The Role of Brain Opioid Transmission in the Conditioned Ejaculatory Preference of the Male Rat

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Abstract

The Role of Brain Opioid Transmission in the Conditioned Ejaculatory Preference of the Male Rat

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The experiments presented in this thesis examined the role of opioids in sexual pleasure, particularly during early formative sexual experiences, on the ability of male rats to associate an olfactory or somatosensory cue with their ejaculation-induced sexual reward state. The results show that opioids are an important substrate of the sexual reward state, and that this state feeds forward to form a conditioned ejaculatory preference (CEP), in which male rats ejaculate preferentially with females bearing or wearing the cue associated with sexual reward. This suggests that sexual reward (a presumably "proximate" cause of behavior) lies at the root of monogamous mate choice in the rat. These data also contribute to a growing body of evidence that the species' specific sexual strategies of the male rat, often described as promiscuous, evidence a degree of plasticity that can move toward a monogamous mate choice when features of a familiar female (odor or jacket) are associated with sexual pleasure. This conditioned ejaculatory preference for familiar cues suggests that proximate pleasure-related states act as a Pavlovian US that can reinforce cue-related CSs to create partner and mate preferences. Such preferences bring proximate reward states into synch with reproductive choice, potentially enhancing reproductive success. Findings are discussed, as well as further directions.

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Contribution of Authors

Chapter 1 - General introduction.

- Gonzalo R. Quintana = Manuscript writing.
- James G. Pfaus = Manuscript writing.

Chapter 2a - First sexual experiences determine the development of conditioned ejaculatory preference in male rats.

- Gonzalo R. Quintana = Study design, data collection and analyses, manuscript writing.
- Andrés Guizar & Sarah Rassi = Data collection.
- James G. Pfaus = Study design, data analyses, manuscript writing.

Chapter 2b - Effect of CS pre-exposure on the conditioned ejaculatory preference of the male rat: Behavioral analysis and neural correlates.

- Gonzalo R. Quintana = Study design, data collection and analyses, manuscript writing.
- Misha Jackson & Mojdeh Nasr = Data collection.
- James G. Pfaus = Study design, data analyses, manuscript writing.

Chapter 3 - Conditioned partner preference in male and female rats for a somatosensory cue.

- Gonzalo R. Quintana = Study design, data collection and analyses, manuscript writing.
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Chapter 4 - Naloxone disrupts the development of a conditioned ejaculatory preference based on a somatosensory cue in male rats.

- Gonzalo R. Quintana = Study design, data collection and analyses, manuscript writing.
- Brunella González, Yvonne Bachoura, Eric Borduas, Valérie Lemay & Francisco Yarur = Data collection and analyses.
- James G. Pfaus = Study design, manuscript writing.

Chapter 5 - Differential disruption of conditioned ejaculatory preference of the male rat based on different sensory modalities by micro-infusions of naloxone to the mPOA or VTA.

- Gonzalo R. Quintana = Study design, data collection and analyses, manuscript writing.
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- James G. Pfaus = Study design, data analyses, manuscript writing.

Chapter 6 - General discussion.

- Gonzalo R. Quintana = Manuscript writing.
- James G. Pfaus = Manuscript writing.

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List of Abbreviations

Fos-IR: Fos Inmunoreactivity

DA: Dopamine

NE: Norepinephrine

ME: Melanocortin

OT: Oxytocin

5-HT: Serotonin

ECB: Endocannabinoid

PEI: Post-ejaculatory interval

CPP: Conditioned partner preference

CEP: Conditioned ejaculatory preference

NAL: Naloxone hydrochloride

PIM: Pimozide

mPOA: medial preoptic area

VTA: Ventral tegmental area

NAc: Nucleus accumbens

CEP: Conditioned ejaculatory preference

GABA: Gamma-aminobutyric acid

CS: Conditioned stimulus

US: Unconditioned stimulus

CR: Conditioned response

UR: Unconditional response

ScF: Scented female

UnScF: Unscented female

5t: 5 times

1t: 1 time

i.p.: intra-peritoneal

IHC: Immunohistochemistry

TBS: Tris-buffered solution

H_2O_2: Hydrogen peroxide

NGS: Normal goat serum

DAB: 3,3'-diaminobenzidine

CeA: central nucleus of the amygdala

BLA: basolateral nucleus of the amygdala

Pir Ctx: Piriform cortex

VMH: ventromedial hypothalamic nucleus

Cg1: cingulate cortex area 1

PrL: Prelimbic

IL: Infralimbic

ANOVA: Analysis of variance

HSD: Honest significant difference

CPC: Conditioned partner choice

SAL: Saline

AMPH: Amphetamine

Chapter 1 - General Introduction

General introduction

Sexual behavior in the male rat encompasses a wide range of biological functions that regulate genital response, arousal, motivation, and performance directed to exert, maintain, and terminate copulation (Hull, 2009). Thus, in order to be able to successfully find a potential mate, an animal must be able to respond effectively to internal and external cues that signal neurochemical and hormonal changes that indicate different probabilities to copulate. All these processes are controlled by different brain areas, neurotransmitters, and hormones in the brain and the periphery of the nervous system, regulated by memory and reward systems that allow the animal to establish associations and valence to them that will help them navigate current and future encounters.

Beyond the priming role of steroid hormones to respond to sexual incentives (for a review see Pfaff, 1999), the orchestration of sexual behavior is a fine-tuning process between excitation and inhibition of the central and peripherical nervous system, regulated differently by several neurotransmitters. The main neurotransmitters involved in excitation are dopamine (DA), norepinephrine (NE), melanocortin (MC), and oxytocin (OT) acting mainly in hypothalamic and limbic regions controlling sexual arousal, attention, and motivated behavior, whereas the ones involved in inhibition are serotonin (5-HT), endocannabinoids (ECB), and opioids believed to be responsible mainly for regulating adaptative response of termination of sexual behavior, satiety, sedation, and reward (Pfaus, 2009).

Mesolimbic, nigrostriatal, and hypothalamic DA facilitate general attention to incentive stimuli, copulatory proficiency, and genital reflexes (Dominguez & Hull, 2005). Systemic administration of DA agonist facilitated male sexual behavior (Melis & Argiolas, 1995), induced copulation in sexually-sluggish males (Tagliamonte *et al*., 1974), as well as in sexually-exhausted males (Rodríguez-Manzo, 1999); whereas DA antagonist has shown to impair sexual behavior in male rats (Pfaus & Phillips, 1989). The NE system has been shown to play a role in general arousal

and control of autonomic outflow, shown to facilitate as well as inhibit male sexual behavior depending to which receptor they may bind (Hull, 2009; Pfaus, 2009). For instance, yohimbine, an adrenergic receptor antagonist, has shown to enhance mounting scores in male rats (Clark, 1995), while lesions of noradrenergic cells bodies from the locus ceruleus increased the postejaculatory refractory period (McIntosh & Barfield, 1984). Finally, OT, also known as the "bonding hormone", has been implicated with sexual arousal, orgasm, satiety, and bonding (Carter, 1992). Located mainly in the paraventricular and supraoptic nuclei of the hypothalamus, infusions of OT into the paraventricular nucleus of male rats stimulated penile erection, while systemic administration facilitated ejaculation in male rats treated with fluoxetine, a serotonin selective reuptake inhibitor shown to reduce sexual behavior (Kita, Yoshida & Nishino, 2006).

Three mainly neurotransmitters are associated with sexual inhibition: serotonin (5-HT, endocannabinoids (ECB), and endogenous opioids (Pfaus, 2009). 5-HT originates in the Raphé nuclei sending projections toward brain areas located in the brainstem, mid and forebrain, as well as in several other structures in the periphery (Hull, 2009; Pfaus, 2009). 5-HT in the brain regulates satiety through a complex mechanism at many levels of processing, including the hypothalamus and prefrontal cortex (REFS). Facilitation of serotonin turnover inhibits sexual behavior in male rats, whereas inhibiting its synthesis, release, or receptor binding, facilitates male sexual behavior (Meyerson, 1964; Pfaus, 2009). Moreover, systemic injections of a serotonin inhibitor restored sexual behaviors in sexually sluggish, as well as in gonadally intact or castrated male rats (Dalló, 1977). ECBs have been well-known for their sedative effects (see Pazos *et al*., 2005). The brain endocannabinoid systems are located mainly in cortical, limbic, hypothalamic, and motor regions (Pazos, Núñez, Benito, Tolón & Romero, 2005). In the context of male rat sexual behavior, the CB1 receptor agonist Δ^9 tetrahydrocannabinol increased the ejaculation latency, whereas an antagonist facilitated erection and ejaculation (Gorzalka, Moris & Hill, 2008).

In addition, Mellis *et al*., (2004) demonstrated that CB1 receptors antagonists (SR141716A or AM251) in the paraventricular nucleus of the male rat induced penile erection in a dose dependent manner and facilitated ejaculation. Finally, opioids are well known for being involved in the rewarding aspects of sex (Parédez, 2014) and as a necessary substrate of sexual refractoriness in the male rat (Garduño-Gutiérrez, Guadarrama-Bazante, León-Olea, & Rodríguez-Manzo, 2013). Accordingly, copulation to ejaculation increased whole brain ß-endorphin content (Szechtman, Hershkowitz & Simantov, 1981) and μ-opioid receptor activation in the medial preoptic area (mPOA; Coolen *et al*., 2004) and ventral tegmental area (VTA; Balfour *et al*., 2004), regions implicated in the control of sexual behavior and reward (Hull, 2009). Moreover, opioid use dramatically reduced sexual arousal, increases the ejaculation latency, and/or inhibits sexual responding altogether (Pfaus & Gorzalka, 1987), and infusions to the mPOA mimicked a postejaculatory state behaviorally (Hughes, Everitt, & Hervert, 1987).

As briefly described above, the organization, control, and termination of sexual behavior in males, as well as the rewarding sensations derived from it, are a complex orchestration of different neurochemical process in different brain regions. Particularly, the present thesis focused on the role of opioids and examined their role in sexual reward in the context of the development of conditioned ejaculatory preference (CEP) in the male rat.

The rewarding properties of opioids

During sexual intercourse, and after several intromissions, male rats ejaculate falling into a period of sexual quiescence in which another erection cannot be achieved in the span of a few minutes, a period known as post-ejaculatory interval (PEI) or refractory period (for a review see Hull, 2009). To evaluate the rewarding properties of ejaculation, Ågmo & Berenfeld (1990) used sexual reward resulting from ejaculation in the conditioned place preference (CPP) paradigm. In

their study, male rats were placed in a CPP box that has three compartments. Initially, male 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 and background color). The time spent in each determines the animal's natural preference. Subsequently, male rats were given several different training trials varying depending on group membership. All groups received their treatment and later on placed in their non-preferred side of the box, while in alternative days they were injected with a vehicle and put in their preferred side. One group was allowed to copulate until ejaculation with a receptive female in a different chamber, and immediately after put in the non-preferred side of the box and left for 30 minutes. Similarly, two more groups were given the same treatment, yet injected with either the opioid receptor antagonist naloxone hydrochloride (NAL) or the dopamine receptor antagonist pimozide (PIM) five min before copulation. Moreover, other groups were injected with either NAL, PIM or the μ opioid agonist morphine without subsequent copulation. Finally, male rats were tested for place preference 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, just like 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 who did not copulate or were only injected with vehicle before copulation did not show any preference for either of the sides of the box (Ågmo & Berenfeld, 1990). 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 associations with cues of the immediate context or environment, and that these associations may be founded in the action of opioid, not dopamine, neurotransmission.

Similar results were found when analyzing the role of opioids and rewarding properties of ejaculation in the development of a conditioned ejaculatory preference (CEP). Ismail, Girard-Bériault, Nakanishi & Pfaus (2009) trained males to develop a preference to for receptive females bearing an olfactory cue (*i.e.,* almond odor) previously paired with copulation to ejaculation (see below). Before each of the 10 conditioning trials, males were injected either with a vehicle, NAL, or the dopamine receptor antagonist flupenthixol (FLU), and subsequently placed into a unilevel pacing chamber where they had access for 30 min. to a sexually-receptive female bearing the odor. Finally, their preference was tested in an open field arena where they had access to two sexually-receptive females, one scented and the other not scented. NAL-treated males did not show a preference for the scented females, whereas FLU-treated and control males did show a partner preference for the scented females. These findings corroborate the rewarding properties of ejaculation and that the activation of opioid, but not dopamine, neurotransmission is necessary for the development of these associations.

The inhibitory properties of opioids

Clearly, opioid transmission is critical in the establishment of sex reward-related associations. However, somewhat paradoxically, the action of opioids in the control of both male and female sexual behavior is inhibitory (reviewed in Pfaus & Gorzalka, 1987; Pfaus, 2009). Similar results have been found in the human sexual behavior when opiates like heroine or morphine are involved (reviewed in van Ree *et al.,* 1999) showing an inhibitory symptoms-like drowsiness, sedation, or lethargy. Although the acute effect of opioids and opiates has been associated with intense euphoria, relaxation, and sedation, chronic opiate use and abuse has been linked to serious deterioration of sexual function, like elimination of sexual dreams, delayed ejaculation, decrease of ejaculation volume, anorgasmia, and erectile dysfunction (Hull & Dominguez, 2007; Pfaus & Gorzalka, 1987).

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, pro-enkephalin, and pro-dynorphin (Goldstein *et al*., 1979; Lewis *et al*., 1980; Nakanishi *et al*., 1978). For these endogenous opioids, there are three types of opioid receptors, μ, κ, δ. These receptors are distributed all along the central nervous system, predominantly located hypothalamic, limbic, and cortical areas (Desjardins, Brawer & Beaudet 1990; Mansour *et al*., 1994), having different roles in the control of sexual behavior. For example, β-Endorphin infused into the mPOA of sexually-experienced male rats impaired their copulatory behaviors in a dose-dependent fashion, where the highest dose (40 pmol) completely abolished mounts, intromissions, and ejaculations (Hughes *et al*., 1987). Furthermore, compared to sexually-active males, sexually-inactive males have shown an increment of endogenous opioid octapeptide Met-Arg^{6}-Gly^{7}-Leu^{8} in the hypothalamus (Rodríguez-Manzo *et al.,* 2002), as well as an increment of pro-enkephalin and pro-dynorphin mRNA expression in the paraventricular nucleus (Arletti *et al.,* 1997). At the receptor level, infusions of morphiceptin, a μ opioid agonist, into the mPOA of male rats produced a delay in their initiation of copulation compared to the control animals infused with a vehicle solution (Matuszewich & Dornan, 1992). Moreover, inhibition of copulatory behaviors (*e.g*., copulation latency and ejaculation) was found when infusing a κ opioid receptor agonist (U-50,488H), an effect that disappeared differentially when a κ opioid receptor antagonist (nor-binaltorphamine) was infused into the ventral tegmental area (VTA), mPOA, and nucleus accumbens (NAc, Leyton & Stewart, 1992). Similarly, facilitation of male sexual behavior has been found when NAL or the longer-acting opioid receptor antagonist, naltrexone, has been administered in sexually-inactive males (Gessa *et al.,* 1979), sexually-naïve males (Pfaus & Wilkins, 1995), and sexually-satiated males (Rodríguez-Manzo & Fernández-Gausti, 1995), and also reduced the ejaculation latency and increased ejaculation frequency in sexually active male rats (Myers & Baum, 1979).

The actions of the different neurotransmitters on sexual behavior vary across brain areas and behaviors, and opioids, in particular, have more than just an inhibitory role in sexual behavior. As previously stated, it has been shown that copulation and ejaculation are crucial rewarding aspects in the establishment of CPP (Ågmo & Gómez, 1991) and conditioned ejaculatory preference (CEP; Ismail *et al.,* 2009; Kippin & Pfaus, 2001). These phenomena are known to be the result of the synthesis of opioid released during copulation, an effect blocked by NAL, thus reducing opioid levels in the midbrain (Szechtman *et al.,* 1981); and more importantly during ejaculation, where an increase concentration of enkephalins in the midbrain and hypothalamus has also been found (Rodriguez-Manzo *et al.,* 2002). For instance, mating and ejaculation induces μ-receptor internalization (a marker of ligand-induced receptor activity) in the mPOA (Coolen *et al.,* 2004) and VTA (Balfour *et al.,* 2004). More specifically, it has been shown that different number ejaculations renders different μ-receptor and δ-receptor internalization in the VTA, where the internalization of both receptors increased as a result of ejaculation, yet only μ-receptor internalization was correlated to the amount of times a male ejaculated (Garduño-Gutiérrez, León-Olea & Rodríguez-Manzo, 2013). Furthermore, micro-infusions of morphine and dynorphin into the VTA increased DA transmission into the NAc, which ultimately facilitated male sexual behavior (Mitchell & Stewart, 1990). Also, male rats developed a CPP when D-Ala^2-Met^5-enkephalin (DALA) was infused bilaterally into their mPOA (Ågmo & Gomez, 1991). Moreover, opioid antagonists have been shown to have inhibitory effects, like increasing the length of the PEI (*e.g.,* McConnell *et al.,* 1981). This facilitation of opioid activity may be possible through the action of opioid neurons disinhibiting DA neurons in the mesolimbic pathway by acting on VTA GABAergic neurons that exert constant inhibition on DA transmission (Balfour *et al.,* 2004; Fields & Margolis, 2015). The variation and complexity are thought to be the result of different factors so far known including dose-base, biphasic effects of agonists and antagonists, brain area, time of the day where testing occurs, and

sexual activity level of the animal (reviewed in Hull, 2009). Regardless of their inhibitory or excitatory action, opioid activity plays a vital role in the establishment of rewarding associations. Thus, opioids and their modulation of mesolimbic DA in response to sexual incentive cues, which focus the attention towards directed behaviors towards reward-predicting stimuli, lay out the foundation of how sexually rewarding experiences and critical periods shape how animals would foster preferences of one mate over the other among the pool of possibilities (Pfaus *et al*., 2012).

Learning and sexual experience

Almost every behavior is the product of the interaction between the animal's central nervous system and learned experiences. Yet, in order to determine which one prevails over the other when it comes to partner preference, or the classical question "nature vs. nurture", studies have shown that experience appears over rides biological predetermination. Previously, it has been shown that inbreed mice prefer to copulate with partners of a different haplotype (or genetic parents; Yamazaki *et al*., 1976). To evaluate whether this is a natural or learned preference, Yamazaki and collaborators took a litter of newborn mice and transferred to foster parents whose litters were removed approximately at the same time after born. 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 (Yamazaki *et al*., 1976), 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 will resemble more an adoptive mother rather than the genetic mother (Kendrick, Hinton & Atkins, 1998). In their

experiment, Kendrick and colleagues separated males and females sheep and goat 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 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 (Kendrick *et al*., 1998). These findings provide 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.

Classical conditioning of sexual behavior

One of the fundamental form of learning by which animals can acquire these preferences from their environments and experiences was described by Pavlov (1927), coined as signal 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.,* Rescorla & Wagner, 1972).

As previously mentioned, the reinforcing properties of sexual reward resulting from copulation, and in particular from ejaculation, are able to be associated with neutral cues like of the environment and of any sensory modalities, depending on their salience. Thus, male rats have shown to display a CPP for the compartment in which copulation or only PEI was paired with (*e.g*., Ågmo & Berenfeld, 1990). Furthermore, Domjan & Hall (1986) showed that male Quail will remain around the vicinity where they could see a female Quail, a conditioned association that was

established only if those male Quails were able to have access to copulate with that female. As for olfactory cues, pairing a wintergreen odor with copulation to ejaculation in male rats has been shown to elevate levels of serum luteinizing hormone and testosterone relative to the same odor when it was not paired with sexual reward (Graham & Desjardins, 1980). Similar findings have been found when an odor cue is beard by a receptive partner whom animals copulate repeatedly.

Male rats develop a conditioned ejaculatory preference (CEP) towards females bearing an odor that has been previously associated with sexual reward (*e.g.,* Kippin, Talianakis, Schattmann, Bartholomew & Pfaus, 1998). In their experiment, Kippin and colleagues trained males in three different groups: the paired group was trained with receptive-scented females; the unpaired group was trained with both receptive-unscented females and non-receptive scented females; and finally the random group was randomly paired with receptive and non-receptive scented females. In the preference test, where males are given the choice to copulate with two sexually-receptive females, one scented and the other not, males in the paired group developed a CEP for the females bearing the odor. Conversely, males in the unpaired group displayed a CEP for the unscented females. Finally, males in the random group did not prefer any female in particular (Kippin *et al.,* 1998). This CEP effect is believed to be the product of rewarding properties of ejaculation and neural processes occurring mainly after it during the post-ejaculatory interval (PEI, Kippin & Pfaus, 2001). In their experiment, Kippin & Pfaus trained male rats for nine trails with sexually-receptive scented females in different conditions. Two groups of males were allowed to ejaculate plus the first intromission after the PEI, one of those groups was allowed to ejaculate twice and the other once. Another group was allowed to ejaculate, yet not to spend their PEI around the scented females. The last group was only allowed to display five intromissions. Results showed that only males who were allowed to ejaculate and spend their PEI around the scented female developed a CEP, demonstrating that events during the PEI are crucial for the establishment of the CEP.

Although rats rely heavily in their sense of smell, similar finding have been found with somatosensory and visual cues to those found with smells. Domjan, Huber-McDonald & Holloway (1992) used Japanese quail males and divided them into two groups. Both groups 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 towards the fully covered inanimate object than the males trained in the non-fading group in the form of grabs, mounts, and cloacal contacts. These data demonstrated that sexual behavior is able to be conditioned towards an inanimate stimulus object that has no natural connection with sexual reward (Domjan, Huber-McDonald & Holloway, 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.

A neutral somatosensory CS can also be conditioned to modulate sexual arousal. Pfaus, Erikson 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 non-receptive female.

These findings on CPP and conditioned partner preference have also been demonstrated in female rats. Previous studies have shown that females develop a preference for a particular chamber of a box if this was paired with paced copulation, yet not when they were not in control of the rate of copulation (Parédez & Alonso, 1997). This was achieved and tested in unilevel pacing chambers bisected with a 4-hole divider, which only allowed only females to cross and to escape from the male at will. Furthermore, females are also able to develop a conditioned partner preference based on an olfactory cue on a male rat if the neutral cue was paired enough times with sexually rewarding experiences (Coria-Ávila *et al.,* 2005). Using a similar conditioning training procedure as the in Kippin *et al*., (1998) and allowing females to control the rate of copulating using pacing chambers bisected with a 4-hole divider, females developed a preference for males bearing the scent over another that did not bear a scent. Similar findings were found when condition a preference in female rats strain of males (Coria-Ávila *et al.,* 2006). Furthermore, subsequent studies showed 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 field test (Parada *et al.,* 2011).

Instrumental learning and sexual behavior

Depending on the US that an animal is exposed to, they will experience a physiological response that is, as the terms coins, unconditional. Yet, the rules of behavior behind how an animal behaves upon being presented with stimuli are described by a whole different type of learning called instrumental conditioning (*e.g*., Ferster & Skinner, 1957).

Instrumental or Operant learning originates with Thorndike's law of effect (Thorndike, 1911) that states that if a behavior in the presence of a stimulus is followed by a satisfying stimulus, the established association between the behavior and the stimulus becomes strengthened. Similarly, when the behavior is followed by an aversive stimulus, the association weakens. The term operant was coined after B.F. Skinner (1938), 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, the animal is free to perform and repeat the operant response over and over. Thus, the behavior can be shaped based on different schedules of reinforcement or punishment (Fester & Skinner, 1957).

Several demonstrations of male needing to perform lever-pressing operants to gain access to sexually receptive females have been documented (Beck, 1971; Jowaises, Taylor, Dewsbury & Malagodi, 1971), along with overcoming obstacles (Sheffield, Wulff & Backer, 1951), or crossing shock grids or other aversive tasks (Anderson, 1938; Meyerson & Lindstrom, 1973; Warner, 1927). Other preparations that manipulated brain neurochemistry and anatomy have also been explored. For example, lesions in 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 (Everitt, Cador & Robbins, 1989). In contrast, lesions in the mPOA disrupted copulation, leaving conditioned lever pressing virtually unaffected at the time of testing (Everitt & Stacey, 1987)

Within the literature of sexual behavior, several factors have been analyzed from the scope of learning, like sexual excitement, copulatory experience, overcoming obstacles to locate a mate, courtship, arousal, copulation, partner preference, etc (see Pfaus, Kippin & Centeno, 2001). Particularly, among the vast leaning variables that come into play in sexual behavior, and particularly for the interest of this thesis, early experiences appear to play an important role in how animals come to choose with whom they will have sex. Similar to Kippin *et al*., (1998) findings,

Ménard *et al.,* (in preparation) found that pairing a lemon odor in the bedding while applying gentle strokes on rat pups after separation from the mother, would imprint a preference towards this odor, leading these rat when adults to ejaculate preferentially with a lemon-scented female partner in their first sexual experience, compare 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 on how first *sexual* experiences may shape the individual's future partner choice.

First sexual experiences

Several studies have shown the importance of first experiences and critical periods on sexual behavior (reviewed in Pfaus *et al.,* 2012). When it comes to partner preference, a critical period can be traced all the way back to the perinatal hormone-induce brain differentiation (see McCarthy & Arnold, 2011). The following periods of gender differentiation and socio-emotional attraction and bonding lead into an awareness of physiological genital changes experienced mainly at puberty to be linked with external cues (Pfaus *et al.,* 2012). It is mostly during this period where individuals are exposed to their first sexual experiences with sexual arousal and reward that can potentially set the stage of new, more, and distinctive paths that would lead different individual to prefer different partners, practices, or even objects. Krafft-Ebing (1886/1929) was perhaps the first one to write about critical periods in sexual context, especially those associated with attraction to other- or same sex, as well as cases of sexual arousal trigger by fetish-like objects and practices in men. However, what exactly constitutes as a 'first sexual experience'?

More controlled manipulations of first experiences have been tested experimentally. In order to elucidate what do previous and first experiences with neutral and biologically relevant stimuli, researchers have exposed animals to either the CS or US before training resulting in different outcomes depending what is being pre-exposed and for how long.

US pre-exposure is the phenomenon in which there is 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 & LoLordo, 1979). For instance, 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 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 have been 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.*, Randich & LoLordo, 1979; Rescorla & Wagner, 1972). 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.*, Rankin *et al*., 2009).

The pre-exposure of a CS before conditioning, phenomenon known as latent inhibition, is the disruption or retardation of a subsequent trained association with the same CS (*e.g.,* Lubow, & Moore, 1959). 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 who was not exposed to it previously (Rodríguez & Alonso, 2002). Most theories coincide in that latent inhibition is the result of a reduction in associability or attention to CS during pre-exposure (Schmajuk, 2002). 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 when trained to learn new associations with neutral cues. Zamble,

Mitchell & Findlay (1986) 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.

First experiences with biologically-relevant stimuli, or stimuli that take over these systems, like drugs of abuse, can further impact how animals develop a preference for a partner. Liu *et al*., (2010) demonstrated that prairie voles injected systemically with amphetamine (AMPH) at medium-to-high doses developed a CPP, an effect specific to the D1-like DA receptor activity. In a subsequent experiment, sexually-naïve AMPH-treated males did not develop a preference for a mate through copulation until ejaculation unlike the saline-treated males at doses previously shown to foster AMPH-based CPP. Further treatment with a D1-like DA receptor antagonist was able to recover the mate preference through copulation until ejaculation in AMPH-treated males (Liu *et al*., 2010). In a follow-up study, Liu *et al*., (2011) demonstrated that D1-like DA, but not D2-like, receptor antagonist in the NAc blocked the AMPH-induce in saline-treated males, while D1-like DA receptor antagonist before AMPH-induced CPP in paired-bonded males (Liu *et al*., 2011). Altogether, these results demonstrate that first experiences with biologically relevant stimuli like drugs or sex can either trump the development for pair bonding preferences or serve as a modulator, hindering the development of drug addiction. Furthermore, these findings deepen the understanding on the DA neurochemical mechanism underlying pair bonding. Yet, an exciting rather-young field of research shows that the effect of first experiences (and also others) can go beyond the neurotransmitter level into how genes are expressed.

Epigenetics

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 (Crews, 2008; Elvir *et al*., 2017). By manipulating the way DNA is unfolded and expressed, either by silencing or expressing certain parts of the gene, scientists have observed behavioral changes in different animal models. Epigenetics is the scientific study of how environmental changes modify the way the genome is expressed through the manipulation of enzyme responsible for the addition or removal of epigenetic tags in histone proteins, without altering the DNA sequence (Roth, 2013). For instance, through the administration of a histone deacetylase inhibitor (HDACi), a drug that promotes transcriptional activation, sexually naïve female voles developed a partner preference by simply being exposed to a male vole partner in the absence of mating (Wang *et al*., 2013). More recently, by administering a lysine specific inhibitor demethylase 1 (LSD1) inhibitor targeting the action of LSD1 demethylase enzymes, Holley (2015) was able to block the development of mate guarding behavior in the female rat (Holley *et al*., 2014, 2015). In her experiment, female rats were divided into two groups, paired and unpaired. The paired group copulated with the same male across all training trials, whereas unpaired group copulated with different males across trials, a training procedure found to promote mate guarding behaviors (*e.g*., female-to-female mount) over other competitor females in the presence of the partner male. One hour prior to training, each group of females was halved and given an intraperitoneal injection of LSD1 or saline. Finally, in the mate guarding test, females were given access to copulate freely with a sexually-receptive male in an open field, while another competitor female was also introduced. Females in the paired groups were tested with the same male they were trained, whereas females in the unpaired groups were tested with a random male. Results showed that females in the paired group failed to display mate guarding behaviors, unlike in the saline paired group. Double-label cell count for oxytocin and vasopressin analyses showed a higher Fos-IR induction, a neuronal marker for non-specific activation, in the saline-treated paired females in their supra-optic and paraventricular hypothalamic nuclei, brain areas that have shown a higher Fos-IR in females who

develop mate guarding behaviors than the ones who did not (Holley *et al*., 2014, 2015).

Considerations of first sexual experiences in humans

When it comes to first sexual experiences in humans, studies show that it is common to have experience with sexual content material (*e.g*., pornography) and masturbation before they have their first sexual encounter with another person, even if it is by accident (see Flood, 2007; Wright, 2013). Thus, it is common to find in the literature both concepts, first sexual experiences and critical periods, blended together or used indistinctively. Both concepts appear to share two main components: timing and relevance. Timing, referring to the very first (and perhaps a few subsequent) experience in the sexual domain involving, but not limited to, copulation. Relevance, referring to the crucial period of sexual development that sets a milestone from which some changes or crystallization of certain associations that may be more difficult to modify, in comparison to subsequent experiences. It is still a matter of research to determine the valence (*i.e*., emotional intensity, significance, or strength of the reward) component of the first and subsequent early experiences. Thus, disentangling how the very first sexual experience (whichever one that may be) can be different (or not) to other subsequent "early" experiences, as well as when these may lose relevance. Also, it is important to determine if relevance is solely or mainly influenced by reward of a sexual nature, and if it susceptible to be manipulated, either by being (re)opened or closed to an experience.

Summary and thesis objectives

Learning plays a pivotal role in how animals generate new association between their environment and internal states. This flexibility extends to all behaviors, including those more traditionally thought to be "fixed" and determined by hormonal priming, such as sexual behavior and partner preferences (see Pfaus, Kippin & Coria-Ávila, 2003). Previous findings have

demonstrated that animals are not a blank slate when it comes to sexual behavior, showing that biological predeterminations are not the only factors influencing partner preference (*e.g.,* Bateson, 1978). Other studies highlighted the importance of early experiences in partner preference (Kendrick, Hinton & Atkins, 1998), just like partner preferences can be the result of rewarding experiences with neutral stimuli (*e.g.,* Kippin *et al.,* 1998), highlighting the role of the post-ejaculatory interval in the development of CEP (*e.g*., Kippin & Pfaus, 2001a) where the opioid receptor system has shown to play a crucial role for these associations to be fostered (*e.g.,* Ågmo & Berenfeld, 1990; Ismail, Girard-Bériault, Nakanishi & Pfaus, 2009). However, at this point, several aspects on how animals develop a partner preference remain unclear, especially on first sexual experiences. Some of these factors are: how do first experiences biologically significant (*i.e.*, US) and neutral (*i.e.*, CS) cues may alter the development of partner preference; can a CEP be based on CS of different sensory nature than olfactory (*e.g*., somatosensory), what is the role of opioid receptors in different brain areas associated with the development of a CEP? Evidence gathered for these questions would shed some light on how first sexual experiences influence, or perhaps even determine, future mate choices. Expanding on using a different sensory cues than olfactory, as traditionally used in the CEP literature (for a review see Pfaus *et al*., 2012) would allows to understand the flexibility of the neural system in term of which neutral cues can predict sexual reward. Finally, assessing the role of opioid receptors in particular brain areas associated with the development of CEP would deepen our understanding on the neural mechanism behind this phenomenon, and how these areas could interact with each other. Therefore, this thesis investigated the rewarding mechanisms, particularly those related to opioids transmission, involved in first sexual experiences, and their role in the development of partner preferences in the male rat. Thus, first experiences were examined using two paradigms of US pre-exposure and CS pre-exposure or latent inhibition. It was predicted that early experiences would play a role in the development of

rewarding associations in the male rat (*i.e.,* CEP) based on neutral cues. Particularly, if first experiences with sexual reward matter, then a primacy effect should be shown with US pre-exposure over CS pre-exposure. Additionally, it was also hypothesized that the system would be flexible to other cue from different sensory modalities (*i.e.,* somatosensory) from the ones already studied (*i.e*., olfactory). Furthermore, NAL was used to generate a non-reward state that, if opioid reward is key to forming the association of an olfactory cue as shown previously (Ismail *et al*., 2009), NAL should block the development of CEP with a somatosensory cue, as well. Finally, to evaluate where in the brain does this conditioning take place, we micro-infused NAL into the mPOA and VTA, expecting a differential effect depending where in the brain the drug is infused.

Previous studies of CEP (*e.g*., Kippin *et al*., 1998; Kippin & Pfaus, 2001a,b; Ismail *et al*., 2009) have used neutral olfactory cues (*e.g*., almond or lemon odor) as CSs paired with the sexual reward state (the US) induced by ejaculation. After several pairings of the CS with the reward state in either bilevel or unilevel pacing chambers, male rats are given a final choice test in a large open field between two sexually receptive females, one scented and the other unscented. Males that were trained to associate the odor with the reward state mount and intromit the two females almost randomly, but ejaculate preferentially with the scented female (Figure 1, notably middle panel showing the necessity of the male being with the scented female during the post-ejaculatory interval or PEI). Conversely, if the odor is explicitly associated with sexually nonreceptive females and no odor associated with sexually receptive females (Figure 1, top: explicitly unpaired-trained group), males will ejaculate preferentially with unscented females. Accordingly, the two main components of CEP are the percent choice for first ejaculation between the two females and the distribution of ejaculations with either female throughout the final open-field test. Although paired conditioning of the odor CS and post-ejaculatory US typically does not induce a preference to copulate with a particular female (*e.g*., examining the distribution of mounts or intromissions), the inhibitory

conditioning (*e.g.*, pairing the odor with a sexually nonreceptive females) compels the male to avoid the ScF on the final open field test, despite her obvious sexual receptivity, often with an associated increase in mounts and intromissions distributed to the UnScF (Figure 1, top panel).

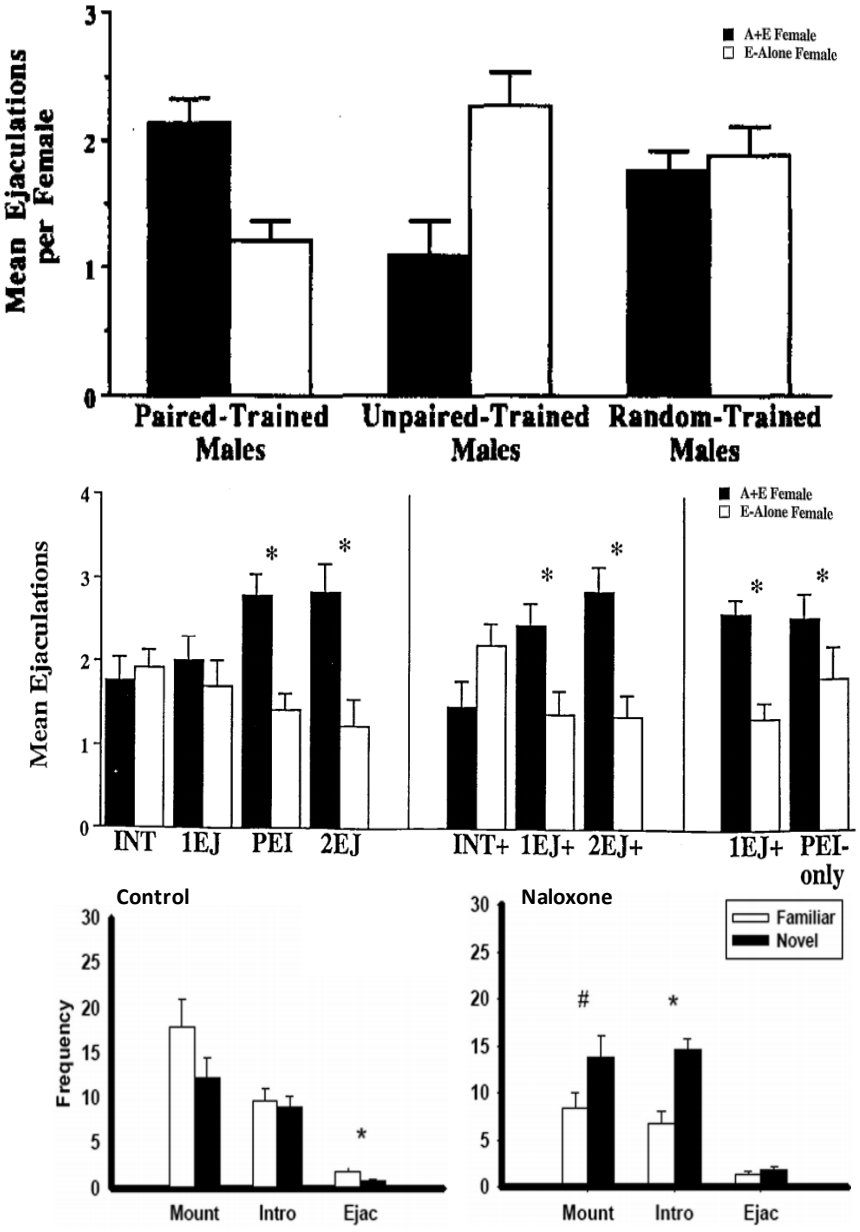

Figure 1. Previous results on conditioned ejaculatory preference of the male rat in the literature. Adapted (with permission) from Kippin *et al*., (1998, top panel), Kippin & Pfaus (2001a, middle panel), and Ismail *et al*., (2009, lower panel). INT = Intromission, PEI = Post-Ejaculatory Interval, EJ/Ejac = Ejaculation, + = plus PEI.

In the studies reported in this thesis, it is hypothesized that male rats trained in the control conditions will develop a CEP towards their familiar female (*e.g*., scented females). However, either by pre-exposing the US or CS, it is expected that the longer the pre-exposure to either of these cues, the more likely it would be for the CEP to be disrupted. Furthermore, by replacing the olfactory cue with a somatosensory cue (*i.e*., the jacket), we expect to find in males a preference towards the jacketed female. Finally, by injecting NAL intra-peritoneally or directly into the brain regions involved in the development of a CEP (*i.e*., mPOA and VTA), it is also expected that CEP will be disrupted compared to the saline control groups, regardless of the neutral cue used.

Although other measures of male sexual behavior, *e.g*., mounts, intromissions, or their latencies along with the and latencies of ejaculations, typically are not altered by CEP training, they are analyzed along with the ejaculation measures noted above to make sure the copulatory behavior of each male is within a normal range on the final open field test. These measures are not correlated with the ejaculatory measures used (see Pfaus, Mendelson, & Phillips, 1990), and thus are not providing overlapping information from a statistical perspective. Similar conclusions have been found in other studies analysing the theoretical components of male sexual behavior (e.g., Dewsbury, 1979; Sachs, 1978).

Finally, the present thesis used both significance testing and effect size estimates to test individual hypotheses. Comparisons between groups are deemed *statistically significant* when p values are below 0.05, and *marginally significant* when the p value ranges from 0.05–0.1. Effect size estimates are used as measures of potential replicability with magnitudes of small (0 to 0.1), moderate (0.1 to 0.3), or large (0.3 and above), as discussed by Kline (2013).

Chapter 2a - First sexual experiences determine the development of conditioned ejaculatory preference in male rats

Abstract

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, pre-exposing 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) pre-exposure, in which male rats were pre-exposed to the ejaculatory reward state either 1 or 5 times with scented (ScF) versus unscented (UnScF) females prior to multiple ejaculatory trials with females in the opposite condition (*e.g.*, ScF pre-exposure received 10 subsequent ejaculatory trials with UnScF, whereas UnScF pre-exposure 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 pre-exposure did not display a CEP for either female. Conversely, males pre-exposed once to a ScF, and later trained with UnScF developed a preference for the latter, whereas males pre-exposed 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.

Key words: sexual behavior, conditioned ejaculatory preference, us pre-exposure, first sexual experiences, critical period.

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Introduction

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 & Westbrook, 1978). Likewise, male rats are naturally driven towards odors from sexually receptive versus nonreceptive females (*e.g.,* Bakker, van Ophemert & Slob, 1996). These cues appear to be hardwired and driven by hormonally-mediated neural systems within hypothalamic and limbic structures (see Pfaus, 2009; Pfaus, Kippin & Coria-Ávila, 2003). 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 (Carr, Loeb & Dissinger, 1963; Kagan & Beach, 1953; Pfaus, Kippin & Centeno, 2001; Stern, 1970). Habituation appears to develop in male rats to natural sex odors from familiar females (Carr, Crames & Costanzo, 1970), although presenting males with novel females can result in the reinitiation of anogenital investigation of the female (Carr, Carmes & Costanzo, 1970; Stern, 1970). Similarly, although presenting male rats with a different receptive female during each multi-ejaculatory test results in a precipitous decline in anogenital investigations prior to the initiation of copulation, subsequent presentation of sexually non-receptive females results in a vigorous reinitiation of anogenital investigations (Pfaus & 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 & Wagner, 1972) in

which neutral cues that become conditioned stimuli (CS's), like neutral odors, acquire associative strength after being paired with biologically relevant cues, or unconditioned stimuli, like the sexual arousal and reward states induced before and after ejaculation, respectively (Kippin & Pfaus, 2001; Tenk *et al*., 2009; Zamble, Mitchell & Findlay, 1986). 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 & Domjan, 1993). Furthermore, male rats have been shown to develop a conditioned ejaculatory preference (CEP) towards females bearing an neutral odor like almond or lemon that has been associated previously with the post-ejaculatory reward state (*e.g.,* Kippin *et al*., 1998; Pfaus & Kippin, 2001) 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 & Berenfeld, 1990; Mehrara & 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 pre-exposure, 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 & LoLordo, 1979). Significant US pre-exposure effects have been demonstrated for conditioned taste aversions (*e.g*., Classen, Wetzell & Riley, 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 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 hypotheses have been offered to explain this phenomenon, 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. 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.*, Randich & LoLordo, 1979; Rescorla & Wagner, 1972). 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.*, Rankin *et al*., 2009). In terms of the sexual reward state induced by ejaculation in male rats, the associative explanation predicts that 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 non-associative 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 pre-exposure to the neutral odor (almond) used as the CS in the development of CEP could alter conditioning via latent inhibition (Quintana, Jackson, Nasr & Pfaus, in press). Males were pre-exposed 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, partner preferences is 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, 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 pre-exposure trial in the medial preoptic area (mPOA), ventral tegmental area (VTA), and nucleus accumbens core (NAc Core) relative to the group given five CS pre-exposure trials was found. In contrast, the group with one CS pre-exposure 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, Ismail & Coria-Ávila, 2010).

The present study sought to evaluate whether US pre-exposure would block the subsequent conditioning of CEP in male rats. Sexually naïve male rats were given either 1 or 5 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, 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.

Methods

Subjects

Males. 92 Long-Evans rats were sexually naïve and weighing approx. 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. 120 Long-Evans rats sexually naïve and weighing approx. 200g 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 (4mg/ml) anesthesia, mixed at a ratio of 4:3 respectively, approximately 2 weeks before the beginning of the experiment. Sexual receptivity was induced by subcutaneous injections of 10 μg estradiol benzoate (Steraloids, injected sc in 0.1ml of sesame oil) 48 hours prior each training session, and 500 μg of progesterone (Steraloids, injected sc in 0.1ml of sesame oil) four hours prior to each training session. Stimulus 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. Different females were assigned to each male randomly for every training session.

Apparatus

All conditioning sessions were conducted in Plexiglas unilevel pacing chambers (38 x 60 x 38 cm) with bedded floors and bisected by a transparent Plexiglas divider with 1-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. The final copulatory preference test took place in a large open field (123 x 123 x 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 Meisel & Sachs, 1995; Pfaus *et al*., 1990; Sachs & Barfield, 1976).

Procedure

The common procedure of the experiment is depicted in Figure 1.

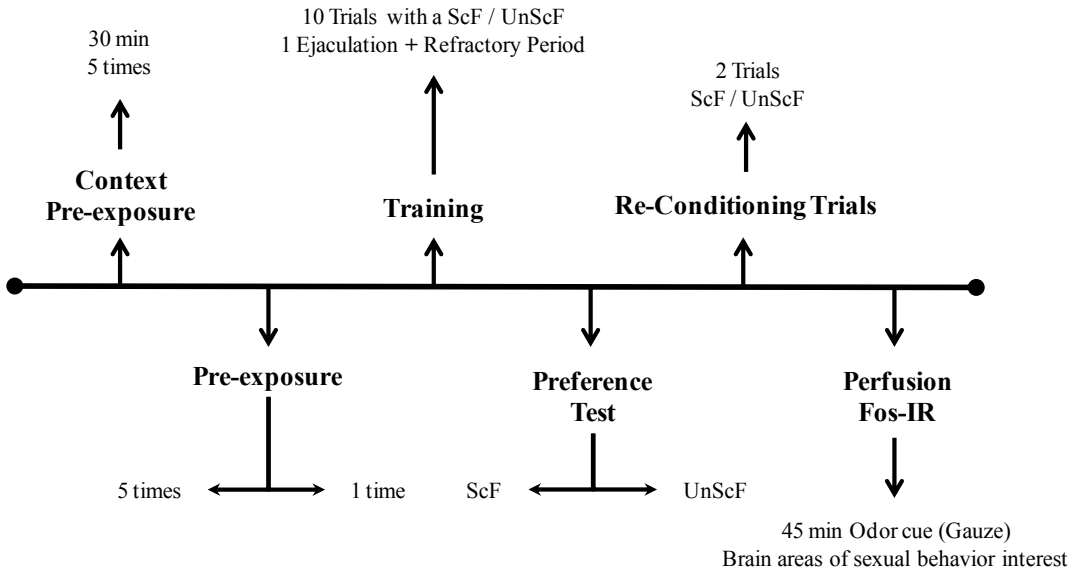


Figure 1. General experimental procedure.

Groups. Males were assigned to one of three main groups (see Table 1): 5 trials of US pre-exposure (5t), 1 trial of pre-exposure (1t), or control groups. During pre-exposure 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 pre-exposure phase. Males in the control group were trained with either ScF or UnScF, only.

Table 1. Pre-exposure and training group distribution.

Group	Type of female	
	Pre-exposure	Training
Control	-	ScF
	-	UnScF
5t	ScF	UnScF
	UnScF	ScF
1t	ScF	UnScF
	UnScF	ScF

Context pre-exposure. All animals were exposed five times to the chamber for approximately 30 minutes for seven days straight prior to the pre-exposure 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 & Wilkins, 1995).

US pre-exposure. Pre-exposure trials consisted of each male copulating in a unilevel pacing chamber with 1-hole divider with a receptive female scented or unscented (depending their group), until the first intromission after the 1st ejaculation. Pre-exposure trials were conducted every 4 days.

Conditioning. Following the pre-exposure phase, all animals were given CEP training with sexually receptive females either with (ScF) or without (UnScF) an almond odor (depending on the group) as described in Kippin & Pfaus (2001b). Rats were trained with ScF or UnScF for 10 conditioning sessions at 4-day intervals during the middle third of the dark phase of the light/dark cycle.

Depending on which type of female the males were pre-exposed 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 pre-exposed to UnScF, or UnScF for the males pre-exposed 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.

Copulatory Preference Test. Four days after the last conditioning trial, each male was placed in a large open field (123 x 123 x 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 minutes. The test was video recorded and subsequently scored for the male's choice of female in the form of mounts, intromissions, and ejaculations.

Perfusion. Following the preference test, males were given two reconditioning trials, after which they were exposed for 45 minutes to 1ml. 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 hours, to be later on stored for 36 hours in a 30% sucrose solution. Finally, the brains were frozen, covered in aluminum foil and stored at -80 °C.

Fos Inmunohistochemistry. This analysis was performed as in previous studies (*e.g*., Kippin, Cain, & Pfaus, 2003). Coronal brain sections were incubated sequentially with 30% w/w hydrogen peroxide (H_2O_2) in Tris-buffered saline (TBS) for 30 min at room temperature, 3% normal goat serum (NGS) in .05% Triton TBS for 90 min at 4 °C, rabbit polyclonal anti-Fos (Oncogene Science, Boston, MA; diluted 1:75,000) in .05% Triton TBS with 3% NGS for 72 h at 4 °C, biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, 1:200) in .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 2h 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 (.1 ml of DAB/Tris buffer, pH 7.8) for 10 min, DAB/3% H_2O_2 in 50-mM Tris for 10 min, and 8% nickel chloride (400 µl per 100 ml of DAB/Tris buffer H_2O_2). 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 40x, and the number of Fos-positive cells was counted bilaterally from each region from five different sections per rat 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 the

similar to the ones analyzed in Kippin, Cain & Pfaus (2003), and were defined using the borders in Paxinos and Watson (1986) and included the medial preoptic area (mPOA, -.35 mm from bregma), nucleus accumbens shell and core (NAc Shell and NAc Core respectively, 1.65 mm from bregma), ventral tegmental area (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 for each brain area from each randomly selected subject (five subjects in each group). No subject was lost during perfusion.

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 between males in the 4 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 compassions of the means were made using the Tukey HSD correction to ensure to ensure maximal statistical power while correcting for family-wise error. Furthermore, partial eta square (η_p^2) was calculated as effect size for each comparison. Additionally, a 1x2 chi square (χ^2) analysis was conducted for the percentage of first ejaculation choice for each group, and a 2x2 χ^2 analysis to contrast the ejaculatory preference between the control groups. Furthermore, Cramer's V and Phi (φ) effect sizes were conducted as effect size for the 1x2 and 2x2 χ^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 t tests with a Bonferroni correction of the alpha level. Since four comparisons were conducted within each brain area, the alpha level was established at .0125 (.05/4 = .0125) for statistically significant differences. Only in the comparisons

between the control groups, the alpha level was kept at .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 vs. 1t ScF, 5t UnScF vs. 1t UnScF, 5t ScF vs. 5t UnScF, 1t ScF vs. 1t UnScF, and ScF control vs. UnScF control. The ScF control vs. UnScF control comparisons were ran 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 period for males before the open field test (*e.g*., Kippin & Pfaus, 2001b), it is believe 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: UnScF 1t, UnScF 5t, ScF 1t, ScF 5t, Control ScF, and Control UnScF) x 2(Female: ScF, UnScF) repeated measure ANOVA. No statistically significant interaction between Female x Group was found for mounts, $F(5,86) = .609$, $p = .693$, $\eta_p^2 = .034$; nor for intromissions, $F(5,86) = 1.524$, $p = .191$, $\eta_p^2 = .081$.

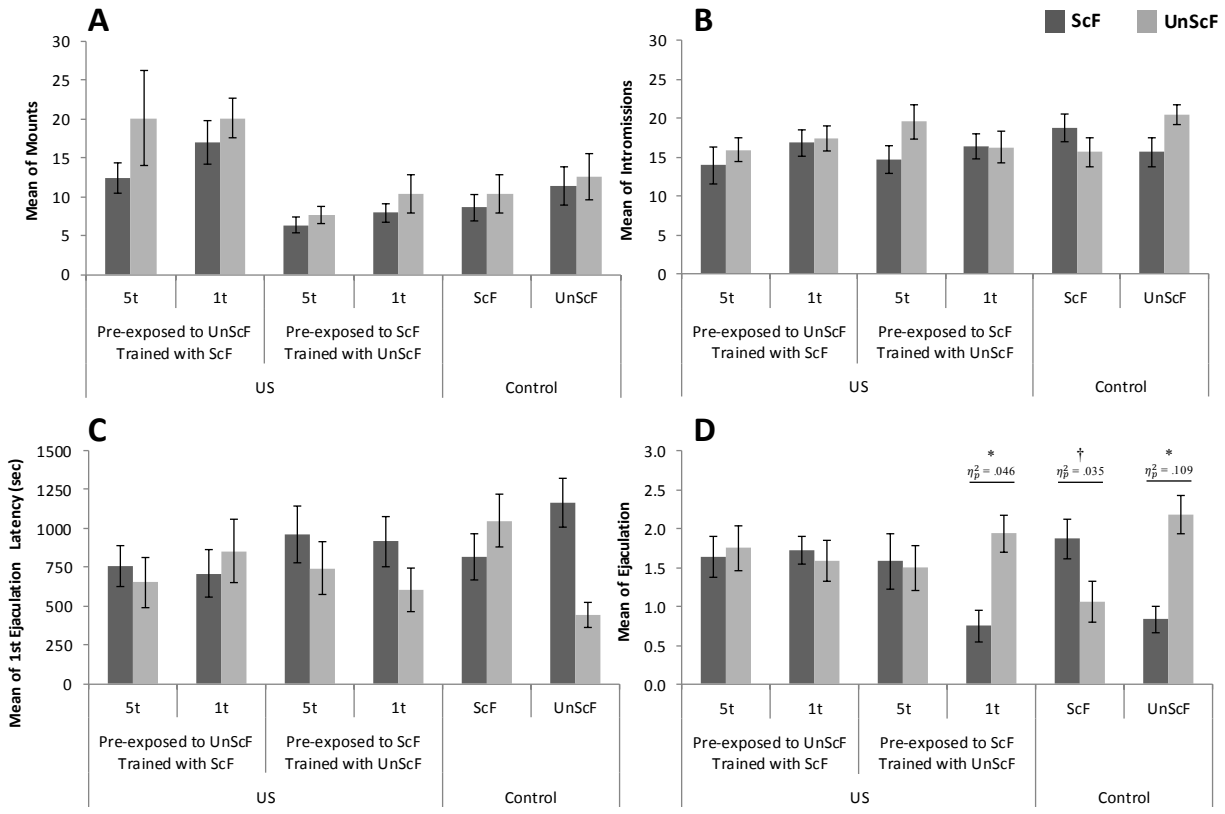


Figure 2. Mean of copulatory behaviors (±SEM) per group during the open field test. † = $p < .01$; * = $p < .05$; η_p^2 = partial eta square.

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 familiar female and chose to ejaculate more with their familiar female. 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) x 2(Female: ScF, UnScF) repeated measure ANOVAs. On one hand, no statistically significant interaction between Female x Group was found for the Latency of 1st ejaculation mean, $F(5,86) = 1.796$, $p = .122$, $\eta_p^2 = .095$. However, a significant interaction between Female x Group was found for the mean of ejaculations, $F(5,86) = 3.173$, $p = .011$, $\eta_p^2 = .156$. Post hoc comparisons using the Tukey HSD correction revealed that the control UnScF group displayed a statistically significantly higher mean ejaculation towards the UnScF ($M = 2.19$) than to the ScF ($M = .688$, $p < .05$, $\eta_p^2 =$

.109), whereas the ScF control displayed only marginally more ejaculations towards the ScF (M = 1.63) than to the UnScF (M = 1.06, $p < .1$, $\eta_p^2 = .035$). Also, the 1t group pre-exposed to ScF and later on trained with UnScF displayed a statistically significantly higher mean ejaculation towards the UnScF (M = 1.94) than the ScF (M = 1.0, $p < .05$, $\eta_p^2 = .046$).

Figure 3. shows the percentage of the males in the different groups that delivered their first ejaculation to one of the two females during the open field test. Males in both 5t groups, and in the 1t UnScF group that was subsequently trained with ScF, did not show a significant choice for first ejaculation. However, males in ScF 1t group trained with UnScF and both control groups chose to ejaculate first with the female they received the most training with. These observations were partially confirmed by χ^2 analyses. No significant differences were found in percentage of first ejaculation choice for the UnScF 5t group trained with ScF group, $\chi^2(1) = .25$, $p = .617$, $V = .125$; nor for the ScF 5t group trained with UnScF, $\chi^2(1) = .25$, $p = .617$, $V = .125$; neither for the UnScF 1t trained with ScF, $\chi^2(1) = 0$, $p > .9$, $V = 0$. However, statistically significant differences were found in the control UnScF group, $\chi^2(1) = 4.0$, $p = .046$, $V = .5$; yet no significant differences were found for the ScF 1t trained with UnScF group, $\chi^2(1) = 2.25$, $p = .134$, $V = .375$; on the Control ScF, $\chi^2(1) = 1$, $p = .317$, $V = .25$. However, a 2x2 χ^2 analysis between the two control groups revealed that, overall, they preferred different females to ejaculate first with, $\chi^2(1) = 4.571$, $p = .033$, $\varphi = .315$.

Fos-IR

Figures 4. and Figure 5. show the Fos-IR in each of the brain area of interest for each of the groups. As shown in figure 4, following exposure to the odor cue in the group of males pre-exposed 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.

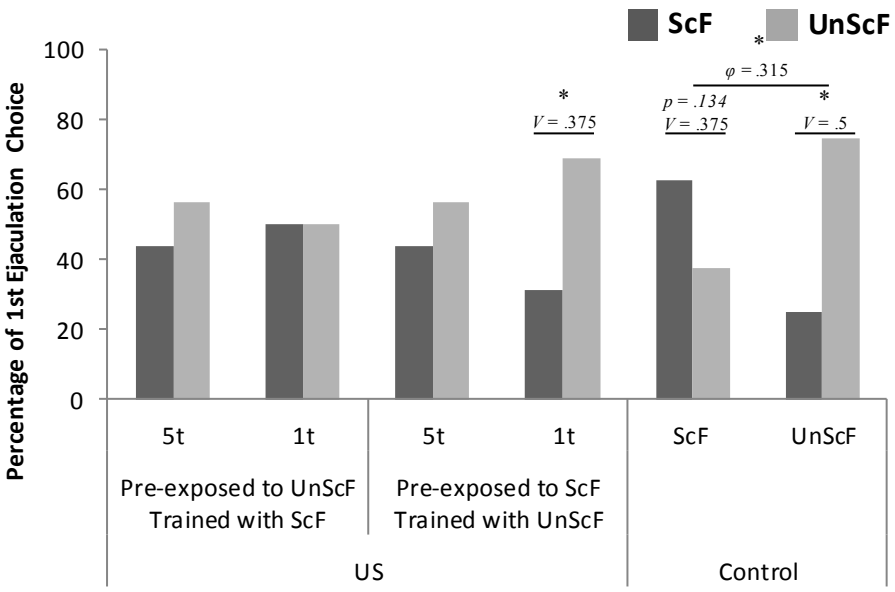


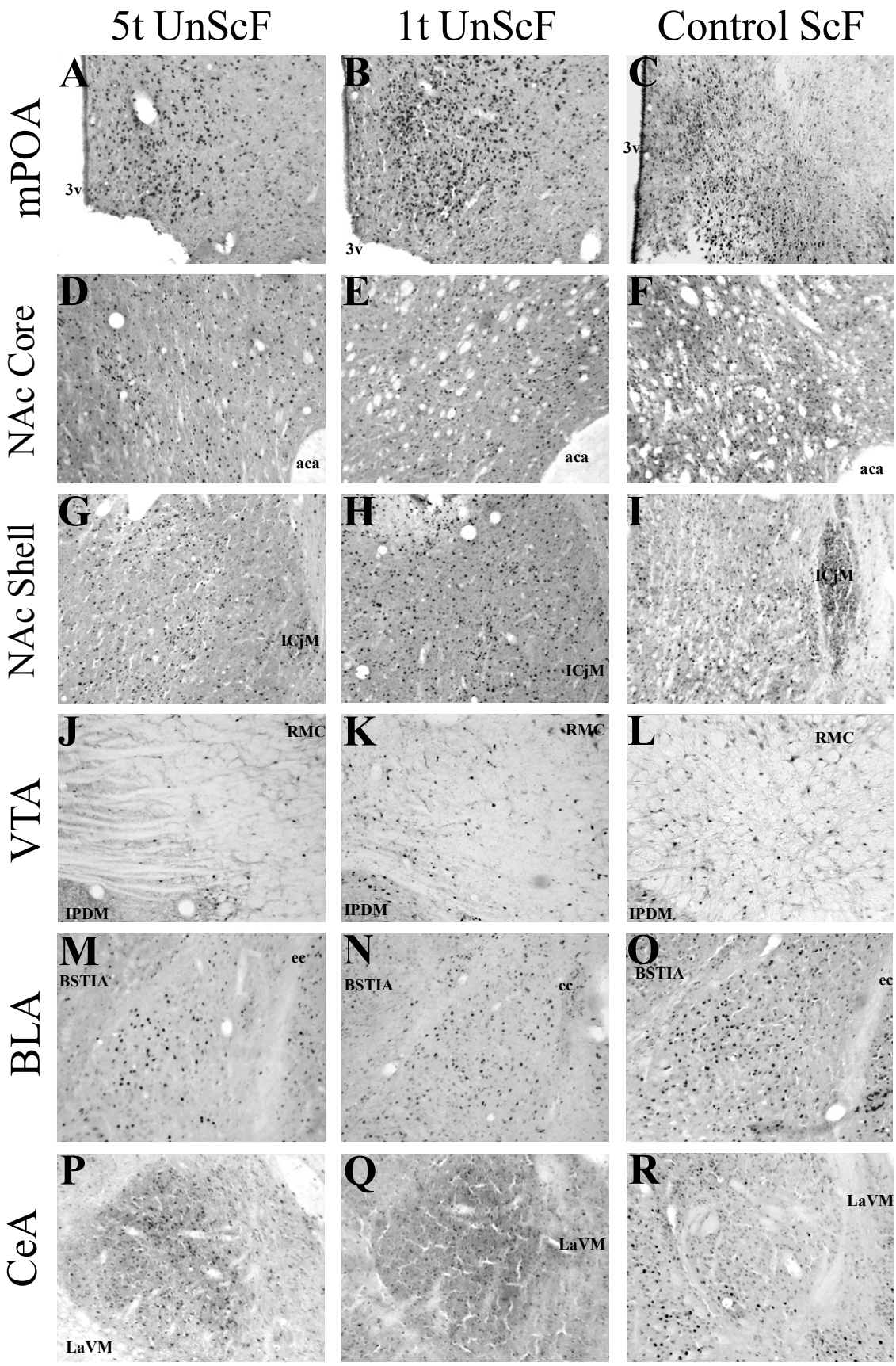
Figure 3. Percentage of 1st ejaculation choice per group during the open field test. * = $p < .05$; V = Cramer's V; φ = phi.

As shown in figure 5, following exposure to the odor cue in the group of males pre-exposed 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 independent t tests with a Bonferroni correction and *d* effect sizes. Table 2. displays the mean Fos-IR positive cell numbers (±SEM) for each group in the brain areas of interest.

Table 2. Mean Fos-IR positive cell numbers (±SEM) for each group in the brain areas of interest.

	US 5t UnScF*		US 1t UnScF*		US 5t ScF*		US 1t ScF*		Control ScF		Control UnScF	
Brain area	*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

* = Type of female to which males were pre-exposed. 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.


5t UnScF
1t UnScF
Control ScF
mPOA
NAc Core
NAc Shell
VTA
BLA
CeA
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
3v
3v
3v
aca
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RMC
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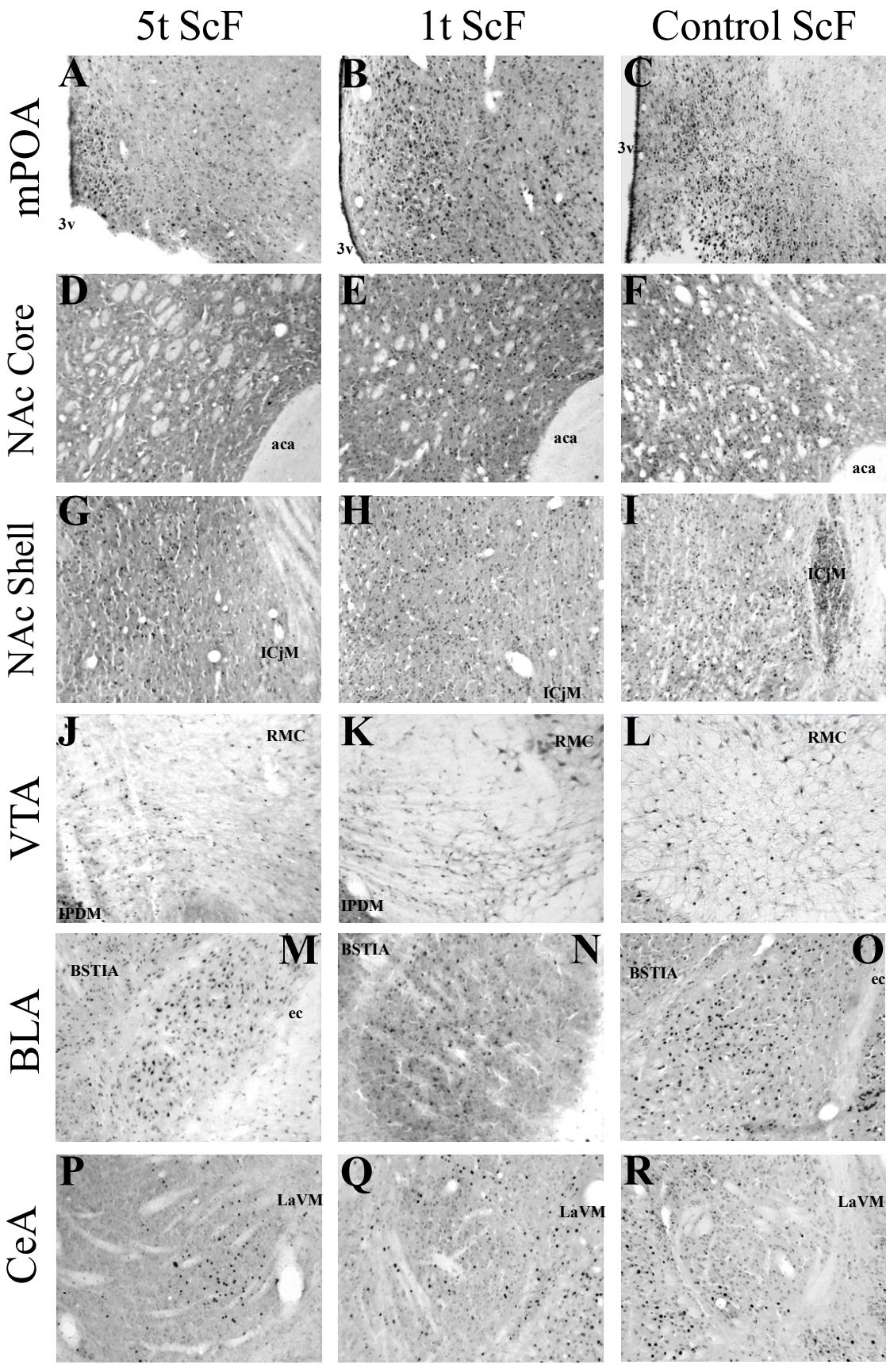
Figure 4. Fos immunoreactivity (Fos-IR) following exposure to the sexually conditioned odor before perfusion in males pre-exposed 5 times to a unscented female before being trained with scented females (5t UnScF), males pre-exposed 1 time to an unscented female before being trained with scented females (1t UnScF), and control males trained with scented females (Control ScF), in brain areas of interest. Pictures were taken accordingly to Paxinos & Watson (1998) coordinates in the medial preoptic area (mPOA): ±-0.40 mm from Bregma (A–C); Nucleus accumbens Core and Shell (NAc Core/Shell): ± 1.70 mm from Bregma (core: D–F, shell: G–I); Ventral tegmental area (VTA): ±-6.04 mm from Bregma (J–L); the Basolateral amygdala (BLA): ±-3.14 mm from Bregma (M–O), and in the Central nucleus of the amygdala (CeA): ±-2.80 mm from Bregma (P–R). Abbreviations used in the figure: 3v, 3rd ventricle; aca, anterior commissure; ICjM, major islands of Calleja; RMC, magnocellular part of red nucleus; IPDM, dorsomedial interpeduncular nucleus; ec, external capsule; BSTIA, intraamigdaloid division of the bed nucleus of the stria terminalis, and LaVM: ventromedial part of the lateral amygdaloid nucleus. See Table 2 for *M*±S.E.M.

mPOA: Males in the 5t ScF group had a statistically significantly lower Fos-IR than the 1t ScF group, $t(8) = -5.28$, $p < .01$, $d = 7.462$; while males from the 5t UnScF also had a statistically significantly lower Fos-IR than the 1t UnScF group, $t(8) = 16.686$, $p < .05$, $d = 1.082$. No statistically significant differences were found comparing males in the 5t ScF vs. 5t UnScF, $t(8) = -1.78$, $p > .05$, $d = 2.23$; nor for males from the 1t ScF vs. 1t UnScF groups, $t(8) = -1.12$, $p > .05$, $d = 1.585$.

VTA: No statistically significant differences were found between males in the 5t ScF group compared to males in the 1t ScF group, $t(8) = -.13$, $p > .05$, $d = .182$; nor did males in the 1t UnScF compared to males in the 5t UnScF group, although the effect size revealed a difference of a high magnitude, $t(8) = -2.26$, $p = .053$, $d = 3.207$. Males in the 5t ScF group had a statistically significantly higher Fos-IR than the 5t UnScF group, $t(8) = -2.76$, $p = .024$, $d = 3.913$; whereas no statistically significant differences were found comparing males from the 1t ScF vs. 1t UnScF groups, $t(8) = 1.31$, $p > .05$, $d = 1.519$.

NAc Shell: No statistically significant differences were found comparing males in the 5t ScF vs. 1t ScF, $t(8) = 1.22$, $p > .05$, $d = .502$; nor for males from the 5t UnScF vs. 1t UnScF groups, $t(8) = 1.55$, $p > .05$, $d = .059$. Males in the 5t ScF group had a statistically significantly higher Fos-IR than the 5t UnScF group, $t(8) = 3.27$, $p < .01$, $d = 4.631$; just like males in the 1t ScF group had a statistically significantly higher Fos-IR than the 1t UnScF group, $t(8) = 5.67$, $p < .001$, $d = 4.161$.


5t ScF
1t ScF
Control ScF
mPOA
NAc Core
NAc Shell
VTA
BLA
CeA
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3v
aca
ICjM
RMC
IPDM
BSTIA
ec
LaVM

Figure 5. Fos immunoreactivity (Fos-IR) following exposure to the sexually conditioned odor before perfusion in males pre-exposed 5 times to a scented females before being trained with unscented females (5t ScF), males pre-exposed 1 time to a scented female before being trained with unscented females (1t ScF), and control males trained with scented females (Control ScF), in brain areas of interest. Pictures were taken accordingly to Paxinos & Watson (1998) coordinates in the medial preoptic area (mPOA): ±-0.40 mm from Bregma (A–C); Nucleus accumbens Core and Shell (NAc Core/Shell): ± 1.70 mm from Bregma (core: D–F, shell: G–I); Ventral tegmental area (VTA): ±-6.04 mm from Bregma (J–L); the Basolateral amygdala (BLA): ±-3.14 mm from Bregma (M–O), and in the Central nucleus of the amygdala (CeA): ±-2.80 mm from Bregma (P–R). Abbreviations used in the figure: 3v, 3rd ventricle; aca, anterior commissure; ICjM, major islands of Calleja; RMC, magnocellular part of red nucleus; IPDM, dorsomedial interpeduncular nucleus; ec, external capsule; BSTIA, intraamigdaloid division of the bed nucleus of the stria terminalis, and LaVM: ventromedial part of the lateral amygdaloid nucleus. See Table 2 for *M*±S.E.M.

NAc Core: No statistically significant differences were found comparing males in the 5t ScF vs. 1t ScF, $t(8) = -0.35$, $p > .05$, $d = 1.727$; nor for males from the 5t UnScF vs. 1t UnScF groups, $t(8) = .04$, $p > .05$, $d = 1.637$. Males in the 5t ScF group had a statistically significantly higher Fos-IR than the 5t UnScF group, $t(8) = 3.28$, $p < .01$, $d = 4.643$; whereas males in the 1t ScF group had a significantly higher number of Fos-IR cells than the 1t UnScF group, $t(8) = 2.94$, $p = .0185$, $d = 8.031$.

BLA: No statistically significant differences were found comparing males in the 5t ScF vs. 1t ScF, $t(8) = -1.6$, $p > .05$, $d = 0$; males from the 5t UnScF vs. 1t UnScF groups, $t(8) = -.01$, $p > .05$, $d = 2.257$; males in the 5t ScF vs. 5t UnScF, $t(8) = 0.2$, $p > .05$, $d = .287$; nor between males from the 1t ScF vs. 1t UnScF groups, $t(8) = -1.4$, $p > .05$, $d = 1.986$.

CeA: Males in the 5t ScF group had a statistically significantly lower Fos-IR than the 1t ScF group, $t(8) = -6.07$, $p < .001$, $d = 8.589$; whereas no statistically significant differences were found comparing males from the 5t UnScF vs. 1t UnScF groups, $t(8) = 1.25$, $p > .05$, $d = 1.772$. No statistically significant differences were found comparing males in the 5t ScF vs. 5t UnScF, $t(8) = -0.69$, $p > .05$, $d = .985$; whereas males in the 1t ScF group had a statistically significantly higher Fos-IR than the 1t UnScF group, $t(8) = 6.15$, $p < .001$, $d = 8.714$.

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 the effect size revealed a difference of a high magnitude, $t(8) = 1.99$, $p = .08$, $d = 1.18$. In the *NAc Shell*, males in the ScF control group had a statistically significantly higher Fos-IR than the UnScF group, $t(8) = 3.11$, $p < .01$, $d = 4.392$. In the *CeA*, males in the ScF control group had a statistically significantly lower Fos-IR than the UnScF group, $t(8) = -4.56$, $p < .001$, $d = 3.823$. No statistically significant differences were found in other areas, $ps < .05$.

Discussion

The present study evaluated the impact of US pre-exposure 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 pre-exposure was given, but only when the female was not bearing an odor. If the female was scented then males developed a CEP for the familiar female. The Fos-IR analyses demonstrated a differential pattern of neural activation regarding the amount of pre-exposure 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 & Pfaus, 2001a; Kippin & Pfaus, 2001b). This effect can be impaired or inhibited when the odor cue is pre-exposed five times, but not one time, before conditioning (Quintana, Jackson, Nasr & Pfaus, in press). Together these studies show that the development of CEP follows Pavlovian rules regarding latent

inhibition and US pre-exposure (*e.g.*, Randich & LoLordo, 1979).

Behavioral analyses

One of the main findings of the present study was the disruption of the CEP towards the familiar female in the 5t groups pre-exposed to the US. As previously mentioned, there are two perspectives to interpret the behavioral results on US pre-exposure, the associative and the non-associative. Moreover, Mis & Moore (1973) concluded that the decremental effect of pre-exposing 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 pre-exposure trial and the first conditioning trial. Either through blocking or the reduction of the initial emotional reaction towards the US, these findings showed that pre-exposing sex five times before training a CEP is enough to disrupt it corroborating similar findings on the effect of pre-exposure of cues related to the training conditions before the conditioning for CEP (Quintana, Guizar, Rassi & Pfaus, in press). This was expected given that it has been shown previously that five multi-ejaculatory trials of 30 min. are the minimal amount of conditioning experience necessary to establish a CEP based on neutral odor cue in male rats (Kippin *et al.,* 2001a). 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 pre-exposure 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 days 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 disruption of US or CS pre-exposure might last. Nevertheless, the pre-exposure effect on the CEP

findings, particularly the disruption of the CEP in the 1t group pre-exposed to an UnScF, confirm the powerful impact of first sexual experiences on the ability to associate a CS with sexual reward.

Although 1 copulatory pre-exposure to the ScF did not block subsequent conditioning to the UnScF, it may be surprising that 1t pre-exposure to the UnScF blocked later training with ScF. This may be an example of belongingness or preparedness, a phenomenon where certain CS-US associations are easier to be established than others (Garcia & 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 (Bressler & Baum, 1996; Carr, Loeb & Dissinger, 1965), eliciting general and sexual arousal (Sachs, 1997) and increasing testosterone and luteinizing hormones levels in plasma (Graham & Desjardins, 1980). Therefore, it appears that rats may have evolved to display specific "prepared" association between estrous odors and sexual partner receptivity (Cook *et at.,* 1986). The absence of a disruption in CEP in the males given 1 pre-exposure to the ScF and trained subsequently with UnScF suggests that a neutral odor requires enough conditioning in order to reach critical salience, unlike "prepotent" 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).

Finally, the strength of the US pre-exposure effect on the development of a CEP is considered even stronger than a regular US pre-exposure manipulation due to the context pre-exposure conducted before the beginning of the US pre-exposure phase. Casey Cole *et al*., (1996) demonstrated an attenuation of the US-exposure effect or simply fail to show a significant US pre-exposure effect in animals that were familiarized with the training context compared to the ones for

which the pre-exposure context was new.

Fos-IR

Analysis of Fos-IR in the brain areas of interest showed a differential pattern of activation depending on the amount of pre-exposure and training conditions.

As depicted on Figure 4, following exposure to the odor cue, on one hand, males in the 5t group pre-exposed to UnScF and later on trained with ScF had a lower mean of Fos positive cells in the mPOA and CeA than the 1t group pre-exposed 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, also as depicted in Figure 5, males in the 5t group pre-exposed to ScF and later on trained with UnScF did not significantly differ from the 1t group pre-exposed and trained in the same conditions in their mean of Fos positive cells in any of the brain areas of interest, expect for the VTA, where the former had a lower mean of Fos positive cells than the later. 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, Cain & Pfaus (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 the exposure to the estrous odors, there was an increase 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 set of neurons in that brain area (Kippin, Cain & Pfaus, 2003). In the present study, US pre-exposure 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 to mention that Kippin, Cain & Pfaus (2003) used bilevel chambers, where chasing dynamic between male and female is completely different from the one in unilevel pacing the chamber like the ones used in this study.

A similar study showing that when CS cue was pre-exposed before training for a CEP based on the same olfactory cue yielded similar decrement in the Fos-IR results in several brain areas (Quintana, Jackson, Nasr & Pfaus, in press). Therefore, a general decrement in the mean of 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 pre-exposure 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 pre-exposure 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 pre-exposure and training are analyzed by brain area as follows.

mPOA. The mPOA is a critical brain region that controls male sexual arousal and behavior, where every sensory modality sends indirect inputs (see Hull & Rodriguez-Manzo, 2009; Dominguez & Hull, 2005). 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 & 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 pre-exposed five times to ScF and later on trained with UnScF than the ones pre-exposed one time may suggest that the odor may have been impaired from fostering rewarding associations with copulation given the pre-exposure manipulation, thus impairing a CEP otherwise found for the UnScF 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, Samaha, Sotiropoulos & Pfaus, 2001). Furthermore, no decrement of Fos-IR was found in the MPOA in males pre-exposed to UnScF. Interestingly, males pre-exposed 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 pre-potent 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 pre-exposed 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 towards

them enabling animals to engage in copulation, which could ultimately result in ejaculation. This allows the sexual reward state to be cued and predicted by a CS (*e.g*., Pfaus, Ismail & Coria-Ávila, 2010). Lesions of this area increased the refractory period and decreased non-contact erection, yet not impeding males from copulating (Liu, Curtis & Salamone, 1998). Moreover, using excitotoxic lesions in the NAc of male rats, several behaviors like mounts, intromissions, non-contact erections, among others, were partially hindered, yet not abolished (Kippin, Sotiropoulos, Badih & Pfaus, 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, Kosobud & Rebec, 2004). Using microdialysis, another study found that this response was shown to be related to an increase of extracellular dopamine in the NAc (Wenkstern, Pfaus & Fibiger, 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 & Ettenberg (2002) also found a higher Fos-IR activation in the NAc of males exposed to an estrous female versus a non-estrous female. This effect was greater in sexually-experienced males than in naïve ones. Therefore, a reduced Fos-IR in the NAc in males pre-exposed to UnScF and later on trained with ScF may suggest that the almond odor cue used did not have as strong an incentive value as it had for the ScF group, as it can be seen also through the absence of a CEP in both experimental 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 pre-exposed to ScF and later on trained with UnScF provides further evidence for the differential patter of associability of neutral cues depending when they are associated with sexual reward. Namely, the odor incentive value still remains high, although pre-exposing an odor cue 5 times before training a CEP based on the same pre-exposed cue. Finally, as previously mentioned, lesions in this brain area increased the refractory period (Liu, Curtis &

Salamone, 1998), while showing a higher Fos-IR in males trained to associate an olfactory cue with sexual reward (Kippin, Cain & Pfaus, 2003); an effect that corresponds with the general higher latency of ejaculation found in both group of males pre-exposed to a ScF and 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 (reviewed in Hull *et al.,* 2006), whereas electrophysiological stimulation facilitated copulatory behavior in the male rat (Markowsky & Hull, 1995), an effect found to be dependant of which portion of the VTA was stimulated (Rodríguez-Manzo & 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 pre-exposed 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 pre-exposed 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 patter 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 (Quintana *et al.,* submitted).

CeA. The amygdala and its functions have been well documented in the sexual behavior of the male rat (*e.g.,* Swanson & 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 sub-nuclei of the amygdala in the male sexual behavior (see Hull & Rodriguez-Manzo, 2009), yet much less work has been done on the role of the CeA. GABA-like inmunoreactivity in the brain of monkeys revealed a very dense array of predominantly GABA neurons and projections to other brain areas (McDonald & 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 & Petrovich, 1998). The CeA is involved in the modulation of conditioned fear (van de Kar & 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 increase 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 pre-exposed 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 speculations.

BLA. This brain region is another of several sub-nuclei of the amygdala and has been studied extensively in the of classical and operant fear conditioning (*e.g.,* Fanselow & LeDoux, 1999). The BLA sends direct projections to NAc that have been implicated in sexual incentives, yet not with copulation (Everitt, Cador & Robbins, 1989). Lesion studies have shown to impair the operant

response associated with sexual reward, yet copulation remained identical to control animals (Everitt, Cador & Robbins, 1989). As previously mentioned, Fos-IR increased following the exposure of a sexually-conditioned odor in the BLA of male rats (Kippin, Cain & Pfaus, 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 pre-exposed 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 and sexual reward. However, a decrement in both 5t and 1t groups pre-exposed to UnScF and later on trained with ScF may contribute to explain the decrement in the same groups found in their NAcc core (and perhaps NAcc 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 pre-exposure to UnScF and not to ScF may lead to a decrement in Fos-IR in the NAc core and shell.

Altogether, this study provides further understanding on the role of first sexual experiences in the male rat, and how these modulate future sexual preferences. Particularly, five trials of pre-exposure hindered the display of a CEP for either female. Conversely, being pre-exposed once to a ScF, and later trained with UnScF developed a preference for the latter, whereas being pre-exposed once to the UnScF, rendered a similar result as being pre-exposed 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 of further experimentation.

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Chapter 2b - Effect of CS pre-exposure on the conditioned ejaculatory preference of the male rat: Behavioral analysis and neural correlates

Abstract

Early experiences with sexual reward play a pivotal role in the formation of sexual behavior and partner preference. Associations of salient partner cues, or even neutral cues on a partner, with sexual reward states are a product of Pavlovian learning. However, the extent to which first experiences that associate a neutral stimulus with no immediate consequence, and how that association may affect subsequent associability after being paired with a sexual reward state after copulation to ejaculation, remains unclear. To address this question, sexually naïve males were pre-exposed over one or five trials to almond scented gauze pads prior to training during which half of the males were trained 10 times with scented receptive females, and the other half with unscented receptive females. A final test of partner preference was conducted in a large open field containing two sexually receptive females, one scented and the other unscented. Males developed a conditioned ejaculatory preference for the type of female they were trained with, except when they were pre-exposed five times to the odor and then trained with females bearing the same odor, indicating a significant CS preexposure effect. One CS preexposure was not sufficient to inhibit subsequent conditioning. Exposure to the scent before perfusion for inmunohistochemistry, revealed different patterns of brain activation in brain areas previously associated with the development of partner preference, like the medial preoptic area, ventral tegmental area, nucleus accumbens, basolateral amygdala, among other, depending on group membership. Thus, CS pre-exposure results in a subsequent impairment of the association that links the odor cue to sexual reward and preference. This highlights the impact of the first sexual experiences in future partner preference.
Key words: first experiences, conditioned ejaculatory preference, sexual behavior, sexual imprinting, latent inhibition.

Effect of CS pre-exposure on the conditioned ejaculatory preference of the male rat: Behavioral analysis and neural correlates

Introduction

Latent inhibition is the phenomenon in which the pre-exposure to a conditional stimulus (CS) leads to a disruption or retardation of a subsequent trained association with the same CS (*e.g.,* Lubow, & Moore, 1959). For example, 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 who was not exposed to it previously (Rodríguez & Alonso, 2002). Most theories coincide in that latent inhibition is the result of a reduction in associability or attention to CS during pre-exposure (Schmajuk, 2002). Whether by hindering the storage or retrieval of the association, how this process occurs is still a matter of debate (Schmajuk, 2002).

Evidence shows that latent inhibition is a context-specific phenomenon (McLaren, Bennett, Palisted, Aitken & Mackintosh, 1994), that also varies depending on the CS duration (Westbrook, Bond & Feyer, 1981), CS intensity (Schnur & Lubow, 1976), numbers of trials of pre-exposure (Lantz, 1973), total CS pre-exposure time (Ayres, Philbin, Cassidy & Belling, 1992), inter-trial interval (Lanz, 1973), among others. This 'latent' learning phenomenon and its properties highlights the ability of animals to form new associations through passive, non-reinforced pre-exposure of CS's, demonstrating that previous experiences influence when trained to learn new associations with neutral cues. This can have important implication for learning phenomena experienced further on in the life of animals when they reach a certain level of biological maturity, like sexual behavior.

Animals become sexually active after reaching puberty (Hull & Rodriguez-Manzo, 2009). At this point, most have already been pre-exposed to several classes of stimuli that can be contingent when mating. Zamble, Mitchell & Findlay (1986) 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 became latently inhibited. Particularly, when studying how animals choose a sexual partner, the literature has confirmed that early experiences crystallize not only sexual behaviors and responses, but also place and partner preferences (Kippin *et al*., 1998; Paredez & Vasquez, 1999; Tenk *et al*., 2009). For example, it has been shown that animals will choose a partner that resembles more an adoptive mother than the genetic mother (Kendrick, Hinton & Atkins, 1998). In that experiment, Kendrick and colleagues separated male and female sheep and goats and cross-fostered them. These animals were allowed to engage in social contact with members of their genetic species during development. When animals reached adulthood, they 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 to socialize and selectively mate more with partners of their non-genetic 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 (Kendrick, Hinton & Atkins, 1998). Likewise, Fillion & Blass (1986) demonstrated that males exposed early on to a neutral lemon odor cue, ejaculated more readily with females bearing the same cue. In their experiment, male pups were exposed from day 2 until separation (day 28) to dams whose vaginal area and nipples were scented with a lemon odor. Subsequently, between days 90 and 120, males were divided into different groups and allowed to copulate with sexually-receptive females, either bearing the lemon scent or no scent. Males who were exposed to the lemon scent ejaculated faster only with females who bore the scent compared to unscented females (Fillion & Blass, 1986).

Early experience with neutral odors that predict sexual reward can generate a preference for partners bearing those cues (Coria-Ávila, Ouimet, Manzo, Pacheco & Pfaus, 2005; Ismail, Gelez, Lachapelle, & Pfaus, 2009; Kippin, Talianakis, Schattmann, Bartholomew & Pfaus, 1998). Kippin

and colleagues (1998) trained males to copulate with sexually receptive females bearing an almond scent. Finally, males were given an open field test to evaluate their preference in which they were given the choice to copulate with two sexually-receptive females, one scented and the other unscented. Males ejaculated preferentially with females bearing the scent, thus displaying a conditioned ejaculatory preference (CEP) for the familiar cue. Similarly, other studies have shown that the odor can also be made aversive depending on the contingencies of conditioning. For example, Kippin and colleagues (1998) also trained males with both receptive unscented females and non-receptive scented females. Unlike males who were paired with scented females, males trained to associate the odor with non-receptive females avoided scented females and displayed a CEP for the unscented female. A third group where the odor was randomly paired with receptive and non-receptive scented females displayed no preference for either of the females.

The rewarding associations fostered during conditioning can come from multiple sources, including sensory stimuli and behaviors aimed at acquiring partners or sexual reward. These associations have been well documented in the context of sexual behavior (Crawford, Holloway & Domjan, 1993). For instance, Tenk *et al.*, (2009) demonstrated that sexually naïve male rats developed a conditioned place preference towards a particular side of a chamber when this was paired with either intromissions or ejaculations. However, when males were sexually experienced, they developed a conditioned place preference only when that side was paired with ejaculation. Furthermore, Kippin & Pfaus (2001a) trained males allowing them to copulate until reaching five intromissions, one ejaculation without PEI, or one or two ejaculations plus the first intromission following their PEI. Findings showed that only male who achieved one or two ejaculations and were allowed to spend their PEI around the scented female developed a significant CEP towards the scent. In a different experiment, males were allowed to remain in the presence (without access) of a scented female during their PEI after a previous copulatory session with unscented females.

Likewise, only males develop a CEP when they achieved one or two ejaculations, but not after five intromissions. These results clearly show that not only ejaculation was necessary to establish a CEP, but also that it is during the post-ejaculatory interval (PEI) when this association takes place.

Altogether, it is clear that early experiences can modulate partner preferences via Pavlovian associations between discrete partner-related cues that predict a sexual reward state. Yet, it is not clear whether latent inhibition will occur as a result of preexposure to the cue alone. The present study evaluated this by pre-exposing sexually naïve rats to the almond odor either once or five times prior to 10 trials of conditioning where the odor was paired with the post-ejaculatory reward state.

Methods

Subjects

Males. 94 Long-Evans rats were sexually naïve and weighing approx. 250g 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. 120 Long-Evans rats sexually naïve and weighing approx. 200g 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 (4mg/ml) anesthesia, mixed at a ratio of 4:3 respectively, approximately two weeks before the beginning of the experiment. Sexual receptivity was induced by subcutaneous injections of 10 μg estradiol benzoate (Steraloids, injected sc in 0.1ml of sesame oil) 48 hours prior each training session, and 500 μg of progesterone (Steraloids, injected sc in 0.1ml of sesame oil) four hours prior to each training session. Stimulus females were scented with 0.6 ml of pure almond

extract (Blue Ribbon, Etobicoke, Ontario, Canada), split equally in the back of their neck and anogenital region as previously done by Kippin & Pfaus (2001b). Different females were assigned to each male randomly for every training session.

Apparatus. All conditioning sessions were conducted in Plexiglas unilevel pacing chambers (38 x 60 x 38 cm) with bedded floors and bisected by a transparent Plexiglas divider with 1-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. The final copulatory preference test took place in a large open field (123 x 123 x 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 Meisel & Sachs, 1995; Pfaus, Mendelson & Phillips, 1990; Sachs & Barfield, 1976).

Procedure

The common procedure of the experiment is depicted in Figure 1.

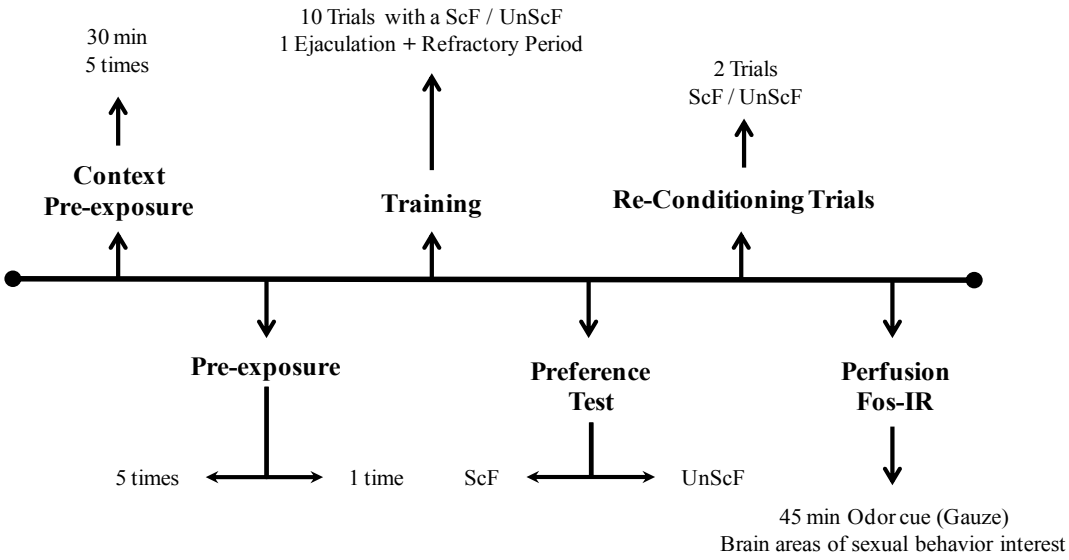


Figure 1. General experimental procedure.

Groups. Males were assigned to one of three main groups: control (no pre-exposure), one trial of pre-exposure, or five trials of pre-exposure. The control group was divided into two subgroups: half of these males were trained with sexually-receptive scented females (ScF), whereas the other half was trained with sexually-receptive unscented females (UnScF). The experimental animals were divided into two groups: males who were pre-exposed to the odor one time (1t), and males who were pre-exposed five times (5t). Furthermore, males in the 1t group were divided into two different conditions: trained with ScF or with UnScF. Males in the 5t were halved and trained similarly.

Context pre-exposure. All animals were exposed five times to the training chamber on a daily basis for 30 min prior to the pre-exposure phase (or the training phase in the case of the control group), 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 & Wilkins, 1995).

CS pre-exposure (latent inhibition procedure). CS pre-exposure trials consisted of animals being placed in one side of the chamber with an almond-soaked gauze pad placed in the other side for 30 minutes. Pre-exposure trials occurred at a 4-day interval, whereas control groups remained in their home cage until the first conditioning trial. Animals in the 1t group were pre-exposed when the 5t group was pre-exposed for the fifth time.

Conditioning. Following the pre-exposure phase, all animals were trained to develop a CEP for a sexually-receptive female with or without bearing an almond odor (depending on group membership), using a similar procedure to the one described in Kippin & Pfaus (2001b). All males were given 10 training conditioning trials at 4-day intervals during the middle third of the dark phase of the light/dark cycle.

During each sexual behavior conditioning trial, males were placed into the chamber for 5 min prior to receiving a sexually receptive female (scented or unscented depending on the group). The pair was allowed to copulate freely until ejaculation was achieved, and the test was terminated once

the male mounted the female after his post-ejaculatory refractory period had dissipated. Thus, although in previous studies rats were allowed to copulate for 30 minutes (to multiple ejaculations) in order to develop a CEP (*e.g.,* Kippin & Pfaus, 2001b), here only one ejaculation was used as the criterion considering that the numbers of ejaculation reached in 30 min and the ejaculation latency varies greatly across male rats during their first sexual experiences. Kippin and colleagues (2001) established that ejaculation creates a rewarding state the male that must have experienced in the presence of the scented female during the refractory period for CEP to occur. Therefore, by allowing males to remain with the females after one ejaculation only, we equated the induction of this rewarding state in males across the groups.

Copulatory Preference Test. Four days after the last sexual behavior conditioning trial, each male was placed in the open field and allowed to explore for 5 min. Subsequently, two females, one ScF and one UnScF, were placed simultaneously into the open field both equally distant from the male. Males were allowed to copulate freely with either female for 30 minutes. The test was video recorded and scored subsequently for the different sexual behaviors (mounts, intromissions, and ejaculations) that each female received from the male.

Perfusion. Following the preference test, males were given two more training trials at 4-day intervals exactly as their training conditions. Four days after, males were exposed for 40 minutes to 1 ml. of the almond odor alone on a gauze pad on the other side of the pacing chamber. Subsequently, males were injected with sodium pentobarbital (Euthanyl, 120 mg/kg, i.p.) and perfused intracardially with 250 ml of ice-cold phosphate buffered saline (PBS) followed by 250 ml of ice cold 4% paraformaldehyde. Brains were extracted and post-fixed in clean 4% paraformaldehyde for 4 hours, to be later on stored for 36 hours in a 30% sucrose solution. Finally, the brains were frozen, covered in aluminum foil and stored at -80 °C.

Fos immunohistochemistry (IHC) and analysis. IHC was conducted as in previous studies (*e.g*.,

Kippin, Cain, & Pfaus, 2003). Coronal brain sections were incubated sequentially with 30% w/w hydrogen peroxide (H_2O_2) in Tris-buffered saline (TBS) for 30 min at room temperature, 3% normal goat serum (NGS) in .05% Triton TBS for 90 min at 4 °C, rabbit polyclonal anti-Fos (Oncogene Science, Boston, MA, USA; diluted 1:75,000) in .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 .05% Triton TBS with 3% NGS for 1 h at 4 °C, and avidin–biotinylate–peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories; diluted 1:55) for 2h 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 (.1 ml of DAB/Tris buffer, pH 7.8) for 10 min, DAB/3% H_2O_2 in 50-mM Tris for 10 min, and 8% nickel chloride (400 µl per 100 ml of DAB/Tris buffer H_2O_2). 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 40x, and the number of Fos-positive cells was counted bilaterally from each region from five different sections per rat using a Leitz Microscope connected to a computerized image-analysis system (ImageJ).

Fos-IR was observed in brain regions previously related to sexual behavior and CEP to evaluate the neural activation evoked by the odor alone. The regions examined were the same as those in Kippin, Cain & Pfaus (2003), and were defined using the borders in Paxinos & Watson (1986). These included the medial preoptic area (mPOA, -.35 mm from bregma), nucleus accumbens shell and core (NAc Shell and NAc Core respectively, 1.65 mm from bregma), piriform cortex (Pir Ctx, 3.70 mm from bregma), ventromedial hypothalamic nucleus (VMH, -2.56 mm from bregma), ventral tegmental area (VTA, -6.04 mm from bregma), prefrontal cortex divided into cingulate cortex area 1 (Cg1, 3.70 mm from bregma), prelimbic (PrL, 3.70 mm from bregma) and infralimbic (IL, 2.70 mm from bregma); the basolateral nucleus of the amygdala (BLA, -2.80 mm

from bregma); and the central nucleus of the amygdala (CeA, -2.80 mm from bregma). An average of Fos-positive cells was calculated from three different slides for each brain area from each randomly selected subject (five subjects in each group). No subject was lost during perfusion.

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 between males in the 4 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 compassions of the means were made using the Tukey HSD correction to ensure to ensure maximal statistical power while correcting for family-wise error. Furthermore, partial eta square (η_p^2) was calculated as effect size for each comparison. Additionally, a 1x2 chi square (χ^2) analysis was conducted for the percentage of first ejaculation choice for each group, and a 2x2 χ^2 analysis to contrast the ejaculatory preference between the control groups. Furthermore, Cramer's V (V) and Phi (φ) effect sizes were conducted as effect size for the 1x2 and 2x2 χ^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 .0125 (.05/4 = .0125) for statistically significant differences. Only in the comparisons between the control groups, the alpha level was kept at .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 vs. 1t ScF, 5t UnScF vs. 1t

UnScF, 5t ScF vs. 5t UnScF, 1t ScF vs. 1t UnScF, and ScF control vs. UnScF control. The latter comparisons were run separately.

Results

Behavioral analyses

Two males that did not copulate during the final open field test were not included in the analyses. Although five min of exploration in the open field have previously been used effectively as a period of acclimation period for males before the open field test (*e.g.*, Kippin & Pfaus, 2001b), it is believed that natural differences in novelty aversion vary in ways that some animals are affected more than others, and likely show fear responses (*e.g.*, hug the walls of the open field) as observed.

The scores for the different copulatory behaviors by female for all groups during the open field test are depicted in Figure 2. As shown on panels A and B, males did not show consistent differences of mounts for either type of female among groups, whereas males in the control groups displayed a higher number of intromissions towards the female they were trained with. For the mean of mounts, the ANOVA analysis did not detected a statistically significant interaction between Female x Group, $F(5,88) = .649$, $p = .663$, $\eta_p^2 = .036$. As for the mean of intromissions, there was a statistically significant interaction between Female x Group, $F(5,88) = 2.56$, $p = .033$, $\eta_p^2 = .128$. Post-hoc analyses with Tukey HSD correction revealed that males in the control ScF displayed a marginally higher mean of intromission for the ScF ($M = 18.81$) over the UnScF ($M = 15.63$, $p < .1$, $\eta_p^2 = .024$), whereas males in the control UnScF displayed a statistically significantly higher mean of intromissions for the UnScF ($M = 20.5$) over the ScF ($M = 15.69$, $p < .05$, $\eta_p^2 = .054$). Similarly, males in the 1t UnScF that displayed a significantly higher mean of intromissions for the UnScF ($M = 18.44$) over the ScF ($M = 14.31$, $p < .05$, $\eta_p^2 = .04$).

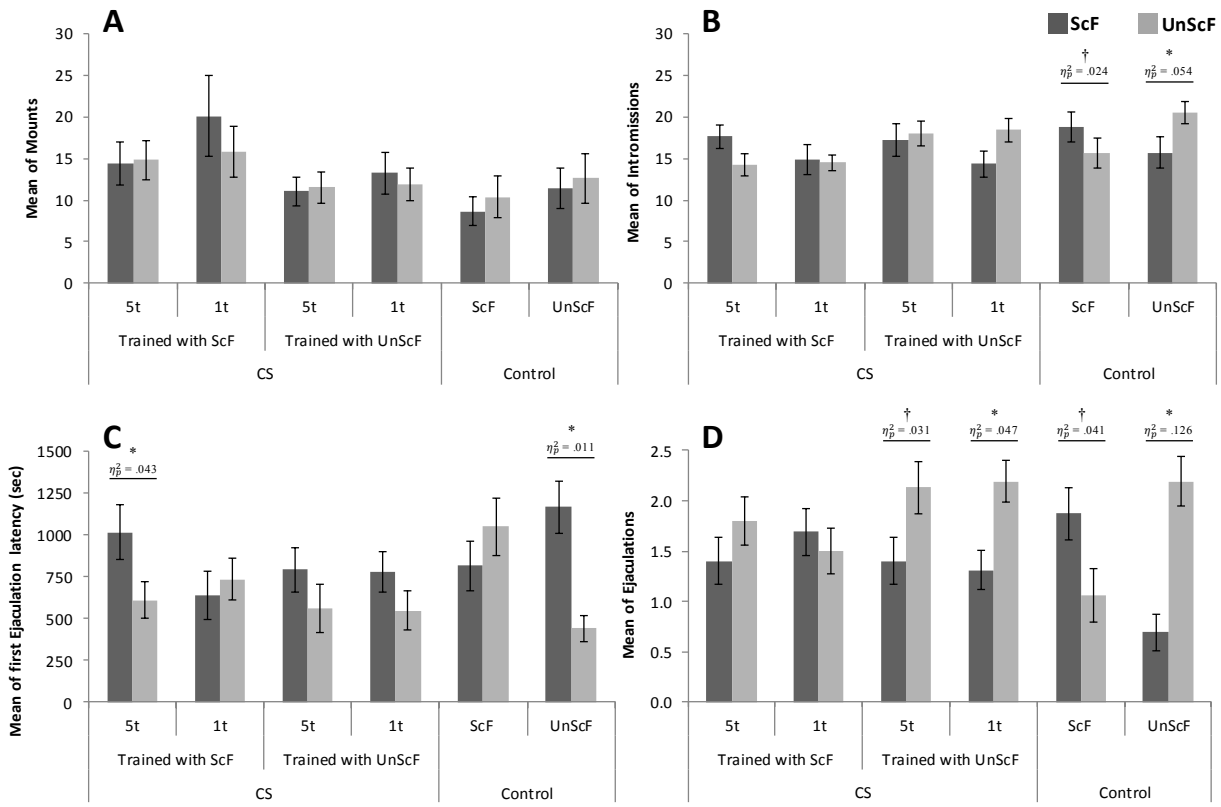


Figure 2. Mean of copulatory behaviors (±SEM) per group during the open field test. † = $p < .01$; * = $p < .05$; η_p^2 = partial eta square.

As shown on panel C of Figure 2, there was no clear general pattern for the mean of 1st ejaculation choice latency, except for the UnScF control group, where males appeared to take less time to ejaculate first with the familiar female. The ANOVA detected a statistically significant interaction between Female x Group, $F(5,88) = 2.548$, $p = .034$, $\eta_p^2 = .12$. Post-hoc analyses with Tukey HDS correction revealed that males in the UnScF control group displayed a statistically significantly lower mean latency to ejaculate first with the UnScF ($M = 440.63$) over the ScF ($M = 1165.13$, $p < .001$, $\eta_p^2 = .11$), just like males in the 5t ScF that displayed a statistically significantly lower mean latency to ejaculate first with the UnScF ($M = 606.06$) over the ScF ($M = 1069.93$, $p < .05$, $\eta_p^2 = .043$).

As shown on panel D of Figure 2 for the mean ejaculations per female, males displayed a

preference to ejaculate more with the familiar female, except for both groups of males in the CS trained with ScF that did not show a preference for either of the females. The ANOVA detected a statistically significant interaction of Female x Group, $F(5,87) = 3.75$, $p = .004$, $\eta_p^2 = .177$. Post-hoc analyses with the Tukey HSD correction revealed that males in the control UnScF displayed a statistically significantly higher mean of ejaculations for the UnScF ($M = 2.19$) over the ScF ($M = .687$, $p < .001$, $\eta_p^2 = .126$), whereas males in the control ScF only approached a marginal trend for higher mean of ejaculations for the ScF ($M = 1.63$) over the UnScF ($M = 1.06$, $p < .1$, $\eta_p^2 = .041$). Furthermore, males in the 1t UnScF group displayed a statistically significantly higher mean of ejaculations for the UnScF ($M = 2.19$) over the ScF ($M = 1.31$, $p < .05$, $\eta_p^2 = .047$), whereas males in the 5t UnScF displayed a statistically marginally higher mean of ejaculations for the UnScF ($M = 2.13$) over the ScF ($M = 1.4$, $p < .088$, $\eta_p^2 = .031$). Neither of the males pre-exposed to the CS and later on trained with ScF displayed a difference in the mean of ejaculations for either of the females (ps > 0.05).

The percentage of males that chose ScF or UnScF for their first ejaculation is shown in Figure 3. Males in all groups, except in the 5t ScF group, clearly preferred their familiar female to ejaculate with first. No statistically significant differences were found in percentage of first ejaculation choice for the 5t ScF, $\chi^2(1) = .067$, $p = .796$, $V = .067$. A non-significant trend towards differences were found for the 5t UnScF, $\chi^2(1) = 2.25$, $p = .134$, $V = .375$; in the 1t ScF group, $\chi^2(1) = 2.25$, $p = .134$, $V = .375$; and 1t UnScF group, $\chi^2(1) = 2.25$, $p = .134$, $V = .375$. Statistically significant differences were found for the UnScF control, $\chi^2(1) = 4.0$, $p = .046$, $V = .50$, yet no statistically significant differences were found in the ScF control, $\chi^2(1) = .60$, $p = .317$, $V = .25$. However, a 2x2 χ^2 analysis between the two control groups revealed that, overall, they preferred different females to ejaculate first with, $\chi^2(1) = 4.571$, $p = .033$, $\varphi = .315$.

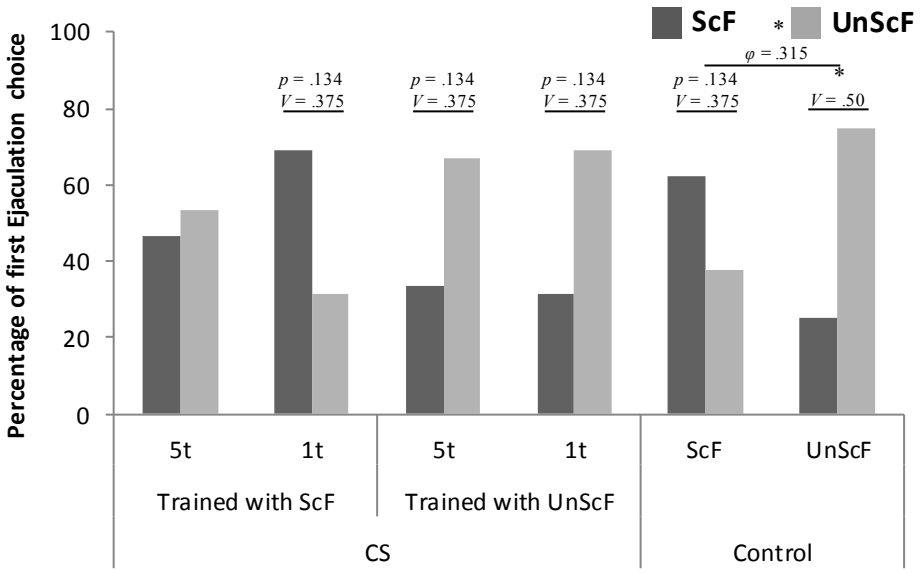


Figure 3. Percentage of 1st ejaculation choice per group during the open field test. * = $p < .05$, V = Cramer's V; φ = phi.

Fos-IR analyses

The mean number of Fos-IR positive cells (±SEM) for each group in the brain areas of interest are presented in Table 1. As depicted in Figure 4. and Figure 5, following exposure to the odor cue in the group trained with ScF, the 1t group had a higher mean of Fos positive cells than the 5t group in the mPOA, Nacc Core, BLA, and VTA, whereas the 1t had a lower activation than the ScF control group in the Nacc Core and Shell, and BLA, yet a higher one on the mPOA. Conversely, following exposure to the odor cue in the group trained with UnScF, the 5t groups had a higher activation than the 1t group in the Nacc Core and Shell, BLA and CeA, whereas the 5t group had a lower mean of Fos positive cells than the ScF control group in the VTA, Nacc Core and Shell, yet higher than the ScF control group in the CeA. The reliability of these observations was partially confirmed by independent t tests with a Bonferroni correction and *d* effect sizes. Table 1. displays the mean Fos-IR positive cell numbers (±SEM) for each group in the brain areas of interest.

Table 1. Fos-IR induction by odor cue (M ± SEM) for each group in the brain areas of interest.

Brain areas	5t Trained with ScF	1t Trained with ScF	5t Trained with UnScF	1t Trained with UnScF	Control ScF	Control UnScF
mPOA	88.4 ± 48.1	320.7 ± 129.3	210.1 ± 64.1	139.1 ± 42.2	127 ± 20.2	82.3 ± 13.1
VTA	1.5 ± 1.2	10.4 ± 6	2.8 ± 1	0.6 ± .3	17.6 ± 7.3	8.3 ± 2
NAc Shell	16.9 ± 12.4	20.3 ± 5.3	103.7 ± 35.8	28.9 ± 19.6	161.6 ± 14.3	101.3 ± 13.2
NAc Core	3.3 ± 1.9	62 ± 50.3	53.2 ± 26.3	11.5 ± 10	114.5 ± 23.1	103.3 ± 17.2
BLA	12.5 ± 5.5	5.8 ± 3.5	18.7 ± 8.8	5.5 ± 3.3	21 ± 7.3	19.6 ± 2.1
CeA	16.4 ± 6.8	7.3 ± 2.6	32.7 ± 13.3	6.1 ± 3.7	7.9 ± 1.8	14.9 ± 4.2
VMH	50.7 ± 13.8	71.3 ± 42.6	94.1 ± 71.9	43.3 ± 20.3	65.8 ± 35.1	41.1 ± 17.8
Pir Ctx	250.9 ± 78.1	189.1 ± 73.2	197.9 ± 54.4	154.9 ± 53.7	170.4 ± 73	115.4 ± 38
Cg1	100.4 ± 50.2	99.7 ± 37	96.9 ± 42.6	98.5 ± 48.2	99.6 ± 32.9	98.7 ± 49.6
PrL	102.4 ± 43.4	144.2 ± 47.3	154.1 ± 86.8	91.3 ± 48.7	143.9 ± 46.5	92.6 ± 50.6
IL	24.6 ± 15.3	64.1 ± 29.7	43.9 ± 25.9	23.6 ± 11.3	65.9 ± 31.6	23.3 ± 11.1

Abbreviations: mPOA: medial preoptic area, NAc Shell: Nucleus accumbens shell, NAc Core: Nucleus acumbens core; VTA: Ventral tegmental area; BLA: Basolateral amygdala; CeA: Central nucleus of the amygdala; VMH: Ventral medial hypothalamus; Pir Ctx: Piriform cortex; Cg1: Cingulate cortex; PrL: Prelimbic cortex; IL: Infralimbic cortex.

mPOA: No statistically significant differences were found comparing males in the 5t ScF vs. 1t ScF, although the effect size revealed a difference of a high magnitude, $t(8) = -1.68$, $p > .05$, $d = 2.381$; males from the 5t UnScF vs. 1t UnScF groups, $t(8) = .092$, $p > .05$, $d = 1.308$; males in the 5t ScF vs. 5t UnScF, $t(8) = -1.52$, $p > .05$, $d = 2.148$; nor between males from the 1t ScF vs. 1t UnScF groups, $t(8) = 1.33$, $p > .05$, $d = 1.888$.

VTA: No statistically significant differences were found comparing males in the 5t ScF vs. 1t ScF, although the effect size revealed a difference of a high magnitude, $t(8) = -1.68$, $p > .05$, $d = 2.057$; males from the 5t UnScF vs. 1t UnScF groups, $t(8) = .092$, $p > .05$, $d = 2.98$; not between males in the 5t ScF vs. 5t UnScF groups, $t(8) = -1.52$, $p > .05$, $d = 1.17$; nor between males from the 1t ScF vs. 1t UnScF groups, although the effect size reveals a difference of a high magnitude, $t(8) = 1.33$, $p > .05$, $d = 2.307$.

NAc Shell: No statistically significant differences were found comparing males in the 5t ScF vs. 1t ScF, $t(8) = .58$, $p > .05$, $d = .357$; not between males from the 5t UnScF vs. 1t UnScF groups, although the effect size revealed a difference of a high magnitude, $t(8) = 1.83$, $p > .05$, $d = 2.592$; not between males in the 5t ScF vs. 5t UnScF, although the effect size also revealed a difference of a

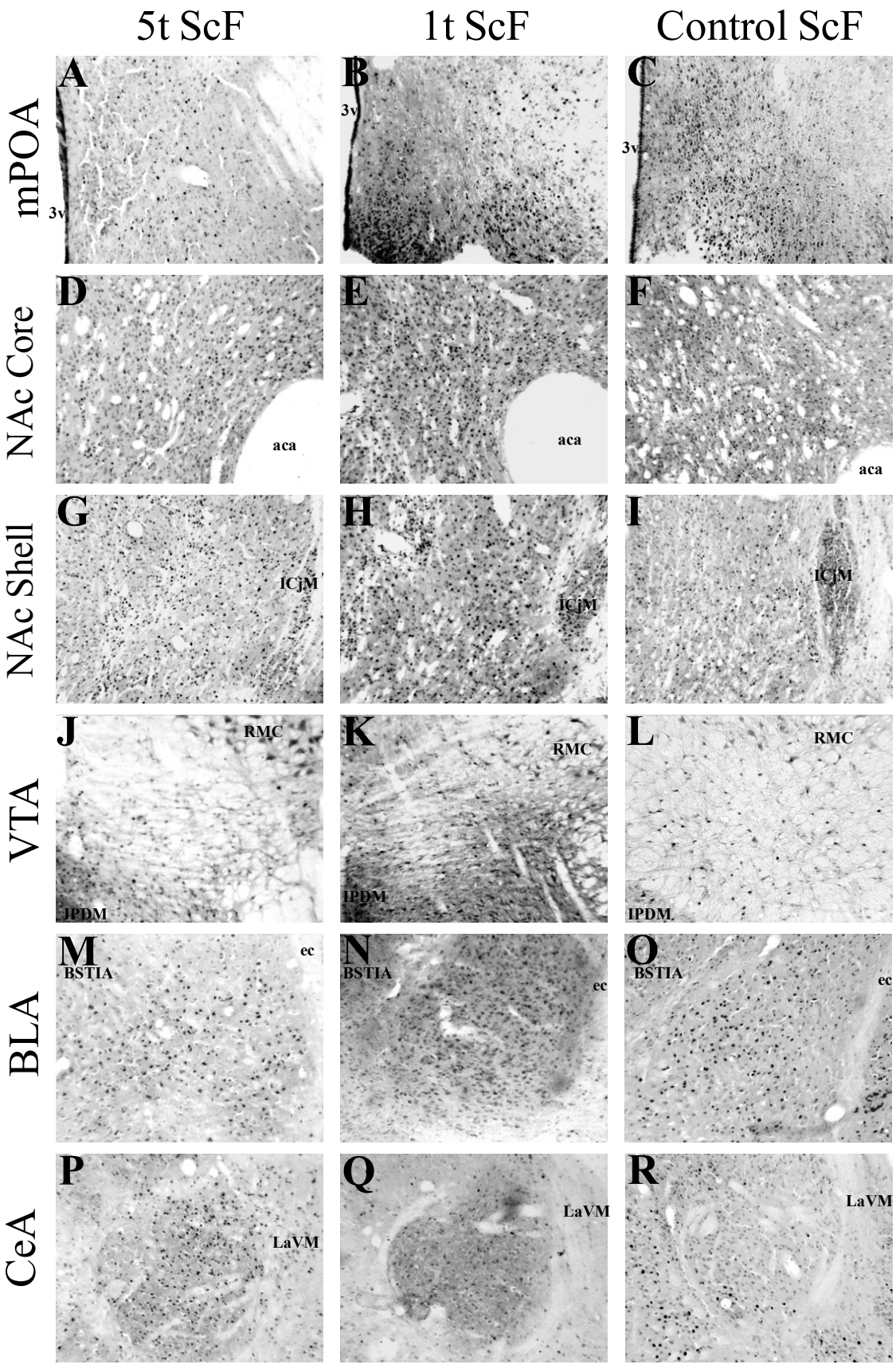

5t ScF
1t ScF
Control ScF
mPOA
NAc Core
NAc Shell
VTA
BLA
CeA
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
3v
aca
ICjM
RMC
IPDM
BSTIA
ec
LaVM

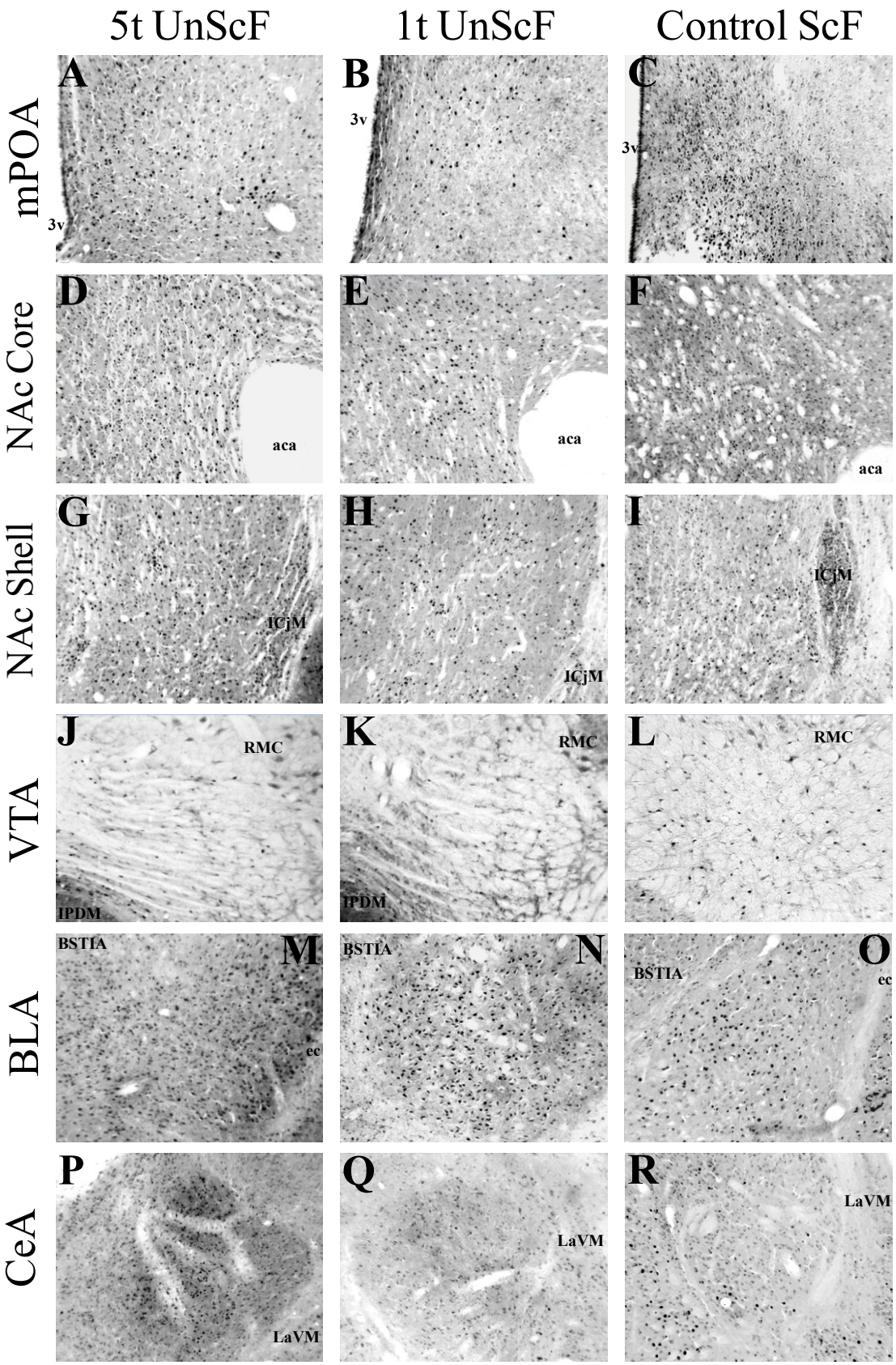
Figure. 4. Fos immunoreactivity (Fos-IR) following exposure to the sexually conditioned odor before perfusion in males pre-exposed 5 times to the odor before being conditioned with scented females (5t ScF), in males pre-exposed 1 time before being conditioned with scented females (1t ScF), and control paired males conditioned with scented females (CC ScF), in brain areas of interest. Pictures were taken accordingly to Paxinos & Watson (1998) in the medial preoptic area (mPOA): ±-0.40 mm from Bregma (A-C); Nucleus accumbens Core and Shell (Nacc Core/Shell): ± 1.70 mm from Bregma (core: D-F, shell: G-I); Ventral tegmental area (VTA): ±-6.04 mm from Bregma (J-L); the Basolateral amygdala (BLA): ±-3.14 mm from Bregma (M-O), and in the Central nucleus of the amygdala (CeA): ±-2.80 mm from Bregma (P-R). Abbreviations used in the figure: 3v, 3rd ventricle; aca, anterior commissure; ICjM, islands of Calleja major islands; RMC, magnocellular part of red nucleus; IPDM, dorsomedial interpeduncular nucleus; ec, external capsule; BSTIA, intraamigdaloid division of the bed nucleus of the stria terminalis, and LaVM: ventrolateral part of the lateral amagindaloid nuleus. See Table 1 for mean±S.E.M. and text for statistical comparisons.

high magnitude, $t(8) = -2.29$, $p > .05$, $d = 3.24$; nor between males from the 1t ScF vs. 1t UnScF groups, $t(8) = .42$, $p > .05$, $d = .599$.

NAc Core: No statistically significant differences were found comparing males in the 5t ScF vs. 1t ScF, $t(8) = -1.16$, $p > .05$, $d = 1.649$; not between males from the 5t UnScF vs. 1t UnScF groups, $t(8) = 1.48$, $p > .05$, $d = 2.096$; not between males in the 5t ScF vs. 5t UnScF groups, although the effect size also revealed a difference of a high magnitude, $t(8) = -1.89$, $p > .05$, $d = 2.676$; nor between males from the 1t ScF vs. 1t UnScF groups, $t(8) = .99$, $p > .05$, $d = 1.393$.

BLA: No statistically significant differences were found comparing males in the 5t ScF vs. 1t ScF, $t(8) = 1.03$, $p > .05$, $d = 1.453$; not between males from the 5t UnScF vs. 1t UnScF groups, $t(8) = 1.4$, $p > .05$, $d = 1.986$; not between males in the 5t ScF vs. 5t UnScF groups, $t(8) = -1.09$, $p > .05$, $d = .845$; nor between males from the 1t ScF vs. 1t UnScF groups, $t(8) = .05$, $p > .05$, $d = .088$.

CeA: No statistically significant differences were found comparing males in the 5t ScF vs. 1t ScF, $t(8) = 1.25$, $p > .05$, $d = 1.768$; nor between the 5t UnScF vs. 1t UnScF groups, although the effect size magnitude is deemed large, $t(8) = 1.93$, $p > .05$, $d = 2.725$; nor between males in the 5t ScF vs. 5t UnScF groups, $t(8) = -.69$, $p > .05$, $d = 1.543$; nor between males from the 1t ScF vs. 1t UnScF groups, $t(8) = -.26$, $p > .05$, $d = .375$.


5t UnScF
1t UnScF
Control ScF
mPOA
NAc Core
NAc Shell
VTA
BLA
CeA
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aca
ICjM
RMC
IPDM
BSTIA
ec
LaVM

Figure 5. Fos immunoreactivity (Fos-IR) following exposure to the sexually conditioned odor before perfusion in males pre-exposed 5 times to the odor before being conditioned with unscented females (5t UnScF), in males pre-exposed 1 time before being conditioned with unscented females (1t UnScF), and control paired males conditioned with scented females (CC ScF), in brain areas of interest. Pictures were taken accordingly to Paxinos & Watson (1998) in the medial preoptic area (mPOA): ±-0.40 mm from Bregma (A-C); Nucleus accumbens Core and Shell (Nacc Core/Shell): ± 1.70 mm from Bregma (core: D-F, shell: G-I); Ventral tegmental area (VTA): ±-6.04 mm from Bregma (J-L); the Basolateral amygdala (BLA): ±-3.14 mm from Bregma (M-O), and in the Central nucleus of the amygdala (CeA): ±-2.80 mm from Bregma (P-R). Abbreviations used in the figure: 3v, 3rd ventricle; aca, anterior commissure; ICjM, islands of Calleja major islands; RMC, magnocellular part of red nucleus; IPDM, dorsomedial interpeduncular nucleus; ec, external capsule; BSTIA, intraamigdaloid division of the bed nucleus of the stria terminalis, and LaVM: ventrolateral part of the lateral amagindaloid nuleus. See Table 1 for mean±S.E.M. and text for statistical comparisons.

All the non-reported comparisons in the rest of the brain areas to be assessed did not reach statistical significance along with low effect sizes. Finally, 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. In the mPOA, males in the ScF control group had a statistically marginally higher Fos-IR than the UnScF group, although the effect size revealed a difference of a high magnitude, $t(8) = 1.99$, $p = .08$, $d = 1.18$. In the NAc Shell, males in the ScF control group had a statistically significantly higher Fos-IR than the UnScF group, $t(8) = 3.11$, $p < .01$, $d = 4.392$. In the CeA, males in the ScF control group had a statistically significantly lower Fos-IR than the UnScF group, $t(8) = -4.56$, $p < .001$, $d = 3.823$. No statistically significant differences were found in other areas, ps > .05.

Discussion

The present study examined whether latent inhibition could develop to a neutral olfactory cue used in the development of CEP in males for a female bearing the cue. Previous studies have found that the pre-exposure of single and contextual cues can hinder their associability with the rewarding aspects of sex, a phenomenon otherwise known as latent inhibition, thus modulating sexual arousal, as well as showing that a CEP develops in male rats when they are given repeated multi-ejaculatory trials with sexually receptive females scented with a neutral almond odor (*e.g.,* Kippin *et al.,* 2001; Kippin & Pfaus, 2001a; Kippin & Pfaus, 2001b). In the present study, male rats pre-exposed five

times to the odor cue before being trained with ScF (5t ScF group) did not developed a CEP for the ScF, unlike when males were pre-exposed five times to the odor and subsequently trained with UnScF (5t UnScF group), or when pre-exposed only once to the odor (1t ScF and 1t UnScF groups). Furthermore, the Fos-IR analysis regarding the odor exposure demonstrated a differential pattern of activation depending on the odor pre-exposure and the conditioning contingencies. Namely, a general decrement in Fos-positive cell counts was found as the number of pre-exposure trials increased compared to the control ScF group. These results show that five trials of pre-exposure of a olfactory CS alone before pairing the it with the post-ejaculatory reward state disrupts the development of CEP, whereas one trial does not. This latent inhibition at the behavioral level was accompanied by a significantly lower Fos-IR induction by the odor alone in brain regions previously shown to be activated by the odor alone following training for CEP (Kippin *et al*., 2003).

Behavioral analyses

Latent inhibition refers to a disruption in associative strength when a neutral cue used as a CS is pre-exposed alone prior to conditioning (*e.g.,* Lubow, & Moore, 1959). Regardless of the paradigm, latent inhibition is reflected in the reduction of novelty in the CS, leading to a slow rate of acquisition of excitation or inhibition, and a small conditioned response (Schmajuk, 2002). Thus, it can be reasoned that that the novelty of the odor was reduced enough to impair the development of CEP after 5 pre-exposures. These data are reminiscent of reports in which the degree of latent inhibition depends on the number of trials of pre-exposure (Lantz, 1973; Zamble, Mitchell & Findlay, 1986), and also in which one trial of pre-exposure may enhance subsequent trained associations (Fanselow, 1990). Latent inhibition has also been shown to be context-specific phenomenon (*i.e.,* Wickens, Tuber & Wickens, 1983). Interestingly, although the training and testing phases were conducted in two different contexts, the latent inhibition effect found in the 5t

ScF group remained the same regardless of this change in context. This can be explained because all males were pre-exposed to the training context before the pre-exposure phase. Latent inhibition is not context-dependent if the training context is pre-exposed before the CS pre-exposition phase (McLaren, Bennett, Palisted, Aitken, & Mackintosh, 1994). Like other type of CSs, a context is conceptualized as an arrangement of exteroceptive physical elements, and prior exposure to a context like a testing chamber would basically reduce the salience of these elements as they are associated with no direct consequence (*e.g.,* McLaren & Mackintosh, 2000).

Finally, previous studies on CEP have associated the CS odor in either an excitatory or inhibitory fashion, after experience with sexually receptive or non-receptive females, respectively (*e.g.,* Kippin *et al.,* 1998). In the present study, however, as a result of the pre-exposure, it is believed the odor became a less relevant or non-relevant stimulus due to a reduction of novelty or the lack of attention given to it after association with no consequences (*e.g*., Schmajuk, 2010). Hence, we argue that the disruption of the CEP due to the latent inhibition procedure is different from, and not simply less intense than, the inhibition found when Kippin *et al* (1998) paired the odor with sexual non-reward.

Fos-IR analyses

The Fos-IR analysis of the brain areas of interest showed different patterns of activation depending on the amount of pre-exposure and the type of female the males were trained with. Generally speaking, the differential patters of Fos-IR are most likely due to different levels of sensory activation (Pfaus & Heeb, 1997). Therefore, we expected to find lower Fos-IR expression in all groups compared to previous studies of Fos-IR expression regarding a cue associated with sexual reward (*e.g.,* Kippin *et al.,* 2003) because males were allowed to ejaculate only one time per trial, unlike previous studies where they were allowed to ejaculate *ad lib* within a 30 min test (*e.g.,* Ismail

et al., 2009; Kippin *et al.,* 1998). This effect was observed most notably in the 5t groups.

Figure 4 shows the Fos-IR following exposure to the sexually conditioned odor before perfusion in the ScF 5t and 1t groups, and control ScF groups in the brain areas of interest. Pre-exposing the odor cue before conditioning in the ScF groups lead to a lower activation of the 5t group compared to the 1t group in the mPOA, Nacc Core, BLA, and VTA, whereas the 1t group had a lower Fos-IR compared to the control ScF group in the BLA, Nacc Core and Shell. These brain areas have been described previously as part of the neural system responsible for olfactory conditioning in the rat (Pfaus *et al.,* 2012). The pathways are constituted by three main interactive systems that process the olfactory cue or CS, the sexual reward or US, and the integration of both, including brain regions like the mPOA, VTA, Nacc Core and Shell, the amygdala, VMH, and the arcuate nucleus of the hypothalamus. These regions are activated unconditionally by olfactory/pheromonal or genitosensory stimulation during copulation (Pfaus & Heeb, 1997). In the present study, the conditioned odor likely activated the representation of the sexual reward state, consistent with Pavlov's (1927) account of cortical processing of CSs. Thus, previous findings have shown a higher Fos-IR count in males trained with ScF after the presentation of the paired odor in the Pir Ctx, BLA, and Nacc Core, and also a higher Fos-IR in the olfactory bulb, amygdala, mPOA, Nacc Core and Shell, and VTA in response to estrous odors (Kippin, Cain & Pfaus, 2003). Therefore, a lower Fos-IR count in the areas mentioned above for the 5t and 1t ScF group compared to the control ScF is consistent with latent inhibition of the neutral odor as a CS.

Fos-IR following 1t of ScF is consistent with what previous studies have shown for ScF control group Fos-IR for the odor cue alone when it is associated with sexual reward in regions like the BLA and NAcc Core (Kippin *et al*., 2003; de Jones *et al*., 1992; West *et al*., 1992). In contrast, the number of Fos positive cells in the ScF 1t group was higher in the mPOA compared to the ScF control group. The mPOA is a brain region that regulates sexual arousal, copulatory responses, and

integrates biologically relevant cues (*e.g.*, estrous odors) with sexual reward (see Hull & Rodriguez-Manzo, 2009). For instance, lesions have shown to abolish copulation in a wide range of male species, whereas electrical or chemical stimulation has shown to facilitate it (Hull, Wood & McKenna, 2006). Thus, regarding the higher Fos-IR activation in the VTA and mPOA in the 1t ScF group compared to the control ScF, it has been shown previously that to pre-expose a CS before conditioning facilitates the formation of further trained associations if there is enough time between the pre-exposure and training (Fanselow, 1990). Thus, it is possible that 1t for the ScF group may have led to an enhanced representation of the odor due to the one trial of pre-exposure leading to a higher Fos-IR mPOA and the VTA.

Figure 5 shows the Fos-IR following exposure to the sexually conditioned odor before perfusion in the UnScF 5t and 1t, and the control ScF groups in the brain areas of interest. Following exposure to the odor cue in the group of males trained with UnScF, the 5t group had a higher number of Fos positive cells than the 1t group the Nacc Core and Shell, BLA, and CeA, yet still lower than the ScF control group in the VTA, Nacc Core, and Shell. These results were expected given that the odor would lead to lower Fos positive cells in the UnScF 5t group in comparison to the ScF control group, considering that not only did the males undergo five trials of pre-exposure to the odor without any consequence, but also because they were trained with UnScF as predictor of the reward. This may explain why, although the 5t UnScF had a higher number of Fos positive cells than the 1t UnScF, it was still lower than the ScF control group in brain areas associated with the control or mediation of appetitive behaviors, attention towards reward-related stimuli, and their own incentive salience (Berridge, 2007).

Another interesting result was the higher Fos-IR activation in the UnScF 5t to the ScF control group in the CeA. The amygdala and its subnuclei process affective information from different modalities and are critical for reward- or avoidance-related associative learning (Amunts *et al.*,

2005). Different studies on specific nuclei of the amygdala have shown different roles on the male sexual behavior like copulation, arousal, post-ejaculatory quiescence, among others (see Hull & Rodriguez-Manzo, 2009). More recently, the BLA has being identified as a main regulation area for positive and negative associations, where projection from the BLA towards the CeA are at the base for negative conditioning associations, whereas projections from the BLA towards the NAc are at the base for positive associations (Namburi *et al.,* 2015). It could be that higher CeA Fos-IR in the UnScF 5t group is at the base of a negative association with the odor due to its prolonged exposure compared to the all other experimental groups. However, replication and more specific studies are required to corroborate this hypothesis. Doable-labeling IHC for GABA, glutamate, or other neurotransmitters that may exert an inhibitory action in the CeA, either directly or indirectly, may elucidate this finding.

In conclusion, the present study extends the findings on latent inhibition to the realm of conditioned sexual responses, and demonstrates how the pre-exposure of a neutral cue before conditioning for a CEP can disrupt the preference found in the control groups. These findings indicate that the pre-exposure results in a subsequent impairment of the association that links the odor cue to sexual reward and preference, leading to a differential pattern of neural activation compared to a control group given the amount of pre-exposure and the contingencies of training. Thus, how animals establish their preference of a sexual partner appears to be not only an orchestration of their own physiological internal state, but also the behavioral and neural mechanisms during first sexual experiences that follow Pavlovian rules of conditioned associations between the internal reward state that serves as the US and external CSs that predict it.

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Chapter 3 - Conditioned partner preference in male and female rats for a somatosensory cue

Abstract

Male and female rats form a conditioned preference to copulate and/or mate with conspecifics bearing an odor that was paired with either the post-ejaculatory 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 tethering jacket, could act also as a discrete cue to establish a conditioned partner choice (CPC). Two experiments were conducted to evaluate this. In the first, sexually-naïve Long-Evans males and females underwent 14 trials for 30 min with their opposite-sex partner. Males were trained in a unilevel pacing chamber with a preferred 1-hole Plexiglas divider with sexually receptive females wearing the rodent tethering jacket, whereas females were trained using the same chambers with a preferred 4-hole divider with jacketed males. On the final test, each experimental male or female was placed into an open field with two sexually receptive opposite-sex 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 for either of the males. In the second study, a new group of males and females underwent to similar training conditions except that they were exposed sequentially to jacketed, sexually receptive partners, and unjacketed, sexually non-receptive partners. The final open field test was run identically to the first. Results showed that both experimental 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.

Key words: Conditioned partner preference, fetishism, sexual behavior

Conditioned partner preference in male and female rats for a somatosensory cue

Introduction

In order 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 develop a conditioned sexual partner choice (CPC) for a potential partner bearing an odor cue (e.g., almond or lemon) that has been paired previously with sexual reward (*e.g.,* paced copulation for females, as in Coria-Ávila, *et al.,* 2005, or the postejaculatory reward state in males, as in Kippin, Talianakis, Schattmann, Bartholomew & Pfaus, 1998, respectively). In these experiments, animals were assigned to three groups. A paired group, where males underwent 10 multi-copulatory trials with scented sexually receptive females; whereas females underwent four multi-copulatory trials with pacing copulation (*i.e.,* 4-hole divider) and four multi-copulatory 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 non-sexually 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.,* 2005; Kippin Talianakis, Schattmann, Bartholomew & Pfaus, 1998). Subsequent studies showed 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 field test (Parada *et al.,* 2011). Even unconditionally aversive odors can become less aversive and conditionally appetitive following pairing with a sexual reward US. Males that underwent multi-ejaculatory training trials with a sexually receptive female scented with cadaverine rapidly approached and displayed appetitive responses towards 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 & 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 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 towards the fully covered inanimate object than the males trained in the non-fading group in the form of grabs, mounts, and cloacal contacts. These data demonstrated that sexual behavior is able to be conditioned towards an inanimate stimulus object that has no natural connection with sexual reward (Domjan, Huber-McDonald & Holloway, 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 CSs 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 sec. before copulation for fertilization with a live female quail. These males, although slower and less efficient in achieving cloacal contact with the female compared to 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).

A neutral somatosensory cue (rodent tethering jacket) can also become a CS for sexual arousal. Pfaus, Erikson and Talianakis (2013) trained male rats to have their first and subsequent 9 sexual experiences wearing or not wearing the rodent tethering jacket. On the final test, rats in both groups were randomly assigned to have the jacket on or jacket off during the test. 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 non-receptive female, but off when paired with a sexually receptive female. Males trained to associate the jacket with a sexually non-receptive female had fewer ejaculations, large ejaculation latencies, and fewer conditioned level changes compared to 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.

Methods

Subjects

75 Females Long-Evans rats, weighing 150–200 g, and 60 male Long-Evans rats, weighing

200–250 g, were obtained from Charles River Canada, Inc. (St-Constant, QC) at six weeks of age. All animals were kept in a 12 h. 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. Non-experimental females and males were obtained from the same distributor, house, fed, and kept under similar regime than the experimental ones.

Females were pair-housed in Plexiglas cages with wood-chip bedding. Sexual receptivity was induced after the ovariectomy by subcutaneous injections of 10 μg (in 0.1ml of sesame oil) of estradiol 48 hours prior each training session, and 500 μg (in 0.1ml of sesame oil) of progesterone four hours prior to each training session. Non-sexually 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).

Surgeries

Ovariectomy

Females were ovariectomized via bilateral lumbar incisions under general anesthesia induced with ketamine (50 mg/ml)/xylazine (4mg/ml) at a ratio of 4:3 respectively, around 2 weeks before the beginning of the experiment to allow at least a week of recovery. This procedure allowed for hormone levels to be controlled under hormonal replacement throughout training sessions.

Orchidectomy

Six male rats were gonadectomized via testicular incision through inhalation anesthesia with 5% isoflurane and used as studs for the female experiments. The surgery was conducted approximately 2 weeks before the beginning of the experiment to allow at least a week of recovery and for testosterone in plasma to completely washout.

Materials and testing apparatus

The somatosensory cue was a tethering rodent 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. All conditioning trials were conducted in Plexiglas unilevel pacing chambers (38 x 60 x 38 cm) with bedded floors and bisected by a transparent Plexiglas divider. Males had a 1-hole divider considering the facilitation for CPC in males over a 4-hole divider (Ismail *et al.,* 2009), whereas females had either no divider or a 4-hole divider big enough to allow only the female to cross through, depending of the experiment. Copulatory preference tests took place in an open field (123 x 123 x 46 cm) filled with clean bedding. All testing trials were recorded and subsequently scored with specialized counting software.

Procedure

Conditioning

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 group of males and females.

Female experiments

Each female experiment had groups of 19 subjects, except for the explicitly paired experimental group which had 18 subjects. There were two female experimental groups. The paired group underwent seven trials with jacketed males with a 4-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 4-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 4-hole divider, and seven trials with no divider and non-sexually-receptive unjacketed males; whereas for the unpaired group, the jacketed males were paired with no divider and non-sexual receptivity, and the unjacketed males were paired with a 4-hole divider and sexual receptivity. Animals were randomly assigned to each group prior to the experiment.

Male experiments

Each male experiment had groups of 15 subjects. 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.

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 non-sexually-receptive unjacketed females, whereas for the unpaired group, the sexually-receptive females did not wear jackets, and the non-sexually-receptive females did wear jackets. Animals were randomly assigned to each group.

Copulatory preference test

Four days after the 14th conditioning trial, each male or female was placed in an open field for five minutes 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 minutes. In the case of female experiments, the open field had two 4-hole dividers that would allow only the female in the middle compartment to cross though into where the males were.

Statistical analyses

A series of repeated measures ANOVA 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 compassions were conducted for each single variable to compare Jacket ON and Jacket OFF using the Tukey HSD test. Furthermore, partial eta square (η_p^2) was calculated as effect size for each comparison. Additionally, chi square (χ^2) analyses were conducted for the percentage of first ejaculation choice for each group, and another to contrast the ejaculatory preference between the groups. Furthermore, Cramer's V (V) and Phi (φ) effect sizes were conducted as effect size.

Results

Females

Experiment 1

Figure 1. shows the mean frequency for the different 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 partners 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)x 2(Male: jacket ON, jacket OFF) repeated measures ANOVA for each of the aforementioned sexual behaviors.

For hops and darts, there was no statistical significantly main effect of Male, $F(1,36) = .042$, $p = .838$, $\eta_p^2 = .001$, nor an interaction between Group x Male, $F(1,36) = 1.434$, $p = .239$, $\eta_p^2 = .038$. For solicitations, there was no statistical significantly main effect of Male, $F(1,36) = .72$, $p = .402$,

η_p^2 = .02, nor an interaction between Group x Male, $F(1,36) = 1.481$, $p = .231$, η_p^2 = .04. For kicks, there was no statistically significant main effect of Male, $F(1,36) = .032$, $p = .858$, η_p^2 = .001, nor an interaction between Group x Male, $F(1,36) = .129$, $p = .722$, η_p^2 = .004.

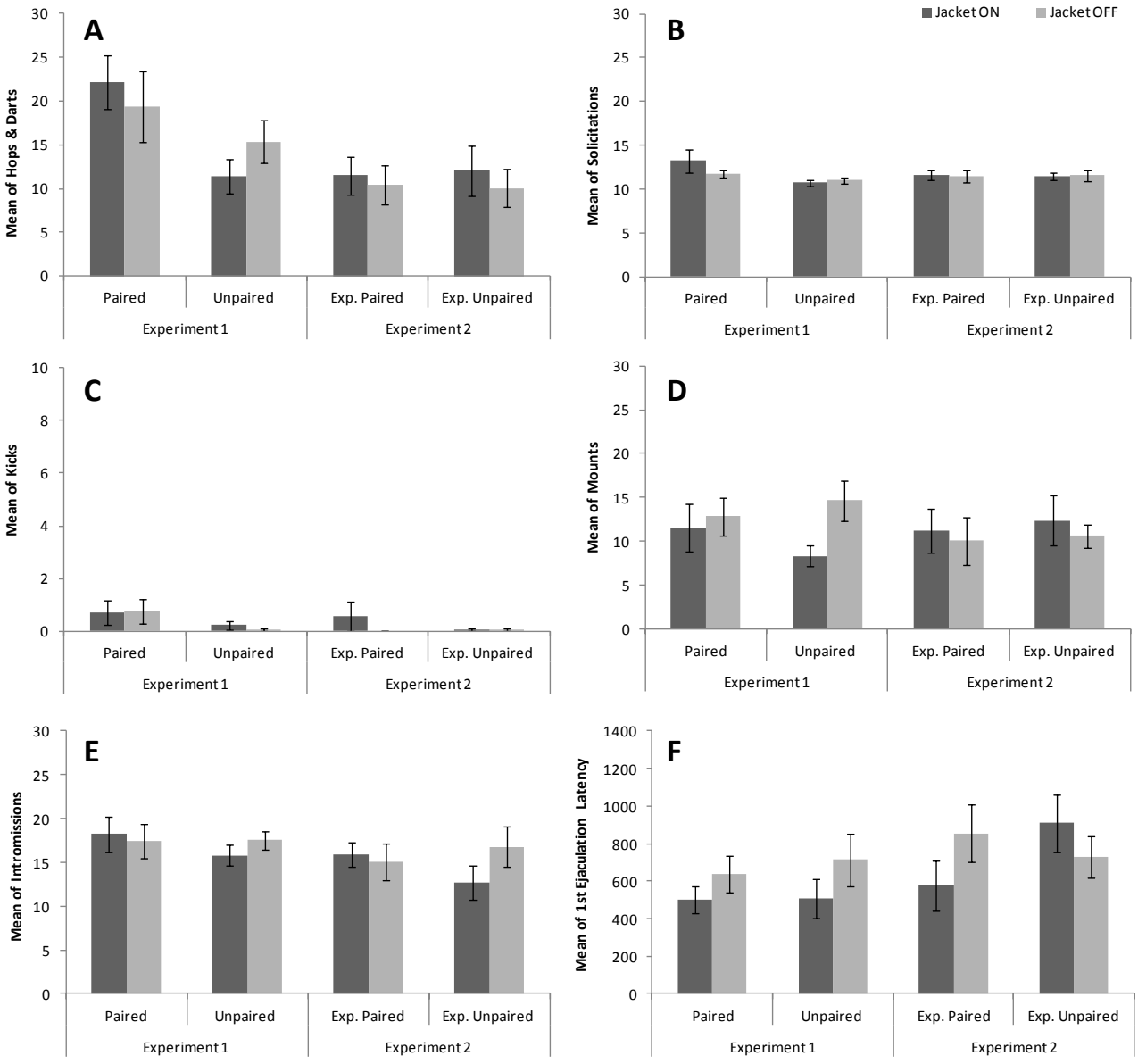

Figure 1. Mean frequency for the different sexual behaviors for females during the open field by condition (Jacket ON vs. Jacket OFF), group (Paired vs. Unpaired), and experiment (Experiment 1 and Experiment 2). Panel A. Hops and darts, Panel B. Solicitations, Panel C. Kicks, Panel D. Mounts, Panel E. Intromissions, Panel F. First ejaculation latency.

For mounts, there was no statistically significant main effect of Male, $F(1,36) = 2.753$, $p = .106$, $\eta_p^2 = .071$, nor an interaction between Group x Male, $F(1,36) = 1.182$, $p = .284$, $\eta_p^2 = .032$. For intromissions, there was no statistically significant main effect of Male, $F(1,36) = .101$, $p = .752$, $\eta_p^2 = .003$, nor an interaction between Group x Male, $F(1,36) = .72$, $p = .402$, $\eta_p^2 = .02$. For mean of 1st ejaculation latency, there was no statistically significant main effect of Male, $F(1,36) = 2.16$, $p = .15$, $\eta_p^2 = .057$, nor an interaction between Group x Male, $F(1,36) = .099$, $p = .755$, $\eta_p^2 = .003$.

Figure 2. shows the preference scores of mean of ejaculation and first ejaculation choice of females during the open field by male, group, and experiment. As panel A shows, for the mean of ejaculation, there was a marginally significantly main effect of Male, $F(1,36) = 4.034$, $p = .052$, $\eta_p^2 = .101$, yet no interaction between Group x Male, $F(1,36) = .161$, $p = .69$, $\eta_p^2 = .004$. Post-hoc comparisons conducted with the Tukey HDS correction showed that, although both group of females displayed a higher mean of ejaculation towards the jacketed males ($M = 2.42$), than to the unjacketed males ($M = 1.95$), this difference was not statistically significant, $p > .05$, $\eta_p^2 = .101$.

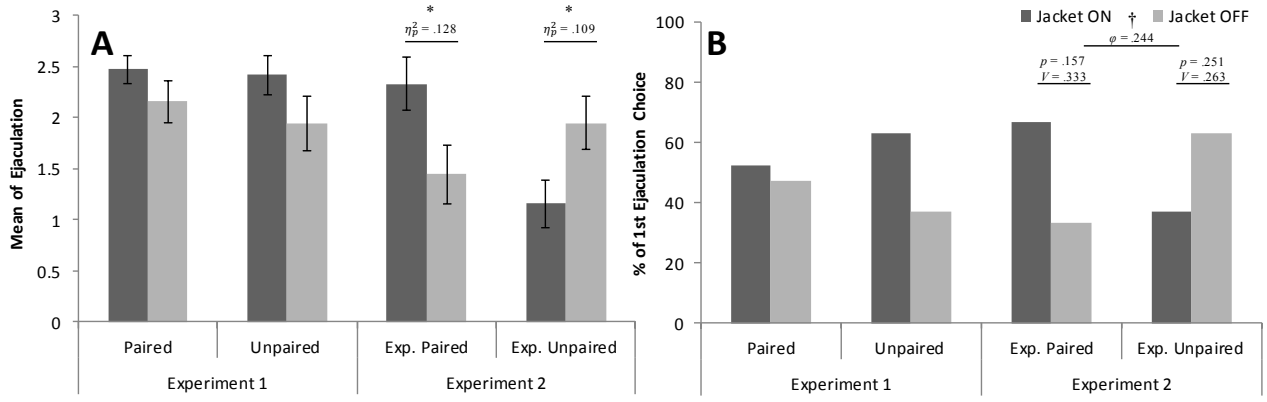

Figure 2. Female preference scores during the open field by condition (Jacket ON vs. Jacket OFF), group (Paired vs. Unpaired), and experiment (Experiment 1 and Experiment 2). Panel A. Mean of ejaculations, Panel B. Percentage of first ejaculation choice. † = $p < .01$; * = $p < .05$; η_p^2 = partial eta square; V = Cramer's V; φ = phi.

As shown in Panel B of Figure 2, the χ^2 square analysis for the 1st ejaculation choice revealed

no statistically 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 1. shows the mean frequency for the different 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 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)x 2(Male: jacket ON, jacket OFF) repeated measures ANOVA for each of the aforementioned sexual behaviors.

For hops and darts, there was no statistically significant main effect of Male, $F(1,35) = .435$, $p = .514$, $\eta_p^2 = .012$, nor a statistically significant interaction between Group x Male, $F(1,35) = .042$, $p = .84$, $\eta_p^2 = .001$. For solicitations, there was no statistically significant main effect of Male, $F(1,35) = .002$, $p = .961$, $\eta_p^2 < .001$, nor a statistically significant interaction between Group x Male, $F(1,35) = .047$, $p = .83$, $\eta_p^2 = .001$. For kicks, there was no statistically significant main effect of Male, $F(1,35) = 1.035$, $p = .316$, $\eta_p^2 = .029$, nor a statistically significant interaction between Group x Male, $F(1,35) = 1.035$, $p = .316$, $\eta_p^2 = .029$. For mounts, there was no statistically significant main effect of Male, $F(1,35) = .487$, $p = .49$, $\eta_p^2 = .014$, nor a statistically significantly interaction between Group x Male, $F(1,35) = .022$, $p = .884$, $\eta_p^2 = .001$. For intromissions, there was no statistically significant main effect of Male, $F(1,35) = .485$, $p = .491$, $\eta_p^2 = .014$, nor a statistically significant interaction between Group x Male, $F(1,35) = 1.106$, $p = .30$, $\eta_p^2 = .031$. For mean of 1st ejaculation latency, there was no statistically significant main effect of Male, $F(1,35) = .106$, $p = .746$, $\eta_p^2 = .003$, nor a statistically significant interaction between Group x Male, $F(1,35) = 2.288$, p

= .139, η_p^2 = .061.

Figure 2. shows the preference scores of mean of ejaculation 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 statistically significant main effect of Male, $F(1,35) = .033$, $p = .857$, $\eta_p^2 = .001$, there was a statistically significant interaction between Group x Male, $F(1,35) = 9.401$, $p = .004$, $\eta_p^2 = .212$. Post-hoc comparisons conducted with the Tukey HSD correction showed that females in the paired experimental group took a statistically significantly higher mean of ejaculation from the jacketed male ($M = 2.33$, $SD = 1.09$), than from the unjacketed male ($M = 1.44$, $SD = 1.20$, $p < .05$, $\eta_p^2 = .128$), whereas females in the unpaired experimental group took a statistically significantly higher mean of ejaculation from the unjacketed male ($M = 1.95$, $SD = 1.13$), than from the jacketed male ($M = 1.16$, $SD = 1.02$, $p < .05$, $\eta_p^2 = .109$).

As shown in panel B of Figure 2, the χ^2 square analysis for the 1st ejaculation choice revealed a trend towards a differential 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 2x2 χ^2 square analysis revealed that both of these groups have a marginally significant different preference for their 1st ejaculation, $\chi^2(1) = 3.29$, $p = .069$, $\varphi = .244$.

Males

Experiment 1

Figure 3. shows the scores for the different copulatory male behaviors on the open field by female and group test in experiment 1. As shown in panels A and B, both paired and unpaired groups displayed a higher mean of mounts and intromissions for the jacketed female. The reliability of these observations was corroborated by 2(Group: paired, unpaired)x 2(Female: jacket ON, jacket OFF) repeated measures ANOVA for each of the aforementioned sexual behaviors. For mounts,

there was a statistically significant main effect of Female, $F(1,28) = 12.764$, $p = .001$, $\eta_p^2 = .313$, yet there was no statistically significant interaction between Group x Female, $F(1,28) = .911$, $p = .348$, $\eta_p^2 = .031$. Post-hoc comparisons conducted with the Tukey HSD correction showed that males in the paired group displayed a marginally higher mean of mounts towards the unjacketed female ($M = 10.87$, $SD = 7.98$), than to the jacketed female ($M = 6.93$, $SD = 6.62$, $p < .1$, $\eta_p^2 = .109$), whereas males in the unpaired group displayed a statistically significantly higher mean of mounts towards the unjacketed female ($M = 11.67$, $SD = 9.69$), than to the jacketed female ($M = 4.87$, $SD = 5.74$, $p < .05$, $\eta_p^2 = .268$). For intromissions, there was no statistically significant main effect of Female, $F(1,28) = 1.553$, $p = .223$, $\eta_p^2 = .053$, nor a statistically significant interaction between Group x Female, $F(1,28) = .02$, $p = .888$, $\eta_p^2 = .001$.

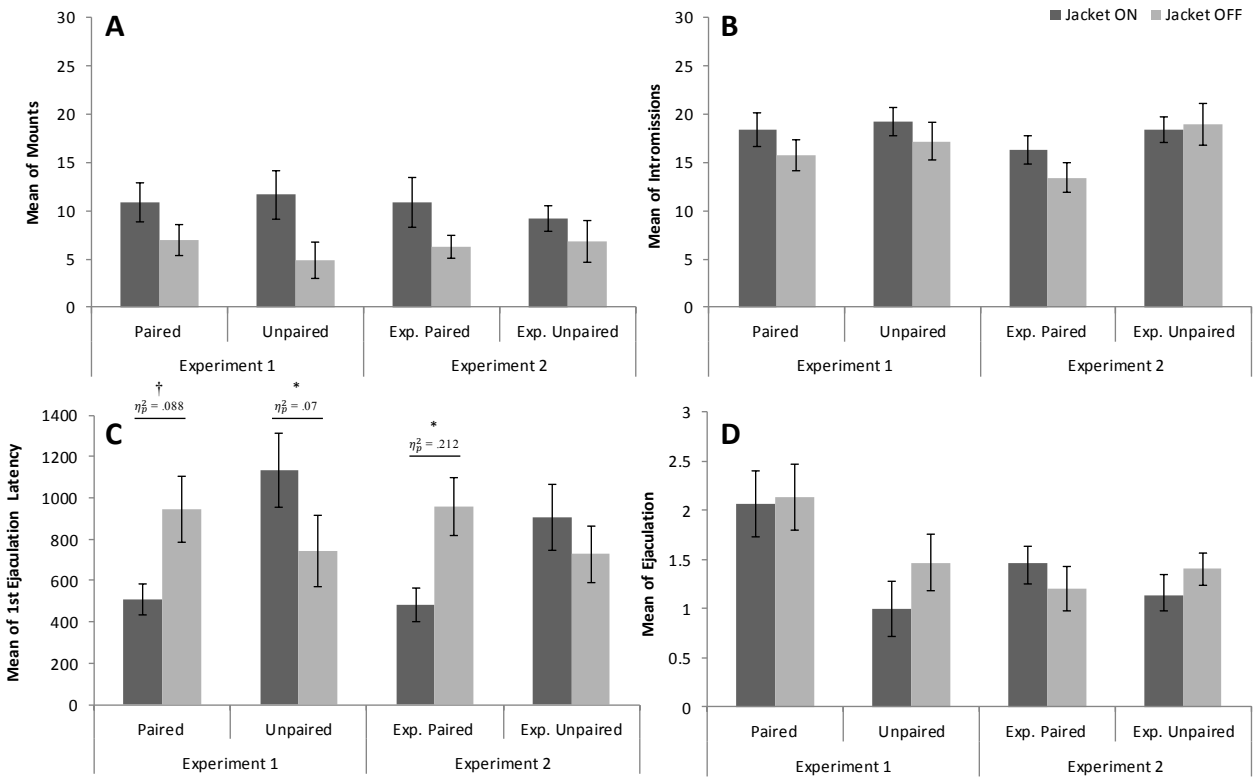

Figure 3. Mean frequencies for copulatory behaviors for males during the open field test by condition (Jacket ON vs. Jacket OFF), group (Paired vs. Unpaired), and experiment (Experiment 1 and Experiment 2). Panel A. Mounts, Panel B. Intromissions, Panel C. First ejaculation latency, Panel D. Ejaculations. † = $p < .01$; * = $p < .05$; η_p^2 = partial eta square.

As shown in panel C of Figure 3, the ANOVA for the 1st ejaculation latency revealed no statistically significant main effect of Female, $F(1,28) = .019$, $p = .893$, $\eta_p^2 = .001$, yet there was a statistically significant interaction between Group x Female, $F(1,28) = 4.772$, $p = .037$, $\eta_p^2 = .146$. Post-hoc comparisons conducted with the Tukey HSD correction showed that only males in the unpaired group displayed a statistically significantly lower mean of latency of 1st ejaculation towards the unjacketed female ($M = 743.2$, $SD = 659.81$), than to the jacketed one ($M = 946.67$, $SD = 696.45$, $p < .05$, $\eta_p^2 = .07$).

As shown in panel D of Figure 3, the ANOVA for the mean of ejaculation revealed no statistically significant main effect of Female was found, $F(1,28) = .472$, $p = .498$, $\eta_p^2 = .017$, nor a statistically significant interaction between Group x Female, $F(1,28) = .265$, $p = .611$, $\eta_p^2 = .009$.

Figure 4 shows the 1st ejaculation choice per female in experiment one. The χ^2 square analysis for the 1st ejaculation choice revealed no statistically significant preference in the paired group for either of the females, $\chi^2(1) = .6$, $p = .439$, $V = .2$, a non-significant trend in the unpaired group to ejaculate first with unjacketed females, $\chi^2(1) = 1.667$, $p = .197$, $V = .333$. Finally, a 2x2 χ^2 square analysis revealed a marginal trend of these groups having a different preference for their 1st ejaculation choice, $\chi^2(1) = 2.143$, $p = .143$, $\varphi = .2$.

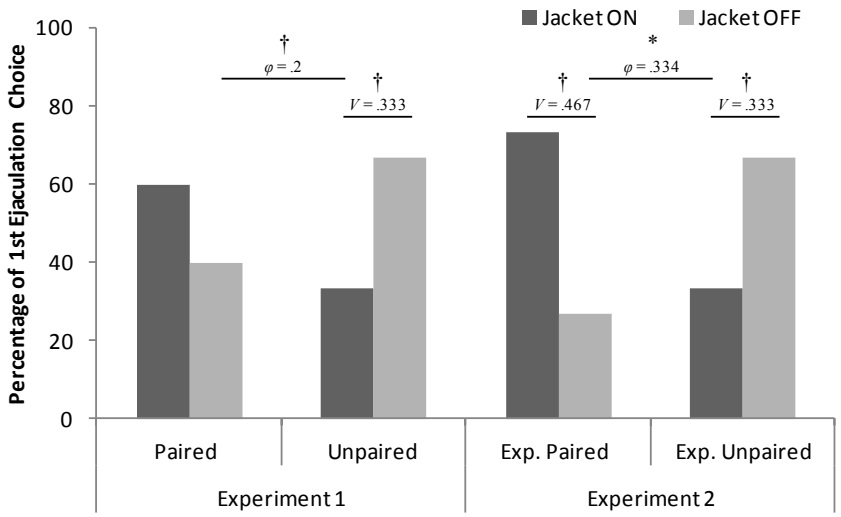

Figure 4. Percentage of first ejaculation choice for males during the open field test by condition (Jacket ON vs. Jacket OFF) and group (Paired vs. Unpaired), and experiment (Experiment 1 and Experiment 2). † = $p < .01$; * = $p < .05$; η_p^2 = partial eta square; V = Cramer's V; φ = phi.

Experiment 2

Figure 3. 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 of mounts and intromissions for the jacketed female. The reliability of these observations was corroborated by 2(Group: paired, unpaired)x 2(Female: jacket ON, jacket OFF) repeated measures ANOVA for each of the aforementioned sexual behaviors. For mounts, there was a statistically significant main effect of Female, $F(1,28) = 6.392$, $p = .017$, $\eta_p^2 = .186$, yet there was no statistically significant interaction between Group x Female, $F(1,28) = .656$, $p = .425$, $\eta_p^2 = .023$. Post-hoc comparisons conducted with the Tukey HSD correction showed that only males in the paired group displayed a statistically significantly higher mean of mounts towards the jacketed female ($M = 10.87$, $SD = 10.11$), than to the unjacketed female ($M = 6.33$, $SD = 5.5$, $p < .05$, $\eta_p^2 = .166$). For intromissions, there was no statistically significant main effect of Female, $F(1,28) = 1.553$, $p = .223$, $\eta_p^2 = .036$, nor a statistically significant interaction between Group x Female, $F(1,28) = 1.792$, $p = .192$, $\eta_p^2 = .06$.

As shown in panel C of Figure 3, the ANOVA for 1st ejaculation latency revealed no statistically significant main effect of Female, $F(1,28) = 1.491$, $p = .232$, $\eta_p^2 = .051$, yet there was a statistically significant interaction between Group x Female, $F(1,28) = 7.075$, $p = .013$, $\eta_p^2 = .202$. Post-hoc comparisons conducted with the Tukey HSD correction showed that only males in the paired group displayed a statistically significantly lower mean of latency of 1st ejaculation towards the jacketed female ($M = 483.67$, $SD = 308.89$), than to the unjacketed female ($M = 957.87$, $SD = 539.15$, $p < .05$, $\eta_p^2 = .212$).

As shown in panel D of Figure 3, the ANOVA for the mean of ejaculation revealed no statistically significant main effect of Female was found, $F(1,28) < .001$, $p > .9$, $\eta_p^2 = 0$, nor a statistically significant interaction between Group x Female, $F(1,28) = 1.665$, $p = .207$, $\eta_p^2 = .056$.

Figure 4 shows the 1st ejaculation choice per female in experiment one. The χ^2 square analysis for the 1st 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 non-significant trend in the unpaired group for the unjacketed females, $\chi^2(1) = 1.667$, $p = .197$, $V = .333$. However, a 2x2 χ^2 square analysis revealed that both of these groups have a statistically significant different preference for their 1st ejaculation choice, $\chi^2(1) = 4.82$, $p = .028$, $\varphi = .334$.

Discussion

The purpose of this study was to examine whether a neutral somatosensory cue (rodent tethering jacket) on a partner during a male or female rat's first experiences of sexual reward could accrue enough associate 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 non-jacketed female for their first ejaculation and had

significantly more ejaculations with 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 non-jacketed males. In the second experiment, the jacket on the partner was paired explicitly with sexual reward, with the non-jacketed partner paired explicitly with sexual non-reward (as the partner was sexually non-receptive). When this differentiation was made, both male and female subjects showed a CPC for the jacketed versus non-jacketed partner on the final choice test. Thus, a neutral somatosensory cue can be used as a discrete, partner-related cue (*i.e.*, CS) that predicts the sexual reward state (*i.e*., US).

Previous studies have shown that CPCs develop in both male and female rats if a neutral cue like an odor is paired enough times with sexually rewarding experiences (Coria-Ávila et *al.,* 2005; Kippin *et al.,* 1998). Also, male Japanese quail display more conditioned approach responses towards a light CS or an inanimate terrycloth object after being paired with the opportunity to copulate with a female (Domjan, Huber-McDonald & Holloway, 1992). The present findings add to this by showing that cues in another sensory domain can be paired with sexual reward and modulate partner preference in male and female rats, but also that the response or associability may differ between the sexes given the ease with which conditioning occurred in both experiments in males, but had to be made explicit in terms of its prediction of sexual reward in females. Unlike previous findings (*e.g.,* Coria-Ávila *et al.,* 2006), females did not show a partner preference for the somatosensory cue when it was associated only with paced versus non-paced copulation. Previously, Parédez & Alonso (1997) showed that paced copulation induces a rewarding state in the female rat that is sufficient for the female to associate contextual cues of a distinctive conditioned place preference (CPP) environment and show subsequent CPP to the side associated with paced copulation. Paced copulation, either induced in unilevel or bilevel pacing chambers, was also necessary and sufficient to induce a CPC in female rats given multiple paced copulations with males

scented with a neutral almond odor (Coria-Ávila *et al.,* 2005), or with pigmented versus albino strains of male rat (Coria-Ávila *et al.,* 2006).

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 different 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 non-paced 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 non-receptive 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

non-reward, and its absence may well have led females on the final test to avoid copulatory attempts with non-jacketed males.

To develop 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; Weisel-Barth, 2013). To the extent that a rodent jacket can become a contextual CS for sexual arousal in male rats (Pfaus, Erickson & Talianakis, 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 labelled it as sexual (Abel, Coffey & Osbon, 2008; Lowenstein, 1997). How such learning crystallizes the object into a sexual cue requires further study, although we have argued that this must be the product of neural plasticity, where epigenetic changes within neurochemical pathways that link sexual reward to sexual desire occur during a rat's first sexual experiences (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, Guizar, Rassi & Pfaus, in press; Quintana, Jackson, Nasr & Pfaus, in press, respectively). 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.

Acknowledgments

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Chapter 4 - Naloxone disrupts the development of a conditioned ejaculatory preference based on a somatosensory cue in male rats

Abstract

We have shown previously that male rats develop a conditioned ejaculatory preference (CEP) toward females based on neutral external cues like an odor or a somatosensory cue (rodent jacket) when those stimuli are paired with the reward state induced by ejaculation. As with a copulatory conditioned place preference, CEP for an odor cue depends on endogenous opioid transmission after ejaculation. The non-selective opioid receptor antagonist naloxone (NAL) disrupts CEP for an odor cue on female rats when injected systemically to males prior to each conditioning trial. Here we evaluated whether NAL would have the same effect the development of a CEP for the somatosensory cue on female partners. Long-Evans males were assigned randomly to two groups, and underwent 14 multi-ejaculatory trials for 30 min each, spaced every 4 days, and consisting of sequential pairing of jacket on a sexually receptive female and no jacket on a sexually nonreceptive female. The saline group was always injected with saline in both conditions throughout training, whereas the experimental group was injected with NAL when females were receptive and wore a jacket, and with saline when they were not receptive and did not wear a jacket. On the final test, all males were injected with saline and placed into an open field with two sexually receptive females, one with the jacket on and the other with the jacket off. Males injected with saline throughout training displayed a significant CEP for females with the jacket on, whereas males injected with NAL during sexually receptive jacket conditions displayed a significant CEP for the non-jacketed female. This study corroborates further that opioid transmission is necessary for the establishment of a CEP, and demonstrates the flexibility of the sexual system in associating external cues with the post-ejaculatory reward state.

Key words: Conditioned partner preference, opioid, naloxone, fetish, sexual behavior.

Naloxone disrupts the development of a conditioned ejaculatory preference based on a somatosensory cue in male rats

Introduction

Previous studies have shown that male rats are able to develop a conditioned ejaculatory preference (CEP) towards females based on external cues like an odor when paired with the post-ejaculatory refractory period (*e.g*., Kippin *et al*., 1998). These Pavlovian associations are flexible with regard to the sensory modality being used as an external cue. For instance, Quintana *et al*., (submitted) trained a CEP for a somatosensory cue – a rodent tethering jacket – on sexually receptive females. In that study, males trained with jacketed females displayed more ejaculations and chose to ejaculate first with the females with the jacket on, compared to females with the jacket off. Similarly, female rats also displayed a conditioned partner choice (CPC) for males wearing the jacket, but only if the jacket had been paired with a sexual receptive male partner and no jacket with a sexually nonreceptive male partner.

In male rats, the post-ejaculatory refractory phase is accompanied by increases in endogenous opioids such as ß-endorphin and enkephalin, along with serotonin and endocannabinoids (Fernández-Guasti & Rodríguez-Manzo, 1997; Garduño-Gutiérrez *et al*., 2013; Hueletl-Soto, Carro-Juárez & Rodríguez-Manzo, 2012; Pfaus, 2009; Rodríguez-Manzo & Fernández-Guasti, 1995; Szechtman, Hershkowitz & Simantov, 1981), along with significant internalization of μ opioid receptors in the medial preoptic area (mPOA; Coolen, Fitzgerald, Yu & Lehman, 2004) and ventral tegmental area (VTA; Garduño-Gutiérrez, León-Olea & Rodríguez-Manzo, 2013). This latter study showed that μ-opioid receptor activity, but not δ-opioid receptor activity, was correlated with the number of ejaculations.

Systemic injections of the nonselective opioid receptor antagonist naloxone hydrochloride (NAL) disrupts the opioid reward state, and in turn, disrupts the establishment of a conditioned

place preference (CPP; Ågmo & Berenfeld, 1990; Mehrara & Baum, 1990; Miller & Baum, 1987; Parédez & Martinez, 2001), and CEP (Ismail *et al*., 2009). Ismail *et al*. trained a CEP in male rats based on an almond odor on female rats while being injected systemic NAL, whereas the control group was injected the same way with saline. All males were injected with saline prior to a final trial in an open field with two receptive females, one scented and the other unscented. Results showed that males previously injected with NAL displayed a CEP for the unscented female, and in fact avoided copulation with the scented female. In contrast, males injected with saline throughout conditioning formed a CEP for the scented female. Male prairie voles injected with the longer-acting antagonist naltrexone during their first sexual experiences do not form partner preferences (Burkett *et al*., 2011), nor do female rats trained for a CPC based on discrete odor cues paired with paced copulation when injected with NAL relative to females injected with saline (Coria-Ávila *et al*., 2008).

The present study examined whether NAL would disrupt the CEP for a somatosensory cue that predicts the postejaculatory sexual reward state in the male rat. Two groups of males were assigned randomly to receive either saline (SAL) or naloxone (NAL) prior to 14 trials with sexually receptive females wearing a rodent jacket. Between positive trials, males in both groups were injected with SAL prior to interaction with sexually nonreceptive females not wearing the rodent jacket.

Methods

Subjects

34 Long-Evans male rats weighing 150–200 g, and 30 female Long-Evans rats, weighing 200–250 g, were obtained from Charles River Canada, Inc. (St-Constant, QC) at six weeks of age. All

animals were kept in a 12 h. 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.

Males were housed in groups of four in plexiglas cages with *ad lib* access to water and food (Purina Rat Chow), whereas female rats were pair-housed in plexiglas cages with wood-chip bedding. Sexual receptivity was induced by subcutaneous injections of 10 μg (in 0.1ml of sesame oil) of estradiol 48 hours prior each training session, and 500 μg (in 0.1ml of sesame oil) of progesterone four hours prior to each training session. The somatosensory cue were tethering rodent jackets (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.

Ovariectomy

Females used for training were ovariectomized via bilateral lumbar incisions under general anesthesia induced with ketamine (50 mg/ml)/xylazine (4mg/ml) at a ratio of 4:3 respectively, around 2 weeks before the beginning of the experiment to allow at least a week of recovery. This procedure allowed for hormonal levels to be controlled under replacement throughout training sessions.

Drug

Naloxone hydrochloride (Sigma: St. Louis, MO), a non-selective opioid antagonist, was dissolved in 0.9% physiological saline, and injected intraperitoneally in a dose of 5 mg/kg in a volume of 1 ml/kg, 5 min before the conditioning trial started, as previously done by Ismail *et al*., (2009). The saline group rats were injected in a similar way with only 0.9% physiological saline.

Behavioral training

Males were halved into two groups of 17 subjects each, and underwent 14 multi-ejaculatory

randomized trials for 30 min., 7 with sexually receptive jacketed females, and 7 with non-sexually receptive unjacketed females. The saline group was always injected with saline throughout the training, whereas the experimental group was injected with NAL when females were receptive and worn a jacket, and with saline when they were not receptive and did not wear a jacket. All conditioning sessions were conducted in Plexiglas chambers (38 x 60 x 38 cm) with bedded floors and bisected by a transparent Plexiglas divider with 1-hole pacing chambers, big enough for the female to be able to cross through, yet not the male. The 1-hole pacing dividers were chosen considering the facilitation for CEP in males over the 4-hole (Ismail *et al.,* 2009). The copulatory preference test took place in an open field (123 x 123 x 46 cm) filled with clean bedding, where males had access to copulate freely for 30 min with two sexually receptive female, one jacketed and another without a jacket. If a male did not ejaculate with either of the females during the open field, the latency for 1st ejaculation was considered 1800 sec, namely, the equivalent of 30 min of the test. All behaviors during the open field test were recorded and later on scored using a computerized event recorder (Cabilio, 1996).

Statistical analyses

A series of repeated measures ANOVA 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 compassions were conducted for each single variable to compare Jacket ON and Jacket OFF using Tukey HSD's correction. Furthermore, partial eta square (η_p^2) was calculated as effect size for each comparison. Additionally, a 1x2 chi square (χ^2) analysis was conducted for the percentage of first ejaculation choice for each group, and a 2x2 χ^2 analysis to contrast the ejaculatory preference between the groups. Furthermore, Cramer's V (V) and Phi (φ) effect sizes were conducted as effect size for the 1x2 and 2x2 χ^2 analyses, respectively.

Results

The scores for the different copulatory behavior by female for both groups during the open field test are depicted in Figure 1. As shown in panel A, males did not show consistent differences of mounts for any of the females between the groups. The reliability of these observations was corroborated by a 2(Group: NAL and SAL) x 2(Female: Jacket ON and Jacket OFF) repeated measure ANOVA. There was not statistically significant interaction between Female x Group, $F(1,32) = .05$, $p = .825$, $\eta_p^2 = .002$.

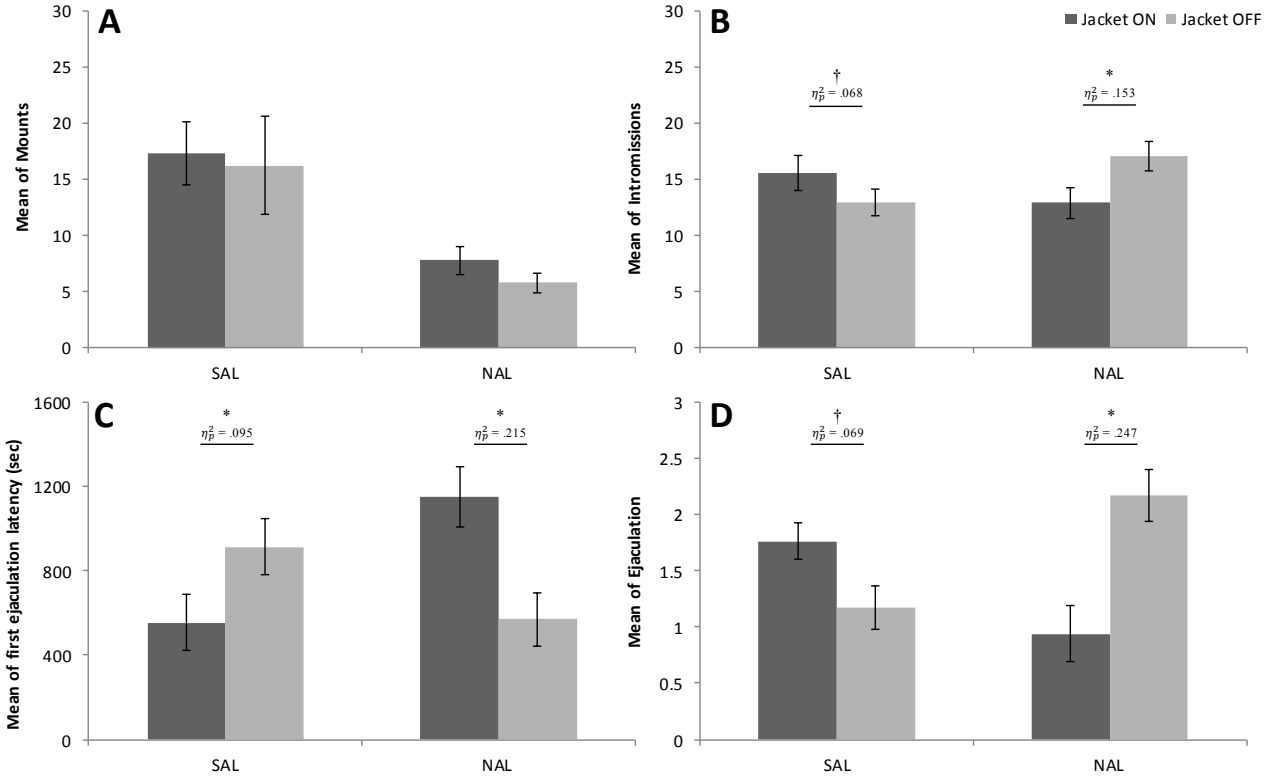

Figure 1. Mean of copulatory behaviors (±SEM) per group during the open field test. † = $p < .01$; * = $p < .05$; η_p^2 = partial eta square.

As shown in panel B, males in the NAL group displayed more intromission towards the unjacketed female, whereas males in the SAL group displayed more intromissions towards the

jacketed female. The reliability of these observations was corroborated by a 2(Group: NAL and SAL) x 2(Female: Jacket ON and Jacket OFF) repeated measure ANOVA. There was a statistically significant interaction between Female x Group, $F(1,32) = 7.721$, $p = .009$, $\eta_p^2 = .194$. Post-hoc analysis with Tukey HSD correction showed that males in the NAL group displayed a statistically significantly higher mean of intromissions for the unjacketed female ($M = 17.06$) over the jacketed female ($M = 12.88$, $p < .05$, $\eta_p^2 = .153$), whereas males in the SAL group displayed a statistically marginally higher mean of intromissions for the jacketed female ($p < .1$, $\eta_p^2 = .068$).

Furthermore, as shown in panel C, males in the NAL group displayed a lower 1st ejaculation latency mean towards the unjacketed female, whereas males in the SAL group displayed a lower 1st ejaculation latency mean towards the jacketed female. The reliability of these observations were corroborated by a 2(Group: NAL and SAL) x 2(Female: Jacket ON and Jacket OFF) repeated measure ANOVA. There was a statistically significant interaction between Female x Group, $F(1,32) = 11.51$, $p = .002$, $\eta_p^2 = .265$. Post-hoc analysis with Tukey HSD correction showed that males in the NAL group displayed a statistically significantly lower latency of 1st ejaculation mean for the unjacketed female ($M = 569.41$) over the jacketed female ($M = 1152.12$, $p < .05$, $\eta_p^2 = .215$), whereas males in the SAL displayed a statistically significantly lower latency of 1st ejaculation mean for the jacketed female ($M = 554.18$) over the unjacketed female ($M = 914.47$, $p = .05$, $\eta_p^2 = .095$).

As shown in panel D, males in the NAL group displayed a higher mean of ejaculation towards the unjacketed female, whereas males in the SAL group displayed a higher mean of ejaculation towards the jacketed female. The reliability of these observations was corroborated by two 2(Group: NAL and SAL) x 2(Female: Jacket ON and Jacket OFF) repeated measure ANOVA. There was a statistically significant interaction between Female x Group, $F(1,32) = 11.42$, $p = .002$, $\eta_p^2 = .263$. Post-hoc analysis with Tukey HSD correction showed that males in the NAL group displayed a

statistically significantly higher mean of ejaculation for the unjacketed female ($M = 2.18$) over the jacketed female ($M = .94$, $p = .003$, $\eta_p^2 = .247$), whereas males in the SAL group only displayed a marginally significantly higher mean of ejaculation towards the jacketed female ($M = 1.76$) over the unjacketed female ($M = 1.18$, $p < .1$, $\eta_p^2 = .069$).

Finally, as shown Figure 2, the χ^2 analyses for the percentage of first ejaculation choice confirmed the same pattern of mean of ejaculation. Males in the NAL group chose to statistically significantly ejaculate first more with the female with jacket off, $\chi^2(1) = 7.118$, $p = .008$, $V = .647$, whereas the saline group chose to statistically marginally ejaculate first more with the female with the jacket on, $\chi^2(1) = 2.882$, $p = .09$, $V = .412$. However, a 2x2 χ^2 analysis corroborated that these two groups had a statistically significant different patter of preference in their first ejaculation choice, $\chi^2(1) = 9.66$, $p = .002$, $\varphi = .474$.

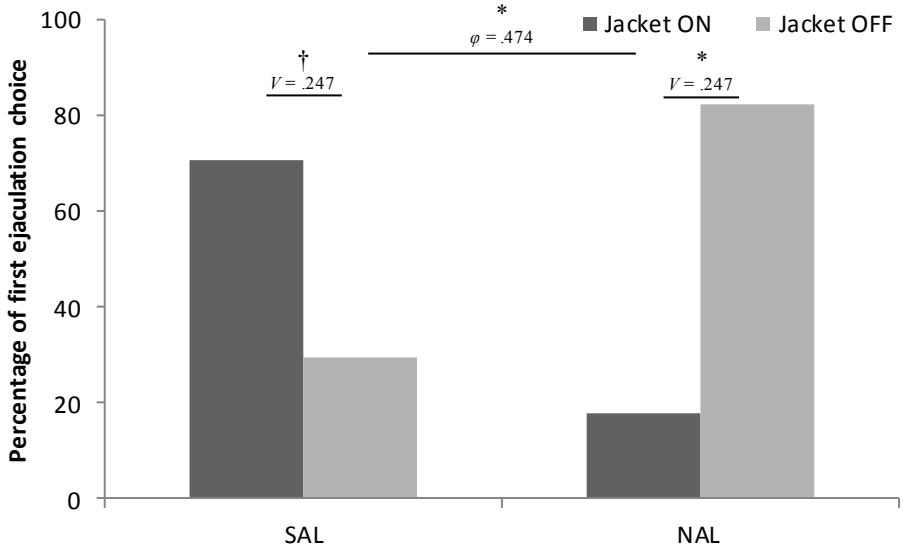

Figure 2. Percentage of 1st ejaculation choice per group during the open field test. † = $p < .01$; * = $p < .05$; V = Cramer's V; φ = phi.

Discussion

The phenomenon of conditioned ejaculatory preference is a result of Pavlovian associations between neutral cues with the rewarding aspects of sex (*e.g*., Ågmo & Berenfeld, 1990; Kippin *et al*., 1998). In the present study, male rats injected systemically with saline rats displayed more ejaculations and chose to ejaculate first more with females wearing a rodent tethering jacket, indicating that the jacket became a reliable predictor of sexual reward. On the contrary, male rats trained to associate the jacket with sexually receptive females and non-sexually receptive females without a jacket while being injected systemically with NAL shifted their preference to ejaculate with females not wearing the jacket. This indicates that nonspecific blockade of opioid receptors inhibited the sexual reward state US during the postejaculatory interval, thus inhibiting the association of the jacket with sexual reward. It is also possible that the experience of naloxone was aversive to the males, thus creating an aversion to the jacket that transferred to the development of the CEP (*e.g*., Ismail *et al*., 2009). The results of the present study are in accordance with previous findings where a neutral odor or somatosensory cue paired with the sexual reward state is able to predict sexual reward in male rats, thus creating a CEP for females bearing that odor (*e.g*., Kippin *et al*., 1998) or wearing a rodent jacket (*e.g*., Quintana *et al*., 2018). Furthermore, the disruption of CPP (Ågmo & Berenfeld, 1990) or CEP (Ismail *et al.,* 2009), along with the present findings, suggest that these two conditioned responses are control by similar neurochemical mechanisms of opioid reward regardless of the sensory system in which they may be based.

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, pro-enkephalin, and pro-dynorphin (van Ree *et al.,* 2000). These endogenous opioids bind with different affinity to the three different types of receptors, μ, κ, and δ. These

receptors are distributed all along the central nervous system, predominantly located hypothalamic, limbic and cortical areas (Pfaus, 2009; van Ree *et al.,* 1999). Although the action of endogenous opioids in the control of male sexual behavior is inhibitory (reviewed in Pfaus & Gorzalka, 1987; Pfaus, 2009), opioids also sensitize mesolimbic dopamine systems (*e.g*., Fields & Margolis, 2015), thereby increasing the likelihood of appetitive responding toward stimuli associated with reward (*e.g*., West, Clancy & Michael, 1992). Whole brain endogenous opioid activity (Szechtman, Simantov & Hershkowitz, 1980) and μ opioid receptor internalization in the mPOA and VTA (Coolen, Fitzgerald, Yu, & Lehman, 2004; Garduño-Gutiérrez, León-Olea, & Rodríguez-Manzo, 2013) increase with ejaculation and decrease pain sensitivity, and it is a pivotal component in the development of CPP (Ågmo & Berenfeld, 1990), as well as CEP (Kippin & Pfaus, 2001). These findings indicate that ejaculation induces an opioid reward state during the postejaculatory refractory period that can act as an appetitive US (Kippin & Pfaus, 2001).

An alternative hypothesis based on the devaluation instead of an inhibition of the US by NAL injections can also explain the present results. It is possible that NAL produced a dysphoric state that made the stimuli associated with it aversive. This phenomenon is known as the reinforcer devaluation effect (*e.g*., Holland & Rescorla, 1975). Through different experimental manipulations, the association unpleasant conditions or sensations immediately after the US delivery in a classical conditioning paradigm results in a shift of preference rather than no preference per se. For instance, food has been devalued as US after rats were given an intraperitoneal injection of lithium chloride (Colwill & Motzkin, 1994) or by making the rats spin at 120 rpm for five min immediately after food was provided (Holland & Rescorla, 1975). Consequently, by blocking the action of opioid receptors through NAL injections, the rewarding aspects of sex and ejaculation would be devaluated, reflected in an avoidance of the jacketed female, thus ultimately displaying a preference for the female who is not wearing a jacket.

Interestingly, previous findings on US devaluation have also shown be sensitive to time. Kraemer, Hoffmann, Randall & Spear (1992) devaluated the reward provided by heat in 10-day-old rats by pairing heat (used as US) with a foot shock. As expected, the devaluation reduced the conditioned response (*i.e.,* mean time the rats spent around an odor cue) in comparison to a control group who did not get a devaluated US. Surprisingly, the devaluation effect dissipated after a sufficiently long interval between the devaluation treatment and the test (*i.e.,* 48 hours), and also retaining the original odor-US association over that interval. Sadly, the present study did not conduct a second open field test, nor establishes a former association between the trained cue (*i.e.,* jacket) and the US before the experimental manipulation. Nevertheless, such data could shed light as to whether NAL may foster an aversion or a devaluation of the US. It is possible that these processes may differ in the magnitude, showing differences in how long the preference for the opposite female may last (given repetitive open field tests). For instance, if NAL devalues the US, repetitive open field tests may show a progression into no preference for either of the females. Conversely, avoidance may be a bit more resistant, perhaps hinted by the differences in magnitude of the effect between the SAL and NAL groups.

Altogether, these findings provide further evidence for a CEP based on a somatosensory cue on a female partner, and that opioid receptor antagonist not only can disrupt a CEP, but also shift it towards the opposite female. Further experiment should explore the nature of opioid antagonism, along with the duration of these associations.

Chapter 5 -- Differential disruption of conditioned ejaculatory preference of the male rat based on different sensory modalities by micro-infusions of naloxone to the medial preoptic area or ventral tegmental area

Abstract

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-treated males distribute their ejaculations first and more often to females without the cue, relative to saline (SAL)-treated males that show the cue-preferred CEP. 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. 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 (200, 500, or 1000 ng/µl, 0.5µl/side) or SAL (0.5 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. Males previously conditioned with SAL in either region showed significant CEP. In contrast, prior infusions of NAL to the mPOA shifted their 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. We conclude that opioid antagonism in the mPOA produces a state of non-reward whereas in the VTA it produces a state in which the odor loses incentive properties.

Key words: conditioned ejaculatory preference, opioid, mPOA, VTA

Differential disruption of conditioned ejaculatory preference of the male rat based on different sensory modalities by micro-infusions of naloxone to the medial preoptic area or ventral tegmental area

Introduction

The effects of opioids have been well documented in the display of both conditioned and unconditioned sexual behaviors (see Halloway, 2012; Parédez, 2014; Pfaus & Gorzalka, 1987; van Furth, Wolterink, & van Ree, 1995). The reinforcing properties 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 (Ågmo & Berenfeld, 1990; Miller & Baum, 1987). Male rats either copulated in the initially non-preferred side of the CPP box (Miller & Baum, 1987) or were placed into the initially non-preferred side immediately after one ejaculation (Ågmo & 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-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, transmission is an important neurochemical substrate of sexual reward induced by ejaculation in male rats. Indeed, opioid transmission is highest during and after ejaculation (Tenk, Wilson, Zhang, Pitchers & Coolen, 2009; Szechtman, Simantov & Hershkowitz, 1980) when male rats are behaviorally quiescent and sleep for a short period. This makes the postejaculatory period essentially an "orgasm-like' sexual reward state (Pfaus *et al*., 2016) during which conditioned sexual learning occurs (Georgiadis, Kringelbach & Pfaus, 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 (*e.g*., Kippin, Talianakis, Schattmann, Bartholomew & Pfaus, 1998). Similarly, males trained to associate a jacket on the female with sexual receptivity and copulation develop a preference toward the jacketed female over an unjacketed one (Quintana, Desbiens, Marceau, Kalantari, Bowden, Bachoura & Pfaus, submitted). As with CPP, systemic administration of NAL disrupts the acquisition of CEP for both odor (Ismail *et al*., 2008) and the jacket (Quintana *et al*., submitted) relative to males injected with SAL. However, instead of no preference, prior experience with NAL during the training trials shifted the preference toward the unscented or unjacketed female on the final open-field test, when all males were injected with SAL. In female rats, NAL disrupts the development of a conditioned partner preference for males bearing an odor or for a different strain of male (pigmented vs. non-pigmented) associated with paced copulation (Coria-Ávila *et al.,* 2008), relative to SAL-treated females, but did not shift the preference to the other male.

Among the different areas of the brain were 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 (*e.g*., van Ree *et al*., 2000; Fields & Margolis, 2015). Both have been regarded as critical in the control and execution of the male sexual behavior and sexually-motivated behaviors in both males and females (*e.g.*, Hull, 2006; Pfaus *et al*., 2009), and particularly in the development of CEP for an olfactory cue in the male rat (see Pfaus *et al*., 2012).

The activity and manipulation of opioid receptors has revealed a differential role of opioids in sexual behavior, depending of the brain area in which they are located. For instance, infusions of the selective μ-opioid receptor agonist 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 (Matuszewich & Dornan, 1992), whereas infusions of morphine or dynorphin into the VTA increased dopamine transmission into the NAc, which ultimately facilitated male sexual behavior (Mitchell & Stewart, 1990). Copulation to ejaculation induces μ-receptor internalization (a marker of ligand-induced receptor activity) in the mPOA (Coolen *et al.,* 2004), as well as in the VTA (Balfour *et al.,* 2006). Furthermore, Garduño-Gutiérrez, León-Olea & Rodríguez-Manzo (2013) found that μ- and δ-receptor internalization as a consequence of copulation to ejaculation in the VTA, where only the μ-receptor, but not δ-receptor activity, correlated with the amount of sexual activity. Compared to sexually-active males, sexually-inactive males have an augmentation of the endogenous opioid octapeptide Met-Arg^{6}-Gly^{7}-Leu^{8} in the hypothalamus (Rodríguez-Manzo *et al.,* 2002), as well as an augmentation of pro-enkephalin and pro-dynorphin precursor polypeptide mRNA expression in the paraventricular nucleus (Arletti *et al.,* 1997). On the other hand, NAL infused into the mPOA significantly reduced the time spent with an estrous female over the time spent with an anestrous female, while also reducing ejaculation latency and post-ejaculatory periods (Hughes, Everitt & Herbert, 1990); whereas NAL infusions to the VTA prevented the increase of anticipatory level changes and reduced significantly the amount of ejaculations over the course of training (van Furth & van Ree, 1996). Infusions of the quaternary opioid antagonist methylnaloxonium (which does not rapidly penetrate the blood-brain barrier) to the mPOA abolished ejaculation-induced CPP, whereas infusions to the nucleus accumbens (NAc), an important terminal region of the mesolimbic dopamine system that originates in the VTA, was without effect (Ågmo & Gómez, 1993).

Taken together, these findings suggest a differential role of opioids on different aspects of male sexual responding depending where in the brain they act. Opioids in the mPOA appear to modulate the reward value of sexually-related cues, whereas in the VTA, opioids appear to regulate

motivational state or the incentive properties of cues associated with reward. Thus, the current study aimed to evaluate if opioids exert a differential modulation of the CEP in the male rat when an opioid-non-specific antagonist was micro-infused into their mPOA and VTA in the context of the CEP. For that, male rats were micro-infused with NAL HCL into either the mPOA or the VTA, in a dose dependent fashion, or a saline control. The first experiment evaluated this hypothesis using 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. Furthermore, Fos-IR was used to evaluate how NAL would affect the neural patter of activation towards the trained cue in both brain areas.

Methods

Subjects, steroids, and conditioned cues

112 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 hr reversed light/dark cycle in a colony room at 21°C, with ad lib tap water and Purina® rat chow available in each cage. 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. Males were housed in groups of four in Plexiglas gang cages with wood-chip bedding and *ad lib* access to water and food until cannulation, after which they were housed individually in a single cage. Female rats were housed in pairs in Plexiglas shoebox cages with wood-chip bedding. Sexual receptivity was induced by subcutaneous injections of 10 µg estradiol benzoate 48 hours prior each training session, and 500 µg of progesterone four hours prior to each training session. 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.

Groups

Male rats were divided into two main groups: odor cue group and jacket cue group. The former group was assigned 80 males (40 to wear an implant into the mPOA and 40 into the VTA) to be trained with scented females; and 40 males (20 to wear an implant into the mPOA and 20 into the VTA) to be trained with jacketed females. Males in the odor cue group were equally divided into four groups (*i.e*., SAL, 200ng, 500ng, 1000ng; 10 each) for each brain area, while for the jacket cue group males were equally halved into two groups (*i.e*., SAL, 1000ng; 10 each) for each brain area.

Surgeries

Ovariectomy

Females were ovariectomized via bilateral lumbar incisions under general anesthesia induced with ketamine (50 mg/ml)/xylazine (4 mg/ml) mixed at a ratio of 4:3 respectively, approximately 2 weeks before the beginning of the experiment to allow at least a week of recovery. This procedure allowed for hormone levels to be controlled under hormonal replacement throughout the training.

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 minutes. 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 and secured in a stereotax apparatus (Kopf instruments). Under aseptic conditions, stainless steel guide cannulae (22G, Plastic One) were implanted bilaterally 1mm above the regions of interest (mPOA, AP: -.5 from bregma; MD: ±.5;DV, -8 mm; VTA, AP: -6.04 from bregma; MD: ±2 at 10°; DV, -8 mm; Paxinos & Watson, 1998). Figure 1. depicts the implant metrics and coordinates per brain area. A stainless steel dummy of the same length was placed in each cannula to ensure clearance.

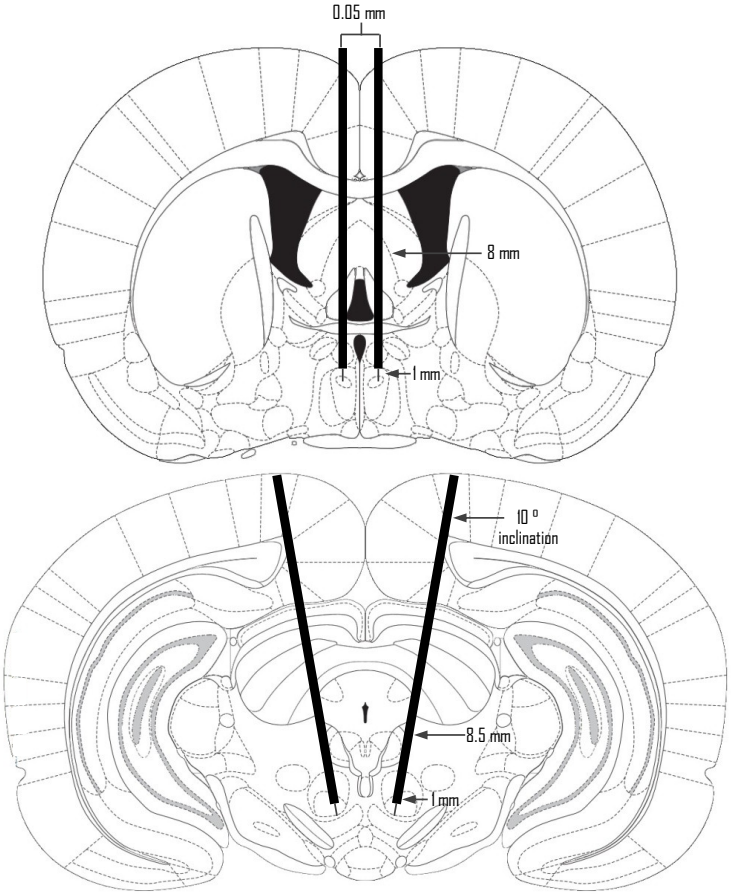

Figure 1. mPOA and VTA implant coordinates and cannulae details

Behavioral training

After recovery, males were given multi-ejaculatory trials for 30 min with sexually receptive females in unilevel one-hole pacing chambers. Males trained with scented females underwent 10 training trials, whereas males trained with jacketed females underwent 14 trials. All conditioning

sessions were conducted in Plexiglas chambers (38 x 60 x 38 cm) with bedded floors and bisected by a transparent Plexiglas divider with one-hole pacing chambers, big enough for the females to be able to cross through, yet 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 in every trial, females were randomly assigned to males.

Infusions of NAL (200, 500, or 1000µg, infused in 1 µl/side over the course of 1 min) or equivolume 0.9% SAL were made to each male in each group prior to conditioning trials for the males trained with the odor cue, whereas males trained with the jacket cue were infused either with 1000µg NAL or equivolume SAL. All males were infused with SAL prior to a final choice test in which males were placed into an open field (123 x 123 x 46 cm) filled with clean bedding to copulate freely for 30 min with two sexually receptive females, once 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 (Cabilio, 1996). 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 inmunohistochemistry

4 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 extracted and then frozen at -80°C until sliced. Slices were coronally on a sliding microtome at 30 µm, and cannula placements were confirmed and marked in

an atlas by a blind, 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. Cannula placements are shown in Figure 2.

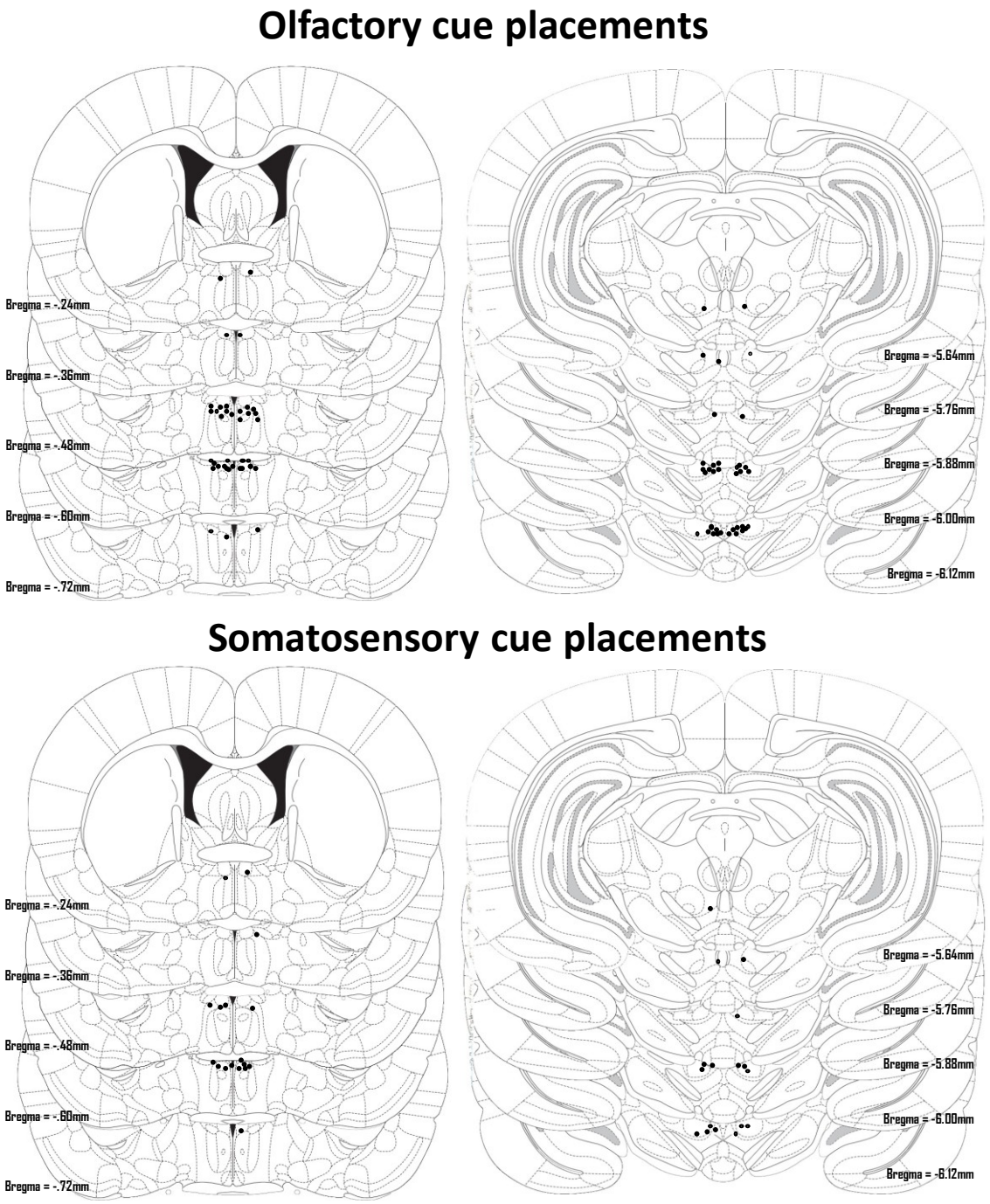

Figure 2. mPOA and VTA placement results

Fos immunocytochemistry was performed as reported previously (Kippin, Cain & Pfaus, 2003). Coronal brain sections were incubated sequentially with 30% w/w hydrogen peroxide (H_2O_2) in Tris-buffered saline (TBS) for 30′ at room temperature, 3% normal goat serum (NGS) in .05% Triton TBS for 90 min at 4 °C, polyclonal rabbit anti-c-Fos (Cedarlane labs, CAN; diluted 1:500) in .05% Triton TBS with 3% NGS for 72 hours at 4 °C, biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; 1:200) in .05% Triton TBS with 3% NGS for one hours at 4 °C, and avidin–biotinylate–peroxidase complex (Vectastain *Elite*® ABC Kit, Vector Laboratories; diluted 1:55) for two hours 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 (.1 ml of DAB/Tris buffer, pH 7.8) for 10 min, followed by DAB/3% H_2O_2 in 50-mM Tris for 10 min, with 8% nickel chloride (400 µl per 100 ml of DAB/Tris buffer H_2O_2) added to turn the reaction product blue-black. Sections were mounted on gel-coated slides and allowed to dry, then dehydrated in ethanol, cleared in Hemo-D, coverslipped, and examined under a microscope (Leitz). Brain sections were examined at 40x, and the number of Fos-positive cells was counted bilaterally from each region from five different sections per rat using a computerized image-analysis system (ImageJ, NIH, Bethesda, MD).

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. The count of the Fos-positive cells within the brain area was performed at coordinates previously mentioned, whereas when there was an implant in the brain area, the Fos counting was performed as close as possible to the coordinates given. Other brain areas known to be involved in sexual behaviors, and particular partner preference, were not considered in this study.

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, 200ng, 500ng, and 1000ng), separately. Analysed behaviors included mounts, intromissions, mean of ejaculation, and latency of first ejaculation. In each analysis, we evaluated the main effect of type of female (*i.e.,* ScF vs. UnScF or Jacket ON vs. Jacket OFF) and the interaction between the type of female by group (*i.e.,* SAL, 200ng, 500ng, and 1000ng). Given statistically significant differences, Post-hoc multiple compassions were conducted for each single variable to compare ScF vs. UnScF or Jacket ON vs. Jacket OFF using Tukey HSD's correction. Furthermore, partial eta square (η_p^2) was calculated as effect size for each comparison. Additionally, a 1x2 chi square (χ^2) analysis was conducted for the percentage of first ejaculation choice for each individual group, and a 2x2 χ^2 analysis to contrast the ejaculatory preference with the control group. Furthermore, Cramer's V and Phi (φ) effect sizes were conducted as effect size for the 1x2 and 2x2 χ^2 analyses, respectively.

To evaluate the differences among the experimental groups in Fos-IR, a series of one-way ANOVA with post-hoc Tukey HSD's correction were conducted for the animals trained with the odor cue, for each brain area of interest (mPOA or VTA), among the different groups (SAL, 200ng, 500ng, and 1000ng), separately. As for the animals trained with the jacket cue, independent t-tests were conducted to compare the two groups (SAL and 1000ng) for each brain area of interest (mPOA or VTA) separately. Partial eta square (η_p^2) and Cohen's d were calculated as a measure of effect size.

Results

Olfactory 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 all groups using the odor as neutral cue during the open field test. As shown in the top panel, males infused into the mPOA did not display a tendency to mount more either of the females. The reliability of these observations was corroborated by a 4(Group: SAL, 200ng, 500ng, 1000ng)x 2(Female: ScF vs. UnScF) repeated measures ANOVA. No main effect of Female, $F(1,37) = 1.78$, $p = .190$, $\eta_p^2 = .046$; and no interaction between Group x Female were found, $F(3,37) = 1.82$, $p = .16$, $\eta_p^2 = .129$.

As for intromissions, males in the NAL group had a preference to intromit more the UnScF, especially in the 500ng and 1000ng groups. The reliability of these observations was corroborated by 4(Group: SAL, 200ng, 500ng, 1000ng)x 2(Female: ScF vs. UnScF) repeated measures ANOVA. A statistically marginal effect of Female was found, $F(1,37) = 3.246$, $p = .08$, $\eta_p^2 = .081$; yet no interaction between Group x Female, $F(3,37) = 1.453$, $p = .243$, $\eta_p^2 = .105$. Post hoc analysis with Tukey HSD revealed that males displayed a statistically marginally higher mean of intromissions for the UnScF ($M = 16.823$, $SD = 6.419$) than for the ScF ($M = 14.02$, $SD = 6.523$, $p = .08$, $\eta_p^2 = .081$).

As for the mean latency of the first ejaculation, no particular patter to ejaculate faster with either of the female was observed. The reliability of these observations was corroborated by 4(Group: SAL, 200ng, 500ng, 1000ng)x 2(Female: ScF vs. UnScF) repeated measures ANOVA. No main effect of female was found, $F(1,37) = .135$, $p = .716$, $\eta_p^2 = .004$; nor an interaction between Group x Female, $F(3,37) = .517$, $p = .637$, $\eta_p^2 = .04$.

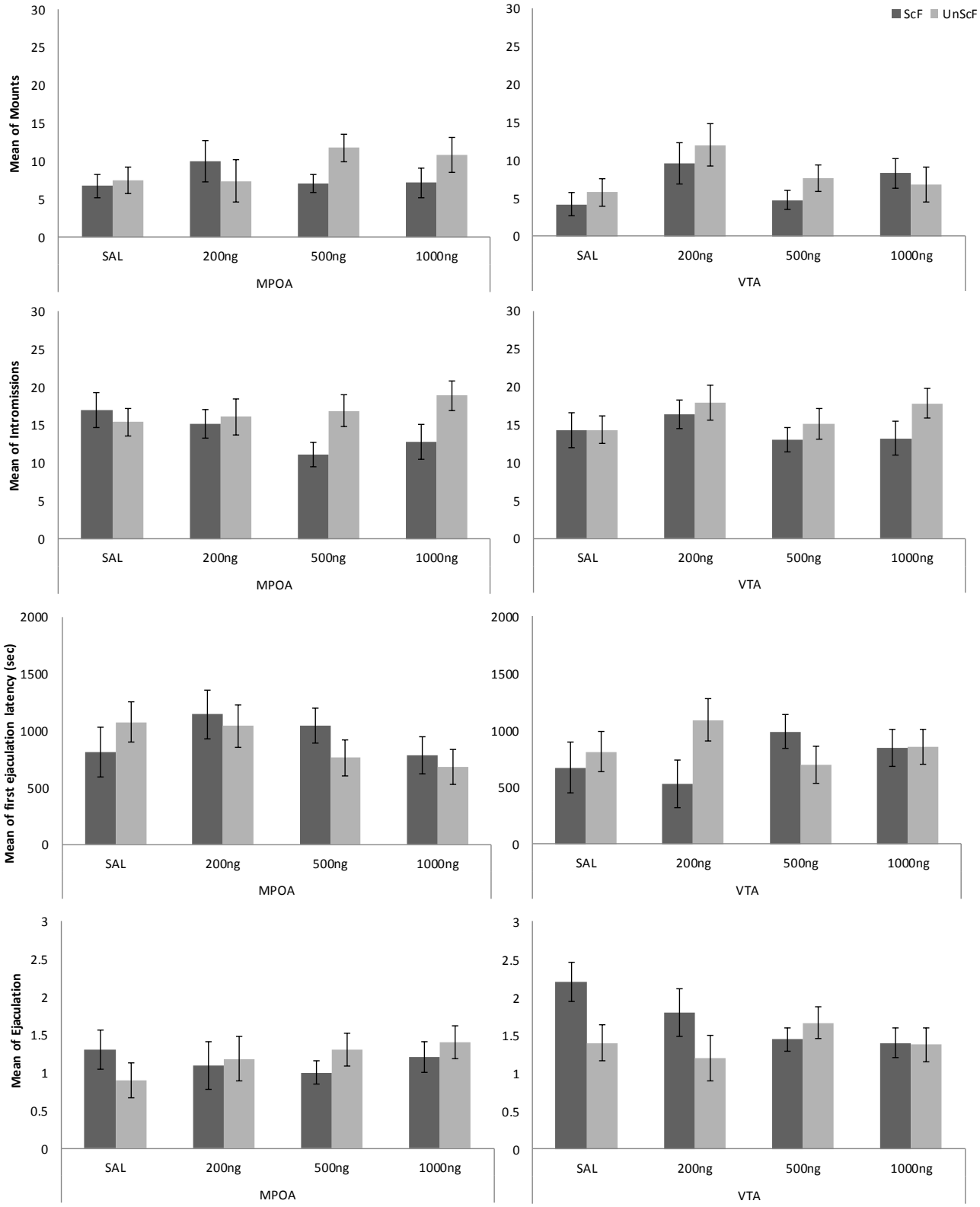

Figure 3. Mean of copulatory behaviors (±SEM) per brain area (columns) and experimental manipulation during the open field test for the odor cue.

As for the mean of ejaculations, males in the SAL had a slight preference to ejaculate more with the ScF, whereas males in the NAL groups had a slight preference to ejaculate more with the UnScF. The reliability of these observations was corroborated by 4(Group: SAL, 200ng, 500ng, 1000ng)x 2(Female: ScF vs. UnScF) repeated measures ANOVA. No statistically significant main effect of Female, $F(1,37) = .046$, $p = .831$, $\eta_p^2 = .001$; nor interaction between Group x Female was found, $F(3,37) = .467$, $p = .701$, $\eta_p^2 = .037$.

Figure 4. 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. Mainly, males in the SAL group chose to ejaculate first more with the ScF, whereas males in the NAL groups tended to ejaculate first more with the UnScF, although none of these differences appear to be of a strong magnitude. The following χ^2 analyses compared the two females during the open field test. Males in the SAL group did not significantly prefer to ejaculate first with either of the females, $\chi^2(1) = 1.6$, $p = .206$, $V = .4$. Nor did the 200ng group, $\chi^2(1) = .818$, $p = .366$, $V = .273$, the 500ng group, $\chi^2(1) = .4$, $p = .527$, $V = .2$, or the 1000ng group, $\chi^2(1) = 1.6$, $p = .206$, $V = .4$. However, a 2x2 between groups χ^2 revealed a statistically marginal trend for the SAL and the 1000ng groups to prefer different females to ejaculate first with, $\chi^2(1) = 3.2$, $p = .074$, $\varphi = .3$.

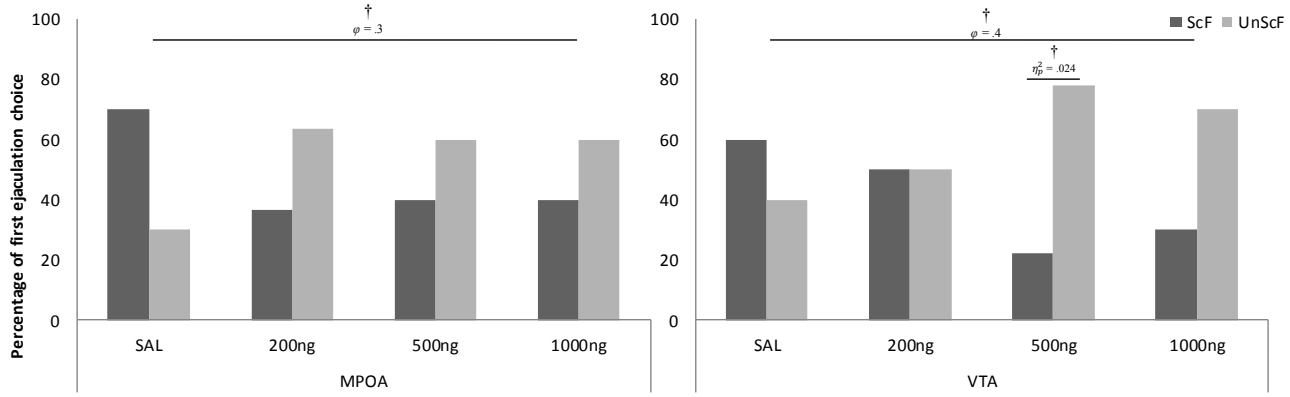

Figure 4. Percentage of 1st ejaculation choice per brain area (columns) and experimental manipulation during the open field test for the odor cue. † = $p < .01$; η_p^2 = partial eta square; φ = phi.

Fos-IR analyses

Figure 5. shows an example of the group results for the Fos-IR results regarding the exposition to the odor and jacket cues before perfusion for each infused brain area (NAL into mPOA, and VTA), group (SAL, 200ng, 500ng, and 1000ng), and brain area analyzed (mPOA and VTA). As depicted in Figure 5, males infused with NAL into the mPOA showed in their mPOA a lower Fos-IR mean count than the SAL control group, regardless of the concentration. The reliability of these observations was corroborated by an ANOVA analysis, where a marginal main effect of Group was found, $F(3,19) = 2.516$, $p = .095$, $\eta_p^2 = .321$. As corroborated with Tukey HSD post-hoc analysis, this effect was mainly driven by the statistically marginally higher mean of Fos-IR of the SAL group ($M = 152.53$, $SD = 82.95$) than the 1000ng group ($M = 70.73$, $SD = 7.68$, $p = .096$, $d = 1.389$).

As depicted in Figure 5, all males infused with NAL into the mPOA showed in their VTA a lower Fos-IR mean count than the SAL control group, regardless of the concentration. The reliability of these observations was corroborated by an ANOVA analysis, where a statistically significant effect of Group was found, $F(3,19) = 4.615$, $p = .016$, $\eta_p^2 = .464$. Post-hoc analysis with Tukey HSD correction showed that the SAL group had in their VTA a statistically significantly higher Fos-IR count ($M = 55.47$, $SD = 38.69$) than the 200ng group ($M = 16.87$, $SD = 7.96$, $p = .042$, $d = 1.382$); statistically marginally higher than the 500ng group ($M = 21.47$, $SD = 10.27$, $p = .081$, $d = 1.201$); and statistically significantly higher than the 1000ng group ($M = 11.53$, $SD = 6.37$, $p = .019$, $d = 1.585$).

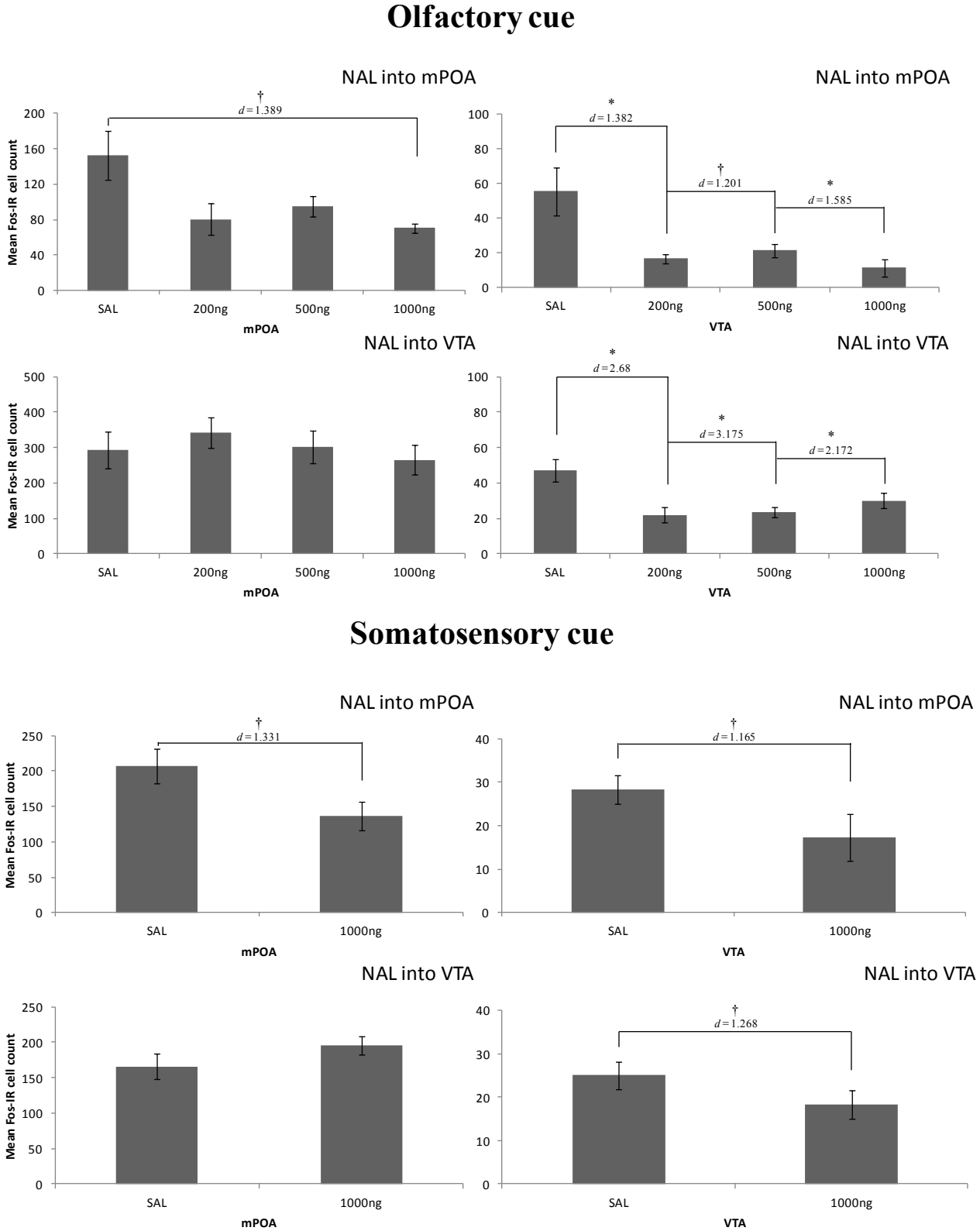

Figure 5. Fos-IR positive cells summary by brain area, group, and experimental cue. *d* = Cohen's *d* effect size; † = $p < .01$; * = $p < .05$.

Infusions of NAL or SAL to VTA

Behavioral analyses

Two males were removed from the analysis due to bad placement of the implant, one in the 200ng and one in the 500ng.

As shown also in Figure 3, males infused into the VTA did not display a particular pattern to mount more with either of the females. The reliability of these observations was corroborated by 4(Group: SAL, 200ng, 500ng, 1000ng)x 2(Female: ScF vs. UnScF) repeated measures ANOVA. No main effect of Female was found, $F(1,35) = 1.657$, $p = .206$, $\eta_p^2 = .045$; and no interaction between Group x Female, $F(3,35) = .892$, $p = .455$, $\eta_p^2 = .071$.

As for intromissions, males in the NAL group had a preference to intromit more the UnScF. The reliability of these observations was corroborated by 4(Group: SAL, 200ng, 500ng, 1000ng)x 2(Female: ScF vs. UnScF) repeated measures ANOVA. A statistically marginal effect of Female was found, $F(1,35) = 3.467$, $p = .071$, $\eta_p^2 = .09$; yet no interaction between Group x Female, $F(3,35) = .766$, $p = .515$, $\eta_p^2 = .062$. Post hoc analysis with Tukey HSD correction revealed that males displayed a statistically marginally higher mean of intromissions for the UnScF ($M = 16.278$, $SD = 6.027$) than for the ScF ($M = 14.225$, $SD = 5.648$, $p = .071$, $\eta_p^2 = .09$).

As for the mean latency of the first ejaculation, no particular pattern to ejaculate faster with either of the female was seen, except for the 200ng group who appeared to ejaculate first faster with the ScF. The reliability of these observations was corroborated by 4(Group: SAL, 200ng, 500ng, 1000ng)x 2(Female: ScF vs. UnScF) repeated measures ANOVA. No main effect of Female was found, $F(1,35) = .355$, $p = .555$, $\eta_p^2 = .01$; nor an interaction between Group x Female, $F(3,35) = 1.016$, $p = .397$, $\eta_p^2 = .08$.

As for the mean of ejaculations, males in the SAL tended to ejaculate more with the ScF,

whereas males in the NAL groups did not show a preference for either of the females. The reliability of these observations was corroborated by 4(Group: SAL, 200ng, 500ng, 1000ng)x 2(Female: ScF vs. UnScF) repeated measures ANOVA. No statistically significant main effect of Female, $F(1,35) = 1.001$, $p = .323$, $\eta_p^2 = .028$; nor interaction between Group x Female was found, $F(3,35) = .523$, $p = .669$, $\eta_p^2 = .043$.

Finally, as shown Figure 4, males in the SAL group chose to ejaculate first more with the ScF, whereas males in the NAL groups tended to ejaculate first more with the UnScF, except the 200ng group where no differences were found. Thus, males in the SAL group did not significantly prefer to ejaculate first with either of the females, $\chi^2(1) = .4$, $p = .527$, $V = .2$, nor did the 200ng group, $\chi^2(1) = 0$, $p > .99$, $V = 0$. Conversely, the 500ng group displayed a statistically marginally preference to choose to ejaculate first more with the UnScF, $\chi^2(1) = 2.778$, $p = .096$, $V = .554$, yet not did the 1000ng group, $\chi^2(1) = 1.6$, $p = .206$, $V = .4$. However, a 2x2 χ^2 along with the phi effect size revealed a trend for the SAL and the 1000ng groups to prefer different females to ejaculate first with, $\chi^2(1) = 1.818$, $p = .178$, $\varphi = .201$.

Fos-IR analyses

As depicted in Figure 5, males infused with NAL into the VTA showed in their mPOA a similar Fos-IR mean count than the SAL control group, regardless of the concentration. The reliability of these observations was corroborated by an ANOVA analysis, where no statistically significantly differences were found, $F(3,19) = .079$, $p = .971$, $\eta_p^2 = .015$.

As depicted in Figure 5, males infused with NAL into the VTA showed in their VTA a lower Fos-IR mean count than the SAL control group, regardless of the concentration. The reliability of these observations was corroborated by an ANOVA analysis, where a statistically significant main effect of Group was found, $F(3,18) = 11.653$, $p < .005$, $\eta_p^2 = .015$. Post-hoc analysis with Tukey

HSD correction showed that the SAL group had in their VTA a statistically significantly higher Fos-IR count (M = 47.13, SD = 10.85) than the 200ng group (M = 22.07, SD = 3.17, $p < .001$, d = 2.68); higher than the 500ng group (M = 23.33, SD = 3.17, $p < .001$, d = 3.175); and higher than the 1000ng group (M = 30.03, SD = 4.49, $p < .01$, d = 2.172).

Somatosensory conditioning

Infusions of NAL or SAL to the mPOA

Behavioral analyses

Three males were removed from the analysis due to bad placement of the implant, two in the SAL and one in the 1000ng.

Figure 6. shows the scores for the different copulatory behavior by female and by brain area for all groups during the open field test. With regard to mounts, no clear tendency to mount more with either of the females was found in either group. The reliability of these observations was assessed by 2(Group: SAL vs. NAL)x 2(Female: Jacket ON vs. Jacket OFF) repeated measures ANOVA. No main effect of Female, $F(1,15) = 1.713$, $p = .210$, $\eta_p^2 = .103$; and no interaction between Group x Female were found, $F(1,15) = 2.519$, $p = .133$, $\eta_p^2 = .144$.

As for intromissions, no clear tendency to intromit more with either of the females was found in either group. The reliability of these observations was corroborated by 2(Group: SAL vs. NAL)x 2(Female: Jacket ON vs. Jacket OFF) repeated measures ANOVA. No main effect of Female, $F(1,15) = .213$, $p = .651$, $\eta_p^2 = .014$; and no interaction between Group x Female were found, $F(1,15) = 2.427$, $p = .14$, $\eta_p^2 = .139$.

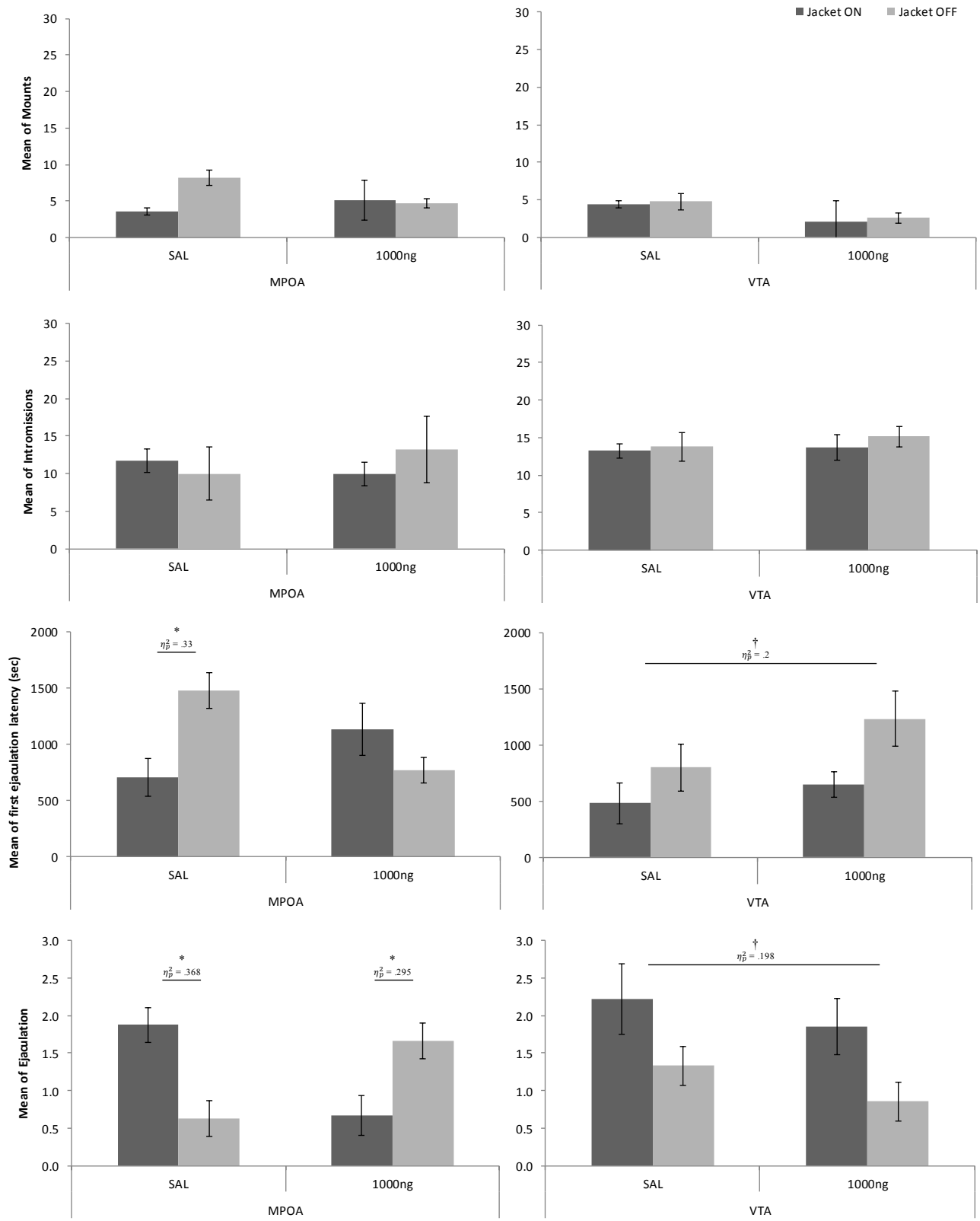

Figure 6. Mean of copulatory behaviors (±SEM) per brain area (columns) and experimental manipulation during the open field test for the jacket cue. † = $p < .01$; * = $p < .05$; η_p^2 = partial eta square.

As for the mean of first ejaculation latency, males in the SAL group ejaculated first faster with the unjacketed females, whereas the opposite was true for males in the NAL group. The reliability of these observations was corroborated by 2(Group: SAL vs. NAL)x 2(Female: Jacket ON vs. Jacket OFF) repeated measures ANOVA. No main effect of Female was found, $F(1,15) = 1.094$, $p = .312$, $\eta_p^2 = .068$; yet a statistically significant interaction between Group x Female was found, $F(1,15) = 8.478$, $p = .011$, $\eta_p^2 = .316$. Post hoc analysis with Tukey HSD correction 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 ($M = 1129.11$, $SD = 67.017$, $p = .016$, $\eta_p^2 = .33$).

As for the mean of ejaculations, males in the SAL tended to ejaculate more with the jacketed female, whereas males in the NAL groups tended to ejaculate more with the unjacketed female. The reliability of these observations was corroborated by 2(Group: SAL vs. NAL)x 2(Female: Jacket ON vs. Jacket OFF) repeated measures ANOVA. No statistically significant main effect of Female was found, $F(1,15) = .185$, $p = .673$, $\eta_p^2 = .012$; yet a statistically significant interaction between Group x Female was found, $F(1,15) = 14.959$, $p = .002$, $\eta_p^2 = .499$. Post hoc analysis with Tukey HSD correction revealed that males in the SAL displayed a statistically significantly higher mean of ejaculation towards the jacketed female ($M = 1.88$, $SD = .641$) than for the unjacketed one ($M = .67$, $SD = .707$, $p = .01$, $\eta_p^2 = .368$), whereas males in the NAL group displayed a statistically significantly higher mean of ejaculation towards the unjacketed female ($M = 1.67$, $SD = .707$) than for the jacketed one ($M = .63$, $SD = .744$, $p = .024$, $\eta_p^2 = .295$).

Finally, as shown Figure 7, the χ^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 preference pattern for animals in the NAL groups vary depending the brain area micro-infused.

Indeed, males in the SAL group significantly prefer to ejaculate first more with the jacketed female, $\chi^2(1) = 4.5$, $p = .034$, $V = .75$, yet this trend did not reach statistical significant in the NAL group, $\chi^2(1) = 1$, $p = .317$, $V = .333$. However, a 2x2 χ^2 revealed a statistically significantly different patter of preference to ejaculate first for these groups, $\chi^2(1) = 5.13$, $p = .024$, $\varphi = .43$.

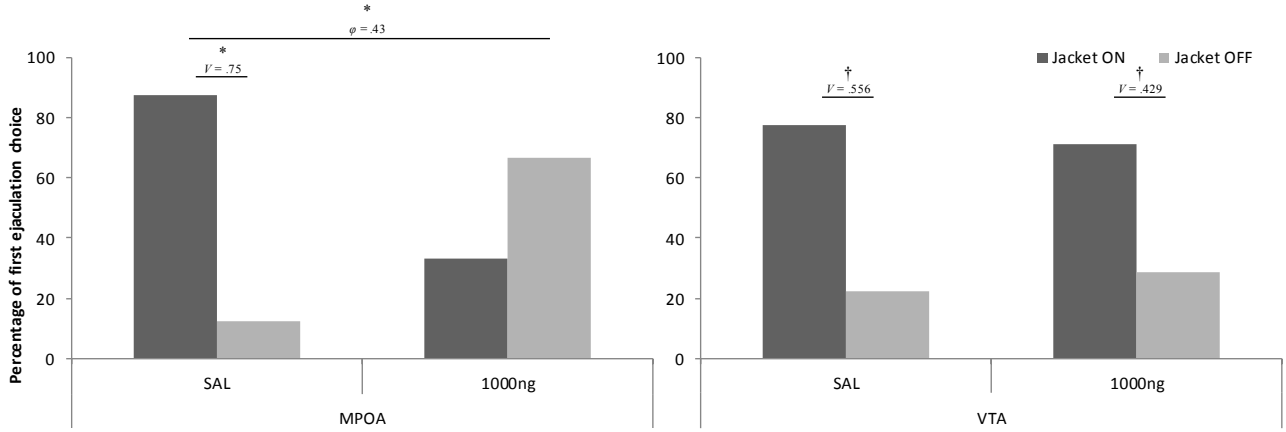

Figure 7. Percentage of 1st ejaculation choice per brain area (columns) and experimental manipulation during the open field test for the jacket cue. † = $p < .01$; * = $p < .05$; V = Cramer's V; φ = phi.

Fos-IR analyses

As depicted in Figure 5, males infused with NAL into the mPOA showed in their mPOA a lower Fos-IR mean count than the SAL control group. An independent samples t test analysis revealed that the SAL group ($M = 206.58$, $SD = 70.21$) had a statistically marginally higher Fos-IR count than the NAL group ($M = 136.53$, $SD = 17.53$, $t(9) = 2.157$, $p = .059$, $d = 1.313$).

As depicted in Figure 5, males infused with NAL into the mPOA showed in their VTA a lower Fos-IR mean count than the SAL control group. An independent samples t test analysis revealed that the SAL group ($M = 28.33$, $SD = 7.5$) had a statistically marginally higher Fos-IR count than the NAL group ($M = 17.3$, $SD = 11.46$, $t(9) = 1.925$, $p = .086$ $d = 1.165$).

Infusions of NAL or SAL to the VTA

Behavioral analyses

Four males were removed from the analysis due to bad placement of the implant, two in the SAL and three in the 1000ng. As shown in Figure 6, neither group infused into the VTA showed a preference to mounts more either of the females. The reliability of these observations was corroborated by 2(Group: SAL vs. NAL)x 2(Female: Jacket ON vs. Jacket OFF) repeated measures ANOVA. No main effect of Female, $F(1,14) = .22$, $p = .647$, $\eta_p^2 = .015$; and no interaction between Group x Female were found, $F(1,14) = .003$, $p = .954$, $\eta_p^2 = 0$.

As for intromissions, the same pattern of mounts was found where no tendency to intromit more with either of the females was found in either group. The reliability of these observations was corroborated by 2(Group: SAL vs. NAL)x 2(Female: Jacket ON vs. Jacket OFF) repeated measures ANOVA. No main effect of Female, $F(1,14) = .624$, $p = .443$, $\eta_p^2 = .043$; and no interaction between Group x Female were found, $F(1,14) = .121$, $p = .733$, $\eta_p^2 = .009$.

As for the mean of first ejaculation latency, males in both groups ejaculated first faster with the jacketed females. The reliability of these observations was corroborated by a 2(Group: SAL vs. NAL)x 2(Female: Jacket ON vs. Jacket OFF) repeated measures ANOVA. A significantly marginal effect of Female was found, $F(1,14) = 3.501$, $p = .082$, $\eta_p^2 = .2$; yet no a significant interaction between Group x Female was found, $F(1,14) = .298$, $p = .594$, $\eta_p^2 = .021$. Post hoc analysis with Tukey HSD correction corroborated the marginal trend of both groups to ejaculate first faster with the jacketed female ($M = 564.444$, $SD = 426.277$) over the unjacketed one ($M = 1015.833$, $SD = 638.329$, $p = .082$; $\eta_p^2 = .2$).

As for the mean of ejaculations, a similar pattern as in the mean of first ejaculation latency was found, where both group appeared to ejaculate more with jacketed female. The reliability of

these observations was corroborated by 2(Group: SAL vs. NAL)x 2(Female: Jacket ON vs. Jacket OFF) repeated measures ANOVA. A statistically marginal main effect of Female was found, $F(1,14) = 3.457$, $p = .084$, $\eta_p^2 = .198$; yet no interaction between Group x Female was found, $F(1,14) = .012$, $p = .914$, $\eta_p^2 = .001$. Post-hoc analysis with Tukey HSD correction revealed that males in both groups displayed a statistically significantly higher mean of ejaculation towards the jacketed female ($M = 2.04$, $SD = 1.042$) than for the unjacketed one ($M = 1.095$, $SD = .904$, $p = .084$, $\eta_p^2 = .198$).

Finally, as shown Figure 7, the χ^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 preference pattern for animals in the NAL groups vary depending the brain area micro-infused. Indeed, males in the SAL group statistically marginally prefer to ejaculate first more with the jacketed female, $\chi^2(1) = 2.778$, $p = .096$, $V = .556$, yet this trend did not reach statistical significant in the NAL group, $\chi^2(1) = 1.286$, $p = .257$, $V = .429$. A 2x2 χ^2 analysis revealed that both groups have a similar pattern of preference to ejaculate first more with the jacketed female, $\chi^2(1) = .085$, $p = .771$, $\varphi = .073$.

Fos-IR analyses

As depicted in Figure 5, males infused with NAL into the VTA showed no difference in their mPOA in their Fos-IR mean count compared to the SAL control group. An independent samples t test analysis corroborated that there were no statistically significant differences in the Fos-IR count between the SAL group ($M = 166.07$, $SD = 55.91$) and the NAL group ($M = 195.5$, $SD = 35.9$, $t(8) = -.991$, $p = .351$, $d = .626$).

As also shown in Figure 5, males infused with NAL into the VTA showed in their VTA a lower Fos-IR mean count than the SAL control group. An independent samples t test analysis

revealed that the SAL group (M = 25, SD = 6.01) had a statistically marginally higher Fos-IR count than the NAL group (M = 18.27, SD = 4.5, $t(8) = 2.005$, p = .08, d = 1.268).

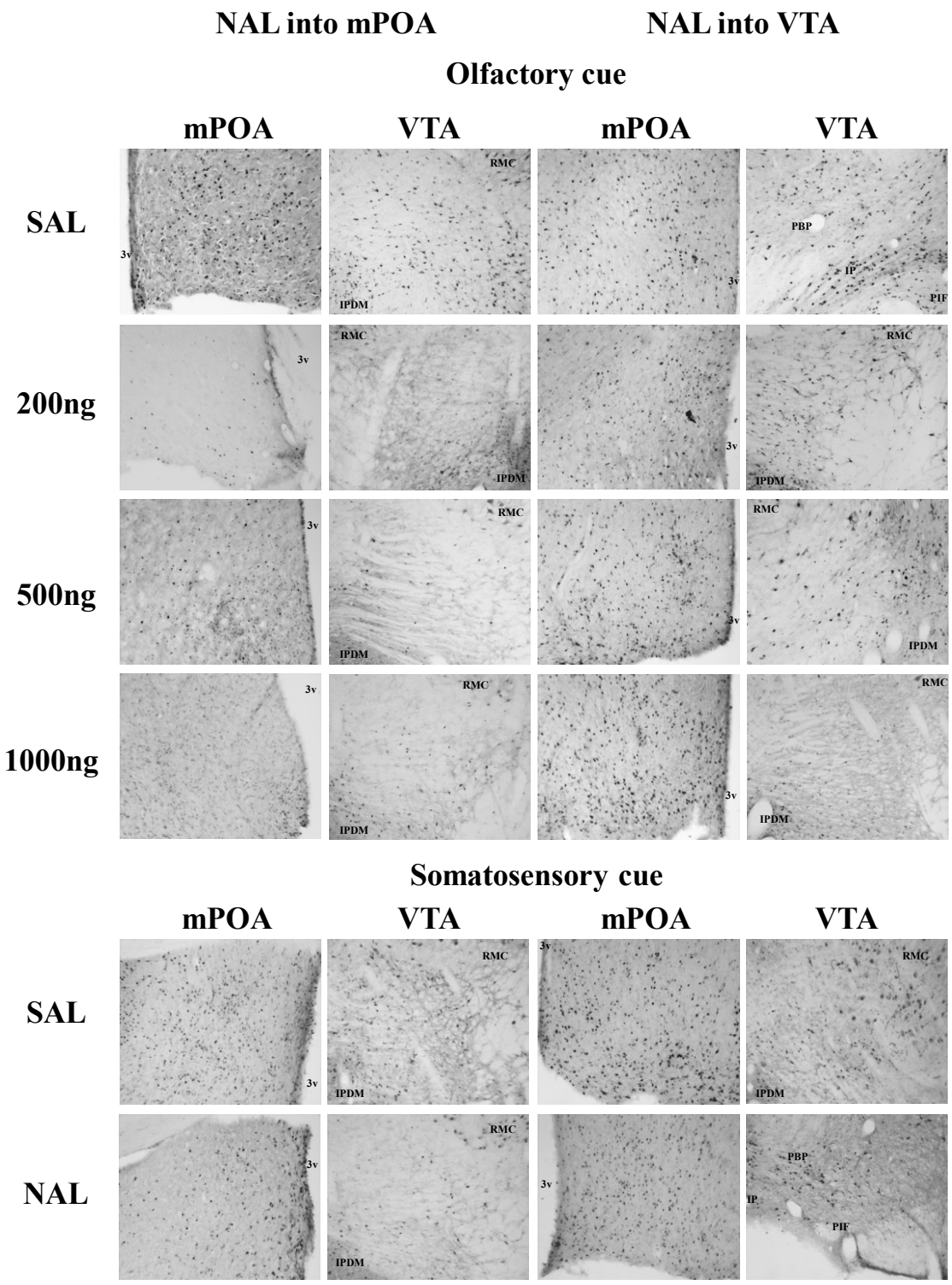

Figure 8. Fos-IR positive cell group example of each brain area by group for the odor and jacket cues.

Discussion

The present study evaluated the role of opioid transmission 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 immunohistochemical 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 has shown to increase opioid release in the midbrain, caudate, and hypothalamus in male rats (Szechtman, Simantov & Hershkowitz, 1981). Ågmo & Benefeld (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 & Benefeld, 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 would lead to a CEP (Kippin & Pfaus, 2001; Szechtman, Simantov & Hershkowitz, 1980; Tenk, Wilson, Zhang, Pitchers & Coolen, 2009). This trained preference has been also shown to shift towards the unfamiliar female in males who are injected systemically with NAL using an olfactory (Ismail *et al.,* 2009) and a somatosensory cue (Quintana *et al.,* submitted). Similar findings have been reported for the development of pair bonds in prairie voles through cohabitation. Systemic injections of a long-lasting non-selective opioid antagonist naltrexone to male prairie voles during

an 18 hr cohabitation period with a female resulted in a preference for the novel female relative to the familiar female they cohabitated with during a subsequent partner preference test (Burkett Spiegel, Inoue, Murphy & Young, 2011). This indicates that blockade of opioid transmission 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 (*e.g.*, Parker & Rennie, 1992).

μ-opioid receptors exist in high density in the mPOA and VTA (Simerly, McCall & Watson, 1988). The mPOA is regarded as one of the major integrative sites in vertebrates for sexual behavior and motivation (Hull & Rodríguez-Manzo, 2009). For example, copulation to ejaculation has shown to increase μ-receptor activation in the mPOA, and prevented by injection of NAL before mating (Coolen, Fitzgerald, Yu & Lehman, 2004). Infusion of morphine to the mPOA inhibits male rat sexual behavior (Band & Hull, 1990; Matuszewich, Ormsby, Moses, Lorrain & Hull, 1995). Furthermore, micro-infusions of NAL to the mPOA, but not the NAc, disrupt a sexually conditioned place preference in male rats (Ågmo & Gómez, 1993). Likewise, micro-infusions of β-endorphin into mPOA impaired sexual performance in male rats in a dose-dependent fashion, while a NAL treatment prevented this impairment (van Furth, van Emst & van Ree, 1995). Furthermore, although systemic and intraventricular injections of two enkaphalinase inhibitors, SCH 34826 and phosphor-leu-phe, facilitated male sexual behavior (*i.e.*, reduce latency to ejaculation, PEI), they did not lead to produce a CPP (Ågmo, Gómez & Irazabal, 1994). Taken together with the present findings, it would appear that opioids activation of mu receptors in the mPOA encodes sexual reward states.

The VTA is the origin of the mesocorticolimbic DA circuit which plays a major role in motivated behaviors (see Hull & Rodriguez-Manzo, 2009) through the control of the attention towards reward-related stimuli and their incentive salience (Berridge, 2007). For instance, conditioned placed preference was observed in rats micro-infused into the VTA with met-enkephalin analogue or an enkephalinase inhibitor, yet not with NAL or the drug vehicle (Glimcher,

Giovino & Margolin, 1984). Furthermore, Devine & Wise (1994) trained male rats to lever-press for VTA micro infusions of morphine, the selective μ-receptor agonist DAMGO, the selective δ-receptor agonist DPDPE, or a drug vehicle. All drugs were effective in establishing and maintaining the lever-press response relative to the vehicle indicating that both μ and δ receptor activation reinforces self-administration of compounds that bind to them. However, the effective dose of DAMGO was 100 times lower than DPDPE, suggesting a major role of μ-opioid receptors in the VTA mechanisms involved in reward. Although infusions of drugs that activate μ receptors, like morphine or ß-endorphin, to the mPOA produce a refractory-like state in male rats (van Furth, van Emst & van Ree, 1995), infusions of μ agonists to the VTA either facilitate sexual behavior in sexually naïve males (Mitchell & Stewart, 1990) or have no effect in sexually-experienced males (van Furth & van Ree, 1996). This suggests that the sensitization of DA systems that occurs as male rats acquire sexual experience (*e.g.*, Fiorino & Phillips, 1999a,b) may be mediated by opioid turnover in general, and binding to μ receptors in particular (Balfour *et al.,* 2004; Fields & Margolis, 2015), ultimately regulating which cue the animal pays attention to. Thus, taking into considerations the findings of the present experiment on the differential role of opioid receptors in the development of CEP, 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 the sensory modality of the neutral cue in which the CEP is based.

A differential modulation of Fos induction by the cues following NAL infusions to the mPOA versus the VTA, and in particular, the directionality of the Fos induction is in accordance with the previous literature. Connections from the mPOA to the VTA have been established in both male and female rats (Brackett, Iuvone & Edwards, 1986; Edwards & Einhorn, 1986; Graham, Payne, Germé & Pfaus, *in press*) and are responsible for drug reward-related locomotion in both sexes (Tobiansky *et al*., 2013; Will, Martz, & Dominguez, 2016). Indeed, it was suggested by Edwards and Einhorn

(1986) that outputs from the mPOA to the midbrain (including both dorsolateral tegmentum and VTA) modulated the sexual reward value of a sexually receptive female. The origin of these connections was localized mainly to 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, the ovarian hormone 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*). Similar mPOA-VTA connections have been linked to the regulation of other naturally rewarding behaviors like proactive maternal behaviors related to pup stimuli (Numan & Numan, 1997; Numan & Stolzenberg, 2009), as well as appetitive, but not consummatory, sexual behaviors in the male rat (Lyilikci, Balthazart & Ball, 2017). Considering that other studies have vastly shown the role of the mPOA in the execution of copulatory behaviors (Hull & 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 & Ball, 2007). Ultimately, these findings support the idea that the mPOA modulates the mesolimbic DA system in the VTA which can ultimately impacts the DA connections between the VTA-NAc, known to be responsible of the modulation of the incentive value of the reward-related cue (Fields & Margolis, 2015; Micevych & Meisel, 2017; Wise, 2009), like the behavioral and Fos-IR outcomes of the present study suggests.

Most of the mPOA-VTA connections in the hypothalamic regions appeared in medially located portion, and that the concentration of GABA and glutamatergic neurons varies among the sub regions with a predominance of glutamatergic marker VGLU2 (Kallo *et al.,* 2015). The differences between the localization and physiology of GABAergic afferences from hypothalamic regions towards the VTA between studies may be explained due to sexually-dimorphic differences in the anatomical (*e.g.,* Northcutt & Nguyen, 2014) and biochemical composition of these brain regions (*e.g.,* Lee & Ho, 2013), as well as differences in hormonal regulation of the behaviors analysed (Tobiansky *et al*., 2016).

A crucial difference between the olfactory and somatosensory cue was found in the behavioral analyses, yet not through the Fos-IR. On 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, 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, Ogawa, Zhu, Vamanrao & Uchida (2012) delineated a whole-brain map of the direct excitatory inputs to the midbrain DA neurons, VTA and the substantia nigra past compacta. Using immunoflourescence, Watabe-Uchida and colleagues 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 and related outputs) to render a similar behavioral output to the one found with the olfactory cue. Opioid receptors are also located in somatosensory cortex in different quantities depending on the subtype of receptor (Mansour *et al*., 1994; Mansour, *et al*., 1988). Particularly, the pattern of receptor binding in the neocortex of the rat brain demonstrated that both μ and δ receptor are more prevalent than κ receptors, while their precise distributions differ greatly among the different layers. μ-subtype receptor appeared most prominent in layers I and III/IV of the frontal, parietal, and temporal cortex, whereas δ-subtype receptor 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 is 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. Indeed, the disruption and shift of CEP towards the unjacketed female in male rats trained to prefer jacketed female when NAL was injected intra-peritoneally before each training trial suggests that opioid receptors in regions other than the mPOA and/or VTA are required (Quintana, Desbiens, Marceau, Kalantari, Bowden, Bachoura, & Pfaus, submitted).

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.

Chapter 6 - General Discussion

General discussion

The experiments presented in this thesis examined the role of opioids in sexual pleasure, particularly during early formative sexual experiences, on the ability of male rats to associate an olfactory or somatosensory cue with their ejaculation-induced sexual reward state. The results show that opioids are an important substrate of the sexual reward state, and that this state feeds forward to form a CEP, in which male rats ejaculate preferentially with females bearing the cue associated with sexual reward. This suggests that sexual reward (a presumably "proximate" cause of behavior) lies at the root of monogamous mate choice in the rat. These data also contribute to a growing body of evidence that the species'-specific sexual strategies of the male rat, often described as promiscuous, evidence a degree of plasticity that can move toward a monogamous mate choice when features of a familiar female (odor or jacket) are associated with sexual pleasure. This conditioned ejaculatory preference for familiar cues suggests that proximate pleasure-related states act as a Pavlovian US that can reinforce cue-related CSs to create partner and mate preferences. Such preferences bring proximate reward states into synch with reproductive choice, potentially enhancing reproductive success.

The second chapter explored how first exposure to either a biologically significant US, sexual reward with an unscented sex partner, or exposure to the neutral almond odor CS prior to their pairing retards or eliminates the development of a CEP. The chapter was divided into two main manipulations: US and CS pre-exposure.

Finding from the US pre-exposure experiment revealed also a differential pattern of disruption of the CEP due to the amount of trials of pre-exposure and the characteristics of the US in combination with the training conditions. More specifically, males pre-exposed five times to the US did not display a CEP for the familiar female, nor did males pre-exposed one time a UnScF and subsequently trained with ScF; whereas males pre-exposed one time to a ScF and subsequently

trained with UnScF displayed a CEP for the later. Furthermore, Fos-IR data revealed a differential pattern of activation in several brain areas related to the development of a conditioned partner preference based on an odor cue. Following exposure to the odor cue, on one hand, males in the 5t group pre-exposed to UnScF and later on trained with ScF had a lower Fos-IR activation in the mPOA than the 1t group pre-exposed and trained under the same conditions. Additionally, males in the 5t group had a lower Fos-IR count in the mPOA compared to the ScF control group, whereas males in the 1t group had a higher Fos-IR count in the CeA compared to the ScF control group. On the other hand, males in the 5t group pre-exposed to ScF and later on trained with UnScF did not significantly differ from the 1t group pre-exposed and trained in the same conditions in their Fos-IR activation in any of the brain areas of interest, expect for the VTA, where the former had a lower Fos-IR activation than the later. Furthermore, both of them had a lower Fos-IR mean count than the ScF control group in the NAc Core and Shell, and only did the 5t group have a lower Fos-IR activation in the VTA compared to the ScF control group.

Findings from the CS pre-exposure revealed a differential pattern of disruption of the CEP due to the amount of trials of pre-exposure and the type of female with which the animals were trained. More specifically, males pre-exposed five times and subsequently trained with scented females (ScF) did not display a CEP for the ScF, whereas males pre-exposed five times and subsequently trained with unscented females (UnScF) did display a CEP for the UnScF. Both groups of males pre-exposed one time developed a CEP for their familiar female. Furthermore, Fos-IR data corroborated a differential pattern of cell activation in the mPOA and VTA given the amount of pre-exposure trials and the training contingencies. Based mostly on the effect size differences, there was a complex patter of Fos-IR activation due to the amount of pre-exposure and the training conditions. The 5t groups both groups of males pre-exposed five times and the ones pre-exposed one time and trained with UnScF had a reduced Fos-IR in the VTA compared to the ScF control group, whereas

males pre-exposed one time and trained with ScF had a similar VTA Fos-IR pattern to the ScF control group. As for the Fos-IR in the mPOA, males pre-exposed five times and trained with ScF also had a lower activation compared to the ScF control group, whereas both groups trained with UnScF has a similar activation to the control group. However, only males pre-exposed one time and trained with ScF had a higher Fos-IR than the ScF control group.

The third chapter explored the flexibility of these associations in the CEP using a CS from a different sensory modality. Thus, one group of male rats was trained to copulate with females wearing a jacket, whereas another group copulated with unjacketed females. Results revealed that males showed a trend to prefer the familiar female. For instance, males from the group that was trained with females wearing jackets showed a trend to prefer the jacketed female over the unjacketed one. Consequently, the second experiment extended the results of the previous one using an explicitly-paired design, where male rats were trained in different trials with sexually receptive jacketed females and non-sexually receptive unjacketed females. A control group with the opposite pattern of association for the jacket was also included. The results showed that males displayed a CEP towards the familiar female. Altogether, these findings demonstrate the flexibility of the system to associate neutral cue of different sensory modalities with sexual pleasure.

The fourth chapter aimed to evaluate the role of opioids in the establishment of the association between the jacket and sexual pleasure found in the previous chapter. Two groups of males were trained using the same explicitly-paired design of experiment two of chapter three. One group of males was always injected intra-peritoneally with saline, whereas the other group was injected with NAL when females were sexually receptive and with saline when females were not-sexually receptive. The results showed that males in the control group displayed a CEP towards the jacketed female over the unjacketed one, whereas males injected with NAL displayed a CEP toward the unjacketed female. These findings replicated the previous findings of chapter three about the usage

of the jacket as neutral cue in which a CEP can be based on. Also, it replicated the findings of chapter two, and other previous findings, about the role of opioids in the development of neutral cues associated with sexual pleasure, in the form of the shift of the preference from the familiar female towards the non-familiar one.

Finally, the fifth chapter aimed to evaluate the specific role of opioid transmission in the development of a CEP based on both, the olfactory and the jacket cues, in the mPOA and VTA. To do so, the experiment used similar methodology as in chapter two and three. Two groups of males were micro-infused into either, the mPOA or VTA, with either saline or NAL while being trained to develop a CEP. These training conditions were assessed separately for the olfactory, as well as for the somatosensory cue. Furthermore, Fos-IR activation toward the neutral cue was also assessed after the preference test. Behavioral finding for the olfactory cue revealed that NAL micro-infused into the mPOA shifted the preference from the ScF towards the UnScF in a dose dependent fashion, whereas males NAL into the VTA only disrupted the CEP for the ScF found otherwise males infused with the saline. The Fos-IR data revealed that both brain areas may be processing the odor differently, where the mPOA may evaluate its reward value, while the VTA may be tagging it as biologically important; along with the directionality going from the mPOA to the VTA, but not the other way around. Prior experience with NAL infusion to the mPOA suppressed Fos-IR in both the mPOA and VTA, whereas when infused into the VTA, NAL only suppressed Fos in the VTA alone. Results for the somatosensory cue replicated the findings previously found for the olfactory cue where either saline or NAL were infused into both brain area using the jacket as a CS. Namely, males infused with saline into the mPOA developed a CEP for the jacketed female, whereas males infused with NAL into the mPOA displayed a CEP for the unjacketed female. Males infused with saline into the VTA developed a CEP for the jacketed female, whereas males infused with NAL into the VTA also displayed a CEP for the jacketed female. Furthermore, the Fos-IR in the infused brain

areas regarding the jacket exposure showed a similar pattern of activation than the olfactory cue did in both brain areas. Thus, generally speaking, it is believed that opioid receptors in the mPOA may convey the rewarding properties of the reward-related cue, whereas in the VTA opioids may convey its incentives properties.

Overall, the present thesis demonstrated that first (or early in general) experiences with reward, or the lack of thereof, play a role in how rewarding associations are established with neutral stimuli. Furthermore, these results were extended into a different CS from the ones previously used in the literature of CEP. It was also corroborated that opioid receptors play a key role in the establishment of these associations, modulating different aspects (incentive and valence) depending on which area of the brain they act.

Optimality and reward: setting the stage

Despite popular perceptions of rat promiscuity and its evolutionary history and value, this thesis along with an extensive literature (for a review see Pfaus *et al*., 2012) show that rats –and animals in general– are equipped with a neurochemical machinery of monogamy available to them, and the selected strategy depend on their –first– experiences with the sexual reward state and the environment. Perhaps the question to be asked is *why* such machinery would be residing in their brains if it was not also selected for over evolutionary time, and if it was, then what advantage in terms of response flexibility and the generation of partner/mate preferences might that give male rats (or indeed males of any other species)?

Bateson (1978) demonstrated experimentally that animals being raised without any con-specific still preferred to spend more time around and copulate with the ones that were phenotypically different from them. The degree of optimality in choosing a sexual partner varies due to biological predetermination that appear to determine the boundaries of inbreeding and

outbreeding, whereas experiences with reward may provide the fine-tuning assessment as to what predict pleasure, also depending the subject's internal state and availability. Yet, what may not seem rewarding or optimal in certain occasions or contexts may be the first choice in others. Michel Cabanac (1971) called this phenomenon *alliesthesia* to describe how the same stimulus can induce a pleasant or unpleasant sensation, depending on the internal state of the individual. The internal drive of the organism –fundamentally and circumstantially biased– is complex and relates to variables of what is called "*milieu interieur*", which describes what is important or vital for the animal at any given moment, like food would be for a hungry animal, yet no so in a satiety state. Thus, alliesthesia describes the behavior as a goal to reach what is pleasurable for the subject at a given moment (Cabanac, 1971), probably capable of overriding certain degrees of in- or out-breeding, fear, and even pain, explaining to a certain extend why vice entices. This dynamic process of circumstantial and experienced-biased reaction provides the bases at which the dual control model of sexual responding (Perelman, 2006) that describes sexual responding as net balance on a continuum between excitation and inhibition. Thus, at any given moment, our brains integrate the *milieu interieur* based on genetic makeup, hormonal profile, and previous experiences computing the right balance of excitation/inhibition that set the stage for arousal, motivation, and desire that build upon, and are triggered by, internal or external cues that signal pleasure. This ultimately drives sexual behavior toward the desired incentive(s) and reward(s).

Assuming this underlying rationale on how animals drive their behaviors towards external cues that predict sexual reward, and ultimately choosing one mate over the other, the findings of the present thesis provide further evidence that first experiences with sexual pleasure by the action of opioid reward are at the substrate of CEP. Thus, the orchestration of conditioned partner preference is a process where first experiences with reward and opioid receptors transmission in the mPOA and VTA, and their interplay, play a key role in CEP. These brain areas interact in a way that appears to

create a comprehensive picture of the US-CS relationship between the odor or jacket and their individual reward value. Although the condition which the role of first experiences and opioid reward were tested are highly contrived relative to the wild or more natural settings, the provided manipulations also represents a model of proximate conditioning with sexual reward translatable to human behavior and sexual experience.

A common reward system

There is no doubt that opioids play an important role in sexual reward. As previously stated, opioids modulation properties modulation vary depending where in the brain they act. In the present thesis, we focused on two main brain areas –mPOA and VTA– known to be involved in sexual behavior, reward, and motivation (see Pfaus, 2009), to evaluate the role of opioid receptors in the orchestration of sexual reward at the base of a CEP in the male rat.

The mPOA is a critical brain region that controls male sexual arousal and behavior, where every sensory modality sends indirect inputs (see Dominguez & Hull, 2005; Hull, 2009). Administration of opioid antagonist into the mPOA of male rats blocked CPP induced by sexual reward (Ågmo & Gomez, 1993), whereas Band & Hull (1990) found inhibition of sexual behavior in the male rat when a μ-opioid receptor agonist was infused into the mPOA. While opioids have an inhibitory effect on behavior, they are also critical for the establishment of the reward state (see Pfaus, 2009). However, although lesions of the mPOA impair sexual behaviors (*i.e.*, mounts, intromissions, and ejaculations) in the male rat, it did not diminish operant responses associated with a getting access to a female in a second-order scheduled of reinforcement (*i.e.*, pressing a lever, Everitt & Stacey, 1987), which indicate a dissociation, to some extent, between consummatory vs. instrumental aspects of sexual behaviors.

The VTA has been described as a main brain structure part of the mesolimbic dopaminergic pathway, thought to the control or mediate of appetitive behaviors and attention towards reward-related stimuli and their own incentive salience (Berridge, 2007). Infusion of opioid agonist morphine into the VTA resulted in a CPP (Olmstead & Franklin, 1997), while infusion of selective μ-opioid receptor antagonist in this same brain region blocked such effect (Zhang *et al.,* 2009). Moreover, mating and ejaculations have shown to induce μ-receptor internalization in both, the mPOA (Coolen *et al.,* 2004) and VTA (Balfour *et al.,* 2004). Generally speaking, although opioid agonists infused to the mPOA produce a sexual refractory state (*e.g*., Band & Hull, 1990), blocking opioid receptors has corroborated their role in the development of rewarding associations (*e.g*., Ismail, 2009), whereas infusions into the VTA sensitize mesolimbic DA into the NAc (Mitchell & Stewart, 1990), suggesting a facilitating role over the constant inhibition on DA transmission (Balfour *et al.,* 2004; Fields & Margolis, 2015), ultimately regulating which cue the animal pays attention to. Thus, and particularly in the context of sexual behavior, taking into considerations the findings of this thesis on the differential role of opioid receptors in the development of CEP produced by infusions of NAL into the mPOA and VTA, particularly the shift in preference found in the mPOA, and not necessarily in the VTA; it is believed that opioid receptor effect in the mPOA may be regulating the valence of the reward, whereas in the VTA they may modulate the incentive value of the reward-related cue, regardless of the sensory modality of the neutral cue in which the CEP is based. These findings are consistent with previous data that indicates that systemic injections of NAL reduced sexual reward resulting from ejaculation, and even anticipatory level-changes (a behavior believed to indicate motivation) in high doses (van Furth *et al*., 1994); as well as the facilitation of sexual performance in sexually sluggish males, but impairing it in normal copulators when NAL was micro-infused into the mPOA (van Ree *et al*. 2000), whereas into the VTA, NAL prevented the increase of anticipatory level changed, while sexual performance remain unchanged

(van Furth & van Ree, 1996).

Complex and widespread neuronal circuits control different reward sensations in the brain (Bassereo & Di Chiara, 1999; Wright, Beijer & Groeneweger, 1996). There are several natural rewarding reinforcers beside sex like food, drugs, etc. All of these appear to be controlled by several common brain areas in which opioid peptides and receptors have been shown to be involved (Le Merrer *et al.,* 2009). One of these systems involved in sexual reward that is also present in the modulation of food and drug reward is the mesolimbic system (*e.g.,* Fields & Margolis, 2015). DAergic cell bodies of the mesolimbic pathway are located in the VTA projecting to several different limbic and cortical structures, including the amygdala, nucleus accumbens (NAc), medial pre-frontal cortex, among others (Wise, 2009). Particularly, studies have shown that VTA-NAc connections are known to regulate incentive salience for reward related cues, connections which μ-opioid receptor modulate in two different ways: by an excitatory effect (directly by increasing Ca^{2+} channel conductance, or indirectly by inhibiting γ-aminobutyric acid [GABA] release), or by an inhibitory effect (directly by activating K+ channels, or indirectly by inhibiting glutamate release; Fields & Margolis, 2015).

As reviewed thus far, these VTA-NAc connections play a crucial role in the development of new rewarding associations in sexual behavior of male rats (West, Clancy & Michael, 1992), as well as food intake (Baldo & Kelley, 2007), and drug addiction (Terashvili *et al.,* 2004). These common pathways, brain areas, and neurotransmitters that orchestrate sexual reward mediated by the action of opioid receptors appear to be a global reward system in the brain mediating other natural reinforcers (see Le Merrer *et al.,* 2009). Evidence of this comes from results that show that ghrelin, the main hormone that trigger the hunger sensation, inhibits sexual receptivity in food-restricted female rats (Bertoldi *et al.,* 2011), as well as findings that showed that leptin, the main hormone that trigger the food satiety sensation, facilitated sexual behavior in *ad-libitum* female hamsters (Wade,

Lempicki, Panicker, Frisbee & Blaustein, 1997). Moreover, 'compulsive' sexual behavior neural correlates overlap neural circuits related to drug reward in humans (Voon *et al.,* 2014). Finally, the regulation of other basic physiological functions that keeps the body in a homeostasis like sleep, thermoregulation, circadian rhythms, and some aspects of pain are mainly concentrated in brain areas around the hypothalamus (reviewed in Kandel, Schwartz & Jessell, 2000). Therefore, it is clear that the hypothalamus is an important brain region in the orchestration of reward in the brain (*e.g.*, Aston-Jones, Smith, Moorman & Richardson, 2009), and that several (perhaps all) rewarding stimuli and main physiological functions are processed by subareas in this cortical structure, particularly where the mPOA has shown to play a pivotal role in the orchestration of sexual reward. It is important to mention that these communalities in common structures and neurotransmitters do not represent that these phenomena are or processed the same way.

Perhaps motivation is also mPOA-mediated. After the reward value is encoded, it is believed that the mPOA also may modulate the motivation toward reward-related cues through the mesolimbic DAergic pathway. Finding of this thesis demonstrated that the Fos-IR based on the discrete cue paired with vary depending where the opioid antagonist was micro-infused in the brain of male rats. That is, male rats micro-infused into the mPOA had a lower Fos-IR count than the control group in their VTA, yet not the other way around when other animals were micro-infused into the VTA. These alleged reward-related connections from the mPOA to the VTA have been recently shown in female rats, where the mPOA innervates the VTA. These connections were mainly localized in the rostral portion of the mPOA, and they were shown to be mainly GABAergic and also receptive to DA (Tobiansky *et al.,* 2013).

An interesting aspect of a global system that encodes reward for several different phenomena and reinforcers is how the system prioritizes for one over the other. For example, how or when would an animal choose to mate while being food-deprived, or choose to eat even when it is cold or

sleep-deprived? As previously stated, the sensations of hunger and satiation interact with sex through the actions of hormones that control appetite. Both leptin and ghrelin travel through the vagal nerve or bloodstream into the hypothalamus, where both stimulate and suppress hypothalamic neurons provoking a downstream orexic (+) or anorexic (-) effects controlling energy balance, effect that antagonize each other (Klok, Jakobsdottir & Drent, 2006). As for leptin, a lower estrogen receptor activity in the ventromedial hypothalamus (VMH) was found in food-deprived females, changes that were not detectable in *ad libitum* animals (Wade, Lempicki, Panicker, Frisbee & Blaustein, 1997). This is because food deprivation decreased detectable estrogen receptor inmunoreactivity in the VMH, yet the sexual behavioral changes due to the administration of leptin were not accompanied by changes in the estrogen receptor immunoreactivity in that brain region. Thus, it is believed that leptin facilitates sexual behaviors in the female hamster, yet it does not overcome the inhibition of copulatory behaviors such as lordosis produced by acute food deprivation (Wade, Lempicki, Panicker, Frisbee & Blaustein, 1997). Administration of ghrelin has shown to inhibit sexual behavior, particularly mount, intromission, and ejaculation by inhibiting the hypothalamo-pituitary-gonadal (HPG) axis, presumably by decreasing testosterone (Babaei-Balderlou & Khazali, 2016). Male rats with a truncated ghrelin receptor (FHH-GHSR$^{m1/Mcwi}$) showed deficits in anticipatory sex behaviors, just like normal food-deprived male rats, compared to their wild type littermates (Hyland *et al.,* 2018). Furthermore, in their same study, Hyland and collaborators showed that central ghrelin transmission modulates sex motivation in a site-dependent manner. Specifically, infusions of ghrelin into the VTA did not alter sex anticipation (*i.e.*, number of level changes and active seeking in a bilevel chamber), whereas infusions of the ghrelin receptor antagonist (D-Lys-GHRP6) decreased sex anticipation in food-deprived males. Infusions of ghrelin into the mPOA decreased sex anticipation, whereas infusion of its antagonist had no effect (Hyland *et al.,* 2018). Babaei-Balderlou & Khazali (2016) suggested that ghrelin may also affect sexual

behavior performance through mechanisms that regulate energy balance preventing the loss or investment of it, considering that ghrelin was involved in the reduction of receptivity in females after food scarcity (Bertoldi *et al*., 2011). Furthermore, both of these hormones have been shown to modulate cue-induced DA activity in the VTA. van der Plasse and colleagues (2015) trained rats in an operant task to associate a signal with access to two different types of food (bacon or fruit). Subsequently, these rats underwent through food derivation. During testing, animals performed increased due to food deprivation, while food-cue DA firing also increased in food-deprived animals. This increment was attenuated by leptin pretreatment, whereas ghrelin pretreatment only affected baseline DA firing, but not cue-induced activity, showing that leptin, yet not ghrelin, plays a key role in linking metabolic information with reward-related neuronal activity in the VTA. Altogether, both feeding hormones regulate sexual hormones in the brain, showing the interaction or reciprocal relationship between both types of behaviors, and their role in the full orchestration of sexual behaviors when other vital functions are also at play, such as hunger.

Another well-known interaction between two reward-related mechanisms is the relationship between drug use/abuse and socio-sexual behavior (Le Merrer *et al.,* 2009; Young *et al.,* 2011). Previous studies have shown that amphetamine (AMPH) experience can alter partner preference in male prairie voles (Liu *et al.,* 2010). In their study, three groups were established, intact, saline pre-treated control, and AMPH pre-treated animals. Males were given either injections of saline, AMPH, o no injection at all for three days. On the fourth day, subjects were paired with a sexually-receptive female partner for 24h. Finally, males were tested for partner preference with their familiar partner (female mate of each subject), and a stranger female (that they never have previously encountered). Results showed that intact and saline-treated males developed a partner preference, whereas AMPH-treated males did not. Further experiments in the same study demonstrated that this effect is a DA receptor-dependent, particularly in the NAc (Liu *et al.,* 2010). A following-up study

demonstrated that sex can become a 'protector' factor in the development of drug-related associations. Two different groups of male prairie voles were trained to develop an AMPH-induced CPP, a sexually naïve and an already paired-bonded group. Results showed that sexually-naïve males developed an AMPH-induced CPP, whereas paired-bonded males did not. Further analyses demonstrated that this effect is specific to DA D_1 receptor in the NAc (Liu *et al.,* 2011). DA transmission in the NAc has also been implicated in partner preference, particularly in its rostral portion (Aragona *et al*., 2006). Aragona and colleagues showed that mating-induced DA release selectively activates D2-like receptors to promote pair bonding, whereas D1-like receptor activity prevented it.

The interactions between the different systems, and particularly the ones related to pair bonding and drugs, provides further evidence of this common reward system that regulates the hedonic sensations, associative learning, and incentive motivation related to natural and learned reinforcing cues. Different brain areas like the mPOA and VTA, and neurotransmitters like opioid and dopamine, have shown to play a pivotal role in the orchestration of what feels good, and therefore who or what gets the attention. Also, the system also encodes for priority or hierarchy among the rewarding cues and other physiological homeostatic states, where reinforcers appear to compete for who *gets first* to set the stage or bias the animal towards particular CSs, making animal more susceptible to associate these cues with different kinds of reward. This could explain the 1 trial US pre-exposure effect found in the firth chapter. Perhaps, epigenetic changes occurring very early on setting the stage for reward-related cues (like the estrous odors) to be easily associated with sexual reward, establishing a rudimentary *blueprint* of partner preference that can be crystallized or shaped with experience. Associations that can also be based on external neutral cue based on experience (Ménard *et al.,* in preparation).

How does the system encode preference based on neutral cues?

The present experiments involved the conditioning of discrete cues, the odor or the jacket, paired with sexual reward. However, there are several other cues present at the point of training, just like the jacket is a cue that stimulates the visual and the somatosensory systems. Other cues can be contextual (time, background light and sounds, chamber dimensions, and smells of the room, etc), or discrete (olfactory, anatomical, and behavioral female features). All of these come into play when the odor or the jacket is being paired with sexually-receptive females. In all experiments, both contextual and discrete variables were controlled for. Namely, before the beginning of each experiment, all animals were pre-exposed to the context alone, without any consequence. This is believed to reduce the salience of those contextual cues, decreasing their associability with the US when the discrete cue is being paired directly as they have been previously associated with no consequences (McLaren, Bennett, Palisted, Aitken & Mackintosh, 1994). Furthermore, any female's feature is believed to be controlled for given that throughout conditioning, females assigned to the males were randomly selected from a large cohort. Thus, the only constant in any given trial was the odor or the jacket. However, it is perhaps naïve from a Pavlovian perspective to believe that the odor or the jacket were the *only* cues that the males directed their attention to (and therefore associated with sexual reward). Not all animals in their respective groups followed strictly the average preference of the group, and even some had the complete opposite preference than expected. Therefore, male rats could have potentially associated any of the other contextual and discrete cues present throughout conditioning with sexual reward. In consequence, they way compound stimuli are processed is what researchers have debated for more than 40 years.

One of the oldest controversies in the associative learning literature is how do animals process compound stimuli, either as an arrangement of elements (*i.e.,* McLaren & Mackintosh, 2002;

Rescorla & Wagner, 1972), or as a configuration different from the sum of the elements (*i.e.,* Pearce & Hall, 1980). The 'elemental' approach assumes that the response towards a compound stimulus (*e.g.,* AB) would be a function of the sum of all element's associative learning which is made of (A + B), whereas the 'configural' approach assumes that a compound is a new identity independent from the elements that is made of, and that the response would be a sum of the associative learning of the configuration itself and some generalization value from its elements (Wagner & Vogel, 2009). Although both approaches assume configurational and elemental processes in the way their algorithms work, when elements are presented in a compound, the elemental approach assumes the sum of all elements, while the configural approach predicts some degree of subtraction of the associative learning of each element when presented together (Wagner & Vogel, 2009). The contradictory evidence (*e.g.,* Collins & Shanks, 2006; Soto, Vogel, Castillo & Wagner, 2009) has lead authors to believe that both approaches are valid, and the processing strategy would vary depending on certain variables related to a task, the stimuli used, and the subjects. Melchers, Shanks & Lachnit (2008) described five variables that would influence whether a particular problem is solved or processed elementally or configurally: task demands, prior experiences, experimental instructions, and stimuli organization and properties.

One of the variables by which the processing strategy shifts is previous experiences. The evidence shows that organisms will solve a problem of discrimination in the same way in which they have solved this problem before (Alvarado & Rudy, 1992), where subjects followed an elemental strategy after having had previous experience with another problem that required an elemental solution, whereas exactly the opposite occurred when they had been previously trained with a strategy that required a configural solution. Previous experience has shown to modify preferences in rats when it comes to sexual behavior. For instance, sexually-naïve female rats have shown to develop a conditioned place preference (CPP) using clitoral stimulation as a US (Parada *et*

al., 2010). However, clitoral stimulation was not able to establish a CPP when females were sexually-experienced (exposed to repeated copulation experiences, Parada, Jafari & Pfaus, 2013). Similar results were found in sexually-naïve males who established a CPP when given copulatory experience based exclusively on intromissions. However, intromissions were able to establish a CPP in sexually-experienced males (*i.e.,* repeated copulation until ejaculation (Tenk, Wilson, Zhang, Pitchers & Coolen, 2009). These findings demonstrate that previous experiences can alter or modulate the rewarding properties of different types of US or their intensity, which ultimately can lead to different type of associations. Thus, it is of particular interest how the rewarding associations were fostered through pre-exposure of the US or CS in the first chapter. In those experiments, the pre-exposure of both, CS and US, showed that a previous experience with neutral and biologically significant cues can alter the future partner preference in male rats. The results showed that this pre-exposure effect is able to disrupt a CEP when the cue is pre-exposed enough times, and particularly in the case of the US, in the conditions in which it is pre-exposed, given that even one trial of US pre-exposure was enough to disrupt the CEP when animals were pre-exposed to an UnScF and later on trained with ScF.

Experimental instructions are another set of variable that influence the type of processing, where evidence has shown that human participants solve tasks elementally when given instructions led the participants to see the keys as independent entities (Williams *et al.,* 1994), and in a configural way when these instructions were not given (control group). Although these studies did not include verbal instructions, the evidence suggests that variations in experimental conditions between subjects could alter the processing, and therefore the preference. For instance, particular details in the training like the pacing conditions have shown to contribute to the development of a CEP for a familiar female in male rats (Ismail *et al.,* 2009). In their experiments, two groups were trained to develop a CEP for a scented female, one with 1-hole pacing divider, and the other with a

4-hole pacing divider. Results showed that only males in the 1-hole pacing divider developed a CEP towards the familiar female, demonstrating that the possible higher arousal level experienced by male due to the longer wait a 1-hole pacing divider creates contributes to the development of a CEP. Although each experiment used only 1-hole pacing dividers, female behaviors, and particularly their rate of copulation, vary among them. Not all of them stay with the male the same time, come back from the other side at the same time, or copulate the same way (some females kick the males more), and even some may climb up to pass through the holes in the top of the 1-hole divider (anecdotal observation). All of that interacts with what each male may like or develop a preference for, and when that occurs (early or later on) in their experience. Therefore, although the training conditions were the same across the studies of the present thesis, animals made use of them differently. Little differences like this may have lead animals to foster associations differently, or with more cues than just the odor or jacket, which could potentially explain why some males deviated from the preference they were trained for.

Another factor that contributes on how animals process compound stimuli is the organization of stimuli, where it is assumed that there is a perceptual interaction between the components of a compound, appealing to the spatial arrangement of the keys. Glautier (2002), through a block experiment where the keys were symbols in letters, when participants were presented spatially close and grouped, subjects opted for a configural strategy, whereas when the keys were presented spatially separated and not grouped, subjects opted for an elemental strategy. It is hard to believe or fathom how this factor may have played any role in how males established the associations.

The variable stimulus type has been explored through an orthogonal distinction of stimuli based on their sensory modality: unisensory compounds (*e.g.,* two different frequency tones) and multisensory compounds (*e.g.,* a tone and a light). Myers *et al.,* (2001) with stimuli of different modalities have found evidence for elemental processing, while Readhead & Pearce (1995) with

visual stimuli found evidence of configural processing. This is of particular interest for the odor and the jacket, because both stimuli interact with stimuli from other or the same sensory modalities. In the case of the olfactory cue, several other odors are present in the room during conditioning. Particularly, female estrous odors can become a major complication, perhaps even more during the first trial, as seen in the disrupting effect US pre-exposure can have. In the case of the jacket cue, different features of the jacket (visual or somatosensory) can come into association for different male rats, just like other visual/somatosensory cues of the female itself (*i.e.,* fur). To control for this, different females were assigned to each male in each trial, so only either the almond odor or the jacket were the only fixed predictive cue for them, while contextual remained constant.

Of rats and men: evolutionary considerations

Human sexual behavior and that of other animals, especially the rat, have much in common. Compared to other forms of behavior and learning, it is also believed that certain aspect of an animal's sexual behavior, like attraction and preference, are hardwired or fixed to certain attributes (*e.g.*, certain body types, face shape, social features, personality traits, etc) that are universally desirable or are related to reproductive success (*e.g.*, Buss & Schimdt, 1993; Townsend & Levy, 1990). More specifically, males and females evolved distinctive mechanisms that would explain why they engage in short-term or long-term strategies. Due to different circumstances, one or the other strategic would become "activated". Contextual variables include: sexual accessibility, fertility assessment, commitment seeking and avoidance, immediate and enduring resource procurement, paternity certainty, assessment of mate value, and parental investment (Buss & Schimdt, 1993). Other evolutionary formulations that highlight "optimality" and "trade-offs" between mating and child-rearing postulate that women tend to prefer men with physical features that would benefit the offspring genetically as a short-term mate, yet this selection post trade-offs in willingness to help

raise the offspring (Gangestad & Simpson, 2000). Furthermore, descriptive studies postulate *men's formidability* as the capacity of fighting or holding resources of power (Sell, Lukazsweski & Townsley, 2017), for which modern women "*should have mate choices that respond to ancestral cues of man fighting's abilities*" (pp. 1). Studies like this describe what is more attractive for one of the other sex showing that, for instance, male attractiveness can be explained up to 80% in terms of their phenotype –particularly upper body strength–, where the strongest male in appearance are deemed the most attractive (Sell, Lukazsweski & Townsley, 2017). However, why are there so many people that violate these standards of universal attractiveness? It only takes to look around us to see that variability is what reigns. Evolutionary perspectives on reproductive success not only limit sexual intercourse to reproduction, but also lose perspective on other different levels of analyses, providing poor or no mechanism at all to account for partner preference (see Pfaus, Kippin & Centeno, 2001).

More reproductive success, or perhaps the success of engaging in sex to be able to reproduce, is often confused with the fact that reproduction may not be the ultimate goal of engaging in sex after all. It appears that sex is all about women mechanisms to track cues that predict genetic "quality" for a better offspring, leaving desire and pleasure completely out of the picture. Learning mechanisms that predict how association of discrete and contextual cues are able to be associated with sexual reward post a powerful tool to describe and predict why people choose one partner over another. Furthermore, as shown in this thesis, first sexual experiences can explain anecdotal cases of individuals who look for features that are familiar to previous partners in new ones (*e.g.*, Stendahl, 1821/1959).

Conclusions and future directions

The present finding provided a more detailed picture of factors that modulate the development

of a CEP in male rats, and the opioid-receptor mechanisms underlying this phenomenon. Yet, like any scientific endeavor, these results raise more questions than answers. Therefore, future studies should explore further behavioral and neurochemical underpinnings underlying opioid reward in the male rat.

Given the finding on the first chapter, particularly on the US pre-exposure, future studies can explore the possibility of given male rats at the open field test to choose between the non-familiar female and the very same female rat they were initially pre-exposed to. If stimuli properties is one of the aspects that modulate how compound stimuli are processed (Melchers, Shanks & Lachnit, 2008), given males the opportunity to choose not only the type of female (either ScF or UnScF), but the very same female to which they were pre-exposed, would allow to evaluate the exact same association that was once fostered during that pre-exposure phase. Furthermore, it would be of particular interest to evaluate the neurochemical changes occurred during those first experiences. Microdialysis and other neuroanatomical techniques in 1-trial training experiments could shed light on the very first physiological and anatomical changes occurring during first sexual experiences in different brain areas.

Much more is yet to be known on the neurochemical mechanisms underlying this phenomenon, and even from its opioid-receptor mechanisms. Another venue for future studies given the present findings is the assessment of which type of endogenous opioids, and which opioid receptor are responsible of the findings of chapter 5, and if they vary from brain area to another. As previously mentioned, a likely candidate for the type of receptor is μ-opioid receptor. Mating and ejaculation have shown to induce μ-receptor internalization (a marker of ligand-induced receptor activity) in the mPOA (Coolen *et al.,* 2004) and VTA (Balfour *et al.,* 2004). On the ligand side, endorphins are likely candidate (see Fields & Margolis, 2015; Le Merrer *et al.,* 2009). Male rats developed a CPP when β-endorphin was administered systemically and intracerebroventricularly in

comparison to saline-injected rats (Amalric, Cline, Martinez, Bloom & Koob, 1987). Different fluorescence techniques can assess the specific μ-receptor activity or the specific protein-ligand interaction (Rossi & Taylor, 2011).

Several other aspects remain to be evaluated at a behavioral and a neurochemical level when it comes to CEP. One of particular interest lies on the evaluation of short or long-lasting these associations may be. For instance, no study has evaluated how long a CEP may last after tested (using the established protocol of Kippin & Pfaus, 2001). 10 training trials may be sufficient to establish a conditioned ejaculatory preference based on a neutral cue, yet how many trials does it take for the preference to disappear still remains unknown. Thus, extinction phenomena like spontaneous recovery, reinstatement, and renewal, can be easily explored to assess how CEP is likely to disappear and re-appear with time and experience. Furthermore, new associations can be fostered with sexual reward. Thus, several questions remain to be assessed: how do new associations compete with older ones; do old association extinguish; how do animals respond to a new combination of two different cues trained separately with sex; etc. The answer to these questions could provide valuable insights on how or why animals establish less or more long-lasting partnership. Similarly, that data could shed light on how human establish their sexual partnership bonds, and how to they vary across time and with regard of new and old rewarding experiences.

In summary, how animals choose a sexual mate is not a trivial question in terms of the reproductive aspect of who would father or mother the animal's offspring, nor for the pleasure aspect of who we choose to engage in sexual intercourse. As shown in this thesis, male rats are able to establish associations between sexual reward with different type of neutral stimuli given the action of opioid receptors that modulate their partner preference. Although evolutionary perspective of partner preference put things into perspective of a 'big picture', it is clear that more proximate experiences with reward provide a more accurate approach and replicable mechanisms as to why

animals choose and prefer a mate. It is hoped that this thesis shed light and expand the knowledge on how humans establish these associations, and how certain manipulations or conditions can modify them.

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