or paces the sexual interaction, and males must chase the stimuli, catch them, mount and intromit with them multiple times before the post-ejaculatory reward state is achieved). And this is done within an environment containing contextual cues that have the same stimulus intensity throughout the interaction. Thus, the salience of discrete partner-related cues may differ from contextual cues both in intensity and in terms of *what* is predicted.

Sexual fetishes develop to a wide variety of discrete and contextual cues, and to modes of expression that are highly arousing and perhaps also require effort. Early theories of fetish development specified an association that crystallizes between inanimate objects or body parts and the experience of sexual arousal and/or reward during periadolescence. For example, Binet [24] believed that fetishes develop when there is a simultaneous association of an object or action such as spanking with a sexually arousing stimulus. Krafft-Ebing [25] noted that “Erotic fetishism makes an idol of physical or mental qualities of a person or even merely of objects... because they awaken mighty associations... with sexual pleasure.” (P. 18). The Pavlovian nature of this association remains in more modern interpretations [26–29] and has been maintained throughout subsequent incarnations of the Diagnostic and Statistical Manual of Mental Disorders (DSM) of the American Psychiatric Association [30] and the International Statistical Classification of Diseases (ICD) and Related Health Problems of the World Health Organization [31], especially in terms of the potential for stimuli associated with early sexual arousal and/or gratification to become not only preferred, but necessary in the expression of sexual arousal. This is reminiscent of the way in which contextual cues control sexual arousal in rats, and is critical in the formation of sexual approach and partner preferences, especially for discrete neutral odors associated with sexual reward or inhibition [1].

The present study examined whether a somatosensory cue (rodent jacket) on male rats could come to elicit sexual approach and sexual arousal when paired with their first 10 multi-ejaculatory sexual experiences with sexually receptive females. The first experiment determined whether removal of the jacket after training would impair sexual approach and arousal. The second experiment determined whether the jacket could possess sexual excitatory or inhibitory properties after pairing with sexual receptive vs. nonreceptive partners, respectively.

## 2. Material and methods

### 2.1. Subjects

Long-Evans rats weighing 200–250 g (males) and 150–200 g (females) were obtained from Charles River Canada, Inc. (St-Constant, QC). Males were housed in large Plexiglas cages four to a cage with wood-chip bedding. Females were housed in pairs in standard Plexiglas shoebox cages with wood-chip bedding. All rats were housed in the same reversed 12 hour light/dark cycle colony room (lights off at 0800), given *ad libitum* tap water and regular rat chow, and kept in constant temperature at 21 °C. All animal procedures conformed to the guidelines of the Canadian Council for Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

### 2.2. Female surgeries, hormone priming, and sex training

Bilateral ovariectomy (OVX) was performed on all females via a lumbar incision so that impregnation was impossible. This also allowed for hormone levels to be controlled throughout testing. General anesthesia was induced with ketamine hydrochloride (100 mg/ml) and xylazine hydrochloride (20 mg/ml), mixed in a 4:3 ratio, respectively, and administered intraperitoneally (ip) at a dose of 1 ml/kg. One week of recovery was provided prior to the onset of behavioral testing.

Females were given four 30-minute training sessions of sexual experience paired with a sexually vigorous male in a bilevel chamber [32,33] to equate them for sexual experience. Sessions took place at 4-day intervals to approximate the normal ovulatory cycle of the female. Females were rendered sexually receptive by sequential treatment with estradiol benzoate (EB; 10 µg, Steraloids, Newport, RI) 48 h, and progesterone (P; 500 µg, Steraloids, Newport, RI) 4 h before each test. Steroids were administered subcutaneously (sc) in 0.1 ml of reagent-grade sesame oil (Sigma-Aldrich, St. Louis, MO). Females used as sexually nonreceptive stimuli were given the same sex training; however, after a 2-week

hormone washout period, these were given sc injections of the sesame oil vehicle 48- and 4-h prior to testing.

### 2.3. Materials and testing apparatus

The somatosensory cue was a rodent tethering jacket (Lomir Biomedical, Ile Perrot, QC). Jackets were made from a double layer of Lycra/Spandex fabric that fit the upper torso, with openings for the forearms and fastened across the back with an adjustable Velcro strip (Fig. 1). Wearing the jacket did not impede any male's ability to move or copulate. All tests were conducted in bilevel chambers [32,33] where males could freely chase females from level to level and copulate in a sideways orientation to the viewer, thus allowing for accurate determination of their copulatory behaviors.

### 2.4. Male behavioral training and testing

#### 2.4.1. Experiment 1

Sexually naïve males were given their first and subsequent 8 copulatory training trials at 4-day intervals with either the jacket on ( $N = 20$ ) or jacket off ( $N = 20$ ). During each trial, males were placed singly in a chamber for a 5 min period prior to the introduction of a sexually-receptive female, after which they were allowed to copulate freely with her for 30 min. On the 10th trial, males in each group were assigned randomly to one of two conditions: jacket on or jacket off ( $n = 10$ /group). All training and testing trials were conducted during the middle third of the rats' dark cycle and recorded on video. Male sexual behaviors (latencies and frequencies of level changes in the 5-min period prior to the female, mounts, intromissions, and ejaculations) scored subsequently using a rodent behavioral scoring program [34], from which were derived post-ejaculatory intervals (time from ejaculation to the next intromission), average interintromission interval (ejaculation latency/number of intromissions), intromission ratios (number of intromissions/number of intromissions + mounts without intromission) and the proportion of males that mounted, intromitted, or ejaculated, in each group.

#### 2.4.2. Experiment 2

Sexually naïve males ( $N = 48$ ) were given their first and subsequent 9 sexual discrimination trials with sexually receptive vs. nonreceptive females at 4-day intervals, for a total of 5 alternating trials with each type of female. Discrimination trials were conducted as in Experiment 1, with males placed into the bilevel chamber for 5 min prior to the introduction of a female. The presentation of sexually receptive or nonreceptive females during the first trial was counterbalanced, such that half of the males received their first experience with sexually receptive females and the other half with sexually nonreceptive females. Males were randomly assigned to two somatosensory valence training

conditions. The excitation (E) group ( $n = 24$ ) had the jacket on during trials with sexually receptive females and the jacket off during trials with sexually nonreceptive females. The inhibition (I) group ( $n = 24$ ) had the opposite order of association: jacket on during trials with sexually nonreceptive females and jacket off during trials with sexually receptive females. A final 30-min test was conducted 4 days after the last discrimination trial. During this test, males in both somatosensory training groups had the jacket on and were given access to a sexually receptive female. Male behaviors were recorded on video and scored as in Experiment 1.

### 2.5. Data analysis

#### 2.5.1. Experiment 1

Latency and frequency data from Experiment 1 were analyzed using a 2-way between subjects analysis of variance (ANOVA) with training group and jacket on or off on the final test as independent variables, along with their interaction. For each significant ANOVA, post hoc comparisons of the individual means were made using the Tukey HSD test. Proportions of rats that engaged in different copulatory behaviors were analyzed using  $\chi^2$ .

#### 2.5.2. Experiment 2

Latency and frequency data from Experiment 2 were analyzed using a 2-way between subjects ANOVA, with somatosensory valence (E vs. I) and start condition (jacket on or off) as independent variables, along with their interaction. For each significant ANOVA, post hoc comparisons of the individual means were made using the Tukey HSD test.

## 3. Results

### 3.1. General observations

Males in both experiments developed copulatory behavior at a normal rate, such that by the 10th (Experiment 1) or 11th (Experiment 2) trial, males in excitatory conditions were mounting within 15 s of the presentation of the female, achieving their first ejaculation within 400 s of their first intromission, and having approximately 3 ejaculations in the 30-min test (as is expected for Long-Evans males [33]). Wearing the jacket did not impede the acquisition of baseline rates of copulation. In fact, in Experiment 1, rats trained with the jacket on had overall faster ejaculation latencies than rats trained with the jacket off, despite the same number of intromissions before ejaculation, indicating a faster rate of copulation with the jacket on (data not shown).

### 3.2. Experiment 1

The proportion of males that copulated was affected by the training and testing conditions. All males in the control group trained with the jacket off mounted, intromitted, and ejaculated on the final test, regardless of whether they had the jacket off or on. Likewise, 100% of males trained with the jacket on mounted, intromitted, and ejaculated with the jacket on during the final test. In contrast, males trained with the jacket on but that had the jacket off during the final test displayed a relative disruption of copulatory activity: 80% mounted, 70% intromitted, and 60% ejaculated. This latter proportional difference was statistically significant compared to the males trained with the jacket on that had the jacket on during the final test ( $\chi^2(1) = 5.00, p < 0.03$ ).

Analysis of the latency and frequency data also revealed a significant disruption of both appetitive and consummatory aspects of copulation when males trained with the jacket on had the jacket off during the final test (Fig. 2). The ANOVA detected a significant interaction of training and testing condition for appetitive level changes ( $F(1,36) = 19.25, p < 0.0001$ ). Post hoc Tukey analyses revealed that the males trained with the jacket on but tested with the jacket off made significantly



Fig. 1. A male rat wearing the Lomir® rodent tethering jacket.

fewer level changes compared to males in the other training and testing conditions, which did not differ significantly from one another.

The ANOVA also detected a significant interaction of training and testing condition for the intromission latency ( $F(1,36) = 27.62$ ,  $p < 0.000005$ ). Post hoc Tukey tests revealed that the males trained with the jacket on but tested with the jacket off had a significant increase in the intromission latency compared to males in the other training and testing groups. Males trained with the jacket off, but wearing the jacket during testing had significantly higher intromission latencies than males trained in the same control condition but that had the jacket off.

A significant main effect of testing condition was found for the number of intromissions ( $F(1,36) = 6.82$ ,  $p < 0.02$ ). Post hoc Tukey tests revealed that overall males with the jacket on during the final test had significantly fewer intromissions than males with the jacket off.

Finally, the ANOVA detected a significant interaction of training and testing groups for the number of ejaculations ( $F(1,36) = 18.23$ ,  $p < 0.00001$ ). Post hoc Tukey tests revealed that males trained with the jacket on but tested with the jacket off had significantly fewer ejaculations compared to males in the other groups. Males trained with the jacket and had the jacket on during testing also had significantly more ejaculations than males trained with the jacket off, but tested with the jacket on. No other significant main effects or interactions were found.

### 3.3. Experiment 2

Although we expected male sexual behaviors to vary as a function of the somatosensory valence (e.g., the jacket's association with sexual excitation or inhibition), they also varied as a function of the start condition such that the interaction of start condition with the somatosensory valence often determined the nature and robustness of the effect (Fig. 3). The ANOVA detected a significant main effect of start condition on the number of intromissions ( $F(1,43) = 10.90$ ,  $p < 0.00002$ ). Post hoc Tukey tests revealed that males that started either excitatory or inhibitory training with the jackets on had significantly more intromissions

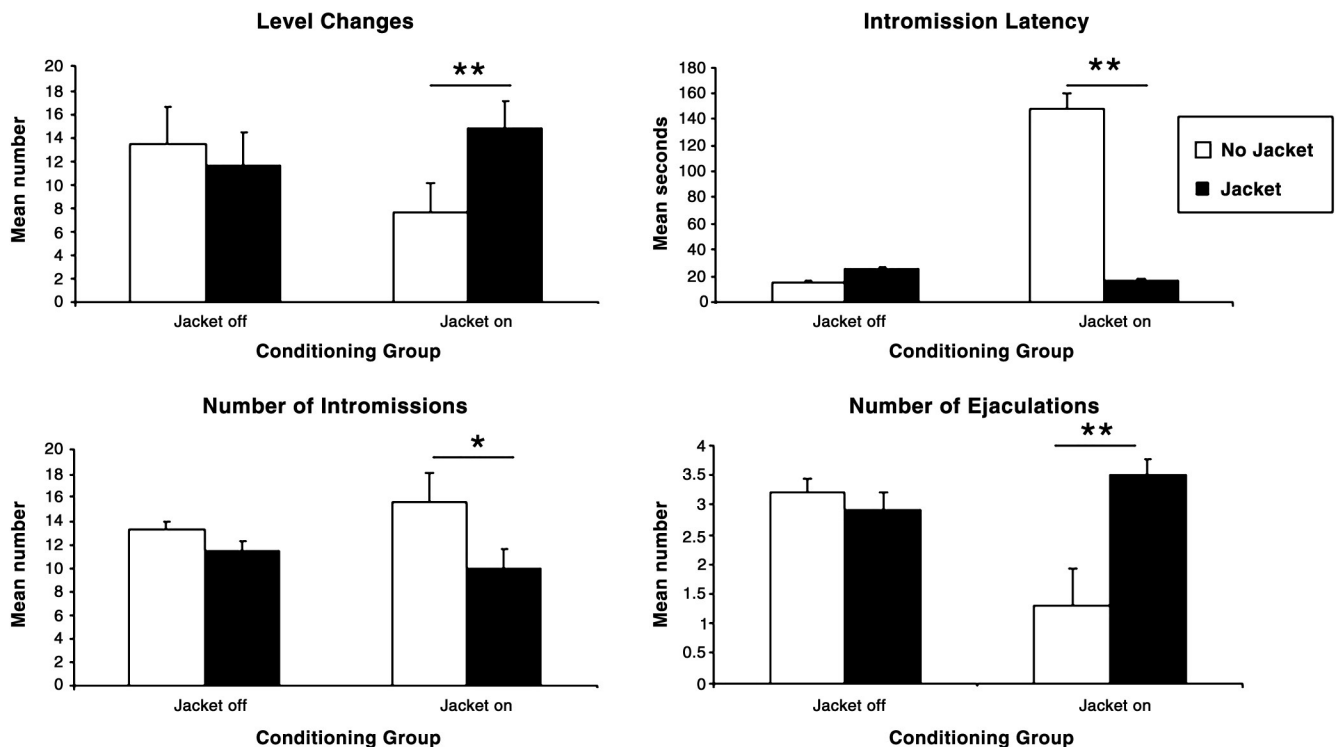
overall on the final test than males that started with the jackets off. The ANOVA also detected a significant interaction of somatosensory valence and start condition for the number of intromissions ( $F(1,43) = 20.05$ ,  $p < 0.00001$ ). Post hoc Tukey tests revealed that males that had the jacket on when the first trial was inhibitory had significantly more intromissions during the test than males that had the jackets off when the first trial was inhibitory.

A significant interaction of somatosensory valence and start condition was found for the number of ejaculations ( $F(1,43) = 27.75$ ,  $p < 0.00001$ ). Post hoc Tukey tests revealed that males that had the jackets on when the first trial was inhibitory, or the jackets off when the first trial was excitatory, had significantly fewer ejaculations than males that had the jackets off when the first trial was inhibitory and males that had the jackets on when the first trial was excitatory.

The ANOVA also detected a significant interaction of somatosensory valence and start condition for the ejaculation latency ( $F(1,43) = 17.36$ ,  $p < 0.0001$ ). Post hoc Tukey tests revealed that males that had the jackets on when the first trial was inhibitory, or the jackets off when the first trial was excitatory, had significantly longer ejaculation latencies than males that had the jackets off when the first trial was inhibitory and males that had the jackets on when the first trial was excitatory. In fact, males that had the jackets on during the first inhibitory trial had longer ejaculation latencies than males in the other three groups.

Finally, the ANOVA detected a significant interaction of somatosensory valence and start condition for the post-ejaculatory refractory period after the first ejaculation ( $F(1,43) = 12.49$ ,  $p < 0.001$ ). Post hoc Tukey tests revealed that males that had the jackets off when the first trial was excitatory had significantly longer PELs than males that had the jackets on when the first trial was excitatory. In fact, males that had the jackets on when the first trial was excitatory had significantly shorter PELs than males in the other three groups on the final test.

There were no significant main effects or interactions for other measures of sexual behavior.



**Fig 2.** Effect of somatosensory conditioning in Experiment 1 on the display of anticipatory level changes, intromission latency, number of intromissions, and number of ejaculations on the final test. \* $p < 0.05$ , post hoc Tukey HSD test. \*\* $p < 0.01$ , post hoc Tukey HSD test.



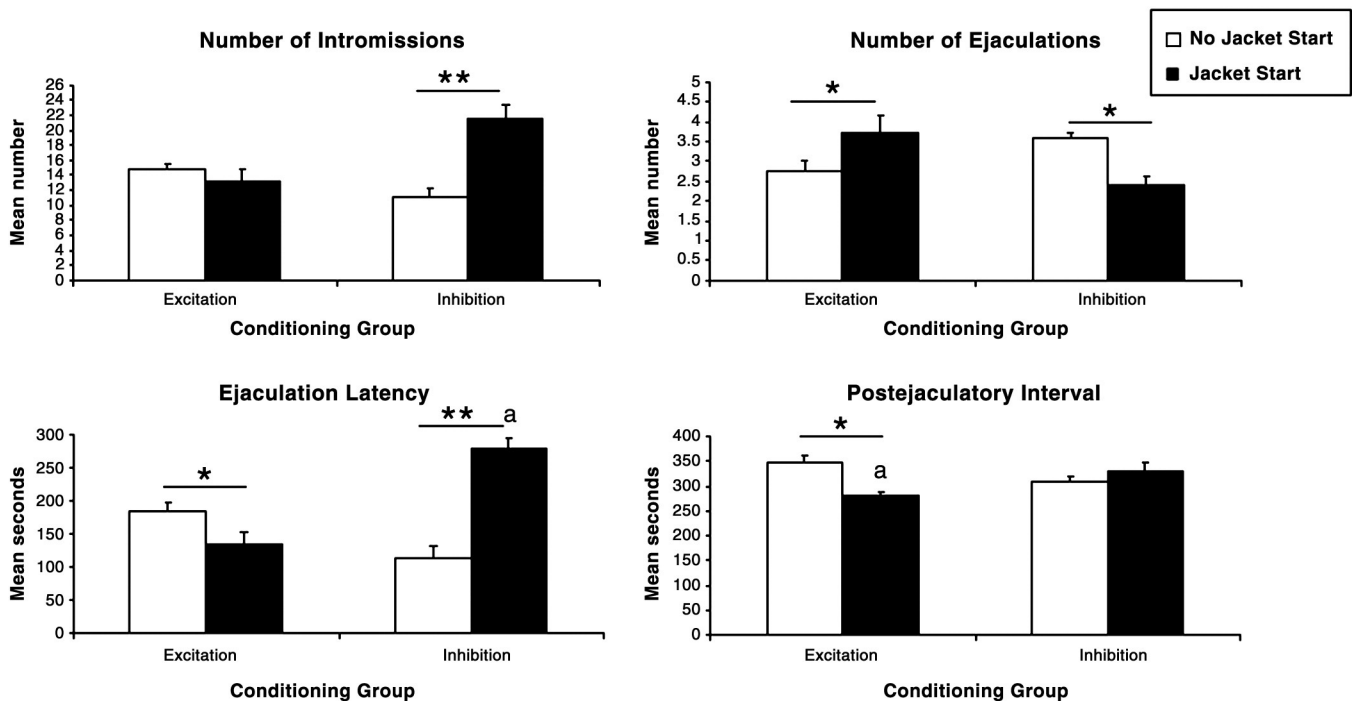


Fig. 3. Effect of somatosensory conditioning in Experiment 2 on the number of intromissions, number of ejaculations, ejaculation latency, and post-ejaculatory intervals. \* $p < 0.05$ , post hoc Tukey HSD test. \*\* $p < 0.01$ , post hoc Tukey HSD test. a = significantly different from all other groups.

#### 4. Discussion

These results show that a rodent jacket can be used as a somatosensory CS to direct sexual arousal and copulatory behavior in the male rat. Experiment 1 demonstrated that rats trained to associate the jacket with multi-ejaculatory copulatory bouts at 4-day intervals displayed normative copulatory behavior with the jacket on during a final test, but relative copulatory deficits with the jacket off, including decreased anticipatory level changes, intromissions, and ejaculations, and increased intromission latencies. Significantly fewer males in this group ejaculated with the jackets off. In contrast, males trained with the jacket off had no copulatory deficit with the jacket on, indicating that the jacket alone, even as a novel stimulus, had no inherent effect on sexual behavior.

Experiment 2 examined whether males could differentially associate the jacket with sexual excitation or inhibition, using a paradigm in which the jacket on was differentially associated with sexually receptive or nonreceptive females, and the jacket off associated with sexually nonreceptive or receptive females, respectively, in males given sequential access to sexually receptive and nonreceptive females. Thus, in one condition the jacket was a CS for excitation and in the other a CS for inhibition. On the final test, males in both groups had the jacket on and were given access to sexually receptive females. Significant interactions were found between the somatosensory valence of the jacket (excitation or inhibition) and the start condition (whether the jacket was on or off during the first conditioning trial) that indicated an unexpected primacy effect of their first sexual experience: Males with the jacket on when the first trial was inhibitory, or the jackets off when the first trial was excitatory, had significantly fewer intromissions, fewer ejaculations, and longer ejaculation latencies and PEIs than males that had the jackets off when the first trial was inhibitory and males that had the jackets on when the first trial was excitatory. This replicates and extends the results of Experiment 1 and indicates that the main effect of the jacket as a CS is on behaviors that reflect measures of sexual arousal, both parasympathetic (intromissions, from which erection can be inferred) and sympathetic (ejaculation latency and frequency). These results also suggest that an animal's initial sexual experiences

are a sensitive or critical period during which neutral stimuli associated with sexual reward can become appetitive sexual CSs, whereas stimuli associated with sexual inhibition can become inhibitory CSs.

We have shown previously that neutral odors (e.g., almond, lemon) on a conspecific that are associated with sexual reward or inhibition come to direct sexual partner preference in both male and female rats [8–14]. The conditioned odors alone activate neurochemical circuits for sexual arousal, desire, reward, and bonding [1]. In the case of male rats, placement of the sexually positive odor on a sexually nonreceptive female stimulates paired-trained male rats (i.e., those that learned to associate the odor exclusively with sexually receptive females) to mount the nonreceptive female significantly more than unpaired-trained males that did not learn this association [35]. Conversely, paired-trained males given access to unscented receptive females displayed slightly longer intromission latencies and lower intromission ratios relative to paired-trained males given access to scented females [35], but did not display longer ejaculation latencies, fewer ejaculations, or decreases in anticipatory level changing, as did males in Experiment 1 of the present study. Thus, it would appear that an olfactory CS on a conspecific may be acting more as a discrete, partner-related stimulus relative to a somatosensory CS on the subject, which, because it has always been present, acts like a contextual cue that is more related to the subject's own sexual arousal than to partner-related cues that stimulate bonding and partner preference.

A model of fetish behavior was reported in male Japanese quail by Köksal et al. [36]. In that study, male quail that had one prior sexual experience with a female in a particular context were given 30 conditioning trials during which half of the males were trained to approach a light CS that signaled copulatory access to a female, or to a soft polyester-filled terrycloth CS that resembled a female and that the birds could copulate with, prior to copulatory access to a live female. In several replications, approximately 44–50% of the birds copulated with the terrycloth CS. Following acquisition, birds in each group were given a series of 30 extinction trials where they were presented with either the light CS or the terrycloth CS but without subsequent copulation with the female. Birds that had previously copulated with the terrycloth CS maintained conditioned approach to the terrycloth CS, whereas birds

that received light-copulation pairings showed a rapid extinction of conditioned approach to the light CS, as did birds that did not copulate with the terrycloth CS. Similar to the expression of human sexual fetishes, the persistence of the conditioned sexual approach indicated resistance to extinction that was likely maintained through conditioned sexual arousal in birds that had previously copulated with the terrycloth CS. This is similar to the idea that the early experience of sexual arousal with fetish objects in humans could be conditioned with masturbation or partnered sexual interaction that leads to orgasm [1,28]. These results also highlight the fact that individual differences exist in the development of sexual fetish behavior, as they did in Experiment 1 in which some paired males did not copulate at all with the jacket off whereas others did, albeit with increased intromission and ejaculation latencies.

The fact that animal sexual behavior comes under stimulus control by contextual and discrete cues associated with states of sexual arousal and/or reward leads us to reiterate that learning is an extremely important variable in the development of human and other animal sexual behavior [1,8,37] specifically because it is tied to mechanisms of reward, reinforcement, and the sensitization of appetitive responses that lead animals to interact with incentives that predict reward [34–40]. Indeed, animals in the wild must be able to identify external stimuli that predict where potential sex partners can be found, to seek out, solicit, court, or otherwise work to obtain sex partners, distinguish external cues and behavioral patterns of potential sex partners from those that are not sexually receptive, and to pursue sex partners once sexual contact has been made. The results of the present study taken together with data from our previous olfactory conditioning studies suggest that contextual and discrete, partner-based cues likely activate different neural pathways. However, some overlap may exist in the activation of sexual arousal, which is necessary as a preparatory state for any type of sexual interaction.

Finally, our data have important implications regarding the development of fetishes. Early and modern theories of fetish development posit a Pavlovian contingency between objects or body parts and early experience with sexual arousal and/or orgasm [24–29]. Typically those early experiences have been reported in case histories to occur during a pre- or peripubertal period [41,42], and have been regarded as “deviant” in the sense that they do not occur in most individuals, and they result in an unusual and obsessive desire for the fetish object which subsequently causes relationship problems, is deemed inappropriate, and can lead to criminal prosecution if revealed. Although these experiences usually did not involve sexual intercourse as the reinforcer, they did involve obsessive fantasies about the fetish and concomitant masturbation to orgasm that served to reinforce the association. We have shown previously that sexually naïve female rats will solicit sex preferentially from a male scented with almond odor during their first sexual experience in an open field with two males, one scented and the other unscented, but only if the odor alone had been paired previously with manual clitoral stimulation [13]. Given that rats do not live in a culture as we know it, and are not subject to socially-constructed notions of sexual morality as we understand them, the development of a sexual preference for a neutral odor or jacket in rats, or copulation with a terrycloth model in quail, by a simple Pavlovian association suggests that fetish development follows a relatively normal pattern that reflects an increased susceptibility to incentive conditioning during all animals’ first experiences with sexual arousal and/or reward.

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## Research

# First sexual experiences determine the development of conditioned ejaculatory preference in male rats

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We have shown previously that male rats develop a conditioned ejaculatory preference (CEP) for females scented with a neutral odor like almond or lemon that is paired with the male's post-ejaculatory reward state during their first and subsequent early sexual experiences. However, preexposing males to the neutral odor alone prior to its pairing with sexual reward results in latent inhibition. Here, we examined the phenomenon of unconditioned stimulus (US) preexposure, in which male rats were preexposed to the ejaculatory reward state either one or five times with scented (ScF) versus unscented (UnScF) females prior to multiple ejaculatory trials with females in the opposite condition (e.g., ScF preexposure received 10 subsequent ejaculatory trials with UnScF, whereas UnScF preexposure received 10 subsequent ejaculatory trials with ScF). As before, mate and partner preference was evaluated in an open field where each male had access to two females, one ScF and the other UnScF. Males that underwent five trials of preexposure did not display a CEP for either female. Conversely, males preexposed once to a ScF, and later trained with UnScF developed a preference for the latter, whereas males preexposed once to the UnScF, and then trained with ScF did not show a preference for any of the females. Subsequent exposure to the odor cue alone revealed different patterns of brain activation in areas related to sexual behavior that depended on the animal's group membership. Altogether, these findings demonstrate the pivotal role of first sexual experiences in the establishment of future sexual partner preference in the male rat, and suggest an innate preference for estrous odors over neutral odors that can become conditioned subsequently as predictors of sexual reward.

There are many cues that naturally and instinctively drive animals toward conspecifics, especially when attempting to recognize a sexually receptive partner. For example, female rats spend more time among gonadally intact males compared to castrated males (e.g., Gilman and Westbrook 1978). Likewise, male rats are naturally driven toward odors from sexually receptive versus nonreceptive females (e.g., Bakker et al. 1996). These cues appear to be hard-wired and driven by hormonally mediated neural systems within certain hypothalamic and limbic structures (see Pfaus et al. 2003; Pfaus 2009). However, the importance of these cues tends to diminish after baseline rates of sexual responding have been achieved. For example, male rats spend more time in olfactory investigation of sexually receptive females during their first sexual experience with them compared to subsequent experiences (Kagan and Beach 1953; Carr et al. 1962; Stern 1970; Pfaus et al. 2001). Habituation appears to develop in male rats to natural sex odors from familiar females (Carr et al. 1970), although presenting males with novel females can result in the reinitiation of anogenital investigation of the female (Carr et al. 1970; Stern 1970). Similarly, although presenting male rats with a different receptive female during each multiejaculatory test results in a precipitous decline in anogenital investigations prior to the initiation of copulation, subsequent presentation of sexually nonreceptive females results in a vigorous reinitiation of anogenital investigations (Pfaus and Pinel 1989).

Animals are also equipped with associative learning mechanisms that allow them to predict and enact biologically relevant changes in their internal state from environmental cues. These associations are formed by Pavlovian conditioning contingencies (e.g., Rescorla and Wagner 1972) in which neutral cues that become conditioned stimuli (CSs), like neutral odors, acquire associ-

ative strength after being paired with biologically relevant cues, or unconditioned stimuli (USs), like the sexual arousal and reward states induced before and after ejaculation, respectively (Zamble et al. 1986; Kippin and Pfaus 2001a; Tenk et al. 2009). For instance, male Japanese quails associated a CS more readily when they had access to copulate with a receptive female than when they were only exposed to it (Holloway and Domjan 1993). Furthermore, male rats have been shown to develop a conditioned ejaculatory preference (CEP) toward females bearing a neutral odor like almond or lemon that has been associated previously with the post-ejaculatory reward state (e.g., Kippin et al. 1998; Kippin and Pfaus 2001a). However, given that the procedure to develop a CEP requires several training trials, the changes that could possibly occur during first sexual experiences and their effects on a subsequent trained CEP are buried in the subsequent trials. Consequently, the impact of first sexual experiences on the development of CEP remains largely unexplored, although blocking opioid transmission by systemic treatment with the opioid receptor antagonist naloxone during training can block the formation of both CEP (Ismail et al. 2009) and sexually conditioned place preference (Ågmo and Berenfeld 1990; Mehrara and Baum 1990).

The impact of the first exposure to a US on the subsequent ability to associate a neutral stimulus with the US, phenomenon known as US preexposure, results in a great retardation or outright blocking of the establishment of a CR if a CS is paired with the US in a context in which the animal was previously exposed to the US alone (e.g., Randich and LoLordo 1979). Significant US preexposure effects have been demonstrated for conditioned taste

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aversions (e.g., Clasen et al. 2017) and conditioned fear (e.g., Frankland et al. 2004), but not learned immunosuppression (Lueckemann et al. 2016). As an early example, Taylor (1956) conditioned the blinking response of human participants using an air puff signaled by a light. Before training, one group received several presentations of the air puff in three different intensities to the cornea of their eyes without the light. The number of eye-blink responses was greater in the group that was not preexposed to the air puff, whereas in the preexposed group the number of eye-blink responses was in an indirect correlation with the intensity of the air puff during the preexposure phase. Two hypotheses have been offered to explain this phenomenon, one associative and the other nonassociative. The former proposes that the US preexposure effect is due primarily to an association between the US and cues in the context in which the initial US preexposure takes place prior to training. The contextual cues trained with the initial exposure to the US blocks the subsequent association of other potential CSs trained in that context (e.g., Rescorla and Wagner 1972; Randich and LoLordo 1979). The latter claims that by preexposing the US, there is a reduction in the initial emotional reactivity of the animal's response due to general habituation that reduces the salience of the US, and thereby attenuates subsequent excitatory conditioning (e.g., Rankin et al. 2009). In terms of the sexual reward state induced by ejaculation in male rats, the associative explanation predicts that contextual cues associated with the first experience (s) of copulation and ejaculation (or other rewarding aspects of sexual interaction with a female) would come to block subsequent attempts to associate a discrete cue, such as a neutral odor, with the ejaculatory reward state. Alternatively, the nonassociative hypothesis would predict that the emotional quality of repeated ejaculations in the same context decreases after the first several experiences, thus driving down the associative strength conferred from the US to the CS as a predictor.

Previously, we assessed if preexposure to the neutral odor (almond) used as the CS in the development of CEP could alter conditioning via latent inhibition (Quintana et al. 2018). Males were preexposed to a neutral odor (almond) either one or five times before they were trained to develop a CEP based on that odor cue. As in our previous studies, mate and partner preferences were evaluated through an open-field test, where males had access to two sexually receptive females, one scented and another unscented. Males that had been given five trials with the odor alone and subsequently trained to develop CEP based on the odor did not show a preference for either female, whereas males exposed only one time showed a preference for the scented female. After two reconditioning trials were given after the open-field test, the odor alone was presented to the males in the different groups and its ability to activate cellular Fos protein (a marker of neuronal activation) in different brain regions was examined. A significantly greater activation of Fos in the group given one CS preexposure trial in the medial preoptic area (mPOA), ventral tegmental area (VTA), and nucleus accumbens core (NAc Core) relative to the group given five CS preexposure trials was found. In contrast, the group with one CS preexposure had smaller numbers of Fos-positive cells compared to a paired control group in the basolateral amygdala (BLA) and NAc Core and NAc shell (NAc Shell), all of which are a subset of the general pathway that underlies olfactory partner preference conditioning in both male and female rats (Pfaus et al. 2010).

The present study sought to evaluate whether US preexposure would block the subsequent conditioning of CEP in male rats. Sexually naïve male rats were given either one or five sexual experiences to one ejaculation each with either sexually receptive females scented with a neutral almond odor (ScF) or left unscented (UnScF). Subsequently, males were given another 10 tests of sexual behavior with ScF if they were preexposed to an UnScF, or vice ver-

sa, after which CEP and other sexual partner preferences were assessed in a large open field with two receptive females, one ScF and one UnScF. As in our previous study, following two reconditioning trials each male was exposed to the almond odor alone for an hour to assess the ability of the CS to activate Fos.

## Materials and Methods

### Subjects

#### Males

Ninety-two Long-Evans rats were sexually naïve and weighing ~250 g at the beginning of the experiment. They were housed in groups of four and two in Plexiglas cages with ad lib access to water and food (Purina Rat Chow). Males were obtained from Charles River Canada (St-Constant, QC, Canada) and kept in a 12 h. reversed light-dark cycle in a room at 21°C.

#### Females

One-hundred and twenty Long-Evans rats sexually naïve and weighing ~200 g at the beginning of the experiment were obtained from the same distributor and housed in pairs in the same conditions as males. Females were ovariectomized via bilateral lumbar incisions under ketamine (50 mg/mL)/xylazine (4 mg/mL) anesthesia, mixed at a ratio of 4:3, respectively, approximately 2 wk before the beginning of the experiment. Sexual receptivity was induced by subcutaneous injections of 10 µg estradiol benzoate (Steraloids, injected sc in 0.1 mL of sesame oil) 48 h prior each training session, and 500 µg of progesterone (Steraloids, injected sc in 0.1 mL of sesame oil) 4 h prior to each training session. Stimulus females were scented with 0.6 mL of pure almond extract (Blue Ribbon), split equally in the back of their neck and anogenital region. Different females were assigned to each male randomly for every training session.

#### Apparatus

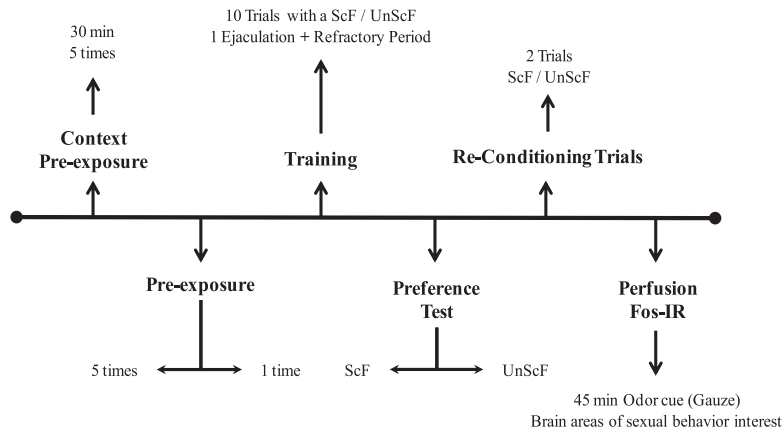
All conditioning sessions were conducted in Plexiglas unilevel pacing chambers (38 × 60 × 38 cm) with bedded floors and bisected by a transparent Plexiglas divider with one-hole large enough for the female to cross but not the male, as it has been previously found that pacing copulation where males have restricted access to a family facilitates the development of a CEP (Ismail et al. 2009). The cage bedding was not changed between conditioning sessions, and animals trained with ScF were trained in separate cages and rooms from the ones trained with UnScF. The final copulatory preference test took place in a large open field (123 × 123 × 46 cm) filled with clean bedding. All sessions were recorded and subsequently scored with using a behavioral scoring program (Cabilio 1996) that counted frequencies and latencies of individual sexual behaviors (e.g., mounts, intromissions, and ejaculations; as in Sachs and Barfield 1976; Pfaus et al. 1990; Meisel and Sachs 1995).

### Procedure

The common procedure of the experiment is depicted in Figure 1.

#### Groups

Males were assigned and equally divided into one of three main groups (see Table 1): five trials of US preexposure (5t), one trial of preexposure (1t), or control groups. During preexposure phase, males in the 5t group copulated five times with receptive females, whereas males in the 1t copulated only once. For this part of the experiment, half of the males in the 1t and 5t copulated with ScF, whereas the other half of males in 5t and 1t copulated with UnScF. Subsequently, during the training trials, males copulated with the opposite female assigned during the preexposure phase. Males in the control group were trained with either ScF or UnScF, only.



**Figure 1.** General experimental procedure.

### Context preexposure

All animals were exposed five times to the chamber in a 4-d interval for ~30 min prior to the preexposure phase (or prior to conditioning in the case of the control groups), in order to habituate them to the training environment, as it has been shown that a novel environment disrupts copulation in sexually naïve rats (Pfaus and Wilkins 1995).

### US preexposure

Preexposure trials consisted of each male copulating in an unilevel pacing chamber with one-hole divider with a receptive female scented or unscented (depending their group), until the first intromission after the first ejaculation.

### Conditioning

Following the preexposure phase, all animals were trained to develop a CEP for a sexually receptive female with or without bearing an almond odor (depending on the group), using a similar protocol to the one described in Kippin and Pfaus (2001b). Rats were trained with ScF or UnScF for 10 conditioning sessions at 4-d intervals during the middle third of the dark phase of the light–dark cycle.

Depending on which type of female the males were preexposed to, half of the animals of each of those groups were given 10 subsequent trials with the other type of female (ScF for the males preexposed to UnScF, or UnScF for the males preexposed with ScF; see Table 1).

During each conditioning trial males were placed into the unilevel pacing chamber for 5 min, after which a sexually receptive female was placed into the chamber to copulate freely with the male. Trials were terminated when the male mounted or intromitted with the female after the refractory period that followed the first ejaculation.

**Table 1.** Preexposure and training group distribution

| Group   | Type of female |          |
|---------|----------------|----------|
|         | Preexposure    | Training |
| Control | -              | ScF      |
|         | -              | UnScF    |
| 5t      | ScF            | UnScF    |
|         | UnScF          | ScF      |
| 1t      | ScF            | UnScF    |
|         | UnScF          | ScF      |

### Copulatory preference test

Four days after the last conditioning trial, each male was placed in a large open-field (123 × 123 × 46 cm) covered with Beta Chip bedding and allowed to explore for 5 min. Subsequently, two females (ScF and UnScF, randomly assigned) were placed into the open field simultaneously, both equally distant from the male, and were allowed to copulate for 30 min. The test was video recorded and scored subsequently for the choice of female for mounts, intromissions, and ejaculations.

### Perfusion

Following the preference test, males were given two reconditioning trials, after which they were exposed for 45 min to 1 mL of the almond odor alone on a gauze

pad on the other side of the pacing chamber with the divider. Subsequently, males were injected with euthanyl (120 mg/kg, i.p.) and perfused intracardially with 250 mL of phosphate buffered saline followed by 250 mL of 4% paraformaldehyde. Brains were extracted and post-fixed in 4% paraformaldehyde for 4 h, to be later on stored for 36 h in a 30% sucrose solution. Finally, the brains were frozen, covered in aluminum foil and stored at –80°C.

### Fos immunohistochemistry

This analysis was performed as in previous studies (e.g., Kippin et al. 2003). Coronal brain sections were incubated sequentially with 30% w/w hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in Tris-buffered saline (TBS) for 30 min at room temperature, 3% normal goat serum (NGS) in 0.05% Triton TBS for 90 min at 4°C, rabbit polyclonal anti-Fos (Oncogene Science; diluted 1:75,000) in 0.05% Triton TBS with 3% NGS for 72 h at 4°C, biotinylated goat anti-rabbit IgG (Vector Laboratories 1:200) in 0.05% Triton TBS with 3% NGS for 1 h at 4°C, and avidin-biotinylated–peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories; diluted 1:55) for 2 h at 4°C. Sections were washed in TBS (35 min) between each incubation. Immunoreactions were stained by sequential treatments at room temperature with 50-mM Tris for 10 min, 3,3'-diaminobenzidine (DAB) in 50-mM Tris (0.1 mL of DAB/Tris buffer, pH 7.8) for 10 min, DAB/3% H<sub>2</sub>O<sub>2</sub> in 50-mM Tris for 10 min, and 8% nickel chloride (400 µL per 100 mL of DAB/Tris buffer H<sub>2</sub>O<sub>2</sub>). Sections were mounted on gel-coated slides and allowed to dry, then dehydrated, cleared in Hemo-D, coverslipped and examined under a microscope. Brain sections were examined at 40×, and the number of Fos-positive cells was counted bilaterally from each region from five different sections per rat (Leitz Microscope) using a computerized image-analysis system (ImageJ).

Brain regions related to sexual behavior and CEP were observed for Fos-IR to evaluate the neural activation evoked by the odor cue used during training. The regions examined were similar to those in Kippin et al. (2003), and were defined using the borders in Paxinos and Watson (1998) including the mPOA (–0.35 mm from bregma), nucleus accumbens shell and core (NAc Shell and NAc Core, respectively, 1.65 mm from bregma), VTA (–6.04 mm from bregma), central nucleus of the amygdala (CeA, –2.80 mm from bregma) and the basolateral nucleus of the amygdala (BLA, –2.80 mm from bregma). An average of Fos-positive cells was calculated from three different slides from each rat (five subjects in each group), for each brain area.

### Statistical analyses

A series of mixed design, between-within repeated-measures ANOVAs were conducted separately for each copulatory measure (mounts, intromissions, ejaculation, and latency to the first ejaculation), displayed among males in the four training groups (1t, 5t, Control ScF, and Control UnScF) with the two receptive females

(ScF or UnScF) on the final open-field test. For each significant ANOVA, post hoc comparisons of the means were made using the Tukey HSD correction to ensure maximal statistical power while correcting for family-wise error. Furthermore, partial eta square ( $\eta_p^2$ ) was calculated as effect size for each comparison. Additionally, a  $1 \times 2$  chi square ( $\chi^2$ ) analysis was conducted for the percentage of first ejaculation choice for each group, and a  $2 \times 2$   $\chi^2$  analysis to contrast the ejaculatory preference between the control groups. Furthermore, Cramer's  $V$  and Phi ( $\phi$ ) effect sizes were conducted as effect size for the  $1 \times 2$  and  $2 \times 2$   $\chi^2$  analyses, respectively.

For the Fos-IR results, the mean of Fos-IR-positive cells for each brain area was compared separately among the experimental groups using independent samples  $t$ -test with a Bonferroni correction of the alpha level to control for the family-wise error (Miller 1966). Since four comparisons were conducted within each brain area, the  $\alpha$  level was set at 0.0125 ( $0.05/4 = 0.0125$ ) for statistically significant differences. Only in the comparisons between the control groups, the  $\alpha$  level was kept at 0.05, since those independent  $t$  tests compared only two groups. Cohen's  $d$  effect size statistics were also calculated as a measure of effect size. The following comparisons between groups were conducted for each brain area of interest: 5t ScF versus 1t ScF, 5t UnScF versus 1t UnScF, 5t ScF versus 5t UnScF, 1t ScF versus 1t UnScF, and ScF control versus UnScF control. The latter comparisons were run separately.

## Results

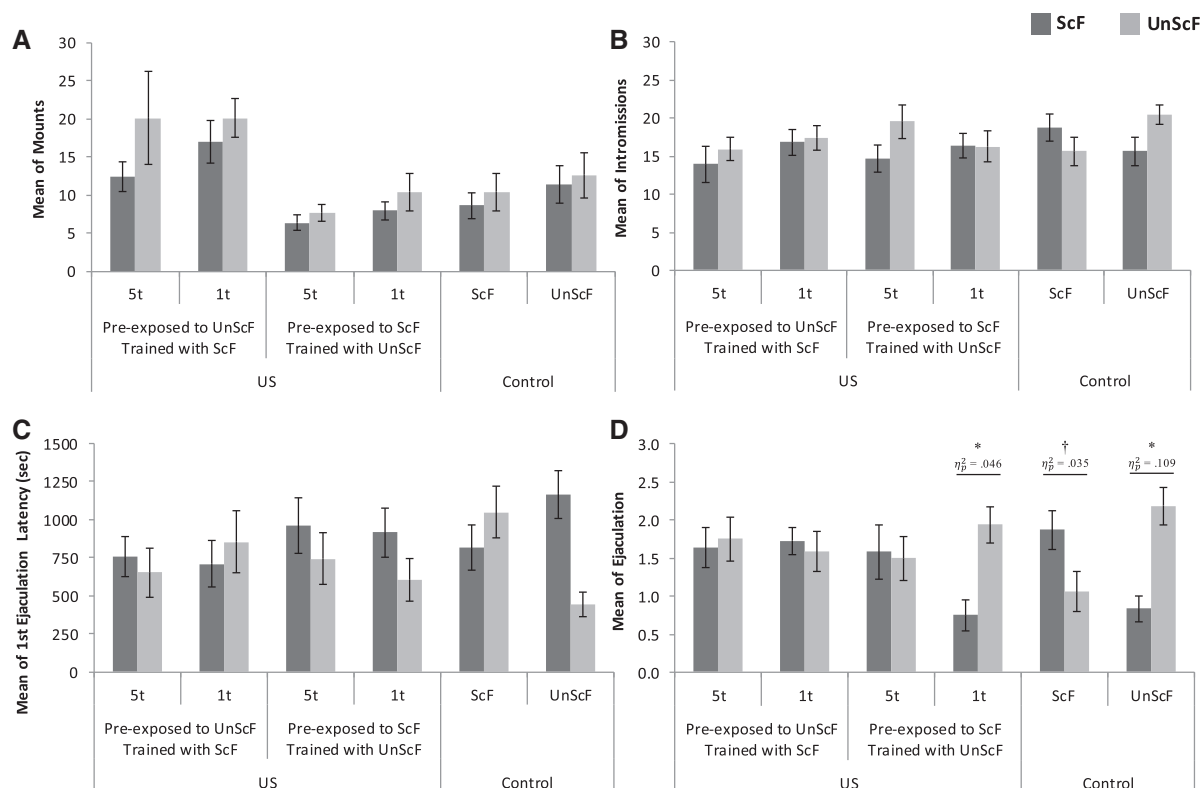
### Behavioral analysis

Four males were not included in the final statistical analyses since they did not copulate in the open-field test. Although five min of exploration in the open field have previously been used effectively as a period of acclimation for males before the open-field test (e.g., Kippin and Pfau 2001b), it is believed that natural differences in novelty aversion vary in such ways that some animals are affected

more than others, and likely show fear responses (such as hugging the walls of an open field) as we observed.

The scores for the different copulatory behavior by female for all groups during the open-field test are shown in Figure 2. Males did not display consistent differences for the distribution of mounts or intromissions between the females among groups. The reliability of these observations was corroborated by two independent 6 (Group: 1t UnScF, 5t UnScF, 1t ScF, 5t ScF, Control ScF, and Control UnScF)  $\times$  2 (Female: ScF, UnScF) repeated-measure ANOVA. No statistically significant interaction between Female  $\times$  Group was found for mounts,  $F_{(5,86)} = 0.609$ ,  $P = 0.693$ ,  $\eta_p^2 = 0.034$ ; nor for intromissions,  $F_{(5,86)} = 1.524$ ,  $P = 0.191$ ,  $\eta_p^2 = 0.081$ .

As shown on panels C and D of Figure 2, the control and the 1t trained with UnScF groups appeared to take less time to ejaculate first with the female they were trained with and chose to ejaculate more with her. The reliability of these observations was partially confirmed by two independent 6 (Group: UnScF 1t, UnScF 5t, ScF 1t, ScF 5t, Control ScF, and Control UnScF)  $\times$  2 (Female: ScF, UnScF) repeated-measure ANOVAs. On one hand, no statistically significant interaction between Female  $\times$  Group was found for the latency of first ejaculation mean,  $F_{(5,86)} = 1.796$ ,  $P = 0.122$ ,  $\eta_p^2 = 0.095$ . However, a significant interaction between Female  $\times$  Group was found for the mean of ejaculations,  $F_{(5,86)} = 3.173$ ,  $P = 0.011$ ,  $\eta_p^2 = 0.156$ . Post hoc comparisons using the Tukey HSD correction revealed that the control UnScF group displayed a statistically significantly higher mean ejaculation toward the UnScF ( $M = 2.19$ ) than to the ScF ( $M = 0.688$ ,  $P < 0.05$ ,  $\eta_p^2 = 0.109$ ), whereas the ScF control displayed a statistically marginally higher mean of ejaculations toward the ScF ( $M = 1.63$ ) than to the UnScF ( $M = 1.06$ ,  $P < 0.1$ ,  $\eta_p^2 = 0.035$ ). Also, the 1t group preexposed to ScF and later on trained with UnScF displayed a statistically significantly higher mean ejaculation toward the UnScF ( $M = 1.94$ ) than the ScF ( $M = 1.0$ ,  $P < 0.05$ ,  $\eta_p^2 = 0.046$ ).



**Figure 2.** Mean of copulatory behaviors ( $\pm$ SEM) per group during the open-field test. ( $\dagger$ )  $P < 0.01$ ; (\*)  $P < 0.05$ ;  $\eta_p^2$  = partial eta square.



The percentage of males that chose ScF or UnScF for their first ejaculation is shown in Figure 3. Males in both 5t groups and in the 1t UnScF group and later on trained with ScF, did not show a significant choice for first ejaculation for either of the females. However, males in ScF 1t group and later on trained with UnScF and both control groups chose to ejaculate first with the female they were training with. These observations were partially confirmed by  $\chi^2$  analyses. No significant differences were found in percentage of first ejaculation choice for the UnScF 5t group trained with ScF group,  $\chi^2(1)=0.25$ ,  $P=0.617$ ,  $V=0.125$ ; nor for the ScF 5t group trained with UnScF,  $\chi^2(1)=0.25$ ,  $P=0.617$ ,  $V=0.125$ ; neither for the UnScF 1t trained with ScF,  $\chi^2(1)=0$ ,  $P>0.9$ ,  $V=0$ . However, statistically significant differences were found in the control UnScF group,  $\chi^2(1)=4.0$ ,  $P=0.046$ ,  $V=0.5$ ; yet no significant differences were found for the ScF 1t trained with UnScF group,  $\chi^2(1)=2.25$ ,  $P=0.134$ ,  $V=0.375$ ; nor on the Control ScF,  $\chi^2(1)=1$ ,  $P=0.317$ ,  $V=0.25$ . However, the effect sizes demonstrated a clear trend for the latter two. Furthermore, a  $2 \times 2$   $\chi^2$  analysis between the two control groups revealed that, overall, they statistically significantly preferred different females to ejaculate first with,  $\chi^2(1)=4.571$ ,  $P=0.033$ ,  $\phi=0.315$ .

### Fos-IR

Figures 4 and 5 show an example of the Fos-IR in each of the brain area of interest for each of the groups. Table 2 displays the mean Fos-IR-positive cell numbers ( $\pm$ SEM) for each group in the brain areas of interest. As shown in Figure 4, following exposure to the odor cue in the group of males preexposed to UnScF and later on trained with ScF, both 5t and 1t groups had a lower Fos-IR activation than the ScF control group in the NAc Core and Shell, VTA, and BLA; whereas only the latter had a higher Fos-IR activation in the mPOA than the ScF control group. As shown in Figure 5, following exposure to the odor cue in the group of males preexposed to ScF and later on trained with UnScF, the 1t group had a higher Fos-IR activation than the 5t group in the mPOA, BLA, and CeA, whereas only in the CeA did the 1t group have a higher Fos-IR activation than the ScF control group. The reliability of these observations was partially confirmed by *t*-test with a Bonferroni correction and *d* effect sizes.

### mPOA

Males in the 5t ScF group had a statistically significantly lower Fos-IR than the 1t ScF group,  $t_{(8)}=-5.28$ ,  $P<0.01$ ,  $d=7.462$ , whereas no statistically significant differences were found between males from the 5t UnScF compared to males in the 1t UnScF group,  $t_{(8)}=$

0.765,  $P>0.05$ ,  $d=1.082$ . No statistically significant differences were found comparing males in the 5t ScF versus 5t UnScF, although the effect size revealed a difference of a high magnitude,  $t_{(8)}=-1.577$ ,  $P>0.05$ ,  $d=2.23$ ; nor for males from the 1t ScF versus 1t UnScF groups,  $t_{(8)}=-1.12$ ,  $P>0.05$ ,  $d=1.585$ .

### VTA

No statistically significant differences were found between males in the 5t UnScF group compared to males in the 1t UnScF group,  $t_{(8)}=-0.13$ ,  $P>0.05$ ,  $d=0.182$ ; nor did males in the 5t ScF compared to males in the 1t ScF group, although the effect size revealed a difference of a high magnitude,  $t_{(8)}=-2.26$ ,  $P=0.053$ ,  $d=3.207$ . Males in the 5t UnScF group had a statistically marginally higher Fos-IR than the 5t ScF group,  $t_{(8)}=-2.76$ ,  $P=0.024$ ,  $d=3.913$ ; whereas no statistically significant differences were found comparing males from the 1t ScF versus 1t UnScF groups,  $t_{(8)}=1.31$ ,  $P>0.05$ ,  $d=1.519$ .

### NAc shell

No statistically significant differences were found comparing males in the 5t ScF versus 1t ScF,  $t_{(8)}=1.22$ ,  $P>0.05$ ,  $d=0.502$ ; nor for males from the 5t UnScF versus 1t UnScF groups,  $t_{(8)}=1.55$ ,  $P>0.05$ ,  $d=0.059$ . Males in the 5t ScF group had a statistically significantly higher Fos-IR than the 5t UnScF group,  $t_{(8)}=3.27$ ,  $P<0.01$ ,  $d=4.631$ ; just like males in the 1t ScF group had a statistically significantly higher Fos-IR than the 1t UnScF group,  $t_{(8)}=5.67$ ,  $P<0.001$ ,  $d=4.161$ .

### NAc core

No statistically significant differences were found comparing males in the 5t ScF versus 1t ScF,  $t_{(8)}=-0.35$ ,  $P>0.05$ ,  $d=1.727$ ; nor for males from the 5t UnScF versus 1t UnScF groups,  $t_{(8)}=0.04$ ,  $P>0.05$ ,  $d=1.637$ . Males in the 5t ScF group had a statistically significantly higher Fos-IR than the 5t UnScF group,  $t_{(8)}=3.28$ ,  $P<0.01$ ,  $d=4.643$ ; whereas males in the 1t ScF group had a statistically significantly higher Fos-IR than the 1t UnScF group,  $t_{(8)}=2.94$ ,  $P=0.0185$ ,  $d=8.031$ .

### BLA

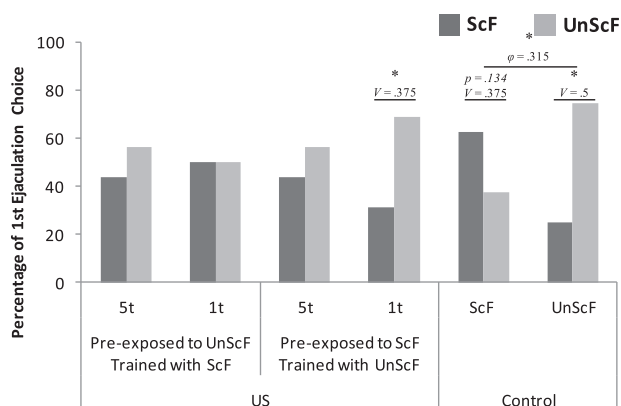
No statistically significant differences were found comparing males in the 5t ScF versus 1t ScF,  $t_{(8)}=-1.6$ ,  $P>0.05$ ,  $d=0$ ; males from the 5t UnScF versus 1t UnScF groups,  $t_{(8)}=-0.01$ ,  $P>0.05$ ,  $d=2.257$ ; males in the 5t ScF versus 5t UnScF,  $t_{(8)}=0.2$ ,  $P>0.05$ ,  $d=0.287$ ; nor between males from the 1t ScF versus 1t UnScF groups,  $t_{(8)}=-1.4$ ,  $P>0.05$ ,  $d=1.986$ .

### CeA

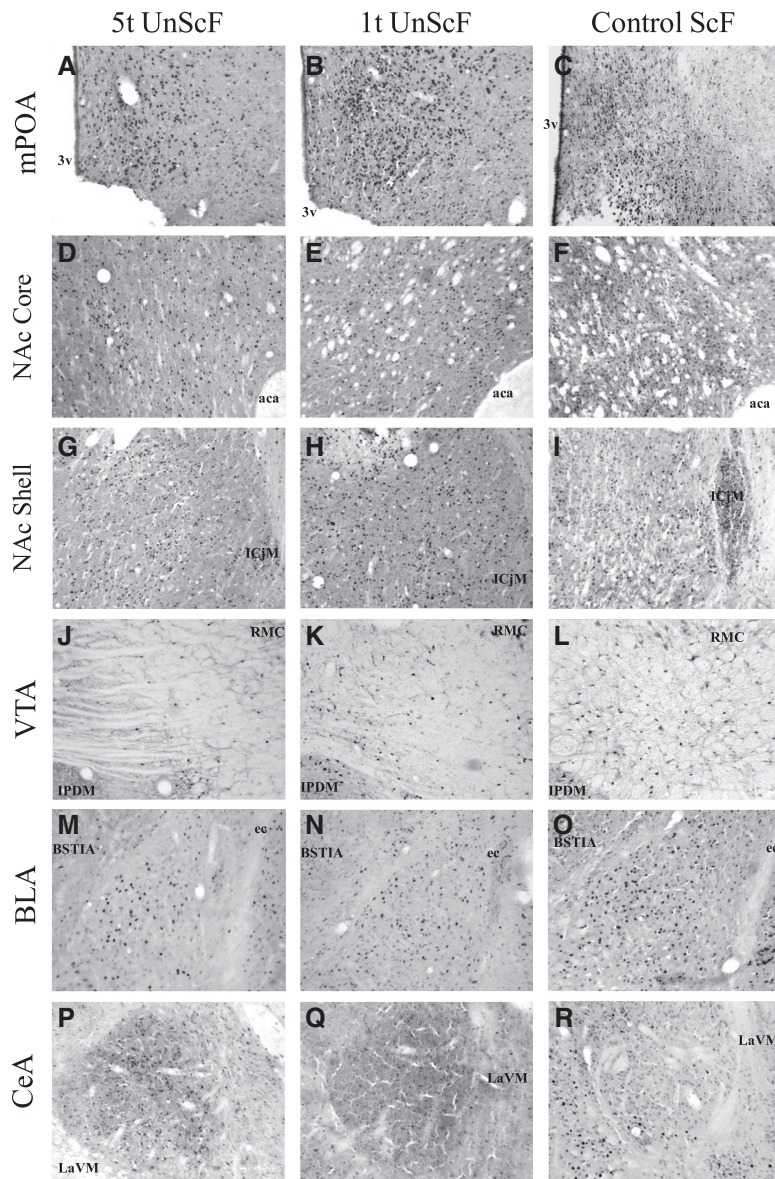
Males in the 5t ScF group had a statistically significantly lower Fos-IR than the 1t ScF group,  $t_{(8)}=-6.07$ ,  $P<0.001$ ,  $d=8.589$ ; whereas no statistically significant differences were found comparing males from the 5t UnScF versus 1t UnScF groups,  $t_{(8)}=1.25$ ,  $P>0.05$ ,  $d=1.772$ . No statistically significant differences were found comparing males in the 5t ScF versus 5t UnScF,  $t_{(8)}=-0.69$ ,  $P>0.05$ ,  $d=0.985$ ; whereas males in the 1t ScF group had a statistically significantly higher Fos-IR than the 1t UnScF group,  $t_{(8)}=6.15$ ,  $P<0.001$ ,  $d=8.714$ .

As shown in Table 2, following exposure to the odor cue, males in the ScF control group had a higher mean of Fos-IR than the UnScF control group in all brain areas, except in the CeA. The reliability of these observations was partially confirmed by an independent *t*-test and *d* effect sizes.

In the mPOA, males in the ScF control group had a statistically marginally higher Fos-IR than the UnScF group, although



**Figure 3.** Percentage of first ejaculation choice per group during the open-field test. (\*)  $P<0.05$ ;  $V$ =Cramer's  $V$ ;  $\phi$ =phi.



**Figure 4.** Fos immunoreactivity (Fos-IR) following exposure to the sexually conditioned odor before perfusion in males preexposed five times to a unscented female before being trained with scented females (5t UnScF), males preexposed one time to an unscented female before being trained with scented females (1t UnScF), and control males trained with scented females (CC+), in brain areas of interest. Pictures were taken according to Paxinos and Watson (1998) coordinates in the mPOA:  $\pm 0.40$  mm from Bregma (A–C); Nucleus accumbens Core and Shell (NAc Core/Shell):  $\pm 1.70$  mm from Bregma (core: D–F, shell: G–I); VTA:  $\pm 6.04$  mm from Bregma (J–L); the BLA:  $\pm 3.14$  mm from Bregma (M–O), and in the Central nucleus of the amygdala (CeA):  $\pm 2.80$  mm from Bregma (P–R). Abbreviations used in the figure: (3v) third ventricle, (aca) anterior commissure, (ICjM) major islands of Calleja, (RMC) magnocellular part of red nucleus, (IPDM) dorsomedial interpeduncular nucleus, (ec) external capsule, (BSTIA) intra-amygdaloid division of the bed nucleus of the stria terminalis, (LaVM) ventromedial part of the lateral amygdaloid nucleus. See Table 2 for  $M \pm S.E.M.$

the effect size revealed a difference of a high magnitude,  $t_{(8)} = 1.99$ ,  $P = 0.08$ ,  $d = 1.18$ . In the *NAc Shell*, males in the ScF control group had a statistically significantly higher Fos-IR than the UnScF group,  $t_{(8)} = 3.11$ ,  $P < 0.01$ ,  $d = 4.392$ . In the *CeA*, males in the ScF control group had a statistically significantly lower Fos-IR than the UnScF group,  $t_{(8)} = -4.56$ ,  $P < 0.001$ ,  $d = 3.823$ . No statistically significant differences were found in any other area,  $P_s < 0.05$ .

## Discussion

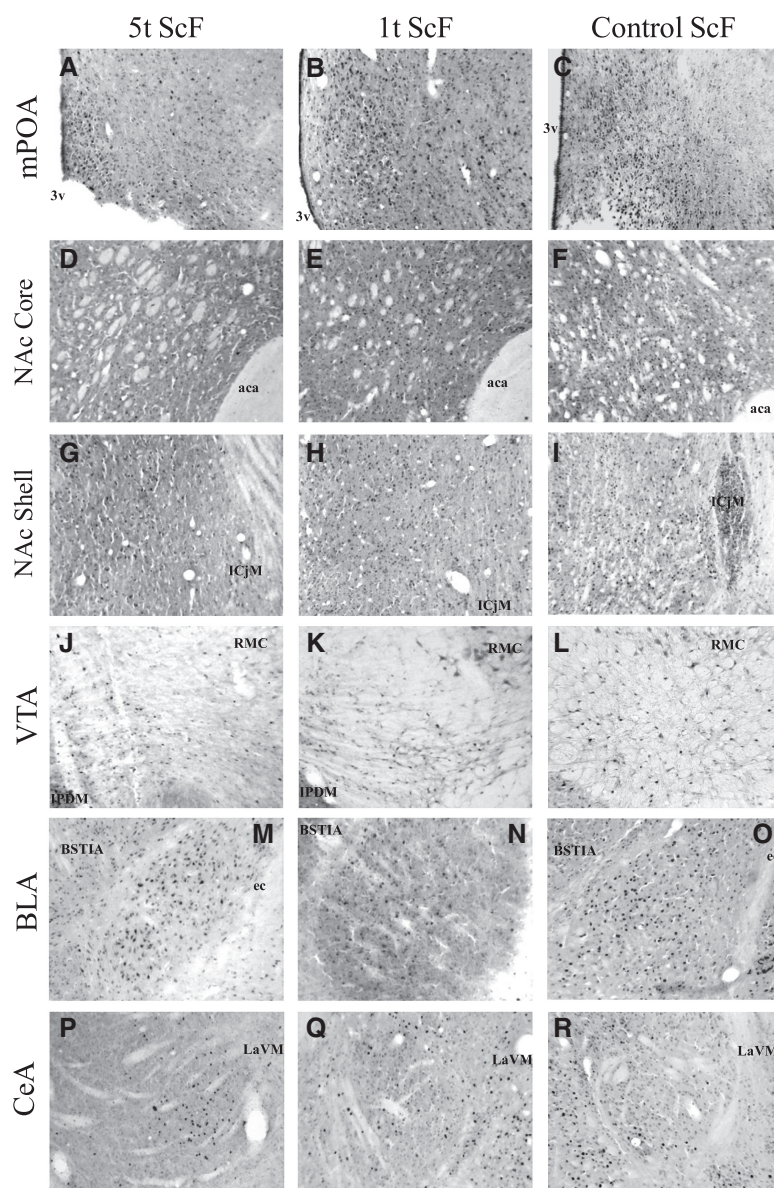
The present study evaluated the impact of US preexposure on the development of CEP in the male rat. Males that were given five sexual experiences with females prior to training (with or without the odor) did not develop a CEP for the subsequently familiar female. Furthermore, the same disruption was found even when only one trial of preexposure was given, but only when the female was not bearing an odor. If the female was scented then males developed a CEP for the female they were trained with. The Fos-IR analyses demonstrated a differential pattern of neural activation regarding the amount of preexposure and the type of female with whom the males underwent the training phase. When compared to the ScF control group, these patterns argue for a differential role on the CS–US associability depending on when these are paired. Previous studies have found that CEP develops when male rats have repeated multi-ejaculatory trials with sexually receptive females bearing a neutral odor such as almond or lemon (e.g., Kippin et al. 2001; Kippin and Pfaus 2001a,b). This effect can be impaired or inhibited when the odor cue is preexposed five times, but not one time, before conditioning (Quintana et al. 2018). Together these studies show that the development of CEP follows Pavlovian rules regarding latent inhibition and US preexposure (e.g., Lubow and Moore 1959; Randich and LoLordo 1979, respectively).

## Behavioral analyses

One of the main findings of the present study was the disruption of the CEP toward the familiar female in the 5t groups preexposed to the US. As previously mentioned, there are two perspectives to interpret the behavioral results on US preexposure, the associative and the non-associative. Moreover, Mis and Moore (1973) concluded that the decremental effect of preexposing animals to a US was in direct correlation to the number of US presentations as well as the US intensity, and inversely correlated with the interval between the last preexposure trial and the first conditioning trial. Either through blocking or the reduction

of the initial emotional reaction toward the US, these findings showed that preexposing sex five times before training a CEP is enough to disrupt it corroborating similar findings on the effect of preexposure of cues related to the training conditions before the conditioning for CEP (Quintana et al. 2018). This was expected given that it has been shown previously that five multiejaculatory trials of 30 min are the minimal amount of conditioning experience necessary to establish a CEP based on neutral odor cue in





**Figure 5.** Fos immunoreactivity (Fos-IR) following exposure to the sexually conditioned odor before perfusion in males preexposed five times to a scented females before being trained with unscented females (5t ScF), males preexposed one time to a scented female before being trained with unscented females (1t ScF), and control males trained with scented females (CC+), in brain areas of interest. Pictures were taken accordingly to Paxinos and Watson (1998) coordinates in the mPOA:  $\pm 0.40$  mm from Bregma (A–C); Nucleus accumbens Core and Shell (NAc Core/Shell):  $\pm 1.70$  mm from Bregma (core: D–F, shell: G–I); VTA:  $\pm 6.04$  mm from Bregma (J–L); the BLA:  $\pm 3.14$  mm from Bregma (M–O), and in the Central nucleus of the amygdala (CeA):  $\pm 2.80$  mm from Bregma (P–R). (3v) third ventricle, (aca) anterior commissure, (ICJM) major islands of Calleja, (RMC) magnocellular part of red nucleus, (IPDM) dorsomedial interpeduncular nucleus, (ec) external capsule, (BSTIA) intraamygdaloid division of the bed nucleus of the stria terminalis, (LaVM) ventromedial part of the lateral amygdaloid nucleus. See Table 2 for  $M \pm S.E.M.$

male rats (Kippin and Pfaus 2001b). Thus, if five trials are enough to establish a CEP, they are also enough to establish an association with the context that could further impair other association trained in the same context. Furthermore, the present study also found a lack of CEP in males preexposure one time to an UnScF and later trained with ScF. It could be possible that the first experience with sexual reward involving ejaculation is strong enough, and an inter trial interval of 4 d is short enough

to establish a Context-US connection that will hinder the subsequent association between any CS-US presentations in which a CEP can be based. However, no available data establish how long lasting these associations may be, nor do they show for how long the disrupting effect of US or CS preexposure might last. Nevertheless, the preexposure effect on the CEP findings, particularly the disruption of the CEP in the 1t group preexposed to an UnScF, confirm the powerful impact of first sexual experiences on the ability to associate a CS with sexual reward.

Considering one copulatory preexposure to the ScF did not block subsequent conditioning to the UnScF, it may be surprising that 1t preexposure to the UnScF blocked later training with ScFs. This may be an example of belongingness or preparedness, a phenomenon where certain CS-US associations are easier to be established than others (Garcia and Koelling 1966; Seligman 1970). This phenomenon was first described in the taste aversion literature, where facilitated acquisition and resistance to extinction was observed between taste and gastrointestinal distress. Likewise, natural pheromonal cues from sexually receptive females are innately preferred by male rats (Carr et al. 1965; Bressler and Baum 1996), eliciting general and sexual arousal (Sachs 1997) and increasing testosterone and luteinizing hormones levels in plasma (Graham and Desjardins 1980). Therefore, it appears that rats may have evolved to display specific “prepared” association between estrous odors and sexual partner receptivity (see Cook et al. 1986). The absence of a disruption in CEP in the males given 1 preexposure to the ScF and trained subsequently with UnScF suggests that a neutral odor requires enough conditioning in order to reach critical salience, unlike prepared cues like estrous odors (also see Kippin et al. 2001) where males did not develop a preference for a ScF after only one training trial. Indeed, estrous odors are able to activate mesolimbic dopamine release unconditionally in the NAc, whereas neutral odors must be paired repeatedly with the post-ejaculatory reward state to induced dopamine release (Pfaus et al. 2012).

## Fos-IR

Analysis of Fos-IR in the brain areas of interest showed a differential pattern of activation depending on the amount of preexposure and training conditions.

As depicted on Figure 4, following exposure to the odor cue, on one hand, males in the 5t group preexposed to UnScF and later on trained with ScF had a lower mean of Fos-positive cells in the



**Table 2.** Mean Fos-IR-positive cell numbers ( $\pm$ SEM) for each group in the brain areas of interest

| Brain area | US 5t UnScF <sup>a</sup> |        | US 1t UnScF <sup>a</sup> |        | US 5t ScF <sup>a</sup> |        | US 1t ScF <sup>a</sup> |        | Control ScF |        | Control UnScF |        |
|------------|--------------------------|--------|--------------------------|--------|------------------------|--------|------------------------|--------|-------------|--------|---------------|--------|
|            | M                        | S.E.M. | M                        | S.E.M. | M                      | S.E.M. | M                      | S.E.M. | M           | S.E.M. | M             | S.E.M. |
| mPOA       | 40.25                    | 2.88   | 104.29                   | 11.79  | 136.47                 | 60.94  | 239.60                 | 120.18 | 127.03      | 20.16  | 82.33         | 13.13  |
| NAC Core   | 55.47                    | 16.69  | 32.00                    | 11.51  | 183.80                 | 35.35  | 137.13                 | 14.50  | 114.46      | 23.15  | 103.29        | 17.23  |
| NAC Shell  | 46.80                    | 22.77  | 48.27                    | 27.00  | 132.51                 | 12.91  | 139.63                 | 15.34  | 161.60      | 14.26  | 101.25        | 13.20  |
| VTA        | 14.29                    | 4.74   | 15.04                    | 3.37   | 1.13                   | 0.39   | 8.80                   | 3.36   | 17.63       | 7.32   | 8.29          | 1.96   |
| BLA        | 9.92                     | 0.97   | 14.23                    | 2.52   | 8.93                   | 4.78   | 8.93                   | 2.81   | 21.03       | 7.33   | 19.63         | 2.10   |
| CeA        | 10.90                    | 4.80   | 4.33                     | 2.11   | 7.42                   | 1.38   | 22.63                  | 2.09   | 7.90        | 1.78   | 14.86         | 1.86   |

mPOA, medial preoptic area; NAC Core, nucleus accumbens core; NAC shell, nucleus accumbens shell; VTA, ventral tegmental area; BLA, basolateral amygdala; CeA, central nucleus of the amygdala.

<sup>a</sup>Type of female to which males were preexposed.

mPOA and CeA than the 1t group preexposed and trained under the same conditions. Additionally, males in the 5t group had a lower mean of Fos-positive cells in the mPOA compared to the ScF control group, whereas males in the 1t group had a higher mean of Fos-positive cells in the CeA compared to the ScF control group. On the other hand, as depicted in Figure 5, males in the 5t group preexposed to ScF and later on trained with UnScF did not significantly differ from the 1t group preexposed and trained in the same conditions in their mean of Fos-positive cells in any of the brain areas of interest, except for the VTA, where the former had a lower mean of Fos-positive cells than the latter. Furthermore, both of them had a lower mean number of Fos-positive cells than the ScF control group in the NAC Core and Shell, and only did the 5t group have a lower mean of Fos-positive cells in the VTA compared to the ScF control group.

Previously, Kippin et al. (2003) examined the Fos-IR in males trained to associate an olfactory cue with sexually receptive females, and compared this to the activation elicited by estrous odors. Following exposure to the estrous odors there was an increase in Fos-IR in the accessory olfactory bulb, medial amygdala, medial bed nucleus of the stria terminalis, mPOA, ventromedial hypothalamus, VTA, and both NAC core and shell. Following exposure to the sexually conditioned odor, Fos-IR increased in the piriform cortex, BLA, NAC core, and the anterior portion of the lateral hypothalamic area. The common activation of the NAC core lead to the authors to suggest that estrous and sexually conditioned odors are processed by a common neuronal system in that brain area (Kippin et al. 2003). In the present study, US preexposure of either, ScF or UnScF, led to a lower Fos-IR compared to the control ScF group in almost all brain areas studied. It is worth mentioning that Kippin et al. (2003) used bilevel chambers, where the chasing dynamic between male and female is completely different from that in the unilevel pacing chambers used in this study (Ismail et al. 2009).

A companion study of CS cue preexposure prior to CEP training yielded similar decrements in Fos-IR in several brain areas (Quintana et al. 2018). Therefore, a general decrement in Fos-positive cells elicited by the odor in comparison to the ScF control group in the brain areas of interest may be due to a different pattern of associability for the odor, depending the contingency between the preexposure and training phase. It is interesting that the mPOA and VTA/NAC appear to be processing the odor differently. First, the mPOA seems sensitive to the odor in a combined function between the number of trials of preexposure and when this was paired with sexual reward, whereas the VTA and NAC seem to be processing the odor mostly according to the training contingencies. This pattern suggests that the mPOA may be balancing the reward prediction value, whereas the VTA and NAC predict the contingencies of when the odor may be predicting the reward. Furthermore, the CeA appears to be doing the opposite than the

ScF control, also doing the opposite than what the mPOA may be doing.

The pattern by which this general decrement varies depending on the contingencies of preexposure and training are discussed by brain area.

### mPOA

The mPOA is a critical brain region that controls male sexual arousal and behavior, where every sensory modality sends indirect inputs (see Dominguez and Hull 2005; Hull and Rodriguez-Manzo 2009). More specifically, it is believed that the mPOA controls erection and copulatory behavior, but not purely motivational aspects of sexual behavior (Everitt 1990). Lesions have shown to impair or completely abolish male sexual behaviors (Hull et al. 2006), and electrophysiological stimulation has shown to facilitate it, yet not reverse sexual satiation (Rodriguez-Manzo et al. 2000). More specifically, Fos-IR has shown to increase in the mPOA in response to copulatory stimulation (Baum and Everitt 1992), and although it has not shown to increase due to the exposition of a neutral cue paired with sexual reward in male rats (Kippin et al. 2003), the ScF control group of this study had a higher Fos-IR than the UnScF control group. Thus, a lower Fos-IR in males preexposed five times to ScF and later on trained with UnScF than the ones preexposed one time may suggest that the odor may have been impaired from fostering rewarding associations with copulation given the preexposure manipulation, thus impairing a CEP otherwise found in the UnScF control group. This highlights the amount of training as a factor that influences trained associations as seen before in the parametric parameters of the development of a CEP (Kippin et al. 2001). Furthermore, no decrement of Fos-IR was found in the mPOA in males preexposed to UnScF. Interestingly, males preexposed 1t and later on trained with ScF had a higher activation than all groups. This suggests that in the mPOA, the pattern of associability for a neutral olfactory cue depends on the contingencies of training, just like the amount of training. However, other prepotent cues like estrous odors would be more readily or easily be associated with the rewarding aspects of sex, as demonstrated by disruption in the CEP and high Fos-IR found in the mPOA of males preexposed 1t and later on trained with ScF.

### NAC core and shell

As one of the terminal brain region of the mesolimbic dopaminergic pathway, the NAC has been associated with reinforcement and appetitive behavior, and attention to sexual incentive cues (e.g., Hull et al. 2006). As a CS-US integrator, the mesolimbic terminal regions focus the necessary attention on conditioned incentive cues to direct motor output toward them enabling animals to engage in copulation, which ultimately results in ejaculation. This

allows the sexual reward state to be cued and predicted by a CS (e.g., Pfaus et al. 2010). Lesions of this area increased the refractory period and decreased noncontact erection, yet not impeding males from copulating (Liu et al. 1998). Moreover, using excitotoxic lesions in the NAc of male rats, several behaviors like mounts, intromissions, noncontact erections, among others, were partially hindered, yet not abolished (Kippin et al. 2004). Recordings from the NAc of male rats after being exposed to novel female estrous odors showed a greater response than the estrous of a familiar female (Wood et al. 2004). Using microdialysis, another study found that this response was shown to be related to an increase of extracellular dopamine in the NAc (Wenkstern et al. 1993). Furthermore, copulation has shown to increase Fos-IR in the NAc of male rats (Robertson et al. 1991), just like estrous female odors did in both, the Shell and Core, whereas a neutral cue paired with copulation did as well, yet only in the Core (Kippin et al. 2003). Similarly, Lopez and Ettenberg (2002) also found a higher Fos-IR activation in the NAc of males exposed to an estrous female versus a nonestrous female. This effect was greater in sexually experienced males than in naïve ones. Therefore, a reduced Fos-IR in the NAc in males preexposed to UnScF and later on trained with ScF may suggest that the odor did not have a strong incentive value as it was for the ScF group, as it can be seen also through the absence of a CEP in both groups. It is believed this effect is driven by US-context associations that blocked the associability. Furthermore, the Fos-IR in the NAc of males preexposed to ScF and later on trained with UnScF provides further evidence for the differential pattern of associability of neutral cues depending when they are associated with sexual reward. Namely, the odor incentive value still remains high, although preexposing an odor cue five times before training a CEP based on the same preexposed cue. Finally, as previously mentioned, lesions in this brain area increased the refractory period (Liu et al. 1998), while showing a higher Fos-IR in males trained to associate an olfactory cue with sexual reward (Kippin et al. 2003); an effect that corresponds with the general higher latency of ejaculation found in both group of males preexposed to a ScF and later on trained with UnScF.

### VTA

As previously stated, the VTA is the source of the mesolimbic dopamine pathway, thought to control or mediate different appetitive behaviors and attention toward reward-related stimuli and their incentive salience (Berridge 2007). The VTA and the NAc are connected largely via dopamine neurons that terminate in the NAc and are activated mainly, but not exclusively, in response to reward-related cues (Berridge 2007; Pfaus 2009). Lesions to this brain area disrupt sexual behaviors and increase the duration of the post-ejaculatory interval, but not to abolish copulation (for review, see Hull et al. 2006), whereas electrophysiological stimulation facilitated copulatory behavior in the male rat (Markowsky and Hull 1995), an effect found to be dependent of which portion of the VTA was stimulated (Rodríguez-Manzo and Pellicer 2007). Fos-IR increases in the VTA of male rats in response to female estrous odors, but not to a conditioned neutral odor paired with sexual reward (Kippin et al. 2003). Therefore, a lower Fos-IR count in the males preexposed five times to ScF and later on trained with UnScF in comparison to the ScF control group may suggest a reduction in the incentive value attributed to the odor cue. Also, as seen with the NAc, there was no reduction in the Fos-IR count in the group of males preexposed to ScF and later on trained with UnScF, corroborating the differential pattern of association. This pattern of association depending on when the odor is paired with sexual reward, and thus having a differential pattern of activation between brain areas, may provide further evidence for the notion of the mPOA as the main brain area that encodes the value of

the reward, and the VTA–NAc as brain areas that encode for the incentive value of the reward (GR Quintana, M Birrel, S Marceau, N Kalantari, J Bowden, Y Bachoura, E Bourdas, V Lemay, C Mac Cionnaith, JG Pfaus, in prep.).

### CeA

The amygdala and its functions have been well documented in the sexual behavior of the male rat (e.g., Swanson and Petrovich 1998). As an arrangement of different nuclei, the amygdala has been regarded as an integrative site between chemosensory, somatosensory, and hormonal cues, projecting to hypothalamic areas playing a role in learning, motivational states, and sexual behavior (Everitt, 1990). Several studies have been conducted exploring the role of the medial and basolateral subnuclei of the amygdala in the male sexual behavior (see Hull and Rodríguez-Manzo 2009), yet much less work has been done on the role of the CeA. GABA-like immunoreactivity in the brain of monkeys revealed a very dense array of predominantly GABA neurons and projections to other brain areas (McDonald and Augustine 1993). CeA inputs come from several cortical, thalamic, and brainstem areas, including the prefrontal insular, temporal and olfactory cortical areas, caudal thalamus, as well as almost all other sub-nuclei of the amygdala (Swanson and Petrovich 1998). The CeA is involved in the modulation of conditioned fear (van de Kar and Blair 1999). For instance, a study done with male prairie voles indicated that there was an increase in Fos-IR in response to cohabitation with an unfamiliar unrelated male (Cushing et al. 2003). This response was explained in terms of an increased response of anxiety and stress, since a reduction in anxiety has been previously found due to lesions of the CeA, but not in the BLA of animals performing anxiety-like tasks (Möller et al. 1997). Therefore, a higher Fos-IR in males preexposed one time to ScF and later trained with UnScF than the ScF control group suggests an inhibitory activation in response to the odor, consistent with the absence of CEP and the completely different pattern found in the mPOA. Also, a higher Fos-IR was found in the UnScF control compared to the ScF control, also the opposite of what was found in the mPOA. Once again, further experiments are necessary to corroborate these suggestions.

### BLA

This brain region is another of several subnuclei of the amygdala and has been studied extensively in the of classical and operant fear conditioning (e.g., Fanselow and LeDoux 1999). The BLA sends direct projections to NAc that have been implicated in sexual incentives, yet not with copulation (Everitt et al. 1989). Lesion studies have shown to impair the operant response associated with sexual reward, yet copulation remained identical to control animals (Everitt et al. 1989). As previously mentioned, Fos-IR increased following the exposure of a sexually conditioned odor in the BLA of male rats (Kippin et al. 2003). Although none of the comparisons resulted in significant differences or meaningful effect sizes, there was a decrement in the Fos-IR pattern in all preexposed groups compared to the ScF control group in the BLA. It is uncertain to explain this general decrement in all groups taking into account the previous findings of other studies and the limited knowledge of this brain area in the context of sexual reward. However, a decrement in both 5t and 1t groups preexposed to UnScF and later on trained with ScF may contribute to explain the decrement in the same groups found in their NAc core (and perhaps NAc shell, as well), considering that the BLA sends direct glutamatergic projections to this brain region that facilitate motivated-behavioral responding (Stuber et al. 2011), like the odor cue is believed to modulate in these males. However, further replication on the differential general pattern of decrement of this

region is needed to properly determine why preexposure to UnScF and not to ScF may have led to a decrement in Fos-IR in the NAC core and shell.

Taken together, the results of this study provide further emphasis on the role of first sexual experiences in the male rat, and how these modulate future sexual preferences. Particularly, five trials of preexposure hindered the display of a CEP for either female. Conversely, being preexposed once to a ScF, and later trained with UnScF developed a preference for the latter, whereas being preexposed once to the UnScF, rendered a similar result as being preexposed five times. Furthermore, the Fos-IR data also argue for a differential role on the associability of neutral cues paired with sexual reward depending on when these come together, either early or later on in sexual experience, in which different areas would code for different aspects of the CS processing. What is the extent of these effects on partner preference, how long do they last, or what constitute as early or late in sexual experience, requires further experimentation.

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## First sexual experiences determine the development of conditioned ejaculatory preference in male rats

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# Conditioned Partner Preference in Male and Female Rats for a Somatosensory Cue

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Male and female rats form a conditioned preference to copulate and/or mate with conspecifics bearing an odor that was paired with either the postejaculatory reward state in males, or paced sexual contact in females, making the odor act as a discrete partner-related cue. Here, we asked whether a somatosensory cue, a rodent jacket, could act as a discrete cue to establish a conditioned partner choice (CPC). In the first study, sexually naïve Long-Evans males and females underwent 14 copulatory conditioning trials for 30 min with their opposite sex partner in unilevel pacing chambers. On the final test, each experimental male or female was placed into an open field with two sexually receptive partners, one jacketed and the other unjacketed. A trend was found for more males to ejaculate first with jacketed females relative to the unjacketed females, whereas the females had no preference. Males and females in the second study were exposed sequentially to jacketed, sexually receptive partners, and unjacketed, sexually nonreceptive partners prior to a final open field test. Both males and females displayed a significant CPC for the jacketed opposite sex partner. This study demonstrates that a somatosensory cue previously used to establish sexual arousal as a contextual cue on rats can be used as a discrete, partner-based cue to establish a CPC for a particular partner wearing the jacket and that stronger conditioning occurs when the jacket is explicitly paired with the sexual reward state.

**Keywords:** conditioned partner preference, fetishism, sexual behavior

To engage sexually with a partner, all animals need to recognize partner-related cues that denote hormonal status and sexual receptivity, along with their own internal state of sexual arousal and desire. Consequently, the mechanisms of sexual arousal, desire, and reward strengthen certain behaviors and weaken others molding the preferences and strategies that will determine copulatory partner preferences (Pfaus et al., 2012).

The modulation of the preferences that animals will establish based on their experiences is a product of Pavlovian learning mechanisms (Pavlov, 1927). In Pavlovian conditioning animals develop a predictive association between a neutral cue, or conditioned stimulus (CS), that is paired with a biologically relevant stimulus, an unconditioned stimulus (US). With sufficient temporal contiguity, the CS will be able to elicit a conditioned response (CR) similar to the one that the US elicits. For example, female

and male rats have shown to develop a conditioned partner choice (CPC) for an odor cue (e.g., almond or lemon) on their partners after being paired with sexual reward (e.g., Coria-Ávila et al., 2006; Kippin, Talianakis, Schattmann, Bartholomew, & Pfaus, 1998). In these experiments, animals were assigned to three groups. A paired group, where males underwent 10 multicopulatory trials with scented sexually receptive females; whereas females underwent four multicopulatory trials with pacing copulation (i.e., four-hole divider) and four multicopulatory trials with no divider, both with sexually receptive males. The unpaired group, who received the opposite contingency of association to the odor than the paired group. Finally, a randomly paired group, where the odor was randomly paired with receptive and nonsexually receptive females for the males and pacing or no pacing copulation for the females. Subsequently, rats were placed in an open field where they had access to two sexually receptive partners, one scented with the almond odor and the other unscented. The results of both studies showed that animals in the paired condition chose to copulate more with the scented partner, whereas in the unpaired group the preference was for the unscented partner. Finally, the randomly paired groups did not prefer one partner over another. More specifically, males chose to ejaculate first and more times with the scented female, whereas females solicited and chose the first ejaculation more from the scented male (Coria-Ávila et al., 2006; Kippin, Talianakis, Schattmann, Bartholomew, & Pfaus, 1998).

Alternatively, other studies have shown that female rats given distributed or paced clitoral stimulation in the presence of an almond-scented gauze pad copulated and solicited more the scented male rat during their first copulatory experience in an open

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field test (Parada, Abdul-Ahad, Censi, Sparks, & Pfaus, 2011). Even unconditionally aversive odors can become less aversive and conditionally appetitive following pairing with a sexual reward US. Males that underwent multiejaculatory training trials with a sexually receptive female scented with cadaverine rapidly approached and displayed appetitive responses toward a cadaverine-scented dowel, whereas males in the control groups that did not copulate with cadaverine-scented females immediately ran away from the dowel or tried to bury it (Pfaus, Kippin, & Centeno, 2001). Thus, neutral or even aversive odors can become appetitive CSs if they are paired with the sexual reward state, leading them to become discrete, partner related cues that an animal forms a preference for.

Other sensory modalities have also been tested to be associated with sexual reward. Domjan, Huber-McDonald, and Holloway (1992) trained Japanese quail males to copulate with an inanimate object. In their experiment, male quail were divided into two groups. Both groups were presented with an inanimate taxidermic female quail with which they could copulate for 30 s. followed by access to a sexually receptive quail hen. In the fading group the taxidermic object was gradually covered with terrycloth over successive trials, until fully covered leaving no quail features in the last trial of training. The nonfading group was always presented with the fully covered inanimate object. After the training, each subject was tested in the training boxes for 5 min with the fully covered inanimate object, except that no live female quail was introduced. Overall, males trained in the fading group spent more time around the object and displayed more copulatory behaviors toward the fully covered inanimate object than the males trained in the nonfading group in the form of grabs, mounts, and cloacal contacts. These data demonstrated that sexual behavior can be conditioned toward an inanimate stimulus object that has no natural connection with sexual reward (Domjan et al., 1992).

Similarly, Köksal et al. (2004) demonstrated persistence in copulation with an inanimate object after an extinction procedure only when the trained CS was the same terrycloth used by Domjan et al. (1992), but not when it was a light. In their experiment, male quails were trained to associate, in different training trials, a terrycloth stimulus or a flashlight with the presentation of a sexually receptive female quail. A different group was used as a control trained without the presentation of the female. After 30 trials of acquisition, all males underwent 30 more trials of extinction in which males were exposed to the CS without having access to a female. The results showed that, during the extinction phase, quail males who were trained with the terrycloth as a CS persisted on copulate (i.e., mounted and displayed cloacal contact responses) with the object, unlike males in which the CS was a light. A second experiment in which the terrycloth and light were paired with food as US did not find difference between the groups during the extinction phase (Köksal et al., 2004). Furthermore, male Japanese quail that were already conditioned to copulate with the inanimate object were presented with it 30 s. before copulation for fertilization with a live female quail. These males, although slower and less efficient in achieving cloacal contact with the female compared with a control group that was not conditioned to prefer the inanimate object, fertilized a greater proportion of eggs than control males, indicating that their fetish-like preference was linked to higher reproductive success (Cetinkaya & Domjan, 2006).

Neutral somatosensory CS can also be conditioned to modulate sexual arousal. Pfaus, Erickson, and Talianakis (2013) tested how a somatosensory cue could affect the sexual arousal of male rats. They trained males to have their first and subsequent 10 sexual experiences with or without wearing a rodent tethering jacket. On the final test, rats in both groups were randomly assigned to have the jacket on or jacket off. Males trained and tested with the jacket on copulated normally, as did males trained without the jacket and tested with it. However, significantly fewer males trained with the jacket copulated to ejaculation with the jacket off; and those that did displayed significantly fewer mounts, intromissions, anticipatory level changes, and ejaculations. A second experiment showed that the jacket could acquire inhibitory properties if it was on the male when he was paired with a sexually nonreceptive female. Males trained to associate the jacket with a sexually nonreceptive female had fewer ejaculations, large ejaculation latencies, and fewer conditioned level changes compared with males for whom the jacket was paired with sexually receptive females and the post ejaculatory sexual reward state. Altogether, these results demonstrated that a somatosensory cue can be conditioned to excite or inhibit sexual arousal in male rats.

Here we asked whether the tethering jacket could be used as a discrete, partner-related cue like the odor in our previous experiments. The first experiment determined if the jacket on the partner during training result in a CPC based on the jacket. The second experiment determined if conditioning sequentially the presence of the jacket with sexual excitation and the absence of it with inhibition, would enhance the associability of the jacket for a CPC.

## Method

### Animals and Surgery

Seventy-five female Long-Evans rats, weighing 150 g to 200 g, and 60 male Long-Evans rats, weighing 200 g to 250 g, were obtained from Charles River Canada (St. Constant, Quebec, Canada) at 6 weeks of age. All animals were kept in a 12 hr reversed light/dark cycle in a room at 21°C with ad libitum tap water and regular rat chow. All animal procedures conformed to the guidelines of the Canadian Council for Animal Care and were approved by the Concordia University Animal Research Ethics Committee. Nonexperimental females and males used as stimuli were obtained from the same breeder and were maintained under identical conditions.

Females were pair-housed in Plexiglas cages with woodchip bedding. Sexual receptivity was induced after the ovariectomy by subcutaneous injections of 10 µg (in 0.1 ml of sesame oil) of estradiol 48 hr prior each training session and 500 µg (in 0.1 ml of sesame oil) of progesterone 4 hr prior to each training session. Nonsexually receptive females did not have hormonal replacement. Males were housed in groups of four in Plexiglas cages with ad lib access to water and food (Purina Rat Chow).

**Ovariectomy.** Females were ovariectomized via bilateral lumbar incisions under general anesthesia induced with ketamine (50 mg/ml)/xylazine (4 mg/ml) at a ratio of 4:3, respectively, around 2 weeks before the beginning of the experiment to allow at least 1 week of recovery. This procedure allowed for hormone levels to be controlled under hormonal replacement throughout training sessions.



**Orchiectomy.** Six male rats used for training were gonadectomized via testicular incision through inhalation anesthesia with 5% Isoflurane. The surgery was conducted 2 weeks before the beginning of the experiment to allow at least a week of recovery and for testosterone in plasma to washout completely.

## Materials and Testing Apparatus

The somatosensory cue was a tethering rodent jacket (Lomir Biomedical, Ile Perrot, Quebec, Canada; see Figure 1.) made of a double layer of Lycra/Spandex fabric. This jacket covered the upper part of the torso, with open holes for the forearms and fastened across the back with Velcro. All conditioning trials were conducted in Plexiglas unilevel pacing chambers (38 cm × 60 cm × 38 cm) with bedded floors and bisected by a transparent Plexiglas divider. Males had a one-hole divider considering the facilitation for CPC in males over a four-hole divider (Ismail, Gelez, Lachapelle, & Pfaus, 2009), whereas females had either no divider or a four-hole divider big enough to allow only the female to cross through, depending of the experiment. Each hole in either of the dividers was 4 cm × 5 cm. The one-hole divider was located in the bottom middle of the divider, while the four-hole divider had holes with the same dimensions equally distributed along the bottom line of the divider, each separated by 20 cm. Copulatory preference tests took place in an open field (123 cm × 123 cm × 46 cm) filled with clean bedding. In the case of female experiments, the open field had two transparent four-hole dividers that divided the open field in three equal parts allowing only the female to cross through to the side where the male was sequestered. Females were placed initially in the middle compartment and returned to it before placing the males into the open field. These partitions did not allow males to cross or interact at any point during the test. All final tests were recorded and subsequently scored with specialized behavioral observation software (Cabilio, 1996).

## Conditioning Procedure

There were two experiments of 14 conditioning trials for 30 min each, at 4-day intervals during the middle third of the dark phase of the light/dark cycle. There were two experiments for each, experimental male and female.

**Females.** There were two female experimental groups. The paired group underwent seven trials with jacketed males with a four-hole pacing divider, and seven trials with no divider and unjacketed males, whereas in the unpaired group, the jacketed males were paired with no divider, and the unjacketed males were paired with a four-hole divider. All trials were randomly assigned.

For the second experiment, there were two new groups of females. The paired group underwent seven trials with sexually

receptive jacketed males with a four-hole divider and seven trials with no divider and nonsexually receptive unjacketed males; whereas for the unpaired group, the jacketed males were paired with no divider and nonsexual receptivity, and the unjacketed males were paired with a four-hole divider and sexual receptivity. Animals were randomly assigned to groups prior to the experiment.

**Males.** In the first experiment, there were two groups of males, a paired group trained with sexually receptive females wearing the rodent tethering jacket, and an unpaired group trained with sexually receptive females not wearing a jacket. All trials were randomly assigned and conducted in unilevel pacing chambers with one-hole dividers.

In the second experiment, there were also two new groups of experimental males. A paired group underwent seven trials with sexually receptive jacketed females and seven trials with nonsexually receptive unjacketed females, whereas for the unpaired group, the sexually receptive females did not wear jackets, and the nonsexually receptive females did wear jackets. Animals were randomly assigned to group.

## Final Copulatory Preference Test

Four days after the 14th conditioning trial, each male or female was placed in an open field for 5 min after which two sexually receptive opposite sex rats were placed simultaneously into the open field, one jacketed and the other one unjacketed. Rats were allowed to copulate freely with both sex partners for 30 min. The criteria used to code male behaviors (mount, intromissions, and ejaculation) were those described by Sachs and Barfield (1976) and Meisel and Sachs (1995). The number of female proceptive sexual behaviors made toward either male was scored as full solicitations (defined as a headwise orientation to the male followed by a runaway), *hops and darts* (defined as intermittent proximal approaches toward and withdrawals from a male that involve hopping over the male or darting by him), and kicks (back kick to the male) as in Hlíňák & Madlafousek (1977) and McClintock, Anisko, and Adler (1982). Consummatory copulatory behaviors associated with female receptivity (lordosis quotients and reflex magnitudes) were determined, along with the proportion of mounts, intromissions, and ejaculations received from either male by the female or given to either female by the male to assess partner preference.

## Statistical Analyses

A series of repeated-measures analyses of variance (ANOVAs) were conducted to explore the interaction between type of partner (jacket on vs. jacket off) and group for different proceptive and copulatory behaviors of male and females separately in each experiment. Given statistically significant differences, post hoc multiple comparisons were conducted for each single variable to compare jacket on and jacket off using Tukey's honestly significant difference correction. Furthermore, partial eta square ( $\eta_p^2$ ) was calculated as effect size for each comparison. Additionally, a 1 × 2 chi-square analysis was conducted for the percentage of first ejaculation choice for each group, and a 2 × 2 chi-square analysis to contrast the ejaculatory preference between the control groups. Furthermore, Cramer's V and phi ( $\phi$ ) effect sizes were conducted

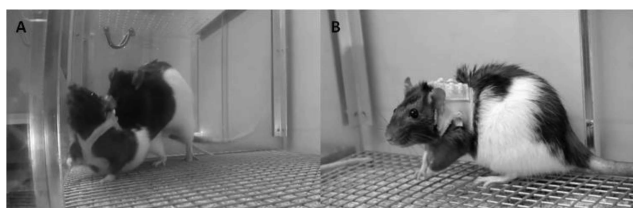


Figure 1. Panel A: Copulation with jacket on. Panel B: Female with jacket on.

as effect size for the  $1 \times 2$  and  $2 \times 2$  chi-square analyses, respectively.

## Results

### Females

**Experiment 1.** Figure 2 shows the averages for the different proceptive and copulatory sexual behaviors of females during the open field by condition, group, and experiment. As shown in Panels A, B, C, D, E, and F, for Experiment 1 there were no apparent trends to prefer either jacket on or jacket off male partner

as seen in the sexual behaviors displayed for either of the groups. The reliability of these observations was corroborated by a 2 (group: paired, unpaired)  $\times$  2 (male: jacket on, jacket off) repeated-measures ANOVA for each of the aforementioned sexual behaviors.

For hops and darts, there was no significant main effect of male,  $F(1, 36) = .042, p = .838, \eta_p^2 = .001$ , nor an interaction between Group  $\times$  Male,  $F(1, 36) = 1.434, p = .239, \eta_p^2 = .038$ . For solicitations, there was no significant main effect of male,  $F(1, 36) = .72, p = .402, \eta_p^2 = .02$ , nor an interaction between Group  $\times$  Male,  $F(1, 36) = 1.481, p = .231, \eta_p^2 = .04$ . For kicks, there was

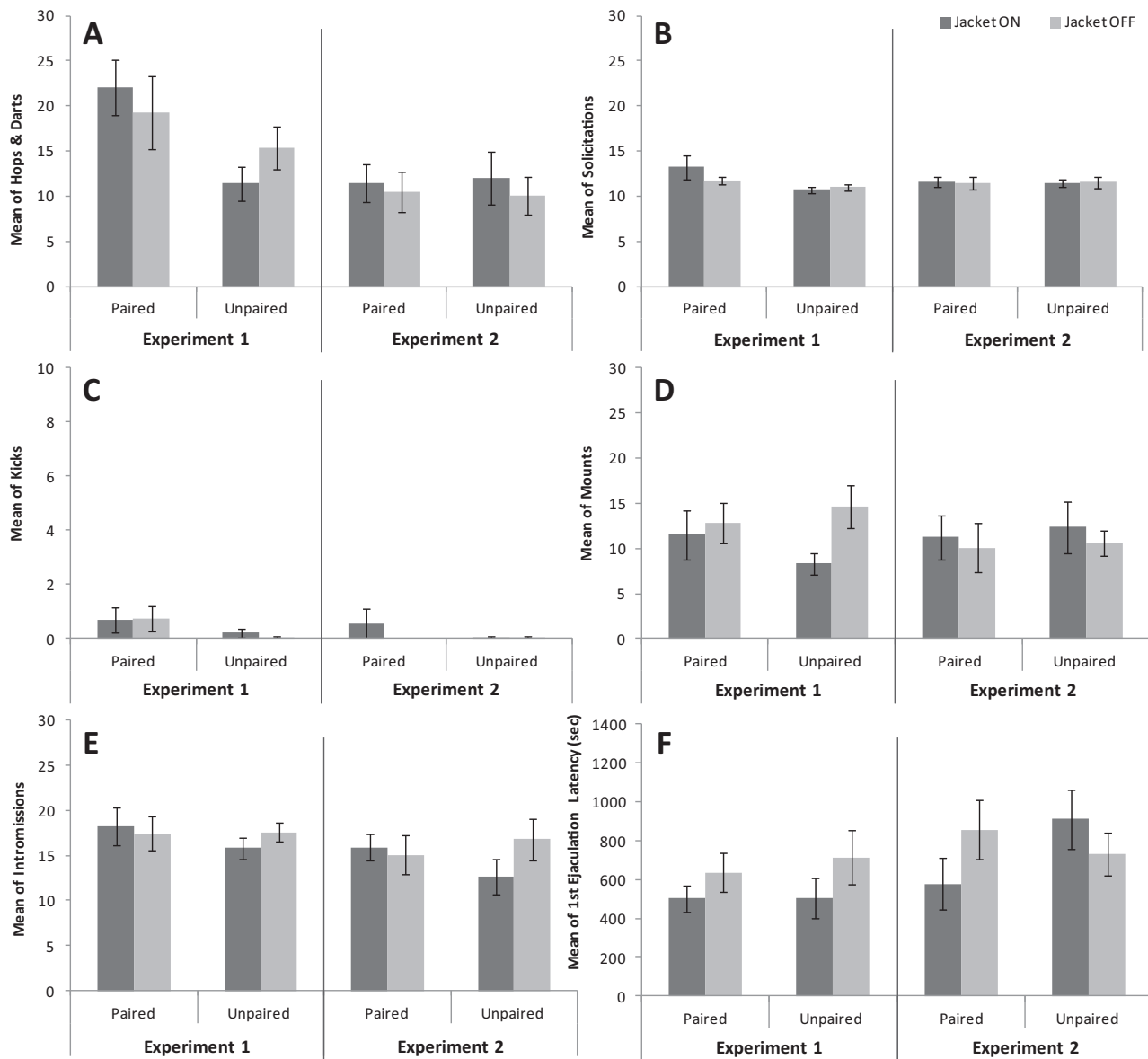


Figure 2. Averages for the different proceptive and copulatory sexual behaviors of females during the open field by condition (jacket on vs. jacket off), group (paired vs. unpaired), and experiment (Experiment 1 and Experiment 2). Panel A: hops and darts. Panel B: solicitations. Panel C: kicks. Panel D: mounts. Panel E: intromissions. Panel F: first ejaculation latency.

no significant main effect of male,  $F(1, 36) = .032, p = .858, \eta_p^2 = .001$ , nor an interaction of Group  $\times$  Male,  $F(1, 36) = .129, p = .722, \eta_p^2 = .004$ . For mounts, there was no significant main effect of male,  $F(1, 36) = 2.753, p = .106, \eta_p^2 = .071$ , nor an interaction of Group  $\times$  Male,  $F(1, 36) = 1.182, p = .284, \eta_p^2 = .032$ . For intromissions, there was no significant main effect of male,  $F(1, 36) = .101, p = .752, \eta_p^2 = .003$ , nor an interaction between Group  $\times$  Male,  $F(1, 36) = .72, p = .402, \eta_p^2 = .02$ . Finally for mean of 1st ejaculation latency, there was no significant main effect of male,  $F(1, 36) = 2.16, p = .15, \eta_p^2 = .057$ , nor an interaction of Group  $\times$  Male,  $F(1, 36) = .099, p = .755, \eta_p^2 = .003$ .

Figure 3 shows the preference scores of average numbers of ejaculations taken from each male and first ejaculation choice taken by females during the open field by male, group, and experiment. As Panel A shows, for the mean of ejaculation, there was a significant main effect of male,  $F(1, 36) = 4.034, p = .052, \eta_p^2 = .101$ , but no interaction of Group  $\times$  Male,  $F(1, 36) = .161, p = .69, \eta_p^2 = .004$ . Post hoc Tukey comparisons showed that, although both group of females displayed a higher preference to receive ejaculations with the jacketed males ( $M = 2.42$ ) relative to the unjacketed males ( $M = 1.95$ ), this difference was not statistically significant ( $p < .05, \eta_p^2 = .101$ ).

As shown in Panel B of Figure 3, the chi-square analysis for the first ejaculation choice revealed no significant preference in the paired group for either of the males,  $\chi^2(1) = .053, p = .819, V = .053$ , nor in the unpaired group,  $\chi^2(1) = 1.316, p = .251, V = .263$ .

**Experiment 2.** Figure 2 shows the average number of different sexual behaviors by females toward the two male partners during the open field by condition, group, and experiment. As shown in Panels A, B, C, D, E, and F, for Experiment 2, once again it appears that there were no apparent trends to prefer either jacket on or jacket off male partner in the sexual behaviors displayed for either of the groups. The reliability of these observations was corroborated by a 2 (group: paired, unpaired)  $\times$  2 (male: jacket on, jacket off) repeated-measures ANOVA for each sexual behavior.

For hops and darts, there was no significant main effect of male,  $F(1, 35) = .435, p = .514, \eta_p^2 = .012$ , nor a significant interaction of Group  $\times$  Male,  $F(1, 35) = .042, p = .84, \eta_p^2 = .001$ . For solicitations, there was no significant main effect of male,  $F(1, 35) = .002, p = .961, \eta_p^2 < .001$ , nor a significant interaction of Group  $\times$  Male,  $F(1, 35) = .047, p = .83, \eta_p^2 = .001$ . For kicks, there was no significant main effect of male,  $F(1, 35) = 1.035, p =$

.316,  $\eta_p^2 = .029$ , nor a significant interaction of Group  $\times$  Male,  $F(1, 35) = 1.035, p = .316, \eta_p^2 = .029$ . For mounts, there was no significant main effect of male,  $F(1, 35) = .487, p = .49, \eta_p^2 = .014$ , nor a significant interaction of Group  $\times$  Male,  $F(1, 35) = .022, p = .884, \eta_p^2 = .001$ . For intromissions, there was no significant main effect of male,  $F(1, 35) = .485, p = .491, \eta_p^2 = .014$ , nor a significant interaction of Group  $\times$  Male,  $F(1, 35) = 1.106, p = .30, \eta_p^2 = .031$ . For mean of 1st ejaculation latency, there was no significant main effect of male,  $F(1, 35) = .106, p = .746, \eta_p^2 = .003$ , nor a significant interaction of Group  $\times$  Male,  $F(1, 35) = 2.288, p = .139, \eta_p^2 = .061$ .

Figure 3 shows the preference scores as average numbers of ejaculations per partner and first ejaculation choice during the open field by male, group, and experiment. As Panel A shows for the mean of ejaculation, although there was no significant main effect of male,  $F(1, 35) = .033, p = .857, \eta_p^2 = .001$ , there was a significant interaction of Group  $\times$  Male,  $F(1, 35) = 9.401, p = .004, \eta_p^2 = .212$ . Post hoc Tukey comparisons showed that females in the paired experimental group took a significantly higher mean number of ejaculations from the jacketed male ( $M = 2.33$ ), than from the unjacketed male ( $M = 1.44, p < .05, \eta_p^2 = .128$ ), whereas females in the unpaired experimental group took a significantly higher mean number of ejaculations from the unjacketed male ( $M = 1.95$ ), than from the jacketed male ( $M = 1.16, p < .05, \eta_p^2 = .109$ ).

As shown in Panel B of Figure 3, the chi-square analysis for the first ejaculation choice revealed marginally significant preference in the paired group for the jacketed males,  $\chi^2(1) = 2, p = .157, V = .333$ , and no statistically significant preference in the unpaired group,  $\chi^2(1) = 1.316, p = .251, V = .263$ . However, a 2  $\times$  2 chi-square analysis revealed that both of these groups have a marginally significant different trend of preference for their first ejaculation choice,  $\chi^2(1) = 3.29, p = .069, \phi = .244$ .

## Males

**Experiment 1.** Figure 4 shows the scores for the different copulatory male behaviors during the final open field test by female and group test in Experiment 1. As shown in Panels A and B, both paired and unpaired groups displayed a higher mean number of mounts and intromissions for the jacketed female. The reliability of these observations was corroborated by 2 (group: paired, unpaired)  $\times$  2 (female: jacket on, jacket off) repeated-

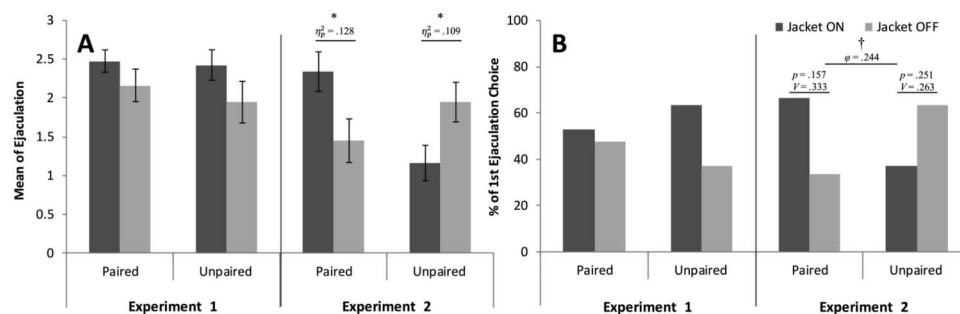


Figure 3. Female preference scores during the open field by condition (jacket on vs. jacket off), group (paired vs. unpaired), and experiment (Experiment 1 and Experiment 2). Panel A: mean of ejaculations. Panel B: percentage of first ejaculation choice.  $^{\dagger} p < .01$ .  $^* p < .05$ .  $\eta_p^2$  = partial eta square;  $V$  = Cramer's  $V$ ;  $\phi$  =  $\phi$ .

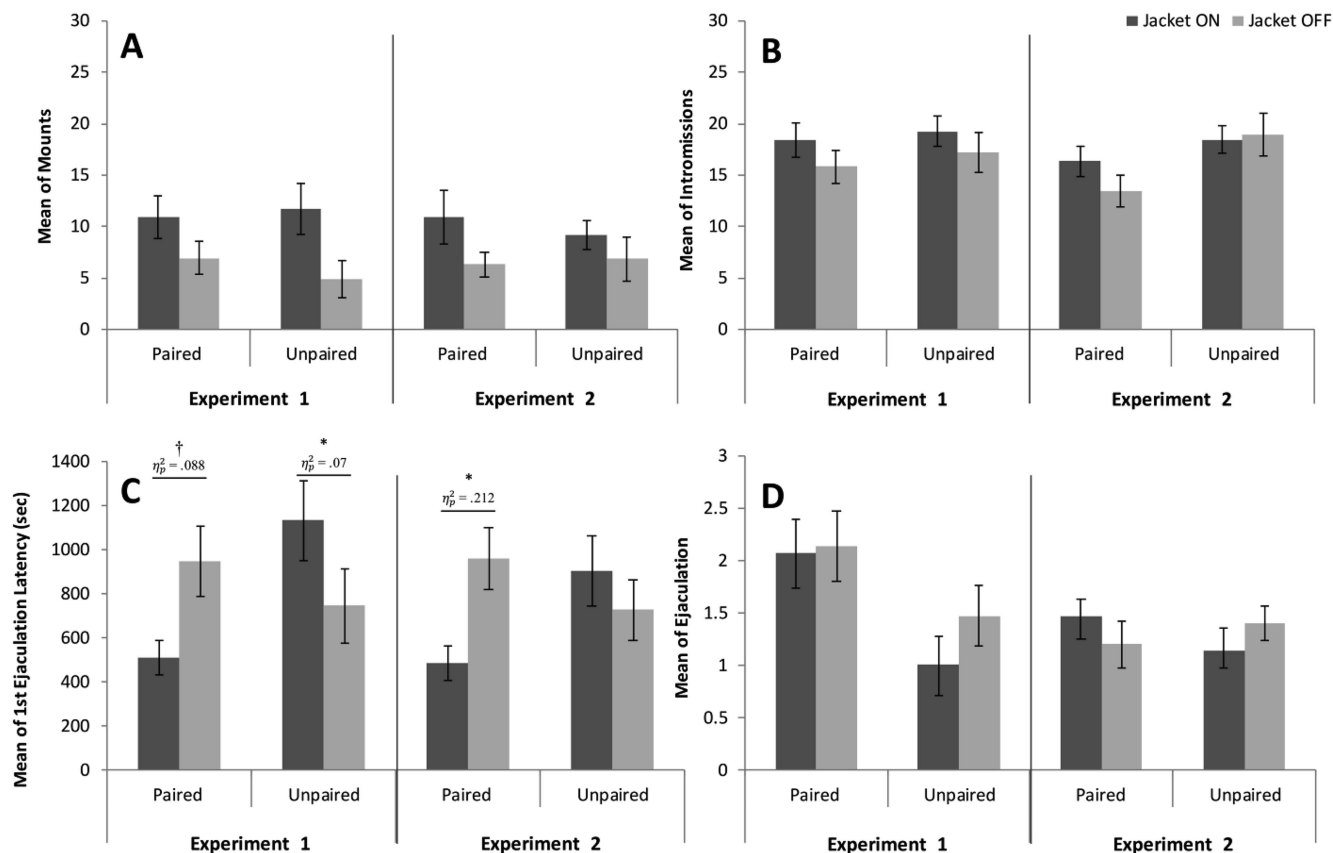


Figure 4. Averages for male copulatory behaviors during the open field test by condition (jacket on vs. jacket off), group (paired vs. unpaired), and experiment (Experiment 1 and Experiment 2). Panel A: mounts. Panel B: intromissions. Panel C: first ejaculation latency. Panel D: ejaculations. †  $p < .01$ . \*  $p < .05$ .  $\eta_p^2$  = partial eta square.

measures ANOVA for each sexual behavior. For mounts, there was a significant main effect of female,  $F(1, 28) = 12.764$ ,  $p = .001$ ,  $\eta_p^2 = .313$ , but no significant interaction of Group  $\times$  Female,  $F(1, 28) = .911$ ,  $p = .348$ ,  $\eta_p^2 = .031$ . Post hoc Tukey comparisons showed that males in the paired group displayed a marginally higher mean of mounts toward the unjacketed female ( $M = 10.87$ ) compared with the jacketed female ( $M = 6.93$ ,  $p < .05$ ,  $\eta_p^2 = .109$ ), whereas males in the unpaired group displayed a significantly higher mean of mounts toward the unjacketed female ( $M = 11.67$ ) compared with the jacketed female ( $M = 4.87$ ,  $p < .05$ ,  $\eta_p^2 = .268$ ). For intromissions, there was no significant main effect of female,  $F(1, 28) = 1.553$ ,  $p = .223$ ,  $\eta_p^2 = .053$ , nor a significant interaction of Group  $\times$  Female,  $F(1, 28) = .02$ ,  $p = .888$ ,  $\eta_p^2 = .001$ .

As shown in Panel C of Figure 4, the ANOVA for the 1st ejaculation latency revealed no significant main effect of female,  $F(1, 28) = .019$ ,  $p = .893$ ,  $\eta_p^2 = .001$ , yet there was a significant interaction of Group  $\times$  Female,  $F(1, 28) = 4.772$ ,  $p = .037$ ,  $\eta_p^2 = .146$ . Post hoc Tukey comparisons showed that males in the paired group displayed a lower mean latency of 1st ejaculation toward the jacketed female ( $M = 507.47$ ) compared with the unjacketed female ( $M = 1130.8$ ,  $p = .112$ ,  $\eta_p^2 = .088$ ), whereas males in the unpaired group displayed a lower mean latency of first ejaculation toward the unjacketed female ( $M = 743.2$ ) compared with the jacketed female ( $M = 946.67$ ,  $p > .05$ ,  $\eta_p^2 = .07$ ).

As shown in Panel D of Figure 4, the ANOVA for the mean of ejaculation revealed no significant main effect of female,  $F(1, 28) = .472$ ,  $p = .498$ ,  $\eta_p^2 = .017$ , nor a significant interaction of Group  $\times$  Female,  $F(1, 28) = .265$ ,  $p = .611$ ,  $\eta_p^2 = .009$ .

Figure 5 shows the 1st ejaculation choice per female in experiment one. The  $\chi^2$  square analysis for the first ejaculation choice revealed no significant preference in the paired group for either of the females,  $\chi^2(1) = .6$ ,  $p = .439$ ,  $V = .2$ , and a nonsignificant trend in the unpaired group to ejaculate first with unjacketed females,  $\chi^2(1) = 1.667$ ,  $p = .197$ ,  $V = .333$ . Finally, a  $2 \times 2$  chi-square analysis revealed a statistically marginal trend of these groups having a different preference for their 1st ejaculation choice,  $\chi^2(1) = 2.143$ ,  $p = .143$ ,  $\phi = .2$ .

**Experiment 2.** Figure 4 shows the scores for the different copulatory male behaviors on the open field by female and group test in Experiment 2. As shown in Panels A and B, both paired and unpaired groups displayed a higher mean number of mounts and intromissions for the jacketed female. The reliability of these observations was corroborated by  $2$  (group: paired, unpaired)  $\times$   $2$  (female: jacket on, jacket off) repeated-measures ANOVA for each sexual behavior. For mounts, there was a significant main effect of female,  $F(1, 28) = 6.392$ ,  $p = .017$ ,  $\eta_p^2 = .186$ , but no significant interaction of Group  $\times$  Female,  $F(1, 28) = .656$ ,  $p = .425$ ,  $\eta_p^2 = .023$ . Post hoc Tukey comparisons showed that only males in the paired group displayed a significantly higher mean of mounts

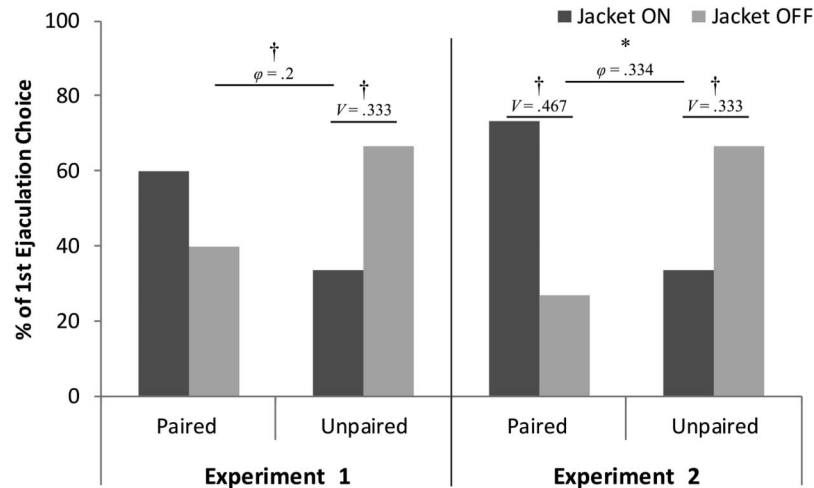


Figure 5. Percentage of first ejaculation choice for males during the open field test by condition (jacket on vs. jacket off) and group (paired vs. unpaired), and experiment (Experiment 1 and Experiment 2). †  $p < .01$ . \*  $p < .05$ .  $\eta_p^2$  = partial eta square;  $V$  = Cramer's  $V$ ;  $\phi = \phi$ .

toward the unjacketed female ( $M = 10.87$ ) compared with the jacketed female ( $M = 6.33$ ,  $p < .05$ ,  $\eta_p^2 = .166$ ). For intromissions, there was no significant main effect of female,  $F(1, 28) = 1.553$ ,  $p = .223$ ,  $\eta_p^2 = .036$ , nor a significant interaction between Group  $\times$  Female,  $F(1, 28) = 1.792$ ,  $p = .192$ ,  $\eta_p^2 = .06$ .

As shown in Panel C of Figure 4, the ANOVA for 1st ejaculation latency revealed no significant main effect of female,  $F(1, 28) = 1.491$ ,  $p = .232$ ,  $\eta_p^2 = .051$ , but there was a significant interaction of Group  $\times$  Female,  $F(1, 28) = 7.075$ ,  $p = .013$ ,  $\eta_p^2 = .202$ . Post hoc Tukey comparisons showed that only males in the paired group displayed a significantly lower mean latency of first ejaculation toward the jacketed female ( $M = 483.67$ ) compared with the unjacketed female ( $M = 957.87$ ,  $p < .05$ ,  $\eta_p^2 = .212$ ).

As shown in Panel D of Figure 4, the ANOVA for the mean of ejaculation revealed no significant main effect of female,  $F(1, 28) < .001$ ,  $p > .9$ ,  $\eta_p^2 = 0$ , nor a significant interaction of Group  $\times$  Female,  $F(1, 28) = 1.665$ ,  $p = .207$ ,  $\eta_p^2 = .056$ .

Finally, Figure 5 shows the first ejaculation choice per female in experiment one. The chi-square analysis for the first ejaculation choice revealed a marginal preference in the paired group for the jacketed females,  $\chi^2(1) = 3.267$ ,  $p = .071$ ,  $V = .467$ , and a nonsignificant trend in the unpaired group for the unjacketed females,  $\chi^2(1) = 1.667$ ,  $p = .197$ ,  $V = .333$ . However, a  $2 \times 2$  chi-square analysis revealed that both of these groups had a significantly different preference for their 1st ejaculation choice,  $\chi^2(1) = 4.82$ ,  $p = .028$ ,  $\phi = .334$ .

## Discussion

This study examined whether a neutral somatosensory cue (rodent tethering jacket) on a partner during male or female rat's early experiences with sexual reward could accrue enough associative strength to direct partner preference during a final choice test. The first experiment showed that males given their first 14 trials of copulation to ejaculation with jacketed females chose the jacketed female over the nonjacketed female for their first ejaculation and directed more ejaculations toward that

female. In contrast, females given their first 14 trials of paced copulation with jacketed males did not show a partner or mate preference for jacketed versus nonjacketed males. In the second experiment, the jacket on the partner was paired explicitly with sexual reward, with the nonjacketed partner paired explicitly with sexual nonreward (as the partner was sexually nonreceptive). When this differentiation was made, both male and female subjects showed a CPC for the jacketed versus nonjacketed partner on the final choice test. Thus, a neutral somatosensory cue can be used as a discrete, partner-related cue (CS) that predicts the sexual reward state (US).

Although sexual behavior encompasses a wide range of partner-related responses, ejaculation frequency toward or from a specific partner, and the choice of partner for first ejaculation and first solicitation, are consistent and reliable indicators of CPC in male and female rats (see Pfaus et al., 2012). In a group-like setting such as the preference test used here rats tend to copulate with both available partners (e.g., Coria-Ávila et al., 2005; Kippin et al., 1998), hence their general acknowledgment as a "promiscuous" species (see also McClintock, 1984). However, when a neutral odor cue (e.g., almond, lemon), is paired with the sexual reward state, the CPC is reflected in a significantly higher number of ejaculations given to a particular female (Kippin et al., 1998) or received from a particular male (Coria-Ávila et al., 2005) along with a significant preference of the partner bearing the conditioned odor for first solicitation and ejaculation in females and first ejaculation preference in males. Thus, for both males and females, ejaculation frequency and first choice for ejaculation are robust reflections of sexual motivation and the underlying hormonal and neurochemical drive triggered by the external cue (Hull & Rodriguez-Manzo, 2009).

It is well known that a CPC based on a discrete cue is controlled by opioid transmission mainly after ejaculation in male rats or during paced copulation in female rats (e.g., Paredes & Vazquez, 1999; Pfaus et al., 2012). Similarly, opioid transmission is critical for the development of a conditioned place preference (CPP) in



both males (Ågmo & Berenfeld, 1990) and females (Paredes & Alonso, 1997). Treatment with the nonspecific opioid receptor antagonist naloxone (NAL) during training or before testing abolished CPP in males (Ågmo & Berenfeld, 1990) and shifted the preference of males from scented to unscented females (Ismail et al., 2009). Treatment with NAL in females abolished both CPP and CPC (Paredes & Martínez, 2001; Coria-Ávila et al., 2008). Likewise, male rats trained to prefer jacketed females as in the present study had this preference shifted to the unjacketed female by concomitant treatment with NAL during training (Quintana et al., *in press*), suggesting that the disruption of opioid transmission blocks CPP similarly in both sexes, but differentially disrupts CPC in males and females.

The neural control of a CPC based on discrete odor cues involves several key brain areas in both males and females (Paredes, 2014; Pfaus, Ismail, & Coria-Ávila, 2010; Pfaus et al., 2012). A crucial role has been attributed to a circuit involving the medial preoptic area (mPOA), the ventral tegmental area (VTA), the nucleus accumbens (NAc), the ventromedial hypothalamus (VMH), and the amygdala (see Paredes, 2009; Pfaus et al., 2010). For instance, following copulation in males and females, immunoreactivity for Fos protein (Fos-IR), a marker of general neuronal activation, was induced in common regions of the mPOA, posteromedial part of the bed nucleus of the stria terminalis, posterodorsal part of the amygdala, and the parvocellular part of the subparafascicular thalamic nucleus, while only in females Fos-IR was also induced in the ventrolateral part and the most caudoventral part of the VMH, and in the premammillary nucleus (Coolen, Peters, & Veening, 1996). More specifically, Fos-IR of males exposed to an olfactory cue previously associated with the postejaculatory reward state was higher than unpaired control males in the piriform cortex (PirCtx), basolateral amygdala, NAc core, and the anterior lateral hypothalamic area (Kippin, Cain, & Pfaus, 2003). A similar study conducted with females that had olfactory cue paired with paced-copulation revealed higher Fos-IR in the PirCtx, mPOA, and VTA compared with nonpaced and unpaired controls (Coria-Ávila & Pfaus, 2007). These brain areas have been associated with different roles in the orchestration of sexual behavior. Broadly speaking, both the mPOA and the VMH are critical brain regions that control sexual arousal and behavior in males and females (see Hull & Dominguez, 2007, 2015), whereas the VTA and amygdala are regarded as sensory integrators modulating the association between external and internal cues, as well as the attention toward reward-related cues (see Berridge, 2007; Everitt et al., 1989).

As demonstrated in this and other studies (e.g., Kippin et al., 1998), an explicitly paired design was more successful at establishing a CPC in males and females than the paired design. Indeed, an explicitly paired design was successful in training albino (Wistar) or pigmented (Long-Evans) female rats to associate paced copulation with one strain of male rat, and copulation with no pacing conditions with the other strain. Thus, regardless of the strain of the male (albino or pigmented), females displayed more solicitations and took the first ejaculation from the pacing-related male, an effect that was facilitated if the male was of the same strain as the female (Coria-Ávila et al., 2006). An experimental design in which animals are trained to associate a neutral cue with sexual reward, as well as trained to associate its absence with no reward, is more likely to result in stronger conditioning compared

with a paired design and a stronger effect than when animals solely trained in a paired design.

In the present study, CPC was denoted as the preference of partner for ejaculation in both male and female rats. Although this has been the consistent dependent measure for males in previous studies using an olfactory or conspecific strain-related cue (Ismail et al., 2009; Ismail, Jones, Graham, Sylvester, & Pfaus, 2011; Ismail et al., 2010; Kippin & Pfaus, 2001a, 2001b; Kippin, Samaha, Sotiropoulos, & Pfaus, 2001; Kippin et al., 1998; Quintana et al., 2018a; Quintana et al., *in press*; Quintana, Guizar, Rassi, & Pfaus, 2018b), it is not consistent with previous studies of CPC in females where the discrete, male-related cue used was an odor (e.g., Coria-Ávila et al., 2005). In those studies, females displayed a significantly higher number of solicitations with the scented male and chose that male for their first and subsequent ejaculations more frequently. A similar pattern of responses was also displayed when the cue was related to the strain of male (pigmented or albino), although ejaculations were selected preferentially only when the male was of the same strain as the female (Coria-Ávila et al., 2006). Although rodents rely most heavily on their olfactory sense, it is not immediately obvious why an olfactory-driven CPC would yield a higher display of solicitations toward, and choice of ejaculations with, the scented male, whereas a somatosensory/visually driven CPC would yield only the higher frequency of ejaculations received from the preferred male. Males become choosy in the last few intromissions before ejaculation (Kippin & Pfaus, 2001b), and they pursue the scented female more during this period, bringing her into almost exclusive physical (and sensory) proximity. Rat visual acuity is far greater if visual stimuli are close, or if the stimuli are farther away but paired with olfactory cues (e.g., Rossier & Schenk, 2003). Being able to discriminate the presence or absence of the jacket may require both close visual and tactile sensation. Thus, paired- or explicitly paired-trained rats may have to identify which partner has the jacket and pursue that partner prior to making or receiving an ejaculation. We note that the hormone priming regimen for the females was the same in the present experiments as in previous studies, thereby obviating the notion that hormonal priming differences could account for the present differences. Finally, although it is possible in the second experiment that animals with a CPC toward the partner with the jacket could have developed an "aversion" toward the partner without the jacket (given the lack of reward experienced with the unreceptive partner), we would have expected to see greater agonistic rejection responses made toward the partner without the jacket. This did not occur.

Although at first glance the lack of conditioning to the jacket in the first experiment in females versus males could indicate a sex difference in associability, this difference may be more apparent than real. It is likely that the ability to physically contact the jacket played a role. Females rarely come into direct headwise contact with the upper torso of the male unless they make olfactory investigations of this area or happen to contact it while soliciting the male. When showing lordosis, the only contact they would have with the male's upper torso would be on the lower half of their backs while the male mounts with pelvic thrusting. In this case, paced versus nonpaced conditions may not have been sufficient for the female to detect a difference. However, when the difference was explicitly paired with sexual reward by pairing the jacket with sexually receptive males and no jacket with sexually nonreceptive males, the female was able to differentiate and show

significant CPC for males wearing the jacket. In contrast, when females wore the jacket during the conditioning of males in both experiments, the males came into direct headwise contact with the jacket during each mount. Given that the associative strength that can accrue to a CS depends on its salience (Pavlov, 1927; Pearce & Hall, 1980), it is likely that the increased contact males had with the jacket allowed for it to be associated in both experiments, whereas the salience of the jacket had to be increased by its explicit pairing with sexual reward in the females in the second experiment. It is worth mentioning that the jacket is also a visual cue, making possible for both males and females to distinguish their preferred partner based in their visual attributes, as found previously for strain of male (Coria-Ávila et al., 2006). Thus, to develop a CPC based on a jacket, there was an interaction of both visual and somatosensory components that was still not salient enough for females until the association with sexual reward was made explicit, in which case no jacket was paired with sexual nonreward, and its absence may well have led females on the final test to avoid copulatory attempts with nonjacketed males.

Finally, the development of a preference in sexual response for a somatosensory cue could also be understood as a conditioned partner preference based on the additional presence of an inanimate object. Sexual arousal and preference based on an inanimate object is at the root of the definition of a fetish, which in the early days of clinical recognition was viewed as having a significant learning component (Krafft-Ebing, 1886) in addition to differences in personality and arousability that may reflect innate genetic causes or reactions to early trauma (e.g., Freud, 1905; Freud, 1927; Weisel-Barth, 2013). To the extent that a rodent jacket can become a contextual CS for sexual arousal in male rats (Pfaus et al., 2013), or a discrete, partner related CS for sexual reward in both male and female rats (present study), Pavlovian associations are clearly critical in fetish development, and may lie at the core of such development during early sexual experiences. Indeed, several human case studies have corroborated this, describing different objects or actions becoming associated with sexually arousing experiences even before the individual labeled it as sexual (Abel, Coffey, & Osborn, 2008; Lowenstein, 1997). How such learning crystallizes the object into a sexual cue requires further study, although we have argued that this must be accompanied by epigenetic changes within neurochemical pathways that link sexual reward to sexual desire (Pfaus, 2009; Pfaus et al., 2012). First experiences with the US alone (e.g., ejaculatory reward experienced with unscented or unjacketed females) or CS alone (e.g., presentations of the odor on a gauze pad to sexually naïve male rats prior to their first sexual experiences to ejaculation with scented females) are able to block the ability of the two to be associated (Quintana et al., 2018a, 2018b). Understanding the nature of sexual reward would aid in determining how such pathways are altered, and whether first experiences in individuals with different capacities for sexual arousal may underlie the development of a true fetish relative to a partner preference.

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# Differential disruption of conditioned ejaculatory preference in the male rat based on different sensory modalities by micro-infusions of naloxone to the medial preoptic area or ventral tegmental area

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## Abstract

**Rationale** Male rats trained to associate a neutral odor or rodent jacket on a female with their post-ejaculatory reward state display a preference to ejaculate with females bearing the odor or jacket. This conditioned ejaculatory preference (CEP) can be shifted by systemic administration of the opioid antagonist naloxone (NAL) during training, such that NAL-trained males distribute their ejaculations to females without the cue, relative to saline (SAL)-trained males.

**Objective** The present study examined two brain sites, the medial preoptic area (mPOA) or ventral tegmental area (VTA), where the opioid reward state might be induced.

**Methods** Sexually naïve Long-Evans males were implanted with bilateral guide cannula aimed at either site before they underwent multi-ejaculatory conditioning trials at 4-day intervals with sexually receptive females that bore either an almond odor or rodent tethering jacket. Infusions of NAL (1 µl/side) or SAL (1 µl/side) were made prior to each conditioning trial. All males were infused with SAL prior to a final open-field choice test with two sexually receptive females, one scented and the other unscented, or one jacketed and the other unjacketed.

**Results** Males previously conditioned with SAL in either region showed significant CEP. In contrast, prior infusions of NAL to the mPOA shifted the preference towards the unfamiliar female, whereas prior infusions to the VTA abolished CEP for the odor. Subsequent detection of Fos protein induced by the cue showed that, relative to SAL-treated males, prior experience with NAL in the mPOA suppressed Fos in both the mPOA and VTA, whereas prior experience with NAL in to the VTA suppressed Fos in the VTA alone.

**Conclusions** Opioid antagonism in the mPOA produces a state of non-reward whereas in the VTA, it produces a state in which the odor does not acquire incentive properties.

**Keywords** Conditioned ejaculatory preference · Opioid · mPOA · VTA

## Introduction

The effects of opioids have been well documented in the display of both conditioned and unconditioned sexual behaviors (Pfaus and Gorzalka 1987; van Furth et al. 1995a, b; Halloway

2012; Paredes 2014). The rewarding effects of opioids that create a preference for contextual cues associated with sexual reward in male rats were first shown using the conditioned place preference (CPP) paradigm (Miller and Baum 1987; Ågmo and Berenfeld 1990). Male rats either copulated in the initially non-preferred side of the CPP box (Miller and Baum 1987) or were placed into the initially non-preferred side immediately after one ejaculation (Ågmo and Berenfeld 1990). In both cases, the contrast was made against the initially preferred side, in which males were not allowed to copulate or experience their post-ejaculatory state. Males injected systemically with saline (SAL) displayed significant CPP for the side associated with copulation to ejaculation, or the post-

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ejaculatory state itself, whereas males injected systemically with naloxone hydrochloride (NAL), but not the dopamine antagonist pimozide, spent significantly less time in the side associated with the copulatory or post-ejaculatory sexual reward state. These data show that opioid, but not dopamine, receptor blockade is an important neurochemical substrate of sexual reward induced by ejaculation in male rats. Indeed, whole brain opioid content and receptor internalization are highest immediately after ejaculation (Szechtman et al. 1981; Tenk et al. 2009) when male rats become behaviorally quiescent and sleep for a short period. This makes the post-ejaculatory period essentially an “orgasm-like” sexual reward state (Pfaus et al. 2016) during which conditioned sexual learning occurs (Georgiadis et al. 2012).

Similar to CPP, male rats trained to associate a neutral odor (e.g., almond, lemon) on a sexually receptive female with their post-ejaculatory sexual reward state display a preference to ejaculate with scented versus unscented females (Kippin et al. 1998; Kippin and Pfaus 2001). Similarly, males trained to associate a jacket on the female with sexual receptivity and copulation develop a preference towards the jacketed female over an unjacketed one (Quintana et al. 2019a). As with CPP, systemic administration of NAL disrupts the acquisition of CEP for both odor (Ismail et al. 2009) and the jacket (Quintana et al. 2019b) relative to males injected with SAL. However, prior experience with NAL during the training trials shifted the preference towards other females for both cues.

Among the different areas of the brain where opioids may play a role in sexual behavior and reward, and specifically in the development of CEP, the medial preoptic area (mPOA) and the ventral tegmental area (VTA) are two of the most widely studied (van Ree et al. 2000; Fields and Margolis 2015). Both have been regarded as critical in the control and execution of male sexual behavior and sexually motivated behaviors in both males and females (Hull et al. 2006; Pfaus 2009), and particularly in the development of CEP for an olfactory cue in the male rat (Pfaus et al. 2012).

The present study examined whether opioids exert a differential modulation of CEP when NAL or SAL were infused into the mPOA or VTA. The first experiment evaluated this with an odor cue on estrous females. The second experiment substituted the odor with a somatosensory cue to evaluate if this effect is dependent on the type of conditioned stimulus used. Finally, Fos-IR was used to evaluate how NAL would affect the neural pattern of activation towards the cue.

## Materials and methods

**Animals, steroids, and conditioned cues** Seventy-three male Long-Evans rats weighing 300–350 g (before surgery), and 100 female Long-Evans rats, weighing 150–200 g, were obtained from Charles River Canada (St-Constant, QC) at

approximately 6 weeks of age. All animals were kept in a 12-h reversed light/dark cycle in a colony room at 21 °C, with unrestricted tap water and Purina® rat chow available in each cage.

Males were housed in groups of four in Plexiglas cages with wood-chip bedding and unrestricted access to water and food until cannulation, after which they were maintained individually in a single cage. Female rats were housed in pairs in Plexiglas (45 × 20 × 20 cm) cages with wood-chip bedding. Sexual receptivity was induced by subcutaneous injections of 10 µg estradiol benzoate (EB, 17β-diol 3-benzoate, ID E0970-000, Steraloids) 48 h prior each training session, and 500 µg of progesterone (P, 4-Pregnen-3, 20-dione, ID Q2600-000, Steraloids) 4 h prior to each training session as detailed in Pfaus et al. (2014). Steroids were dissolved in reagent grade sesame oil and delivered in a volume of 0.1 ml. Stimulus females were either scented or jacketed depending on the experiment. For the odor cue, females were scented with 0.6 ml of pure almond extract (Blue Ribbon, Etobicoke, ON), split equally in the back of their neck and anogenital region. For the somatosensory cue, females wore a rodent tethering jacket (Lomir Biomedical, Ile Perrot, QC) made of a double layer of Lycra/Spandex fabric. This jacket covered the upper part of the torso, with open holes for the forearms and fastened across the back with Velcro (see Quintana et al. 2019a).

**Surgeries Ovariectomy.** Females were ovariectomized via bilateral lumbar incisions under general anesthesia induced with ketamine (50 mg/ml, CDMV, ID UN7919, Wyeth)/xylazine (4 mg/ml, Rompun, DIN 02169592, Bayer) mixed at a ratio of 4:3 respectively (administered based on body weight), approximately 2 weeks before the beginning of the experiment to allow at least a week of recovery (e.g., Kippin et al. 1998; Pfaus et al. 1999). This procedure allowed for hormone levels to be controlled under hormonal replacement throughout training sessions.

**Cannula implantation.** In order to avoid sexually sluggish males, all males were allowed to copulate with a scented or jacketed (depending on the experiment) sexually receptive female in a pacing chamber for 30 min. All males were bilaterally cannulated into the mPOA or VTA, around 2 weeks before the first training trial to allow recovery. Animals were anesthetized with 5% isoflurane (Institute for the Laboratory Animal Research 2011) and secured in a stereotaxic apparatus (Kopf Instruments). Under aseptic conditions, stainless steel guide cannulae (22G, Plastic One) were implanted bilaterally 1 mm above the regions of interest (mPOA, AP, −0.5 from bregma; MD, ±0.5; DV, −8 mm; VTA, AP, −6.04 from bregma; MD, ±2 at 10°; DV, −8 mm (see Coolen et al. 2004). A stainless steel dummy of the same length was placed in each cannula to ensure it remained unblocked.

**Behavioral training** After recovery, males were given multi-ejaculatory trials for 30 min each with sexually receptive females

in one-hole unilevel pacing chambers, where only females could pace through a divider of a two-compartment chamber. Males trained with scented females underwent 10 training trials, whereas males trained with jacketed females underwent 14 trials. As established by Quintana et al. (2019a), the difference in the amount of training trials was necessary since the somatosensory cue does not have the same salience in rats as the olfactory cue in the development of a CEP. The cage bedding was not changed between conditioning sessions and in every trial, females were assigned randomly to males to assure that conditioning accrued only to the odor or jacket.

A NAL concentration that would effectively reverse the CEP as previously established (Ismail et al. 2009) was determined by testing different doses (i.e., 200, 500, and 1000 ng) in an unpublished preliminary study. Although 200 ng and 500 ng disrupted the CEP compared to the control group, the 1000-ng dose reversed it, as was found previously with systemic NAL (Ismail et al. 2009). Thus, infusions of either 1 µg NAL in 0.9% SAL, or the SAL vehicle (infused in 1 µl/side over the course of 1 min), were made to all males in each group prior to conditioning trials, depending on group membership. The distribution of males per group is presented in Table 1. Three males that appeared sick during the experiment were euthanized and their data not included.

All males were infused with SAL prior to a final choice test in which males were placed into an open field (123 × 123 × 46 cm) filled with clean bedding to copulate freely for 30 min with two sexually receptive females, one scented (ScF) and the other unscented (UnScF), or one jacketed (Jacket ON) and the other unjacketed (Jacket OFF), depending on group membership. All behaviors were recorded on video and scored later using a computerized event recorder (Balfour et al. 2006). Following the preference test, males were given two more training trials in the exact same conditions of their training trials before perfusion.

**Perfusion, histology, and Fos immunohistochemistry** Four days after their last reconditioning trial, males were euthanized with sodium pentobarbital (120 mg/kg, i.p.) and perfused intracardially with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were extracted and post-fixed in fresh 4% paraformaldehyde in PBS overnight, then transferred into a hypertonic 30% sucrose solution to extract water from the brain. Brains were then frozen at −80 °C until sliced. Coronal slices were cut and collected through

a sliding microtome at 30 µm, and cannula placements were confirmed and marked in an atlas by a third-party researcher. The criterion to exclude animals from the analysis was set so that males whose injectors placed outside of the boundaries of either the mPOA or VTA were exempt from the study. Therefore, males with uni- or bilateral cannulation on target were included in the analyses. Unilateral placements in these regions were included given that previous studies have shown they are effective. For instance, unilateral infusions of NAL into the mPOA affected the different parameters of song productions in male birds (Kelm-Nelson et al. 2013), as well as maternal behavior (Miranda-Paiva et al. 2003). In the present experiment, four males were ultimately not included in the analyses due to a bad cannula placement (one from the Jacket SAL mPOA group, one from the Jacket NAL mPOA group, and two in the VTA NAL VTA group; see Table 1).

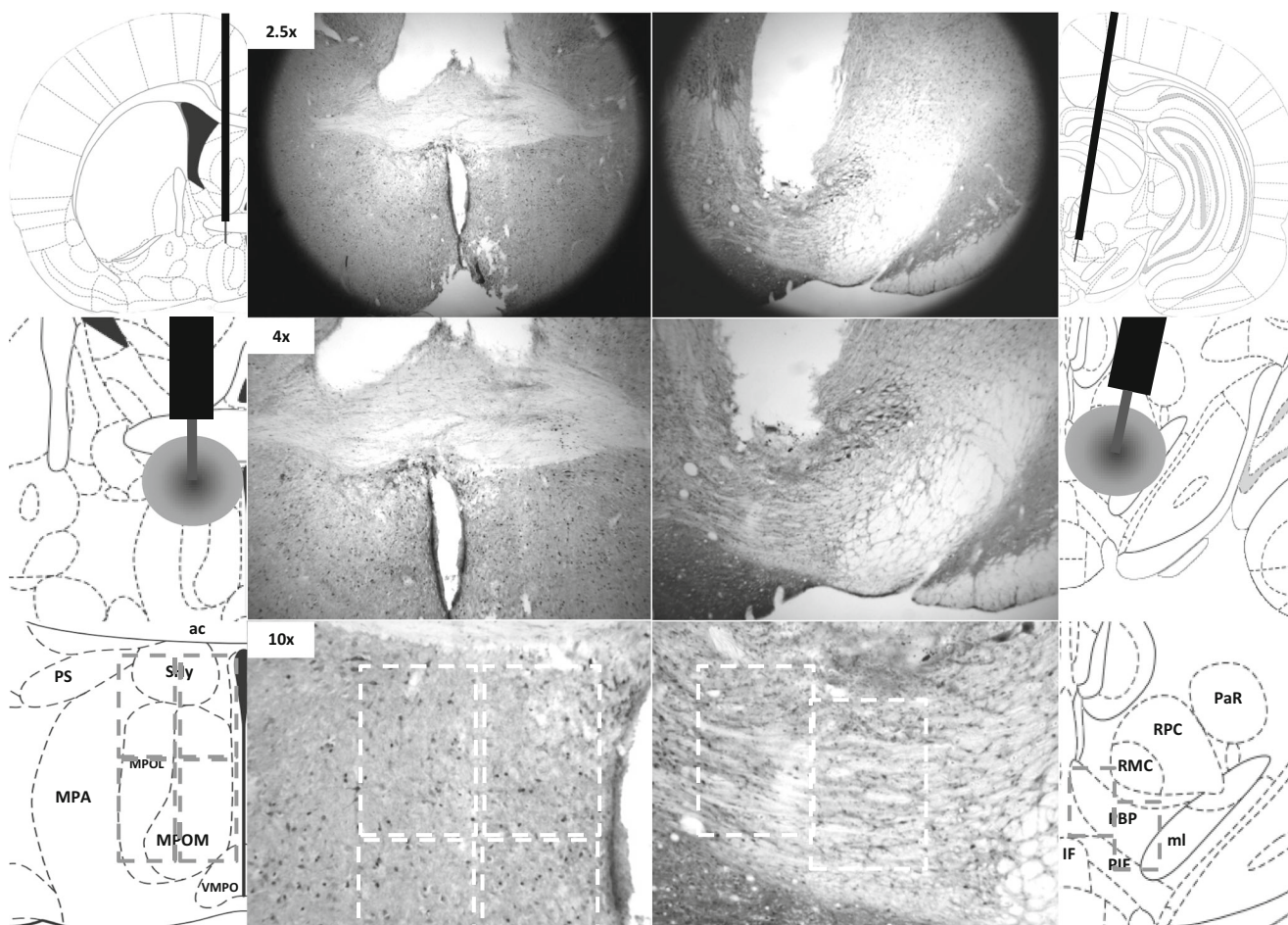
Fos immunocytochemistry was performed as reported previously (Garduño-Gutiérrez et al. 2013). Brain sections were examined at ×40 magnification, and the number of Fos-positive cells was counted bilaterally from each region from five different sections per rat per region using a Leitz Laborlux light microscope connected to a computerized image-analysis system (ImageJ, NIH, Bethesda, MD) as we have done previously (Coria-Avila and Pfaus 2007; Kippin et al. 2003). The selected brain regions were observed for Fos-IR to evaluate the neural activation evoked by the odor or jacket cue used during training. An average of Fos-positive cells was calculated from three different slides from each rat (5 subjects in each group), for each brain area in the control and experimental groups, for each main group depending on where their implants were located (mPOA or VTA), separately.

Figure 1 shows three-magnification examples of the cannula trace, as well as the Fos-IR counting rationale for the mPOA (left panels) and VTA (right panels). For the mPOA, four times a standard area of 84 × 107 mm was utilized to cover the area of interest, whereas for the VTA, two times a standard area of 66 × 99 mm was used (out of pictures of 216 × 66 mm [2560 × 1920 pixels]), distributed as illustrated through the white dash-line areas. As in our previous studies, the threshold to detect Fos-positive cells were set at the lowest range necessary for one Fos-positive cell to be detected and any other nearby highlighted cell-like shape not. For counting purposes, the size of cells detected (in pixels<sup>2</sup>) was set between 0 and 40, while circularity was set between 0.5 and 1.0 pixels. Pictures were taken as close as possible to the injection site, while avoiding the immediate nearby area of the injection to improve sensitivity and specificity of actual Fos-positive cells. Lower numbers of Fos-IR in comparison to other reports, especially in the case of the VTA, were expected, where the detection criteria and the narrow counting area covered could explain the differences.

**Table 1** Group sample distribution

|      |     | Odor | Jacket |
|------|-----|------|--------|
| mPOA | SAL | 10   | 8      |
|      | NAL | 10   | 9      |
| VTA  | SAL | 10   | 9      |
|      | NAL | 10   | 7      |





**Fig. 1** Illustration of the cannula traces as well as the Fos-IR counting rationale for the mPOA (left panels) and VTA (right panels). From top to bottom rows, we illustrate pictures taken with a  $\times 10$ ,  $\times 16$ , and  $\times 40$

magnification. The brain atlas illustrations depict the theoretical cannula trace, injection area, and subregions where the injection was most likely located. The cannula, injector, and rectangles are not set to real scale

**Statistical analyses** A series of repeated measures ANOVA were conducted to explore the influence of training in different sexual behaviors by each experimental cue used (odor or jacket), in each brain area subgroup (mPOA or VTA), for each group (SAL or NAL), separately. Analyzed behaviors included mounts, intromissions, mean of ejaculation, and latency of first ejaculation. In each analysis, we evaluated the interaction between the type of female (ScF vs. UnScF or Jacket ON vs. Jacket OFF) by group (i.e., SAL or NAL). Given statistically significant differences, post hoc comparisons were conducted for each single variable to compare the type of female using the Bonferroni correction. Partial eta square ( $\eta_p^2$ ) was calculated as effect sizes for each comparison. Additionally, a  $1 \times 2$  chi square ( $\chi^2$ ) analysis was conducted for the percentage of first ejaculation choice for each individual group, and a  $2 \times 2$   $\chi^2$  analysis to contrast the ejaculatory preference with the control group. Furthermore, Cramer's V and Phi ( $\phi$ ) effect sizes were conducted as effect size for the  $1 \times 2$  and  $2 \times 2$   $\chi^2$  analyses, respectively.

To evaluate the differences among the experimental groups in Fos-IR, one-way ANOVA analyses were conducted. For each significant ANOVA, post hoc analyses were made using the Tukey HSD test for the animals trained with the odor cue, for each brain area of interest (mPOA or VTA), among the different groups (SAL or NAL), separately. For animals trained with the jacket, only the highest dose of NAL was infused (1000 ng); therefore, independent  $t$  tests were conducted to compare the two groups (SAL and 1000 ng) for each brain area of interest (mPOA or VTA) separately. Partial eta square ( $\eta_p^2$ ) and Cohen's  $d$  were calculated as measures of effect size.

## Results

### Olfactory conditioning

#### Infusions of NAL or SAL to the mPOA: behavioral effects

Figure 2 shows the percentage of first ejaculation choice by

female and by brain area for all groups using the odor as neutral cue during the open-field test. A  $2 \times 2$   $\chi^2$  analysis revealed a statistically marginal trend for the SAL and the NAL groups to prefer different females to ejaculate first with,  $\chi^2(1) = 3.2$ ,  $p = 0.074$ ,  $\varphi = 0.3$ .

The analyses of the remaining variables (i.e., mounts, intromissions, ejaculation latency, or the mean of ejaculation) found no interactions between the factors, as well as no differences in the  $1 \times 2$   $\chi^2$  analyses.

**Infusions of NAL or SAL to the mPOA: Fos-IR analyses** Figure 3 shows the group results for the Fos-IR results regarding the exposition to the odor cue before perfusion for each main (cannulated into mPOA and VTA), group (SAL or NAL), and brain area (mPOA and VTA).

When analyzing the mPOA of males infused with NAL into the mPOA showed a statistically marginally lower Fos-IR mean count than the SAL control group,  $t(8) = 2.196$ ,  $p = 0.059$ ,  $d = 1.389$ . When analyzing, the VTA of males infused with NAL into the mPOA showed a statistically significantly lower Fos-IR mean count than the SAL control group,  $t(8) = 2.505$ ,  $p = 0.037$ ,  $d = 1.585$ .

**Infusions of NAL or SAL to VTA: behavioral analyses** As shown in Fig. 2, males in the SAL group chose to ejaculate first more with the ScF, whereas males in the NAL group clearly preferred more the UnScF. A  $2 \times 2$   $\chi^2$  between groups showed a statistically marginally different pattern to choose different females to ejaculate first with,  $\chi^2(1) = 1.818$ ,  $p = 0.178$ ,  $\varphi = 0.201$ .

The analyses of the remaining variables (i.e., mounts, intromissions, ejaculation latency, or the mean of ejaculation) found no interactions between the factors, as well as no differences in the  $\chi^2$   $1 \times 2$  analyses.

**Infusions of NAL or SAL to VTA: Fos-IR analyses** As shown in Fig. 3, when analyzing, the VTA of males infused with NAL into the VTA showed a lower Fos-IR mean count than the SAL control group,  $t(8) = 3.237$ ,  $p = 0.014$ ,  $d = 2.172$ , whereas no differences were found in their mPOA,  $t(8) = 0.289$ ,  $p = 0.780$ ,  $d = 0.178$ .

## Somatosensory conditioning

**Infusions of NAL or SAL to the mPOA: behavioral analyses** Figure 3 shows the scores for the different copulatory behaviors by female and by brain area for both groups during the open-field test.

Males in the SAL group ejaculated first faster with the unjacketed females, whereas the opposite was true for males in the NAL group,  $F(1,15) = 8.478$ ,  $p = 0.011$ ,  $\eta_p^2 = 0.361$ .

Bonferroni post hoc analyses revealed that males in the SAL displayed a statistically significantly lower mean first ejaculation latency for the jacketed female ( $M = 706.13$ ,  $SD = 463.593$ ) than for the unjacketed one ( $M = 1129.11$ ,  $SD = 617.017$ ,  $p = 0.016$ ,  $\eta_p^2 = 0.33$ ), whereas males in the NAL group had the opposite pattern of first ejaculation latency, yet did not reach statistically significant differences,  $p = 0.194$ ,  $\eta_p^2 = 0.11$ . Males in the SAL group ejaculated more with the jacketed female whereas males in the NAL group ejaculated more with the unjacketed female,  $F(1,15) = 14.959$ ,  $p = 0.002$ ,  $\eta_p^2 = 0.499$ . Bonferroni post hoc analyses revealed that males in the SAL displayed a statistically significantly higher mean of ejaculation towards the jacketed female ( $M = 1.88$ ,  $SD = 0.641$ ) than for the unjacketed one ( $M = 0.67$ ,  $SD = 0.707$ ,  $p = 0.01$ ,  $\eta_p^2 = 0.368$ ), whereas males in the NAL group displayed a statistically significantly higher mean of ejaculation towards the unjacketed female ( $M = 1.67$ ,  $SD = 0.707$ ) than for the jacketed one ( $M = 0.63$ ,  $SD = 0.744$ ,  $p = 0.024$ ,  $\eta_p^2 = 0.295$ ).

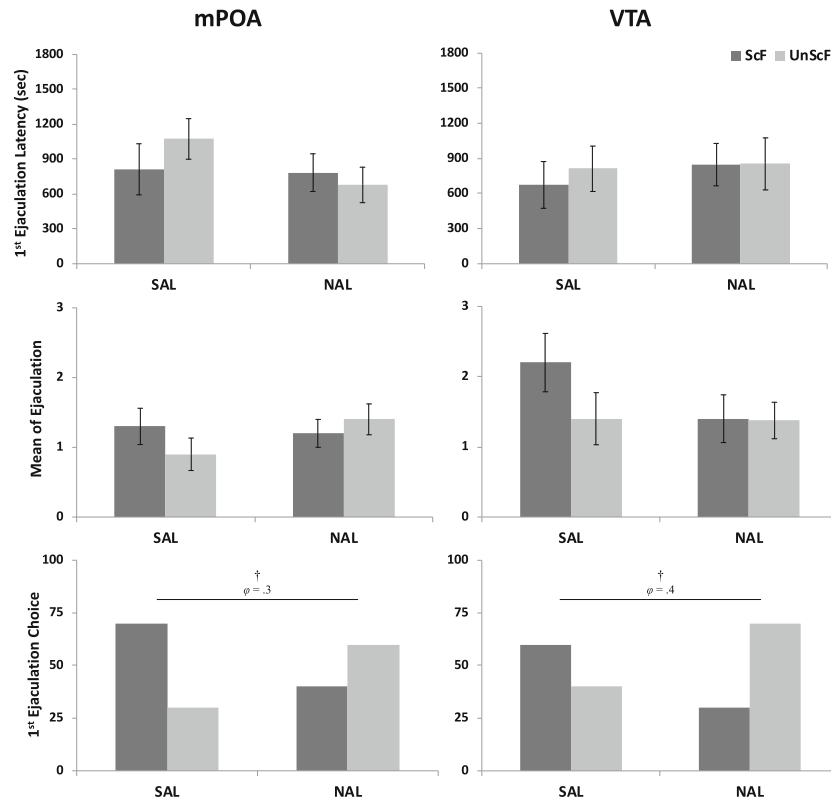
Finally, the  $\chi^2$  analyses for the percentage of first ejaculation choice shows that males in the SAL group chose to ejaculate first more with the jacketed female, whereas the opposite was true for males in the NAL group. Indeed, males in the SAL group significantly preferred to ejaculate first more with the jacketed female,  $\chi^2(1) = 4.5$ ,  $p = 0.034$ ,  $V = 0.75$ . Furthermore, a  $2 \times 2$   $\chi^2$  revealed a statistically significantly different pattern of preference to ejaculate first for both groups,  $\chi^2(1) = 5.13$ ,  $p = 0.024$ ,  $\varphi = 0.43$ .

**Infusions of NAL or SAL to the mPOA: Fos-IR analyses** As depicted in Fig. 3, when analyzing, the mPOA of males infused with NAL into the mPOA showed a marginally lower, yet of large magnitude, Fos-IR mean count than the SAL control group, where the SAL group ( $M = 206.58$ ,  $SD = 70.21$ ) had a higher Fos-IR count than the NAL group ( $M = 136.53$ ,  $SD = 17.53$ ,  $t(9) = 2.157$ ,  $p = 0.059$ ,  $d = 1.313$ ). When analyzing, the VTA of males infused with NAL into the mPOA showed a marginally lower, also of large magnitude, Fos-IR mean count than the SAL control group, where the SAL group ( $M = 28.33$ ,  $SD = 7.5$ ) had a higher Fos-IR count than the NAL group ( $M = 17.3$ ,  $SD = 11.46$ ,  $t(9) = 1.925$ ,  $p = 0.086$ ,  $d = 1.165$ ).

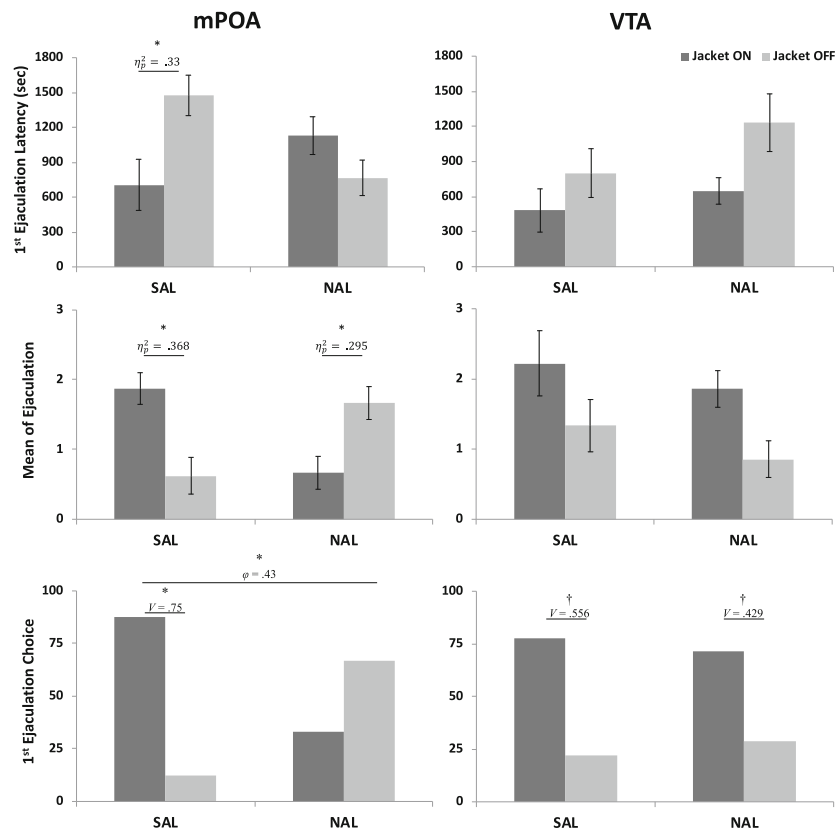
**Infusions of NAL or SAL to the VTA: behavioral analyses** As shown Fig. 2, the  $\chi^2$  analysis for the percentage of first ejaculation choice shows that both groups have a similar pattern of preference to ejaculate first more with the jacketed female,  $\chi^2(1) = 0.085$ ,  $p = 0.771$ ,  $\varphi = 0.073$ . The analyses of the remaining variables (i.e., mounts, intromissions, ejaculation latency, or the mean of ejaculation) found no interactions between the factors, as well as no differences in the  $1 \times 2$   $\chi^2$  analyses.



## Olfactory Cue

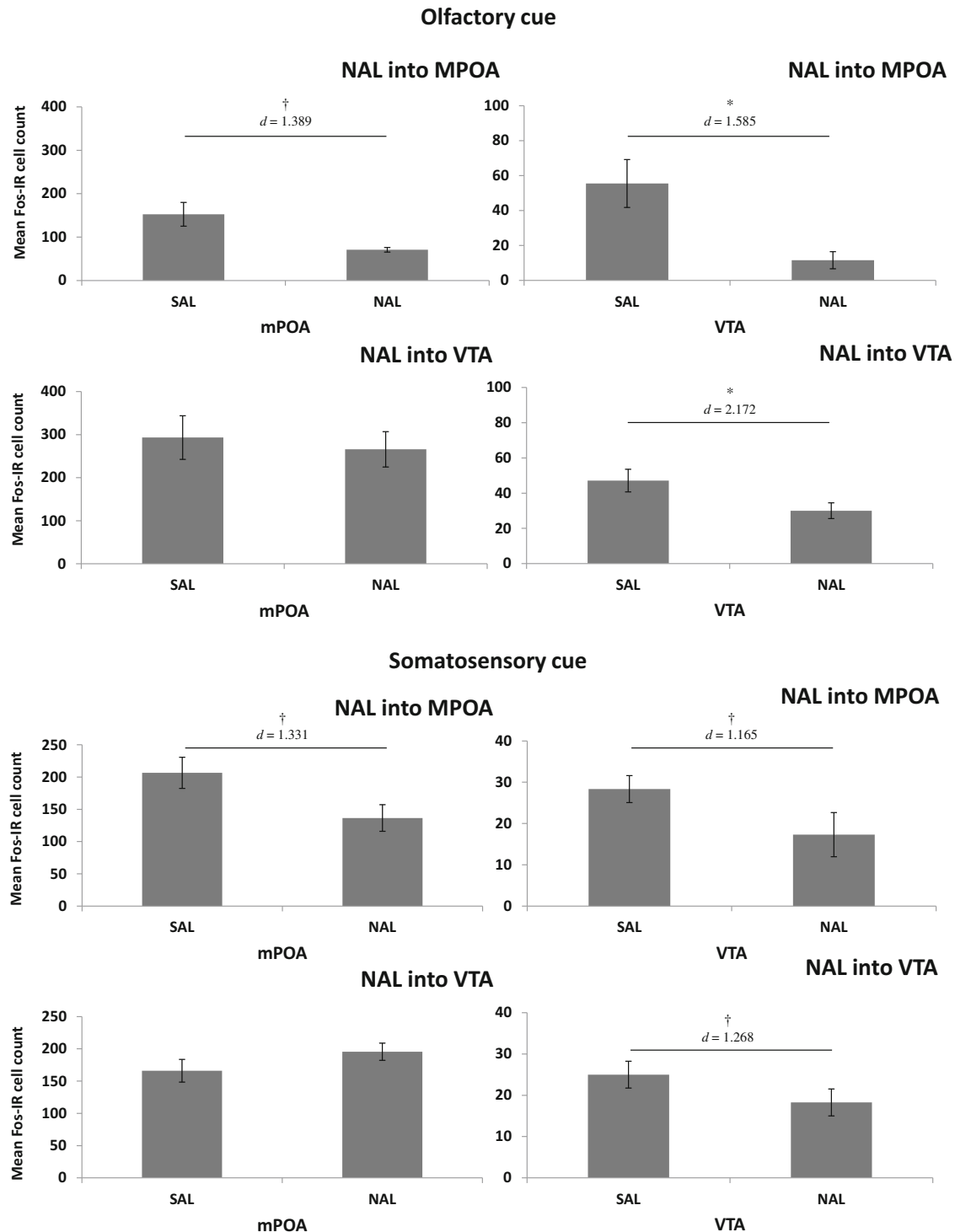


## Somatosensory Cue



◀ **Fig. 2** Mean of 1st ejaculation choice, mean of ejaculation, and 1st ejaculation choice per brain area (columns), group, and experimental cue during the open-field test.  $^{\dagger}p < 0.01$ ;  $*p < 0.05$ .  $V$  = Cramer's  $V$ ,  $\eta_p^2$  = partial eta square,  $\phi$  = phi

**Infusions of NAL or SAL to the VTA: Fos-IR analyses** As depicted in Fig. 3, when analyzing, the mPOA of males infused with NAL into the VTA showed no difference in their Fos-IR mean count than the SAL control group. An independent sample  $t$  test analysis revealed there were no statistically significant differences in the Fos-IR count between the SAL



**Fig. 3** Fos-IR-positive cells summary by brain area, group, and experimental cue.  $^{\dagger}p < 0.01$ ;  $*p < 0.05$ .  $d$  = Cohen's  $d$

group ( $M = 166.07$ ,  $SD = 55.91$ ) and the NAL group ( $M = 195.5$ ,  $SD = 35.9$ ,  $t(8) = 0.991$ ,  $p = 0.351$ ,  $d = 0.626$ ).

When analyzing, the VTA of males infused with NAL into the VTA showed a marginally lower, yet of large magnitude, Fos-IR mean count than the SAL control group, where the SAL group ( $M = 25$ ,  $SD = 6.01$ ) had a higher Fos-IR count than the NAL group ( $M = 18.27$ ,  $SD = 4.5$ ,  $t(8) = 2.005$ ,  $p = 0.08$ ,  $d = 1.268$ ).

## Discussion

The present study evaluated the role of opioid receptor antagonism in the mPOA and VTA of male rats trained to associate either an olfactory cue (almond) or somatosensory cue (rodent jacket) with their post-ejaculatory reward state. Males given SAL during training showed significant CEP for the odor or jacket, whereas males given NAL during training showed a differential response depending on the site of infusion: NAL infusions to the mPOA during training shifted the preference to the novel (unscented or unjacketed) female, whereas NAL infusions to the VTA abolished CEP altogether. Subsequent detection of Fos protein induced in these regions by the odor alone showed that prior experience with NAL infusions to the mPOA suppressed Fos in both the mPOA and VTA compared to males infused with saline, whereas prior experience with NAL infusions to the VTA suppressed Fos in the VTA alone.

Copulatory behaviors and particularly ejaculation increase whole brain opioid content and receptor internalization in the caudate, hypothalamus, and midbrain of male rats (Szechtman et al. 1981; Arletti et al. 1997; Rodríguez-Manzo et al. 2002; Coolen et al. 2004). Ågmo and Berenfeld (1990) showed that male rats who associated their post-ejaculatory period with a particular side of a three-compartment chamber later on spend more time in it over other sides of that chamber just like males who were only injected with morphine, an effect that was reversed with NAL (Ågmo and Berenfeld 1990). Furthermore, copulation to ejaculation has also been used as an unconditional stimulus to train a neutral stimulus as a predictor of sexual reward, which ultimately leads to a CPP or CEP (Tenk et al. 2009; Kippin and Pfau 2001). This trained preference shifts towards the unfamiliar female in males who are injected systemically with NAL using an olfactory (Kippin and Pfau 2001) or somatosensory cue (Quintana et al. 2019b). Together, these data indicate that blockade of opioid receptors results in a shift of male preference to cues not associated with the antagonist state, further reinforcing the notion that opioid receptor antagonism induces an aversive state (Parker and Rennie 1992).

Opioid receptors exist in high density in the mPOA and VTA (Simerly et al. 1988). Copulation to ejaculation increases  $\mu$ -receptor internalization in the mPOA (Coolen et al. 2004) and VTA (Balfour et al. 2006), effects that are prevented by

injections of NAL before copulation. Although both  $\mu$ - and  $\delta$ -receptor internalization occur in the VTA as a consequence of copulation to ejaculation (Garduño-Gutiérrez et al. 2013), only  $\mu$ -receptor internalization was correlated with the amount of sexual activity. Infusions of  $\mu$  agonists like morphine or morphiceptin to the mPOA inhibit male rat sexual behavior (Band and Hull 1990; Matuszewich and Dornan 1992; Matuszewich et al. 1995), and infusions of NAL to the mPOA, but not the NAc, disrupt a sexually conditioned place preference in male rats (Ågmo and Gómez 1993). Likewise, infusions of  $\beta$ -endorphin into mPOA impaired sexual behavior in male rats (van Furth et al. 1995a, b) and produced a refractory-like state (Hughes et al. 1987, 1990) NAL treatment prevented this impairment. Taken together with the present findings, opioid activation of  $\mu$  receptors in the mPOA appears to produce a sexual reward state.

The VTA is the origin of the mesocorticolimbic DA system which plays a major role in motivated behaviors (Fibiger and Phillips 1988; Le Moal and Simon 1991; Blackburn et al. 1992; Wise 2005; Hull and Rodríguez-Manzo 2009) through the control of the attention towards reward-related stimuli and their incentive salience (Balfour et al. 2004; Berridge 2007). In contrast to their effects in the mPOA, infusions of  $\mu$ -receptor antagonists to the VTA either facilitate sexual behavior in sexually naïve males (Mitchell and Stewart 1990) or have no effect in sexually experienced males (van Furth and van Ree 1996). This suggests that the sensitization of DA systems as male rats acquire sexual experience (Fiorino and Phillips 1999a, b) may be mediated by opioid turnover in general, and binding to  $\mu$  receptors in particular (van Ree et al. 2000; Balfour et al. 2004). Moreover, connections from the mPOA to the VTA (Brackett et al. 1986; Edwards and Einhorn 1986) are responsible for drug reward-related locomotion (Tobiansky et al. 2013; Will et al. 2016) and modulate the sexual reward value of a sexually receptive female (Lyilikci et al. 2017). Indeed, DA release patterns in the mPOA and NAc are virtually identical (Blackburn et al. 1992), and this activation increases the incentive value of the reward-related cue (Berridge 2007; Pfau et al. 2012). Thus, we suggest that opioid activation in the mPOA underlies both refractoriness and the sexual reward state, whereas opioid activation in the VTA modulates the incentive value of the reward-related cue, regardless of its sensory modality.

A differential modulation of Fos induction by the cues following NAL infusions to the mPOA versus the VTA is in accordance with the previous literature. Connections from the mPOA to the VTA have been established in both male and female rats (Brackett et al. 1986; Edwards and Einhorn 1986; Graham et al. in press), and Edwards and Einhorn (1986) suggested that outputs from the mPOA to the midbrain in male rats (including both dorsolateral tegmentum and VTA) modulated the sexual reward value of a sexually receptive female. The origin of these connections was localized mainly in the

rostral portion of the mPOA, traced back to the mesolimbic DA system, and shown to be mainly GABAergic and also sensitive to DA (i.e., co-localization of DA receptors, Tobiansky et al. 2013). Another study in males that mapped the GABAergic and glutamatergic projection from hypothalamic nuclei to the VTA through retrograde labelling showed that nearly 24% of the projecting neurons were found in the preoptic area, and nearly 77% in the anterior tuberal and mammillary areas of the hypothalamus (Kallo et al. 2015). Furthermore, estradiol benzoate has also shown to regulate GABA cell containing D1-like DA receptors in the mPOA projecting into the VTA of female rats, which indicates a modulating effect on the expression of DA receptors that ultimately regulates copulatory and proceptive behaviors in the female rat (Graham et al. *in press*). Considering that other studies have shown the role of the mPOA in the execution of copulatory behaviors (Hull and Rodriguez-Manzo 2009), these differences in the control of appetitive vs. consummatory mPOA-VTA connections likely depend on subregions of the mPOA. For example, rostral regions appear to modulate appetitive behaviors, whereas more caudal regions appear to control consummatory behaviors in the male rat (Balthazart and Ball 2007). Ultimately, these findings support the idea that the mPOA modulates the mesolimbic DA system by controlling the activation of dopamine neurons in the VTA, which in modulate the incentive value of the reward-related cue (Fields and Margolis 2015; Micevych and Meisel 2017; Wise 2009), as both the behavioral and Fos-IR outcomes of the present study suggest.

A crucial difference between the olfactory and somatosensory cue was found in the behavioral analyses, yet not through the Fos-IR. On the one hand, male rats micro-infused with NAL into their VTA while being conditioned to prefer females with the jacket still displayed a CEP towards the jacketed females, just like males micro-infused with SAL. In contrast, micro-infusion of NAL into the mPOA disrupted the CEP relative to males infused with SAL to the mPOA. On the other hand, while using the same training conditions with the olfactory cue, males micro-infused with NAL into either the VTA or mPOA did not display a CEP for the scented females, unlike their respective SAL control groups. It was expected that this would have resulted in a different pattern of Fos activation. However, the pattern of Fos-IR results between the two cues was exactly the same, where males in the NAL groups yielded a lower Fos-IR count when infused either into the mPOA or VTA compared to males infused with SAL, but also a lower Fos-IR count in the VTA when NAL was infused into the mPOA, yet not vice versa. Watabe-Uchida et al. (2012) delineated a whole-brain map of the direct excitatory inputs to the midbrain DA neurons, VTA, and the substantia nigra pars compacta. This work demonstrated that, among several other brain regions, the somatosensory and motor cortex send direct inputs (i.e., higher density and number of

neurons) mainly to the SNc. Thus, the fact that males micro-infused with NAL into their VTA still displayed a CEP towards the jacketed female suggests that this cue may require input from other brain areas (e.g., somatosensory cortex). Opioid receptors are located in somatosensory cortex in different quantities depending on the subtype of receptor (Mansour et al. 1988, 1994). Particularly, the pattern of receptor binding in the neocortex of the rat brain demonstrated that both  $\mu$  and  $\delta$  receptor are more prevalent than  $\kappa$  receptors, while their precise distributions differ greatly among the different layers. The  $\mu$ -subtype receptor appeared most prominent in layers I and III/IV of the frontal, parietal, and temporal cortex, whereas  $\delta$  receptors tend to be diffusely localized in layers II, III, V, and VI (Mansour et al. 1994). Therefore, to disrupt the development of a CEP based on an olfactory cue, micro-injections of NAL into either the mPOA or VTA are necessary and sufficient, while for a somatosensory cue, the disruption of a CEP by NAL micro-infusions is sufficient and necessary in the mPOA, while in the VTA is not, suggesting the involvement of other brain regions.

A possible alternative explanation to account for the disruption of the CEP by NAL is a hindering of familiarity and not reward. In previous studies using conditioned place preference (CPP), NAL not only disrupts, but also shifts the preference towards the opposite side of the chamber (e.g., Lett et al. 2001). To test this hypothesis properly, CPP boxes would need a middle “neutral” compartment that is not associated with any training condition. Thus, a CPP disrupted by NAL based on reward should result in a higher time spent in the opposite side, whereas a disruption based on familiarity should result in a more evenly distributed time between the opposite side and the middle compartment of the chamber. Many drug reward studies use a ratio between the time spent in the drug-associated side over the time spent in the other side of the chamber, assessing only the time spent in the “opposite” compartment (e.g., Hasenöhl et al. 1991; Spyraiki et al. 1985; Trujillo et al. 1991). We note that NAL disrupted a copulatory reward-based CPP in male rats (Mehrer and Baum 1990), and animals in that study spent very little time in the middle compartment during the test phase. It is thus most likely that NAL disrupts preference by disrupting reward rather than familiarity. Indeed, males injected with NAL throughout the development of a CEP based on either an odor (Ismail et al. 2009) or a jacket cue (Quintana et al. 2019b) also displayed a preference for females without the cue during the preference test rather than no preference for either female.

In summary, opioid antagonism in the mPOA appears to produce a state of non-reward, whereas in the VTA, such antagonism produces a state in which the odor has no incentive properties. Thus, opioids modulate CEP by multiple pathways that convey both the incentive value and valence of the reward-related odor CS, regardless of the neutral cue used, but depending where in the brain they act.

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## Compliance with ethical standards

The authors declare that all animal procedures conformed to the guidelines of the Canadian Council for Animal Care. All procedures were approved by the Concordia University Animal Research Ethics Committee.

**Conflict of interest** The authors declare they have no conflicts of interest.

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# Differential role of oxytocin and vasopressin in the conditioned ejaculatory preference of the male rat

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Supraoptic

## ABSTRACT

The neuropeptides oxytocin (OT) and vasopressin (AVP) are critical in the formation of pair bonding in the vole, and potentially in other species. The finding that normally promiscuous male rats display a conditioned ejaculatory preference (CEP) for females that bear an odor paired with the sexual reward state induced by ejaculation prompted us to examine whether similar mechanisms may mediate this rudiment of pair bonding. Sexually naïve Long-Evans male rats were given 9 multi-ejaculatory copulation trials at 4-day intervals with either almond-scented (paired) or unscented (unpaired) sexually receptive females. CEP was examined in an open field, in which each male had unrestricted access to two receptive females, one scented with almond and the other unscented. Males in both groups were given two reconditioning trials and presented with the almond odor on gauze for 1 h prior to sacrifice. Neuronal activation was assessed by immunohistochemical detection of Fos protein within OT or AVP neurons. Exposure to the odor induced significantly greater activation of OT neurons in parvocellular neurons of the paraventricular nucleus (PVN) and of AVP in magnocellular neurons of the supraoptic nucleus (SON) in the paired group compared to the unpaired group. The second experiment examined whether oxytocin or vasopressin could enhance the acquisition of a CEP. Sexually naïve male Long-Evans rats received a subcutaneous injection of OT, AVP, or the saline vehicle, prior to their first sexual experience with an almond-scented female. CEP was examined 4 days later in the open field. Males injected with OT, but not AVP or saline, displayed significant CEP. The selective activation of OT neurons by the conditioned odor in the paired group, and the ability of OT injections to enhance the association of the odor and sexual reward, indicates that enhanced OT transmission is critical in the formation of CEP in male rats.

## 1. Introduction

The neuropeptides oxytocin (OT) and vasopressin (AVP) play critical roles in a variety of integrated vertebrate neuroendocrine processes and behavioral states, including general arousal, homeostatic regulation, learning and memory, cognition, social recognition, sexual behavior, bonding, affiliation, parental care and lactation, territorial aggression, pain modulation, and stress reactivity [1–14]. The role of OT and AVP on sexual partner preferences has been examined in detail in several species, notably socially monogamous prairie voles (*Microtus ochrogaster*) that display partner preferences after their first experiences of cohabitation and/or copulation [15–20]. Intracerebroventricular (icv) administration of OT to female prairie voles, or AVP to male prairie voles, prior to cohabitation with an opposite-sex partner enhanced partner preference for the familiar female relative to a novel

female [15,20,21]. Conversely, pair-bond formations induced by copulation or cohabitation were inhibited in prairie vole females by icv administration of the OT antagonist ornithine vasotocin, or in prairie vole males by icv administration of the vasopressin V1 receptor antagonist d(CH<sub>2</sub>)<sup>5</sup>[Tyr-Me]AVP [15,20]. Relative to promiscuous male meadow voles (*Microtus pennsylvanicus*), male prairie voles have increased vasopressin 1a (V1a) receptor densities in several brain regions, notably the ventral pallidum [9], a site that relays incentive partner information from limbic and cortical structures that are sensitized during the male prairie vole's first sexual experiences. Indeed, viral transfection of the V1a receptor into the male meadow vole ventral pallidum leads to overexpression of the receptor and a subsequent enhancement of the ability of first sexual experiences to condition sexual partner preferences [22].

Like the male meadow vole, the mating strategies of male and

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female rats are considered promiscuous [23,24] with both sexes copulating relatively indiscriminately in large groups with opposite sex conspecifics. However, dominant female rats have been observed to compete with other females in group-mating situations and to intercept certain males selectively immediately prior to the males' ejaculations [25]. This suggests that some aspects of selective mate choice exist naturally in female rats. Indeed, using paradigms that ensure the induction of optimal sexual reward states during paced copulation [26,27], female rats can be conditioned to show both sexual partner and mate preferences for males of their same strain bearing neutral odors (e.g., almond, lemon) or for males of different strains associated with optimal sexual reward [28–31]. Moreover, female rats given their first rewarding (paced) sexual experiences with one particular male display mate-guarding behaviors when paired with that male and a competitor female in an open field [32,33]. These behaviors include hovering near the male, blocking access to the competitor by placing herself between the male and competitor, and intercepting and mounting the competitor (an agonistic dominance behavior that prevents the competitor female from soliciting the male, and prevents the male from mounting the competitor). These conditioned effects are eliminated if the nonselective opioid receptor antagonist naloxone is administered during each conditioning session, suggesting that opioid activation is critical for the sexual reward state [30,34,35].

Male rats can also be conditioned to show a rudimentary form of mate choice. If males receive their first sexual experiences with sexually receptive females scented with a neutral odor, such as almond or lemon, they will ejaculate first and more times with a scented receptive female over an unscented receptive female in an open field partner preference choice test [36–45]. This conditioned ejaculatory preference (CEP) depends on pairing the postejaculatory reward state with the presence of the odor cue [42] and can be eliminated if conditioning trials occur under the influence of the opioid receptor antagonist naloxone [39]. Similarly, although less robust, males that receive their first multiejaculatory sexual experiences with unscented females show a greater number of ejaculations with unscented relative to scented females in the partner preference test [41,42].

What underlies the ability of a neutral olfactory stimulus to induce CEP? Studies utilizing the immediate-early gene product Fos as a marker of neuronal activation following exposure to the odor in paired vs. unpaired males have shown significantly greater activation in regions associated with bonding and incentive motivation, including the nucleus accumbens (NAc) core, olfactory tubercle and main olfactory (piriform) cortex, lateral hypothalamus, supraoptic nucleus (SON), paraventricular nucleus (PVN), and basolateral amygdala (BLA) [46]. A similar pattern of activation has been shown in female rats that display a conditioned partner preference for males bearing a neutral olfactory stimulus associated with paced copulation [47]. The activation of Fos in the PVN and SON were of interest in the present study as these are regions contain cell bodies of OT and AVP neurons that project both to the posterior pituitary and to other brain regions like the ventral pallidum [48–52]. Given the critical role played by both OT and AVP in the development of social and sexual partner preferences of female and male prairie voles, and of OT in the conditioned mate guarding behavior shown by female rats [33], we asked in the first experiment whether the conditioned olfactory cue itself might activate Fos within OT and/or AVP neurons in the PVN and SON in paired versus unpaired control animals. In the second experiment we asked whether a peripheral injection of OT or AVP during a male rat's first copulatory experience with an almond-scented female might enhance CEP during a choice test 4 days after the conditioning. This followed the methodology in Holley et al. [33].

## 2. Materials and methods

### 2.1. Subjects

Long-Evans male (200–250 g) and female (150–200 g) rats were obtained from Charles River Canada, Inc. (St-Constant, QC). Females were pair-housed with wood-chip bedding in Plexiglas cages. Males were housed in large Plexiglas cages four to a cage, also with wood-chip bedding. All rats were housed in the same colony room on a reversed 12-h light/dark cycle (lights off at 0800), given ad libitum tap water and regular rat chow, and kept in a constant temperature of 21 °C.

### 2.2. Surgeries and hormone administration (females)

To prevent pregnancy and induce common hormonal priming, bilateral ovariectomies were performed on all females via lumbar incision. General anaesthesia was induced with ketamine hydrochloride (100 mg/ml) and xylazine hydrochloride (20 mg/ml), mixed in a 4:3 ratio, respectively, and administered intraperitoneally (ip) at a dose of 1 ml/kg. Females received daily subcutaneous (sc) injections of enrofloxacin (5 mg/kg) and flunixin meglumine (2.5 mg/kg) to prevent post-surgical infections and nociception, respectively. One week of recovery was provided prior to the onset of behavioral testing.

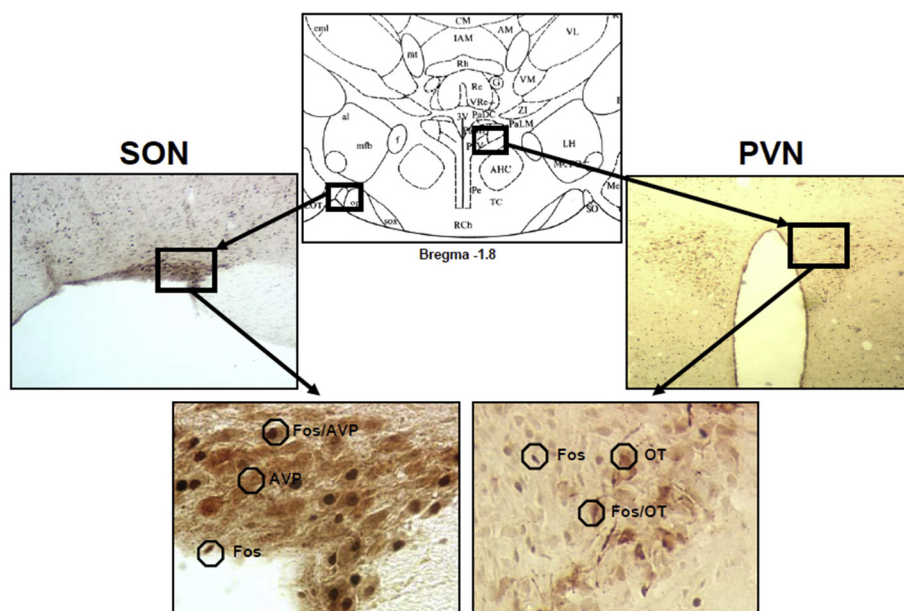
Females were given four 30-min multi-ejaculatory training sessions of sexual interaction with random sexually vigorous males in bilevel chambers to equate them for sexual experience. Sessions took place at 4-day intervals to approximate the normal ovulatory cycle of the female. Females were primed with EB (10 µg) 48 h and P (500 µg) 4 h before each test. Steroids were administered subcutaneously (sc) in 0.1 ml of reagent-grade sesame oil (Sigma-Aldrich, St. Louis, MO).

All animal procedures conformed to the guidelines of the Canadian Council for Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

### 2.3. Odor conditioning and brain preparation (males)

In the first experiment, sexually naïve males ( $N = 20/\text{group}$ ) that had been handled extensively and preexposed to bilevel chambers during 5 daily sessions for 15 min each, were given nine 30-min multi-ejaculatory conditioning sessions of sexual interaction with fully primed females in bilevel chambers at 4-day intervals. Each male was placed into a bilevel chamber for 5 min after which a sexually receptive female was introduced for the conditioning session. Males in the Paired Group copulated exclusively with females scented with almond extract (Blue Ribbon, Etobicoke, ON). Almond extract was applied to the back of the neck and anogenital region of the females using a cotton swab [41]. Males in the Unpaired control group copulated exclusively with unscented females that had distilled water smeared to the back of their necks and anogenital regions. These males thus served as the control for exposure to almond odor and any conditioned reinforcement that accrued to the odor after repeated pairing with the postejaculatory reward state. Four days after the ninth conditioning trial, males were placed into a large open field (123 cm × 123 cm × 46 cm) for 5 min, after which two sexually receptive females were introduced, one scented with almond and the other unscented. Males were allowed to copulate freely with both females for 30 min. All sexual behavior trials were recorded on video during the test and scored subsequently using the Behavioral Observation Program [53]. Latencies and frequencies for mounts, intromissions, and ejaculations per female were calculated, along with the proportion of males that made first mount, first intromission, and first ejaculation with either female.

Because the final open field test exposed males in both conditioning groups to copulation with females of a different olfactory status (e.g., Paired males with unscented females, and vice-versa), the males were given two reconditioning trials to reinforce the original learning (as we have done previously for both males and females; see [46,47]). These



**Fig. 1.** Location of AVP and OT neurons in the SON and PVN that were analyzed in the present study. The immediate-early gene product Fos appears as a black dot within the nucleus of activated cells. This was either surrounded by clear cytoplasm or by brownish cytoplasm indicating the co-labeling of AVP or OT, both of which were analyzed in the SON and PVN of alternating sections taken from paired and unpaired males. Hypothalamic structures at Bregma  $-1.8$  mm modified from Plate 28 of Paxinos and Watson [54].

were conducted as above for the Paired and Unpaired control groups at 4-day intervals. Four days after the second reconditioning trial, males were placed singly into a Plexiglas shoebox cage with wood-ship bedding and a cotton gauze pad that had been immersed in almond extract was placed into the chamber for 1 h [46,47]. Males were allowed to interact freely with the gauze pad. After the hour, males were anesthetized deeply with an intraperitoneal (ip) injection of sodium pentobarbital (Euthanyl®, Bimeda-MTC Animal Health, Lavaltrie, QC, diluted to 120 mg/kg/ml), and perfused intracardially with 350 ml of phosphate-buffered saline followed by 350 ml of 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 4 h and stored overnight in 30% sucrose solution. Brains were then covered in aluminum foil and stored at  $-80^{\circ}\text{C}$  prior to sectioning.

In the second experiment, sexually naïve males ( $N = 60$ ) were handled and preexposed to bilevel chambers as in the first experiment. Males were then assigned to one of three groups and injected subcutaneously (sc) with OT (5  $\mu\text{g/kg}$ ; Bachem Cat #H2510, Torrance, CA), AVP (5  $\mu\text{g/kg}$ ; Sigma-Aldrich Cat # V9879, Oakville, ON), or the 0.9% saline vehicle, in a volume of 1 ml/kg, 4 h, 2 h, and 15 min prior to their first sexual experience with a scented receptive female in bilevel chambers. Four days after their first experience, males were placed into the open field for 5 min, after which two sexually receptive females were introduced, one scented with almond and the other unscented. Males were allowed to copulate freely with both females for 30 min. All sexual behavior trials were recorded on video during the test and scored subsequently using the same Behavioral Observation Program [53]. Latencies and frequencies for mounts, intromissions, and ejaculations per female were calculated, along with the proportion of males that made first mount, first intromission, and first ejaculation with either female.

#### 2.4. Immunohistochemistry

Brains from 5 Paired and 5 Unpaired males in the first experiment were selected randomly, blocked around the area of the anterior hypothalamus containing the PVN and SON (corresponding to Plates 23 to 27 in Paxinos and Watson [54]), mounted on a chuck, and frozen 30  $\mu\text{m}$  coronal sections were cut on a cryostat. Serial sections were allocated to two groups, one for staining of oxytocin and the other for staining of vasopressin. Sections were washed in ice-cold 0.9% TBS, incubated for 30 min in a mixture of TBS and 30% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) at  $4^{\circ}\text{C}$ , rinsed, and transferred for 90 min to a preblocking serum consisting of

0.05% triton TBS (50  $\mu\text{l}/100$  ml) and Normal Goat Serum (NGS; Vector Laboratories Canada, Burlington, ON, diluted 1:200) at  $4^{\circ}\text{C}$ . The sections were rinsed and incubated in a mixture of NGS, 0.05% triton TBS, and a rabbit polyclonal primary antibody raised against residues 4–17 of the Fos protein (Fos ab5, Calbiochem, Mississauga, ON; diluted 1:40,000) in 0.05% Triton-TBS with 3% NGS for 72-h at  $4^{\circ}\text{C}$ . The sections were then rinsed and transferred to a secondary antibody solution consisting of 0.05% triton TBS, NGS, and a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories Canada, Burlington, ON, diluted 1:200) for 1 h at  $4^{\circ}\text{C}$ . Sections were rinsed and transferred to a solution of 0.2% triton TBS, and Vectastain Elite ABC solutions (diluted 1:55) for 2 h at  $4^{\circ}\text{C}$ . Finally, the sections were rinsed in TBS, rinsed in 50 mM Tris buffer (pH 7.6) for 10 min, and incubated on an orbit shaker for 10 min in 0.05% 3,3'-diaminobenzidine (DAB) in 50 mM Tris buffer with 3%  $\text{H}_2\text{O}_2$  (solution buffered to pH 7.8 with drops of 6 M NaOH), with the addition of  $\text{NiCl}_2$  to catalyze the DAB and color the chromagen product blue-black. The reaction was stopped by rinsing in cold TBS. Sections from one group were then incubated in a rabbit polyclonal primary antibody raised against OT (Chemicon ab #911, diluted 1:5000) while sections from the other group were incubated with a rabbit polyclonal primary antibody raised against AVP (Chemicon ab #937, diluted 1:5000). Both antibodies have  $< 1\%$  cross-reactivity with each other's antigen. Following secondary antibody and tertiary Vectastain Elite ABC reactions, sections were incubated in the DAB/Tris buffer/ $\text{H}_2\text{O}_2$  solution to color the DAB chromagen product reddish brown. Sections were rinsed in TBS to stop the reaction, mounted onto 75x25mm gel coated slides (Serum International), dehydrated in  $\text{H}_2\text{O}$  and ethanols (70%, 95%, and 100%, 10 min per solution) followed by immersion in Xylenes (Fisher Scientific) overnight to clear them. Slides were coverslipped with Permount, allowed to dry, cleaned, and subsequently examined under a Leitz Laborlux microscope.

Fos-positive cells, OT- and AVP-positive cells, and double-labeled Fos/OT or Fos/AVP cells were counted bilaterally per slice in regions of the PVN and SON that contained the most Fos, as we have done previously for double-labeled Fos/glutamate neurons in the ventromedial hypothalamus [55]. Counts were made on 5 slices per animal, and a mean of the counts was determined per animal. Single-labeled Fos cells were observed as a black nucleus surrounded by a colorless cytoplasm, whereas OT or AVP cells were observed as a light brown cytoplasmic stain and clear nucleus. Double-labeled Fos/OT or Fos/AVP cells had a black nucleus surrounded by a light brown cytoplasm (Fig. 1, Fig. 3,

top) in one plane of view under the microscope. For all double-labeled cells counted, care was taken to focus the plane of view in and out to make sure the cells were not single-labeled and simply adjacent to one another (as can occur in a 30  $\mu$ m section that is approximately 3 cell bodies' deep). All counts were made at 400 x. Mean numbers of double-labeled cells per section for each rat were obtained by dividing by 2 the sum of the labeled cells counted on both sides for all 5 sections counted per rat (Fig. 3, bottom).

### 2.5. Statistical analyses

For the first experiment, latency and frequencies of mounts, intromissions, ejaculations during the open-field tests were analyzed using a mixed between-within  $2 \times 2$  analysis of variance (ANOVA) with the between variable being conditioning group (Paired vs. Unpaired) and the within variable being the female (scented vs. unscented). The proportion of males that chose the scented or unscented female for their first mount, first intromission, or first ejaculation was analyzed using a 2-way  $\chi^2$  test for goodness of fit. The average number of double-labeled cells per section was analyzed using a one-way, between-subjects ANOVA for counts of Fos/OT or Fos/AVP cells in the PVN or SON between Paired and Unpaired control groups. For all significant interactions, posthoc Tukey HSD tests were conducted on the individual means. In the second experiment, latency and frequencies of mounts, intromissions, ejaculations during the open-field tests were analyzed using a mixed between-within  $3 \times 2$  analysis of variance (ANOVA) with the between variable being treatment group (OT, AVP, or saline) and the within variable being the female (scented vs. unscented). Posthoc Tukey HSD tests were conducted for all significant main effects and interactions. Effect size was calculated using partial  $\eta^2$ . The proportions of males that chose the scented or unscented female for their first mount, first intromission, or first ejaculation were analyzed using two 2-way  $\chi^2$  tests for goodness of fit, OT vs saline and AVP vs. saline.

## 3. Results

### 3.1. Conditioned ejaculatory preference

Fig. 2 shows the proportion of males that chose the scented vs. unscented females for first mount, intromission, and ejaculation, and the distribution of mounts, intromissions, and ejaculations per female during the open field choice test. The ANOVA detected a significant interaction of conditioning group by female for the number of ejaculations,  $F_{1,38} = 26.40$ ,  $p < .0001$ , partial  $\eta^2 = 0.59$ . Posthoc Tukey tests revealed that males in the Paired group ejaculated significantly more times with the scented female relative to the unscented female, and males in the Unpaired group ejaculated significantly more times with the unscented female relative to the scented female (Fig. 2). Although more males in the Paired group chose the scented female for their first ejaculation relative to males in the Unpaired group, this effect did not reach statistical significance,  $\chi^2(1) = 2.56$ ,  $p = .11$ . However, males in both groups chose their respective familiar females (scented for paired, unscented for unpaired) for first mount,  $\chi^2(1) = 6.40$ ,  $p = .01$ .

### 3.2. Fos induction in oxytocin and vasopressin neurons by the conditioned odor

Consistent with previous observations (Kippin et al., 2003), male rats in the Paired group reacted intensely with the almond-scented gauze pad by sniffing and picking up the pad to rub it over their snouts and/or bite it. Males in the Unpaired group did not display these reactions and most ignored the pad altogether.

Fos induction by the odor was found differentially within OT and AVP neurons in the PVN and SON of males in the two groups, with Fos found largely in parvocellular neurons in the PVN and magnocellular

neurons in the SON (Fig. 3). For the PVN, the ANOVA detected a significant difference between Paired and Unpaired groups in the number of double-labeled Fos/OT neurons,  $F_{1,8} = 7.35$ ,  $p = .026$ , partial  $\eta^2 = 0.91$ . No significant difference was found for the number of double-labeled Fos/AVP cells in the PVN,  $F_{1,8} = 3.49$ , ns, partial  $\eta^2 = 0.44$ . In contrast, for the SON, the ANOVA found a significant difference in the number of double-labeled Fos/AVP cells,  $F_{1,8} = 6.17$ ,  $p = .038$ , partial  $\eta^2 = 0.77$ . No significant difference was found for the number of double-labeled Fos/OT cells in the SON,  $F_{1,8} = 1.33$ , ns, partial  $\eta^2 = 0.17$ .

### 3.3. Potentiation of CEP by OT or AVP

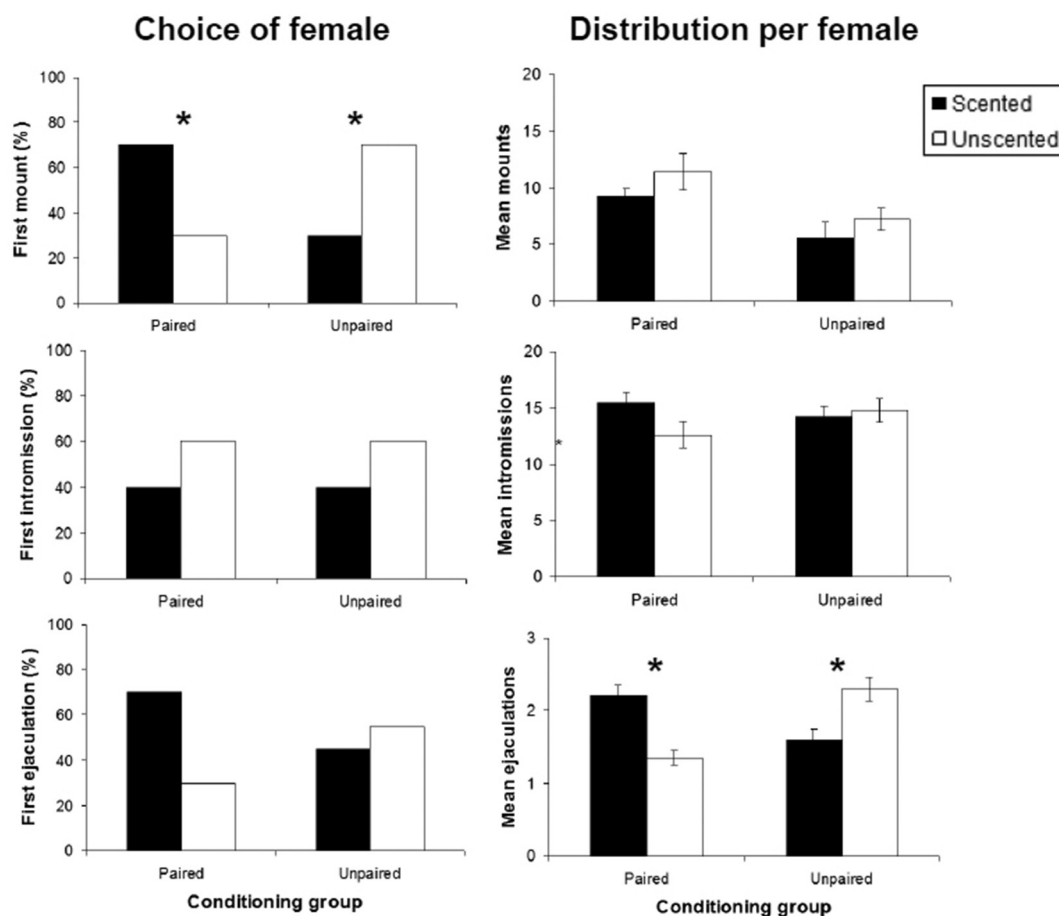
Fig. 4 shows the proportion of males that received SAL, AVP, or OT treatment prior to their first sexual experience that chose the scented vs. unscented female during their second sexual experience for first mount, intromission, or ejaculation (left side), and the total number of mounts, intromissions, and ejaculations received by the scented vs. unscented female from males in each treatment group (right side). Injection of OT, but not AVP or SAL, during the males' first sexual experience potentiated the distribution of ejaculations in males during their second experience. Although the total number of ejaculations made by males in each treatment group did not differ significantly, the ANOVA detected a significant within-subjects effect for female,  $F_{1,57} = 4.20$ ,  $p < .05$ , partial  $\eta^2 = 0.07$ , with scented females receiving more ejaculations overall than unscented females. A significant interaction of group by female was also found,  $F_{2,57} = 4.13$ ,  $p < .03$ , partial  $\eta^2 = 0.14$ . Post-hoc Tukey tests of the individual means in the interaction effect revealed that the injection of OT before the first sexual experience produced a significant difference in the distribution of ejaculations between the scented and unscented females ( $p < .05$ ), whereas the injections of AVP or SAL did not (Fig. 4, right). Although more animals in the OT-treatment groups ejaculated first with scented females relative to unscented females (Fig. 4, left), this effect was not statistically significant relative to SAL,  $\chi^2(1) = 1.60$ , ns. No significant effects were observed for mounts or intromissions.

## 4. Discussion

This study examined the involvement of OT and AVP in the conditioned ejaculatory preference displayed by male rats for sexually receptive female rats scented with a neutral odor (almond) that had been paired repeatedly with the males' post-ejaculatory reward state. The first experiment found a significant activation of the immediate-early gene product Fos within largely parvocellular OT neurons in the PVN of paired relative to unpaired males, but no significant activation of AVP neurons in this region. Conversely, there was a significant increase in Fos within largely magnocellular AVP neurons in the SON, but no significant activation of OT neurons in this region by conditioning. The second experiment found that peripheral administration of OT, but not AVP, to sexually naïve paired males during their first multi-ejaculatory experience with almond-scented females enhanced the number of ejaculations made with scented relative to unscented females during an open field test 4 days later. Given that parvocellular OT neurons in the PVN project to other hypothalamic, limbic, and cortical structures [51,56,57], we suggest that the sensitized activation of central OT neurons is an important mediator of CEP.

In humans OT increases the salience of social cues, which are themselves mediated by context [58]. In rodents, both OT and AVP modulate conspecific social recognition at the level of the olfactory system, and in particular the formation of both short- and long-term odor memories [13], and OT facilitates both conditioned place and social preferences for one female over another in female mice [59]. Given that CEP is expressed as a preference to ejaculate selectively with a female bearing a conditioned cue such as odor [37,41–43], it is possible that part of the behavioral sensitization of the odor cue is





**Fig. 2.** Conditioned ejaculatory preference (expressed as the choice of female for first ejaculation, or increased distribution of ejaculations). Males in the paired and unpaired groups mounted first, and ejaculated more times with, scented and unscented females, respectively. \* $p < .05$ .

mediated by the activation of central OT systems that project from the PVN to hypothalamic, limbic, and cortical regions that process olfactory input, such as medial preoptic area, bed nucleus of the stria terminalis, medial amygdala, and piriform cortex [60–62]. The selectivity for the scented female is strongest as the male approaches ejaculation [43], thus we would predict that OT transmission in some or all of those regions may be highest at that time. Interestingly, OT rises dramatically in the plasma of heterosexual men after they have masturbated to orgasm while watching an erotic video [63]. It is not yet known to what extent the rise peripherally might match with increases that occur centrally; however, it is clear from the present results that systemic pharmacological priming with OT during the male's first multi-ejaculatory sexual experience augments the learning of the odor discrimination necessary for CEP. OT could also accomplish this by augmenting the salience of the ejaculatory reward state.

Although systemic administration of AVP prior to the males' first sexual experience did not produce a significant CEP during the second experience, more Fos was found co-expressed in magnocellular AVP neurons in the SON, but not PVN, of paired relative to unpaired males following exposure to the conditioned almond odor in the first experiment. In that experiment, males had far more extensive experience with the odor paired with sexual reward. Magnocellular AVP neurons in the SON project largely to the posterior pituitary vasculature and could, in this case, participate in the maintenance of social memory at the level of the olfactory bulb (via circumventricular feedback; [64]) and/or mediate the relatively rapid increases in blood pressure typically observed in male rats and humans in response to highly arousing first- or second-order sexual cues [65–67]. However, Krüger et al. [63] did not observe significant increases in AVP in plasma of heterosexual men

masturbating to orgasm while watching an erotic video. Given that samples were taken at 2-min intervals in that study it is likely that any early focused pulsatile release of AVP was spread throughout the sample(s) making it difficult to detect. It was also noted that participants showed transient increases in heart rate and systolic and diastolic blood pressure during masturbation which reached a peak prior to orgasm and then returned to baseline. Thus, it may be the case that watching the erotic video utilized in that study, watching it in a laboratory setting, and/or masturbating as a means of achieving orgasm, did not raise blood pressure in an appreciable way. In that study epinephrine and norepinephrine levels both rose significantly in at least two samples before orgasm. This suggests that sympathetic activation did occur (presumably to move more blood into erectile tissue and muscle), but may well have been specific to the activation of peripheral sympathetic arousal leading to orgasm and not induced in response to the stimuli used per se.

How might OT transmission become sensitized to a neutral odor that predicts a sexual reward state? Certain long-term changes occur in both OT receptor density and OT synthesis and release that are a function of changes to OT receptor and synthesis genes in response to stimulation [68,69]. Ejaculation in male rats results in opioid release [70,71] and induces the reward state necessary for both conditioned place preference (CPP) and CEP [42,72]. The nonselective opioid receptor antagonist naloxone blocks the development of both post-ejaculatory CEP and CPP [34,39,73,74], indicating that opioid binding is critical for these effects. OT is also released from the posterior pituitary immediately prior to and during ejaculation [3]. Presentation of the conditioned odor on gauze to paired male rats results in both noncontact erections and enhanced dopamine release in the nucleus

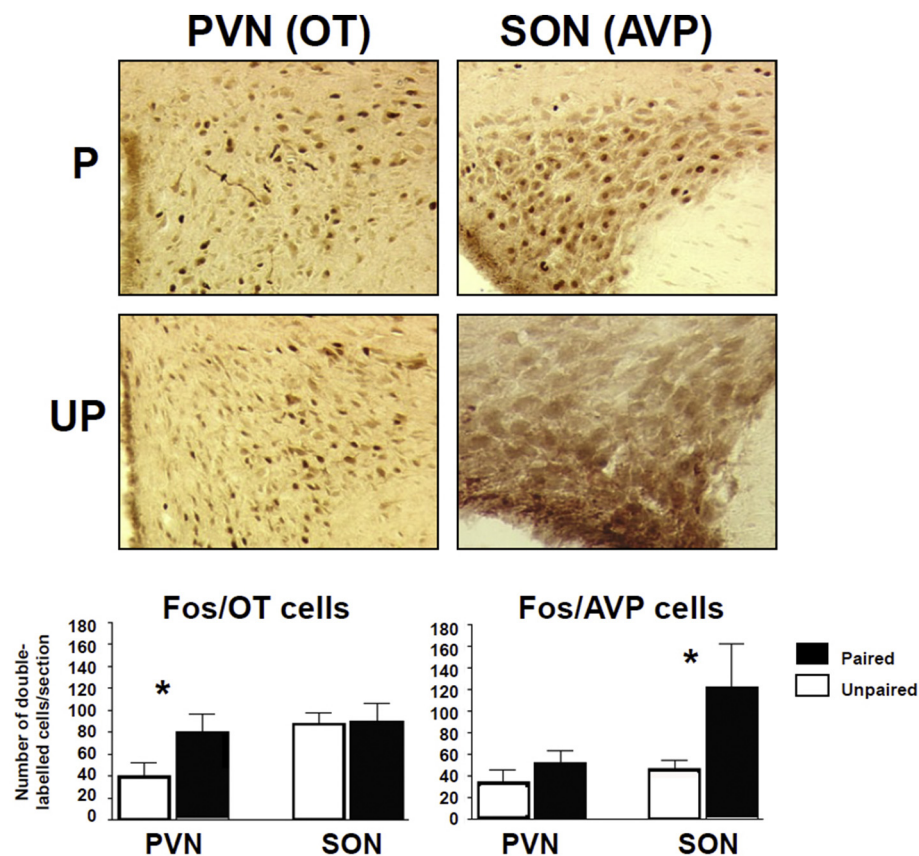


Fig. 3. Induction of Fos within AVP or OT neurons in the SON or PVN of paired (P) and unpaired (UP) males by the conditioned odor. \* $p < .05$ .

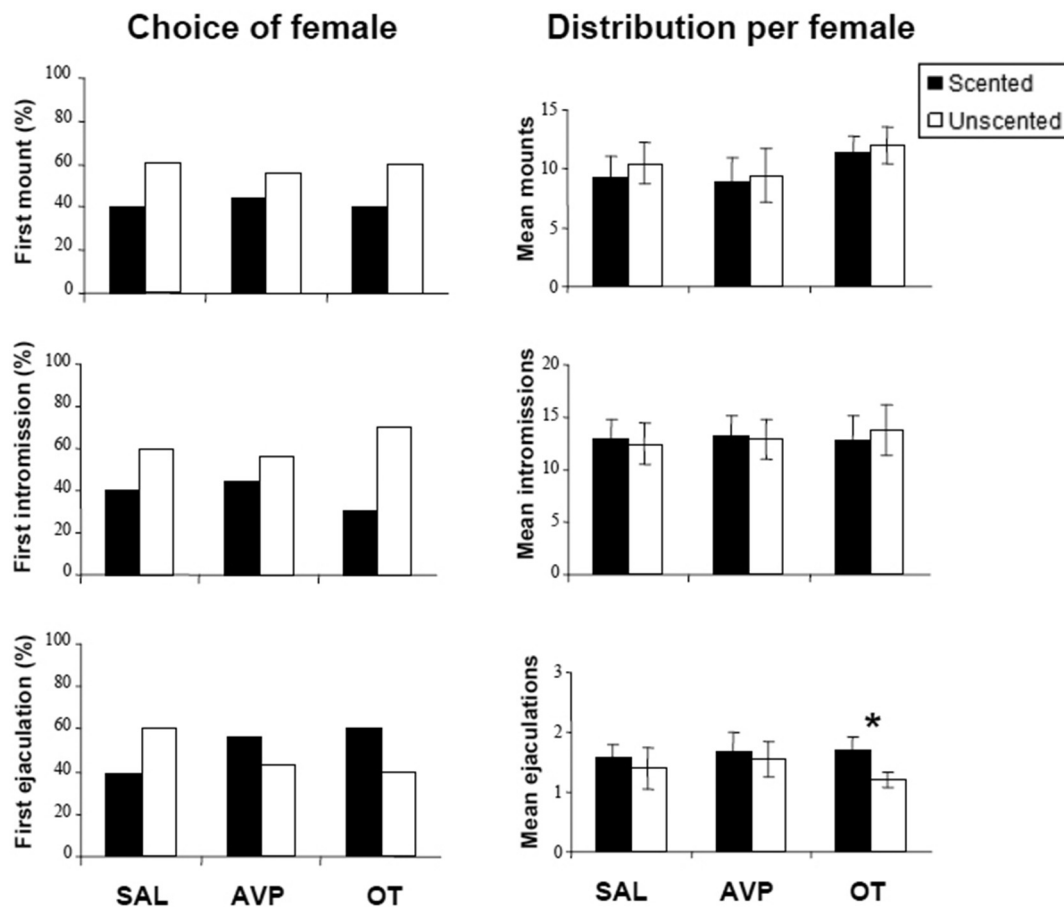
accumbens [75], an effect that may well be driven by conditioned OT release in the VTA [34,76–80], which activates dopamine (DA) neurons that have already been sensitized by opioid actions. This effect could be enhanced by the stimulation of OT neurons in the PVN [80], which stimulates penile erection in male rats and, via projections to the VTA [81], activates DA neurons and leads to further enhancement of extracellular DA in the nucleus accumbens [80,82,83]. It may also be the case that early augmentation of OT during conditioning acts at spinal circuits to enhance ejaculation. In the present study, the administration of OT during the first conditioning trial increased the proportion of males that ejaculated first with the scented females during the second test and augmented the number of ejaculations displayed by those males with the scented females, although the former effect did not reach statistical significance. As in the present study, Kippin and Pfau [42] reported that the number of ejaculations was a more sensitive measure of CEP during early phases of conditioning than was the choice for first ejaculation. However, following a full complement of conditioning trials, Kippin and Pfau [43] found that paired males focus exclusively on the scented female a few intromissions before their first and subsequent ejaculation(s). This suggests that the full expression of CEP depends on a differential time-course of coordinated action of OT in both spinal and supraspinal circuits.

Although CEP for females bearing a conditioned odor cue was augmented by OT and not AVP in the present study, we note that the partner preferences displayed by male prairie voles following their first copulation with sexually receptive females are driven by both OT and AVP, and in particular AVP binding to the V1aR in the ventral pallidum [9,16,22]. Promiscuous male meadow voles have relatively reduced expression of V1aR in this region, and gene transfer of the V1aR by viral vector to this region in meadow vole males to induce over-expression of the receptor results in those males forming pair bonds following their first copulation with females. AVP has a strong role in olfactory

recognition [13] and both the main and accessory olfactory bulb contain AVP neurons that project to limbic and cortical sites, along with the anterior olfactory nucleus of the cortex. Both main and accessory olfactory pathways project to striatopallidal neurons [84], thus it is possible that AVP in this region promotes recognition of social or individual cues that subserve partner recognition and preference.

In addition to the actions of OT and AVP in limbic and hypothalamic systems that promote partner recognition and physical closeness, mesolimbic dopamine (DA) is critical for the activation of attention to rewarding partner-related cues [75,85–88]. DA transmission in the nucleus accumbens increases in male prairie voles during their first copulation with receptive females, as it does in male rats [89]. Micro-infusion of the nonspecific DA receptor agonist apomorphine to the nucleus accumbens of male prairie voles during a 24-h cohabitation period with receptive females promotes partner preference formation, as does systemic administration of the D2-selective agonist quinpirole, but not the D1-selective agonist SKF-38393. We have found that systemic administration of quinpirole to male rats during a 24-h cohabitation period with an almond-scented, sexually non-receptive female, produces a significant CEP for an almond-scented receptive female over an unscented receptive female during the males' first sexual experience in an open field 4 days later [90]. In fact, quinpirole-treated males that cohabit for 24 h with an almond-scented male will display a social partner preference and spend more time near that male relative to a sexually receptive female [91]. Connections between the nucleus accumbens and ventral pallidum are critical for incentive motivation and adaptive behavior [92–96], and output is controlled in part by DA release in the nucleus accumbens [97,98]. The conditioned odor activates DA release selectively in the nucleus accumbens of paired but not unpaired males [75] and activates Fos selectively in the piriform cortex, nucleus accumbens core, lateral hypothalamic area, and basolateral amygdala of paired relative to unpaired male rats [46]. It is possible





**Fig. 4.** Conditioned ejaculatory preference (choice of female for first ejaculation, or increased distribution of ejaculations) induced by SAL, AVP, or OT injections prior to the males' first sexual experience with almond-scented sexually receptive females. Prior experience with OT during sex produced a significant increase in the number of ejaculations directed toward the scented female during the second test. \* $p < .05$ .

that the activation of these regions marks the priming cue as a sexual incentive and the addition of OT activation prompts physical closeness to the female bearing the familiar cue prior to ejaculation.

Finally, sexually receptive female rats that have had their only experiences of rewarding paced copulation with one male display conditioned mate-guarding behaviors in the presence of their familiar male and a competitor female [32,33]. Mate guarding was expressed by females as hovering and presenting next to the male, blocking the competitor female when she came close to the male, and agonistic mounting of the competitor female if she solicited the male. When presented with their preferred familiar males behind a screen, these paired females showed a very similar activation of OT and AVP neurons [33]. Analogous to the present results, administration of OT during the paired female's first rewarding (paced) sexual experience with the male potentiated the hovering and presenting response 4 days later; however, administration of AVP during the first experience potentiated the competitor blocking response during that second trial. We have also shown previously that female rats given their first rewarding sexual experiences with male rats scented with an almond or lemon odor not only solicit scented males over unscented males, but they select scented males significantly more for their first and subsequent ejaculations [29,83]. As with males, the sexual partner and mate preference by the females is blocked by the opioid antagonist naloxone during conditioning [30], and the odor alone induces Fos in many similar regions, including the PVN of paired relative to unpaired females [47]. Indeed, systemic administration of the longer-lasting opioid receptor antagonist naltrexone, or infusions of the  $\mu$ -selective antagonist CTAP to the dorsal striatum, but not nucleus accumbens, of female prairie voles that cohabitated with males for 18 h, blocked the development of pair bonding

[99]. We suggest that the conditioning procedures used here activate a bonding system common to many mammals and indeed other vertebrates [7,100] in response to sexual reward states. This system interacts with general limbic and cortical mechanisms of incentive motivation to direct behaviors toward cues that predict reward and pleasure [82,101], thus allowing animals a great deal of flexibility in the generation of sexual partner preferences.

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# Acute ethanol disrupts conditioned inhibition in the male rat

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## Abstract

**Rationale** Alcohol can disrupt conditioned sexual inhibition (CSI) established by first-order conditioning in male rats. CSI can also be induced using second-order conditioning, during which male rats are trained to associate a neutral odor with a nonreceptive female. As a result, when given access to two receptive females (one scented and one unscented) during a copulatory preference test, they display CSI toward the scented female.

**Objective** The present study examined the effect of low-to-moderate doses of alcohol on CSI and brain activation following exposure to alcohol and the olfactory cue alone.

**Methods** Sexually-naïve Long-Evans rats received alternate conditioning sessions with unscented receptive or scented (almond extract) non-receptive females. Following the conditioning phase, males were injected with saline, alcohol 0.5 g/kg or 1 g/kg, 45 min before a copulatory test with two receptive females, with one bearing the olfactory cue. Fos activation was later assessed, following exposure to alcohol and the olfactory cue alone, in several brain regions involved in the expression and regulation of male sexual behavior.

**Results** While males in the saline group displayed sexual avoidance towards the scented female, those injected with alcohol before the copulatory test, regardless of the dose, copulated indiscriminately with both females. Subsequent exposure to alcohol and the olfactory cue alone induced different Fos expression between groups in several brain regions.

**Conclusions** Low to moderate doses of alcohol disrupt conditioned sexual inhibition in male rats and induce a differential pattern of neural activation, particularly in regions involved in the expression and regulation of sexual behavior.

**Keywords** Conditioned sexual inhibition · Alcohol · Fos expression

## Introduction

Inhibitory and disinhibitory effects of alcohol on sexual behavior have been well-documented in both humans and animals (Abel 1980; Berry and Johnson 2018; Crowe and George 1989; Dolder et al. 2017; Garcia et al. 2019; George 2019; Pfaus et al. 2010; Scott-Sheldon et al. 2016). The effects appear to be dose-dependent: high, intoxicating doses

inhibit physiological sexual arousal and motor function (e.g., Briddell and Wilson 1976; Farkas and Rosen 1976; Rubin and Henson 1976; Wilson 1977), whereas low to moderate doses induce subjective or psychological disinhibition that is observed especially when inhibition of sexual response is present prior to intoxication (e.g., Rubin and Henson 1976; Wilson and Niaura 1984). Low levels of alcohol intoxication increase sexual risk-taking in terms of haphazard condom use (MacDonald et al. 1996), sex with multiple partners (Santelli et al. 1998), and an increased likelihood of contracting a sexually transmitted illness (Buffum 1988; Ross and Williams 2001; Shafer et al. 1993). From a cognitive perspective, low-to-moderate doses of alcohol facilitate subjective sexual arousal and the intent to have sexual intercourse (Prause et al. 2011) and increase the preference to view sexual images that had been rated less pleasant overall than neutral pictures in control participants not given alcohol (Dolder et al. 2017).

In animals, acute administration of low to high doses of alcohol dose-dependently delayed or blocked erection,

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ejaculation, and mounting behavior in both male dogs (Gantt 1940; 1952; 1957; Hart 1969; Teitelbaum and Gantt 1958) and male rats (Ferraro and Kiefer 2004; Hart 1969; Menéndez Abraham et al. 1991; Pfaus and Pinel 1989). No facilitation of sexual responding was observed in studies where sexually experienced male rats were paired with sexually receptive females or following nonreinforced extinction trials in the operant paradigm by Scott et al. (1994). However, a moderate dose of alcohol (1 g/kg, ip, w/v) increased conditioned level-changing behavior of sexually experienced male rats in bilevel chambers (Ferraro and Kiefer 2004), suggesting the facilitation of anticipatory sexual motivation. Interestingly, injection of a higher dose (2 g/kg, ip, w/v) was reported to increase brain testosterone synthesis within 30 min in male rats (Alomary et al. 2003), an effect due to ethanol oxidation.

The early lack of evidence for disinhibition in animal models led Wilson (1977) to suggest that “...it is difficult to extrapolate from animal studies to human behavior because...in contrast to the laboratory rat or dog, it is generally accepted that cognitive mediation – psychogenic sources of stimulation and inhibition – are paramount in the control of human sexual arousal and behavior” (p. 239). What psychogenic inhibition exists in animals? One strong source of inhibition for male rats is the behavioral state of anestrus females. Gonadally intact, naturally cycling female rats display sexual solicitations, pacing behaviors, and lordosis approximately once every four to five days during their fertile periovulatory period (Pfaus et al. 2015). This effect can be mimicked in ovariectomized (OVX) rats given steroid replacement with estradiol and progesterone. However, sexually nonreceptive females (naturally cycling or OVX without hormone priming) respond to attempted mounts with rejection responses, including kicks, boxing postures, and threat postures (Barnett 1963). Pfaus and Pinel (1989) gave sexually experienced male rats alternating experience with OVX, hormone-primed, sexually receptive females and hormone-unprimed nonreceptive females every 48 h in bilevel chambers where the males had previously acquired sexual experience. Although all males attempted to mount the nonreceptive females on their first trial, none attempted to mount the nonreceptive females by the fifth trial. However, they copulated to multiple ejaculations on intermittent days with the receptive females. Subsequent acute administration of 0.5 and 1.0 g/kg (w/v) of 95% alcohol delayed ejaculation and inhibited copulation when males were presented with sexually receptive females. However, the 0.5 g/kg dose disinhibited mounting behavior in 70% of the males when presented with sexually nonreceptive females. Moreover, in males that mounted, 50% achieved ejaculation despite ex copula mounting and persistent rejection responses by the females. Thus, disinhibition of sexual behavior could be

revealed with low doses of alcohol in male rats, but only in circumstances of active, first-order sexual inhibition.

The study by Pfaus and Pinel used the natural behavior of sexually nonreceptive females, which thwarted the copulatory attempts by the males as primary, or first-order, inhibitors. Using a similar paradigm, Kippin et al. (1998) found that a neutral odor (almond extract) placed on the neck and anogenital region of sexually nonreceptive females that males attempted to copulate with could subsequently inhibit copulatory advances toward sexually receptive females scented with the odor relative to unscented females that received preferential mounts, intromissions, and ejaculations from the males. The present study utilized this paradigm of second-order conditioned inhibition to examine the acute effect of a low (0.5 g/kg) or moderate (1.0 g/kg) dose of alcohol (w/v) relative to a saline control. In addition, the brain regions potentially involved were examined using Fos immunohistochemistry (IR) (Pfaus and Heeb 1997) to identify regions activated by the odor alone, with or without acute alcohol treatment.

## Materials and methods

### Animals

**Males** Sexually naive male Long-Evans rats ( $N=44$ ) were obtained from Charles River, Canada (St. Constant, Quebec). The males weighed approximately 250 g at the start of the experiment and were housed in groups of 4 in Plexiglas cages with ad libitum access to food (Purina Rat Chow) and water.

**Females** Female Long-Evans rats were obtained from the same supplier. They were bilaterally ovariectomized via lumbar incisions using intraperitoneal (ip) injections (1 ml/kg body weight) of ketamine hydrochloride (50 mg/ml; Ketaset<sup>®</sup>, Wyeth Canada) and xylazine hydrochloride (4 mg/ml; Rompun<sup>®</sup>, Bayer Healthcare) anesthetic mixed at a ratio of 4:3, respectively. After a week of recovery, sexual receptivity was induced by subcutaneous injections of estradiol benzoate (10 µg) 48 h and progesterone (500 µg) 4 h before each conditioning trial and copulatory test.

All rats were maintained on a reverse 12-h dark–light cycle (lights off at 08:00) at  $21 \pm 1$  °C with ad lib access to food and water. Behavioral tests were performed during the middle third of their dark cycle.

### Apparatus

Conditioning trials took place in bilevel chambers made of Plexiglas (18 cm × 25 cm × 65 cm) with a platform (40 cm in length) dividing the chamber into two levels (Mendelson



and Gorzalka 1987; Pfaus et al. 1990). The final copulatory preference test took place in a large open field (123 cm × 123 cm × 96 cm) with bedding covering the floor, as in the original study by Kippin et al. (1998). All conditioning trials and the preference test were recorded on a video camera and later scored using a PC-based behavioral observation program (Cabilio, 1996).

## Ethanol treatment

Ethyl alcohol (95%) was diluted in saline to obtain doses of 0.5 g/kg and 1 g/kg in a 25% v/v solution. To avoid bias due to the amount of liquid injected, males in the saline group received an ip injection of saline solution in the same volume as the highest dose of alcohol. On the copulatory test day, 45 min before testing, each male was injected with their respective dose of alcohol or saline and was placed individually in a shoebox until testing began.

## Procedure

**Habituation and conditioning phase** Males were preexposed to the bilevel chambers for 15 min each day over seven days before the beginning of the experiment to increase the proportion of males that become vigorous copulators (Pfaus and Wilkins 1995). Following this habituation phase, males were given a total of 20 conditioning trials at 2-day intervals, ten with an unscented receptive female and ten with a scented nonreceptive female. Female rats were scented with approximately 1 ml of pure almond extract (Blue Ribbon, Etobicoke, Ontario, Canada) on the back of their neck and the anogenital region. The access to an unscented receptive female or a scented nonreceptive female was alternated on each conditioning day. For all the conditioning trials, each male was placed alone in a bilevel chamber for five minutes, after which a female in the appropriate sexual condition was introduced into the chamber for a 30-min conditioning trial. Criteria for mounts, intromissions, and ejaculations were those previously described by Sachs and Barfield (1976), Pfaus et al. (1990), and Sachs and Meisel (1994).

**Copulatory preference test** Each male received an injection of 2 ml of saline 45 min before the last two conditioning trials to ensure that the i.p. injection would not stress the animals on the final test day. Four days after the last conditioning trial with receptive females, male rats were randomly assigned to one of the three ethanol treatment groups: 0 g/kg alcohol (saline), 0.5 g/kg alcohol (A0.5) or 1 g/kg alcohol (A1). Respective doses of alcohol or saline were injected ip 45 min before the test. Each male was then placed in the large open field and allowed to habituate for 5 min, after which two receptive females, one scented and one unscented, were placed simultaneously into the open

field at an approximately equal distance from the male. During the 30-min test, all copulatory behaviors and the females to which they were directed were recorded.

## Behavioral and statistical analyses

The last two conditioning sessions and the copulatory test were recorded and later scored. The frequency of appetitive level changes during the 5-min habituation was recorded for the final conditioning sessions with the receptive and the nonreceptive females. Frequencies and latencies of mounts, intromissions and ejaculations were recorded. All statistical analyses were performed using SPSS Statistics 25.0 for PC.

**Conditioning** A paired sample t-test was performed to compare the frequency of level changes during the last conditioning with a receptive female and the last with a nonreceptive female. To ensure that the results of the open field were not biased by the performances of the male rats during the conditioning phase, a one-way ANOVA was used to compare group differences in sexual behaviors on the final conditioning day with a receptive female. Following a significant overall main effect, group differences were analyzed using Tukey's post-hoc analysis. Eta squared ( $\eta^2$ ) was reported as a measure of effect size.

**Open field** A 3 (alcohol treatment) × 2 (female) mixed design ANOVA was used to determine differences in the number of mounts, intromissions and ejaculations during the copulatory test, followed by Tukey's post-hoc comparisons. Partial eta squared ( $\eta_p^2$ ) was reported as a measure of effect size. Chi-square analyses assessed the proportions of males who chose one of the two females for their first mount, intromission, and ejaculation on the copulatory preference test. For all analyses, the significance level was set to  $p < 0.05$ .

## Olfactory stimulation test

Following the copulatory test, males were given four reconditioning sessions using the same procedure as during the training phase. Four days after the last reconditioning trial with a receptive female, each male was injected with the previously assigned ethanol treatment and exposed 45 min later either to 1 ml of distilled water (No odor condition) or 1 ml of almond extract (Odor condition) on cotton gauze in an exposure chamber (individual shoe box cage). Males were allowed to remain undisturbed in the chamber for one hour. Following this exposure period, males were injected with a lethal dose of sodium pentobarbital (120 mg/kg, i.p.) and perfused intracardially with 300 ml of phosphate-buffered saline (PBS) followed by 300 ml of 4% paraformaldehyde (4% PFA). Brains were extracted and post-fixed in 4% PFA for four hours, then switched into a solution of 30% sucrose for 24 to 48 h. Brains were then stored at -80 °C until slicing.



## Fos immunohistochemistry

Each brain was sliced in 30 µm coronal sections and collected in a 0.9% tris-buffered saline (TBS). Brain sections were always rinsed in three 5-min periods in 0.9% TBS between incubations during the immunohistochemistry process. Sections were incubated in 30% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in TBS for 30 min at room temperature, followed by a 2-h incubation in a pre-blocking solution of 3% normal goat serum (NGS) in 0.2% Triton TBS at room temperature. Sections were then incubated in a primary antibody solution made of 3% NGS and rabbit polyclonal anti-fos (1:40,000; Fos ab5, Calbiochem, Mississauga, ON) in 0.05% Triton TBS for 72 h at 4 °C. Following this incubation, brain sections were incubated for one hour in a secondary antibody mixture of 3% NGS and biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories Canada, Burlington, ON) in 0.05% triton TBS, then in a solution of 3% NGS and avidin-biotinylated peroxidase complex (1:55; Vectastain ELITE ABC Kit, Vector Laboratories Canada) at 4 °C for 2 h. Immunoreactions were stained by incubation, at room temperature, in 50 mM tris buffer (Tris) for 10 min, then in a solution of 3,3'-diaminobenzidine (DAB) in 50 mM Tris for 10 min and finally in a mixture of 8% nickel chloride, 3% H<sub>2</sub>O<sub>2</sub> and DAB for 10 min. Finally, brain sections were mounted on gel-coated slides and allowed to dry, then dehydrated in increasing alcohol concentrations, cleared in xylene, cover-slipped and examined under a light microscope.

## Histological and statistical analyses

Brain sections were examined at 40× magnification. Images of Fos immunoreactive (Fos-IR) cells were captured using Q-Capture Pro. They were counted bilaterally from 3 to 5 sections per region per rat using Image J. Using the rat brain atlas of Paxinos and Watson (1998), counts of Fos-IR cells were made in the prelimbic cortex (PL; Plates 7 to 10), infralimbic cortex (IL; Plates 8 to 10), piriform cortex (PIR; Plates 11 to 18), nucleus accumbens core (NAcCore) and shell (NAcShell; Plates 11 to 14), medial preoptic area (mPOA; Plates 18 to 20), medial amygdala (MEA), central amygdala (CeA), and basolateral amygdala (BLA; Plates 26 to 30), and the ventral tegmental area (VTA; Plates 40 to 44).

The total number of Fos-IR cells in each area was calculated bilaterally in 3 to 5 slices taken randomly from each region for each rat and summed into means per rat. Those means were then summed per treatment group. A one-way ANOVA compared the mean number of Fos-IR cells between alcohol treatments when rats were not exposed (No Odor condition) or exposed to the almond odor (Odor condition) followed by planned contrasts. Partial eta squared ( $\eta^2$ )

was reported as a measure of effect size for the ANOVAs. Unpaired t-tests assessed differences between No Odor and Odor conditions (No Odor vs. Odor) for each alcohol treatment. Cohen's d was reported as a measure of effect size. For all analyses, the significance level was set to  $p < 0.05$ .

## Results

### Final conditioning session

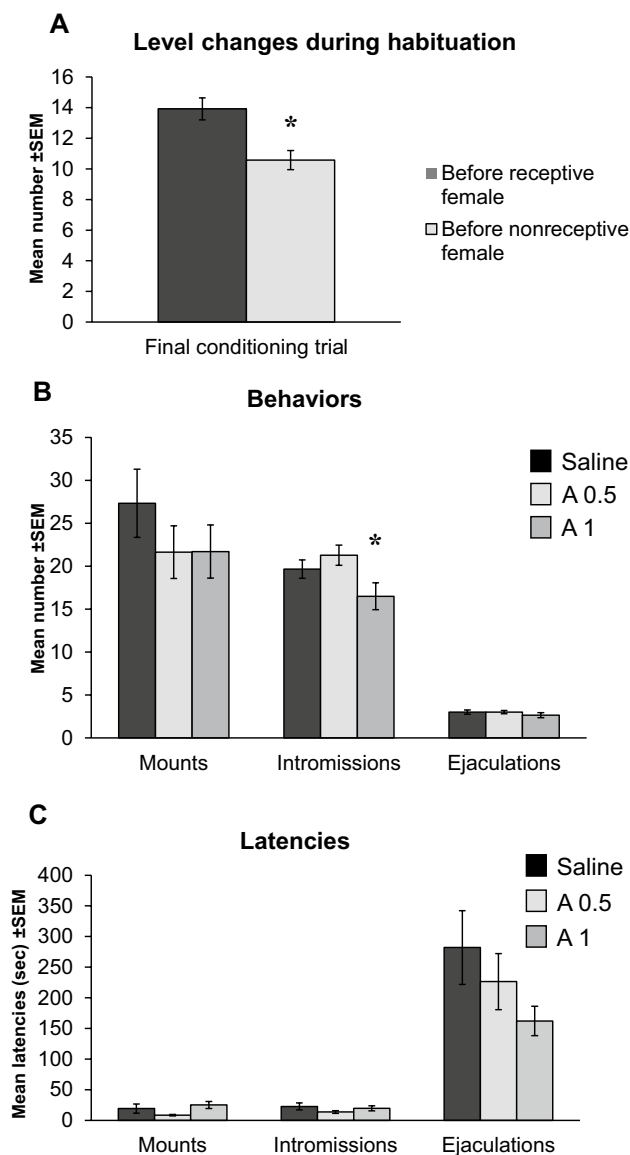
During the last trial, males displayed significantly more level changes before the trial with the receptive unscented female than before the trial with the nonreceptive scented female  $t(34) = 4.639$ ,  $p < 0.001$ , Cohen's  $d = 4.263$  (Fig. 1A). When males are assigned to their respective groups (saline, A0.5 or A1), there were no significant differences between groups in the number of mounts ( $F(2, 39) = 0.898$ ,  $p = 0.416$ ,  $\eta^2 = 0.046$ ) and the number of ejaculations ( $F(2, 39) = 0.735$ ,  $p = 0.486$ ,  $\eta^2 = 0.038$ ). However, there was a significant difference between groups for the number of intromissions ( $F(2, 39) = 3.603$ ,  $p = 0.037$ ,  $\eta^2 = 0.163$ ). Tukey post hoc tests revealed that the males selected for the injection of 0.5 g/kg of ethanol intromitted more with the receptive female on the last conditioning trial compared to the rats selected for the injection of 1 g/kg of ethanol (Fig. 1B).

No significant differences between groups were observed for the latency for the first mount ( $F(2, 39) = 2.787$ ,  $p = 0.075$ ,  $\eta^2 = 0.131$ ), the latency for the first intromission ( $F(2, 39) = 1.230$ ,  $p = 0.304$ ,  $\eta^2 = 0.062$ ), and the latency for the first ejaculation ( $F(2, 39) = 1.795$ ,  $p = 0.180$ ,  $\eta^2 = 0.088$ ) (Fig. 1C).

### Open field partner preference test

Of the 44 male rats, more males from the A1 group did not ejaculate ( $n = 4$ ) compared to the saline group ( $n = 1$ ) and the A0.5 group ( $n = 1$ ). Those males were excluded from the data analyses. Thus, the number of rats included in each group was as follows: saline:  $n = 13$ , A0.5:  $n = 14$ , A1:  $n = 11$ .

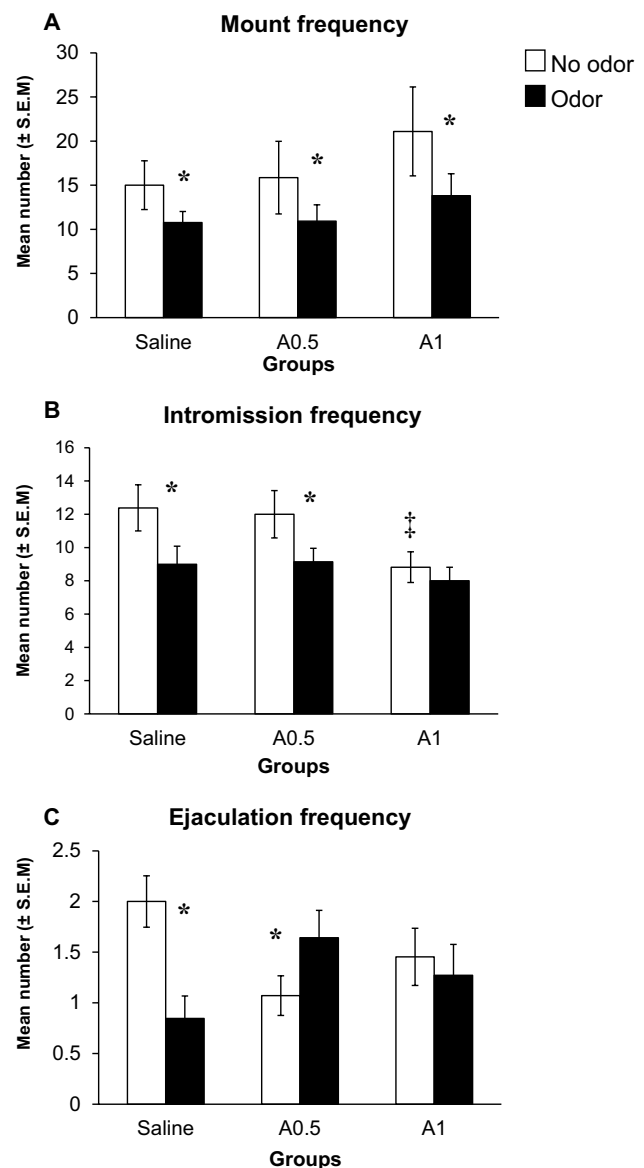
Figure 2 displays the mean number of mounts, intromissions and ejaculations per female over the 30-min preference test. Overall, males mounted the unscented females more frequently than the scented ones (Fig. 2A). A 3 × 2 mixed ANOVA confirmed a main effect of the female type ( $F(1, 35) = 8.02$ ,  $p = 0.008$ ,  $\eta^2 = 0.19$ ). Post-hoc Tukey tests revealed that, regardless of their groups, males mounted the unscented female significantly more often than the scented female. Neither the main effect of alcohol treatment nor the interaction met statistical significance. The unscented females also received more intromissions than the scented females (Fig. 2B). The mixed ANOVA confirmed this



**Fig. 1** Mean level changes during the 5-min habituation period before the final conditioning trial with a receptive and a nonreceptive female (A). Mean number of male sexual behaviors (B) and mean latencies in seconds (C) during the last conditioning trial with a receptive female. \* $p < 0.05$  (A: between groups; B: A1 vs. A0.5)

observation's significance as there was a main effect of female type ( $F(1,35) = 4.88$ ,  $p = 0.034$ ,  $\eta^2 = 0.12$ ). Post-hoc Tukey tests revealed that all males intromitted significantly more with the unscented females. There was no main effect of group, and the interaction group  $\times$  female type did not reach significance.

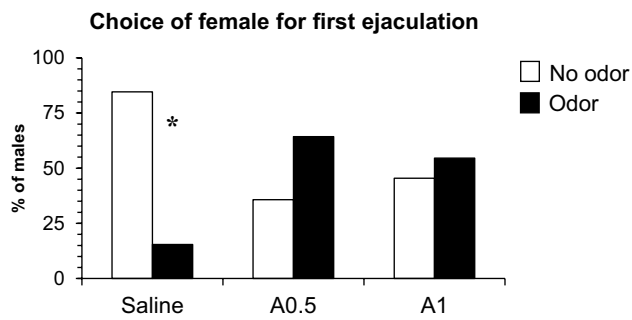
Finally, neither the alcohol treatment nor the female type affected the frequency of ejaculation as there were no significant main effects of group or female type. However, there was a significant interaction (alcohol treatment  $\times$  female) ( $F(2,35) = 3.56$ ,  $p = 0.039$ ,  $\eta^2 = 0.17$ ). As shown



**Fig. 2** Mean number of mounts (A), intromissions (B) and ejaculations (C) during the 30-min open field test with two receptive females, one scented (Odor) and one unscented (no odor). Saline:  $n = 13$  males, A0.5:  $n = 14$  males, A1:  $n = 11$  males. \* $p < 0.05$  (odor vs. no odor)

in Fig. 2C, males in the saline group displayed significantly more ejaculations with the unscented female compared to the scented one. There were no significant differences between groups or females type in latencies for first mount, first intromission and first ejaculation.

Regardless of the groups, males did not show a preference in their choice of female for the first mount or intromission. However, males in the saline group chose more frequently the unscented female for their first ejaculation compared to the scented one, as confirmed by chi-square analysis  $\chi^2(1, N = 13) = 6.23$ ,  $p = 0.013$ . Males in the alcohol groups did



**Fig. 3** Choice of female for the first ejaculation during the open field test. Saline:  $n=13$  males, A0.5:  $n=14$  males, A1:  $n=11$  males. \* $p<0.05$  (odor vs. no odor)

not show this preference in the choice of female for their first ejaculation (Fig. 3).

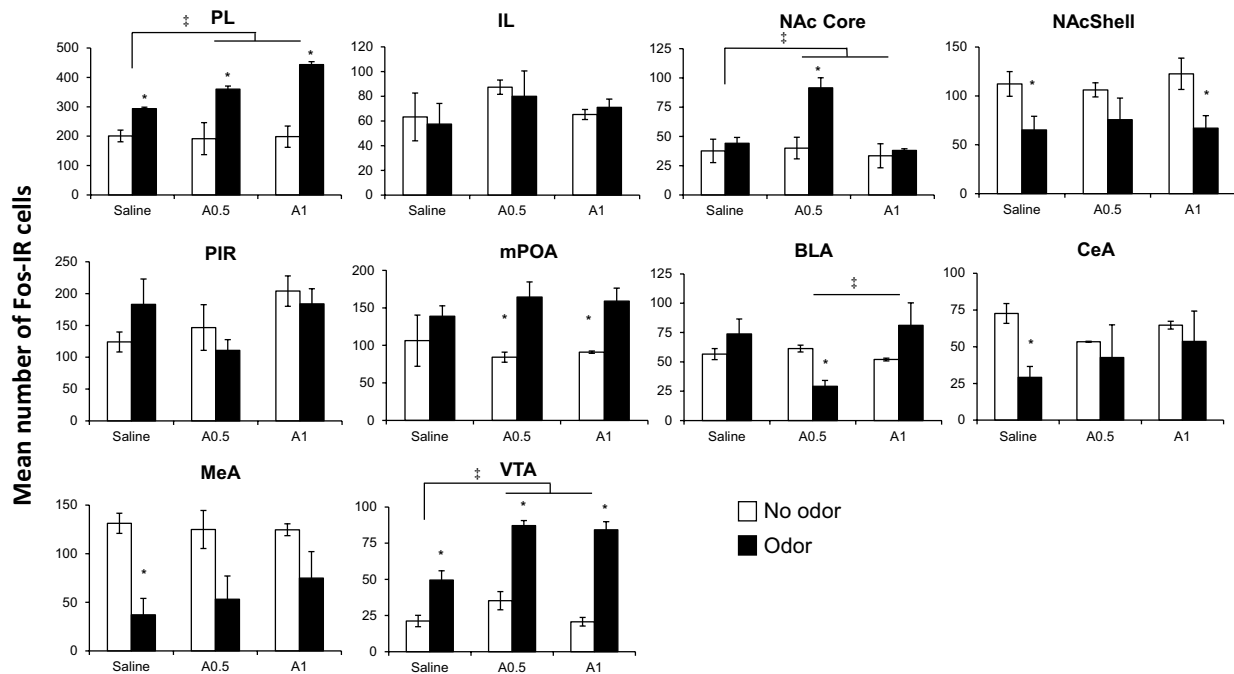
### Odor induced Fos-IR

Figure 4 depicts the mean numbers of Fos-IR cells in different brain regions of 33 male rats (No Odor condition:  $n=6$  per group, Odor condition:  $n=5$  per group).

**Saline vs. alcohol** Overall, the ANOVA revealed that there were significant differences between groups in

the PL ( $F(5,32)=16.40$ ,  $p<0.001$ ,  $\eta^2=0.75$ ), NAc-Core ( $F(5,11.32)=6.46$ ,  $p=0.005$ ,  $\eta^2=0.53$ ), BLA ( $F(5,32)=3.89$ ,  $p=0.009$ ,  $\eta^2=0.42$ ), and the VTA ( $F(5,32)=52.75$ ,  $p<0.001$ ,  $\eta^2=0.91$ ). Planned contrasts revealed that alcohol overall increased the mean number of Fos-IR cells compared to saline in PL ( $t(27)=-3.31$ ,  $p=0.002$ ), NAcCore ( $t(8.104)=-3.17$ ,  $p=0.013$ ), and VTA ( $t(27)=-6.91$ ,  $p<0.001$ ). Furthermore, males in the A0.5 group had significantly more Fos-IR cells in the NAc-Core ( $t(4.26)=6.21$ ,  $p=0.003$ ), but significantly less in the BLA ( $t(27)=-3.96$ ,  $p<0.001$ ), compared to males in the A1 group. The planned contrasts also revealed that, when male rats were not exposed to the olfactory cue (No Odor condition), no significant difference was observed between saline and alcohol groups for any of the brain regions analyzed.

**No Odor vs. Odor** Male rats from the saline group exposed to the odor showed significantly more Fos-IR in PL ( $t(6)=-3.48$ ,  $p=0.013$ , Cohen's  $d=2.90$ ), and VTA ( $t(6)=-3.18$ ,  $p=0.019$ , Cohen's  $d=2.55$ ), compared to males from the saline group not exposed to the odor. Conversely, Fos-IR was significantly lower in NAcShell ( $t(9)=2.50$ ,  $p=0.034$  Cohen's  $d=1.51$ ), MEA ( $t(6)=3.96$ ,  $p=0.007$  Cohen's  $d=3.17$ ), and CeA ( $t(6)=3.94$ ,  $p=0.008$  Cohen's  $d=3.02$ ) in this subgroup of rats compared to those not exposed to the almond odor.



**Fig. 4** Mean number of Fos-IR cells in different brain regions compared to saline (No Odor and Odor conditions) or compared to no odor (No odor vs. Odor). PL: prelimbic cortex; IL: infralimbic cortex; NAcCore: nucleus accumbens core; NAc Shell: nucleus accumbens shell; PIR: piriform cortex; mPOA: medial preoptic area; BLA: baso-

lateral amygdala; CeA: central nucleus of the amygdala; MeA: medial nucleus of the amygdala; VTA: ventral tegmental area. Error bars represent the standard error of the mean. ‡ Odor condition  $p<0.05$ . \* No odor vs. Odor condition  $p<0.05$

When males were injected with 0.5 g/kg of alcohol and exposed to the odor, the number of Fos-IR cells was significantly higher in PL ( $t(4.306) = -3.04$ ,  $p = 0.035$ , Cohen's  $d = 1.93$ ), NAcCore ( $t(9) = -4.03$ ,  $p = 0.003$ , Cohen's  $d = 2.46$ ), mPOA ( $t(6) = -2.93$ ,  $p = 0.026$ , Cohen's  $d = 2.43$ ), and the VTA ( $t(6) = -7.96$ ,  $p < 0.001$ , Cohen's  $d = 5.49$ ) but significantly lower in the BLA ( $t(6) = 4.59$ ,  $p = 0.004$ , Cohen's  $d = 3.69$ ) compared to those not exposed to the odor.

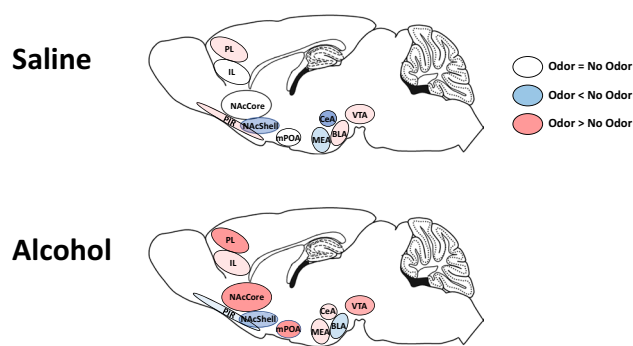
Finally, males in the A1 group exposed to the almond odor showed a number of Fos-IR cells significantly higher in PL ( $t(6) = -5.05$ ,  $p = 0.002$ , Cohen's  $d = 4.20$ ), mPOA ( $t(6) = -2.94$ ,  $p = 0.026$ , Cohen's  $d = 2.47$ ), and VTA ( $t(6) = -8.06$ ,  $p < 0.001$ , Cohen's  $d = 6.55$ ), but significantly lower in NAcShell ( $t(9) = 2.62$ ,  $p = 0.028$ , Cohen's  $d = 1.61$ ), compared to males in the A1 group exposed to the inhibitory cue.

## Discussion

Results from the present study indicate that low to moderate doses of alcohol can disinhibit copulatory behavior in male rats that has come under inhibitory control by an odor that predicts sexual nonreceptivity in females, and, therefore, sexual frustration in the male. This disruption of conditioned sexual inhibition appears to occur through the effects of alcohol on brain regions such as the mPFC, NAc, mPOA, amygdala, and VTA (Fig. 5).

The present behavioral results replicate those of Kippin et al. (1998). Only males in the saline group showed a conditioned ejaculatory preference for the unscented female, which indicates a conditioned inhibition toward the odor on the scented female. However, this inhibition was abolished following a single injection of alcohol, regardless of the dose. Thus, the disruption of preference during the open field was due to the alcohol treatment. It has been previously demonstrated that an acute treatment of alcohol, even at doses higher than the ones used in the present study, does not disrupt responding to conditioned olfactory aversion. Rats conditioned to associate an olfactory stimulus with foot shock develop an aversion to this odor. As a single injection of alcohol does not abolish odor aversion (Lopez et al. 1996; Molina et al. 1987), this suggests that the absence of preference in the present study is not due to a lack of olfactory distinction between the two females but to the disinhibitory effects of alcohol on sexual behavior.

These results also agree with previous findings by Pfaus and Pinel (1989). In their study, male rats were injected with alcohol before an inhibitory test with nonreceptive females. The dose of 0.5 g/kg of alcohol induced a disinhibition of sexual behavior in male rats. This phenomenon could also be observed in the present experiment with the absence of



**Fig. 5** Summary of the Fos results between Odor and No-Odor following Saline or Alcohol. Open circles indicate no significant difference in Fos induction between Odor and No-Odor. Blue circles indicate less Fos for Odor relative to No Odor. Red circles indicate more Fos for Odor relative to No Odor

preference or avoidance towards the female bearing the inhibitory odor. Although more male rats injected with 1 g/kg of alcohol did not ejaculate during the open field, no other aspect of their sexual behavior was impaired. This is not consistent with previous findings showing that a dose of 1 g/kg of alcohol injected to male rats can affect sexual performances (Ferraro and Kiefer 2004) and even reduce the proportion of males mounting or ejaculating with females (Pfaus and Pinel 1989). An explanation for this discrepancy could be the presence of two receptive females in an open field that might have increased the male's arousal relative to a single female in a smaller testing apparatus in previous studies (Pfaus and Pinel 1989). The enhanced arousal may have counteracted the inhibitory effects of the 1 g/kg dose of alcohol.

The second phase of the study examined the induction of Fos-IR in regions of the brain associated with behavioral inhibition (e.g., PFC, BLA) or sexual motivation (e.g., NAcCore and Shell, mPOA, VTA) in response to the odor or no odor, with or without alcohol treatment. It was found that exposure to the No Odor condition did not induce changes in Fos activation in any region, nor did the acute injection of alcohol alone. Although these findings may differ from previous research, they could be attributed to the lower alcohol dosage used in the present study compared to previous studies examining the effect of acute alcohol (from 1.5 g/kg up to 3 or 4 g/kg). As previously reviewed by Chang et al. (1995), among others, Fos activation by alcohol can be dose-dependent; therefore, without the presence of another stimulus, higher doses of alcohol might be necessary to induce Fos in the brain regions analyzed.

In contrast, exposure to the inhibitory cue alone (No Odor vs Odor) or in the combination alcohol + inhibitory odor (Odor condition) induced a significant increase in Fos in the PL, NAcCore, mPOA and VTA and a significant decrease

in the NAcShell, BLA, MeA, and CeA. The different patterns of Fos activation between groups, when exposed to the olfactory cue and even when under the influence of alcohol, confirm the ability of the male rats to discriminate the inhibitory olfactory cue. Accordingly, the differences in Fos expression observed between the saline and alcohol groups within the same brain area indicate that the almond odor might be interpreted and processed differently.

Male rats exposed to the olfactory cue display significantly more Fos-IR cells in PL and VTA. This is true when comparing exposure to no odor vs. odor and saline vs. alcohol. As part of the mPFC, the PL may play a role in the regulation of sexual behavior (Ågmo et al. 1995; Balfour et al. 2006; Davis et al. 2010; Hernández-González et al. 1998) and in behavioral inhibition and disinhibition (Aron 2007; Huey 2020). Although the activation of PL does not seem required for the normal expression of sexual behavior, it appears necessary to suppress maladaptive sexual behaviors (Davis et al. 2010). A recent study reported that the inactivation of PL by GABA agonists could disrupt active avoidance in situations where male rats had to distinguish different cues, suggesting the role of PL in discriminating between conflicting cues (Capuzzo and Floresco 2020). As alcohol is known to act on GABA neurotransmission, this would suggest that the increase in Fos activation in PL observed in male rats exposed to the olfactory cue and alcohol would reflect the inactivation of this region by GABA transmission, possibly leading to the disruption of active avoidance towards the scented female during the open field. The authors further mentioned that the initiation or inhibition of the behavior by recruitment of PL was dependent on the valence of the context (Capuzzo and Floresco 2020). This would also explain the increased Fos activation in the saline group exposed to the olfactory inhibitory cue. The fact that the inhibitory cue was on a receptive female during the open field (as opposed to a nonreceptive female during the conditioning phase) likely created a conflicting context requiring the recruitment of the PL.

Despite the known actions of alcohol on the VTA (reviewed in You et al. 2018), Fos activation was increased in all groups and only in the presence of the olfactory cue, revealing that the increased activation in this region is not alcohol-related. The VTA plays an important role in processing rewarding and aversive stimuli (Lammel et al. 2014) and experiences through different subpopulations of neurons (Bariselli et al. 2016). The increased VTA activation while exposed to the olfactory cue as opposed to no odor and the fact that the alcohol groups show significantly more Fos-IR cells than the saline group suggests that the groups might process and interpret the olfactory cue differently. This distinction in Fos activation between the alcohol and saline group only in the presence of the olfactory cue would explain the behavioral results observed during the open field

when male rats in the alcohol groups copulated with the scented females, whereas males in the saline group did so to a significantly lesser extent.

An increase in Fos activation is also observed in NAcCore when comparing the alcohol to saline groups. However, this increase was mainly detected in the male rats injected with 0.5 g/kg of alcohol. Previous research has shown that responses to sexually conditioned odors were enhanced in neurons of the NAc (West et al. 1992). As stated by Kippin et al. (2003), the NAcCore might be important in assessing the incentive value of the conditioned cue. During the open field test, male rats were exposed for the first time to alcohol and the odor on a receptive female. It is possible that the inhibitory odor acquired an incentive value that is state-dependent (under the influence of alcohol). Therefore, this subsequent exposure to the olfactory cue under the influence of alcohol could lead to this increase in Fos expression in this brain region. This increase is further supported by the increase in the other brain regions of the mesolimbic pathway presented above. Despite the lack of behavioral differences between the two alcohol groups during the open field test, potentially explained by the presence of two females, the fact that only the A0.5 group showed this significant increase might be dose-dependent. Pfaus and Pinel (1989) showed that this dose was high enough to induce disinhibition of sexual behavior and that a higher dose (e.g. 1 g/kg) could disrupt sexual performances. As for Fos expression, males were only exposed to the alcohol and the odor without the presence of any females; the cue alone might not have been enough to induce changes in Fos activation.

Males in both alcohol groups exposed to the inhibitory cue displayed significantly higher Fos in the mPOA compared to no odor. As the mPOA is an essential region for the regulation of sexual behavior in male rats (Hull and Dominguez 2019; Pfaus 2009), activation of this region would agree with the behavioral results observed during the open field as males in the alcohol groups copulated with the scented females. However, they are inconsistent with previous studies in which mPOA failed to be activated by a sexually conditioned cue but was rather activated by estrous odors (Kippin et al. 2003). An explanation could be that the olfactory cue might not be perceived as a new conditioned cue per se but as part of a context (alcohol + cue) that predicts sexual activity. Again, the open field is the first time male rats are intoxicated and presented with a receptive female bearing the olfactory cue. The olfactory stimulation test is the second time the males are in this “alcohol + odor” context. Therefore, Fos activation in this region in the alcohol groups exposed to the olfactory cue could be state-dependent instead of cue-conditioned.

On the other hand, exposure to the olfactory cue induced a significant decrease in Fos activation in the NAcShell and several amygdala nuclei. Male rats exposed to the olfactory



cue and injected with saline or alcohol 1 g/kg showed a significant decrease in Fos. This pattern, which is opposite to what is observed in the NAcCore, reinforces the findings of previous studies that suggest functional differences (Di Chiara 2002; Ito and Hayen 2011) and selective responding (Pezze et al. 2001) between the two subregions of the NAc. It also suggests that they may have distinct functional roles in the disinhibition of sexual behavior by alcohol. The significant decrease in Fos expression could even indicate its involvement in the expression of sexual inhibition. Further investigations would be of interest to disentangle the role of both subregions of the expression of sexual inhibition and disinhibition.

Alcohol alone did not alter Fos expression in the BLA, as there was no difference between saline and any alcohol group in the No Odor condition. However, the association between alcohol, particularly at 0.5 g/kg, and the olfactory cue significantly decreased Fos expression in this brain region. This would be consistent with previous findings reporting that BLA inactivation prevents the retrieval and expression of odor memory (Sevelinges et al. 2009). This would also suggest that the inactivation of the BLA might be part of the brain mechanisms required for the disruption of conditioned sexual inhibition by alcohol. BLA is known to be involved in the integration and representation of both positive and negative stimuli with their respective valence (Janak and Tye 2015; Namburi et al. 2015; Wassum and Izquierdo 2015). Conversely, it is not involved in the expression of sexual behavior in male rats, but lesions can impair conditioned bar-pressing to gain access to receptive females (Everitt 1990). It would be of interest to further investigate if the BLA is also inactivated by other drugs that disrupt conditioned sexual inhibition.

A significant decrease in Fos activation was observed in the CeA and MeA in the saline group exposed to the olfactory cue. While the CeA is known for its involvement in alcohol-related behaviors, the MeA is involved in emotional responses (LeDoux 2012) and sexual behavior (Kondo and Arai 1995; Lehman et al. 1980). The fact that both regions have decreased Fos expression in the saline group suggests their potential involvement in the inhibition of sexual behavior. Previous findings suggest that the MeA is important in olfactory cue-elicited sexual arousal (De Jonge et al. 1992). Further, the inactivation of MeA neurons results in an impairment of copulation in male rats (Dominguez et al. 2001). It is not yet known whether alcohol disinhibited the MeA directly in the presence of the olfactory cue or whether that disinhibition occurred downstream of the activation of the PL, NAc Core, and/or mPOA.

This study demonstrates that low to moderate doses of alcohol can disrupt conditioned sexual inhibition in male rats. Further, exposure to the inhibitory olfactory cue revealed a differential pattern of Fos activation between

the saline and alcohol groups, particularly in brain regions involved in sexual behavior and encoding the valence of a cue. One interesting finding in the present study is that the olfactory cue is needed in addition to alcohol to elicit changes in Fos expression, suggesting that in this context, disruption of conditioned sexual inhibition is not only drug-related but rather drug + cue-related. This could explain why changes in Fos expressions are mainly perceived in areas involved in sexual behavior or conditioning responses. Further studies are needed to disentangle the connections and neurotransmitters involved in this phenomenon and if this could be replicated with other drugs, such as cocaine, known to disinhibit sexual behavior.

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**Declarations** The authors declare that all animal procedures conformed to the guidelines of the Canadian Council for Animal Care and were approved by the Concordia University Animal Research Ethics Committee (Protocol #30000300 to JGP).

**Conflict of interest** The authors declare they have no conflicts of interest.

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**C.**

**Original studies on the conditioning of sexual  
behavior in female rats**



## Olfactory Conditioned Partner Preference in the Female Rat

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Paced copulation induces conditioned place preference in female rats. The authors examined whether associating almond-scented males with paced copulation induces conditioned partner preference. The paired group received 4 paced copulations with almond-scented males and 4 nonpaced copulations with unscented males sequentially at 4-day intervals. The unpaired group received the opposite order of association, whereas the randomly paired group received random associations. A 4th group received a single pairing. On the final test, females were placed into an open field with 2 males, 1 scented and 1 unscented. Females in the paired group solicited the scented male more frequently, and most chose the scented male for their 1st ejaculation. Thus, an odor paired with paced copulation elicits conditioned partner preference in female rats.

**Keywords:** pacing, sexual reward, odor, appetitive, conditioned place preference

Olfactory stimuli play an important role in the expression and modulation of sexual behavior in the rat (Cain & Paxinos, 1974; Larsson, 1971; McClintock, 1978, 1984a; McClintock & Adler, 1978; Schank & McClintock, 1997). Pheromonal and olfactory stimuli emitted naturally from sexually receptive females (i.e., estrous odors) are attractive to males (Bressler & Baum, 1996) and produce sexual arousal (Sachs, 1997). Estrous odors also increase serum luteinizing hormone and testosterone levels in male rats (Graham & Desjardins, 1980) and increase Fos activation within a common set of brain structures in several rodent species, including the accessory olfactory bulb, the nucleus accumbens core and shell, the medial preoptic area, the bed nucleus of the stria terminalis, the medial amygdala, and the ventral tegmental area (Bialy & Kaczmarek, 1996; Kippin, Cain, & Pfaus, 2003; Newman, Parfitt, & Kollack-Walker, 1997; Pfaus & Heeb, 1997; Swann & Fiber, 1997; Veening & Coolen, 1998). Likewise, female rats find

the odors of male rats attractive (Bermant, 1961; Bermant & Westbrook, 1966; Eliasson & Meyerson, 1975; Pfaus, Smith, & Coopersmith, 1999), and exposure to male odors increases Fos activation in similar brain regions (Pfaus, Kleopoulos, Mobbs, Gibbs, & Pfaff, 1993; Wersinger, Baum, & Erskine, 1993).

Neutral odors (e.g., almond, lemon, wintergreen) associated with copulation to ejaculation in male rats elicit conditioned elevations in serum luteinizing hormone and testosterone (Graham & Desjardins, 1980), increases in sexual arousal, and a phenomenon referred to as *conditioned ejaculatory preference*, in which males display a partner preference for ejaculation but not copulation per se (Kippin & Pfaus, 2001a, 2001b; Kippin, Samaha, Sotiropoulos, & Pfaus, 2001; Kippin, Talinakis, Schattmann, Bartholomew, & Pfaus, 1998). In the Kippin et al. studies, males in the paired group copulated with sexually receptive females that had the odor smeared on the back of their neck and anogenital region. Several Pavlovian control groups were used in which males received either no copulatory experience with the odor (unpaired), random pairing of the odor with copulation, or the odor explicitly unpaired (i.e., paired with exposure to nonreceptive females). The final test took place in an open field with two receptive females, one scented and the other unscented. Although all males mounted and intromitted indiscriminately with the two females, those in the paired group ejaculated preferentially with the scented females and did so for at least the first two ejaculations. Males in the unpaired group did not show a significant preference for either female. Males in the randomly paired group switched females for each subsequent ejaculation, reminiscent of a Coolidge effect (Beach & Jordan, 1956), whereas males in the explicitly unpaired group displayed an early avoidance of the scented female. Conditioned ejaculatory preferences did not develop if males did not ejaculate (Kippin & Pfaus, 2001a), and significant preferences were observed following a paired male's first ejaculation (Kippin et al., 2001).

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Currently, more is known about the role of learning in the sexual behavior of males relative to females (reviewed in Pfaus, Kippin, & Centeno, 2001). In the case of females, however, it is known that they display complex patterns of behavior that allow them to control virtually all aspects of sexual interaction with males (Erskine, 1989; McClintock, 1984b; Pfaus et al., 2001). Females attract and solicit males (Beach, 1976; Erskine, 1989; McClintock, 1984b) and control, or pace, the rate of sexual interaction (Erskine, Kornberg, & Cherry, 1989; Pfaus et al., 1999). Like males, females display a variety of conditioned appetitive behaviors, including a conditioned place preference (CPP) for distinctive environments associated with sexual reward (Oldenburger, Everitt, & de Jonge, 1992; Paredes & Alonso, 1997). As with conditioned ejaculatory preferences, significant CPP develops in males only if they ejaculate during the conditioning trials. What constitutes the precise rewarding effect of copulation for females remained elusive until Paredes and Alonso (1997) and Paredes and Vazquez (1999) found that female rats show CPP for distinctive sides associated with paced copulation, relative to nonpaced copulation. As with male rats, this copulatory CPP in female rats can be disrupted by injections of the opioid receptor antagonist naloxone (Paredes & Martinez, 2001), which suggests that elevated levels of endogenous opioids induced by paced copulation form an important part of the neurochemical reward substrate, as they do in male rats following ejaculation (Ågmo & Berenfeld, 1990; Miller & Baum, 1987). Thus, paced sexual interactions induce a positive affective state of sufficient intensity and duration to induce CPP.

In the present study, we ask whether the association of almond odor with pacing will induce a preference for almond-scented males, similar to the preference observed in males for females bearing an almond odor paired with ejaculation.

## Method

### *Subjects and Surgery*

Long-Evans male (300–350 g) and female (200–250 g) rats were purchased from Charles River Canada (St. Constant, Quebec, Canada). They were housed in groups of 4 in large hanging wire mesh cages in a colony room maintained on a reversed 12-hr light–dark cycle (lights off at 0800) at approximately 21 °C. Food and water were continuously available.

The females were anesthetized with a mixture of ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (4 mg/ml), mixed at a ratio of 4:3, respectively, and injected intraperitoneally in a volume of 1 ml/kg of body weight. Anesthetized females were then ovariectomized bilaterally via a lumbar incision. All females were given a week of postsurgical recovery prior to odor conditioning trials. Intact Long-Evans male rats that served as stimulus males had at least 10 tests of sexual behavior in bilevel chambers prior to the start of these experiments. These males were sexually vigorous and initiated copulatory activity with females within 15 s of being placed into the chambers. For all behavioral tests, sexual receptivity was induced in all females by subcutaneous injections of estradiol benzoate (10 µg) 48 hr before and progesterone (500 µg) 4 hr before each test. If scented, males received almond extract (Blue Ribbon, Etobicoke, Ontario, Canada) applied to the back of their neck and anogenital region with a cotton swab. Unscented males received similar applications of distilled water to the same areas.

### *Odor Conditioning*

Odor conditioning trials occurred at 4-day intervals during the middle third of the rat's dark circadian cycle following hormone priming. Paced

copulation occurred in semicircular chambers (38<sup>2</sup> cm × 60 cm × 38 cm) bisected by a clear Plexiglas divider with four holes cut into the bottom (4 cm × 4 cm), which rested on bedding. These holes were large enough for the female to crawl through but too small for the males to crawl through. Nonpaced copulation occurred in identical chambers, but with the divider removed. Females in the paired group copulated with almond-scented males in the paced condition and unscented males in the nonpaced condition. Females in the unpaired group copulated with unscented males in the paced condition and scented males in the nonpaced condition. Females in the randomly paired group copulated with scented and unscented males paired randomly with paced and nonpaced copulation. Females received a total of eight sequential conditioning trials (four in each condition). The order of conditioning was counterbalanced for all groups, such that half of the rats in each group had their first sexual experience in the paced condition and the other half had their first experience in the nonpaced condition. During each test, females were allowed to copulate for 30 min, after which they were transferred to another testing room with CPP boxes and placed in one of the distinctive sides for an additional 30 min before being taken back into their home cage. All testing with scented and unscented males took place in different rooms to ensure containment of the scent.

### *Place Preference Conditioning*

Before sex testing began, females were tested in CPP boxes to determine any unconditioned preference (Oldenburger et al., 1992). The boxes were constructed of wood with three compartments (Pfaus, Kippin, & Coria-Avila, 2003). The two distinctive environments were rectangular in shape and were connected by guillotine doors to either side of a smaller start compartment. One of the goal compartments (darker) was painted with black and white stripes and had a grid floor. The other compartment (lighter) was painted exclusively in white. Most of the rats showed a significant unconditioned preference for the darker side with the stripes. For all animals, the initially nonpreferred side was then paired with the paced condition, whereas the preferred side was paired with the nonpaced condition. Thus, any shift in preference toward the initially nonpreferred side was taken as evidence of reward associated with those place cues.

The CPP test was conducted 4 days after the eighth odor-conditioning test. Females did not copulate prior to this test but instead were placed into the start compartment for 5 min, after which the guillotine doors were opened to allow free access to both goal compartments. The amount of time rats spent in each side was recorded by computer. Females received a ninth odor-conditioning trial 4 days after the CPP test.

### *Copulatory Preference Test*

Four days after the final odor-conditioning test, each female was placed into a large open field (123 cm × 123 cm × 46 cm) with a thin layer of wood chip bedding. At two diagonal corners of this open field were 2 sexually vigorous males, one scented with almond and the other unscented. Each male wore a rodent jacket connected on the back to a spring 30 cm in length. This allowed the males to roam freely within the 45-cm radius but not beyond. The females were placed in the center of the open field and allowed to copulate freely with both males for a 30-min period. These tests were videotaped and scored.

### *Behavioral Measures and Statistical Analyses*

Place preference was determined by changes in preference ratio for the reinforced compartment (time spent in the compartment divided by total time of test) before and after conditioning and by the difference between time spent in the nonreinforced and reinforced compartments of the CPP box. Female copulatory preference was determined by latency and frequency measures for female solicitation (defined as a headwise orientation

to the male followed by a runaway, forcing the male to chase the female), hops and darts, pacing (defined as intermittent approaches and withdrawals from a male, as in Hlinák & Madlafousek, 1977; McClintock, Anisko, & Adler, 1982), lordosis magnitude (on a scale from 1 to 3, with 1 representing low magnitude, 2 representing moderate magnitude, and 3 representing high magnitude, as in Hardy & DeBold, 1972), and the amount of time spent in quadrants of the open field that contained each of the males. Olfactory investigation was considered as a single event from the moment a female started sniffing the male's body until the female moved its head away. The male chosen for first mount, first intromission, and ejaculation for each ejaculatory series was also recorded. Chi-square analyses were used to determine differences in the proportion of females in the conditioning groups that chose the scented or unscented male for copulation. One-way analyses of variance (ANOVAs) were used to determine differences in the frequencies and latencies of copulatory behaviors among the conditioning groups and to analyze the preference ratio and difference between the time spent in the two distinctive sides of the CPP apparatus. For all significant main effects, Tukey's post hoc tests were conducted to assess differences between individual means. The level of significance for all comparisons was .05.

## Results

### Place Preference Test

The CPP test confirmed that females in all groups found the pacing condition rewarding. In the preconditioning test, 39 of the 60 females preferred the darker side of the CPP apparatus,  $\chi^2(1, N = 60) = 5.40, p = .02$ .

In the postconditioning test, 38 of the 60 females spent more time in the reinforced side, and 22 spent more time in the nonreinforced side,  $\chi^2(1, N = 60) = 4.20, p = .03$ . The ANOVA detected a significant shift in the overall preference ratio,  $F(1, 56) = 23.5, p < .01$ . Tukey's post hoc tests revealed that this shift was a significant increase for all groups after conditioning (Figure 1A): paired (before:  $M = 0.20$ ; after:  $M = 0.35$ ), unpaired (before:  $M = 0.22$ ; after:  $M = 0.32$ ), and randomly paired (before:  $M = 0.28$ ; after:  $M = 0.35$ ). The ANOVA also detected an overall difference between the time spent in the nonreinforced and reinforced compartments (Figure 1B),  $F(1, 56) = 70.0, p < .01$ . Tukey's post hoc tests revealed that the time spent in these compartments shifted significantly in all three groups. Before conditioning there was a positive difference in all groups: paired ( $M = 213$  s), unpaired ( $M = 155$  s), and randomly paired ( $M = 142$  s). This difference became negative after conditioning: paired ( $M = -46$  s,  $SD = 32$  s), unpaired ( $M = -31$  s,  $SD = 19$  s), and randomly paired ( $M = -19$  s,  $SD = 15$  s).

### Copulatory Preference Tests

Behavioral results during the copulatory preference test are shown sequentially in Figures 2, 3, 4, and 5. Table 1 lists means and significance levels. Figure 2 shows the latency and frequency of solicitations and the proportions of females that solicited the scented or unscented males. The ANOVA detected a significant effect of conditioning on solicitation latency,  $F(2, 56) = 5.0, p = .01$ . Tukey's post hoc tests revealed that females in the paired group showed a shorter first solicitation latency for scented males than for unscented males, whereas no differences were found in first solicitation latency between these males in the unpaired or randomly paired groups. In the paired group, 75% of the females

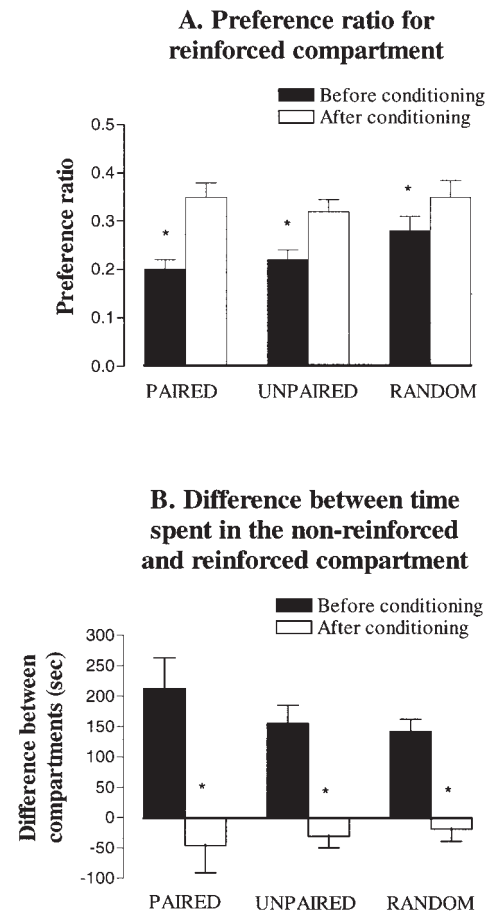


Figure 1. A: Preference ratio for the reinforced compartment (time spent in the compartment divided by the total time of test) in three groups: paired ( $n = 20$ ), unpaired ( $n = 20$ ), and randomly paired ( $n = 20$ ). There was a significant increase in preference ratio after the reinforced side was associated with paced copulation. B: Difference between the time spent in the nonreinforced and reinforced compartments (nonreinforced minus reinforced). The positive graph before conditioning and negative graph after conditioning indicate an increase in time spent in the reinforced compartment. Data are means plus SEM. \* $p < .05$ .

directed their first solicitation toward the scented male (Figure 2B), compared with the unscented male,  $\chi^2(1, N = 20) = 5.00, p = .025$ . No significant preferences were detected in the unpaired or randomly paired groups for first solicitation. The analysis of proportions, frequencies, and latencies throughout the 30-min test revealed more differences among groups. The ANOVA detected a significant effect of conditioning on number of solicitations,  $F(2, 56) = 3.3, p < .05$ , and hops and darts,  $F(2, 56) = 3.8, p < .05$ . Post hoc analyses revealed that females in the paired group performed more solicitations (Figure 2C) toward scented males than toward unscented males and displayed more hops and darts (Figure 3A) toward scented males than toward unscented males. Females in both the unpaired group and the randomly paired group did not display these preferences.

The analysis of olfactory investigations revealed a main effect of scented males regardless of the association with pacing or no pacing (Figure 3B). In all groups, females showed a higher fre-

Table 1  
Sexual Behaviors Displayed Toward Scented or Unscented Male During the Copulatory Preference Test

| Behavior                                    | Paired group  |              | Unpaired group |               | Randomly paired group |              | One conditioning trial |             |
|---------------------------------------------|---------------|--------------|----------------|---------------|-----------------------|--------------|------------------------|-------------|
|                                             | Scented       | Unscented    | Scented        | Unscented     | Scented               | Unscented    | Scented                | Unscented   |
| Solicitation latency                        | 150.0 ± 33.0* | 495.0 ± 98.0 | 410.0 ± 90.0   | 385.0 ± 54.0  | 378.0 ± 60.0          | 377.0 ± 56.0 | 22.5 ± 4.0             | 20.3 ± 4.1  |
| Solicitation frequency                      | 19.0 ± 3.0*   | 8.0 ± 3.0    | 12.0 ± 2.0     | 14.0 ± 2.0    | 10.0 ± 4.0            | 14.0 ± 3.0   | 2.2 ± 0.22             | 3.7 ± 0.35  |
| Choice of male for 1st solicitation (n) (%) | 15 (75%)*     | 5 (25%)      | 9 (45%)        | 11 (55%)      | 11 (55%)              | 9 (45%)      | 10 (50%)               | 10 (50%)    |
| Hops and darts                              | 27.0 ± 3.5*   | 15.7 ± 1.2   | 19.4 ± 3.3     | 17.6 ± 3.7    | 18.0 ± 2.0            | 17.5 ± 1.0   | 12.0 ± 3.0             | 15.0 ± 2.0  |
| Olfactory investigation frequency           | 10.0 ± 1.3*   | 7.0 ± 1.0    | 6.9 ± 0.7*     | 5.6 ± 0.8     | 9.0 ± 0.7*            | 6.0 ± 0.5    | 2.4 ± 1.0              | 2.3.0 ± 1.0 |
| Olfactory investigation latency             | 104.0 ± 29.0* | 246.0 ± 59.0 | 140.0 ± 35.0*  | 328.0 ± 126.0 | 148.0 ± 55.0          | 70.0 ± 30.0  | 41.9 ± 29.0            | 47.2 ± 25.0 |
| Choice of male for 1st mount (n) (%)        | 14 (73%)*     | 5 (27%)      | 11 (56%)       | 8 (44%)       | 11 (55%)              | 9 (45%)      | 7 (45%)                | 13 (65%)    |
| Mount frequency <sup>a</sup>                | 1.9 ± 0.2*    | 0.3 ± 0.2    | 2.8 ± 0.2      | 1.5 ± 0.2     | 1.3 ± 0.5             | 1.5 ± 0.5    | 5.1 ± 1.0              | 5.6 ± 1.8   |
| Intromission frequency                      | 10.0 ± 0.8    | 9.8 ± 0.8    | 9.2 ± 0.5      | 7.4 ± 1.0     | 9.0 ± 0.8             | 8.0 ± 0.5    | 6.2 ± 1.3              | 6.5 ± .6    |
| Intromission latency                        | 75.0 ± 17.9   | 174.0 ± 42.0 | 206.7 ± 84.5   | 183.0 ± 60.0  | 48.0 ± 8.8            | 92.0 ± 22.0  | 80.0 ± 20.0            | 67 ± 49     |
| Mean interintromission interval             | 53.5 ± 15.0   | 77.0 ± 27.0  | 55.0 ± 19.5    | 61.0 ± 8.0    | 46.0 ± 2.7            | 42.0 ± 1.8   | 45.0 ± 14.0            | 48 ± 17     |
| Ejaculation frequency                       | 2.3 ± 0.2     | 1.9 ± 0.2    | 1.9 ± 0.1      | 1.7 ± 0.2     | 2.1 ± 0.2             | 2.4 ± 0.1    | 1.7 ± 0.3              | 1.9 ± .31   |
| Hit rate                                    | 0.6 ± 0.1     | 0.7 ± 0.2    | 0.6 ± 0.1      | 0.7 ± 0.0     | 0.7 ± 0.0             | 0.6 ± 0.0    | 0.7 ± 0.0              | .60 ± .02   |
| Choice of male                              |               |              |                |               |                       |              |                        |             |
| 1st ejaculation (n) (%)                     | 14 (73%)*     | 5 (26%)      | 8 (45%)        | 10 (55%)      | 7 (35%)               | 11 (55%)     | 9 (45%)                | 11 (55%)    |
| 2nd ejaculation (n) (%)                     | 9 (50%)       | 9 (50%)      | 10 (62%)       | 6 (38%)       | 8 (45%)               | 10 (55%)     | 9 (45%)                | 11 (55%)    |
| 3rd ejaculation (n) (%)                     | 9 (53%)       | 8 (47%)      | 8 (50%)        | 8 (50%)       | 10 (55%)              | 8 (45%)      | 9 (50%)                | 9 (50%)     |
| Lordosis Magnitude 1                        | 9.8 ± 1.5     | 7.0 ± 1.5    | 10.4 ± 1.4*    | 3.7 ± 1.5     | 10.0 ± 1.4            | 8.0 ± 1.5    | 8.5 ± 1.1              | 7 ± 1       |
| Lordosis Magnitude 2                        | 5.9 ± 1.0     | 1.9 ± 1.5    | 7.3 ± 1.2      | 4.9 ± 0.9     | 6.2 ± 0.9             | 9.3 ± 1.6    | 10.0 ± 1.5             | 9.2 ± .9    |
| Lordosis Magnitude 3                        | 10.4 ± 2.7*   | 4.9 ± 1.5    | 11.9 ± 3.0     | 12.7 ± 2.1    | 4.1 ± 1.2             | 6.8 ± 1.2    | 4.5 ± 0.9              | 3 ± 1       |
| Mean number of visits                       | 40.1 ± 2.9    | 38.6 ± 3.1   | 37.2 ± 2.5     | 34.5 ± 2.5    | 27.7 ± 4.1            | 28.4 ± 3.4   | 38.7 ± 3.0             | 40 ± 3      |
| Mean intervisit interval                    | 44.8 ± 3.0    | 46.6 ± 2.1   | 48.3 ± 2.0     | 52.1 ± 4.0    | 64.9 ± 5.1            | 63.3 ± 3.0   | 46.5 ± 0.9             | 45 ± 1.6    |
| Time spent with each male                   |               |              |                |               |                       |              |                        |             |
| 5 min of test                               | 97.4 ± 8.8    | 96.5 ± 11.7  | 87.7 ± 4.7     | 102.8 ± 9.3   | 95.0 ± 8.0            | 100.0 ± 11.0 | 80.0 ± 25.0            | 75 ± 20     |
| 10 min of test                              | 192.0 ± 10.9  | 159.6 ± 11.0 | 188.0 ± 11.3   | 190.6 ± 15.0  | 185.0 ± 11.0          | 171.0 ± 20.0 | 150.0 ± 16.0           | 140 ± 18    |
| 30 min of test                              | 545.9 ± 50.0  | 454.4 ± 30.0 | 538.5 ± 37.0   | 511.3 ± 44.0  | 543.0 ± 40.0          | 470.0 ± 35.0 | 503.0 ± 50.0           | 486 ± 49    |

Note. Four groups are shown. In the paired group ( $n = 20$ ), a scented male was associated with paced copulation. In the unpaired group ( $n = 20$ ), a scented male was associated with nonpaced copulation. In the randomly paired group ( $n = 20$ ), the association of paced copulation was randomly distributed between scented and unscented males. In the one conditioning trial group ( $n = 20$ ), females received only one exposure to a scented male in the paced condition, and an unscented male in a nonpaced condition, before the partner preference test. Data are  $M \pm SEM$  during the 30-min test, except where pointed out. Latencies are expressed in seconds.

<sup>a</sup> During first ejaculatory series.

\*  $p < .05$  between scented and unscented males within a group.

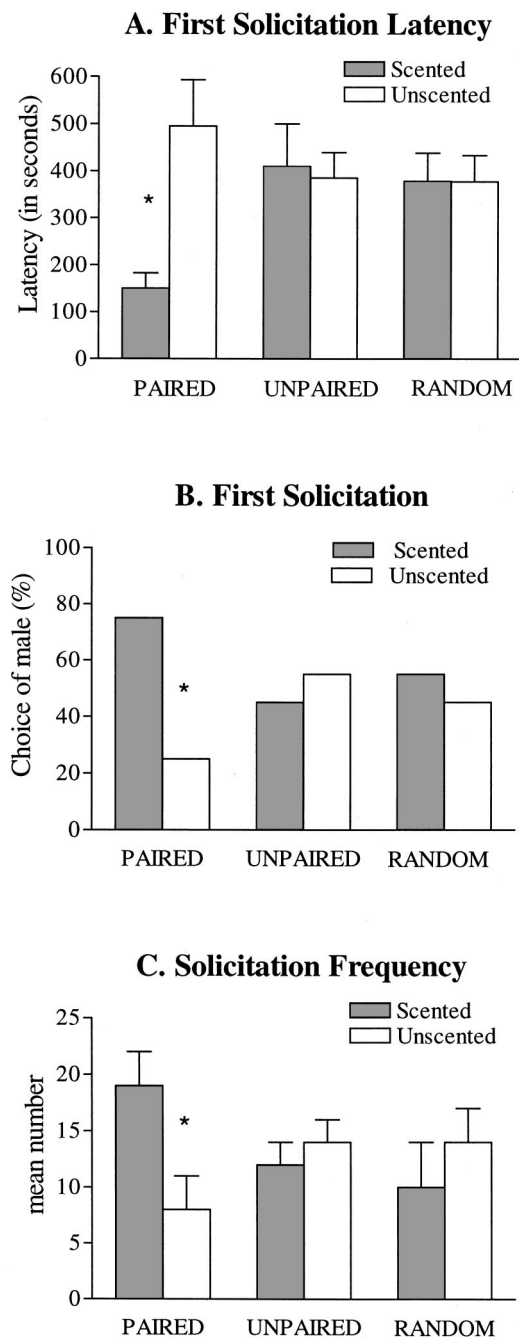


Figure 2. A: Latency to perform the first solicitation in three groups: paired ( $n = 20$ ; scented male associated with paced copulation), unpaired ( $n = 20$ ; scented male associated with nonpaced copulation), and randomly paired ( $n = 20$ ; scented males associated randomly with paced and non-paced copulation). B: Choice of male for first solicitation. C: Number of solicitations during 30-min test. Latency and frequency data are means plus SEM. \* $p < .05$ .

quency of olfactory investigations toward scented than toward unscented males,  $F(2, 56) = 4.6$ ,  $p < .05$ . The latency for first olfactory investigation in the paired group (Figure 3C) was shorter toward scented males than toward unscented males. This was also

found for scented males in the unpaired group relative to unscented males,  $F(2, 56) = 4.0$ ,  $p < .05$ . This difference, however, did not occur for the randomly paired group.

Differences in proportion and frequency of mounts (see Figure 4) were also detected. Significantly more females in the paired group received their first mount from a scented male,  $\chi^2(1, N = 19) = 4.20$ ,  $p = .03$ , whereas no significant differences in proportion were detected for either the unpaired or the randomly

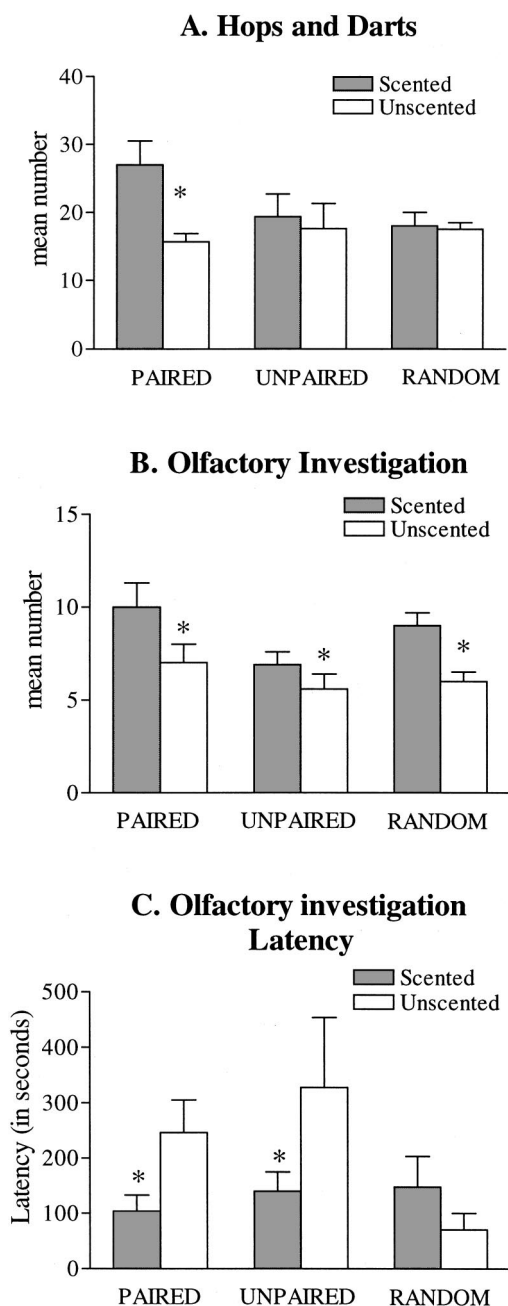


Figure 3. A: Number of hops and darts toward scented and unscented males. Females displayed a preference for scented males only in the paired group. B: Number of olfactory investigations. C: Latency to first olfactory investigation. Data are means plus SEM. \* $p < .05$ .

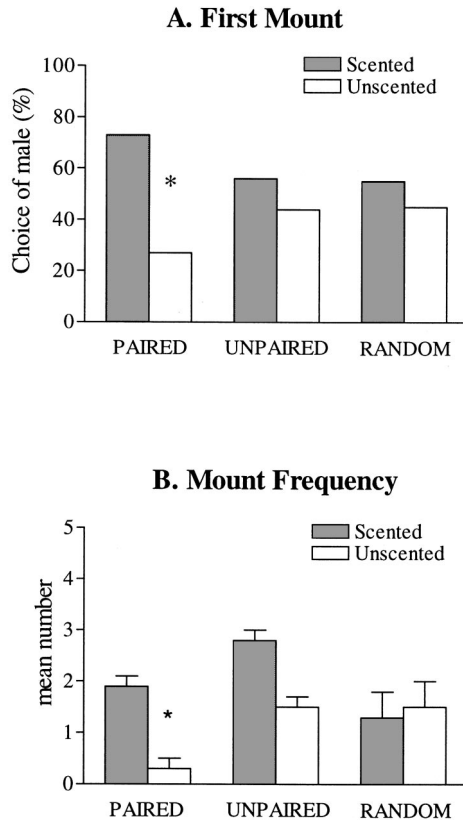


Figure 4. A: Choice of male for first mount. B: Number of mounts with each male. Frequency data are means plus SEM. \* $p < .05$ .

paired group. The ANOVA detected a significant effect of conditioning on the number of mounts received from either male during the first ejaculatory series,  $F(2, 56) = 3.9$ ,  $p = .02$ . Post hoc analyses revealed that females in the paired group received significantly more mounts from the scented males relative to the unscented males, whereas these differences were not significant for females in the other groups.

Figure 5 shows females' choice of male for the first three ejaculations and the number of lordoses with low, moderate, and high magnitude toward scented and unscented males. Of the 60 females, all but 5 copulated to ejaculation with 1 or both males on the final copulatory preference test. Of the females that did not receive an ejaculation, 1 was in the paired group, 2 were in the unpaired group, and 2 were in the randomly paired group. Accordingly, these females were not included in the analysis of ejaculation data. A significant proportion of females in the paired group (75%) received their first ejaculation from the scented male, relative to the unscented male,  $\chi^2(1, N = 19) = 4.26$ ,  $p = .03$ .

No significant differences in the choice of male for ejaculation were found in the unpaired,  $\chi^2(1, N = 18) = 0.22$ ,  $p = .63$ , or randomly paired,  $\chi^2(1, N = 18) = 0.88$ ,  $p = .30$ , groups (Figure 5A). During the second ejaculatory series, 6 females did not receive a second ejaculation (2 in the paired group, 4 in the unpaired group, and 2 in the randomly paired group). No preferences were detected for either male for second ejaculation in any group. Likewise, during the third ejaculatory series, 9 females did

not receive a third ejaculation (3 in the paired group, 4 in the unpaired group, and 2 in the randomly paired group; Figure 5C). The analysis of proportions of females that switched partner for consecutive ejaculations revealed no differences. Ten of the females (55%) in the paired group switched partners for the second ejaculation,  $\chi^2(1, N = 18) = 0.22$ ,  $p = .63$ ; 7 females (44%) in the unpaired group switched partners,  $\chi^2(1, N = 16) = 0.25$ ,  $p = .61$ ; and 9 females in the randomly paired group switched partners,  $\chi^2(1, N = 18) = 0.20$ ,  $p = .65$ . For the third ejaculation, 11 females (68%) in the paired group switched partners,  $\chi^2(1, N = 17) = 2.20$ ,  $p = .13$ ; 8 females (50%) in the unpaired group switched partners, and 10 females (55%) in the randomly paired group switched partners.

The ANOVA detected a significant effect of conditioning on the number of high-magnitude lordoses,  $F(2, 56) = 3.6$ ,  $p < .05$ . Post hoc analyses revealed that only females in the paired group displayed a higher number of high-magnitude lordoses with scented males than with unscented males. The ANOVA also detected a significant effect of conditioning on the number of low-magnitude lordoses,  $F(2, 56) = 6.9$ ,  $p < .01$ . Post hoc analyses detected that females in the unpaired group showed a higher number of low-magnitude lordoses with the scented male than with the unscented male. No differences in the number of low-magnitude lordoses were found in the paired or randomly paired groups between the scented and the unscented males, and no group showed differences in frequency of moderate lordosis (Figures 5D, 5E, and 5F).

#### Effect of Single Paired Conditioning Trials

An additional group, Paired 1, was added to the experiment and analyzed independently to examine whether a single pairing of odor and paced copulation was sufficient to induce a conditioned partner preference. Females copulated in the same conditions as did the paired group, but with only one exposure to a scented male in the paced condition and an unscented male in a nonpaced condition before the final copulatory preference test. The analysis of frequencies, latencies, and proportions, overall, did not reveal any significant difference in the choice between scented and unscented males (see Table 1).

#### Discussion

As with male rats, female rats develop olfactory conditioned partner preference for cues associated with sexual reward. Relative to nonpaced copulation, paced copulation induced a place preference in the paired, unpaired, and randomly paired groups, replicating previous findings by Martinez and Paredes (2001), Paredes and Alonso (1997), and Paredes and Vazquez (1999) and confirming that the three groups were capable of discriminating these conditions. However, subsequent partner preferences were displayed by females in the paired but not the unpaired or randomly paired groups. Paired females chose the scented male for their first solicitation, first mount, and first ejaculation and displayed more solicitations with the scented male, shorter solicitation latencies, and more hops and darts, relative to the unscented male. In contrast, females in the unpaired and randomly paired groups did not display a significant preference for either male, which indicates that the almond scent alone does not carry unconditional appetitive value.



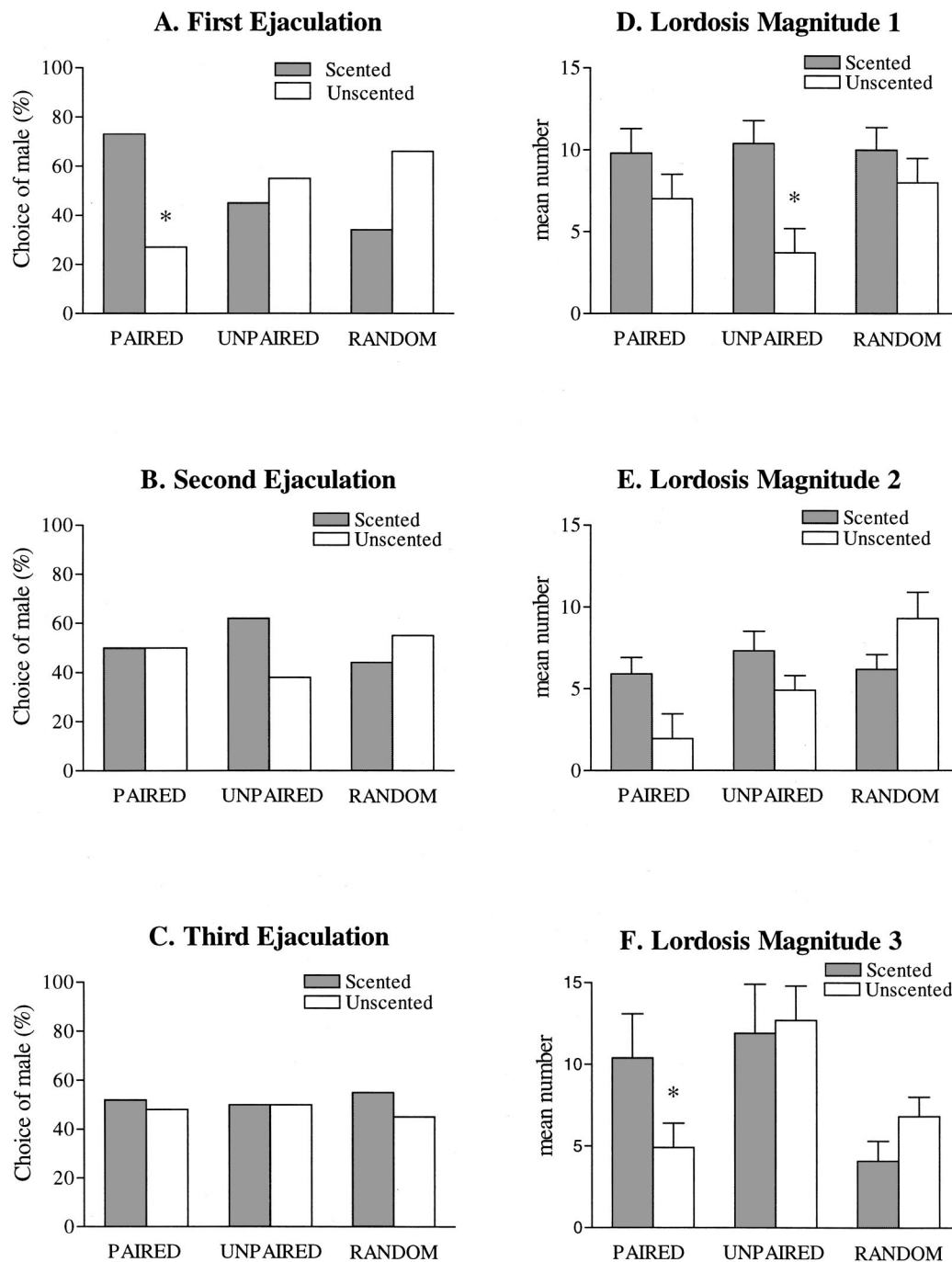


Figure 5. A, B, and C: Preference of male for first, second, or third ejaculation, respectively. D: Number of low-magnitude lordoses (Magnitude 1, flat back). E: Number of medium-magnitude lordoses (Magnitude 2, either head or tail pointing upward). F: Number of high-magnitude lordoses (Magnitude 3, head and tail pointing upward). Frequency data are means plus SEM. \* $p < .05$ .

Kippin et al. (1998) demonstrated that repeated pairing of a neutral almond odor with copulation to ejaculation in male rats produces a subsequent conditioned ejaculatory preference for females bearing that odor. However, in that study, males in the paired group did not display a copulatory preference for any

female with regard to mounts and intromissions prior to ejaculation. Subsequent experiments determined that males in the paired group became choosy only during the mounts immediately preceding ejaculation (Kippin & Pfaus, 2001b), which suggests that they were choosing the female specifically to receive their ejacu-



lation and that they differentiated copulation from mating. Moreover, the necessary unconditioned stimulus for this conditioning was exposure to the scented female during the postejaculatory refractory period during each conditioning trial (Kippin & Pfaus, 2001a), which indicates that a postejaculatory reward state is critical for the induction of partner preference in the male rat, as it is for the induction of CPP (Ågmo & Berenfeld, 1990). In contrast, paired females in the present study displayed both copulatory (regarded as those behaviors that are needed but not sufficient to induce pregnancy) and mate (i.e., ejaculatory) preferences, which suggests that the reward state induced by paced copulation occurs throughout the conditioning trial. Another important difference concerns the magnitude of the reward state. A single pairing of the odor and the postejaculatory reward state was sufficient to induce significant partner preferences in male rats (Kippin et al., 2001), although multiple trials were more effective. We and others have argued previously that early experience with sexual reward may form a flexible critical period in which salient features of the first partner come to be preferred in subsequent partners (e.g., Pfaus et al., 2001; Storms, 1981). In contrast, a single set of exposures of odor with pacing and no odor with no pacing was not sufficient in the present study to induce any conditional preference in females. It may be that the reward state induced by pacing in females is of a smaller magnitude than that induced by ejaculation in males, such that multiple conditioning trials are necessary to induce the conditioned preference in females. Although the activation of endogenous opioid systems in both male and female rats is necessary for the induction of CPP (Ågmo & Berenfeld, 1990; Miller & Baum, 1987; Paredes & Martinez, 2001), it is not yet known whether similar mechanisms control partner or mate preferences in male and female rats.

Partner preferences can be viewed as a rudiment of pair bonding, in which familiar cues are preferred or chosen relative to novel cues (Wickler & Seibt, 1983; Young & Wang, 2004). Pair bonding occurs in putatively monogamous species, such as cichlid fish (Taylor, Morley, Rico, & Balshine, 2003); certain birds, such as budgerigars (Hile, Plummer, & Striedter, 2000); and mammals such as prairie voles (Carter & Getz, 1993). In contrast, the general mating strategy of rats has been described as polygamous and opportunistic (McClintock et al., 1982). Indeed, male and female rats both display a Coolidge effect, in which a novel partner produces more sexual arousal and copulatory behavior relative to a familiar one (Bermant, Lott, & Anderson, 1968; Dewsbury, 1981). However, McClintock (1984b) and Tiefer (1969) have shown that during group mating female rats compete actively while copulating with a male and that such competition is greatest when the male is about to ejaculate. McClintock (1984b) has also observed sexual choice strategies between dominant and subordinate female rats in group-mating conditions in which the dominant females are more likely than the subordinates to intercept males and take their early ejaculations. Although rats are not observed to form pair bonds in such group-mating conditions, our data suggest that they are capable of doing so if the conditions are different. This suggests a degree of experience-dependent plasticity in the brain of both male and female rats regarding mate strategies. It may be the case that similar neural mechanisms that underlie the formation of partner preferences in other species—for example, oxytocin or vasopressin release (Bales, Kim, Lewis-Reese, & Sue, 2004; Carter, DeVries, & Getz, 1995; Insel, Winslow, Wang, &

Young, 1998; Insel, Winslow, Wang, Young, & Hulihan, 1995; Winslow, Hastings, Carter, Harbaugh, & Insel, 1993)—may also be necessary for the formation of conditioned partner preference in rats.

In addition to facilitative effects on sexual arousal and partner preference, an odor paired with sexual reward can also enhance reproductive function in male rats. Graham and Desjardins (1980) reported a conditioned increase in serum testosterone and luteinizing hormone in male rats following access to a wintergreen odor previously paired with copulation to ejaculation. Males in an unpaired control group did not show any increase in hormone levels following exposure to the odor. However, males in both groups exposed to estrous vaginal secretions showed identical increases in these hormones, which suggests that unconditioned hormone release is mimicked reliably following pairing of odor with sexual reward. In female rats, pacing serves an important reproductive function, allowing females to control the rate of vaginocervical stimulation (VCS). Such control serves to distribute VCSs in time, which, in turn, enhances both sperm transport and the induction of nightly prolactin surges that support the maintenance of progesterone release by the corpora lutea (Erskine, 1989; Matthews & Adler, 1978). Accordingly, paced copulation is more effective than nonpaced copulation in inducing luteal function, a faster termination of estrus, and pregnancy (Bermant & Westbrook, 1966; Erskine, 1989; Lodder & Zeilmaker, 1976). Moreover, paced copulation increases litter size compared with nonpaced copulation (Coopersmith & Erskine, 1994), and females are more likely to become pseudopregnant following exposure to nonpaced relative to paced copulation (Frye & Erskine, 1990). Thus, copulating with a male in a paced situation is more rewarding and has positive effects on reproduction. It is not yet known whether odors paired with paced copulation could induce luteal function or a faster termination of estrus or enhance pregnancy.

In summary, female rats can learn to associate a neutral odor with sexual reward. An odor paired with the ability of females to pace copulation becomes a sexual incentive, such that males bearing the odor are preferred when females are given a choice between scented and unscented males. Thus, just like male rats, female rats can learn to modify their sexual behavior and partner preferences on the basis of experience with sexual reward. This confirms that early sexual experiences have particularly powerful influences on subsequent sexual preferences and that the development of sexual preferences is influenced by interactions between conditioned stimulus–unconditioned stimulus pairings and motivational variables.

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## Conditioned partner preference in female rats for strain of male

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### Abstract

Female rats show conditioned place preference following paced copulation, and we have recently demonstrated that pairing almond odor with paced copulation induces a conditioned partner preference for almond-scented males. The present study examined whether cues of two different strains of male (albino and pigmented) induce a conditioned partner preference for the strain of male associated with paced copulation. Ovariectomized, hormone-primed Wistar (W) or Long-Evans (LE) female rats received 10 conditioning trials at 4-day intervals. In the Wistar-pacing group females copulated with W males in a chamber bisected by a 4-hole partition that only the female could pass through. Four days later, they copulated with LE males without the partition. The Long-Evans-pacing group received the opposite association. In the final preference test all females chose freely between two males tethered in opposite corners of an open field, one W and one LE. Regardless the strain of male, females displayed more solicitations toward the pacing-related male, and most of the females received their first ejaculation from that male. The preference was facilitated if the pacing-related male was of the same strain as the female. These results suggest that female rats have an unconditioned preference for males of the same strain, but this preference can be switched towards males of a different strain if that male is associated with the sexual reward induced by paced copulation.

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**Keywords:** Pacing; Appetitive; Sexual reward; Conditioning; Sexual behavior; Wistar; Long-Evans

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### 1. Introduction

Conditioned stimuli (CSs) can activate appetitive sexual behaviors and neuroendocrine functions [17,21,19,35,36,43], or suppress them [1], depending on their association with rewarding or aversive experiences, respectively. For example, male rats perform anticipatory behaviors in the presence of stimuli that predict copulation [34,37], but will display avoidance responses toward sex-related stimuli such as estrous odors associated with malaise (e.g., paired with the gastrointestinal distress induced by injections of LiCl) [1]. In male rats, ejaculation induces a state of reward sufficient to induce a conditioned place preference (CPP) [2]. Males spend more time in sides of a CPP chamber associated with the ejaculation-induced reward state. This can occur even if males are placed into the CPP box after they have ejaculated in another chamber. Systemic administration of the opioid receptor antagonist naloxone during conditioning blocks ejaculation-induced CPP [2],

indicating that activation of endogenous opioids is an important component of the reward state, and of the conditioning mechanism that links the contextual CS to the reward. The post-ejaculatory reward state is also critical for conditioned partner preference. For example, Kippin et al. [20] found that male rats develop a preference to ejaculate with females bearing and odor (almond), but only if they had experienced the odor during the postejaculatory refractory period.

Females display complex sexual behaviors that allow them to control or “pace” their sexual interaction with males [35]. They solicit males [4,13,27] and also pace the rate of sexual interaction with approaches or withdrawals if they are given the opportunity [15,37]. Female rats develop CPP for distinctive sides of a chamber associated with paced copulation, relative to non-paced copulation [31,33]. This copulatory CPP in female rats is blocked by injections of naloxone [32], suggesting that increased levels of endogenous opioids induced by paced copulation form a significant part of the neurochemical reward substrate, as they do in male rats following ejaculation. Accordingly, paced sexual interactions induce a positive affective state of sufficient intensity and duration to induce conditioning.

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We have recently reported that females given the ability to pace copulations with almond-scented males develop a conditioned partner preference for almond-scented males [9]. In our paired group, female rats copulated with almond-scented males in chambers bisected by a Plexiglas divider with four small holes that allowed the female to run back and forth from the side with the male, but that were too small for the male to pass through. In the unpaced condition, females copulated in the same chamber with unscented males, but without the divider. After 5 sequential pairings of paced and unpaced copulation (with and without the odor, respectively), females were tested in a large open field with two sexually vigorous copulating males, one scented and the other unscented. Paired females showed a significant preference for the almond-scented male for their first solicitation, first mount, and first ejaculation, and displayed shorter first solicitation latencies and a higher frequency of solicitations and hops and darts throughout the test with those males compared to females in an unpaired group (that had the odor on males presented in the unpaced condition, and no odor on males presented in the paced condition) or in a random-paired group (that had the odor presented equally in paced and unpaced conditions). This study demonstrated that neutral odors can acquire sexual incentive value and modulate partner preference when paired with the rewarding effects of paced copulation.

Ersine [14] has argued that conditioned place and partner-preference paradigms offer important glimpses into female sexual motivation and mate choice, but raised the question of how such preferences are exhibited in natural conditions (i.e., without the addition of almond or other foreign odors). Female rats are polyandrous, and descriptions of group mating in the wild [6] suggest that females copulate indiscriminately with many males. However, laboratory modeling of group mating in large open fields has revealed that females show serial preferences throughout the mating sequence [29]. It is not clear what mate strategies or choices may underlie these shifts in preference, and individual differences in mate strategies exist, especially between dominant and subordinate females.

Albino and pigmented strains of rats have been used in experiments of sexual competition, and females in particular appear to react differently toward males of their same or other strain [3,10,30]. This suggests that female rats can differentiate different strains of male rat, although the exact cues the females attend to have not been determined (e.g., olfactory, visual, and/or auditory). The present study examined whether pairing a particular strain of male (albino or pigmented) with paced copulation would condition subsequent appetitive sexual behaviors and partner preferences in female rats. Based on our previous findings [9] we hypothesized that regardless of the strain of male, female rats would show a partner preference for those males paired with paced copulation.

## 2. Materials and methods

### 2.1. Animals and surgery

Wistar (W) and Long-Evans (LE) female rats (200–250 g) and W and LE male rats (350–400 g) were purchased from

Charles River Canada (St-Constant, QC). Males were housed in groups of four in Plexiglas cages and females were housed in groups of 4 in large wire mesh cages in a colony room maintained on a reversed 12:12 h light/dark cycle (lights off at 08:00 h) at approximately 21 °C. Commercial food and water were continuously available.

The females were anesthetized with a mixture of ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (4 mg/ml), mixed at a ratio of 4:3, respectively, and injected intraperitoneally in a volume of 1 ml/kg of body weight. Anesthetized females were then ovariectomized bilaterally via a lumbar incision. Post-surgical treatment included single subcutaneous injections of Flunixin meglumine 2.5 mg/kg (Banamine®) for analgesia, and Enrofloxacin 5 mg/kg (Baytril®) to prevent post-surgical bacterial infections. All females were given a week of post-surgical recovery prior to conditioning trials. Gonadally intact W and LE male rats that served as stimuli had at least 4 tests of sexual behavior in the same conditioning chambers prior to the start of these experiments. These males initiated copulatory activity with females within 15 s of being placed into the chambers. For all behavioral tests, sexual receptivity was induced in all ovariectomized females by subcutaneous injections of estradiol benzoate (10 µg) 48 h and progesterone (500 µg) 4 h before each test.

### 2.2. Conditioning

The experiment included two counterbalanced groups. Females in the Wistar-pacing (W-P) group copulated with Wistar males in the paced condition and Long-Evans males in the non-paced condition. Females in the Long-Evans-pacing (LE-P) group copulated with Long-Evans males in the paced condition and Wistar males in the non-paced condition. Conditioning trials occurred at 4-day intervals during the middle third of the rat's dark circadian cycle following hormone priming. Paced copulation occurred in semicircular chambers (38<sup>2</sup> × 60 × 38 cm) bisected by a clear Plexiglas divider with 4 holes cut into the bottom (4 × 4 cm) that rested on bedding [36]. These holes were large enough for the female to crawl through, but too small for the males to pass through. Non-paced copulation occurred in the same chambers, but with the divider removed. Females received a total of 10 sequential experiences of 30-min each in paced and non-paced conditions at 4-day intervals (5 each). The order of condition for the first conditioning trial was counterbalanced so that half of each group started with paced condition and the other half with non-paced copulation. Animals were allowed to copulate for 30 min before being taken back into their home cages.

### 2.3. Copulatory preference test

Four days after the 10th conditioning trial, each female was placed into a large open field (123 × 123 × 46 cm) with a thin layer of woodchip bedding. At two diagonal corners of this open field were two males, one W and the other LE. Both males had received their last copulatory test 4 days before the open field test. Each male wore a rodent jacket connected on the back

to a spring wire 30 cm in length. This allowed the males roam freely within a 45-cm radius. The females were placed in one neutral corner of the open field and allowed to copulate freely with both males for a 30-min period. These tests were videotaped and scored.

#### 2.4. Behavioral measures and statistical analyses

Female copulatory preference was assessed as in previous olfactory conditioned experiments [9] and determined by latency and frequency measures for female solicitations, hops and darts (H&D), visits to the males, lordosis magnitude (on a scale of 1 to 3, with 1 representing low magnitude, 2 representing moderate magnitude, and 3 representing high magnitude, as in [18]), and the amount of time spent in quadrants that contained either male. A  $2 \times 2$  (group  $\times$  strain) mixed design ANOVA was used to determine differences in the frequencies and latencies of copulatory behaviors towards the two males. All significant main effects and interactions were followed by a Tukey least significant difference (LSD) post hoc test to detect specific differences between individual means. The level of significance for all comparisons was  $p < 0.05$ . The proportion of ejaculations received from either male was assessed with a Chi-square test. The level of significance for all comparisons was  $p < 0.05$ .

### 3. Results

Some females were eliminated from statistical analysis because one or both of the males became free from their tethered jackets during the final preference test. Of a total of 60 W females this left 57 ( $n = 30$  in the W-P group;  $n = 27$  in the LE-P group), and of a total of 24 LE females this left 22 ( $n = 10$  in the W-P group;  $n = 12$  in the LE-P group). Table 1 shows the means + standard errors for all the behaviors assessed during the first ejaculatory series and throughout the 30-min test.

#### 3.1. Wistar females

##### 3.1.1. Olfactory investigations and visits

The analysis of olfactory investigations during the first ejaculatory series showed a main effect of strain of male [ $F(1,55) = 6.06$ ,  $p = 0.01$ ], and an interaction between group and strain of male [ $F(1,55) = 7.1$ ,  $p = 0.009$ ]. Post hoc analyses indicated significant differences between W ( $M = 8.6$ ) and LE males ( $M = 12.8$ ) in the LE-P group, but not in the W-P group. Throughout the 30-min test, there was also a main effect of strain of male [ $F(1,55) = 8.09$ ,  $p = 0.006$ ]. Post hoc analyses indicated significant differences between W ( $M = 21.1$ ) and LE males ( $M = 27.1$ ) in the LE-P group, but not in the W-P group, indicating that overall females displayed more olfactory investigations towards LE males from the beginning of the final preference test, and this effect was facilitated when LE males were associated with paced copulation. The ANOVA did not detect any main effects or interactions for olfactory investigation latency, indicating that females performed their first olfactory investigation towards W and LE males with the same latency. Likewise, the ANOVA did not detect any

significant effects regarding the number of visits during the first ejaculatory series, or during the 30-min test, as similar lack of effect was found for the first visit latency. This indicates that the females in both conditioning groups visited the W and LE males equally throughout the 30-min test.

##### 3.1.2. Appetitive sexual behaviors

The analysis of appetitive behaviors revealed significant differences between the conditioning groups (Fig. 1). With regard to solicitations during the first ejaculatory series, the ANOVA detected an interaction between group and strain of male [ $F(1,55) = 6.07$ ,  $p = 0.01$ ]. However, regardless of this significance, the LSD post hoc test failed to reveal significant differences between W and LE males in the W-P ( $p = 0.10$ ) or LE-P groups ( $p = 0.07$ ). The analysis of solicitations during the 30-min test revealed a significant interaction between group and strain of male [ $F(1,55) = 13.7$ ,  $p = 0.0004$ ], and the post hoc test indicated a significant difference between W ( $M = 13.6$ ) and LE males ( $M = 9.3$ ) in the W-P group, and between W ( $M = 10.4$ ) and LE males ( $M = 17.5$ ) in the LE-P group, indicating that, overall, females displayed more solicitations towards the pacing-related male. With regard to the first solicitation latency, there was a significant interaction between group and strain of male [ $F(1,47) = 5.5$ ,  $p = 0.02$ ]. However, the post hoc test that failed did not reveal significant differences between W ( $M = 162$  s) and LE males ( $M = 223$  s) in the W-P group ( $p = 0.15$ ) or between W ( $M = 271$  s) and LE males ( $M = 121$  s) in the LE-P group ( $p = 0.06$ ). This suggests that there was a trend for females to perform their first solicitation with a shorter latency towards the pacing-related male.

With regard to H&D, although no significant main effects or interaction were detected during the first ejaculatory series, the analysis during the 30-min test indicated a main effect of strain of male [ $F(1,55) = 6.2$ ,  $p = 0.01$ ]. Post hoc analyses revealed a significant difference between W ( $M = 28.4$ ) and LE males ( $M = 43.1$ ) in the LE-P group ( $p = 0.007$ ), but not between W ( $M = 35$ ) and LE males ( $M = 38.4$ ) in the W-P group ( $p = 0.49$ ), indicating that females displayed more H&D towards LE exclusively in the LE-P group. No significant main effects or interaction were found for first H&D latency. Together, these results indicate that, regardless of the strain of male, females displayed more appetitive behaviors towards the pacing-related males, relative to non-pacing-related males.

##### 3.1.3. Lordosis

With regard to the different magnitudes of lordosis during the first ejaculatory series, the ANOVA found a significant interaction of between-group and strain of male for lordosis magnitude 3 [ $F(1,55) = 4.3$ ,  $p = 0.04$ ]. The post hoc test showed that females in the W-P group displayed higher number of lordosis magnitude 3 towards W males ( $M = 4.1$ ) than towards LE males ( $M = 1.8$ ). In the LE-P group, however, no significant differences were detected between W ( $M = 3$ ) and LE ( $M = 3.7$ ) males. No significant main effects or interaction were found for the other lordosis magnitudes, nor did the analysis of lordosis magnitude 1, 2 and 3 during the 30-min test reveal any main effects or interactions.



Table 1

Sexual behaviors displayed by Wistar or Long-Evans females during the final partner-preference test with two males, one Wistar and one Long-Evans

| Behavior                            | Wistar females             |                 |                                |                 | Long-Evans females         |                 |                                |                 |
|-------------------------------------|----------------------------|-----------------|--------------------------------|-----------------|----------------------------|-----------------|--------------------------------|-----------------|
|                                     | Wistar-pacing group (n=30) |                 | Long-Evans-pacing group (n=27) |                 | Wistar-pacing group (n=10) |                 | Long-Evans-pacing group (n=12) |                 |
|                                     | Wistar male                | Long-Evans male | Wistar male                    | Long-Evans male | Wistar male                | Long-Evans male | Wistar male                    | Long-Evans male |
| 1st visit latency                   | 19.2±3.9                   | 16±7.9          | 61.9±44                        | 10.8±1.5        | 43.4±18                    | 26.2±9.5        | 15.9±5.1                       | 18.3±4.9        |
| Visits frequency                    |                            |                 |                                |                 |                            |                 |                                |                 |
| First ejaculatory series            | 22.1±2.2                   | 23.1±2.5        | 22.6±2.9                       | 25±2.5          | 14.4±2.3                   | 14.3±2.6        | 17.6±2.4                       | 20.2±2.9        |
| 30 min of test                      | 56.5±6.1                   | 58.9±6.1        | 67.7±8.7                       | 71.5±8.4        | 63.6±6.3                   | 59.2±7.2        | 85.5±8.9                       | 88.6±4.9        |
| 1st olfactory investigation latency | 32.5±5.2                   | 29.0±8.7        | 81.9±47.6                      | 14.0±1.8        | 56.7±19                    | 75.8±35         | 102.8±33                       | 104.6±45        |
| Olfactory investigation frequency   |                            |                 |                                |                 |                            |                 |                                |                 |
| First ejaculatory series            | 11.2±1.1                   | 11.0±1.0        | 8.6±1.1*                       | 12.8±1.7        | 1.8±.41                    | 2.9±.73         | 1.7±.39                        | 2.2±.60         |
| 30 min of test                      | 22.5±1.2                   | 24.2±1.9        | 21.1±2.0*                      | 27.1±2.0        | 11.9±1.5                   | 11.1±2          | 9.2±1.3                        | 12.5±1.5        |
| 1st hop and dart latency            | 117.7±62                   | 83.1±36         | 146.04±51                      | 52.0±9.4        | 74±16                      | 77.6±22         | 76.8±27                        | 79.4±26         |
| Hops and darts                      |                            |                 |                                |                 |                            |                 |                                |                 |
| First ejaculatory series            | 15.2±2.9                   | 13.8±2.3        | 10.1±1.5                       | 14.9±2.7        | 7.8±1.8                    | 10.5±2.3        | 8.9±1.8                        | 9.9±1.6         |
| 30 min of test                      | 35.0±4.7                   | 38.4±4.3        | 28.4±3.4*                      | 43.1±5.4        | 46.6±4.9                   | 34.8±6.9        | 40.5±7.2                       | 37.3±6.9        |
| 1st solicitation latency            | 162.2±41                   | 223.3±52        | 271.4±81                       | 121.7±20        | 138.7±17.3                 | 133.6±32        | 155.9±28                       | 119.3±23        |
| Solicitation frequency              |                            |                 |                                |                 |                            |                 |                                |                 |
| First ejaculatory series            | 5.7±1.63                   | 3.6±.72         | 3.2±.68                        | 5.7±1.2         | 3.4±.92                    | 3.2±.80         | 2.08±.52*                      | 5±1.1           |
| 30 min of test                      | 13.6±2.4*                  | 9.3±1.7         | 10.4±1.8*                      | 17.5±2.8        | 14.1±2.5                   | 9.8±1.6         | 13.7±2.3*                      | 21±2.6          |
| 1st mount latency                   | 236.8±55                   | 178.0±44        | 450±122                        | 211.4±97        | 128.2±33                   | 188.1±75        | 373.4±152                      | 108±24          |
| Mount frequency                     |                            |                 |                                |                 |                            |                 |                                |                 |
| First ejaculatory series            | 4.3±1.2                    | 1.7±.50         | 2.5±.78                        | 3.5±1.0         | 2.3±.84                    | 2.4±.85         | 1.5±.33                        | 2.5±.54         |
| 30 min of test                      | 9.5±1.7                    | 7.1±1.6         | 7.5±1.3                        | 9.0±1.7         | 11±1.5                     | 8.5±2.2         | 9.2±1.6                        | 8.6±1.3         |
| 1st intromission latency            | 193.7±44                   | 217.6±85        | 274.8±83                       | 187.3±72        | 136.3±40                   | 57.3±16         | 94.7±18.6                      | 94.1±16         |
| Intromission frequency              |                            |                 |                                |                 |                            |                 |                                |                 |
| First ejaculatory series            | 4.1±.65                    | 2.3±.55         | 2.9±.65                        | 3.4±.56         | 3.7±1.08                   | 5.5±1.1         | 3.5±.59                        | 4.2±.82         |
| 30 min of test                      | 8.5±1.1                    | 6.3±1.2         | 7.9±1.1                        | 6.8±1.0         | 18.2±1.6                   | 14.9±3          | 16±2.1                         | 14.2±1.4        |
| 1st ejaculation latency             | 592.0±84                   | 618.0±103       | 575.0±96                       | 485.0±99        | 429±93                     | 230±54          | 564±85*                        | 315±34          |
| Ejaculation frequency               |                            |                 |                                |                 |                            |                 |                                |                 |
| 30 min of test                      | 1.3±.21                    | 1.2±.23         | 1.3±.22                        | 1.1±.23         | 2.4±.42                    | 2.4±.45         | 1.7±.27                        | 3.08±.25        |
| Choice of male for                  |                            |                 |                                |                 |                            |                 |                                |                 |
| First ejaculation (n)               | 83% (20)*                  | 16% (4)         | 50% (12)                       | 50% (12)        | 30% (3)                    | 70% (7)         | 8.3% (1)*                      | 91.6% (11)      |
| Second ejaculation (n)              | 60.8% (14)                 | 39.2% (9)       | 56.5% (13)                     | 43.5% (10)      | 40% (4)                    | 60% (6)         | 16.6% (2)*                     | 83.3% (10)      |
| Third ejaculation (n)               | 25% (4)*                   | 75% (12)        | 63% (12)                       | 37% (7)         | 33.3% (3)                  | 66.6% (6)       | 20% (2)*                       | 80% (8)         |
| Lordosis magnitude 1                |                            |                 |                                |                 |                            |                 |                                |                 |
| First ejaculatory series            | 1.3±.38                    | 1.03±.25        | 1.0±.33                        | 1.6±.54         | .3±.15                     | .1±.1           | .16±.11                        | .08±.08         |
| 30 min of test                      | 3.7±.68                    | 4.5±1.0         | 3.8±.84                        | 4.4±1.0         | .5±.30                     | .6±.33          | 1.9±.63                        | .66±.22         |
| Lordosis magnitude 2                |                            |                 |                                |                 |                            |                 |                                |                 |
| First ejaculatory series            | 2.7±.94                    | 1.1±.28         | 1.3±.46                        | 1.6±.58         | 2.5±1.3                    | 5±1.05          | 2.6±.51                        | 4±.69           |
| 30 min of test                      | 5.3±1.1                    | 3.6±.84         | 3.6±.78                        | 4.1±1.0         | 15±3                       | 16.7±3.9        | 14.8±1.9                       | 13.3±1.6        |
| Lordosis magnitude 3                |                            |                 |                                |                 |                            |                 |                                |                 |
| First ejaculatory series            | 4.1±.81*                   | 1.8±.50         | 3.0±.72                        | 3.7±.71         | 2.6±.89                    | 2.7±.65         | 2.08±.46                       | 2.08±.71        |
| 30 min of test                      | 10.2±1.6                   | 6.1±1.2         | 8.8±1.3                        | 8.2±1.5         | 14.3±2.2*                  | 7.6±2           | 9.2±1.7                        | 10.3±1.9        |

In Wistar-pacing groups, females were trained to associate Wistar males with paced copulation and Long-Evans males with nonpaced copulation. In Long-Evans-pacing groups, females received the opposite association. \* $p < 0.05$  within groups.

### 3.1.4. Choice of male for ejaculation and male responses

Chi-square analyses for proportions of ejaculations indicated significant differences between males (Fig. 2). With regard to the choice of male to receive the first ejaculation, 20 (83%) of the 24 females in the W-P group that received an ejaculation received their first from the W male and 4 (16%) from the LE male [ $\chi^2(1, N=24)=10.6$ ,  $p=0.001$ ]. However, in the LE-P group 12 (50%) of 24 females that received an ejaculation received their first from the W male and 12 (50%) from the LE male [ $\chi^2(1, N=24)=0.0001$ ,  $p=1.0$ ]. For the choice of male to receive the second ejaculation, in the W-P group, 14 (60.8%) of 23 females received the second ejaculation from the W male and

only 9 (39.2%) from the LE male [ $\chi^2(1, N=23)=1.08$ ,  $p=0.29$ ]. In the LE-P group 13 (56.5%) of 23 females received the second ejaculation from the W male and 10 (43.5%) from the LE male [ $\chi^2(1, N=23)=0.39$ ,  $p=0.53$ ]. For the choice of male to receive a third ejaculation, in the W-P group, 4 (25%) of 16 females, received a third ejaculation from the W male and 12 (75%) from the LE male [ $\chi^2(1, N=16)=4$ ,  $p=0.04$ ]. In the LE-P group, 12 (63%) of 19 females received their ejaculation from the W male and 7 (37%) from the LE male [ $\chi^2(1, N=19)=1.3$ ,  $p=0.25$ ].

No significant main effects or interactions were found for the number of mounts, intromissions, or ejaculations, received by the females from the W or LE males, nor any main effects or

interactions for the mount, intromission, or ejaculation latencies during the first ejaculatory series. These results indicate that females allowed both strains of male to initiate copulation at comparable times, and that they received a comparable number of ejaculations from each male.

### 3.2. Long-Evans females

#### 3.2.1. Visits and olfactory investigations

The analysis of visit frequencies during the 30-min test revealed a significant main effect of group [ $F(1,20)=8.05$ ,  $p=0.01$ ]. The post hoc test indicated that females in the LE-P group visited both males with a higher frequency overall than did females in the W-P group. With regard to the first visit latency, there was a trend for a main effect of group [ $F(1,20)=3.9$ ,  $p=0.059$ ], but no trends were detected for strain of male or the interaction of group and strain of male. With regard to number of visits during the first ejaculatory series, the ANOVA failed to detect any main effect or interaction. No significant main effects or interactions were found for the analysis of

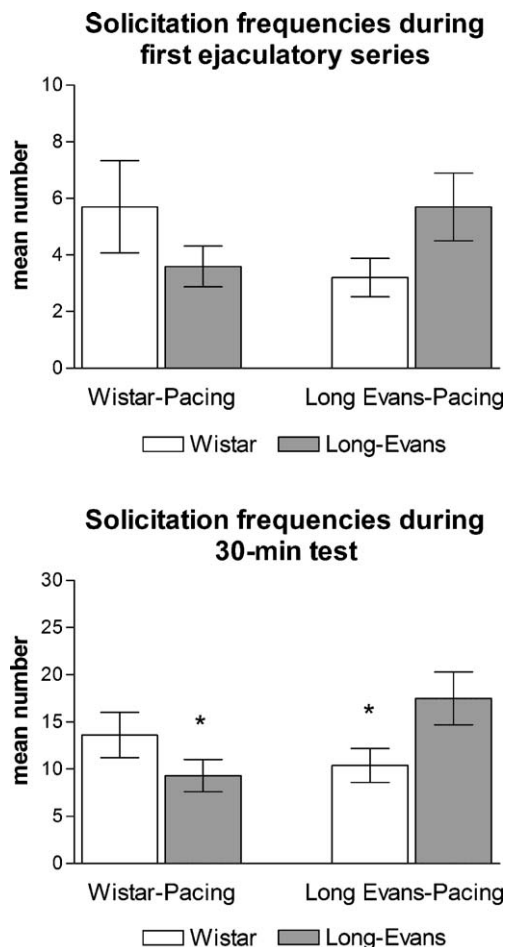


Fig. 1. Solicitation frequencies of Wistar females during first ejaculatory series (top) and during the 30-min partner-preference test (bottom). In the Wistar-pacing group females were conditioned to associate Wistar males with paced copulation, and Long-Evans males with nonpaced copulation. In the Long-Evans-pacing group, females received the opposite associations. Data are means  $\pm$  S.E.M. \* $p < 0.05$  for differences within group for Wistar and Long-Evans males.

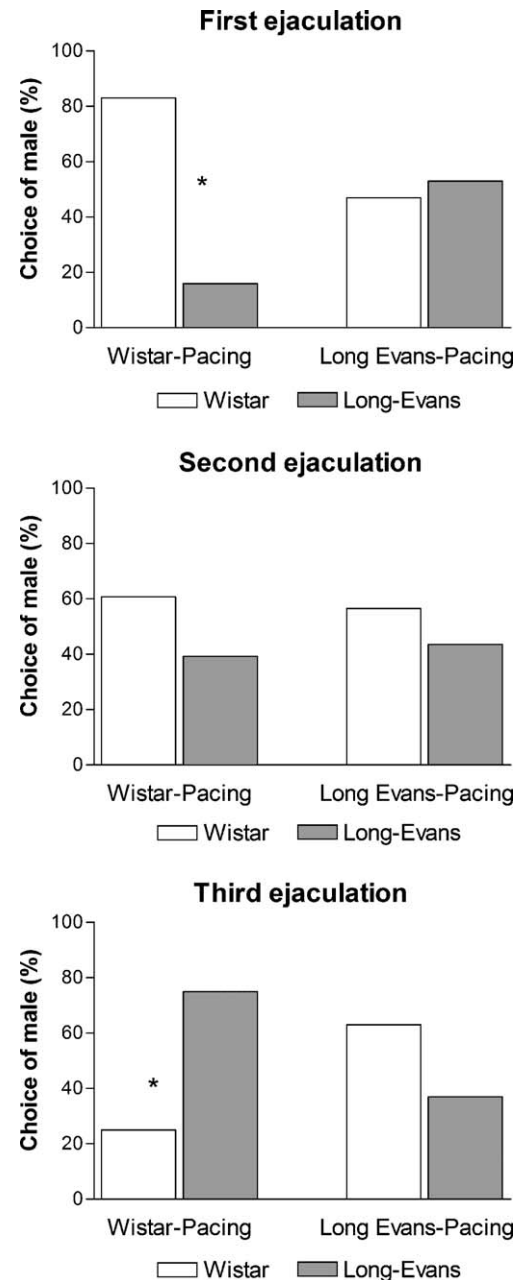


Fig. 2. Wistar females' choice of male to receive their first (top), second (middle), and third (bottom) ejaculation during the 30-min partner-preference test. \* $p < 0.05$  for differences within groups for Wistar and Long-Evans males.

olfactory investigations. This suggests that females investigated both strains of male equally.

#### 3.2.2. Appetitive sexual behaviors

With regard to the number of solicitations during the first ejaculatory series (Fig. 3), the ANOVA detected an interaction between group and strain of male [ $F(1,20)=5.9$ ,  $p=0.02$ ]. Post hoc analyses indicated that in the LE-P group, females displayed a higher number of solicitations towards LE males ( $M=5$ ) than towards W males ( $M=2.08$ ), but no differences between W and LE males were detected in the W-P group. The analysis of solicitations frequency during the 30-min test

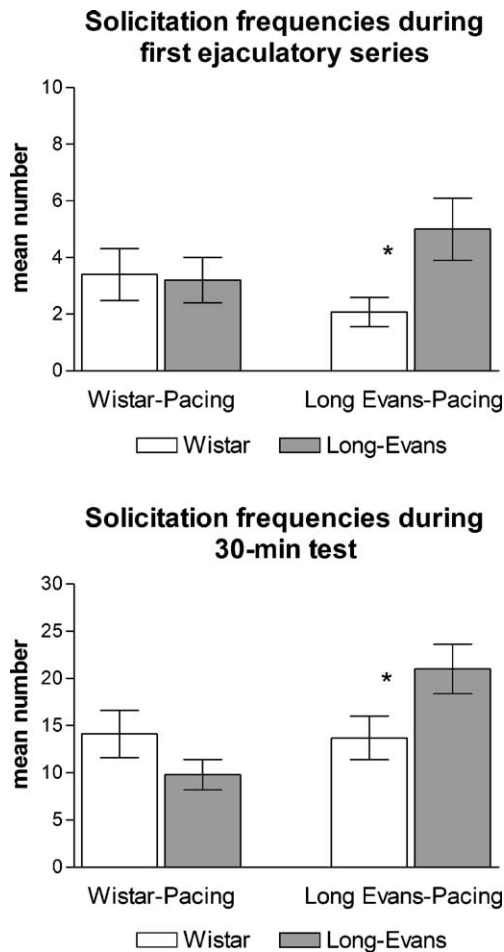


Fig. 3. Solicitation frequencies of Long-Evans females during first ejaculatory series (top) and during the 30-min partner-preference test (bottom). Data are means  $\pm$  S.E.M. \* $p < 0.05$  for differences within group for Wistar and Long-Evans males.

detected a main effect of group [ $F(1,20)=4.4$ ,  $p=0.04$ ] and an interaction between group and strain of male [ $F(1,20)=7.7$ ,  $p=0.01$ ]. Post hoc analyses indicated that females displayed more solicitations towards LE males in the LE-P group ( $M=21$ ) than towards W males ( $M=13.7$ ) of the same group, and also more than W ( $M=3.4$ ) and LE ( $M=3.2$ ) males in the W-P group. This indicates that, overall, females displayed more solicitations towards the pacing-related males, but this effect was facilitated significantly if the pacing males was LE. No main effects or interaction were found for first solicitation latency, nor were significant effects found for H&D frequencies or latencies.

### 3.2.3. Lordosis

The ANOVA detected a main effect of strain of male [ $F(1,20)=5.3$ ,  $p=0.03$ ], and an interaction between group and strain of male [ $F(1,20)=10.3$ ,  $p=0.004$ ] for the frequency of lordosis magnitude 3 for the entire 30-min test. Post hoc analyses indicated that in the W-P group, females displayed higher numbers towards W males ( $M=14.3$ ) than towards LE males ( $M=7.6$ ). In the LE-P group, no differences were detected in the frequency of lordosis magnitudes

1, 2, or 3. No differences were detected for the frequency of lordosis magnitudes during the first ejaculatory series.

### 3.2.4. Choice of male for ejaculation and male responses

Chi-square analyses for proportions of ejaculations indicated significant differences between males (Fig. 4). Regarding the choice of male to receive the first ejaculation, 11 (91.6%) of the 12 females in the LE-P group that received an ejaculation received their first from the LE male and 1 (8.3%) from the W male [ $\chi^2(1, N=12)=8.3$ ,  $p=0.04$ ]. In the W-P group, the choice of male was not significantly different from chance [ $\chi^2(1, N=10)=1.6$ ,  $p=0.20$ ]. With regard to the choice of male to receive the second

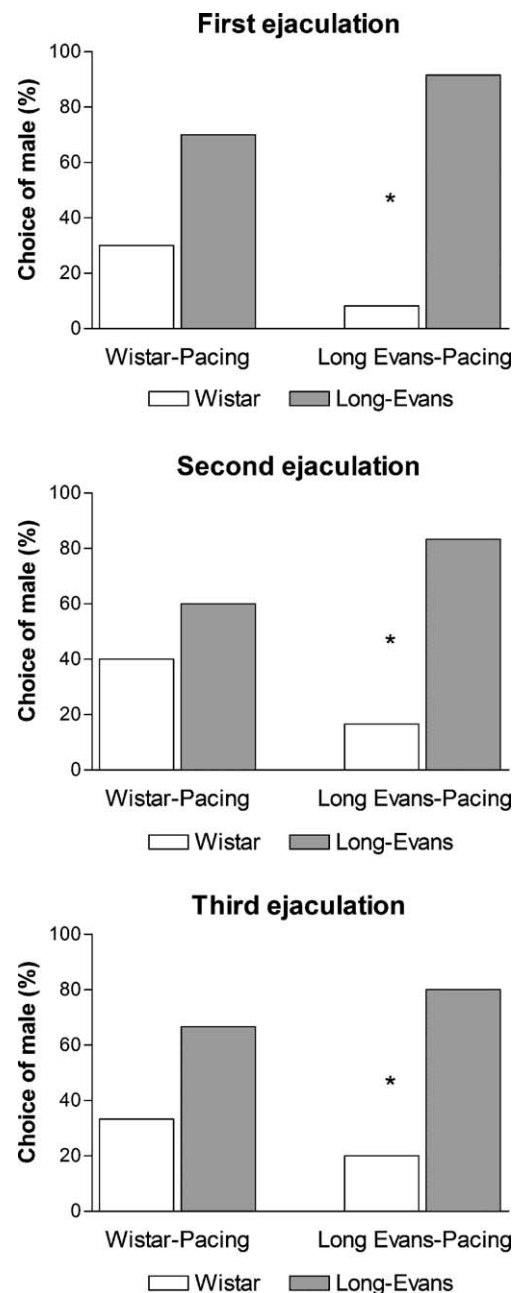


Fig. 4. Long-Evans females' choice of male to receive their first (top), second (middle), and third (bottom) ejaculation during the 30-min partner-preference test. \* $p < 0.05$  for differences within groups for Wistar and Long-Evans males.

ejaculation, 10 (83.3%) of 12 females in the LE-P group received their second ejaculation from the LE male, and 2 (16.6%) from the W male ( $\chi^2(1, N=12)=5.3, p=0.02$ ). In the W-P group the proportion was not different from chance [ $\chi^2(1, N=12)=0.4, p=0.52$ ]. For the choice of male to receive the third ejaculation, 8 (80%) of 10 females in the LE-P group receive their third ejaculation from the LE male, and 2 (20%) from the W male [ $\chi^2(1, N=10)=5.3, p=0.02$ ]. In the W-P group the proportion of ejaculations from either male was not different from chance [ $\chi^2(1, N=10)=1, p=0.3$ ]. Overall, the analysis of ejaculations indicated that in the LE-P group, most of the females chose LE males to receive the first three ejaculations. In the W-P group, however, there was no preference for either male.

No significant main effects or interactions were found for the number of mounts, intromissions, or ejaculations, received by the females from the W or LE males, nor any main effects or interactions for the mount, intromission, or ejaculation latencies during the first ejaculatory series. These results indicate that, like the W females, the LE females allowed both strains of male to initiate copulation at comparable times, and that they received a comparable number of ejaculations from each male.

#### 4. Discussion

The present study demonstrates that W and LE female rats can develop a conditioned partner preference for a strain of male previously associated with paced copulation. This preference was expressed most strongly and consistently by an increase in solicitations toward the pacing-related male, regardless of strain. W, but not LE, females also displayed a higher frequency of lordosis magnitude 3 toward the pacing-related male. Thus, natural cues of different strains of male can acquire learned significance as predictors of sexual reward for female rats. However, this study also showed that the conditioned partner preference was more robust when the pacing-related male was of the same strain as the female. This facilitation of the partner preference was evident in both appetitive and consummatory behaviors, and in particular with the choice of male for first ejaculation. This suggests that both W and LE females have an unconditioned preference for males of the same strain that can be enhanced by an association with paced copulation. However, when the cues of the dissimilar male were paired with pacing, both W and LE females altered their preference with regard to solicitation. These results suggest that solicitations (and possibly also hops and darts) are the most sensitive appetitive measures of female sexual motivation and choice, and reflect the reward characteristics of paced copulation.

The choice of male for the second and third ejaculation indicated a difference in copulatory strategies between W and LE females. Although W females displayed a significant preference for the pacing-related male of the same strain for their first ejaculation, this preference was not displayed for second ejaculation, and for the third ejaculation, the preference was switched for the originally nonpreferred male. This phenomenon resembles a Coolidge effect, in which animals switch their preference from a familiar partner to a novel one. In this case, the preferred male was chosen for the first ejaculation and the non-preferred male for the third ejaculation. However, a Coolidge effect implies increased

sexual desire for a novel partner relative to a familiar one, and females continued to solicit the pacing-related male more throughout the 30-min test, indicating a willingness to copulate more with that male. In contrast, LE females did not show any shift in preference of male for the second or third ejaculation. This preference, however, was not evident when LE males were associated with nonpaced copulation, indicating further that innate preferences can be devaluated or bypassed by the development of a conditioned preference.

In our previous study using an almond odor paired with paced copulation [9], LE females in the paired group displayed significant preferences for scented LE males over unscented LE males in the same open field test, including increased solicitations, hops and darts, higher magnitude lordoses, and also a clear mate preference in obtaining their first ejaculation from the scented male. Taken together with the present study, these results suggest that neutral cues such as almond odor paired with sexual reward, in the presence of naturally preferred cues from a male of the female's own strain, induce a robust conditioning that is reflected in appetitive behaviors such as solicitations and hops and darts, consummatory behaviors such as high magnitude lordoses, and in the choice to receive that male's ejaculations. Interestingly, although the LE females in that study chose the scented male for their first ejaculation, they switched males for their second and third ejaculations, similar to the W females in the present study. It may be that when strain cues of the males are held constant for LE females, the odor has a greater conditioned effect on solicitations relative to the choice of male for ejaculations.

This study also shows that the relationship and expression of innate and conditioned partner preferences are of different magnitude in W and LE females. Regarding the distribution of ejaculations during the 30-min test, W females expressed a choice for the same strain of male only for the first ejaculation, whereas LE females expressed it for three ejaculations. In the case of solicitations, W females clearly displayed a higher frequency of solicitations for the pacing-related males, regardless of the strain of male. However, in the case of LE females, the significance was reached exclusively when the pacing-related male was LE. Interestingly, the mean number of solicitations made toward W males during the 30-min test was nearly identical for W and LE females in the W-P condition, and the lower mean number of solicitations made toward the LE males was also nearly identical in this conditioning group (Figs. 1 and 3). It is likely that the lower number of LE relative to W females contributed to the lack of significance on this measure. Therefore, we suggest that this difference may be more apparent than real. However, taken together, those data suggest that the synergy between innate and conditioned preferences was stronger in LE females than in W females.

One possible explanation for the magnitude differences in conditioning between W and LE females concerns the inherent differences between the two strains with regard to their sensory abilities. Females might have distinguished the males visually, although differences in natural odors or inherent vocalizations by each strain of male cannot be ruled out. Albino rats have diminished visual acuity [38], but no auditory disadvantage [42]



relative to pigmented rats. If visual differences between W and LE males were the most salient cues, conditioning may have been weaker in W females relative to LE females. Other physiological differences between albino and pigmented rats could account for differences in sensitivity to the partner cues. For example, it has been shown that pigmented male rats exhibit noncontact erections in response to remote odor cues from sexually receptive females [41,40], whereas albino males do not [39] unless treated with the serotonin synthesis inhibitor *p*-chlorophenylalanine [25]. Those data indicate that albino rats have higher circulating serotonin levels compared to pigmented rats, and it is conceivable that high serotonin transmission in the hypothalamus or limbic system may lead to greater sexual inhibition [36]. Albino and pigmented females may also be differentially affected by the environmental circumstances that allow them to pace copulatory contact. In a previous pilot study [8], we reported that Wistar females under the same conditioning protocol, but tested in a smaller, three-compartmented chamber (rather than a large open field), received more mounts, intromissions, and ejaculations from the pacing-related males, regardless of strain. The ability to express these additional copulatory choices may depend on certain features of the testing chamber that allow females greater control over the copulatory responses of the males, in addition to the strength of the conditioned cue (e.g., olfactory vs. visual).

The present results add to a growing body of evidence that paced copulation induces both conditioned place and partner preferences in female rats [9,24,31,33]. At present, it is not clear what component of paced copulation produces the reward necessary to support these conditioned associations. The positive effect of paced copulation may depend on a cascade of events that start with the ability of the female to control the rate of vaginocervical stimulation (VCS) she receives from male intromissions. In female rats, pacing serves a significant reproductive purpose, allowing females to control VCS at their preferred intervals. Such preferred intervals may not only be species-specific, but individual-specific, and serve to distribute VCSs in time, which in sequence enhances both sperm transport and the induction of prolactin surges that support the maintenance of progesterone release by the corpora lutea [15,26,28]. Accordingly, paced copulation is more efficient than nonpaced copulation for inducing luteal function, a faster termination of estrus, and pregnancy or pseudopregnancy [5,15,23], and increases litter size compared to nonpaced copulation [7].

Finally, the present study confirms previous findings that early experiences, in particular early sexual experiences, have powerful influences on subsequent sexual preferences [9,21,20,19,35]. Such influences may begin when animals are young and learn to identify their own strain. For example, early postnatal experiences induce social preferences for an animal's own strain and lead to assortative mating [12], in which animals choose to mate selectively with members of their own strain relative to members of a different strain of a species that are genetically less similar. Assortative mating is believed to maintain homozygosity in a strain and thereby keep strains from outbreeding positive characteristics. However, cross-fostering studies have revealed a high degree of plasticity in this phenomenon. For example, mice raised by rat mothers

engage in more sexual contact with rats and less with mice, whereas the opposite occurs in mice raised by mouse mothers [22]. Early postnatal experience of male rats with a neutral odor (citral) smeared on the teats and anogenital area of female rat mothers increased sexual arousal in those males as adults: more paired males ejaculated with citral-scented estrous females compared to paired males that copulated with unscented females [16]. Taken together with our previous studies, the current results suggest that an animal's initial sexual experiences represent a second "critical period" in which place and strain characteristics become preferred, and in which the effects of early postnatal experience can be modified. These sexual preferences depend strongly on the contingency between rewarding or aversive unconditioned stimuli and the cues available during those initial sexual experiences. In the case of females, it is not yet known how the spinal cord and brain integrate the VCS during paced copulation with the available sensory cues to induce place or partner preferences. However, both are disrupted by the administration of the opioid antagonist naloxone during conditioning [11,32], suggesting the involvement of endogenous opioids as a primary substrate of sexual reward in both males and females.

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## NEURONAL ACTIVATION BY STIMULI THAT PREDICT SEXUAL REWARD IN FEMALE RATS

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**Abstract**—Conditioned stimuli (CSs) associated with paced copulation induce a conditioned partner preference for males bearing the CS. Here we examined the activation of Fos immunoreactivity (Fos-IR) following exposure to a CS previously paired with either paced or nonpaced copulation. Ovariectomized, hormone-primed rats received 10 sequential conditioning trials at 4-day intervals. In experiment 1, females in the odor-paired group learned to associate an almond odor on a male with paced copulation and an unscented male with nonpaced copulation. In the odor-unpaired group, females received the opposite association. In experiment 2, females associated two different strains of male, Long-Evans or Wistar, with paced or nonpaced copulation, respectively. A preference test indicated that females in both experiments developed a conditioned preference for the pacing-related males, as indicated by significantly more solicitations toward the male and a preference to copulate with the pacing-related male. Subsequently, females were exposed to the CS (odor or strain) alone for 1 h prior to kill and preparation of their brains for immunocytochemistry. In both experiments, the CS associated with paced copulation produced significantly more Fos-IR in the piriform cortex, medial preoptic area, and ventral tegmental area, relative to the same odor or strain cues associated with nonpaced copulation. These findings provide evidence that the state associated with paced copulation can be conditioned to environmental stimuli such as neutral odors or strain cues, which earn an incentive value via classical conditioning. The significance of the brain areas activated is discussed with regard to their role in sexual and other motivated behaviors. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** Fos, partner preference, pavlovian conditioning, odor, strain, reward.

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**Abbreviations:** ACC, anterior cingulate cortex; aLHA, anterior lateral hypothalamic area; ANOVA, analysis of variance; ArcN, arcuate nucleus; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; BNSTpm, posteromedial region of the bed nucleus of the stria terminalis; CoA, corticomedial amygdala; CPu, caudate-putamen; CS, conditioned stimulus; DAB, 3,3'-diaminobenzidine; IR, immunoreactivity; LE, Long-Evans; LS, lateral septum; MeApd, posteriodorsal region of the medial amygdala; mPOA, medial preoptic area; NAc, nucleus accumbens; NAcc, nucleus accumbens core; NAcSh, nucleus accumbens shell; NGS, normal goat serum; OVX, ovariectomized; PirCtx, piriform cortex; PVN, paraventricular nucleus of the hypothalamus; TBS, Tris-buffered saline; Tu, olfactory tubercle; UCS, unconditioned stimulus; VCS, vaginocervical stimulation; VMH, ventromedial hypothalamus; VMHvl, ventrolateral region of the ventromedial hypothalamus; VTA, ventral tegmental area; W, Wistar.

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Learning can affect many aspects of sexual behavior in animals (see Pfaus et al., 2001, for review). In rodents, for example, these include changes in the expression of sexual excitement (Mendelson and Pfaus, 1989; Pfaus, 1996, 1999), in the capacity to locate and recognize a place associated with mating (Everitt, 1990), in courtship behaviors and mate-directed vocalizations (Nyby et al., 1983), in copulatory parameters (Larsson, 1956; Dewsbury, 1969), and in partner preference (Kippin and Pfaus, 2001b; Coria-Avila et al., 2005, 2006).

The effects of learning on conditioned partner preference can be observed when an individual has the opportunity to choose between two or more potential partners. Typically, the conditioned preference is displayed as more time spent with, or more directed sexual behavior toward, an individual that bears recognizable features associated with sexual reward. For instance, olfactory cues (e.g. almond odor) paired with the reward state induced by ejaculation in males can induce males to ejaculate preferentially with almond-scented females when males are given a choice to copulate freely with scented and unscented, sexually receptive females in an open field (Kippin et al., 2001; Kippin and Pfaus, 2001a,b). In the case of females, almond odor paired with their ability to “pace” or control the rate of copulation induces females to solicit and receive ejaculations selectively from an almond-scented male when they are given a choice to copulate freely with scented and unscented, sexually vigorous males in an open field. This preference is not displayed if the odor is paired with nonpaced copulation (Coria-Avila et al., 2005). Similarly, female rats can learn to associate a strain of male (pigmented Long-Evans (LE) versus albino Wistar (W) rats) with pacing, and display a conditioned preference to solicit males of the pacing-related strain when given a free choice between the two different strains in an open field (Coria-Avila et al., 2006).

Like ejaculation in males, paced copulation induces a reward state of sufficient intensity to induce conditioning in females (Paredes and Alonso, 1997; Paredes and Vazquez, 1999). The rewarding effects of paced copulation are believed to be the result of the appropriate vaginocervical stimulation (VCS) distributed over time at the female's preferred interval. This is supported by the fact that female rats will develop conditioned place preference when they were actively pacing, but also when the males are removed by the researcher from the copulatory arena at the female's preferred interval (Jenkins and Becker, 2003) or when females are given manual VCS with a lubricated glass rod distributed in clusters at preferred intervals (Afonso and Pfaus, submitted for publication).

Brain regions sensitive to sexual stimulation have been identified using Fos immunoreactivity (IR) as a marker of neuronal activation in females (Pfaus and Heeb, 1997). A number of hypothalamic and limbic structures are sensitive to VCS, either from multiple intromissions by the male or following manual application with a lubricated glass rod (Pfaus et al., 1993, 1996, 2006). These regions include the piriform cortex (PirCtx), anterior cingulate cortex (ACC), nucleus accumbens (NAc), lateral septum (LS), medial nucleus of the preoptic area (mPOA), posteromedial region of the bed nucleus of the stria terminalis (BNSTpm), the paraventricular nucleus of the hypothalamus (PVN), ventrolateral portion of the ventromedial hypothalamus (VMHvl), the arcuate nucleus (ArcN), posteriodorsal region of the medial amygdala (MeApd), and the ventral tegmental area (VTA). Females that receive intromissions express a higher number of Fos-labeled cells in these regions compared with females that receive mounts only, indicating that the expression of Fos in these areas during copulation is not due to olfactory input from the male or cutaneous somatosensory input received from flank stimulation during mounts only (Erskine, 1993). However, Erskine and Hanrahan (1997) found that when females were allowed to pace either 5 or 15 intromissions, there was more Fos-IR in the MeApd relative to females that received 5 or 15 non-paced intromissions. In addition, females that received 5 paced intromissions had as much Fos-IR as those that receive 15 nonpaced intromissions, indicating that the MeApd is particularly sensitive to the type and frequency of VCS. These differences were not detected in the mPOA. Furthermore, they reported that the BNSTpm and the VMHvl expressed more Fos-positive neurons following paced copulation relative to females that received mounts alone, or to those that remained in their home cages. Such differences were not observed following nonpaced copulation.

Kippin et al. (2003) trained males to associate an almond odor on females with the postejaculatory reward state, or to have the almond odor unpaired with that state. Subsequently, males in the paired and unpaired groups were presented with the almond odor alone in bedding. The odor activated Fos selectively in the PirCtx, nucleus accumbens core (NAcc), basolateral amygdala (BLA), and the anterior region of the lateral hypothalamic area, in paired relative to unpaired males. Conversely, estrous odors alone in bedding activated Fos within the accessory olfactory bulb, NAcc and nucleus accumbens shell (NAcSh), mPOA, ventromedial hypothalamus (VMH), MeApd, and VTA of males in either the paired or unpaired groups, indicating that the conditioned odor activated main olfactory terminals whereas unconditionally appetitive estrous odors activated accessory olfactory terminals. Given that a neutral odor such as almond, or the particular strain of a male, can gain conditioned incentive value by being associated with paced copulation, and thus direct the copulatory preferences of female rats, this study examined the pattern of Fos induction following exposure to a conditioned odor alone, or a pacing-related male behind a wire-

mesh screen, that had previously been associated with the ability to pace copulation.

## EXPERIMENTAL PROCEDURES

### Animals and surgery

LE and W male (300–350 g) and female (200–250 g) rats were purchased from Charles River Canada (St-Constant, QC, Canada). They were housed in groups of four in large semi-enriched plastic gang cages in a colony room maintained on a reversed 12-h light/dark cycle (lights off at 08:00 h) at approximately 21 °C. Food and water were continuously available. All animal procedures were approved by the Concordia University Animal Research Ethics Committee in compliance with the guidelines of the Canadian Council on Animal Care. Every effort was made to minimize the number of animals used and their suffering.

Females were anesthetized with a mixture of ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (4 mg/ml), mixed at a ratio of 4:3, respectively, and injected intraperitoneally in a volume of 1 ml/kg of body weight. Females were then ovariectomized (OVX) bilaterally via a lumbar incision. All females were given a week of post-surgical recovery prior to odor or strain conditioning trials. Gonadally intact LE or W male rats that served as stimulus males had at least 10 tests of sexual behavior in bi-level chambers prior to the start of these experiments to ensure comparable copulatory activity (Coria-Avila et al., 2004). These males were sexually vigorous and initiated copulatory activity with females within 15 s of being placed into the chambers. For all behavioral tests, sexual receptivity was induced in all females by s.c. injections of estradiol benzoate (10 µg) 48 h and progesterone (500 µg) 4 h before each test. If scented, males received almond extract (Blue Ribbon, Etobicoke, ON, Canada) applied to the back of their necks and anogenital regions with a cotton swab. If unscented, males received distilled water to the same areas.

### Odor conditioning

The training procedures were identical to those in Coria-Avila et al. (2005). Odor conditioning trials occurred at 4-day intervals during the middle third of the rats' dark circadian cycle following hormone priming. Paced copulation occurred in semicircular chambers (382×60×38 cm) bisected by a clear Plexiglas divider with four holes cut into the bottom (4×4 cm) that rested on bedding. These holes were large enough for the female to crawl through, but too small for the males to crawl through. Nonpaced copulation occurred in identical chambers, but with the divider removed. Females in the odor-paired group copulated with almond-scented males in the paced condition and unscented males in the non-paced condition. Females in the odor-unpaired group copulated with unscented males in the paced condition and scented males in the nonpaced condition. Females received nine sequential experiences in paced and non-paced conditions at 4-day intervals. The order of presentation of paced and non-paced conditions was counterbalanced for the odor-paired and odor-unpaired groups. During each test, females were allowed to copulate freely for 30 min with the male, after which they were transferred to their home cages. All testing with scented and unscented males took place in different rooms to assure containment of the scent.

### Strain conditioning

The training procedures were identical to those of Coria-Avila et al. (2006). Conditioning trials occurred at 4-day intervals during the middle third of the rat's dark circadian cycle following hormone priming. Paced copulation occurred in the same semicircular chambers as odor conditioning. There were two counterbalanced groups. In the W-pacing (W-P) group females copulated with W males in the paced condition and with LE males in the nonpaced

condition. In the LE-pacing (LE-P) group females copulated in pacing chambers with LE males and in non-pacing chambers with W males. Females received 10 sequential experiences in paced and non-paced conditions at 4-day intervals. The order of presentation of paced and non-paced conditions was counterbalanced for both groups, so that half of the group had the first sexual experience in a pacing condition and the other half in non-pacing condition. During each test, females were allowed to copulate for 30 min, after which they were transferred back to their home cages.

### Activation of Fos-IR by odor or strain cues

Each odor-conditioned female was placed in a Plexiglas chamber containing wood shavings as bedding and a cotton gauze pad saturated with almond odor for 60 min undisturbed. Each strain-conditioned female was paired with a male of the strain associated with either paced or nonpaced copulation. The pairing occurred in a chamber divided by a wire mesh screen that allowed olfactory, visual, and auditory cues to be detected, but not physical contact, between the male and the female (e.g. Pfaus et al., 1990). Females were exposed to the cues 4 days after their final conditioning trial. In all cases, the females were allowed to remain undisturbed in the chamber for 60 min before being injected with an overdose of sodium pentobarbital (120 mg/kg, i.p.) and perfused intracardially with 350 ml of phosphate-buffered saline followed by 350 ml of 4% paraformaldehyde. Brains were removed and post fixed in 4% paraformaldehyde for 4 h and stored overnight in 30% sucrose solution. Brains were then covered in aluminum foil and stored at  $-80^{\circ}\text{C}$  prior to sectioning.

### Fos immunocytochemistry

Coronal brain sections ( $30\ \mu\text{m}$ ) were taken from each brain from the region of the anterior NAC to the VTA, corresponding to plates 13–41 of Paxinos and Watson (1998), using a freezing microtome. Sections were incubated sequentially with 30% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in Tris-buffered saline (TBS) for 30 min at room temperature, with 3% normal goat serum (NGS) in 0.05% Triton TBS for 90 min at  $4^{\circ}\text{C}$ , with rabbit polyclonal anti-Fos (Oncogene Science, Boston, MA, USA; diluted 1:40,000) in 0.05% Triton TBS with 3% NGS for 72 h at  $4^{\circ}\text{C}$ , with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; 1:200) in 0.05% Triton TBS with 3% NGS for 1 h at  $4^{\circ}\text{C}$ , and avidin–biotinylated–peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories; diluted 1:55) for 2 h at  $4^{\circ}\text{C}$ . Sections were washed in TBS ( $3\times 5\ \text{min}$ ) between each incubation. Immunoreactions were stained by sequential treatments at room temperature with 50-mM Tris for 10 min, 3,3'-diaminobenzidine (DAB) in 50-mM Tris (0.1 ml of DAB/Tris buffer, pH 7.8) for 10 min, DAB/3%  $\text{H}_2\text{O}_2$  in 50-mM Tris for 10 min, and 8% nickel chloride (400  $\mu\text{l}$  per 100 ml of DAB/Tris buffer +  $\text{H}_2\text{O}_2$ ). Sections were mounted on gel-coated slides and allowed to dry, then dehydrated in alcohol 70%, 90% and 100% 10 min each respectively, cleared in Xylenes (2 h), coverslipped, and examined under a Zeiss light microscope.

### Histological and statistical analyses

Tissue sections were examined at  $40\times$  and the number of Fos-positive cells was counted bilaterally for each region from six sections per rat using NIH Image connected to a Leitz Laborlux microscope. The regions were defined by using the atlas of Paxinos and Watson (1998): NAcc (plates 13 and 14), NAcSh (plates 13 and 14), olfactory tubercle (Tu, plates 13 and 14), PirCtx (plates 13 and 14), ACC (plates 13 and 14), caudate-putamen (CPU; plates 13 and 14), mPOA (plates 20 and 21), LS (plates 13 and 14), BNSTpm (plates 21 and 22), PVN (plates 22–25), anterior lateral hypothalamic area (aLHA) (plates 23 and 24), VMHvl (plates 27 and 28), ArcN (plates 27–30), MeApd (plates 28 and

29), BLA (plates 28 and 29), corticomedial amygdala (CoA; plates 28 and 29), and VTA (plates 40 and 41). A mean was calculated for each area in each rat from five sections per area, and statistical analyses were conducted for rats in each group ( $N=5\text{--}6/\text{condition}$ ). For the odor conditioning, Student's *t*-test for independent samples was used to compare the mean number of Fos-positive neurons in the odor-paired vs. odor-unpaired conditions. The level of significance for all comparisons was  $P<0.05$ . For the strain conditioning, a two-way (pacing $\times$ strain of male) between-subjects analysis of variance (ANOVA) was performed to assess differences in Fos induction between females exposed randomly to four conditions: Fos-IR following exposure to 1) W male associated with paced copulation, 2) LE male associated with nonpaced copulation, 3) W male associated with nonpaced copulation, and 4) LE male associated with paced copulation ( $N=5\ \text{females/condition}$ ). For each significant ANOVA, post hoc analysis of mean differences were made using the least significant difference (LSD) method,  $P<0.05$ .

## RESULTS

### Brain activation by an odor paired with sexual reward

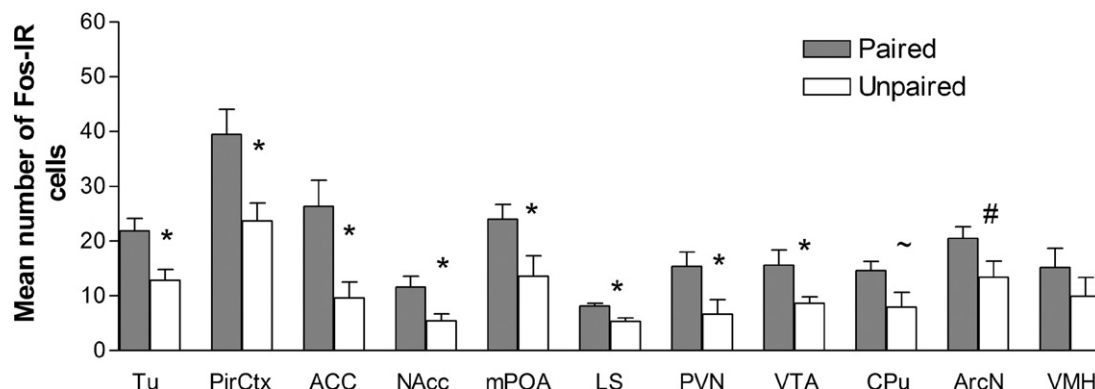
More Fos-positive neurons were found in the Tu, PirCtx, ACC, NAcc, mPOA, LS, PVN, VTA, CPu and ArcN of odor-paired relative to odor-unpaired females (Figs. 1, 4, and Table 1). For the MPOA, the statistical analysis revealed that females in the paired group there was more Fos-IR cells relative to the unpaired group,  $t(10)=2.22$ ,  $P<0.05$ . For the NAcc, more Fos-IR cells were found in the odor-paired relative to the odor-unpaired group,  $t(10)=2.5$ ,  $P<0.02$ . For the PirCtx, more Fos-IR cells were found in the odor-paired relative to the odor-unpaired group,  $t(10)=2.7$ ,  $P<0.02$ . For the ACC more Fos cells were observed in the odor-paired relative to the odor-unpaired group,  $t(10)=2.7$ ,  $P<0.02$ . For the PVN more Fos cells were observed in the odor-paired relative to the odor-unpaired group,  $t(9)=2.68$ ,  $P<0.02$ . For the VTA more Fos cells were found in the odor-paired relative to the odor-unpaired group,  $t(10)=2.2$ ,  $P=0.049$ . The statistical analysis detected a trend for significance in the analysis of the CPu, with more Fos-IR cells in the odor-paired relative to the odor-unpaired group,  $t(10)=2.04$ ,  $P=0.06$ . A trend toward significance was also detected for the ArcN, with more Fos-IR cells in the odor-paired relative to the odor-unpaired group,  $t(10)=1.9$ ,  $P=0.08$ . There were no significant differences in the NAcSh, MeApd, CoA, BLA, lateral hypothalamus, bed nucleus of the stria terminalis (BNST), and VMH (Table 1).

### Brain activation by strain cues associated with sexual reward

Regardless of strain, males associated with paced copulation induced greater Fos-IR in PirCtx, mPOA, VMH, and VTA (Figs. 2, 4, and Table 2). For the PirCtx, the statistical analysis revealed a main effect of pacing  $F(1, 16)=4.57$ ,  $P=0.04$ , but no main effect of strain of male or interaction. For the mPOA, the ANOVA revealed a main effect of pacing  $F(1, 16)=4.1$ ,  $P=0.05$ , but no main effect of strain or interaction. For the VMH there was a main effect of pacing  $F(1, 16)=4.1$ ,  $P=0.05$ , but no main effect of strain



### Neural Activation by Conditioned Odors



**Fig. 1.** Brain areas of female rats that expressed significant differences in Fos-IR following exposure to a conditioned odor alone. For females in the paired group, the odor (almond extract) was associated with paced copulation during previous conditioning trials. In the unpaired group, the odor was associated with nonpaced copulation. The bars represent the mean  $\pm$  S.E.M. number of Fos-IR cells within a sample area of the same dimensions for all the regions. \*  $P < 0.05$ ; ~  $P = 0.06$ ; #  $P < 0.08$ , between paired and unpaired groups.

or interaction. Finally, for the VTA there was a main effect of pacing  $F(1, 16) = 6.2$ ,  $P = 0.02$ , but no main effect of strain or interaction.

Exposure to LE males induced more Fos in the PVM, MeApd, CoA, aLH, and BNST relative to exposure to W males (Fig. 3 and Table 2). For the PVN the ANOVA confirmed a main effect of strain of male,  $F(1, 16) = 4.9$ ,  $P = 0.04$ , but no main effect of pacing or interaction. For the MeApd there was a main effect of strain of male,  $F(1,$

16) = 6.25,  $P = 0.02$ , and a trend for significant main effect of pacing  $F(1, 16) = 3.99$ ,  $P = 0.06$ , but no interaction. For the CoA there was a main effect of strain of male  $F(1, 16) = 4.6$ ,  $P = 0.04$ , but no effect of pacing or interaction. For the aLHA there was a main effect of strain of male  $F(1, 16) = 6.33$ ,  $P = 0.02$ , but no effect of pacing or interaction. For the BNST there was a main effect of strain of male  $F(1, 16) = 6.51$ ,  $P = 0.02$ , but no main effect of pacing or interaction.

Finally, no differences were detected between the groups in Fos induction in the ACC, NAcc, NAcSh, CPu, ArcN, and BLA.

**Table 1.** Brain areas of female rats that expressed Fos-IR following exposure to a conditioned odor alone

| Area              | Paired            | Unpaired        |
|-------------------|-------------------|-----------------|
| BLA               | 6.10 $\pm$ 0.60   | 4.90 $\pm$ 1.35 |
| CoA               | 26.8 $\pm$ 3.70   | 21.0 $\pm$ 6.79 |
| Amygdala (medial) | 17.6 $\pm$ 3.54   | 20.5 $\pm$ 4.07 |
| ArcN              | 20.5 $\pm$ 2.15#  | 13.4 $\pm$ 2.90 |
| BNST              | 7.09 $\pm$ 0.85   | 7.81 $\pm$ 3.91 |
| CPu               | 14.6 $\pm$ 1.71~  | 7.92 $\pm$ 2.78 |
| ACC               | 26.4 $\pm$ 4.75** | 9.60 $\pm$ 2.99 |
| aLHA              | 9.23 $\pm$ 2.33   | 6.25 $\pm$ 1.88 |
| LS                | 8.14 $\pm$ 0.55** | 5.35 $\pm$ 0.67 |
| mPOA              | 24.0 $\pm$ 2.72*  | 13.6 $\pm$ 3.78 |
| NAcc              | 11.6 $\pm$ 2.00** | 5.45 $\pm$ 1.26 |
| NAcSh             | 10.2 $\pm$ 1.63   | 7.60 $\pm$ 1.87 |
| Tu                | 21.8 $\pm$ 2.35** | 12.8 $\pm$ 2.04 |
| PVN               | 15.5 $\pm$ 2.66** | 7.3 $\pm$ 1.5   |
| PirCtx            | 39.4 $\pm$ 4.60** | 23.7 $\pm$ 3.27 |
| VTA               | 15.6 $\pm$ 2.80*  | 8.70 $\pm$ 1.1  |
| VMH               | 15.2 $\pm$ 3.51   | 9.92 $\pm$ 3.44 |

In the paired group, the odor (almond extract) was associated with paced copulation during previous conditioning trials. In the unpaired group, the odor was associated with nonpaced copulation. The data are expressed as means  $\pm$  S.E.M. The mean number of Fos-IR cells was taken from a sample area of the same dimensions for all the regions.

\*  $P < 0.05$ , paired versus unpaired.

\*\*  $P < 0.02$ , paired versus unpaired.

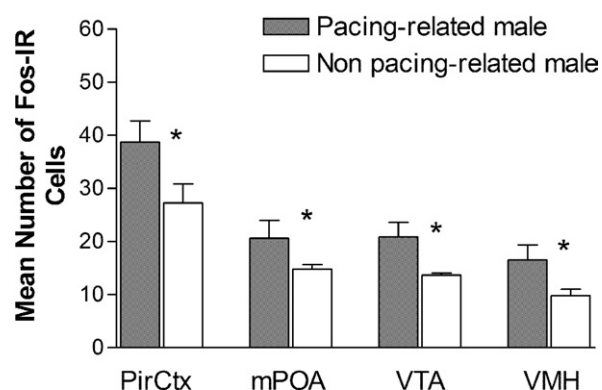
~  $P = 0.06$ , paired versus unpaired.

#  $P < 0.08$ , paired versus unpaired.

### DISCUSSION

We have reported previously that neutral odors or strain cues paired with paced copulation can become condi-

### Neural Activation by Pacing-related cues



**Fig. 2.** Brain areas of W female rats that expressed significant differences in Fos-IR following exposure to a strain of male associated with paced or nonpaced copulation, respectively. Regardless of the strain, males associated with paced copulation induced more Fos-IR than males associated with nonpaced copulation. The bars represent the mean  $\pm$  S.E.M. number of Fos-IR cells within a sample area of the same dimensions for all the regions. \*  $P < 0.05$ .

**Table 2.** Brain areas of female rats that expressed Fos-IR following exposure to a W or LE male, associated with paced or nonpaced copulation

| Area                | W-pacing  |            | LE-pacing |           |
|---------------------|-----------|------------|-----------|-----------|
|                     | W         | LE         | W         | LE        |
| BLA                 | 5.8±0.1   | 6.2±1.3    | 4.8±1.7   | 6.4±2     |
| CoA*                | 19.8±3.4  | 22.6±2.4   | 12.2±3.5  | 23.7±1.8  |
| Amygdala (medial)*  | 15.8±1.5  | 16.9±2.22  | 9.3±1.4   | 19.1±3.79 |
| ArcN                | 28.6±8    | 18.8±3.5   | 18.6±5.8  | 31±0.31   |
| BNST*               | 5.8±0.82  | 8.9±0.84   | 6.7±1.6   | 11.2±3.2  |
| CPu                 | 10.1±3    | 8.01±1.2   | 5.7±0.95  | 6.4±1.5   |
| ACC                 | 15±2.9    | 10.17±2.22 | 8.5±1.37  | 10.92±3.5 |
| aLHA*               | 9.2±1.8   | 14.7±2.2   | 7.4±0.96  | 12.2±2.1  |
| LS                  | 8.09±1    | 9.6±2.08   | 6.81±0.51 | 6.45±0.47 |
| mPOA <sup>#</sup>   | 18.7±3.9  | 15.3±1.2   | 14.2±1.4  | 24.5±6.8  |
| NAcc                | 13.7±3.9  | 7.77±0.87  | 4.9±0.65  | 7.4±0.7   |
| NAcSh               | 8.9±2.7   | 7.67±1.4   | 4.6±0.64  | 5.7±1.03  |
| Tu                  | 26.3±3.3  | 26.0±3.06  | 24.2±2.7  | 21.6±3.5  |
| PVN*                | 9.9±1.9   | 12.8±1.84  | 8.2±1.9   | 15.9±4.7  |
| PirCtx <sup>#</sup> | 40.6±5.7  | 34.8±5.3   | 20±4.8    | 35.1±4.3  |
| VTA <sup>#</sup>    | 21.7±4.1  | 14.2±0.43  | 13.2±0.57 | 19.2±1.19 |
| VMH <sup>#</sup>    | 17.8±4.15 | 11.68±1.92 | 7.6±0.99  | 13.9±2.9  |

In the W-pacing group, females were trained to associate a W male with paced copulation and an LE male with nonpaced copulation. In the LE-pacing group, females received the opposite training. Data are expressed as means±S.E.M. The mean number of Fos-IR cells was taken from a sample area of the same dimensions for all the regions.

\* Main effect of strain of male,  $P<0.05$ .

<sup>#</sup> Main effect of pacing,  $P<0.05$ .

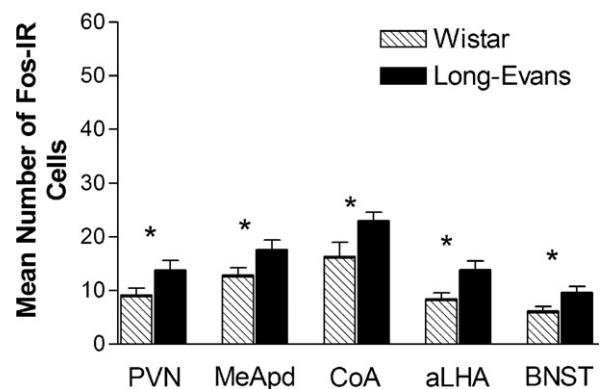
tioned stimuli (CSs) that predict sexual reward in female rats, and that lead to the development of a conditioned partner preference for males possessing those characteristics (Coria-Avila et al., 2005, 2006). The present study shows that exposure to the CS generates greater Fos-IR in brain areas associated with olfactory processing, appetitive motivation, and reward-related learning, in paired versus unpaired females. The almond odor associated with pacing induced more Fos-IR in the Tu, PirCtx, ACC, NAcc, mPOA, LS, PVN, and VTA, and there was a trend for significance in the CPu and ArcN. These data suggest that those areas are involved in the development and/or expression of olfactory-conditioned partner preference. The strain of male associated with paced copulation induced more Fos-IR in the PirCtx, mPOA, VTA and the VMH. These results suggest that there at least three common neural areas that express Fos-IR as a main effect of exposure to stimuli associated with pacing: PirCtx, mPOA, and the VTA. In addition to these regions, the activation of the VMH, and lack of activation in the NAc and LS, by strain cues associated with pacing is consistent with the Fos activation patterns in monogamous prairie vole females during their first copulatory contact with a male (Curtis and Wang, 2003; Cushing et al., 2003). Thus the differential effects of olfactory and strain conditioning noted above may result from a different pattern of activation in main and accessory olfactory pathways.

More Fos was induced in the BNST, PVN, MeApd, CoA, and aLHA of W females as a function of exposure to

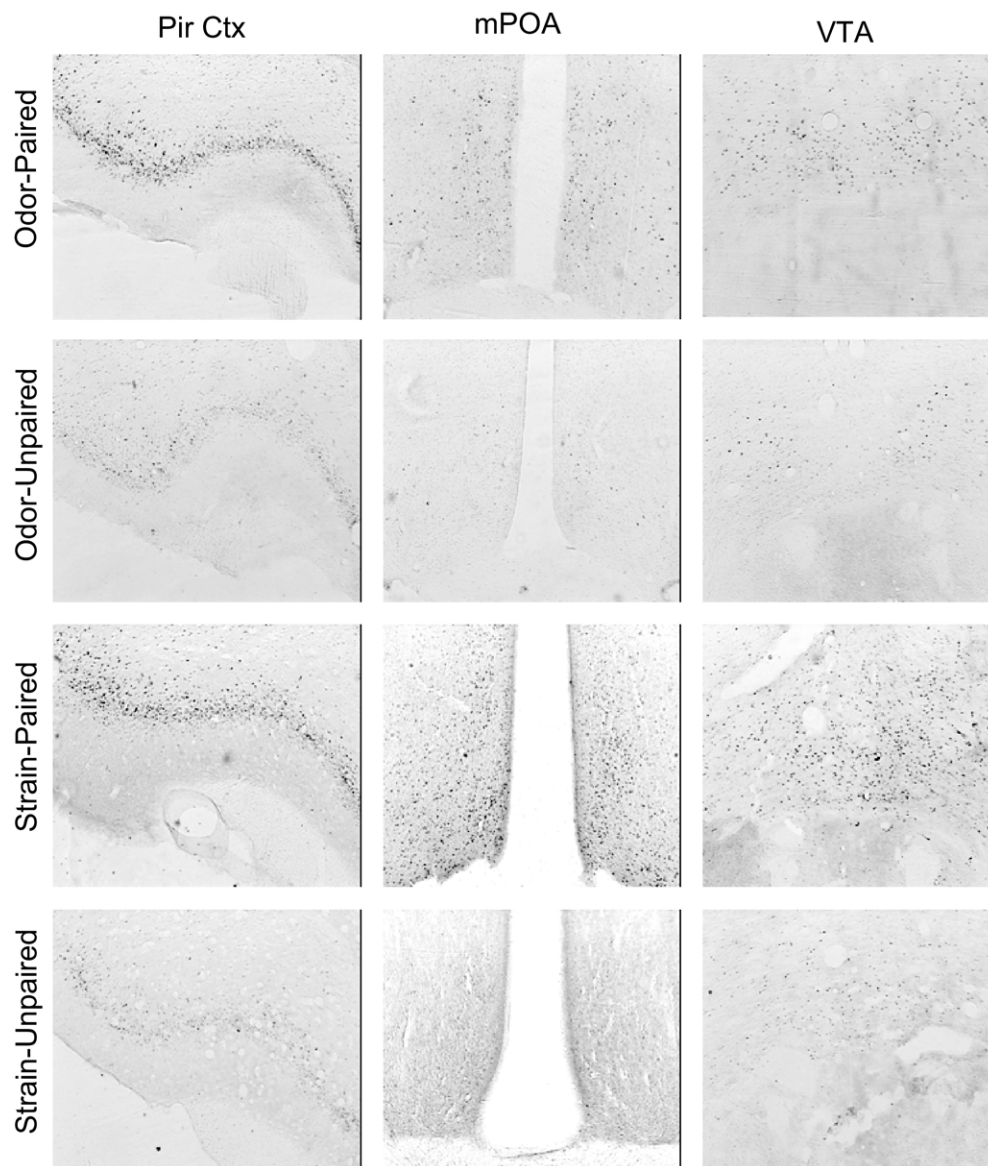
LE males overall relative to W males. We have shown previously that if the strain of male associated with sexual reward is the same as the female subject, stronger conditioning emerges (Coria-Avila et al., 2006). For example, W females that associate W males with paced copulation not only solicit the W male more than the LE male, but also are more likely to choose the W male to receive their first ejaculation. Although W females solicit the LE male significantly more than the W male if LE is paired with paced copulation, they do not display a significant ejaculatory preference toward the LE male. However, W females display significantly more anogenital investigation of LE males in general, and had more Fos in secondary and tertiary terminals of the accessory olfactory pathway, including the BNST and MeApd, by exposure to LE males regardless of conditioning history. Our use of extensive sexual training produced similar, if not identical, copulatory behavior between the two strains of male. No obvious differences in male behavior were noted by the observers or in the behavioral responses of the females, other than an increased anogenital investigation by W females of the LE males. It may be the case that LE males smell sufficiently different from W males, and that this or other differences led to the increased activation of Fos.

Both olfactory and strain cues associated with paced copulation induced similar activation of the PirCtx, mPOA, and VTA. These three regions form an interconnected circuit that is well-suited to translate olfactory and genito-sensory information into motivated behavioral responses by way of outputs through the VTA and ventral pallidum. All three regions are activated by VCS (Pfaus et al., 1993, 1996), and receive inputs from both main and accessory olfactory bulbs (Coolen and Wood, 1998; Rosin et al., 1999; Swanson, 1976; Wilson, 2001). The differential effect of olfactory versus strain conditioning may be determined in part by multiple outputs to the VTA, especially from the mPOA in the case of olfactory conditioning

### Neural Activation by Strain-related cues



**Fig. 3.** Brain areas of W female rats that expressed significant differences in Fos-IR following exposure to a W or LE male associated with paced or nonpaced copulation. Overall, exposure to LE males induced more Fos-IR than exposure to W males. MeA, medial amygdala. The bars represent the mean±S.E.M. number of Fos-IR cells within a sample area of the same dimensions for all the regions. \*  $P<0.05$ .



**Fig. 4.** Common brain areas that express significantly more Fos-IR in female rats exposed to conditioned odors or strains of male associated with paced (paired) or nonpaced (unpaired) copulation.

(Swanson, 1976; Brackett and Edwards, 1984), and additional outputs from the BNST that may modulate those of the mPOA in the case of strain conditioning (Holstege et al., 1985). Given the relatively equivalent activation of the PirCtx, mPOA, and VTA in the two experiments, it is unlikely that the differences observed in some brain regions between the two conditions, such as ACC, NAcc, VMH, or PVN, were due to the use of different strains of female in the two experiments. In our previous studies, no differences were observed between LE and W females in the number of solicitations or time spent with the pacing-related male as a function of olfactory or strain conditioning (Coria-Avila et al., 2005, 2006), suggesting that females of both strains process CSs that predict sexual reward in a similar manner.

Systemic administration of the dopamine antagonist flupenthixol during training blocked olfactory conditioning but not conditioning by strain (Coria-Avila et al., submitted for publication). Similarly, in the present study the odor induced significantly more Fos in the NAcc of odor-paired versus odor-unpaired rats, but no differences were detected in Fos induction in this region following exposure to strain cues. This suggests that mesolimbic dopamine transmission may be critical for olfactory conditioning, which involves a truly pavlovian contingency between a neutral stimulus and a rewarding unconditioned stimulus (UCS), relative to an already biased CS (strain cues that naturally activate the accessory olfactory pathway) and the rewarding UCS. Previous studies have shown a modest increase in extracellular concentrations of NAc dopamine



when female rats are presented with a sexually vigorous male behind a screen (Pfaus et al., 1995) whereas much higher concentrations are reported during copulation in those studies, and especially in situations where females are allowed to pace their copulatory interactions (Mermelstein and Becker, 1995). In the latter study, significant extracellular dopamine concentrations were also reported in the CPU, and we note a trend toward a significant increase in Fos induction by the conditioned odor in the CPU in the present study. In contrast, systemic administration of the opioid antagonist naloxone to female rats during training blocks the development a conditioned partner preference for odor or strain (Coria-Avila et al., submitted for publication). Although it is not known where the inhibition of opioid receptors may inhibit the development of conditioned partner preference, there is evidence to suggest it may occur in the mPOA.

### Role of the PirCtx

The PirCtx constitutes primary olfactory cortex, and its activation may be critical for learning the odor- and strain-conditioned partner preference. In addition to receiving direct inputs from the main olfactory bulb, the PirCtx is reciprocally connected to other brain regions, including the entorhinal cortex, amygdala, thalamus, and NAc (Kowianski et al., 1999), and thus can be activated indirectly by the accessory olfactory system. Accordingly, the information that represents the incentive value of a conditioned odor or strain cue may be transmitted to limbic areas that will mediate sexual motivation. The PirCtx has been heavily investigated for its role in olfactory discrimination, and several studies have observed synaptic and neuronal alterations in PirCtx associated with learning olfactory discrimination tasks, or tasks in which electrical stimulation of the olfactory inputs is used as a discriminative stimulus for positive reward (Datiche et al., 2001; Roman et al., 1987, 1993; Saar et al., 1999). Piriform cell firing in an eight-odor discrimination task is also influenced by whether odors were associated with reward (Schoenbaum and Eichenbaum, 1995). Fos is elevated in the PirCtx of male rats exposed to almond-scented bedding previously paired with copulation to ejaculation, but not by unconditionally rewarding estrus odors (Kippin et al., 2003) or in response to the almond odor unpaired with copulation. The PirCtx may therefore be part of a distinct neural pathway for the learning of contingencies between neutral odors and sexual reward, and sends that information to the rest of the limbic system. Although it is unclear what olfactory or other stimuli the females were responding to during strain conditioning, it is clear that they can easily distinguish pigmented LE males from albino W males at a distance (Coria-Avila et al., 2006), suggesting that olfactory and/or pheromonal differences between the strains of male were salient.

### Role of the mPOA

The mPOA receives inputs from both the primary and accessory olfactory systems (Shipley and Ennis, 1996). The number of neurons expressing Fos, along with extracellular dopamine concentrations, is increased in the

mPOA of male or female rats by sex-related olfactory stimuli, along with genitosensory stimuli that may travel along the spinohypothalamic pathway in females via the pelvic and hypogastric nerves (Baum and Everitt, 1992; Blackburn et al., 1992; Cliffer et al., 1991; Hull and Dominguez, 2006; Paredes et al., 1998; Pfaus et al., 2006; Wersinger et al., 1993; Xiao et al., 2005). Lesions of the mPOA abolish appetitive solicitations and hops and darts (Whitney, 1986; Hoshina et al., 1994), but can increase lordosis in OVX, steroid-primed rats during paced or non-paced copulation (Whitney, 1986; Xiao et al., 2005). The mPOA also receives inputs from the ArcN (Zaborszky and Makara, 1979), a significant proportion of which contain opioids or melanocortins (Chronwall, 1985; Eskay et al., 1979; Mills et al., 2004; Piekut and Knigge, 1984; Roselli-Rehffuss et al., 1993). Dopamine release in the mPOA increases during copulation (Matuszewich et al., 2000), and the mPOA contains populations of neurons that increase their firing rate when female rats solicit sex; these neurons are inhibited by dopamine antagonists such as pimozide, which also inhibit solicitations (Kato and Sakuma, 2000). Indeed, the mPOA is an important site of action for melanocortin and dopamine stimulation of solicitation (Gelez et al., submitted for publication). Given that treatment with the opioid antagonist naloxone during training also reduces solicitations and hops and darts significantly (Coria-Avila et al., submitted for publication), and that melanocortin actions stimulate solicitations, the mPOA would appear to be a critical site that integrates the VCS and olfactory stimulation received during paced copulation with opioid reward and melanocortin actions that trigger solicitations toward the preferred male on subsequent trials. In the present study, the conditioned odor and the strain cues activated a cluster of Fos in and around the median nucleus of the mPOA. A nearly identical distribution of Fos-positive neurons was activated by systemic administration of the melanocortin agonist bremelanotide (Gelez et al., submitted for publication), suggesting that a common population of melanocortin-sensitive neurons in the mPOA may be critical for solicitation. Efferent projections of the mPOA to the ACC and PirCtx (Gaykema et al., 1990), and to the VTA (Brackett and Edwards, 1984), may be critical for the expression of conditioned solicitation.

### Role of the VTA

The medial VTA is the site of dopamine cell bodies that project throughout the limbic system (Fallon and Moore, 1978; Phillipson, 1979), including regions of the NAc, Tu, and ACC. The mesolimbic dopamine system is thought to mediate attention toward reward-related stimuli, and in particular the incentive salience of stimuli (Berridge, in press; Robinson and Berridge, 1993), whether they relate to reward, punishment, or general arousal (Horvitz, 2000; Insel, 2003). Although the dopamine system that projects to the mPOA arises from a different population of cell bodies in the zona incerta (Bjorklund et al., 1975), nearly identical patterns of dopamine release during copulation have been found in the mPOA and NAc of male rats (Blackburn et al., 1992) and female rats (Matuszewich et

al., 2000; Mermelstein and Becker, 1995; Pfaus et al., 1995). Outputs from the mPOA to the VTA may link inter-hypothalamic and mesolimbic dopamine turnover to coordinate goal-directed behaviors with the hypothalamic control of sympathetic and parasympathetic activation during sexual behavior (Dominguez and Hull, 2005). In prairie voles, blockade of oxytocin or dopamine D2 receptors in the NAc blocks copulation-induced partner preference (Liu and Wang, 2003; Young et al., 2001). However, activation of D1 dopamine receptors in the NAc blocks partner preferences, and these receptors are upregulated in male prairie voles that bond after their initial copulatory experiences (Aragona et al., 2006), suggesting a mechanism for turning off plasticity once the bond has been established.

### Other regions

Odor conditioning induced Fos in several cortical, limbic, motor, and hypothalamic regions. Some of those regions, such as the NAc, PVN and LS, have been implicated in pair bonding in prairie voles (Young and Wang, 2004; Young et al., 2005), whereas others, such as the ACC, are involved in discrimination of CS and tagging their emotional meaning (Bussey et al., 1997; Cardinal et al., 2002). Interestingly, these regions did not display significant Fos induction following exposure to strain cues associated with paced copulation. It is possible that activation of accessory olfactory inputs from the strain cues alone is sufficient to stimulate motor and autonomic outputs without significant processing, and that their association with sexual reward in regions such as the mPOA is prepotent. It may well be that a truly neutral stimulus requires more activation of attentional mechanisms, such as mesolimbic dopamine release, to solidify the CS–UCS relationship between stimulation and reward. Indeed, Hernandez et al. (2006) have shown that low trains of electrical stimulation of the medial forebrain bundle in rats result in sustained dopamine release in the NAc, whereas dopamine release is transient during higher trains of stimulation. The rewarding impact of the stimulation was reduced if high trains were administered prior to testing, relative to low trains, suggesting that stimuli of high unconditional incentive value diminish the need for sustained dopamine release. Thus, strain cues may have been of sufficient intensity to bypass the activation of associative mechanisms required to link neutral olfactory stimulation to sexual reward. Such a “dual system” has been found previously in male rats exposed to estrous vaginal secretions relative to the same almond odor associated with copulation to ejaculation (Kippin et al., 2003). Although the pattern of Fos activation throughout the forebrain and midbrain was different following exposure to vaginal secretions or the conditioned odor, comparable Fos activation was found in the NAc regardless of the stimuli. However, in that study, odors and vaginal secretions were presented in bedding rather than on gauze as in the present study. It is not clear whether the odor or pheromonal intensity was similar between the two presentation methods, or whether the different activation patterns between our former and present studies reflect sex differ-

ences in sexual reward intensity or the way odor or strain cues are linked to sexual reward.

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# Neurochemical Basis of Conditioned Partner Preference in the Female Rat: I. Disruption by Naloxone

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The effects of the opioid antagonist naloxone were examined on the development of conditioned partner preference induced by paced copulation in female rats. In Experiment 1, ovariectomized, hormone-primed rats were conditioned to associate scented and unscented male rats with paced and nonpaced copulation, respectively. Female rats in Experiment 2 associated albino or pigmented male rats with paced or nonpaced copulation. Naloxone or saline was administered before each conditioning trial. During a final drug-free preference test, female rats could choose to copulate with either a pacing related or unrelated male. Saline-trained female rats in the paired group copulated preferentially with the pacing-related male rat, whereas naloxone-trained female rats did not show a preference. The authors concluded that opioids mediated the conditioned partner preference induced by paced copulation.

**Keywords:** paced copulation, odors, sexual behavior and reward, conditioned partner preference, opioids

Different types of environmental stimuli can become sexually conditioned stimuli (CSs) if they are associated in contingency with rewarding sexual experiences. Accordingly, CSs can become predictors of reward during sexual encounters and increase appetitive sexual behaviors, indicative of sexual desire (Pfaus, Kippin, & Centeno, 2001; Pfaus, Kippin, & Coria-Avila, 2003). In male rats, for example, it has been shown that the state of reward that follows ejaculation is of sufficient intensity to induce conditioned place preference (CPP; Ågmo & Berenfeld, 1990). Specifically, male rats learn to prefer one distinctive side of a CPP box if it had been previously associated with the postejaculatory reward state. The underlying process of this conditioning is poorly understood, but pharmacological studies have shed light on the role of different substances involved. For example, systemic injections of the opioid antagonist naloxone effectively block such CPP (Ågmo & Berenfeld, 1990), demonstrating that opioids form part of the neurochemical substrate that mediates the conditioning process that occurs after sexual reward induced by ejaculation.

In female rats, a similar conditioning process occurs after paced copulation. Females that are able to control, or *pace*, the rate of

sexual interaction with approaches or withdrawals from the males (Erskine, Kornberg, & Cherry, 1989; Pfaus, Smith, & Cooper-smith, 1999) develop CPP for distinctive sides associated with paced copulation (Paredes & Alonso, 1997; Paredes & Vazquez, 1999). This copulatory CPP in females is also disrupted by administration of naloxone (Paredes & Martinez, 2001), indicating that the rewarding properties induced by paced copulation depend on endogenous opioid turnover, similar to the rewarding effects of ejaculation in males.

The rewarding effects of copulation can also induce conditioned partner preferences toward individuals that bear CSs predictive of sexual reward. For example, male rats can display a conditioned ejaculatory preference toward almond-scented females when the almond odor predicts sexual receptivity, but it does not occur when the odor predicts no receptivity (Kippin & Pfaus, 2001a, 2001b; Kippin, Samaha, Sotiropoulos, & Pfaus, 2001). In the case of female rats, we have demonstrated that they can display a conditioned partner preference toward males that bear an odor associated with paced copulation, compared with males associated with nonpaced copulation (Coria-Avila, Ouimet, Pacheco, Manzo, & Pfaus, 2005). Females in the paired group copulated with an almond-scented male in a pacing chamber, which was divided by a Plexiglas partition that had four small holes in the bottom to allow the females exclusively to pass freely from one side to the other. Four days later, the females copulated with an unscented male in the same chambers but with the partitions removed (non-paced). These two conditions were alternated for a total of eight conditioning trials (four in each condition). Four days after the final conditioning trial, females were tested for a conditioned partner preference in an open field with two tethered males, one scented and the other unscented. This procedure allowed the females to choose freely between the two males. Females in the paired group displayed significantly more solicitations, hops and darts, and high magnitude lordosis with the scented male, from whom they also chose to receive more mounts and their first

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ejaculation. In contrast, the preference toward scented males was not observed in the unpaired group, in which the scented males were associated with nonpaced copulation, or in a randomly paired group, in which scented and unscented males were randomly paired with paced copulation.

Female rats can also develop a conditioned partner preference for strain cues that differentiate an albino male from a pigmented male (Coria-Avila et al., 2006). In that study, Wistar (W) and Long-Evans (LE) females were conditioned to associate one of these two strains of males with paced copulation and the other with nonpaced copulation. After 10 conditioning trials (5 in each condition), females were tested for conditioned partner preference with 1 W and 1 LE male tethered in opposite corners of an open field. Regardless of the strain, females displayed more solicitations toward the pacing-related male and chose him to receive the first ejaculation. Thus, despite the fact that the mating strategy of rats has been described as polygamous, (McClintock, 1984; McClintock, Anisko, & Adler, 1982), both male and female rats showed conditioned partner preferences that were based on CSs associated with sexual reward. This conditioned partner preference may be considered a rudiment of the pair-bonding behavior observed in other species of rodents and in humans.

Given evidence that opioids are part of the underlying reward mechanism that facilitates CPP in female rats after paced copulation; the present study attempted to clarify the role of opioids in the development of conditioned partner preference. We hypothesized that blockade of opioid receptors with the antagonist naloxone during the conditioning process would disrupt the rewarding properties of paced copulation. Thus, in the absence of sexual reward, the development of conditioned partner preference would not occur.

## General Method

### *Subjects and Surgery*

LE male (300–350 g) and female (200–250 g) rats were purchased from Charles River Canada (St.-Constant, Quebec, Canada). They were housed in groups of 4 in large, hanging wire mesh cages in a colony room maintained on a reversed 12:12-hr light/dark cycle (lights off at 0800) at approximately 21°C. Food and water were continuously available. The females were anesthetized with a mixture of ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (4 mg/ml) mixed at a ratio of 4:3, respectively, and injected intraperitoneally in a volume of 1 ml/kg of body weight. Anesthetized females were then ovariectomized bilaterally through a lumbar incision. After ovariectomy, females were given daily subcutaneous injections of 2.5 mg/kg flunixin meglumine (Banamine) for analgesia and 5 mg/kg enrofloxacin (Baytril) for 3 days to prevent postsurgical bacterial infections. All females were given a week of postsurgical recovery before conditioning trials.

Gonadally intact LE male rats that served as stimulus males had at least 10 tests of sexual behavior in bilevel chambers before the start of these experiments. These males were sexually vigorous and initiated copulatory activity with females within 15 s of being placed into the chambers. For all behavioral tests, sexual receptivity was induced in all females by subcutaneous injections of estradiol benzoate (EB; 10 µg) 48 hr before each test and progesterone (P; 500 µg) 4 hr before each test. If scented, males received

almond extract (Blue Ribbon; Etobicoke, Ontario, Canada) applied to the back of their necks and anogenital regions with a cotton swab. Unscented males received similar applications of distilled water to the same areas.

### *Drugs*

Naloxone hydrochloride (Sigma; St. Louis, MO), a general opioid receptor antagonist, was dissolved in 0.9% physiological saline and injected intraperitoneally in a dose of 4 mg/kg in a volume of 1 ml/kg, 1 min before every conditioning trial (as in Paredes & Martinez, 2001). Control rats were injected with physiological saline 1 min before testing.

## Experiment 1

The first experiment tested the effect of injections of naloxone versus saline on the development of olfactory-conditioned partner preference induced by paced copulation with scented males, as in the paired group reported by Coria-Avila et al. (2005). In this experiment, only LE females and males were used.

### *Method*

**Odor conditioning.** Conditioning trials occurred every 4 days in semicircular chambers (38 cm<sup>2</sup> × 60 cm × 38 cm). For paced copulation, the chambers were divided by a middle Plexiglas partition with four equidistant holes cut into the bottom (4 cm × 4 cm) resting on bedding. The holes were large enough for the female to pass through but too small for the males. Nonpaced copulation occurred in the same chambers but without the partition. Females copulated with scented males in the pacing condition and copulated 4 days later with unscented males in the nonpacing condition. If scented, almond extract (Blue Ribbon, Etobicoke, Ontario, Canada) was applied on the back of the neck and anogenital area of the males with a cotton swab. Females received a total of 10 conditioning trials (5 paced and 5 nonpaced). The order of conditioning was counterbalanced so that half of the rats in every condition had their first sexual experience in a paced situation and the other half in a nonpaced situation. During each conditioning trial, experimental females were injected with naloxone 1 min before the start of the session, and females in the control group were injected with saline. They were allowed to copulate for 30 min before being returned to their home cages. All tests with scented and unscented males took place in different rooms to prevent noncontingent odor associations.

**Copulatory preference test.** Four days after the final conditioning trial, females were placed into a large open field (123 cm × 123 cm × 46 cm) with a thin layer of wood chip bedding. At two diagonal corners, there were 2 males unfamiliar to the female, 1 scented and 1 unscented. Each male wore a rodent jacket connected on the back to a spring wire 30 cm in length, which allowed the males to roam freely in a radius of approximately 45 cm. For this final partner preference test, females in the experimental and control groups were injected exclusively with saline, so that all behavioral differences between naloxone- and saline-trained rats were the result of the conditioning process and not because of any drug effect during the final test. Females were allowed to copulate freely with both males during the 30-min test.



Conditioned partner preference was determined as in Coria-Avila et al. (2005, 2006) by latency and frequency measures of female visits, olfactory investigations, solicitations (described as a headwise orientation of the female to the male followed by a runaway), hops and darts, and lordosis magnitudes (on a scale ranging from 1 [*low magnitude*] to 3 = [*high magnitude*]) during interactions with each male. In addition, we assessed the male chosen for the first ejaculation, as well as the frequency of mounts, intromissions, and ejaculations during the final preference test.

**Statistical analysis.** A  $2 \times 2$  (Odor  $\times$  Drug) mixed-design analysis of variance (ANOVA) was used to determine differences in frequencies and latencies. Chi-square analyses were used to determine the differences in proportion of males chosen for first mount, intromission, and ejaculation. All significant main effects in the ANOVAs were followed by a Tukey's least significant difference (LSD) post hoc test to assess differences between individual means. The level of significance for all tests was  $p < .05$ .

## Results

Of the 39 females originally conditioned, 8 were eliminated from the statistical analysis because one or both males became free from their tethered jackets during the final preference test. This left 31 females ( $n = 17$  in the control group;  $n = 14$  in the naloxone-treated group). Table 1 shows means plus or minus standard errors for all the behaviors assessed during the 30-min test for saline- versus naloxone-treated females.

**Visits and olfactory investigations.** With regard to the first-visit latency, the ANOVA detected no main effects of drug,  $F(1, 29) = 0.69$ ,  $p = .41$ ; odor  $F(1, 29) = 0.65$ ,  $p = .42$ ; or Drug  $\times$  Odor,  $F(1, 29) = 0.28$ ,  $p = .59$ , indicating that females in both

groups performed their first visit with approximately the same latency toward scented or unscented males. The analysis of visit frequencies did not reveal any main effect of odor,  $F(1, 29) = 0.0002$ ,  $p = .98$ ; or drug  $F(1, 29) = 0.48$ ,  $p = .49$ , indicating that neither odor nor naloxone alone affected the number of visits toward both males. However, an Odor  $\times$  Drug interaction was found,  $F(1, 29) = 6.90$ ,  $p = .01$ . The post hoc analysis revealed differences only within the naloxone-treated females, indicating that females in this group visited the scented males fewer times than the unscented males. With regard to the number of olfactory investigations, the ANOVA failed to detect a main effect of odor,  $F(1, 29) = 0.04$ ,  $p = .83$ ; or drug  $F(1, 29) = 1.2$ ,  $p = .26$ , but detected an interaction between the two,  $F(1, 29) = 5.05$ ,  $p = .03$ . The post hoc analysis revealed that saline-treated females displayed more olfactory investigation toward scented males than toward unscented males. There were no main effects of drug or odor on first olfactory investigation latency. Together, these results indicate that naloxone-treated females visited the pacing-related male fewer times than non-pacing-related males and that saline-treated females performed more olfactory investigations of pacing-related males than of non-pacing-related males.

**Frequency of solicitations and of hops and darts.** With regard to solicitation frequencies, there was a main effect of drug,  $F(1, 29) = 5.8$ ,  $p = .02$ ; and a Drug  $\times$  Odor interaction,  $F(1, 29) = 4.7$ ,  $p = .03$ ; but there were no effects of odor alone,  $F(1, 29) = 0.000$ ,  $p = .99$ . The post hoc analysis revealed a significant difference in the number of solicitations between scented males in the saline and naloxone groups but failed to detect significant differences within groups. With regard to the latency for the first solicitation, the ANOVA detected a main effect of drug,  $F(1, 29) = 9.4$ ,  $p = .004$ .

Table 1

Means (Plus or Minus Standard Errors) for Sexual Behaviors Assessed During the 30-Min Test for Saline- Versus Naloxone-Treated Female Rats

| Behavior                                           | Odor-pacing group: Saline ( $n = 17$ ) |                  | Odor-pacing group: Naloxone ( $n = 14$ ) |                     |
|----------------------------------------------------|----------------------------------------|------------------|------------------------------------------|---------------------|
|                                                    | Scented                                | Unscented        | Scented                                  | Unscented           |
| First visit latency (in seconds)                   | 11.8 $\pm$ 2.7                         | 16.7 $\pm$ 3.0   | 18.9 $\pm$ 5.7                           | 19.9 $\pm$ 8.2      |
| Visit frequency                                    | 46.0 $\pm$ 2.7                         | 41.5 $\pm$ 2.3   | 40 $\pm$ 2.5*                            | 47.0 $\pm$ 3.8      |
| First olfactory investigation latency (in seconds) | 35.0 $\pm$ 9.3                         | 35.0 $\pm$ 6.4   | 23.6 $\pm$ 6.5                           | 30.9 $\pm$ 9.0      |
| Olfactory investigation frequency                  | 32.8 $\pm$ 2.6*                        | 25.0 $\pm$ 1.4   | 28.2 $\pm$ 2.4                           | 30.8 $\pm$ 4.2      |
| First hop and dart latency (in seconds)            | 34.0 $\pm$ 6.3                         | 74.0 $\pm$ 35.0  | 85.0 $\pm$ 34.0                          | 73.0 $\pm$ 31.0     |
| Hops and darts                                     | 42.9 $\pm$ 5.3*                        | 24.5 $\pm$ 2.5   | 24.3 $\pm$ 1.9**                         | 28.0 $\pm$ 3.4      |
| First solicitation latency (in seconds)            | 88.4 $\pm$ 21.0                        | 122.0 $\pm$ 27.0 | 369.0 $\pm$ 76.0**                       | 301.0 $\pm$ 115.0** |
| Solicitation frequency                             | 19.4 $\pm$ 2.5                         | 14.1 $\pm$ 2.0   | 9.2 $\pm$ 1.4**                          | 14.5 $\pm$ 2.6      |
| First mount latency (in seconds)                   | 68.0 $\pm$ 22.0                        | 47.5 $\pm$ 11.4  | 239.0 $\pm$ 72.0*, **                    | 63.9 $\pm$ 26.0     |
| Mount frequency                                    | 13.1 $\pm$ 2.0                         | 10.6 $\pm$ 1.9   | 5.0 $\pm$ 1.2*, **                       | 12.0 $\pm$ 2.5      |
| First intromission latency (in seconds)            | 333.0 $\pm$ 149.0                      | 82.0 $\pm$ 20.0  | 95.0 $\pm$ 30.0                          | 191.0 $\pm$ 97.0    |
| Intromission frequency                             | 8.5 $\pm$ 1.2                          | 9.5 $\pm$ 0.81   | 6.1 $\pm$ 1.4                            | 10.7 $\pm$ 1.2      |
| First ejaculation latency (in seconds)             | 528.0 $\pm$ 110.0                      | 406.0 $\pm$ 64.0 | 422.0 $\pm$ 115.0                        | 519.0 $\pm$ 95.0    |
| Ejaculation frequency                              | 1.3 $\pm$ 0.3                          | 2.05 $\pm$ 0.23  | 1.2 $\pm$ 0.33                           | 1.7 $\pm$ .24.0     |
| Choice of male for first ejaculation               | 5 (30%)                                | 12 (70%)         | 6 (43%)                                  | 8 (57%)             |
| Lordosis magnitude 1                               | 3.8 $\pm$ 0.63                         | 6.3 $\pm$ 1.4    | 3.2 $\pm$ 0.83                           | 6.0 $\pm$ 1.3       |
| Lordosis magnitude 2                               | 10.2 $\pm$ 1.6*                        | 6.4 $\pm$ 0.9    | 4.0 $\pm$ 1.1**                          | 7.9 $\pm$ 1.8       |
| Lordosis magnitude 3                               | 8.6 $\pm$ 1.2                          | 9.3 $\pm$ 1.1    | 5.0 $\pm$ 1.4*, **                       | 10.1 $\pm$ 1.2      |

*Note.* Female sexual behavior was assessed during an olfactory-conditioned partner preference test with two males, one scented and one unscented. Scented males were associated with paced copulation, and unscented males were associated with nonpaced copulation.

\* $p < .05$  between scented and unscented male rats within groups. \*\* $p < .05$ , compared with controls.

The post hoc analysis revealed that saline-treated females performed their first solicitation significantly faster than naloxone-treated females. There was no main effect of odor or interaction between odor and drug with regard to first solicitation latency.

With regard to frequencies of hops and darts, the ANOVA detected a main effect of drug,  $F(1, 29) = 4.6, p = .03$ ; and a Drug  $\times$  Odor interaction,  $F(1, 29) = 7.9, p = .008$ ; but no main effect of odor alone  $F(1, 29) = 3.5, p = .07$ . The post hoc analysis revealed that saline-treated females performed more hops and darts toward scented males than toward unscented males. This difference was not detected in the naloxone-treated females. The analysis of the first latency of hops and darts did not show a main effect of drug,  $F(1, 29) = .57, p = .45$ ; odor  $F(1, 29) = .31, p = .58$ ; or Drug  $\times$  Odor,  $F(1, 29) = 1.07, p = .30$ , indicating that the first hops and darts occurred at approximately the same time for all females, regardless of the group or the status of the male. Overall, the analysis of solicitations and of hops and darts indicated that the saline-trained females performed more of these proceptive behaviors toward pacing-related males, whereas naloxone-trained females did not (see Figure 1).

**Lordosis.** The analysis of frequencies for lordosis magnitude 1 indicated a main effect of odor  $F(1, 29) = 5.9, p = .02$ ; but there were no main effects of drug,  $F(1, 29) = 0.18, p = .67$ ; or of Drug  $\times$  Odor,  $F(1, 29) = 0.01, p = .90$ . The post hoc analysis failed to show significant differences within or between groups. The analysis of lordosis magnitude 2 showed a significant Drug  $\times$  Odor interaction,  $F(1, 29) = 7.6, p = .009$ ; but there were no main effects of drug or odor alone. The post hoc analysis indicated that saline-treated females displayed more instances of lordosis magnitude 2 toward scented males than toward unscented males within group and scented males in the naloxone group. The analysis of lordosis magnitude 3 indicated a main effect of odor,  $F(1, 29) = 5.4, p = .02$ ; and a trend for a Drug  $\times$  Odor interaction,  $F(1, 29) = 3.13, p = .08$ . The post hoc analysis revealed that naloxone-treated females displayed significantly fewer instances of lordosis magnitude 3 toward scented males than toward unscented males and also scented and unscented males in the saline-trained group.

**Choice of male for ejaculation and male responses.** Of the 17 females in the saline group, 5 (30%) received the first ejaculation from the scented male and 12 females (70%) received the first ejaculation from the unscented male. Chi-square analyses detected a trend toward significance in these proportions,  $\chi^2(1, N = 17) = 2.8, p = .09$ . Of the 14 females in the naloxone-treated group, 6 (43%) received their first ejaculation from scented males and 8 (57%) received the first ejaculation from unscented males. Chi-square analysis failed to detect any significant difference in these proportions,  $\chi^2(1, N = 14) = 0.28, p = .59$ . There were significant differences in frequencies and latencies of male sexual behavior. With regard to mount frequencies, the ANOVA detected a Drug  $\times$  Odor interaction,  $F(1, 29) = 5.2, p = .02$ . The post hoc analysis indicated that naloxone-treated females received fewer mounts from scented males than from unscented males and fewer compared with scented males in the saline group. The analysis of the latency to the first mount showed a main effect of Drug,  $F(1, 23) = 6.1, p = .02$ ; Odor  $F(1, 23) = 9.7, p = .004$ ; and a Drug  $\times$  Odor interaction,  $F(1, 23) = 6.7, p = .01$ . The post hoc test indicated that scented males in the naloxone-treated group had a higher latency relative to unscented males in the same group, and relative to scented and unscented males in the saline group. There

were no significant differences in the latency or frequency for intromissions and ejaculations.

## Experiment 2

This experiment examined the effects of naloxone on the development of conditioned partner preference for strain of male associated with paced copulation, as in Coria-Avila et al. (2006).

### Method

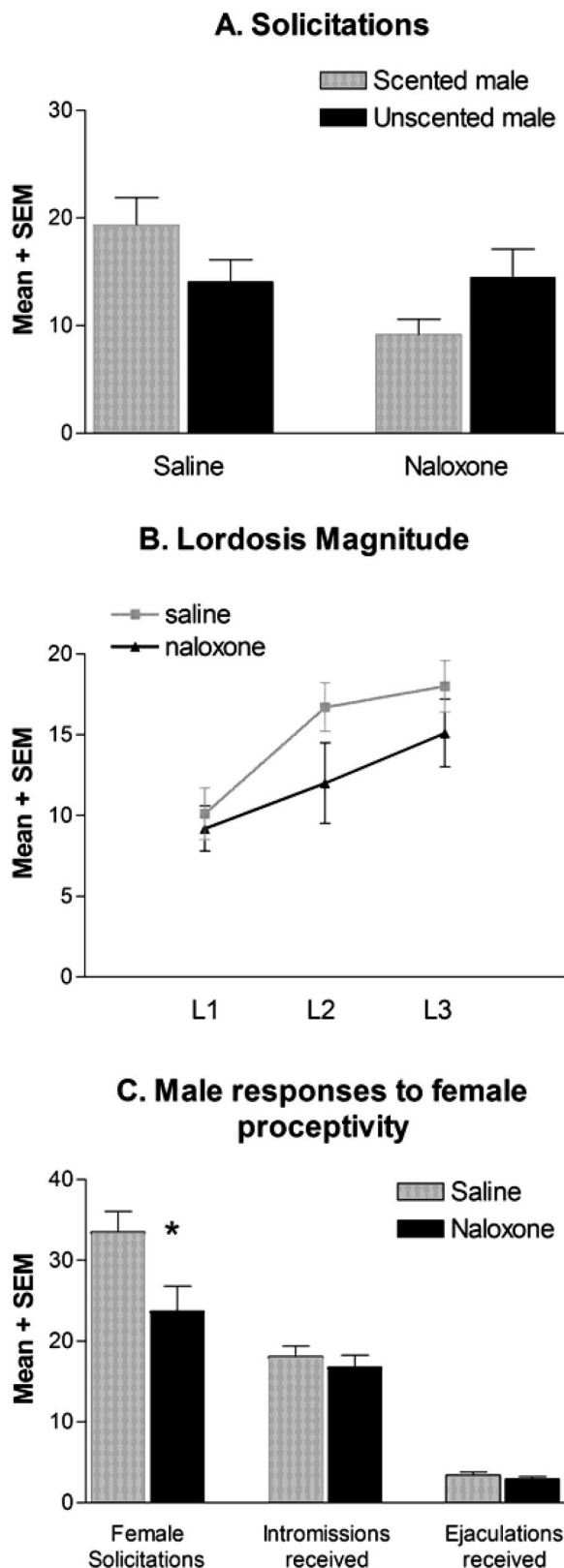
**Strain conditioning.** There were two counterbalanced conditioning groups. Females in the W pacing (W-P) group ( $n = 30$ ) copulated with W males in the same paced condition as in Experiment 1 and with L-E males in the same nonpaced condition as in Experiment 1. Females in the L-E pacing (LE-P) group ( $n = 30$ ) copulated with L-E males in the paced condition and with W males in the nonpaced condition. Half of each group received saline as control, and the other half received naloxone 1 min before every conditioning trial. This procedure led to four subgroups: W-P + saline ( $n = 16$ ), LE-P + saline ( $n = 14$ ), W-P + naloxone ( $n = 16$ ), and LE-P + naloxone ( $n = 14$ ). Females received a total of 10 conditioning trials (5 paced and 5 nonpaced) of 30 min each before being taken back into their home cages.

**Copulatory preference test.** As in Experiment 1, 4 days after the 10th conditioning trial, a final test was carried out for each female into a large open field. At two opposite corners of this open field were 2 males, 1 W and 1 L-E. All the females were injected exclusively with saline intraperitoneally 1 min before being placed in one neutral corner of the open field and were allowed to copulate freely with both males for a 30-min period. The same behavioral measures were assessed as in Experiment 1.

**Statistical analyses.** A  $2 \times 2 \times 2$  (Group  $\times$  Drug  $\times$  Strain of Male) mixed-design ANOVA was used to analyze differences in the frequencies and latencies of copulatory behaviors toward the 2 males. All significant main effects and interactions were followed by a Tukey's LSD post hoc test to detect specific differences between group means.

### Results

**Visits and olfactory investigations.** With regard to the first-visit latency, the ANOVA detected a Group  $\times$  Drug  $\times$  Strain of Male interaction,  $F(3, 56) = 7.45, p = .008$ . Post hoc analyses revealed that, in the W-P + saline group, W males received their first visit with a longer latency than did LE males within group, but no other group showed differences within group (see Table 2). The analysis of visit frequency detected a main effect of strain of male,  $F(3, 56) = 24.9, p = .000006$ ; and a Strain of Male  $\times$  Group interaction,  $F(3, 56) = 12.47, p = .0008$ . Post hoc analyses indicated that, in the W-P + saline and W-P + naloxone groups, females visited both males with the same frequency. In the LE-P + saline and LE-P + naloxone groups, females visited the LE males more, indicating that, overall, females preferred to visit LE males exclusively when they were associated with paced copulation, but this preference in visits did not occur when W males were associated with pacing. With regard to the first olfactory investigation latency, the ANOVA showed no effects of drug, strain of male, or interactions. The analysis of olfactory investigation frequencies



detected a main effect of drug,  $F(3, 56) = 8.22, p = .005$ ; a main effect of strain of male,  $F(3, 56) = 6.3, p = .01$ ; and a Group  $\times$  Strain of Male interaction,  $F(3, 56) = 4.3, p = .04$ . Post hoc analyses indicated that naloxone-treated females displayed more olfactory investigations than saline-treated females and that this effect was more robust toward LE males.

**Frequency of solicitations and of hops and darts.** The ANOVA detected significant differences between the saline- and naloxone-treated females. With regard to solicitation frequencies, there was a main effect of drug,  $F(3, 56) = 10.3, p = .002$ ; a Group  $\times$  Strain of Male interaction,  $F(3, 56) = 15.7, p = .0002$ ; and a Group  $\times$  Strain of Male  $\times$  Drug interaction,  $F(3, 56) = 5.6, p = .02$ . Post hoc analyses revealed that, in the W-P + saline group, females displayed more solicitations toward W males than toward LE males. In the LE-P + saline group, females displayed more solicitations toward LE males than toward W males, indicating that, overall, females solicited the pacing-related males more than nonpacing-related males. In the naloxone-treated females, there were no differences in the amount of solicitations toward either strain of male. In addition, the amount of solicitations in all naloxone-treated females was not different from the amount of solicitations displayed toward no-pacing males. With regard to the latency for the first solicitation, the ANOVA detected a main effect of drug,  $F(3, 56) = 4.3, p = .04$ . The post hoc analysis indicated that females from the W-P + saline group displayed their first solicitation toward W males with a shorter latency than that with W males in the W-P + naloxone group and in the LE-P + naloxone group. This difference was not significant in LE males between groups.

The frequency of hops and darts differed among the groups. The ANOVA detected a main effect of strain of male,  $F(3, 56) = 10.3, p = .002$ ; and a Drug  $\times$  Strain of Male interaction,  $F(3, 56) = 4.3, p = .04$ . The direction of the data indicated that, overall, naloxone-treated females displayed more hops and darts compared with saline-treated females, but this effect was particularly more robust toward the LE males. The post hoc analysis however, only showed differences within groups in the LE-P + naloxone condition but did not in any other group. For the first latency of hops and darts, the ANOVA detected a main effect of strain of male,  $F(3, 56) = 7.03, p = .01$ . The post hoc test however, only detected that the latency toward W males in the LE-P + naloxone group was significantly longer compared with the LE males. These results indicate that females preferred the pacing-related males and that this effect was disrupted by naloxone treatment. Together, the results of solicitations and of hops and darts also showed a dissociation between these two proceptive behaviors and the effects of the drug treatment on them.

**Lordosis.** The analysis of frequencies for lordosis magnitude 1 and 2 failed to detect any significant effect of drug, strain of male,

**Figure 1.** (A) Number of solicitations during 30-min test in two groups: saline ( $n = 17$ ) and naloxone ( $n = 14$ ). Scented males associated with paced copulation, and unscented males associated with nonpaced copulation. (B) Lordosis magnitude of saline- and naloxone-treated females. (C) Total number of solicitations of saline- and naloxone-treated females and the number of intromissions and ejaculations received. See Table 1 for details of behavior. \* $p < .05$ .

Table 2  
Means (Plus or Minus Standard Errors) for All Behaviors Assessed During the 30-Min Test for Saline- Versus Naloxone-Treated Pacing Groups

| Behavior                                           | Wistar pacing group: Saline |               | Long-Evans pacing group: Saline |               | Long-Evans pacing group: Naloxone |               | Long-Evans pacing group: Naloxone |               |
|----------------------------------------------------|-----------------------------|---------------|---------------------------------|---------------|-----------------------------------|---------------|-----------------------------------|---------------|
|                                                    | Wistar                      | Long-Evans    | Wistar                          | Long-Evans    | Wistar                            | Long-Evans    | Wistar                            | Long-Evans    |
| First visit latency (in seconds)                   | 16.2 ± 3.6*                 | 5.6 ± 0.52    | 10.8 ± 1.8                      | 11.1 ± 2.5    | 9.7 ± 1.7                         | 16.6 ± 6.8    | 19.8 ± 4.5                        | 9.7 ± 1.3     |
| Visit frequency                                    | 40.7 ± 4.3                  | 43.1 ± 4.3    | 38.6 ± 5.0*                     | 43.7 ± 5.3    | 38.2 ± 3.9                        | 38.1 ± 3.3    | 35.4 ± 3.5*                       | 43.4 ± 3.3    |
| First olfactory investigation latency (in seconds) | 23.3 ± 5.7                  | 22.4 ± 7.3    | 36.8 ± 17.4                     | 14.3 ± 2.9    | 19.6 ± 5.9                        | 26.8 ± 10.7   | 24.5 ± 4.9                        | 17.7 ± 4.6    |
| Olfactory investigation frequency                  | 22.8 ± 1.7                  | 22.6 ± 2.0    | 19.1 ± 2.7                      | 23.7 ± 2.9    | 29.6 ± 2.8                        | 32.1 ± 3.4    | 21.3 ± 1.7*                       | 30.5 ± 2.5    |
| First hop and dart latency (in seconds)            | 61.0 ± 13.4                 | 91.1 ± 61.0   | 104.0 ± 38.0                    | 63.1 ± 14.4   | 106.7 ± 48.0                      | 80.6 ± 42.0   | 229.0 ± 94.0**                    | 45.0 ± 21.0   |
| Hops and darts                                     | 36.8 ± 6.7                  | 31.8 ± 5.0    | 26.8 ± 5.0                      | 39.2 ± 7.0    | 31.4 ± 8.0*                       | 44.6 ± 8.2    | 30.7 ± 8.0*                       | 52.4 ± 8.6    |
| First solicitation latency (in seconds)            | 155.7 ± 63.0                | 224.0 ± 85.0  | 304.0 ± 115.0                   | 135.0 ± 33.0  | 347.0 ± 165.0**                   | 368.0 ± 107.0 | 351.0 ± 88.0                      | 385.0 ± 143.0 |
| Solicitation frequency                             | 11.5 ± 2.4*                 | 7.2 ± 1.5     | 6.1 ± 1.4*                      | 14.1 ± 2.9    | 4.9 ± 1.6**                       | 4.5 ± 0.95    | 3.4 ± 1.2                         | 6.2 ± 1.4**   |
| First mount latency (in seconds)                   | 246.3 ± 93.0                | 155.0 ± 68.0  | 295.0 ± 153.0                   | 322.0 ± 180.0 | 219.0 ± 70.0                      | 415.0 ± 266.0 | 207.0 ± 102.0                     | 61.9 ± 12.6   |
| Mount frequency                                    | 10.2 ± 2.2                  | 6.0 ± 2.1     | 8.1 ± 2.2                       | 9.1 ± 2.6     | 7.0 ± 2.4                         | 3.2 ± 1.8     | 5.6 ± 2.6                         | 5.3 ± 2.4     |
| First intromission latency (in seconds)            | 214.0 ± 74.0                | 177.0 ± 143.0 | 301.0 ± 124.0                   | 236.8 ± 122.0 | 333.0 ± 112.0                     | 526.0 ± 402.0 | 229.0 ± 67.0                      | 242.0 ± 160.0 |
| Intromission frequency                             | 7.0 ± 1.4                   | 5.4 ± 1.5     | 4.2 ± 1.0                       | 4.9 ± 1.4     | 4.1 ± 1.6                         | 2.2 ± 1.1     | 2.6 ± 1.3                         | 2.6 ± 1.2     |
| First ejaculation latency (in seconds)             | 781.0 ± 161.0               | 494.0 ± 162.0 | 766.0 ± 207.0                   | 639.0 ± 219.0 | 732.0 ± 82.0                      | 489.0 ± 132.0 | 758.0 ± 118.0                     | 353.0 ± 41.0  |
| Ejaculation frequency                              | 0.81 ± 0.24                 | 1.3 ± 0.36    | 0.78 ± 0.26                     | 0.85 ± 0.34   | 0.62 ± 0.23                       | 0.31 ± 0.21   | 0.57 ± 0.3                        | 0.42 ± 0.25   |
| Choice of male for first ejaculation               | 5 (42%)                     | 7 (58%)       | 4 (40%)                         | 6 (60%)       | 5 (71%)                           | 2 (29%)       | 3 (50%)                           | 3 (50%)       |
| Lordosis magnitude 1                               | 3.1 ± 0.74                  | 3.1 ± 1.1     | 2.7 ± 1.1                       | 2.9 ± 1.1     | 1.6 ± 0.75                        | 2.2 ± 1.4     | 2.5 ± 1.3                         | 3.0 ± 1.5     |
| Lordosis magnitude 2                               | 6.0 ± 1.7                   | 3.3 ± 1.0     | 4.0 ± 1.4                       | 4.7 ± 1.6     | 4.1 ± 1.6                         | 1.2 ± 0.62    | 2.8 ± 1.1                         | 2.5 ± 1.1     |
| Lordosis magnitude 3                               | 8.8 ± 2.1                   | 5.6 ± 1.7     | 6.2 ± 1.7                       | 7.2 ± 2.5     | 5.7 ± 2.1                         | 2.1 ± 0.99    | 3.0 ± 1.6                         | 2.4 ± 1.0**   |

Note. The conditioned partner preference test was conducted with two male rats, one Wistar and one Long-Evans. In Wistar-pacing groups, females were trained to associate Wistar males with paced copulation. In Long-Evans pacing groups, females received the opposite association.

\* $p < .05$  between Wistar and Long-Evans males within groups. \*\* $p < .05$ , compared with controls.

or interaction. However, an ANOVA indicated a main effect of drug for lordosis magnitude 3,  $F(3, 56) = 6.27$ ,  $p = .01$ , indicating that, overall, saline-treated females displayed more instances of high-magnitude lordosis than naloxone-treated females (see Figure 2).

**Choice of male for ejaculation and male responses.** From a total of 60 females across conditions, 25 did not receive an ejaculation from either male during the final partner preference test. Most of these females ( $n = 17$ ) belonged to the naloxone group. Of the 12 females in the W-P + saline group that received an ejaculation, 5 (42%) received the first ejaculation from the W male, and 7 females (58%) received it from the LE male. Chi-square analyses failed to detect any significant difference in these proportions,  $\chi^2(1, N = 12) = 0.33$ ,  $p = .56$ . Of the 10 females in the LE-P + saline group that received an ejaculation, 4 (40%) received their first ejaculation from the W male and 6 (60%) received it from the LE male,  $\chi^2(1, N = 10) = 0.40$ ,  $p = .52$ . Of the 7 females in the W-P + naloxone group that received an ejaculation, 5 (71%) received their first ejaculation from a W male and 2 (29%) received it from unscented males,  $\chi^2(1, N = 7) = 1.2$ ,  $p = .25$ . Of the 6 females in the LE-P + naloxone group that received an ejaculation, 3 (50%) received their first ejaculation from the W male and 3 received it from the LE male. There were no significant differences for the proportion of males chosen for the second and third ejaculation.

There were no significant differences in the frequency or latency for mounts, but the analysis of intromission frequencies detected a main effect of drug,  $F(3, 56) = 5.54$ ,  $p = .02$ , indicating that, overall, saline-treated females received more intromissions than naloxone-treated females. The post hoc analysis showed significant mean differences between W males in the W-P + saline group, compared with LE males in the W-P + saline group and with W and LE males in the LE-P + naloxone group.

With regard to ejaculation frequency, the ANOVA detected a main effect of drug,  $F(3, 56) = 6.42$ ,  $p = .01$ , indicating that, overall, naloxone-treated females received fewer ejaculations than saline-treated females. The post hoc test only demonstrated significant differences between LE males in the W-P + saline group and LE males copulating with naloxone-treated females. There were no significant differences for first intromission or first ejaculation latencies.

### General Discussion

The present study shows that the development of a conditioned partner preference is disrupted by treatment with the opioid receptor antagonist naloxone (4 mg/kg) injected before conditioning trials. In agreement with our previous findings (Coria-Avila et al., 2005, 2006), saline-treated females displayed more solicitations toward the pacing-related males, indicating that CSs (e.g., odors, strain cues) associated with paced copulation become predictors of sexual reward. Naloxone-treated females did not show a similar increase in solicitations and, in fact, showed decreases in solicitations overall on the final partner preference test. This suggests strongly that opioids are a necessary substrate of reward in the formation of conditioned partner preference induced by paced copulation, similar to the findings of Paredes and Martinez (2001), in which naloxone blocked the formation of pacing-induced CPP.

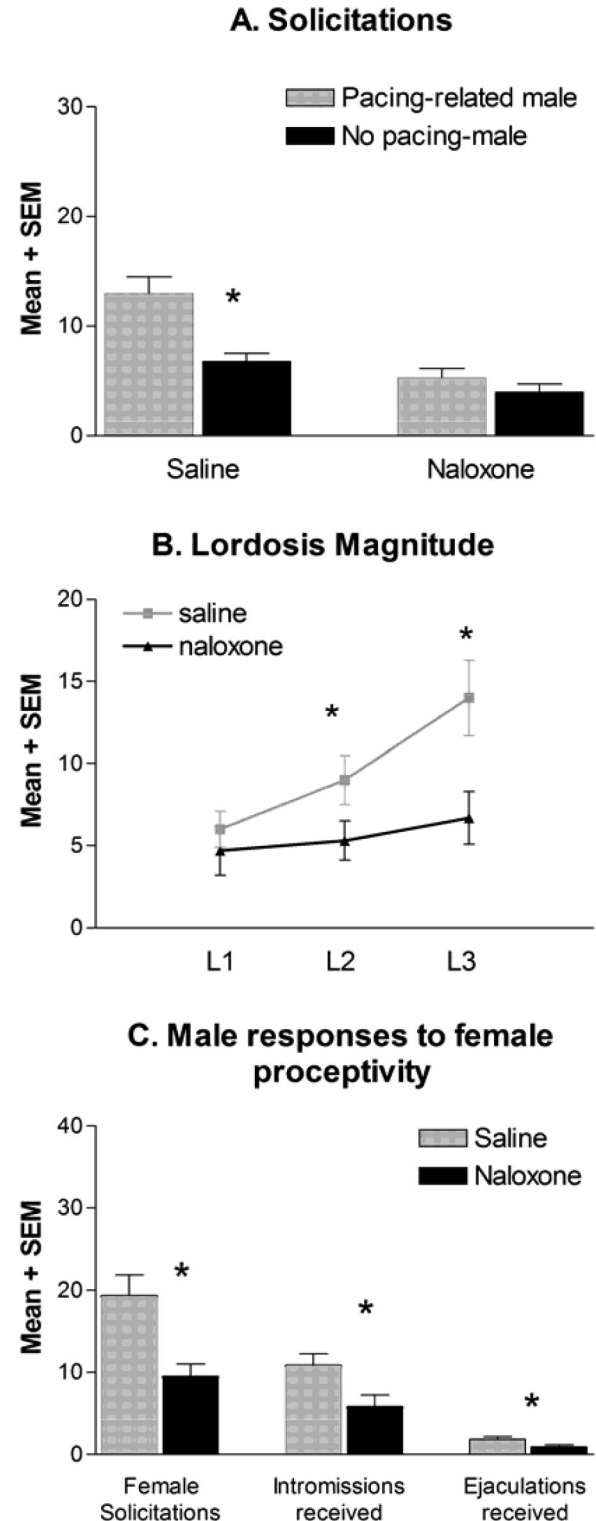


Figure 2. (A) Number of solicitations during 30-min test in two groups: saline and naloxone. Pacing-related were of a strain associated with paced copulation, and nonpacing-related males were of a different strain associated with nonpaced copulation. (B) Lordosis magnitude of saline- and naloxone-treated females. (C) Total number of solicitations of saline- and naloxone-treated females and the number of intromissions and ejaculations received. See Table 2 for details of behaviors. \* $p < .05$ .



In our experimental paradigm, solicitations were the most reliable measure of partner preference, and the measure most sensitive to the effects of naloxone. Solicitations represent the female's desire for sexual interaction (Pfaus et al., 2003). In Experiments 1 and 2, females trained with naloxone performed fewer solicitations on the final test, comparable with the number of solicitations that saline-treated females performed toward the nonpacing males in the saline groups. Fewer solicitations represent a decrease in the motivation to copulate. In addition to soliciting less, females in Experiment 2 received fewer intromissions and ejaculations and displayed lordoses of lower magnitude compared with saline-treated females. Thus, females appeared less motivated to copulate overall, despite the fact that they were primed fully with EB + P. This suggests that the absence of opioid transmission during a female's early sexual experiences induces an expectation of non-reward that decreases sexual motivation.

Expectation can be inferred from responses made in anticipation of reinforcement or punishment (Toates, 1986), thus anticipatory or appetitive sexual responses, including conditioned excitation and preference behaviors that depend on sexual reward, can be taken as measures of a rat's expectancy of sexual reward (Pfaus et al., 2003). For example, male rats display anticipatory locomotion in bilevel chambers during a 5-min period before the introduction of a sexually receptive female rat and copulation to ejaculation (Mendelson & Pfaus, 1989; Pfaus, Mendelson, & Phillips, 1990). This behavior is dependent on the presence of olfactory cues in the chamber (van Furth & Van Ree, 1996a), does not develop if male rats are given access to sexually nonreceptive females or other males (Mendelson & Pfaus, 1989), is inhibited if males are given systemic injections of naloxone or infusions of naloxone directly to the ventral tegmental area (VTA), before copulatory trials (van Furth & Van Ree, 1996b; van Furth et al., 1996a). This suggests that exposure to nonreceptive partners, or antagonism of opioid receptors in the VTA, leads to an expectation of sexual nonreward as inferred from the lack of anticipatory locomotion.

Endogenous opioid actions at mu-opioid receptors in the VTA appear to disinhibit dopamine (DA) neurons by hyperpolarizing inhibitory GABA interneurons (Ford, Mark, & Williams, 2006; Gysling & Wang, 1983; Kalivas, Duffy, & Eberhardt, 1990; Sotomayor, Forray, & Gysling, 2005). Indeed, infusions of morphine to the VTA induce CPP (Olmstead & Franklin, 1997) and stimulate sexually sluggish male rats to mount receptive females (Mitchell & Stewart, 1990). Repeated intermittent exposure of sexually naïve males to estrous vaginal secretions sensitizes DA release in the nucleus accumbens (Mitchell & Gratton, 1992), and this effect is abolished by naloxone treatment before exposure. Release of DA in mesolimbic terminals drives goal-directed locomotion in response to incentive cues (Berridge & Robinson, 1998), although antagonism of DA receptors in either the nucleus accumbens or medial preoptic area (which receives DA inputs from the incerto-hypothalamic DA system) abolish conditioned level changing in anticipation of sexual reward (Pfaus & Phillips, 1991).

In female rats, expectation of sexual reward appears to be important when they desire sex but not when they are not in heat. Barbosa, d'Ostie-Racine, and Pfaus (2005) trained ovariectomized female rats primed with EB and P to associate one side of a CPP box with paced copulation in a racetrack chamber and the other side with nonpaced copulation (with the racetrack insert removed). Females were then primed with EB + P, EB alone, or no steroids,

and were tested for CPP. Females in all three hormone-priming groups displayed significant CPP, as has been observed by previously Paredes and colleagues who test for CPP a day after hormone priming (Paredes & Alonso, 1997; Paredes & Martinez, 2001; Paredes & Vazquez, 1999). The females were then put on a partial extinction schedule, in which they were placed in the side of the CPP box associated previously with paced copulation but without antecedent copulatory contact. Females also remained in the hormone priming condition in which CPP was originally tested: EB + P, EB alone, or no steroids. After five extinction trials, only the females primed with EB + P showed significant extinction of CPP. Females in the EB-alone, and no-steroid conditions maintained their preference for the side previously associated with paced copulation. Because females primed with EB + P display high rates of solicitations and lordosis and low rates of pacing—whereas females primed with EB alone or no steroids display low numbers of solicitations, low lordosis, and high rates of pacing (Pfaus et al., 1999)—it was concluded that the lack of sexual reward produced partial extinction in only the fully primed females that anticipated sexual reward on the basis of their previous experience.

Although paced copulation reliably induces CPP in female rats when contrasted with nonpaced copulation, it can be induced under certain circumstances by nonpaced copulation when contrasted with no copulation (Meerts & Clark, 2007; Yamada, Horibe, & Kondo, 2006) or by manual vaginocervical stimulation with a lubricated glass rod that is distributed in time when contrasted with no stimulation (Afonso & Pfaus, 2006). It is interesting that systemic naloxone treatment did not alter a CPP induced by nonpaced copulation (Yamada et al., 2006). This suggests that the rewarding effects of paced copulation are opioid dependent, whereas the rewarding effects of nonpaced copulation may involve the action of other neurotransmitter systems. Sexual reward may well be a continuum in which chemosensory signals from a potential mate induce small increments of arousal and reward sufficient to trigger motivation (e.g., as in Mitchell & Gratton, 1992), whereas paced copulation (or ejaculation in male rats) induces a maximal level of reward. However, the rewarding properties of nonpaced copulation are not sufficient to induce conditioned partner preference. Thus, learning to prefer place-related cues seems to be neurochemically distinct from learning to prefer partner-related cues.

Paced copulation does not induce conditioning in every female. Using CPP as an example, we have reported previously that approximately 60% of females develop CPP for an originally nonpreferred place if associated with paced copulation, relative to nonpaced copulation (Coria-Avila et al., 2005). Likewise, not all females that undergo nine conditioning trials pairing odor or strain with paced copulation develop a preference for the pacing-related male. There are at least three reasons why this may be so.

First, paced copulation may simply increase the probability of experiencing sexual reward relative to nonpaced copulation, given that females can control the initiation and rate of copulatory contact. In a previous factor analysis of sexual behaviors displayed by female rats in bilevel chambers, three pacing-related factors were detected that determined (a) the chasing behavior of the male, (b) control of the number of mounts and intromissions, and (c) control over the number of ejaculations (Pfaus et al., 1999). Clearly, females adjust their behavioral responses depending on



the stimulation they receive from males. Females that are given access to sexually naïve or sluggish males typically mount those males as a supersolicital behavior (Afonso & Pfaus, 2006; Beach, 1968), which in many cases prompts males to mount the females in return. Likewise, females that copulate in small chambers that do not allow them to escape the male, or with males that do not chase them, are typically observed to solicit copulation using hops and darts in the immediate vicinity of the male rather than soliciting as they would in a bilevel chamber or open field, with a headwise orientation to the male followed by a runaway (McClintock, 1984; Pfaus et al., 2003). Thus, control over the copulatory contact may be a cognitive and emotional mechanism that activates endogenous opioids fully within reward pathways. Females that are given the opportunity to pace copulation efficiently at their preferred rate may therefore be more likely to experience sexual reward. Indeed, male rats that do not have control over their copulatory interactions do not form a sexual CPP, despite ejaculating on each conditioning trial (Camacho, Sandoval, & Paredes, 2004).

Second, paced copulation facilitates reproductive outcome. For example, females that pace a moderate number of intromissions (e.g., 10–15) before ejaculation are significantly more likely to get pregnant than females that are not allowed to pace the intromissions (e.g., Coopersmith & Erskine, 1994; Erskine et al., 1989). Indeed, to the extent that sexual reward and satiety result in sexual inhibition, it is noteworthy that paced copulation (Erskine et al., 1989) or distributed vaginocervical stimulation (Pfaus, Smith, Byrne, & Stephens, 2000) also result in a faster termination of estrus. Intromissions spaced in time facilitate reproductive events such as sperm transport and prolactin surges that support the maintenance of progesterone release by the corpora lutea (Erskine et al., 1989; Matthews & Adler, 1978; McClintock & Adler, 1978). However, it is not known to what extent mechanisms of sexual reward are related to mechanisms of reproduction, although it is likely that the sequential actions of estrogen and P set both into motion at a genomic and functional level (Pfaff, 1999).

Third, individual differences may exist in the propensity of paced copulation to activate reward or bonding mechanisms, such as oxytocin or vasopressin release or binding in the hypothalamus, septum, or ventral forebrain (Carter, Witt, Thompson, & Carlstead, 1988; Insel, Preston, & Winslow, 1995; Young, Murphy Young, & Hammock, 2005). For example, monogamous prairie vole males have greater vasopressin 1a (V1a) receptor density in the ventral pallidum compared with polygamous meadow voles (Young & Wang, 2004). Increasing the number of V1a receptors in this region of meadow voles after viral transfection renders them far more likely to bond after their first copulatory experience (Lim et al., 2004). It is therefore possible that individual differences in this or other mechanisms of bonding may result in differences in propensity to form place or partner preferences.

Some studies indicate that naloxone administration can produce aversive effects. For example, naloxone induces conditioned place aversion in mice (Skoubis, Matthes, Walwyn, Kieffer, & Maidment, 2001) and rats (Azar, Jones, & Schulteis, 2003). Those aversive effects are believed to be due to a disruption of a tonic “hedonic” homeostasis exerted by opioids (Bals-Kubik, Herz, & Shippenberg, 1989). It is unlikely that the behaviors observed in the naloxone-treated females in the present study were the consequence of a conditioned place aversion because the final test did

not occur under the influence of naloxone, nor did it occur in the same chambers as conditioning occurred (e.g., pacing chambers vs. the open field). It is also unlikely that females developed an aversion toward the males, as the number of visits and olfactory investigations were not affected by the drug, only sexual behaviors. In fact, females did not stop displaying solicitations altogether, which would be more suggestive of an aversion. Instead, they showed a reduction in frequency similar to the number of solicitations displayed by saline-trained females toward nonpacing-related males. This suggests a selective disruption of conditioned preference and, in context with the decrease in high-level lordosis, a diminished sexual desire. Daily administration of naloxone can also diminish motor output (Amir, Solomon, & Amit, 1979), the motor activation induced by psychomotor stimulants (Jones & Holtzman, 1994), and olfactory-induced activation of the amygdala in rats (Klemm & Mallari, 1979). However, the 4-day period between conditioning trials in the present experiments was a sufficient time to wash the drug from the central and peripheral nervous systems, as peak levels of naloxone are reached in rat brain in less than 30 min, with a half-life in plasma of 40 min (Berkowitz, Nqai, Hempstead, & Spector, 1975).

In summary, the present study confirms that opioids are an important neurochemical substrate for the development of pacing-induced conditioned partner preference in female rats. This type of preference depends on the contingency between rewarding stimuli (unconditioned stimuli) and the available cues (CSs). The nature of these cues can be olfactory alone (Coria-Avila et al., 2005) or a mixture of olfactory, auditory, and visual, as may differentiate two strains of males (Coria-Avila et al., 2006). In the monogamous female prairie vole, the development of pair bonding depends on DA and oxytocin. It is unknown what the effects of DA and oxytocin are on the conditioned partner preference observed in female rats. It is possible that sexual reward induced by paced copulation (unconditioned stimuli) may be accompanied by an augmentation of endogenous opioid activity that leads in sequence to increased DA and oxytocin activity. It may be that opioid activation underlies both pacing-related CPP and conditioned partner preference, but that DA and oxytocin are necessary for the development of conditioned partner preference, but not for CPP (given that administration of the DA receptor antagonist flupenthixol does not disrupt CPP; (Garcia Horsman & Paredes, 2004). The latter systems may be required to facilitate the contingency of reward and social recognition during exposure to male cues.

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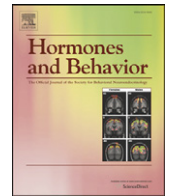
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# Clitoral stimulation induces conditioned place preference and Fos activation in the rat

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## ABSTRACT

The present study examined the ability of clitoral stimulation (CLS) to induce conditioned place preference (CPP) and Fos protein in the brain. Ovariectomized, hormone-primed Long-Evans rats were randomly assigned to receive either distributed CLS (1 stimulation every 5 s for 1 min prior to being placed in one distinctive side of a nonbiased CPP box for 2 min, after which the cycle of stimulation and CPP exposure were repeated for 4 more cycles, totaling 60 stimulations) or continuous CLS (1 stimulation per second for 1 min with 2 min in one side of the CPP box, repeated for 4 more cycles, totaling 300 stimulations). Two days later, females were placed into the other side of the CPP box without prior stimulation. CPP was tested after 5 sequential exposures each of CLS and no stimulation. Females given distributed stimulation developed a significant CPP whereas females given continuous stimulation did not. CLS induced Fos in hypothalamic and limbic structures, including the nucleus accumbens, piriform cortex, arcuate nucleus, and dorsomedial portion of the ventromedial hypothalamus, compared to no stimulation. However, distributed CLS induced more Fos in the medial preoptic area than continuous CLS or no stimulation. In contrast, continuous CLS induced more Fos in the posteroventral medial amygdala compared to no stimulation. These data indicate that CLS induces a reward state in the rat and a pattern of Fos activation in regions of the brain that process genitosensory input, incentive salience, and reward.

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## Introduction

The ability of female rats to control or “pace” the initiation and rate of copulation supports the development of a positive hedonic state that results in a conditioned place preference (CPP; Oldenburger et al., 1992; Paredes and Alonso, 1997; Paredes and Vazquez, 1999). Female rats will also selectively approach, solicit, and receive intromissions and ejaculations from males bearing an odor (e.g., almond) or strain cue (pigmented or albino) associated with paced copulation (Coria-Avila et al., 2005a,b, 2006). Both conditioned place and partner preferences are blocked by treatment with the opioid receptor antagonist naloxone during conditioning (Coria-Avila et al., 2008; Paredes and Martinez, 2001) whereas the dopamine antagonist flupenthixol blocks the development of conditioned partner preference for an odor, but not for strain cues (Coria-Avila et al., 2008) or conditioned place preference in female rats (García Horsman and Paredes, 2004). Naloxone infusions to regions of the hypothalamus (medial preoptic area (mPOA) or ventromedial hypothalamus (VMH)) or limbic system (medial amygdala (MEA) but not nucleus accumbens), block the development of pacing-induced CPP (García Horsman et al., 2008), indicating that opioid actions within those structures play an important role in pacing-induced sexual reward.

Despite knowledge of the putative neurochemical mechanisms involved in linking place or partner cues to sexual reward in female rats, very little is known about the role of sensory stimulation received during copulation in this regard. Such stimulation could be olfactory or genitosensory in nature, and could arise from simple exposure to males or from the distributed stimulation received during each paced (or otherwise controlled) mount with pelvic thrusting and intromission from the male. Indeed, the development of significant CPP for copulation can occur in female rats in conditions where pacing is not optimized (Meerts and Clark, 2007), suggesting that pacing or control per se is not necessarily the rewarding element in copulation, but rather the distribution of stimulation at some preferred interval that is rewarding (Jenkins and Becker, 2003). Moreover, one recent study showed that 15 distributed artificial vaginocervical stimulations (VCSs) induced a reward state in female rats (Meerts and Clark, 2009), indicating that olfactory or visual cues provided by the presence of a male are not necessary for sexual reward. It is not known whether other types of genitosensory stimulation received by female rats during copulation could support the development of a sexual CPP, or whether electrical stimulation of the nerves that innervate the genitosensory tract (pelvic, pudendal, hypogastric, vagus), might stimulate a reward state.

The clitoris is an important organ for genitosensory stimulation. In women, the clitoris is regarded as a gland that relays localized pleasurable sensory input (e.g., Giuliano et al., 2002; Masters et al., 1986; van Netten et al., 2008) and influences vaginal muscle function (Shafik

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et al., 2008). It has been studied in rats with regard to its innervation and vasculature during sexual arousal (Peters et al., 1987; Pacheco et al., 1989; Giuliano et al., 2001; Cruz et al., 2004) and its morphology in response to hormones (Hall, 1938; Munarriz et al., 2003; Pessina et al., 2006). However, its role in carrying sensory information during sexual activity in the female rat is currently unknown. However, the lordosis posture of the female facilitates the male's ability to mount with pelvic thrusting and intromission. This interaction stimulates the female's flanks, rump, tailbase, perineum, and perivaginal surfaces which include the clitoris (Pfaff et al., 1977), in addition to allowing the male to make penile intromission, which provides VCS. It is thus possible that clitoral stimulation in the rat could carry reward-related sensory inputs that are generated externally from the pelvic thrusting of the male during a mount and mounts with intromission.

In addition to CPP, paced copulation with intromission, or artificial distributed VCS, induces a faster termination of estrus (Coopersmith et al., 1996a,b; Erskine and Baum, 1982; Lodder and Zeilmaker, 1976; Pfau et al., 2000) and pseudopregnancy (Coopersmith and Erskine, 1994; Erskine et al., 2004; Lehmann and Erskine, 2004; Terkel, 1986). VCS activates the immediate-early gene product Fos in lumbar and sacral regions of the spinal cord, and in hypothalamic and limbic regions of the brain such as mPOA, posteriodorsal MEA (MEApd), bed nucleus of the stria terminalis (BNST), paraventricular nucleus (PVN), and VMH (Dudley et al., 1992; Erskine 1993; Pfau et al., 1993, 1996; Pfau and Heeb, 1997; Tetel et al., 1993; Wersinger et al., 1993). These regions also show differential activation in response to distributed VCS under different hormone priming regimens (Pfau et al., 1996). Olfactory stimuli associated with paced copulation also induce Fos in those brain regions (Coria-Avila and Pfau, 2007). One study using transneuronal tracing following pseudorabies virus injections to the clitoris has shown afferent input to lumbar and sacral spinal cord, and regions of the hypothalamus such as mPOA, PVN, and VMH (Marson, 1995), suggesting that clitoral stimulation (CLS) and VCS could carry a complex array of information relevant for reward and reproduction.

Given the lack of information on the functional role of the rat clitoris in sexual behavior, the present study asked whether different types of CLS (continuous vs. distributed) could induce a reward state of sufficient intensity to induce CPP. We also examined where those two types of CLS induced Fos within hypothalamic and limbic structures previously shown to be activated by VCS or paced copulation.

## Materials and methods

### *Animals and surgery*

Sexually naive female Long-Evans rats, weighing 200 to 250 g, were obtained from Charles River Canada, Inc., St. Constant, QC. Animals were housed in groups of two in shoebox cages in a colony room maintained on a reversed 12:12 h light/dark cycle (lights off at 08:00 h) at approximately 21 °C. Food and water were continuously available. Females were ovariectomized bilaterally through lumbar incisions under intraperitoneal (i.p.) injections (1 ml/kg of body weight) of ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (4 mg/ml) anesthetic mixed in a ratio of 4:3 respectively. All females were given 1 week of post surgical recovery and maintained for the duration of the experiment on hormone replacement by subcutaneous injections of estradiol benzoate (EB; 10 µg in 0.1 ml of sesame oil) 48 h and progesterone (P; 500 µg in 0.1 ml of sesame oil) 4 h prior to testing.

Long-Evans males from the same breeder were given 10 prior sexual experiences with sexually receptive females to generate maximal baseline rates of sexual responding ( $n = 20$  per group).

All animal procedures conformed to the guidelines of the Canadian Council on Animal Care, and were approved by the Concordia University Animal Research Ethics Committee.

### *CPP apparatus*

Conditioning was conducted in 8 identical tri-colored PVC plastic rectangular boxes (21 × 21 × 68 cm), each containing three chambers separated by guillotine doors (Med-Associates Inc; St. Albans, VT). The two large end chambers were separated by a smaller center choice chamber, which was used on the pre-exposure and test days. One of the main chambers had black walls and a wire mesh floor; the other had a white wall located across from the guillotine door and a stainless steel rod floor with larger spaces. All floors were raised 5 cm to reduce the accumulation of urine and feces. Through the use of a computer interface, time spent in each chamber was recorded by means of infrared beam crossings. In each of the two end chambers, there were two beams separated by 8 cm. A rat was considered to be in an end chamber if the beam furthest from the door was broken. If only the beam closest to the door was broken, the rat was said to be in the center choice chamber. During conditioning and testing, the room was kept in semidarkness with only a single lamp reflecting light off one wall of the room.

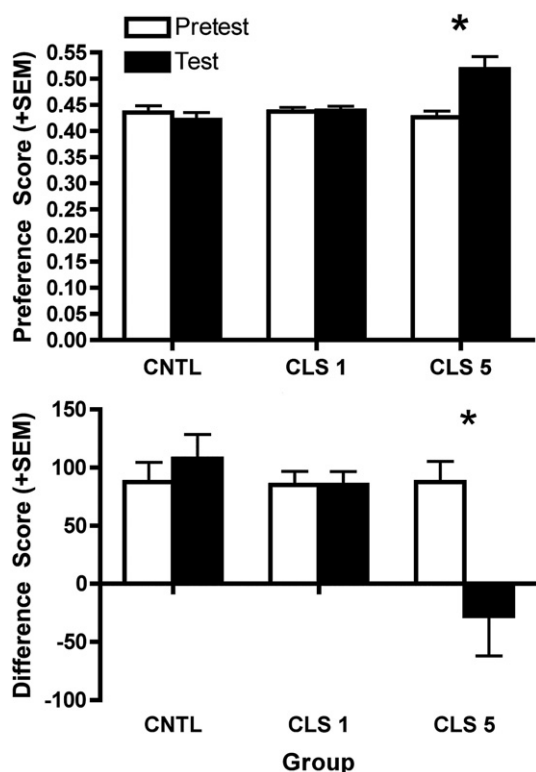
### *Conditioning procedure*

All conditioning and testing was conducted at 4-day intervals, 4 h after P injections, during the middle third of the rats' dark circadian cycle (as in Pfau et al., 1999). The place conditioning procedure consisted of three phases: pre-exposure, conditioning, and a final CPP test. During the pre-exposure phase, each female was placed in the center choice chamber of the CPP box with the guillotine doors removed to allow access to the entire apparatus for 15 min. The amount of time spent in each chamber was monitored and used to assess unconditioned preferences.

During the conditioning phase, females were randomly assigned to one of three stimulation conditions. The first group ( $n = 20$ ) received continuous clitoral stimulation (CLS 1). CLS was applied once per second over the course of 1 min, after which females were placed into the previously non-preferred side of the CPP box for 2 min, after which they were removed and given CLS again. The cycle of CLS for 1 min and placement into the CPP apparatus for 2 min was repeated for a total of 5 cycles which totaled 300 stimulations. A second group ( $n = 20$ ) received distributed CLS (CLS 5). This consisted of 1 CLS every 5 s for 1 min, after which females were placed in the previously non-preferred side of the apparatus for 2 min. The cycle of CLS and placement into the CPP apparatus was repeated for a total of 5 cycles which totaled 60 stimulations. A third group ( $n = 19$ ) received sham (CNTL) clitoral stimulation prior to placement into the CPP apparatus. Each of the conditioning sessions lasted a total of 15 min per animal. On alternate days females received general handling stimulation, but no clitoral stimulation, and were placed into the previously preferred side of the CPP apparatus.

Clitoral stimulations were given by the experimenter. The stimulations were made by lifting the base of the female's tail and lightly brushing the clitoris with one stroke of a small, soft-bristle number 4 paintbrush dabbed with K-Y<sup>®</sup> jelly. As with manually applied stimuli, the intensity was likely not exactly the same every time; however care was taken to avoid brushing too strongly and to avoid stimulating the vagina. Sham stimulations were done by lifting the base of the tail but not touching the clitoris. Stimulation parameters were chosen specifically to examine distributed vs. continuous sensory stimulation of the clitoris. The 5-s interval was chosen on the basis of unpublished observations that fully primed female rats in bilevel chambers receive mounts with pelvic thrusting in bouts with approximately 5 s between successive mounts before an intromission is achieved. The 1-s interval was therefore chosen to provide continuous stimulation relative to the more distributed stimulation that occurs under normal circumstances.

After 10 conditioning sessions, 5 reinforced and 5 non-reinforced, a final 15-minute preference test was conducted 24-hours after the last conditioning trial in the same manner as the initial preference test.



**Fig. 1.** Mean  $\pm$  SEM preference scores (top) and difference scores (bottom) on the pre-test (pre-conditioning, white bars) and test (post-conditioning, black bars) for female rats that received sham stimulation (CNTL), continuous stimulation (CLS 1), or distributed stimulation (CLS 5) ( $n=20$  per group). Rats receiving distributed stimulation displayed CPP for the compartment associated with that treatment. This coincides with a significant decrease in difference score,  $*p<0.05$ .

#### Statistical analysis for CPP

Pre-exposure and CPP test outcomes were determined by the total time spent in each chamber. To ensure that there was no initial bias of the chambers, a within-subjects repeated measures ANOVA was used to assess the effect of Chamber. Based on [Paredes and Alonso \(1997\)](#) there were two criteria used to determine whether place preference conditioning was successful. A preference score (time in the reinforced compartment / (time in the reinforced compartment + time in the non-reinforced compartment)) was expected to increase significantly between pre-test and post-test. To account for the possibility that the rats are spending most of their time in the grey (neutral) chamber, a difference score was also calculated (difference between the time spent in the non-reinforced cage and time spent in the reinforced cage) and was expected to decrease significantly after conditioning.

Preference scores and difference scores were analyzed for each stimulation type across pre-test and post-test using a paired-samples *t*-test to examine the change in scores prior to, and following, clitoral stimulation ([Dominguez-Salazar et al., 2005](#); [Meerts and Clark, 2007](#); [Meerts and Clark, 2009](#)). As per [Paredes and Alonso \(1997\)](#) a significant increase in the preference score and a significant decrease in the difference score are the main criteria when a conditioned place preference is developed.

#### Fos activation by CLS

One week following the CPP experiment the same females were primed with EB and P as above, placed in a Plexiglas cage identical to their home cages (36 cm  $\times$  26 cm  $\times$  19 cm), and allowed to acclimate for 20 min, after which females were randomly re-assigned to one of the three CLS groups described above (to eliminate any previous

effects of conditioning on the activation of Fos by CLS). Females remained in the cage and were sacrificed 1-hour following the initiation of CLS and perfused to prepare tissue for immunocytochemistry.

#### Histology and immunocytochemistry

All rats were sacrificed by overdose of sodium pentobarbital (120 mg/kg, i.p.) and perfused intracardially with phosphate buffered saline (250 ml) and followed by 4% paraformaldehyde in 0.1 M phosphate buffer (250 ml). Brains were extracted and placed in fresh 4% paraformaldehyde for 4 hours and then overnight in 30% sucrose at 4 °C.

Frozen coronal brain sections (40  $\mu$ m) were cut from each brain through the medial prefrontal cortex and to the level of the ventral tegmental area on a cryostat. The sections were washed in cold Tris-buffered saline (TBS) and incubated sequentially with 30% hydrogen peroxide ( $H_2O_2$ ) in TBS for 30 min at room temperature with 3% Normal goat serum (NGS) in 0.05% Triton-TBS for 90 min at 4 °C, with rabbit polyclonal anti-Fos (Fos ab5, Calbiochem, Mississauga, ON; diluted 1:40,000) in 0.05% Triton-TBS with 3% NGS for 72 h at 4 °C, with biotinylated goat anti-rabbit IgG (Vector Laboratories Canada, Burlington, ON; 1:200) in 0.05% Triton-TBS with 3% NGS for 1 h at 4 °C, and avidin-biotinylated-peroxidase complex (Vectastain ELITE ABC KIT, Vector Laboratories Canada; diluted 1:55) for 2 h at 4 °C. Sections were washed in TBS (3  $\times$  5 min) between each incubation. Immunoreactions were stained by sequential treatments at room temperature with 50 mM Tris for 10 min, 3,3'-diaminobenzidine (DAB) in 50 mM Tris (0.1 ml of DAB/Tris buffer, pH 7.8) for 10 min, and 8% nickel chloride (400  $\mu$ l per 100 ml of DAB/Tris buffer +  $H_2O_2$ ). Sections were then mounted on gel-coated slides and allowed to dry, then dehydrated in alcohols (70%, 90% and 100%, 10 min each, respectively), cleared in Xylenes (2 h), coverslipped, and examined under a Leitz light microscope.

#### Histological and statistical analyses

Tissue sections were examined at 40 $\times$ , and the number of Fos-positive cells was counted bilaterally for each region from 3 sections per area per rat using Scion Image 1.63. The regions were defined by using the atlas of [Paxinos and Watson \(1986\)](#): mPOA (medial preoptic area), VMH (ventromedial hypothalamus), MeA (medial amygdala), BLA (basolateral amygdala), NAcc (nucleus accumbens), VTA (ventral tegmental area), PirCtx (piriform cortex), LH (lateral habenula), BNST (basal nucleus of the stria terminalis), and ArcN (Arcuate Nucleus). A mean was calculated for each area in each rat from the 3 bilateral sections per area, and statistical analyses was conducted for 5 rats in each stimulation group (15 sections per group for each brain area, as

**Table 1**

Average numbers of Fos-positive cells in different hypothalamic and limbic structures as a function of sexual stimulation (mean  $\pm$  SEM).

| Area   | Stimulation condition |                   |                 |
|--------|-----------------------|-------------------|-----------------|
|        | CNTL                  | CLS 1             | CLS 5           |
| Nacc   |                       |                   |                 |
| shell  | 45.9 $\pm$ 9.7        | 38.1 $\pm$ 8.9    | 64.9 $\pm$ 17.5 |
| core   | 33.2 $\pm$ 9.5        | 56.6 $\pm$ 18.9   | 53.4 $\pm$ 14.6 |
| mPOA   | 16.8 $\pm$ 1.8        | 17.05 $\pm$ 1.9   | 59.7 $\pm$ 8.2* |
| LS     | 17.7 $\pm$ 5.9        | 28.7 $\pm$ 12.3   | 37.9 $\pm$ 7.3  |
| BNST   | 6.7 $\pm$ 4.5         | 12.8 $\pm$ 5.6    | 19.7 $\pm$ 4.3  |
| VMH    | 32.3 $\pm$ 6.3        | 42.7 $\pm$ 14.6   | 29.1 $\pm$ 2.6  |
| Arc    | 37.0 $\pm$ 6.6        | 40.4 $\pm$ 7.5    | 50.7 $\pm$ 4.5  |
| MEApv  | 39.4 $\pm$ 6.5        | 123.2 $\pm$ 27.2* | 82.6 $\pm$ 15.6 |
| LH     | 20.8 $\pm$ 5.4        | 28.5 $\pm$ 7.7    | 27.7 $\pm$ 5.8  |
| PirCtx | 91.0 $\pm$ 10.8       | 140.6 $\pm$ 26.5  | 83.0 $\pm$ 13.1 |
| VTA    | 57.0 $\pm$ 11.0       | 47.0 $\pm$ 9.2    | 56.4 $\pm$ 20.2 |

CNTL, sham stimulation; CLS 1, continuous clitoral stimulation; CLS 5, distributed clitoral stimulation ( $n=5$  per group).

\*  $p<0.05$  from CNTL.



we have done previously for Fos induction by VCS; Pfaus et al., 1993, 1996). A between-subjects analysis of variance (ANOVA) was performed to assess differences in Fos induction between females that received CLS 1, CLS 5, and sham stimulation. For each significant ANOVA, post hoc analysis of mean differences was made using the least significant difference (LSD) method,  $p < 0.05$ .

## Results

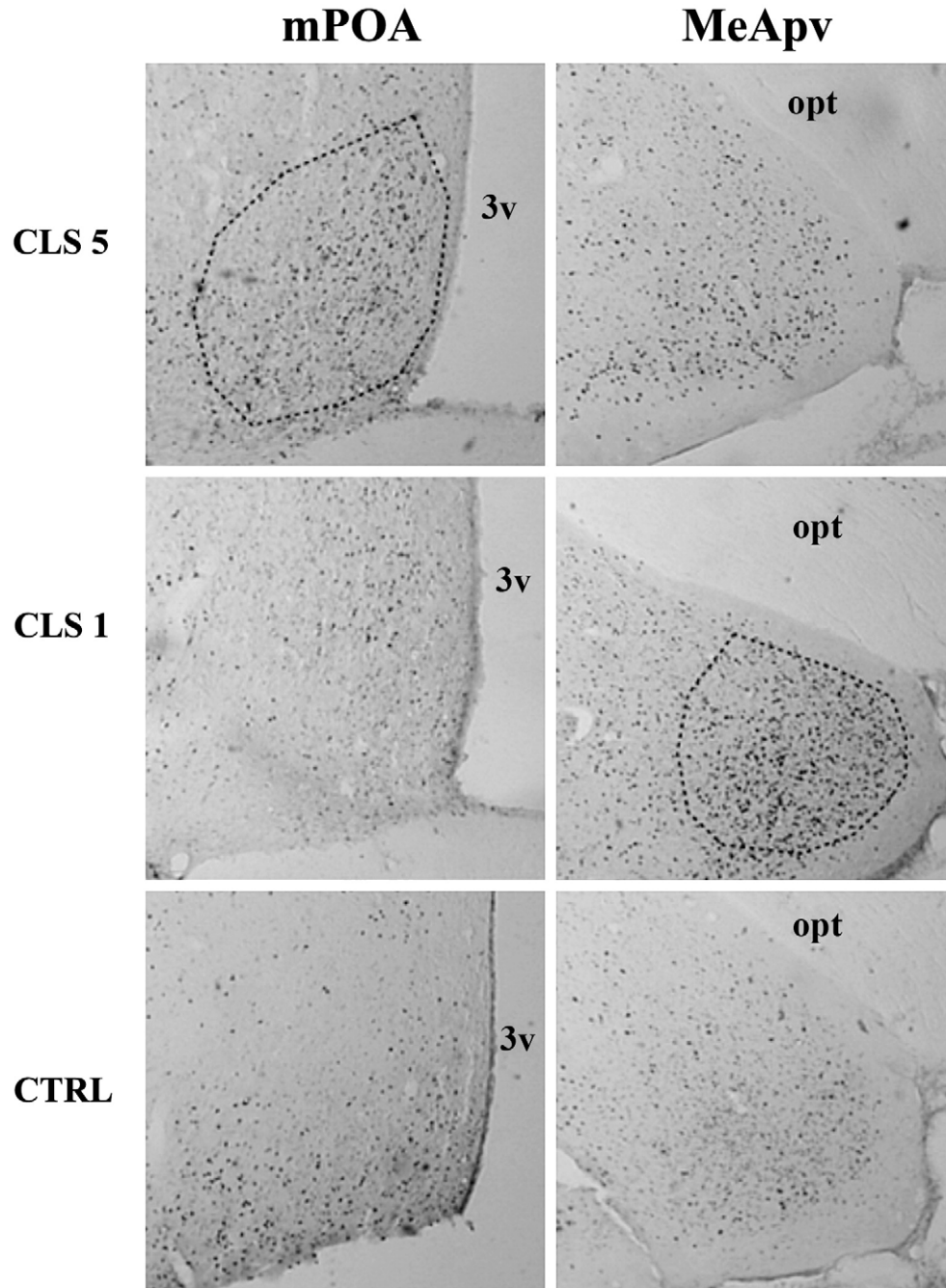
### CPP associated with manual clitoral stimulation

Rats that received CLS 5 (distributed CLS) showed both a significant increase in preference scores at the post-test following

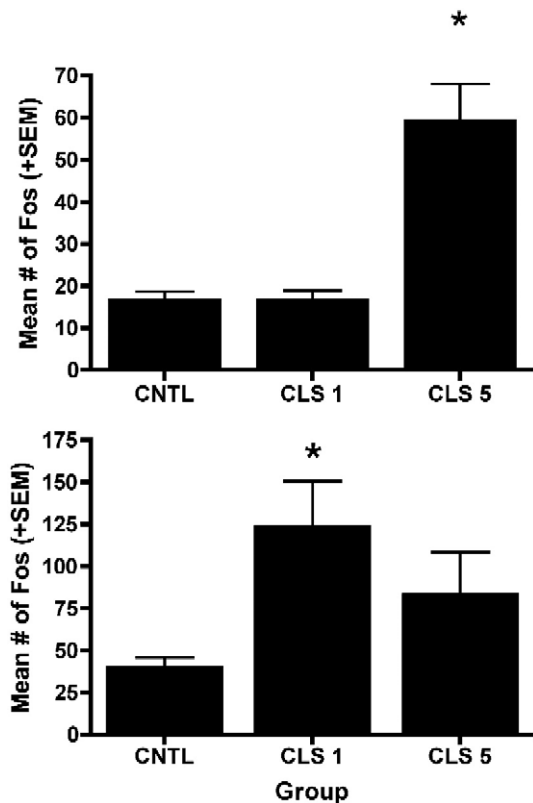
the conditioning trials ( $t(19) = -3.394$ ,  $p < 0.05$ ) and a significant decrease in difference scores ( $t(19) = 3.213$ ,  $p < 0.05$ ) (Fig. 1). Rats that received CLS 1 (continuous CLS) or sham stimulation did not show any significant change in preference or difference scores.

### Fos induction by CLS

Examination of brain sections from the medial prefrontal cortex back to the level of the ventral tegmental area of the midbrain revealed clusters of Fos in areas associated with tactile genitosensory stimulation. Specifically Fos was found in and around the mPOA, VMH, MEA, BLA, NAcc, VTA, PirCtx, LH, BNST, and ArcN (see Table 1). Although clusters of Fos were present in these areas only the mPOA



**Fig. 2.** Representative digitized images of the mPOA (left side) and MEApv (right side) taken at 40 $\times$ . Areas that were analyzed for Fos IR are outlined in black. Fos activation for the mPOA as a function of stimulation type (CLS 5: distributed; CLS 1: continuous, CTRL: sham stimulation) are displayed along the left side and Fos activation for the MEApv is displayed along the right side. 3 V, third ventricle; opt, optic tract.



**Fig. 3.** Mean number of Fos cells/side (+SEM) as a function of different types of CLS are shown for the mPOA (top) and MEApv (bottom) for females receiving no stimulation (CNTL), continuous stimulation (CLS 1), and distributed stimulation (CLS 5) ( $n=5$ /stimulation condition). Rats receiving distributed stimulation showed a significantly greater number of Fos cells in the mPOA than rats receiving continuous stimulation and no stimulation. Rats receiving continuous stimulation showed a significantly greater number of Fos cells in the MEApv compared to no stimulation,  $*p<0.05$ .

and the MEApv had significantly different levels of Fos in response to the type of CLS received.

#### Fos in the mPOA

In the mPOA (Fig. 2), distributed CLS produced a clustered pattern of activation located around the medial, central, and most ventral aspect of the medial preoptic nucleus, with a scattering of induction in the medial, dorsal aspect of this nucleus. This pattern of activation was not induced by continuous or sham CLS. For the induction in the mPOA, the ANOVA detected a significant main effect of CLS,  $F(2,15) = 19.388$ ,  $p<0.0001$ . Post-hoc comparisons revealed that only distributed CLS induced Fos over that observed in the continuous CLS and control groups (Fig. 3).

#### Fos in the MEA

In the MEA (Fig. 2), both distributed and continuous CLS induced a cluster of Fos activation located within the ventromedial portion of the MEApv only, with scattered Fos cells radiating laterally away from this cluster. Little to no Fos cells were observed in other regions of the MEA including the MEApd. For the induction in the MEApv, the ANOVA detected a significant main effect of CLS,  $F(2, 15) = 5.104$ ,  $p<0.05$ . Post-hoc comparisons revealed that continuous CLS produced a significantly greater amount of Fos activation in the MEApv compared to the control group (Fig. 3).

#### Discussion

The present study demonstrates both a behavioral and neural response to clitoral stimulation in the rat. This study identified manual

distributed CLS as a strong inducer of sexual CPP and also that CLS induces Fos protein within regions of the brain generally associated with tactile genitosensory stimulation when females are in a receptive state. Further, the ability of CLS to induce Fos-immunoreactivity in the mPOA and the MEApv is dependant on the type of CLS that is received by the female, with distributed CLS resulting in a significantly larger number of cells with Fos in the mPOA, and continuous CLS resulting in a significantly larger number of cells with Fos in the MEApv.

We suggest that the rat clitoris, like that of the human, carries reward-related tactile stimulation during sexual interaction to the brain. In the rat, however, a distributed form of stimulation is necessary to induce reward of sufficient intensity to support CPP, similar to recent studies using VCS as the tactile cue (Meerts and Clark, 2009). We speculate that the clitoris may well be stimulated by pelvic thrusting during mounts, intromissions and ejaculations during copulation. Thus, together with VCS, CLS may form an important component of sexual reward.

Distributed CLS induced a significantly greater number of Fos cells in the mPOA compared to continuous CLS and controls. It has been shown previously that mechanical stimulation of the cervix excites neurons in the mPOA (Haskins and Moss, 1983). Further, expression of Fos immunoreactivity has also been shown in this region in response to vaginocervical stimulation both from a male partner during copulation and from artificial manual stimulation (Pfaus et al., 1993, 1996; Tetel et al., 1993, 1994a; Wersinger et al., 1993). It is interesting that different subregions of the mPOA appear to be activated by CLS and VCS. Whereas VCS activates a central and medial region (Pfaus et al., 1996), CLS activates a more ventral region of the mPOA. This suggests a specific area of activation for clitoral stimulation. This also suggests that clitoral stimulation during copulation may be independent of other forms of tactile stimulation (e.g., flank stimulation), and may contribute information to the mPOA about the timing of pleasurable anogenital sensations. A similar effect was observed within the MEA. Previous work has shown that VCS activates Fos in cells of the MEApd in a linear fashion, with more activation in response to increasing amounts of stimulation (Erskine, 1993; Pfaus et al., 1993, 1996; Polston and Erskine, 1995; Tetel et al., 1993). However the present study did not find Fos activation in the MEApd, but rather in the posteroventral MEA (MEApv) in response to continuous CLS. This suggests that specific regions of the MEA respond to different forms of genitosensory stimulation with VCS activating the MEApd and CLS activating the MEApv. The idea that separate regions of the amygdala contribute differentially to female sexual behavior is not new. Lesion studies of the corticomedial amygdala have reported a reduction of the lordosis response whereas lesions of the basolateral and lateral nuclei of the amygdala facilitate lordosis (Masco and Carrer, 1983; McGinnis et al., 1978; Nance et al., 1974).

Neural implants of estrogen to the MEApd have been shown to facilitate lordosis (Lisk and Barfield, 1975) and VCS in females primed with estrogen results in an increase in Fos-immunoreactivity in this same region but only to threshold levels in the MEApv (Pfaus et al., 1996). Lidocaine infusions to the medial amygdala, which include regions around the MEApv and MEApd, block the induction of pseudopregnancy when administered both before and after receiving intromissions, which has been suggested to provide evidence that the MEApd may modulate neuroendocrine and behavioral functions that are activated by VCS (Coopersmith et al., 1996a,b; Pfaus et al., 1996). It is possible that activation of the MEApv by a large amount of CLS may also contribute to the modulation of neuroendocrine and behavioral functions.

Like distributed CLS, distributed VCS induces significant CPP (Meerts and Clark, 2009). There is considerable overlap in the sensory innervation of both clitoris and vagina, including the proximal perineal branch of the sacral plexus that innervates the skin between the clitoris and vagina, the viscerocutaneous branch of the pelvic nerve, and the distal perineal branch of the pudendal nerve that

innervates the skin between the vagina and anus, and the distal perineal branch of the pudendal nerve and the proximal perineal branch of the sacral plexus that innervates the skin lateral to the vaginal opening (Cruz et al., 2004). Cruz et al. (2004) suggested that such overlap might help to ensure that somatosensory information necessary to trigger reproductive and non-reproductive reflexes actually reaches the spinal cord and brain. It is therefore possible that a mount with intromission and pelvic thrusting from a male rat activates the clitoris, vagina, and cervix of the female, thus activating reward- and reproductive-related circuits of the spinal cord and brain simultaneously, including the mPOA and VMH. It is important to note that although CLS and VCS are strong inducers of CPP and thus sexual reward during copulation, it is possible that pacing copulation or distributing stimulation to induce pseudopregnancy drives reward. Females may pace the behavior to ensure that enough intromissions occur to induce ejaculation and pregnancy, and that this may be “more” rewarding than the behavior itself. It is also possible that the reward value of the somatosensory stimulation drives the reproductive consequences, or that the two occur by simultaneous activation of reward- and reproductive-related brain systems. It would be interesting to examine pseudorabies virus traces at different times after injection, given that the virus travels approximately 1 synapse per day. It would also be instructive to perform selective unilateral lesions of reward- (e.g., VTA, NAcc, mPOA, VMH) and reproductive- (VMH, mPOA, MEApd) related brain regions prior to the pseudorabies virus injection, to examine the actual synaptic flow of afferent information.

It is also important to note that the stimulations given in this experiment differed not only in terms of distribution but the total number received during a given conditioning session. Females in the distributed group received a total of 60 clitoral stimulations during the 15-minute period in contrast to the 300 stimulations received by females in the continuous group. It is therefore possible that CPP developed as a result of the total number of stimulations rather than the pattern of distribution. However, in this case, the results of the current study would suggest that more is less in terms of the activation of CPP and Fos in the mPOA, a site that plays a critical role in linking sexual reward to appetitive sexual responses, such as solicitations in females (Hoshina et al., 1994; Paredes, 2009). This is in contrast to studies that have revealed a positive relationship between the amount or intensity of VCS and the induction of Fos in various brain regions (Erskine, 1993; Pfau et al., 1993, 1996; Tetel et al., 1993; Wersinger et al., 1993), and strongly suggests that an optimal timing exists for the conduction of reward-related CLS.

Finally, the parameters for the stimulation conditions used in the present study were chosen specifically to examine continuous vs. distributed CLS. They were based on unpublished observations from our laboratory that mounting stimulation often occurs in bouts that consist of several trains of pelvic thrusting prior to intromission and dismount. We do not yet know whether the stimulation parameters used here were in any way optimal for particular behavioral end-points (e.g., CPP, partner preference, estrus termination, etc.). It is important to acknowledge that there is inconsistency throughout the literature regarding artificial VCS and its method of application, the intensity of the stimulation, and the timing which makes it difficult to approximate what a female rat would experience in the real world. Attempts have been made to mimic the timing of intromissions to what is observed on average during a copulatory bout (Meerts and Clark, 2009) or to compare Fos activation from differing amounts of artificial VCS to what is seen following an hour of copulation (Pfau et al., 1993). However, it is not known whether the same temporal parameters used for artificial VCS would induce a similar pattern of Fos induction with CLS. The present study found statistical trends toward activation of other regions (e.g., BNST, LS, PirCtx), and it may be the case that other patterns of CLS will result in significant increases (or decreases) in Fos induction in those regions. Further work will need to address the optimization of CLS, although it may be the case that individual

differences will exist for female rats in the optimization of CLS for reward, as is acknowledged in women (Masters et al., 1986).

In summary, the present study confirms that clitoral stimulation is a form of tactile genitosensory stimulation that has a reinforcing value strong enough to induce CPP. This type of conditioned preference occurs when the stimulation is distributed rather than continuous, and induces Fos-immunoreactivity in areas previously associated with the processing of genitosensory stimulation for reward, estrus termination, and pseudopregnancy. Throughout a copulatory bout females typically receive both CLS and VCS from mounts. CLS likely occurs when the male's testicles or pelvis come into contact with the clitoris during pelvic thrusting, whereas VCS occurs during intromissions and ejaculations. This would stimulate both the vagina and cervix and also the clitoris through internal contact with the nerves that feed into it. Because the innervation of the clitoris and vagina share common sensory pathways in humans and other animals (e.g., Foldes and Buisson, 2009), receiving both distributed CLS combined with VCS may be an important sensory experience that links reward with reproduction.

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# Conditioned mate-guarding behavior in the female rat

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## HIGHLIGHTS

- Female rats can display mate guarding behavior.
- Experience with sexual reward shifts sexual strategies in the female rat.
- Hypothalamic and limbic brain regions are differentially activated.

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## ABSTRACT

Female and male rats are often described as having a promiscuous mating strategy, yet simple Pavlovian conditioning paradigms, in which a neutral odor or strain-related cues are paired with preferred sexual reward states during an animal's first sexual experiences, shift this strategy toward copulatory and mate preferences for partners bearing the familiar odor or strain cue. We examined whether female rats given exclusive rewarding copulation with one particular male would display mate-guarding behavior, a strong index of monogamous mating. Ovariectomized, hormone-primed female Long-Evans rats were given their first 10 paced sexual experiences at 4-day intervals with a particular unscented male of the same strain. A final test was conducted in an open field 4-days later in which the primed, partnered female was given access to the male partner and a fully-primed competitor female. In this situation, the partnered females mounted the competitor female repeatedly if she came near the vicinity of the male. This behavior prevented the male from copulating with the competitor, and was not displayed if partnered females could not pace the rate of copulatory behavior efficiently during the training trials, nor was it displayed by the competitor females. Fos expression was examined in both the partnered and competitor females after the final open field test. Partnered females had significantly higher expression within the supraoptic nucleus and nucleus accumbens shell compared to partnered females that did not develop this behavior or competitor females. These data show that females engaged in paced copulation with the same male display mate-guarding when exposed to that male and a competitor female. Increased activation of the SON and NAc may underlie this behavior.

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## 1. Introduction

Mating strategies are specific sets of interactions that determine with whom and how often an individual mates. Several mating strategies can be observed in mammals, and each is accompanied by its own set of defining behaviors. For example, in nature, the rat is described as having a promiscuous mating strategy that is characterized by cooperative and promiscuous group mating in both males (polygamy) and females (polygyny). On the opposite end of the spectrum are prairie voles, one of the few mammals that display social monogamy. Prairie voles are considered unique in that they form preferential

associations with a specific sexually mature individual of the opposite sex, known as a pair bond. Pair bonds are characterized by selective contact and affiliation with the partner over a stranger vole, biparental care of the young, nest sharing, and mate-guarding behavior [1]. Thus, although voles and rats are closely related, they have adapted very different mating strategies due presumably to differences in environmental factors such as population density, sex ratio, and predator range, factors that are critical for the development of social monogamy in the prairie vole [2,3].

The flexibility of sexual and reproductive strategies in the rat has been revealed in laboratory studies that employ Pavlovian conditioning paradigms [4–7]. When neutral odors (e.g., wintergreen, lemon, almond) are paired with copulation to ejaculation, male rats form a preference to ejaculate with a familiar partner over an unfamiliar one, a phenomena referred to as a conditioned ejaculatory preference [6,7]. Female rats allowed to control or pace the initiation and rate of

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copulation form conditioned place preferences for distinctive environments paired with the postcopulatory reward state [5]. Paced copulation also supports the conditioning of sexual partner and mate preferences in female rats [4] as measured by selective solicitations and copulations with familiar males relative to novel males in an open field, and a selective preference to receive the familiar male's ejaculations. Thus, rats are capable of displaying rudiments of monogamous sexual partner and mate preference despite being an allegedly promiscuous species. Conversely, both male and female rats learn to avoid partners bearing an odor paired with sexual nonreward. In those studies, rats are paired with a sexually nonreceptive partner bearing an odor, or are administered the opioid receptor antagonist drug naloxone, prior to their first several sexual experiences. Rats are given alternating access to unscented sexually receptive partners (or administration of saline prior to unscented sexually receptive partners). On a final test, rats are given access to two receptive partners, one scented and the other unscented. In both cases, males and females copulate selectively with the unscented partner [8]. Thus, rats can learn to modify their sexual strategy depending on early sexual learning. However, how far the mating strategy of the rat can be shifted by early sexual experience remains elusive.

The present study investigated whether female rats that have exclusive access to a single male during their first paced sexual experiences might display mate-guarding behavior when paired with that male and a competitor female in an open field. We also examined whether the development of such behavior alters the pattern of expression of the immediate-early gene product Fos in the brain of those females following this interaction compared to the competitor females or partnered females that received nonpaced early sexual experience with a male. Comparative studies between monogamous and non-monogamous vole species have led to the identification of brain regions that are involved in monogamous responding in voles, including the activation of oxytocin and vasopressin systems in the paraventricular and supraoptic hypothalamic nuclei, the mesolimbic dopamine system, and regions such as the ventral pallidum that integrate those systems into behavioral output [9–14]. The activation of these and other regions related to partner preference, sexual reward, and appetitive sexual responding were examined using Fos immunocytochemistry following the open-field test.

## 2. Materials and methods

### 2.1. Animals and surgery

Sexually naïve Long–Evans female rats (200–250 g) were obtained from Charles River Canada (St-Constant, QC, Canada). Animals were housed in shoebox cages in groups of two in a colony room on a reversed 12:12 h light/dark cycle at approximately 21 °C and given free access to food and water. Female rats were ovariectomized (OVX) bilaterally via lumbar incision. Prior to surgery, female rats were anesthetized using a 1 ml/kg intraperitoneal injection of ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (4 ml/kg), mixed in a ratio of 4:3 respectively. Female rats were given 1 week to recover from the procedure prior to the conditioning trials. Throughout the duration of the experiment, female rats were maintained on hormone replacement by subcutaneous injections of estradiol benzoate (EB; 10 µg in 0.1 ml of sesame oil) 48 h prior to testing and progesterone (P; 500 µg in 0.1 ml of sesame oil) 4 h prior to testing.

Sexually naïve male rats (300–350 g) were also obtained from Charles River Canada (St-Constant, QC, Canada). They were housed in group cages (4 animals per cage) and housed under conditions identical to those of the female rats.

All animal procedures complied with the guidelines of the Canadian Council on Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

### 2.2. Conditioning apparatus

Conditioning occurred in Plexiglas unilevel pacing chambers (38 cm H × 60 cm W × 38 cm deep) with wire-mesh floors covering a layer of bedding [4]. Chambers were bisected by a Plexiglas divider with four holes cut into the bottom which were large enough for the female to crawl through but too small for the male to crawl through [4,15,16].

### 2.3. Conditioning procedure

Conditioning sessions occurred at 4-day intervals, 4 h after P injections, during the middle third of the rats' circadian cycle (lights off at 08:00). Females were assigned randomly to one of 2 cohorts. Cohort 1 consisted of self-paced paired and self-paced unpaired females. Cohort 2 contained non-self-paced paired and non-self-paced unpaired (N = 12/group) females. All paired females copulated with the same male across all trials, whereas unpaired females copulated with a variety of males across all trials. Males were placed onto one side of the conditioning chambers and allowed a 5-minute habituation period before each trial. Females were then placed into the opposite side of the conditioning chamber and allowed to have paced sex with the male for 20 min. Each group received 10 trials, which were all recorded on video and scored for mate-guarding behavior.

A third cohort of animals was run following the same protocol, however, self-paced (N = 15 paired + 15 unpaired) and non-self-paced (N = 16 paired + 16 unpaired) groups were run within the same test session so that a direct comparison could be made between the two conditions.

### 2.4. Mate-guarding test

Four days after the final conditioning trial each mate-guarding was assessed using an open-field (123 cm × 123 cm × 46 cm) with a thin layer of bedding [4]. Each open-field contained a paired female with her corresponding male and an unpaired competitor female. Before the test, males were placed into the open field for a 5-minute habituation period, after which both the paired and unpaired females were placed into the open-field at 2 diagonal corners. Rats were allowed to copulate freely for a 1-h period. After the open-field test half of female rats (N = 6/group) were perfused and their brains were collected to examine Fos induction as a function of the behavior both of these groups displayed. The remaining animals underwent 2 reconditioning trials, which followed the same protocol as the conditioning trials. After the second reconditioning, the female rats were exposed to the pacing chamber alone for 1 h and then perfused so their brains could be examined for Fos expression in response to the contextual cues alone.

All open-field tests were recorded on video and scored afterward using a computerized event recorder customized for rat sexual behavior in an open-field [17]. The frequency of solicitations, hops and darts, and defensive responses were recorded, as were the incidents and reflex magnitudes of lordosis when males mounted. Mate-guarding behavior appeared as female–female mounting, typically initiated by the paired female toward the unpaired female competitor. Interceptions and time spent near the male were also recorded for both females.

### 2.5. Perfusions

Animals were euthanized with an overdose of sodium pentobarbital (120 mg/kg) administered via intraperitoneal injection. They were then perfused intracardially with 250 ml of phosphate-buffered saline (PBS) followed by 250 ml of 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 4 h. After which brains were placed into a 30% sucrose solution for 48 h then flash frozen and stored at –80 °C until slicing.



## 2.6. Tissue preparation and immunocytochemistry

Brains were divided into 4 groups: Both paired and unpaired females perfused after the open-field, and paired and unpaired females perfused after reconditioning ( $N = 5/\text{group}$ ). Using a freezing microtome, sections were cut at 30  $\mu\text{m}$  and divided into three series, one of which was used for immunocytochemistry to label for Fos expression. The sections were washed in cold Tris-buffered saline (TBS) and incubated first with 30% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in TBS for 30 min at room temperature followed by 3% normal goat serum (NGS) in 0.05% Triton-TBS for 90 min at 4 °C, with rabbit polyclonal anti-Fos (Fos ab5, Calbiochem, Mississauga, ON; diluted 1:40,000) in 0.05% Triton-TBS with 3% NGS for 72 h at 4 °C, with biotinylated goat anti-rabbit IgG (Vector Laboratories Canada, Burlington, ON; 1:200). Sections were next incubated sequentially in 0.05% Triton-TBS with 3% NGS for 1 h at 4 °C, and avidin-biotinylated-peroxidase complex (Vectastain ELITE ABC KIT, Vector Laboratories Canada; diluted 1:55) for 2 h at 4 °C. Sections were washed in TBS ( $3 \times 5 \text{ min}$ ) between each incubation. Immunoreactions were stained by sequential treatments with 50 mM Tris for 10 min, 3,3'-diaminobenzidine (DAB) in 50 mM Tris (0.1 ml of DAB/Tris buffer, pH 7.8) for 10 min, and 8% nickel chloride (400  $\mu\text{l}$  per 100 ml of DAB/Tris buffer +  $\text{H}_2\text{O}_2$ ) all at room temperature. Reaction was stopped by rinsing ( $3 \times 10 \text{ min}$ ) in PBS.

Following immunocytochemistry, sections were mounted onto gel-coated slides and cover slipped. Cover slipping procedure started with sequential washes of distilled water followed by washes in 70%, 90%, and 100% alcohol each at room temperature for 1 minute duration. Slides were then submerged in Xylenes for 2 h at room temperature after which they were cover slipped using Permount solution.

Pictures of brain regions were taken using an Olympus light microscope at 10 $\times$  magnification using Q-Capture pro software. Brain regions were defined using the atlas of Paxinos and Watson [18]. Fos positive cells were then counted using Image-J technology within the Ca fields and dentate gyrus of the hippocampus (Hc, plates 28–36), central nucleus of the amygdala (CeC, plates 25–31), nucleus accumbens shell (NAc, plates 10–15), supraoptic nucleus (SON, plates 21–25), paraventricular nucleus (PVN, plates 25–26), medial preoptic area (mPOA, plates 18–24), arcuate nucleus (Arc, plates 26–35), and ventral medial hypothalamus (VMH, plates 26–32). Five slices were counted per animal. These slices represented each area rostrally to caudally and were matched among animals to ensure site specificity. Counts were done bilaterally in a total of 5 animals per group that were chosen at random. Total number of Fos positive cells was counted on each section and added together to represent a mean total count per region per animal.

## 2.7. Statistical analyses

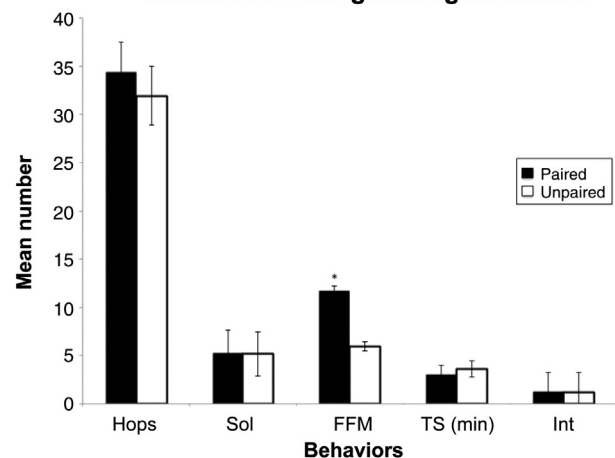
Mate-guarding behavior in the open-field was assessed using independent sample  $t$ -tests since group-specific comparisons were planned ahead of time. Cohen's  $d$  was used to assess effect size. The data collected from Fos-IR cells were analyzed using a one-way analysis of variance (ANOVA). Eta squared was used as a measure of effect size.

## 3. Results

### 3.1. Mate-guarding behavior

An independent sample  $t$ -test revealed that paired female rats in the self-paced condition (cohort 1) showed a significant increase in female–female mounting,  $t(44) = -2.174$ ,  $p = .035$ ,  $d = .641$ , compared to unpaired female rats from the same condition. This increase in female–female mounting was not seen in the non-self-paced condition,  $t(42) = .305$ ,  $p = .762$  (Fig. 1). Other behaviors such as interceptions, solicitations, aggression, time spent in proximity to the male, and the number of times initiating contact with the male, did not reach statistical significance.

## Sexual and mate-guarding behaviors



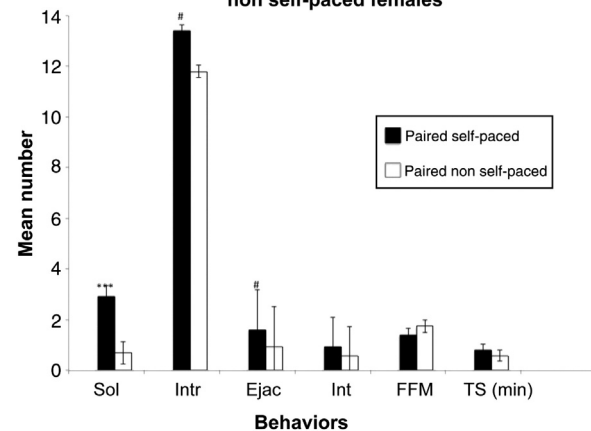
**Fig. 1.** Paired female rats that were allowed to pace the rate of sexual interaction displayed mate-guarding behavior as expressed by agonistic female–female mounting (FFM) during the open-field test. Behaviors include hops, solicitations (Sol), female–female mounting (FFM), time spent in proximity to the male (TS), and interceptions (Int). Data are means  $\pm$  SEM. \* $p < 0.05$ .

Independent sample  $t$ -tests were also used to look at behavioral differences between the paired females in both the self-paced and non-self-paced conditions (cohort 3). This statistical analysis revealed that paired females in the self-paced condition solicited the male more frequently than the paired females from the non-self-paced condition,  $t(29) = 4.650$ ,  $p = .000$ ,  $d = 1.652$ . Although statistical significance was not reached, there was a trend in favor of paired females in the self-paced condition receiving more ejaculations than paired females in the non-self-paced condition,  $p = .056$ ,  $d = .715$ , and also, more intromissions,  $p = .054$ ,  $d = .278$  (Fig. 2). Interceptions, hops and darts, aggression, female–female mounting, time spent in proximity to the male, and number of times initiating contact with the male were also quantified, but did not approach statistical significance.

### 3.2. Brain activation

A one-way ANOVA was used to look at differences in Fos expression between paired and unpaired females after, both, the open-field and exposure to the pacing chamber alone. Paired females from the self-paced condition, perfused after the open-field, showed significantly more Fos

## Sexual behaviors of self-paced versus non self-paced females



**Fig. 2.** Paired females in the self-paced condition selectively solicited the male more frequently than paired females in the non-self-paced condition in the open-field test. Behaviors include: solicitations (Sol), intromissions (Intr), ejaculations (Ejac), interceptions (Int), female–female mounting (FFM), and time spent in proximity to the male (TS). Data are means  $\pm$  SEM. \*\*\* $p < 0.001$ , # $p < 0.1$  (trend).

expression within the supraoptic nucleus than did unpaired females from the same condition,  $F(2,10) = 1475.878$ ,  $p = .001$ ,  $\eta^2 = .78$  (Fig. 3). Although statistical significance was not reached, there was a trend observed in the paraventricular nucleus, with paired females showing more Fos expression than unpaired females,  $F(2,9) = 40795.333$ ,  $p = .073$ ,  $\eta^2 = .44$ . In accordance with our hypothesis, the ventromedial hypothalamus, an area known to be activated by copulation alone, showed no differences between paired and unpaired females in the self-paced group, that were perfused after the open-fields,  $p = .988$  (Fig. 3).

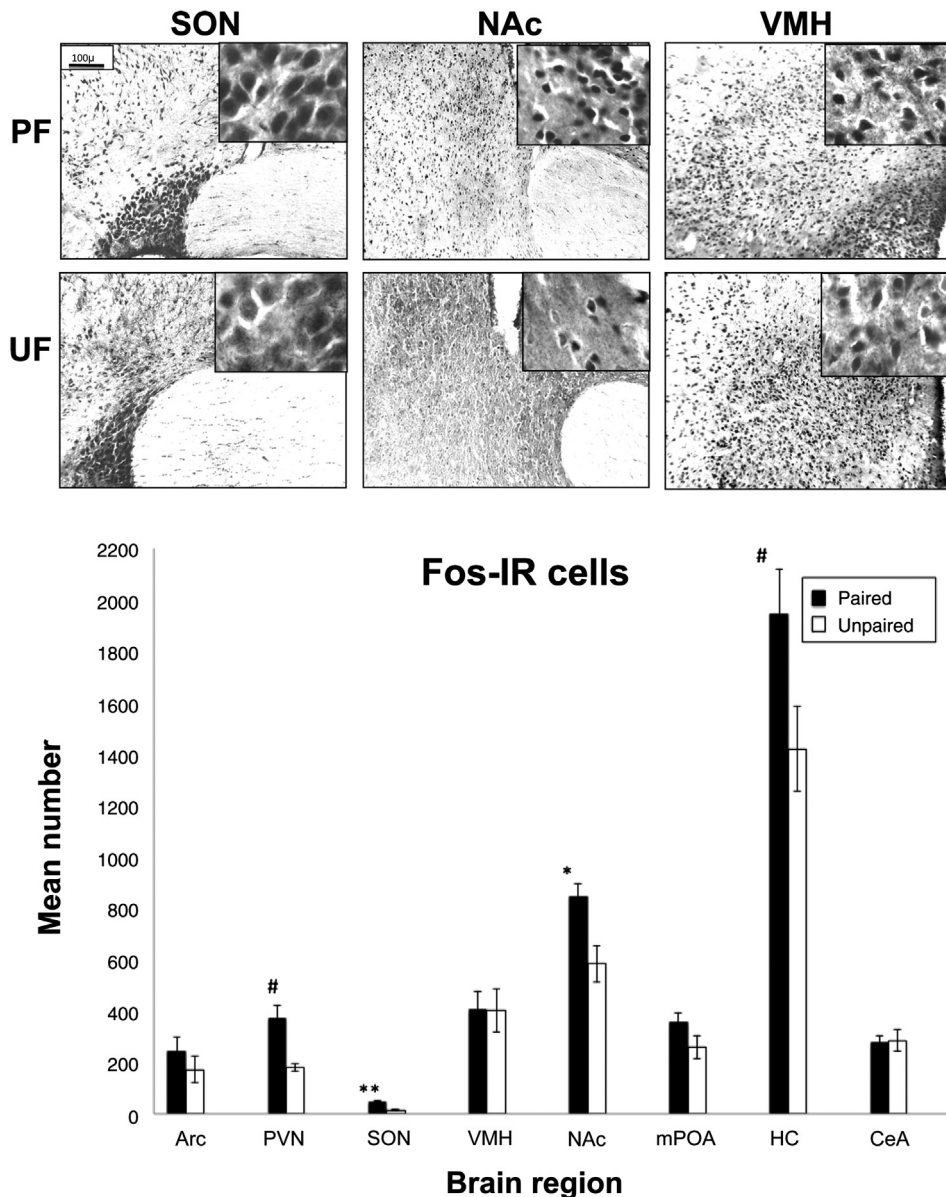
Additionally, paired females from the self-paced condition, whose brains were collected after exposure to the pacing chamber alone, had significantly more Fos induction within the nucleus accumbens shell than did unpaired females from the same condition,  $F(2,12) = 22085.928$ ,  $p = .033$ ,  $\eta^2 = .434$  (Fig. 3). Although statistical significance was not reached, there was a trend in favor of paired females also

having more Fos expression than unpaired females within the hippocampus,  $p = .083$ .

Fos expression was also quantified in other regions including the medial preoptic area, central nucleus of the amygdala, and the arcuate nucleus, however, these results did not reach statistical significance.

#### 4. Discussion

In this experiment, female rats were given their first 10 sexual experiences either with the same male (paired) or with a different male each time (unpaired). When tested in an open-field with the familiar male, paired female rats displayed agonistic mounting of a competitor female, whereas unpaired females did not display this behavior. Female–female mounting is a form of female social behavior, and plays a role in maintaining a dominant social status [21]. Female rats are more likely to mount an unfamiliar female than a familiar one, especially if the other



**Fig. 3.** Top: Photomicrographs (40 $\times$ ) showing Fos-IR in the VMH, SON, and NAc following the open-field test (VMH, SON) or exposure to the context alone (NAc) in paired versus unpaired females. Paired females showed significantly more Fos induction, following the open-field, in the SON than did unpaired females. Following exposure, paired females showed significantly more Fos expression within the NAc than did unpaired females. VMH was measured as a control region; no significant differences in Fos were observed. Magnified inserts taken of selected regions at 100 $\times$  to more clearly display differences in Fos. Bottom: Fos positive cells in different brain regions from paired or unpaired groups. Data are means  $\pm$  SEM. \*\* $p < .001$ , \* $p < .05$ , #trend. Brain regions include: arcuate (Arc), paraventricular nucleus (PVN), supraoptic nucleus (SON), ventral medial hypothalamus (VMH), nucleus accumbens shell (NAc), medial preoptic area (mPOA), hippocampus (Hc), and central nucleus of the amygdala (CeA).

female is in heat; however, this mounting behavior typically decreases in the presence of a male [21]. In this study, in the presence of the male and a competitor female, the paired female feels dominant to the competitor, and mounts her in an attempt to establish dominance and to chase her away from the male before she can potentially mate with him. This mounting behavior would often occur in succession until the unpaired female had left the vicinity of the male. Although unpaired female rats did mount the paired female, the context in which they did so was markedly different. The few times that unpaired females would mount the paired female, were done out of frustration from not receiving sexual contact from the male.

We also found that mate-guarding behavior only develops if female rats are allowed to pace the rate of copulation, as animals in cohort 1, which were allowed to pace the rate of copulation displayed mate-guarding in the presence of a competitor, but females in cohort 2, which were not allowed to self-pace the rate of copulation, did not. This is supported by previous work by Martinez and Paredes [5] showing that rats will only develop CPP for environments they pair with the sexual reward achieved through self-paced mating. Coria-Avilla et al. [4] also demonstrated that female rats will develop a preference to copulate with a scented partner over an unscented one if they are allowed to self-pace the rate of sex with a scented partner. The data from this study extend these findings by showing that the sexual reward induced by self-paced mating is so strong that odor is not necessary for a partner preference to form in female rats. If a female rat receives her first 10 paced sexual experiences with the same male, she will recognize that male without the addition of an odor, and will defend her exclusive access to him as a sex partner if a competitor is present. Furthermore, the behavioral differences between the paired females in the self-paced and non-self-paced condition indicate that only the self-paced paired females selectively solicit sex from the male more frequently than those in the non-self-paced condition (cohort 3). During conditioning, females in the self-paced condition had many more opportunities to move, and could easily solicit the male within the chamber as it was easy for them to have free access to both sides. This was not the case in the non-self-paced condition; the female was often blocked by the male from either entering or leaving his side of the chamber, which likely led to a different and less optimal rate of sexual stimulation. Thus, we argue that females in the self-paced condition experienced a more optimal level of sexual stimulation and reward, leading to a stronger preference for the stimulus male paired with this condition relative to females in the non-self-paced condition or unpaired females.

Brain activation was also examined using Fos as a marker of neuronal activation in two conditions, one in response to the expression of mate-guarding behavior, and the other in response to the context (chamber alone). In line with our hypothesis, after the mate-guarding test, paired females showed significantly more Fos-IR within the SON, and there was a trend in the favor of paired females also showing more Fos-IR within the PVN. Both of these brain regions contain OT and AVP cell bodies, and OT and AVP are known to regulate many social behaviors that are associated with pair bonding, such as sexual behavior, aggression, and maternal care [10,11]. Furthermore, it has been shown that OT and AVP play a critical role in pair bonding in monogamous vole species, and that differences in the receptor distribution for both OT and AVP influence the social structure of monogamous and polygamous vole species [10,11]. Alternatively, the increase in Fos-IR within the SON and PVN could signal a reaction to stress [23]. Considering that we also observed a trend for a larger number of Fos-IR cells in the hippocampus of paired females, another region involved in stress responding, it is possible that some of the neuronal activation was due to the stress of mate guarding in addition to appetitive responses to the paired male or rewarding copulatory stimulation [24]. However, we note that differences in the number of Fos-IR cells between the groups were not observed in other stress-reactive regions, such as the arcuate nucleus or central amygdala, suggesting that the overall pattern of brain activation is not simply due to the induction of a stress response. Finally, although Fos

is used as a marker of neuronal activation, it cannot be used to denote the role of a region or neurochemical system in behavior. As with other forms of brain activation, such as fMRI, the induction of Fos could indicate the activation of either excitatory or inhibitory pathways, and result from the processing of sensory information that excites or disinhibits different aspects of behavior, or that will inhibit behavior following suitable sensory stimulation [25]. More work is thus required to delineate the role of these regions in mate-guarding behavior.

Female rats in the paired group learned to associate the sexual reward induced by paced mating with a specific male. Because all copulation trials took place within the same chamber, paired female rats also learned that placement in the chamber predicted the presence of her paired male, so, we wanted to look at the Fos induction in response to context exposure alone. Paired females from the self-paced condition, whose brains were collected after exposure to the context alone, showed significantly more Fos induction within the nucleus accumbens shell than did unpaired females from the same condition. The nucleus accumbens has been shown to play a vital role in establishing and maintaining pair bonds in voles [22]. Dopamine transmission within the rostral shell of the accumbens promotes pair bond formation, through activation of D2 receptors, and also, plays a role in maintaining pair bonds, by activating D1 receptors, which in turn facilitates selective aggression [22]. However, further investigation is required to see if the brain activation seen here is within the dopamine system of this region.

The question of why monogamous behavior has evolved in rodents, namely prairie voles, remains unanswered although several theories exist. It is thought that monogamy arises from the environmental circumstances in which these animals live. Prairie voles, a socially monogamous rodent, occupy the grasslands of the central United States, which is a very harsh environment limited in resources and vole populations are widely dispersed [19]. The influence of the environment on this behavior is best seen during the shift to winter months, when prairie voles live in closer proximity to one another, called clustering [20]. During this time of year, more extra pair copulations occur, than in the spring/summer months when they disperse [20]. In this study, we recreated this change in the female rat's environment artificially by allowing her to copulate with only one partner. This change in environment and specificity of the male may well have created a change in behavior by shifting the female rat's mating strategy more toward a monogamous pattern that resulted in mate-guarding, a behavior that has not previously been reported in rats, as they are typically described as being a promiscuous species.

In summary, these data demonstrate that the female rat is not hard-wired to be promiscuous. By pairing sexual reward with a specific male, she can recognize that male based on primary partner cues alone (e.g. major histocompatibility complexes, pheromones, natural odors). Furthermore, when a competitor female is present, paired female rats display mate-guarding in the presence of their paired male. That this change in behavior is accompanied by brain activation in regions associated with the neurohypophyseal hormones oxytocin and vasopressin, dopamine, or regions known to be involved in monogamous responding in other rodent species, suggests that changes in environment, such as partner availability, during a rat's primary experiences with sexual reward induce long-term changes in neuroanatomical systems that underlie partner preference.

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# The role of oxytocin and vasopressin in conditioned mate guarding behavior in the female rat



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## HIGHLIGHTS

- Female rats can display mate guarding behavior.
- Experience with sexual reward shifts sexual strategies in the female rat.
- Female rats that display mate guarding show enhanced activation of OT and AVP neurons.
- Injection of OT or AVP facilitates different aspects of mate guarding behavior.

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## ABSTRACT

We have shown previously that female rats given their first copulatory experiences with the same male rat display mate guarding behavior in the presence of that male provided a female competitor is also present. Females given access to the familiar male show more Fos induction within regions of the brain that contain oxytocin (OT) and vasopressin (AVP) cell bodies, notably the supraoptic (SON) and paraventricular nuclei (PVN) relative to females given sexual experience with different males. The present experiments examined whether the Fos induction we previously observed within the SON and PVN occurred within OT and/or AVP neurons, and whether exogenous administration of OT or AVP prior to female rats first sexual experience could potentiate the acquisition of mate guarding behavior. Female rats that display conditioned mate guarding had significantly more double-labeled Fos/OT neurons in both SON and PVN, and significantly more Fos/AVP neurons in the PVN. Peripheral administration of OT or AVP prior to their first sexual experience with the familiar male facilitated different aspects of mate guarding: OT augmented affiliative behaviors and presenting responses whereas AVP augmented interference behavior. These results indicate that female rats' first experiences with sexual reward when paired with the same male induce changes to bonding networks in the brain. Moreover peripheral administration of OT or AVP during their first sexual experience can augment different aspects of mate guarding behavior.

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## 1. Introduction

Socially monogamous animals form enduring associations with one another that are characterized by selective affiliation, contact, and preferential copulation with their partners relative to strangers. These partnerships are called pair bonds [1]. In rodents, the hallmark example of social monogamy is displayed by the prairie vole (*Microtus ochrogaster*), and this rich literature has provided the scientific community with ground breaking and in depth analyses that have laid the groundwork for other studies looking into monogamy, bonding, and mate guarding. Prairie voles inhabit a very harsh environment, with limited food, water, and mate access. It is thought that they adapted a socially monogamous

mating strategy in order to increase the likelihood of their survival and reproductive success [2,3]. For example, if a prairie vole happens to come across a potential mate, it would make sense to bond with this mate, since there is not a large likelihood of encountering another one. Bonding would allow for access to a mate, reproduction, would allow for biparental care of the young, and ultimately enable the couple to defend territory and gather resources more efficiently. However, the influence of the environment on bonding behavior in prairie voles can be most clearly observed during the winter months, when voles cluster together to form communal groups [4]. When living in communal groups, more extra pair copulations occur among pair bonded voles than in spring and summer months when the vole population is more widely dispersed, demonstrating that population density and mate access can create shifts toward promiscuity within the mating strategy of the prairie vole [4]. In the laboratory, pair bonding is determined when male prairie voles display a preference to spend more time with the first female they copulated with relative to a novel female [5], or

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when male/female prairie voles mate guard the first mate they copulated with [5].

The neuroanatomical and neurochemical bases of pair bonding have been elaborately examined in monogamous vs. promiscuous voles. Oxytocin (OT) and vasopressin (AVP) were early targets of this research, as these neuropeptides were known to modulate species-specific social behaviors such as sexual behavior, aggression, maternal care, and olfaction related to conspecific identification [6–12]. Neuroanatomical studies have revealed that, although there are minor species differences in cell bodies and fibers, there are dramatic differences in regional receptor distribution between monogamous and promiscuous vole species [13–15]. Specifically, monogamous prairie voles have higher densities of the vasopressin A1 receptor (V1aR), in the BNST, ventral pallidum (VP), central amygdala (CeA), basolateral amygdala (BLA), and accessory olfactory bulbs (AOB) than promiscuous montane voles [16–18]. Monogamous voles also display higher densities of oxytocin receptor (OTR) in the BNST, medial prefrontal cortex (mPFC), and nucleus accumbens (NaC) than promiscuous vole species [18–20]. These regional differences in V1a and OTR expression have been shown to be stable across lifespan and do not exist in other systems such as the opiate receptor system [20].

Pharmacological studies provided direct evidence for the importance of OT and AVP in the formation and maintenance of pair bonding in monogamous voles [16,21]. By administering a vasopressin 1a receptor (V1aR) antagonist intracerebroventricularly (icv), researchers were able to block the onset of pair bonding in male prairie voles. Similarly, direct icv administration of AVP facilitated pair bonding in males even in the absence of mating, and in females after 1 h cohabitation [21, 22]. Administration of OTR antagonist, similarly, blocked pair bonding in both males and females, and concurrent administration of OT was able to rescue pair bond formation [22]. These results clearly demonstrate that both OT and AVP are essential for pair bonding in both sexes of prairie vole.

Rats (*Rattus norvegicus*), on the other hand, are considered promiscuous. They tend to live in large underground burrow systems and mate freely with many different partners throughout several copulatory series [40]. Despite this, a growing body of evidence shows that both male and female rats have the ability to display rudiments of monogamy such as sexual partner preferences. For example, through Pavlovian conditioning paradigms, where neutral odors are paired with sexual reward states, female rats have been shown to display preferences for scented partners over unscented ones, and vice versa if rewarding sex is paired with unscented partners [25]. We have also reported that changing the conditions under which the promiscuous female rat has her first experiences of sexual reward, notably by shifting mate access, can alter her mating strategies, much like the population density and partner access creates shifts in the prairie vole mating strategy [4,23]. Female rats given their first 10 paced copulatory experiences with the same male displayed mate guarding behavior in the presence of their paired male and a competitor female. In these studies, mate guarding was defined as any behavior displayed by the female that prevented the male from attending to or mating with the competitor female [23]. Under this definition several sexual (solicitations, presenting), aggressive (biting, tackling accompanied by vocalizations from the victim), dominant (female–female mounting), social (time spent in proximity, body positioning), and competitive (interceptions) behaviors were included to describe mate guarding [23]. When female rats mate guarded, they displayed all of these behaviors to varying degrees, however, in our previous study, over time, paired females displayed significantly more female–female mounting relative to unpaired female rats. Paired female rats that were placed into an open field with their paired male and a female conspecific, would mostly remain in the vicinity of the male. However, if the competitor female approached the male, the paired female would run over to her, and mount her in succession until she left the vicinity of the male. We also demonstrated that mate guarding was dependent on paced mating, because females not allowed to pace the rate

of copulations during their first sexual experiences, which decreases the reward value of copulation [24], did not display mate-guarding behavior. Likewise, females that received paced copulation with different males each time did not show mate guarding behavior.

This shift in mating strategy was also accompanied by different patterns of neuronal activation within regions of the brain known to play a role in bonding behavior [23]. For example, female rats conditioned to show mate guarding had significantly more Fos-positive neurons within the PVN and SON (along with other brain regions such as the nucleus accumbens and hippocampus) following exposure to their familiar male relative to females conditioned to show no partner preference. These data suggest that a similar set of neuroanatomic regions are activated by partner cues in monogamous female prairie voles and promiscuous female rats that have been conditioned to show mate guarding behavior. In turn, this opens up the possibility that OT and AVP may play a role in conditioned monogamous behavior.

This purpose of this study was to examine the potential role of OT and AVP in conditioned mate guarding behavior. First we examined whether the Fos induction in the PVN and SON observed in females conditioned to show mate guarding was within OT and/or AVP neurons. We hypothesized that females that had all of their sexual experience paired with the same male would have more Fos induction within oxytocin and vasopressin neurons than females who had received their sexual experience with a variety of males. Second, we tested whether OT or AVP, injected peripherally prior to female rats first rewarding sexual experience, would facilitate the display of mate guarding behavior on their second sexual experience with the same male, relative to females injected with saline in order to elucidate potential roles that OT and AVP might be playing in the early onset of conditioned mate guarding. We hypothesized that peripheral administration of OT or AVP, but not saline, would facilitate different aspects of mate guarding behavior.

## 2. Materials and methods

### 2.1. Animals and surgery

Sexually naïve Long–Evans female rats (200–250 g) were obtained from Charles River Canada (St-Constant, QC, Canada). Animals were housed in pairs in shoebox cages in a colony room on a reversed 12:12 h light/dark cycle at approximately 21 °C. Animals had free access to food and water. Females were anesthetized using a 1 ml/kg intraperitoneal injection of ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (4 ml/kg), mixed in a ratio of 4:3 respectively, prior to bilateral ovariectomy (OVX) using lumbar incisions. Females had 1 week to recover before behavioral tests began. Throughout the duration of the experiment, female rats were maintained on hormone replacement by subcutaneous injections of estradiol benzoate (EB; 10 µg in 0.1 ml of sesame oil) 48 h prior to testing, and progesterone (P; 500 µg in 0.1 ml of sesame oil) 4 h prior to testing.

Sexually naïve male rats (300–350 g) were also obtained from Charles River Canada (St-Constant, QC, Canada). Males were housed in group cages (4 animals per cage) under conditions identical to those of the female rats.

All animal procedures complied with the guidelines of the Canadian Council on Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

### 2.2. Conditioning apparatus

All conditioned took place in Plexiglas unilevel pacing chambers (38 cm × 60 cm × 38 cm) with wire-mesh floors covering a layer of bedding [25,26]. Chambers were bisected by a Plexiglas divider with four holes cut into the bottom. Holes were large enough for the female to crawl through but too small for the male to crawl through [25,26].



### 2.3. Conditioning procedure for animals used for double immunohistochemistry

Females whose brain tissue was used for double IHC in this study were conditioned as follows: Sexually naïve female rats were divided into 2 groups; females who always copulated with the same male (paired females,  $N = 12$ ), and females that copulated with a variety of males across all trials (unpaired females,  $N = 12$ ). All conditioning occurred in a pacing chamber (described above). Five minutes prior to the onset of conditioning, males were placed into one side of the chamber for a 5-minute habituation period. After these 5 min, the female was placed onto the opposite side of the chamber and the 20-minute mating test began. Both groups received a total of 10 trials at 4 day intervals. Four days following the last conditioning session, mate guarding was assessed using an open field ( $123 \text{ cm} \times 123 \text{ cm} \times 46 \text{ cm}$ ) with a thin layer of bedding, containing the paired female, her paired male, and an unpaired female. Males were placed into the apparatus first for a 5-minute habituation. After the conclusion of 5 min, his paired female and an unpaired female were placed into the open field at opposite corners and a 1 h mate guarding test commenced. All trials and open fields were video recorded. Open field videos were coded and then scored blindly for mate guarding behavior.

### 2.4. Perfusions

Animals in the first experiment were euthanized with an intraperitoneal injection of an overdose of sodium pentobarbital (120 mg/kg) immediately following their 1 h mate guarding test. They were then intracardially perfused with 250 ml of phosphate-buffered saline (PBS) followed by 250 ml of 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 4 h. After which brains were placed into a 30% sucrose solution until they began to sink. Brains were removed from sucrose solution, flash frozen in dry ice, and stored at  $-80^\circ\text{C}$  until used for histochemical procedures.

### 2.5. Tissue preparation and immunocytochemistry

Brains from both paired and unpaired females were cut coronally at  $30 \mu\text{m}$  on a freezing microtome and divided into three series, one of which was used for double immunohistochemistry to label for Fos and OT, the second was used to label for Fos and AVP, and the third was stored as a backup. In the primary phase, sections were washed in cold Tris-buffered saline (TBS) and incubated first with 30% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in TBS for 30 min at room temperature followed by 3% normal goat serum (NGS) in 0.05% Triton-TBS for 90 min at  $4^\circ\text{C}$ , with rabbit polyclonal anti-Fos (Fos ab5, Calbiochem, Mississauga, ON; diluted 1:40,000) in 0.05% Triton-TBS with 3% NGS for 72 h at  $4^\circ\text{C}$ . In the secondary phase, sections are incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories Canada, Burlington, ON; 1:200), in 3% NGS, and .2% Triton TBS for 1 h. Following this, sections enter a tertiary phase, where sections are incubated in avidin-biotinylated-peroxidase complex (Vectastain *ELITE*® ABC KIT, Vector Laboratories Canada; diluted 1:55) in 3% NGS, and .05% Triton TBS for 2 h at  $4^\circ\text{C}$ . Sections were washed in TBS ( $3 \times 5 \text{ min}$ ) between each incubation. Immunoreactions were stained by sequential treatments with 50 mM Tris for 10 min, 3,3'-diaminobenzidine (DAB) in 50 mM Tris (0.1 ml of DAB/Tris buffer, pH 7.8) for 10 min, and 8% nickel chloride (400  $\mu\text{l}$  per 100 ml of DAB/Tris buffer +  $\text{H}_2\text{O}_2$ ) all at room temperature. Reaction was stopped by rinsing ( $3 \times 10 \text{ min}$ ) in PBS. Sections were then transferred into the second primary antibody, either rabbit polyclonal anti-oxytocin (Chemicon, AB911, diluted 1:10,000) or rabbit polyclonal anti-vasopressin (Millipore, AB1565, diluted 1:10,000) in 0.05% Triton-TBS with 3% NGS, and incubated for 72 h at  $4^\circ\text{C}$ . After completion of this incubation, secondary and tertiary phases are repeated. However, during the final DAB wash, no nickel chloride was used in order to create a light brown cytoplasmic stain.

Sections were mounted onto gel-coated slides and cover slipped following immunohistochemistry. Cover slipping procedure started with sequential washes of distilled water followed by washes in 70%, 90%, and 100% alcohol each at room temperature for a 1 minute duration. Slides were then submerged in Xylenes for 2 h at room temperature after which they were cover slipped using Permount solution. Slides were untouched for a week to allow for the Permount to dry and then cleaned and coded so that all analysis could be done blind.

Sections were examined using an Olympus light microscope at  $40\times$  magnification. Pictures were taken at  $20\times$  and  $100\times$  using Q-Capture pro software. Double-labeled cells were identified by eye as any cell having a dark black nucleus (Fos positive) and a light brown cytoplasm (OT or AVP positive). Brain regions were defined using the atlas of Paxinos and Watson [27]. Double-labeled cells were counted within the supraoptic nucleus (SON, Plates 21–25), and paraventricular nucleus (PVN, Plates 25–26). Five slices were counted per animal. These slices represented each area rostrally to caudally and were matched among animals to ensure accuracy. Counts were done bilaterally in a total of 5 animals per group. Total number of double-labeled cells was counted in each section, which were then used to derive the mean number of double-labeled cells within the region for each animal.

### 2.6. Drug preparation

Reagent grade OT (Bachem, H2510) or AVP (Sigma, V-9879) was added to .9% saline solution and diluted to a  $5 \mu\text{g/ml}$  concentration.

### 2.7. Conditioning procedure for peripheral administration of OT or AVP

Sexually naïve female rats were divided into 4 groups: paired oxytocin treated females ( $N = 6$ ), paired vasopressin treated females ( $N = 6$ ), paired saline treated females ( $N = 6$ ), and unpaired untreated females ( $N = 18$ ). Although in total there were 18 unpaired untreated females, for the purposes of comparison, unpaired females were only compared to paired females from the treatment group they were run with in the open-field. Females were injected with  $5 \mu\text{g/kg}$  of OT, AVP, or saline subcutaneously 15 min prior to the first conditioning trial. Drugs were administered once, prior to the first trial, in order to examine whether augmenting OT or AVP during the first copulation session would facilitate different aspects of bonding during the second copulation session. Male rats were placed into the conditioning apparatus (described above) for a 5-min habituation period prior to the introduction of each female. Females were placed into the compartment adjacent to the one containing the male and given a 30-minute copulation session with the male. Open-field tests were run 4 days after the conditioning session. All open fields contained a paired female from one of the treatment groups, the male she copulated with during the conditioning session, and an unpaired female. The open field was  $123 \text{ cm} \times 123 \text{ cm} \times 46 \text{ cm}$  with a thin layer of bedding on the floor [25]. Five minutes prior to the start of the open-field, males were placed into the apparatus for a habituation period, after which females were placed in at diagonal corners. Rats were allowed to copulate freely for a 1 h period. After the open-field, female rats were perfused and their brains collected for future use.

All open-field tests were recorded on video, coded, and blindly scored subsequently using a computerized event recorder customized for rat sexual behavior in an open field [28]. The frequency of solicitations (SOL), hops and darts (HOP), defensive or aggressive responses (AGG) were recorded, as were the incidents and reflex magnitudes of lordosis when males mounted. The frequency of female–female mounting (FFM) and interceptions (INT) were also scored, as was the amount of time spent maintaining body contact with the male. Presenting (PRES) behavior was also scored when the female remained in a receptive position in front of the male before or after he mounted or intromitted. We also took into account the number of times that a female would position her body in between the male and the opposing female, thus interfering the male's interaction with the female conspecific (RI).

Lastly, the number of mounts (MOUN), intromissions (INTRO), and ejaculations (EJAC) received by each female were recorded in order to assess the effectiveness of mate guarding. After conclusion of test, females were euthanized and their brains were collected for double IHC to look for Fos colocalization within either oxytocin or vasopressin.

## 2.8. Statistical analyses

Mate guarding behavior in the open-field was assessed using independent samples *t*-tests along with a Bonferroni correction. For animals whose brains were used for IHC, there were only two groups, paired and unpaired, and one level of comparison. For the pharmacological administration, each group (OT, AVP, and Saline) was represented in each cohort, each treatment group was only compared to the untreated control they were tested with in the open-field, therefore there were only 2 groups with one level of comparison. The data collected from immunohistochemistry were also analyzed using independent samples *t*-tests along with a Bonferroni correction, as there were only 2 groups with one level of comparison. Cohen's *D* was used to evaluate effect size.

## 3. Results

### 3.1. Mate guarding behavior after open field of animals used for IHC

Independent samples *t*-tests, along with Bonferroni corrections, were used to assess mate guarding behavior between paired and unpaired females to establish if a shift in mating strategy had occurred post conditioning. We found that paired females did, in fact, display subsets of mate guarding behavior, and showed significantly more female–female mounting,  $t(7.031) = 2.415$ ,  $p = .024$ ,  $d = 3.4099$ , than unpaired females (Fig. 1). This finding is consistent with our previously reported findings [23]. Although other behaviors were examined, they did not approach statistical significance.

### 3.2. Double-labeled Fos and oxytocin or vasopressin cell counts

Independent samples *t*-tests, with Bonferroni correction, were used to look at differences in the average number of double-labeled cells within the SON and PVN between paired and unpaired females. Analysis revealed that paired females showed significantly more Fos + OT positive cells within the SON,  $t(7.031) = 73.14$ ,  $p = .000$ ,  $d = 4.44$ , and PVN,  $t(7.031) = 76.9$ ,  $p = .004$ ,  $d = 3.17$ , than did unpaired females

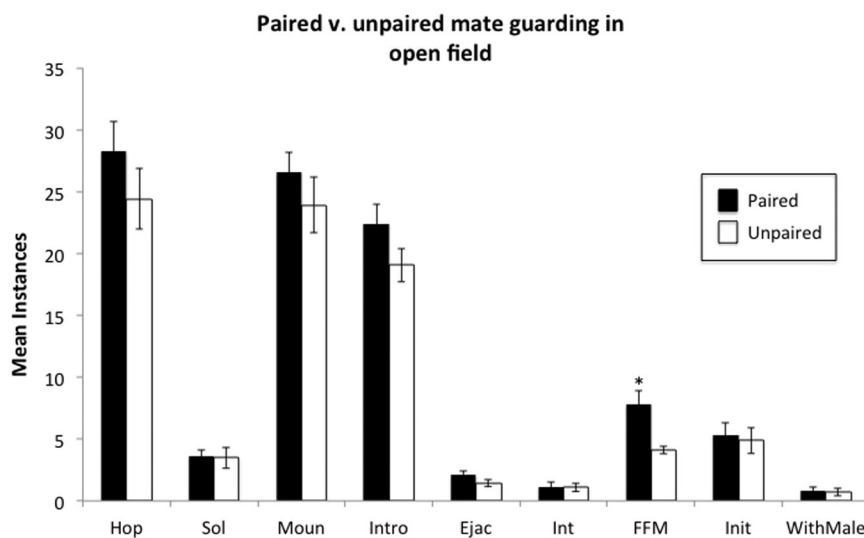
(Fig. 2). Paired females also showed more Fos + AVP positive cells within the SON than did unpaired females,  $t(2.972) = 41.840$ ,  $p = .018$ ,  $d = 1.87$  (Fig. 3).

### 3.3. Mate guarding behavior after peripheral injection of OT, AVP, or Saline

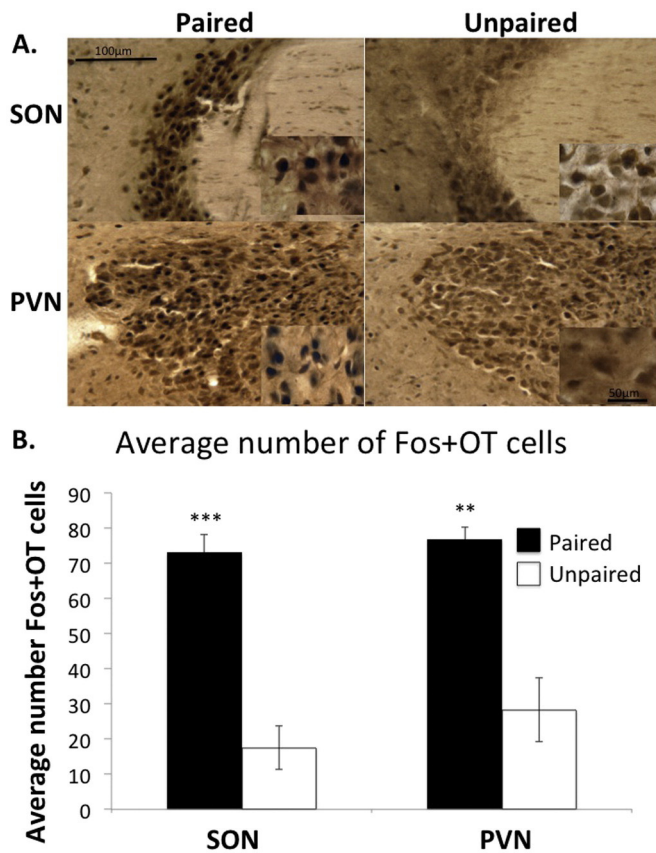
Independent samples *t*-test, with Bonferroni correction, were used to examine differences in mate guarding behavior between oxytocin, vasopressin, and saline treated paired females and their respective unpaired females during the open field. Analysis revealed that paired females treated with oxytocin showed significantly more presenting behavior than unpaired females during the open field,  $t(3.676) = 4.67$ ,  $p = .004$ ,  $d = 2.12$  (Fig. 4a). Consequently, paired females treated with oxytocin also received more intromissions than did their corresponding unpaired females in the open field,  $t(3.101) = 16.5$ ,  $p = .011$ ,  $d = 1.79$  (Fig. 4a). Paired females treated with vasopressin imposed their body between the male and the other female significantly more than did unpaired females in the open-field,  $t(3.873) = 1.00$ ,  $p = .003$ ,  $d = 2.27$  (Fig. 4b). No differences in behavior occurred between paired saline treated females and their corresponding unpaired females in the open-field test (Fig. 4c).

## 4. Discussion

The results of these experiments replicate and extend those of our previous study showing that paired female rats display conditioned mate guarding behavior of their paired male against a competitor female. The paired females had significantly more double-labeled Fos-OT, within the PVN and SON, and Fos-AVP neurons within the SON, in response to the presentation of the preferred male compared to unpaired females that showed no significant partner preference to any male. In the second experiment, we sought to unravel the role that OT and AVP could be playing in the early onset of conditioned mate guarding, by administration of OT or AVP to sexually naïve females during their first sexual experience. Peripheral administration enhanced different aspects of mate guarding on the second trial relative to females injected with saline: Females given OT on the first trial displayed more presenting near their preferred male on the second trial relative to saline-treated females, whereas females given AVP displayed more interference behavior relative to saline-treated females on the second test. Saline treated females did not differ behaviorally from untreated females on the second trial. These data indicate that



**Fig. 1.** Mate guarding behavior displayed in open field by paired female compared to unpaired female rats. Behaviors include hops, solicitations (Sol), mounts received (moun), intromissions received (intro), ejaculations received (ejac), interceptions (int), female–female mounting (FFM), number of times initiating closeness to the male (Init), time spent in proximity to the male (WithMale), interference (RI), and presenting (pres). Data are means  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < .01$ .

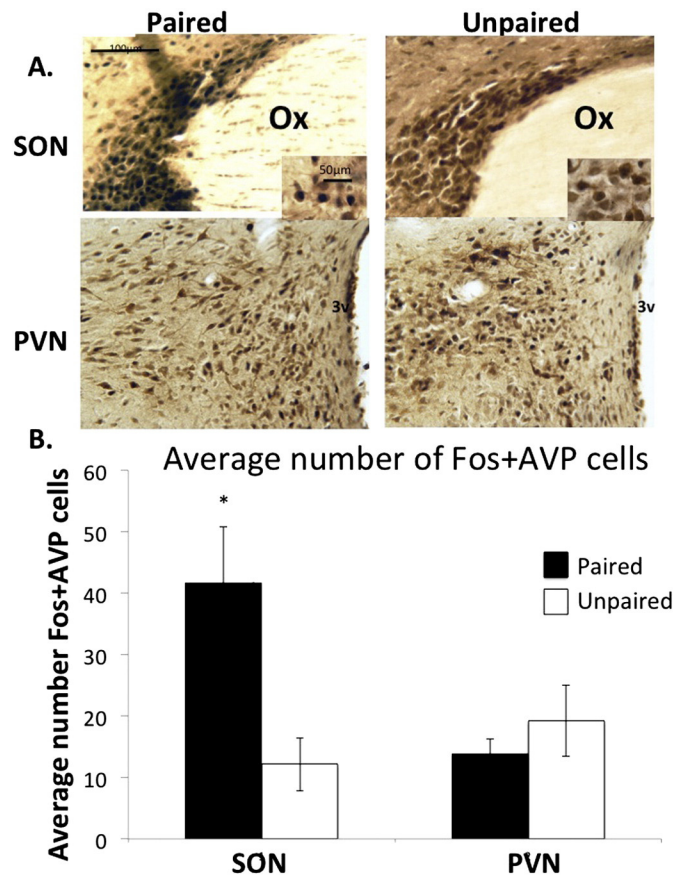


**Fig. 2.** Top: Photomicrographs (40 $\times$ ) showing OT + Fos-IR in the SON and PVN following the open-field test. Cells positive for both markers have a light brown cytoplasmic stain (OT) with a dark black nucleus (Fos). Magnified inserts taken of selected regions at 100 $\times$  to more clearly demonstrate double labeled v. single labeled cells. Bottom: OT + Fos positive cells in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) from paired (black) or unpaired (white) groups. Data are means  $\pm$  SEM. \* $p$  < .05, \*\* $p$  < .01; \*\*\* $p$  < .001, #trend.

OT and AVP neurons are activated in the SON and PVN of female rats that are conditioned to show mate guarding behavior over the course of a rigorous conditioning paradigm. Furthermore, by administering OT and AVP we observed that these two peptides play distinct roles in the early development and manifestation of this behavior.

It has been established that there are remarkably stable regional differences in the distribution of OT and AVP receptors between monogamous and promiscuous vole species that underlie differences in mating strategies between them [16–19]. By limiting a female rat's rewarding sexual experience to a single male partner, we have been able to shift her behavioral mating strategy toward monogamous mate guarding. This is accompanied by changes to neurochemical systems involved in bonding within the brain [23]. Although the findings of the first experiment show that hypothalamic OT and AVP neurons are activated more in paired vs. unpaired female rats, this does not provide any information about receptor distribution, which is known to underlie vole behavior. It could be the case that when female rats are conditioned monogamously, OT and AVP cell bodies become more active, releasing more peptide, which sensitizes the system, thus leading to changes in behavioral output, such as mate guarding. However, further investigation is warranted to examine if this conditioning procedure also alters OT and/or AVP receptor expression.

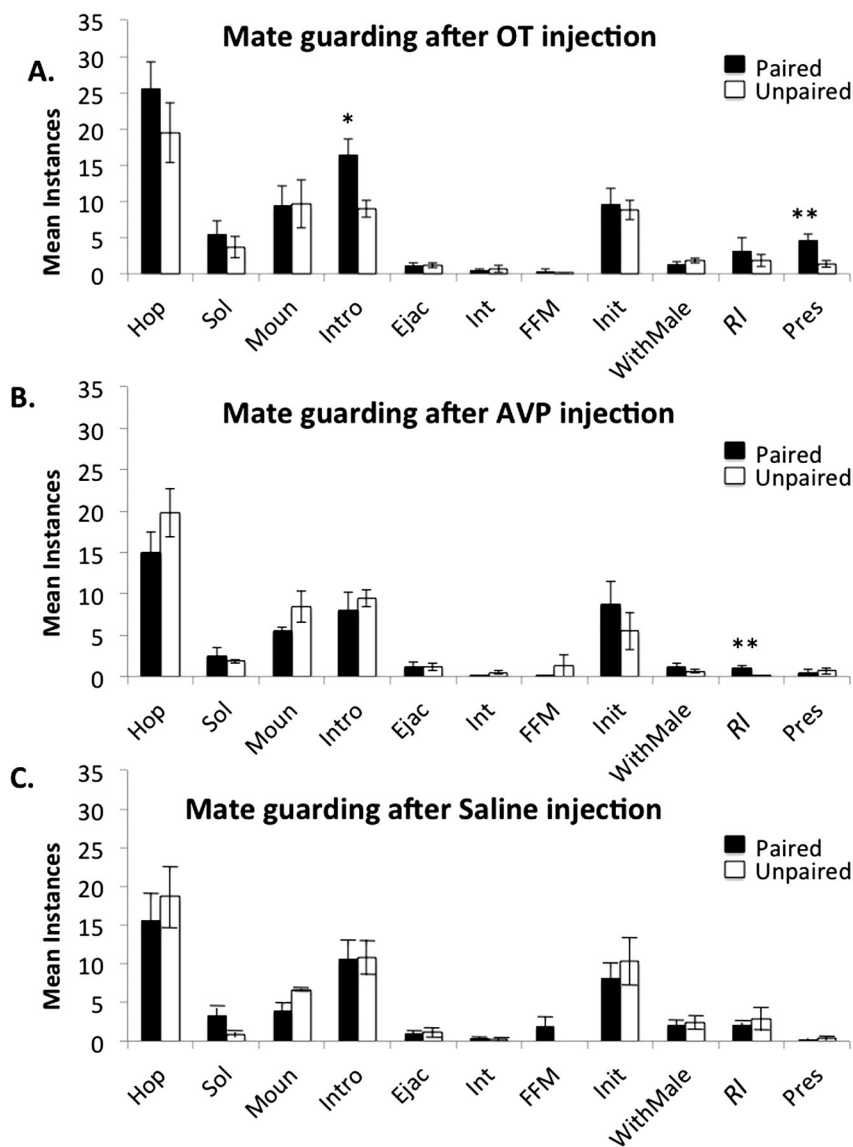
The double immunohistochemistry data demonstrate that OT and AVP neurons are activated over the course of conditioning; however, they do not reveal the role played by OT or AVP in the development of mate guarding behavior. Therefore, in the second study, we administered OT or AVP to female rats prior to their first sexual experience. We have shown that OT and AVP are activated over the 40 day course



**Fig. 3.** Top: Photomicrographs (40 $\times$ ) showing AVP + Fos-IR in the SON and PVN following the open-field test. Cells positive for both markers have a light brown cytoplasmic stain (AVP) with a dark black nucleus (Fos). Magnified inserts taken of selected regions at 100 $\times$  to more clearly demonstrate double labeled v. single labeled cells. Bottom: AVP + Fos positive cells in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) from paired (black) or unpaired (white) groups. Data are means  $\pm$  SEM. \* $p$  < .05, \*\* $p$  < .01; \*\*\* $p$  < .001, #trend.

of testing, however, we were interested to see if OT and AVP come on board sooner, how they would effect the development of conditioned mate guarding. We hypothesized that peripheral administration would facilitate different aspects of conditioned mate guarding. In line with our hypothesis, treatment with both, OT and AVP, facilitated the onset of mate guarding after one trial alone. However, each of these enhanced different subsets of mate guarding behaviors. Paired females treated with OT showed more presenting around the paired male and consequently they received significantly more intromissions than untreated females. Furthermore, neither AVP nor saline treated females showed an increase in this behavior when compared to controls. In contrast, paired females treated with AVP displayed significantly more interference behaviors than untreated females, however, this effect was very small. These behaviors were not enhanced by OT or saline treatment. The control injection of saline did not enhance any aspect of mate guarding behavior. In previous studies, presenting and interference were observed, but only minimally, and not in consistent enough of a manner to score. However, our previous assessment of mate guarding was made after an extensive conditioning paradigm, where females and males were paired for 10 sessions. In this study, females and males were only paired one time and were sexually naïve prior to this. What these data suggest is that mate guarding could develop as presenting and interference, and then mature into the display of more aggressive and dominant behaviors. This phenomenon of behavioral maturation is true of other complex interactions. For example, when male rats first learn to copulate, protracted anogenital investigation is a key component but this behavior dissipates as the males gain





**Fig. 4.** Mate guarding behavior displayed in open field by paired female rats that were treated with; OT, AVP, or Saline. Behaviors include hops, solicitations (Sol), mounts received (moun), intromissions received (intro), ejaculations received (ejac), interceptions (int), female–female mounting (FFM), number of times initiating closeness to the male (Init), time spent in proximity to the male (WithMale), interference (RI), and presenting (pres). Data are means  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < .01$ .

sexual experience. In order to fully understand the roles of OT and AVP in the progression of conditioned mate guarding, further work is warranted to investigate the expression of both neuropeptides at different time points over the conditioning process.

OT and AVP were identified early as neural correlates of bonding in the vole literature due to the fact that they mediate a variety of social behaviors. When we administered OT to female rats in the present study, it mediated presenting behavior, that occurs in proximity to the male, and is described as maintaining a receptive posture in front of the male, allowing her to receive more intromissions. OT and AVP have been conceptualized as having opposing influences on one another [29]. OT is generally linked to passive social behaviors that require proximity and closeness such as maternal care and maternal bonding [9,11,12,30]. In contrast, AVP is known for its role in fear, aggression, social recognition, and sociability [19,30,31]. In the present experiment, AVP treatment appeared to facilitate the female's ability to control the male's access to the competitor female by imposing her own body between the two. This type of behavior requires the female to be able to recognize the male and the threat of the competitor female, and to also maintain bodily awareness to interfere with the conspecific's access to the male.

Although this study clearly demonstrated that OT and AVP are able to facilitate different aspects of conditioned mate-guarding behavior, we are not able to determine if they are exerting their effects centrally or peripherally due to the nature of the injection. Until very recently, the effects of acute IP administration of OT or AVP on social behavior have been largely uninvestigated despite striking evidence that peripherally-administered OT is able to cross the blood brain barrier [32,33]. Taking these findings, and our double label immunohistochemistry data, into account, it is possible that the OT and AVP are exerting their effects on mate-guarding centrally, although we cannot definitively draw that conclusion from this line of work.

Mate guarding is a term broadly used in the literature and can be applied to different behaviors across species. For example, in prairie voles, mate guarding is typically synonymous with selective aggression, whereas in long-tailed macaques, mate guarding refers to a male monopolizing a female [3,34]. To be consistent, we operationally defined mate-guarding as the female taking away the choice of the male to mate with the opposing female, which was achieved through a set of aggressive, dominant, sexual, competitive, and social behaviors [23]. Administration OT or AVP facilitated certain aspects of mate-guarding

behavior, however, we did not see a facilitation of female–female mounting, which we observed in the conditioned group of animals in this study whose brains underwent double IHC, and that we previously reported following 10 sexual experiences with the paired male [23]. There are several explanations as to why this may be the case. Female–female mounting is not synonymous with mate guarding in female rats, it is just one behavior encompassed under our definition of mate guarding. This is reminiscent of other complex behaviors, such as sexual behavior, which is comprised of a range of appetitive and consummatory behaviors that together describe an animal's sequence of copulatory events. Although each paired animal shows higher instances of each behavior in the mate guarding repertoire than unpaired females, it may be the case that each animal also chooses to employ a certain set of behaviors which they find most effective, which then accounts for individual differences seen across groups. It is important to note, that individual differences in behavioral expression occur in nature and in the laboratory quite frequently, yet are less frequently discussed. Prairie voles are used as an ultimate example of social monogamy within the rodent literature and indeed across taxa. However, even within prairie vole populations, some individuals do not show pair bonding, biparental care, or selective aggression at all, or will display only a subset of these behaviors [4,35–38]. Individual differences play a role in how rodents mate in both nature and laboratory, and demonstrate how flexible patterns of reproduction are expressed at an individual level, which provides a plausible explanation for why female rats employ different methods of mate guarding. We have observed differences in FFM in paired females that underwent rigorous conditioning over a 40-day period. It could be that during this time, while the female is learning to associate reward with her mate, and bonding with him during each trial, OT and AVP systems both slowly come online, and both are needed ultimately for the display of FFM. In the present study we artificially administered either OT or AVP but not both. Alternatively, in prairie voles, the onset of mate guarding coincides with an increase in the dopamine D1 receptor (D1R) in the nucleus accumbens [39]. If D1Rs are selectively blocked in this region, voles fail to display mate guarding behavior [39]. Additionally, AVP has also been shown to regulate selective aggression in voles, where central infusion into the lateral ventricles induced aggression toward a conspecific [21]. Taken together, these data suggest that OT and AVP are individual pieces in the mate-guarding puzzle, and that dopamine could potentially be a key component in facilitating other behaviors, such as FFM, which require approach and contact with the competitor. In order to gain a full profile of conditioned mate guarding, much more work is required to examine the behavioral and accompanying neurochemical development of this behavior.

We suggest that OT and AVP are key individual players in the early phase of conditioned mate guarding behavior in female rats. Peripheral administration of OT or AVP is able to facilitate two different aspects of mate guarding behavior typically observed in the early phase, presenting (OT) and interference behavior (AVP). The activation of endogenous OT and AVP systems appears to sensitize following repeated paced copulation with the familiar male. Thus, as with males, patterns of bonding and partner preference are heavily influenced by a female's first experiences with sexual reward. Given that rats are generally reported to have a promiscuous group-mating strategy, we suggest that mating strategies can be shifted from promiscuous toward monogamous by experience with sexual reward that is predicted by a single long-term mate (or cues such as neutral odors worn by potential mates that predict the reward state). The reward state induces long-lasting changes in neuroanatomical structure and neurochemical systems that underlie bonding and attention toward partner cues.

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# Inhibition of lysine-specific demethylase enzyme disrupts sexually conditioned mate guarding in the female rat

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## ABSTRACT

Although female rats are typically described as having a promiscuous mating strategy, if sexually naïve females have their formative sexually rewarding experiences paired with the same male, they will recognize that male and display mate-guarding behavior towards him in the presence of a female competitor. Female rats that display mate guarding behavior also show enhanced activation of oxytocin and vasopressin neurons in the supraoptic and paraventricular hypothalamic nucleus. Here, we examined the potential role that histone demethylation might have in establishing this pair-bonded behavior, and whether the corresponding changes in oxytocin and vasopressin neuronal activation depended on demethylation. To accomplish this, we examined the effect of a lysine-specific demethylase-1 inhibitor to block the action of demethylase enzymes and maintain the methylation state of corresponding genes. Female rats treated with the demethylase inhibitor failed to show any measure of mate guarding, whereas females treated with vehicle displayed mate guarding behavior. Demethylase inhibitor treatment also blocked the ability of familiar male cues to activate oxytocin and vasopressin neurons, whereas vehicle-treated females showed this enhanced activation. These data indicate that histone demethylation is a crucial component in the epigenetic modification of neural circuitry that underlies conditioned mate guarding in female rats. These results are the first to demonstrate the role of histone demethylation underlying changes in mating strategy.

## 1. Introduction

Individual differences in how animals approach biologically significant situations such as mating are acquired through learning and experience. This is essential for adaptability and survival in a complex environment. In the context of mating, much work has been devoted into identifying how excitatory and inhibitory brain systems mediate “normal” mating patterns in a variety of species ([14], [28]; [29]; [27]). However, how the brain produces flexible patterns of mating that result in individual patterns of behavior is less explored, especially in the context of possible epigenetic modifications that underlie alterations in gene expression and protein availability.

Although rats are described as having a promiscuous mating strategy [13], they acquire conditioned mate preferences for a familiar partner through Pavlovian association of critical partner cues with sexual reward (Pfaus et al., 2012). If female rats have their formative sexual experiences paired with the same male, they will mate guard that male in the presence of a competitor female ([6]; [6,7]). In addition to increased sexual solicitations of the male, this conditioned mate-guarding behavior consists of hovering next to the male and presenting a pre-lordosis crouch (PRES), positioning her body between the male and the approaching competitor to block any competitive solicitation (Bet), and female-female mounting (FFM) of the competitor should she

actually solicit the male ([7]; [6]). With increasing experience, FFM sensitizes and becomes the dominant form of the behavior, initiated very quickly after the competitor is introduced into the open field. As in prairie voles, this conditioned mate-guarding behavior depends critically on the activation of bonding circuits in the brain, including oxytocin (OT) and vasopressin (AVP) neurons within supraoptic (SON) and paraventricular (PVN) hypothalamic nuclei, by their preferred male, but not by a novel male. Control females that were given unrestricted access to several males during their formative sexual experiences also did not display any mate guarding behavior, nor did they show the activation patterns of hypothalamic OT or AVP neurons [7]. Peripheral administration of OT or AVP during their first sexual experience facilitated PRES and RI, respectively, further demonstrating the critical nature of OT and AVP in the expression of this behavior [7]. Thus, conditioned sexual reward is a powerful mechanism capable of influencing the mating strategy of female rats to shift from promiscuity towards monogamy.

Memory formation requires gene transcription, and epigenetic modifications to chromatin structure are vital in regulating such gene expression, especially for long-term memory formation ([2,4,5,12,18]; for review see: [11]). Conditioned learning underlies the pair bonding, mate choice, and mate-guarding behavior we have observed in female rats (reviewed in Pfaus et al., 2012), and histone modifications have

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been shown to play a defining role in naturalistic bonding behavior observed in monogamous prairie voles.

Wang et al. [24] demonstrated a role of histone modifications in pair bonding in prairie voles by threatening sexually naïve females with histone deacetylase inhibitors (HDACi) prior to their induction into the pair bonding paradigm. Typically, in a pair bonding paradigm, female voles are allowed a 6 h cohabitation with the male and allowed to mate with him, if not allowed to mate this condition will not produce a pair bond. However, when treated with the HDACi, a 6 h cohabitation without mating was sufficient to induce pair bonding [24]. The facilitated establishment of the pair bond in a presence of the HDACi lead to an up-regulation of OT receptor and vasopressin 1a receptor (V1aR), both of which have been shown to be absolutely essential for pair bonding, in the nucleus accumbens [8,9,24]. Histone deacetylase inhibitors prevent the removal of acetyl groups from histone tails which promotes a more relaxed conformation of the histones. This conformation leads to DNA being more accessible and is therefore associated with an upregulation in gene expression. Therefore, it is consistent that treatment with an HDACi would facilitate the formation of pair bonding and the expression of pair bonding relevant neuropeptides. Although this line of work only explored one type of epigenetic histone modification, it demonstrates a strong role of epigenetic modification on the effects of early sexual experience that augment the activation of OT and AVP neuronal systems to underlie pair-bond formation.

Although a role of histone deacetylation has been demonstrated, epigenetic modifications do not act alone, but rather, in combination to influence gene expression. Therefore, the present study examined whether histone demethylation [15,22] is critical in the formation of conditioned mate-guarding in female rats. To do this, a lysine specific demethylase 1 (LSD-1) inhibitor [16] was used to disrupt the demethylation process during conditioning. LSD1 removed methyl groups from mono and demethylated lysine 4 or 9 residues of H3 histone tails [16]. If histone demethylation is important in conditioned mate guarding, then the maintenance of methylated states by the inhibitor should disrupt the development of this behavior and its neural correlates, in particular the activation of OT and AVP neurons by familiar, partner-related cues.

## 2. Materials and methods

### 2.1. Animals and surgery

Sexually naïve Long-Evans female rats (200–250 g) were obtained from Charles River Canada (St-Constant, QC, Canada). Animals were housed in shoebox cages in groups of two in a colony room on a reversed 12:12 h light/dark cycle at approximately 21 °C and given free access to food and water. Female rats were ovariectomized (OVX) via bilateral lumbar incision. Proceeding surgery, female rats were anesthetized with 1 mL/kg of ketamine hydrochloride (50 mg/mL) and xylazine hydrochloride (4 mL/kg), mixed in a ratio of 4:3 respectively, administered via intraperitoneal injection. Female rats were given 1 week to recover from surgery prior to the conditioning trials. Throughout the duration of the experiment, female rats were maintained on hormone replacement by subcutaneous injections of estradiol benzoate (EB; 10 µg in 0.1 mL of sesame oil) 48 h prior to testing, and progesterone (P; 500 µg in 0.1 mL of sesame oil) 4 h prior to testing.

Sexually naïve male rats (300–350 g) were also obtained from Charles River Canada (St-Constant, QC, Canada). They were housed in group cages (4 animals per cage) and housed under conditions identical to those of the female rats.

All animal procedures complied with the guidelines of the Canadian Council on Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

### 2.2. Conditioning apparatus

Conditioning occurred in unilevel Plexiglas pacing chambers (38 cm H × 60 cm W × 38 cm deep) with wire-mesh floors covering a layer of bedding. Chambers were bisected by a Plexiglas divider with four holes cut into the bottom which were large enough for the female to crawl through but too small for the male to crawl through [6,17,18]. These chambers are designed in a manner that optimizes the ability of the female to associate her paired male with a sexual reward state. The conditioning, testing, and behavioral paradigm follows our previous work on mate-guarding behavior [6,7].

### 2.3. Drug Preparation

The LSD1 demethylase inhibitor (LSD1) was purchased from Calbiochem (LSD1 inhibitor IV; RN-1, HCL; cat# 489479). 10 mg of drug was dissolved in 2 mL of saline to obtain a concentration of 5 mg/mL, aliquoted into Eppendorf tubes, and stored at –20 °C [16].

### 2.4. Conditioning procedure

Conditioning sessions occurred at 4-day intervals, 4 h after P injections, during the middle third of the rats' circadian cycle (lights off at 08:00). Females were assigned randomly to one of 3 groups. Paired females treated with LSD1, paired females treated with vehicle alone, or unpaired females that received no treatment ( $N = 12/\text{group}$ ). All paired females copulated with the same male across all trials, whereas unpaired females copulated with a variety of males across all trials. 1 h prior to testing, paired females were given an intraperitoneal injection of either LSD1 (10 mg/kg) or vehicle (0.9% saline). The chosen dose and 1 h time frame was chosen based off of microdialysis data demonstrating that 1 h is sufficient time for LSD1 to cross the blood brain barrier [16]. Males were placed onto one side of the conditioning chambers and allowed to habituate for 5-min before each trial. Females were then placed into the opposite side of the conditioning chamber and allowed to have paced sex with the male for 20 min. Each group received 10 trials, which were all recorded on video.

Throughout the course of this study, the health of the animals was assessed daily. Rats eating and drinking was monitored and body weights were taken bi-daily to ensure drug treatment did not impair the health of the animals. This was done to ensure the overall health and comfort of the rats, and also, to ensure that any effects were due to the action of the drug and not to failing health or abnormal behavior.

### 2.5. Mate-guarding test

Four days after the final conditioning trial mate guarding was assessed using an open-field (123 cm × 123 cm × 46 cm) with a thin layer of bedding [30]. The open-field apparatus allows enough space for the agonistic interactions between females, and for the display of mate-guarding behavior as observed in our previous studies ([6,7]). Each open-field contained a paired female, from either LSD-1 or vehicle groups, along with her corresponding male and an unpaired competitor female. Before the test, males were placed into the open field for a 5-min habituation period, after which both the paired and unpaired females were placed into the open-field at 2 diagonal corners. Rats were allowed to copulate freely for a 1-h period. After the open-field test female rats were perfused and their brains were collected to examine Fos induction within OT and AVP neurons.

All open-field tests were recorded on video and scored afterward using a computerized event recorder customized for rat sexual behavior in an open-field [1]. Mate guarding was defined operationally as any behavior engaged in by the female that took away the male's ability to mate with the competitor female. Although this can be achieved through a variety of aggressive, dominant, sexual, competitive, and social behaviors, and consisted of one or more of the following: 1)

presenting behavior (PRES) occurred when one female hovered in a receptive position (lordosis or pre-lordosis crouch) after the male had mounted or intromitted, which would more normally be followed by the female hopping and darting away from the male; 2) interference behavior (Bet), characterized by a female positioning her body between the male and the other female in an attempt to block her access to the male. Interference behavior typically preceded 3) female-female mounting (FFM) which occurred when one female mounted the other, typically until the mounted female left the vicinity of the male. We also took into account the number of times female rats initiated body contact with the male (init) and the total amount of time spent in the vicinity of the male (time spent). Similar measures are used commonly to determine pair-bond formation in prairie voles [3]. We also scored typical measures of appetitive and consummatory female sexual behaviors, including 1) solicitations (sol), which were displayed as a head-wise orientation to the male followed by turning, darting away, or a full runaway (as in [14]); 2) hops and darts, and 3) lordosis. Instances of aggression were quantified when one rat attacked another and the victim rat displayed signs of distress and vocalizations. Finally, we assessed the number of intromissions (Intro) and ejaculations (Ejac) received by each of the females on the final open-field test.

## 2.6. Perfusions

Animals were deeply anesthetized with an overdose of sodium pentobarbital (120 mg/kg) administered via intraperitoneal injection. They were then perfused intracardially with 250 mL of ice-cold phosphate-buffered saline (PBS) followed by 250 mL of ice-cold 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 4 h at 4 °C, then placed into a 30% sucrose solution for 48 h at 4 °C, after which they were flash frozen in crushed dry ice, covered with aluminum foil, and stored at –80 °C until slicing.

## 2.7. Tissue preparation and immunocytochemistry

Since no animals were disqualified from behavioral testing due to being outliers, brains were chosen at random from paired and unpaired females ( $N = 5$  brains/group) were cut coronally at 30  $\mu$ m on a freezing microtome and divided into three series, one of which was used for double immunohistochemistry to label for Fos and OT, the second was used to label for Fos and AVP, and the third was stored as a back up. In the primary phase, sections were washed in cold Tris-buffered saline (TBS) and incubated first with 30%  $H_2O_2$  in TBS for 30 min at room temperature followed by 3% Normal goat serum (NGS) in 0.05% Triton-TBS for 90 min at 4 °C, with rabbit polyclonal anti-Fos (Fos ab5, Calbiochem, Mississauga, ON; diluted 1:40,000) in 0.05% Triton-TBS with 3% NGS for 72 h at 4 °C. In the secondary phase, sections are incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories Canada, Burlington, ON; 1:200), in 3% NGS, and 0.2% Triton TBS for 1 h. Following this, sections enter a tertiary phase, where sections are incubated in avidin-biotinylated-peroxidase complex (Vectastain ELITE® ABC KIT, Vector Laboratories Canada; diluted 1:55) in 3% NGS, and 0.05% Triton TBS for 2 h at 4 °C. Sections were washed in TBS (3  $\times$  5 min) between each incubation.

Immunoreactions were stained by sequential treatments with 50 mM Tris for 10 min, 3,3'-diaminobenzidine (DAB) in 50 mM Tris (0.1 mL of DAB/Tris buffer, pH 7.8) for 10 min, and 8% nickel chloride (400  $\mu$ L per 100 mL of DAB/Tris buffer +  $H_2O_2$ ) all at room temperature. Reaction was stopped by rinsing (3  $\times$  10 min) in PBS. Sections were then transferred into the second primary antibody, either rabbit polyclonal anti-oxytocin (Chemicon, AB911, diluted 1:10,000) or rabbit polyclonal anti-vasopressin (Millipore, AB1565, diluted 1:10,000) in 0.05% Triton-TBS with 3% NGS, and incubated for 72 h at 4 °C. After completion of this incubation, secondary and tertiary phases are repeated.

However, no nickel chloride was added to the final DAB wash in

order to create a light brown cytoplasmic stain.

Following staining, sections were mounted onto gel-coated slides and cover slipped following immunohistochemistry. Cover slipping procedure started with sequential washes of distilled water followed by washes in 70%, 90%, and 100% alcohol each at room temperature for 1 min duration each. Slides were then dipped 50 times in Citrisolve at room temperature after which they were cover slipped using Permount solution. Slides were set aside for a week to allow for the Permount to dry and then cleaned and coded so that all analysis would be conducted blind.

Sections were examined using an Olympus light microscope at 400 $\times$  magnification. Pictures were taken at 200 $\times$  and 1000 $\times$  using Q-Capture pro software. Double-labeled cells were identified by eye as any cell having a dark black nucleus (Fos positive) and a light brown cytoplasm (OT or AVP positive). Brain regions were defined using the atlas of [17]. Double-labeled cells were counted within the supraoptic nucleus (SON, Plates 21–25), and paraventricular nucleus (PVN, Plates 25–26) as these two regions are where OT and AVP neuronal bodies reside. Five slices were counted per animal. These slices represented each area rostrally to caudally and were matched among animals to ensure accuracy. Counts were done bilaterally in a total of 5 animals per group. Total number of double-labeled cells was counted in each section, which were then used to derive the mean number of double-labeled cells within the region.

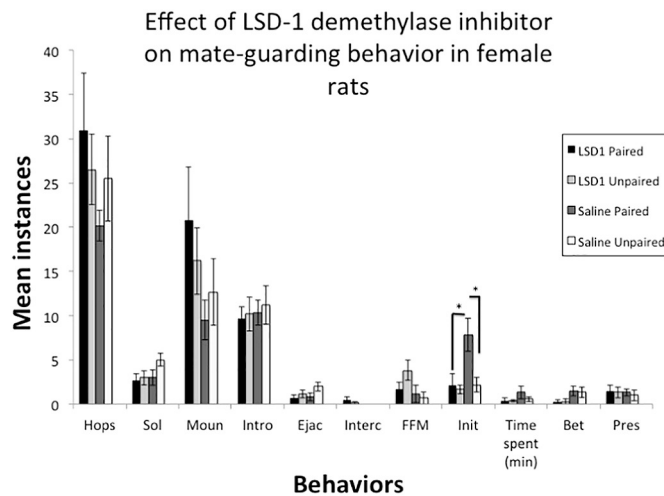
## 2.8. Statistical analyses

Mate-guarding behaviors in the open-field were assessed using independent samples *t*-tests, since we had planned comparisons ahead of time, described below in the results section. In short; only animals that behaved within the same open field test were measured together for statistical purposes. For example, the behavior of LSD1 treated females was compared to the behavior of saline treated females that served as competitor females in their set of open fields (LSD1 unpaired). Likewise, the behavior of untreated paired females was compared to the behavior of the saline treated animals that served as competitors in their set of open fields (Saline unpaired). This design rendered us with two groups and one level of comparison, which made independent samples *t*-tests appropriate statistical tests for this study. Each female in the 4 groups was placed together with an unpaired, saline-treated competitor female. Planned comparisons were made between the subject female and the competitor female and summed into means for each of the four subject treatment groups. Cohen's *d* was used to assess effect size. The data collected from Fos-IR cells were analyzed using a one-way analysis of variance (ANOVA) with post-hoc *t*-tests between individual groups corrected for elevated experiment-wise error using the Bonferroni method. Eta squared was used as a measure of effect size.

## 3. Results

### 3.1. Mate guarding behavior in the open field

During the final open-field test, paired females treated with the vehicle initiated more contacts with the male (INIT) than did the unpaired females they were tested with ( $t(10) = 2.791$ ,  $p = .028$ ,  $d = 1.609$  (Fig. 1), but did not display more FFMs ( $t(10) = 0.422$ ,  $p = .683$ ), Sol ( $t(10) = 0.114$ ,  $p = .115$ ), Pres ( $t(10) = 0.439$ ,  $p = .672$ ), Time Spent ( $t(10) = 1.268$ ,  $p = .234$ ) or RI ( $t(10) = 0.073$ ,  $p = .838$ ). In contrast, paired females treated with LSD-1 inhibitor failed to show any measure of mate guarding behavior and did not differ behaviorally from unpaired, vehicle-treated females. During the open field test, LSD-1 inhibitor treated females did not react protectively towards their partner male, and passively allowed the conspecific to approach and mate with the male. Control behaviors, such as hops and darts (HOP), were assessed in order to evaluate sexual proceptivity to ensure all animals were sexually receptive and behaving normally.

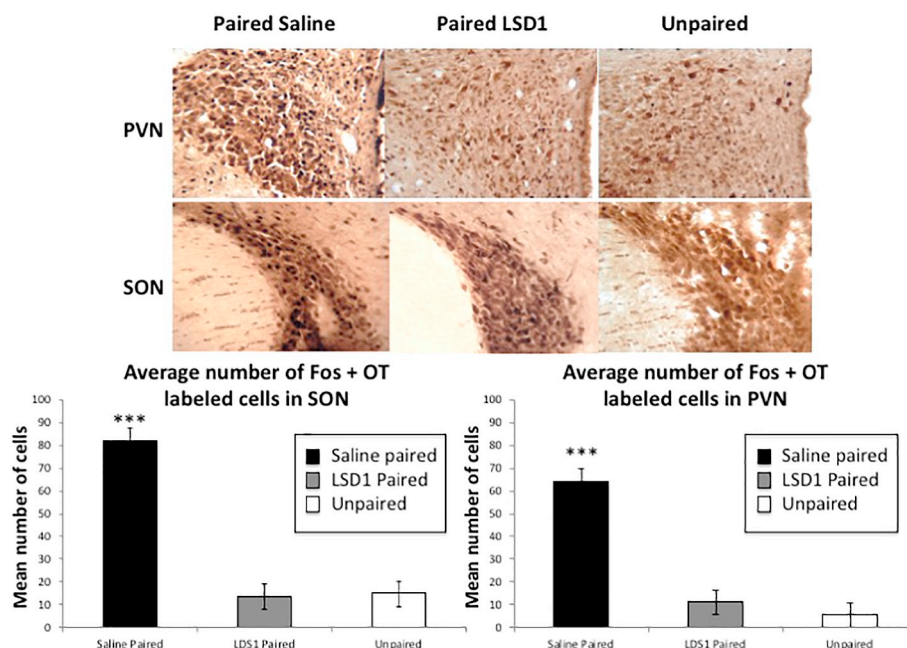


**Fig. 1.** Mate guarding behavior displayed in open field by paired female rats that were treated with either LSD1 or Saline. Behaviors include hops, solicitations (Sol), mounts received (moun), intromissions received (intro), ejaculations received (ejac), interceptions (interc), female-female mounting (FFM), number of times initiating closeness to the male (Init), time spent in proximity to the male (TimeSpent), interference (Bet), and presenting (pres). Data are means  $\pm$  SEM. \* $p < .05$ , \*\* $p < .01$ .

No statistical differences were found in HOP behavior between groups indicating that all animals were sexually receptive and behaving in a comparable manner ( $t(10) = -1.040$ ,  $p = .561$ ).

### 3.2. Double-labeled cell counts

A one-way ANOVA was used to examine differences in Fos induction within OT and AVP neurons between paired LSD-1, paired vehicle, and unpaired untreated females. Vehicle treated females had significantly more double-labeled Fos/OT neurons within both the SON,  $F(2,12) = 97.063$ ,  $p = .000$ ,  $\eta^2 = 0.942$ , and PVN,  $F(2,12) = 70.903$ ,  $p = .000$ ,  $\eta^2 = 0.922$ , compared to females treated with LSD-1 or controls (Fig. 2). Paired vehicle treated females also had more double-labeled Fos/AVP neurons within both the SON,  $F(2,12) = 25.895$ ,  $p = .000$ ,  $\eta^2 = 0.812$ , and PVN,  $F(2,12) = 10.836$ ,  $p = .002$ ,



**Fig. 2.** Top: Photomicrographs (40 $\times$ ) showing OT + Fos-IR in the SON and PVN following the open-field test. Cells positive for both markers have a light brown cytoplasmic stain (OT) with a dark black nucleus (Fos). Magnified inserts taken of selected regions at 100 $\times$  to more clearly demonstrate double labeled v. single labeled cells. Bottom: OT + Fos positive cells in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) from saline paired (black), LSD1 paired (grey), or unpaired (white) groups. Data are means  $\pm$  SEM. \* $p < .05$ , \*\* $p < .01$ ; \*\*\* $p < .001$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

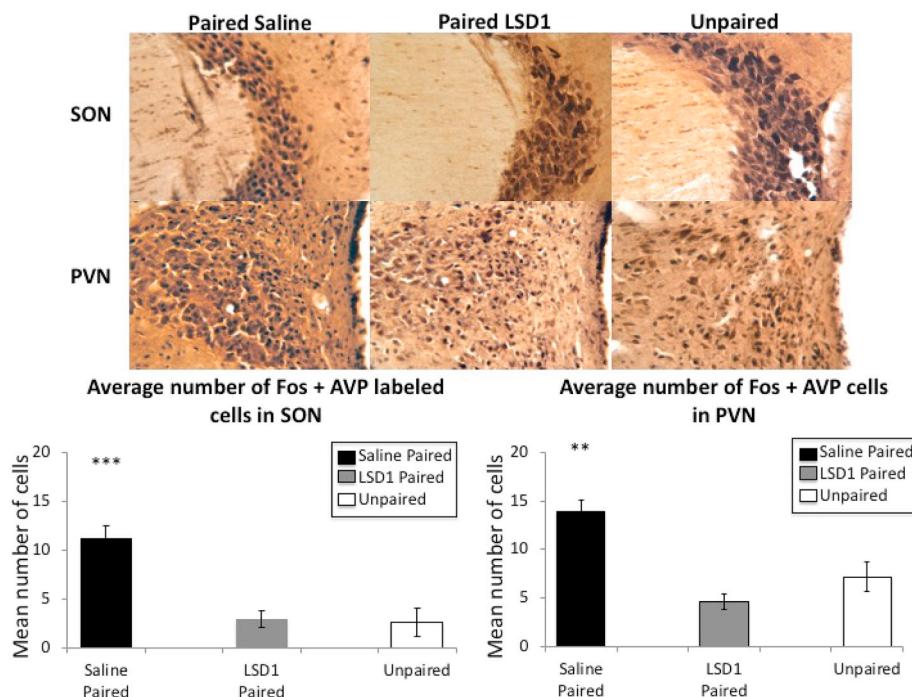
$\eta^2 = 0.644$  compared to treated with LSD-1 and controls (Fig. 3). Fos induction within OT and AVP neurons did not differ between females treated with LSD-1 and controls (Figs. 2 and 3),  $p > .5$  for all measures. Since double-labeling data from the LSD1-unpaired and saline-unpaired groups did not differ, we paired their brains for analysis.

### 4. Discussion

These data demonstrate that conditioned mate guarding behaviors in female rats, along with the activation of at least two important neurochemical mediators, are regulated epigenetically by lysine-specific histone demethylation during a female rat's first experiences of sexual reward. Blockade of the lysine-specific demethylase-1 enzyme blocked the development of conditioned mate guarding behavior in female rats, and blocked the corresponding increase in activation of OT and AVP neurons that underlies different aspects of this behavior. However, this treatment did not block the activation of solicitations or lordosis by estradiol and progesterone, indicating that the effect was specific to the formation of conditioned sexual partner preferences and not to the general display of appetitive or consummatory sexual behaviors. Taken together, these data provide evidence that the conditioned shift in mating strategy from promiscuity towards monogamy that we have observed in female rats in response to sexual conditioning is dependent, in part, on an epigenetically-regulated increase in activation of OT and AVP neurons by individual cues of the familiar male.

The LSD-1 demethylase inhibitor used here has been shown to cross the blood brain barrier in under an hour, and has also been shown to disrupt central processes such as long-term memory formation. Specifically, mice that were given LSD1 failed a novel object recognition task [16]. Although LSD-1 demethylase enzymes have been shown to act centrally they also can act peripherally, so it could be that the effect we observed here is due to the blockade of LSD-1 demethylase enzyme function in a peripheral location [15,16,26]. However, because LSD-1 inhibitor treatment did not create any symptoms of sickness in treated animals (no visible weight loss, tumors, loss of appetite, failure to groom, discoloration of paws), did not disrupt appetitive or consummatory behaviors in general, and blocked the activation of Fos within OT and AVP neurons in both the PVN and SON, the effect on behavior is likely mediated by central actions. Further work is warranted in order to investigate the role of central vs. peripheral contributions.





**Fig. 3.** Top: Photomicrographs (40 $\times$ ) showing AVP + Fos-IR in the SON and PVN following the open-field test. Cells positive for both markers have a light brown cytoplasmic stain (AVP) with a dark black nucleus (Fos). Magnified inserts taken of selected regions at 100 $\times$  to more clearly demonstrate double labeled v. single labeled cells. Bottom: AVP + Fos positive cells in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) from saline paired (black), LSD1 paired (grey), or unpaired (white) groups. Data are means  $\pm$  SEM. \* $p < .05$ , \*\* $p < .01$ ; \*\*\* $p < .001$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Neuroanatomical and pharmacological studies have provided strong evidence that both OT and AVP are crucial for bonding in prairie voles [8,9,19,20,23,25]. Previously, we demonstrated that female rats conditioned to display monogamous behaviors showed an increased activation of OT neurons within the PVN and SON, and increased activation of AVP neurons in the SON, when exposed to their individual partnered males [7]. Control groups of female rats, with the same amount of sexual experience, but with a variety of partners do not show this increase in OT and AVP neuronal activation in response to exposure to potential mate [7]. In the second part of that study, peripheral administration of OT or AVP prior to a female's first paced copulation conditioning trial with a male facilitated the display of presenting or interference behavior, respectively, on the second trial with that male, whereas saline injection did not [7]. Thus, the conditional activation of OT and AVP shapes the natural display of these two aspects of mate guarding in female rats.

That both mate guarding behaviors and the activation of OT and AVP neurons were blocked by LSD-1 demethylase inhibitor treatment in the present study indicates that histone demethylation is an essential mechanistic component that enhances the activation of OT and AVP neurons in response to cues of the partner male, leading to the crystallization of a monogamous-like partner preference and mate-guarding behavior. Although we have not identified the precise mechanism, it is likely that the disruption in histone demethylation by LSD1 is occurring within cells that make up the OT/AVP neural circuitry.

Histone methylation/demethylation does not have straightforward transcriptional outcomes as do other histone modifications, like acetylation, which is almost invariably associated with transcriptional activation (For review: [10]). What is of vital importance in regards to histone methylation/demethylation is the pattern of methylation that is established. Patterns of methylation incorporate both methylated and demethylated residues, and are what ultimately play a role in transcriptional outcomes. Patterns of histone methylation can act bidirectionally, meaning that some patterns result in transcriptional activation, whereas others result in transcriptional repression, and both repressive and activational patterns can occur simultaneously [10,21]. In the present study, inhibiting LSD1 demethylase enzymes disrupted the ability of cells to properly establish histone methylation/demethylation patterns, thus creating a deficit in the cells' ability to transcribe

the gene products necessary for the enhanced induction of OT, AVP, and the subsequent mate-guarding behaviors we observed. It could also be that the disruption in histone demethylation within OT and AVP circuitry is altering the expression of upstream neurochemical inputs that drive the activation of OT or AVP neurons, and/or of other neurochemical pathways that are themselves driven by OT and AVP action or influence downstream OT and AVP action in the brain during a female rat's first rewarding sexual experiences.

In summary, this study is the first to demonstrate a definitive role of epigenetic histone modifications in a conditioned sexual response. Histone modifications are a key element in gene regulation through chromatin remodeling. LSD-1 demethylases have been shown to demethylate repressive histone markers thus leading to transcriptional activation [15]. The up-regulation of OT and AVP neuronal activation in response to monogamous conditioning would require changes in gene expression. Although the data from this study strongly support the role of LSD-1 histone demethylases in this process, further work is required to explore the contribution of other histone modifications. Epigenetic regulation requires the interplay of processes such as methylation, acetylation, and phosphorylation (Kouzarides et al., 2007). It is therefore highly unlikely that only one of these processes works alone to establish the changes to neural circuitry that underlie conditioned mate guarding. Indeed, it has already been established that histone acetylation plays an important role in naturally occurring pair bonding in prairie voles [24]. It would therefore be important to explore the role of other histone modifications in conditioned mate guarding.

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