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ORIGINAL INVESTIGATION

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

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Abstract

Rationale Male rats trained to associate a neutral odor or rodent jacket on a female with their post-ejaculatory reward state display a preference to ejaculate with females bearing the odor or jacket. This conditioned ejaculatory preference (CEP) can be shifted by systemic administration of the opioid antagonist naloxone (NAL) during training, such that NAL-trained males distribute their ejaculations to females without the cue, relative to saline (SAL)-trained males.

Objective The present study examined two brain sites, the medial preoptic area (mPOA) or ventral tegmental area (VTA), where the opioid reward state might be induced.

Methods Sexually naïve Long-Evans males were implanted with bilateral guide cannula aimed at either site before they underwent multi-ejaculatory conditioning trials at 4-day intervals with sexually receptive females that bore either an almond odor or rodent tethering jacket. Infusions of NAL (1 μl/side) or SAL (1 μl/side) were made prior to each conditioning trial. All males were infused with SAL prior to a final open-field choice test with two sexually receptive females, one scented and the other unscented, or one jacketed and the other unjacketed.

Results Males previously conditioned with SAL in either region showed significant CEP. In contrast, prior infusions of NAL to the mPOA shifted the preference towards the unfamiliar female, whereas prior infusions to the VTA abolished CEP for the odor. Subsequent detection of Fos protein induced by the cue showed that, relative to SAL-treated males, prior experience with NAL in the mPOA suppressed Fos in both the mPOA and VTA, whereas prior experience with NAL in to the VTA suppressed Fos in the VTA alone.

Conclusions Opioid antagonism in the mPOA produces a state of non-reward whereas in the VTA, it produces a state in which the odor does not acquire incentive properties.

Keywords Conditioned ejaculatory preference · Opioid · mPOA · VTA

Introduction

The effects of opioids have been well documented in the display of both conditioned and unconditioned sexual behaviors (Pfaus and Gorzalka 1987; van Furth et al. 1995a, b; Halloway 2012; Paredes 2014). The rewarding effects of opioids that create a preference for contextual cues associated with sexual reward in male rats were first shown using the conditioned place preference (CPP) paradigm (Miller and Baum 1987; Ågmo and Berenfeld 1990). Male rats either copulated in the initially non-preferred side of the CPP box (Miller and Baum 1987) or were placed into the initially non-preferred side immediately after one ejaculation (Ågmo and Berenfeld 1990). In both cases, the contrast was made against the initially preferred side, in which males were not allowed to copulate or experience their post-ejaculatory state. Males injected systemically with saline (SAL) displayed significant CPP for the side associated with copulation to ejaculation, or the post-

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ejaculatory state itself, whereas males injected systemically with naloxone hydrochloride (NAL), but not the dopamine antagonist pimozide, spent significantly less time in the side associated with the copulatory or post-ejaculatory sexual reward state. These data show that opioid, but not dopamine, receptor blockade is an important neurochemical substrate of sexual reward induced by ejaculation in male rats. Indeed, whole brain opioid content and receptor internalization are highest immediately after ejaculation (Szechtman et al. 1981; Tenk et al. 2009) when male rats become behaviorally quiescent and sleep for a short period. This makes the post-ejaculatory period essentially an "orgasm-like" sexual reward state (Pfaus et al. 2016) during which conditioned sexual learning occurs (Georgiadis et al. 2012).

Similar to CPP, male rats trained to associate a neutral odor (e.g., almond, lemon) on a sexually receptive female with their post-ejaculatory sexual reward state display a preference to ejaculate with scented versus unscented females (Kippin et al. 1998; Kippin and Pfaus 2001). Similarly, males trained to associate a jacket on the female with sexual receptivity and copulation develop a preference towards the jacketed female over an unjacketed one (Quintana et al. 2019a). As with CPP, systemic administration of NAL disrupts the acquisition of CEP for both odor (Ismail et al. 2009) and the jacket (Quintana et al. 2019b) relative to males injected with SAL. However, prior experience with NAL during the training trials shifted the preference towards other females for both cues.

Among the different areas of the brain where opioids may play a role in sexual behavior and reward, and specifically in the development of CEP, the medial preoptic area (mPOA) and the ventral tegmental area (VTA) are two of the most widely studied (van Ree et al. 2000; Fields and Margolis 2015). Both have been regarded as critical in the control and execution of male sexual behavior and sexually motivated behaviors in both males and females (Hull et al. 2006; Pfaus 2009), and particularly in the development of CEP for an olfactory cue in the male rat (Pfaus et al. 2012).

The present study examined whether opioids exert a differential modulation of CEP when NAL or SAL were infused into the mPOA or VTA. The first experiment evaluated this with an odor cue on estrous females. The second experiment substituted the odor with a somatosensory cue to evaluate if this effect is dependent on the type of conditioned stimulus used. Finally, Fos-IR was used to evaluate how NAL would affect the neural pattern of activation towards the cue.

Materials and methods

Animals, steroids, and conditioned cues Seventy-three male Long-Evans rats weighing 300–350 g (before surgery), and 100 female Long-Evans rats, weighing 150–200 g, were obtained from Charles River Canada (St-Constant, QC) at approximately 6 weeks of age. All animals were kept in a 12-h reversed light/dark cycle in a colony room at 21 °C, with unrestricted tap water and Purina® rat chow available in each cage.

Males were housed in groups of four in Plexiglas cages with wood-chip bedding and unrestricted access to water and food until cannulation, after which they were maintained individually in a single cage. Female rats were housed in pairs in Plexiglas (45 × 20 × 20 cm) cages with wood-chip bedding. Sexual receptivity was induced by subcutaneous injections of 10 μg estradiol benzoate (EB, 17β-diol 3-benzoate, ID E0970-000, Steraloids) 48 h prior each training session, and 500 μg of progesterone (P, 4-Pregnen-3, 20-dione, ID Q2600-000, Steraloids) 4 h prior to each training session as detailed in Pfaus et al. (2014). Steroids were dissolved in reagent grade sesame oil and delivered in a volume of 0.1 ml. Stimulus females were either scented or jacketed depending on the experiment. For the odor cue, females were scented with 0.6 ml of pure almond extract (Blue Ribbon, Etobicoke, ON), split equally in the back of their neck and anogenital region. For the somatosensory cue, females wore a rodent tethering jacket (Lomir Biomedical, Ile Perrot, QC) made of a double layer of Lycra/Spandex fabric. This jacket covered the upper part of the torso, with open holes for the forearms and fastened across the back with Velcro (see Quintana et al. 2019a).

Surgeries *Ovariectomy*. Females were ovariectomized via bilateral lumbar incisions under general anesthesia induced with ketamine (50 mg/ml, CDMV, ID UN7919, Wyeth)/xylazine (4 mg/ml, Rompun, DIN 02169592, Bayer) mixed at a ratio of 4:3 respectively (administered based on body weight), approximately 2 weeks before the beginning of the experiment to allow at least a week of recovery (e.g., Kippin et al. 1998; Pfaus et al. 1999). This procedure allowed for hormone levels to be controlled under hormonal replacement throughout training sessions.

Cannula implantation. In order to avoid sexually sluggish males, all males were allowed to copulate with a scented or jacketed (depending on the experiment) sexually receptive female in a pacing chamber for 30 min. All males were bilaterally cannulated into the mPOA or VTA, around 2 weeks before the first training trial to allow recovery. Animals were anesthetized with 5% isoflurane (Institute for the Laboratory Animal Research 2011) and secured in a stereotaxic apparatus (Kopf Instruments). Under aseptic conditions, stainless steel guide cannulae (22G, Plastic One) were implanted bilaterally 1 mm above the regions of interest (mPOA, AP, − 0.5 from bregma; MD, ± 0.5; DV, − 8 mm; VTA, AP, − 6.04 from bregma; MD, ± 2 at 10°; DV, − 8 mm (see Coolen et al. 2004). A stainless steel dummy of the same length was placed in each cannula to ensure it remained unblocked.

Behavioral training After recovery, males were given multi-ejaculatory trials for 30 min each with sexually receptive females

in one-hole unilevel pacing chambers, where only females could pace through a divider of a two-compartment chamber. Males trained with scented females underwent 10 training trials, whereas males trained with jacketed females underwent 14 trials. As established by Quintana et al. (2019a), the difference in the amount of training trails was necessary since the somatosensory cue does not have the same salience in rats as the olfactory cue in the development of a CEP. The cage bedding was not changed between conditioning sessions and in every trial, females were assigned randomly to males to assure that conditioning accrued only to the odor or jacket.

A NAL concentration that would effectively reverse the CEP as previously established (Ismail et al. 2009) was determined by testing different doses (i.e., 200, 500, and 1000 ng) in an unpublished preliminary study. Although 200 ng and 500 ng disrupted the CEP compared to the control group, the 1000-ng dose reversed it, as was found previously with systemic NAL (Ismail et al. 2009) Thus, infusions of either 1 μg NAL in 0.9% SAL, or the SAL vehicle (infused in 1 μl/side over the course of 1 min), were made to all males in each group prior to conditioning trials, depending on group membership. The distribution of males per group is presented in Table 1. Three males that appeared sick during the experiment were euthanized and their data not included.

All males were infused with SAL prior to a final choice test in which males were placed into an open field (123 × 123 × 46 cm) filled with clean bedding to copulate freely for 30 min with two sexually receptive females, one scented (ScF) and the other unscented (UnScF), or one jacketed (Jacket ON) and the other unjacketed (Jacket OFF), depending on group membership. All behaviors were recorded on video and scored later using a computerized event recorder (Balfour et al. 2006). Following the preference test, males were given two more training trials in the exact same conditions of their training trials before perfusion.

Perfusion, histology, and Fos immunohistochemistry Four days after their last reconditioning trial, males were euthanized with sodium pentobarbital (120 mg/kg, i.p.) and perfused intracardially with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were extracted and post-fixed in fresh 4% paraformaldehyde in PBS overnight, then transferred into a hypertonic 30% sucrose solution to extract water from the brain. Brains were then were frozen at − 80 °C until sliced. Coronal slices were cut and collected through a sliding microtome at 30 μm, and cannula placements were confirmed and marked in an atlas by a third-party researcher. The criterion to exclude animals from the analysis was set so that males whose injectors placed outside of the boundaries of either the mPOA or VTA were exempt from the study. Therefore, males with uni- or bilateral cannulation on target were included in the analyses. Unilateral placements in these regions were included given that previous studies have shown they are effective. For instance, unilateral infusions of NAL into the mPOA affected the different parameters of song productions in male birds (Kelm-Nelson et al. 2013), as well as maternal behavior (Miranda-Paiva et al. 2003). In the present experiment, four males were ultimately not included in the analyses due to a bad cannula placement (one from the Jacket SAL mPOA group, one from the Jacket NAL mPOA group, and two in the VTA NAL VTA group; see Table 1).

Table 1 Group sample distribution

		Odor	Jacket
mPOA	SAL	10	8
	NAL	10	9
VTA	SAL	10	9
	NAL	10	7

Fos immunocytochemistry was performed as reported previously (Garduño-Gutiérrez et al. 2013). Brain sections were examined at × 40 magnification, and the number of Fos-positive cells was counted bilaterally from each region from five different sections per rat per region using a Leitz Laborlux light microscope connected to a computerized image-analysis system (ImageJ, NIH, Bethesda, MD) as we have done previously (Coria-Avila and Pfaus 2007; Kippin et al. 2003). The selected brain regions were observed for Fos-IR to evaluate the neural activation evoked by the odor or jacket cue used during training. An average of Fos-positive cells was calculated from three different slides from each rat (5 subjects in each group), for each brain area in the control and experimental groups, for each main group depending on where their implants were located (mPOA or VTA), separately.

Figure 1 shows three-magnification examples of the cannula trace, as well as the Fos-IR counting rationale for the mPOA (left panels) and VTA (right panels). For the mPOA, four times a standard area of 84 × 107 mm was utilized to cover the area of interest, whereas for the VTA, two times a standard area of 66 × 99 mm was used (out of pictures of 216 × 66 mm [2560 × 1920 pixels]), distributed as illustrated through the white dash-line areas. As in our previous studies, the threshold to detect Fos-positive cells were set at the lowest range necessary for one Fos-positive cell to be detected and any other nearby highlighted cell-like shape not. For counting purposes, the size of cells detected (in pixels^2) was set between 0 and 40, while circularity was set between 0.5 and 1.0 pixels. Pictures were taken as close as possible to the injection site, while avoiding the immediate nearby area of the injection to improve sensitivity and specificity of actual Fos-positive cells. Lower numbers of Fos-IR in comparison to other reports, especially in the case of the VTA, were expected, where the detection criteria and the narrow counting area covered could explain the differences.

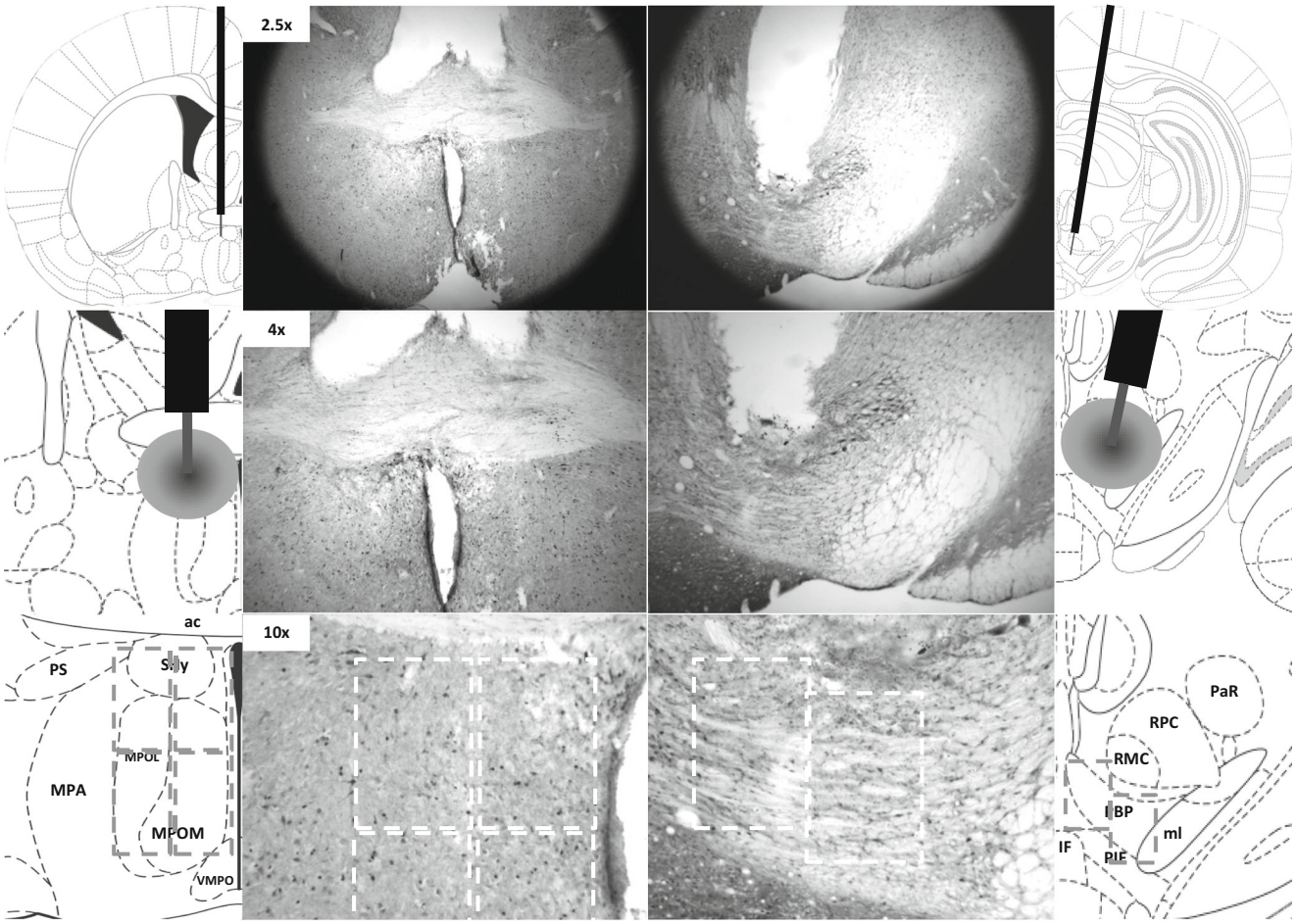


Fig. 1 Illustration of the cannula traces as well as the Fos-IR counting rationale for the mPOA (left panels) and VTA (right panels). From top to bottom rows, we illustrate pictures taken with a × 10, × 16, and × 40 magnification. The brain atlas illustrations depict the theoretical cannula trace, injection area, and subregions where the injection was most likely located. The cannula, injector, and rectangles are not set to real scale

Statistical analyses A series of repeated measures ANOVA were conducted to explore the influence of training in different sexual behaviors by each experimental cue used (odor or jacket), in each brain area subgroup (mPOA or VTA), for each group (SAL or NAL), separately. Analyzed behaviors included mounts, intromissions, mean of ejaculation, and latency of first ejaculation. In each analysis, we evaluated the interaction between the type of female (ScF vs. UnScF or Jacket ON vs. Jacket OFF) by group (i.e., SAL or NAL). Given statistically significant differences, post hoc compassions were conducted for each single variable to compare the type of female using the Bonferroni correction. Partial eta square (η_p^2) was calculated as effect sizes for each comparison. Additionally, a 1 × 2 chi square (χ^2) analysis was conducted for the percentage of first ejaculation choice for each individual group, and a 2 × 2 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 1 × 2 and 2 × 2 χ^2 analyses, respectively.

To evaluate the differences among the experimental groups in Fos-IR, one-way ANOVA analyses were conducted. For each significant ANOVA, post hoc analyses were made using the Tukey HSD test for the animals trained with the odor cue, for each brain area of interest (mPOA or VTA), among the different groups (SAL or NAL), separately. For animals trained with the jacket, only the highest dose of NAL was infused (1000 ng); therefore, independent *t* tests were conducted to compare the two groups (SAL and 1000 ng) for each brain area of interest (mPOA or VTA) separately. Partial eta square (η_p^2) and Cohen's *d* were calculated as measures of effect size.

Results

Olfactory conditioning

Infusions of NAL or SAL to the mPOA: behavioral effects Figure 2 shows the percentage of first ejaculation choice by

female and by brain area for all groups using the odor as neutral cue during the open-field test. A 2 × 2 χ^2 analysis revealed a statistically marginal trend for the SAL and the NAL groups to prefer different females to ejaculate first with, $\chi^2(1) = 3.2$, $p = 0.074$, $\varphi = 0.3$.

The analyses of the remaining variables (i.e., mounts, intromissions, ejaculation latency, or the mean of ejaculation) found no interactions between the factors, as well as no differences in the 1 × 2 χ^2 analyses.

Infusions of NAL or SAL to the mPOA: Fos-IR analyses Figure 3 shows the group results for the Fos-IR results regarding the exposition to the odor cue before perfusion for each main (cannulated into mPOA and VTA), group (SAL or NAL), and brain area (mPOA and VTA).

When analyzing the mPOA of males infused with NAL into the mPOA showed a statistically marginally lower Fos-IR mean count than the SAL control group, $t(8) = 2.196$, $p = 0.059$, $d = 1.389$. When analyzing, the VTA of males infused with NAL into the mPOA showed a statistically significantly lower Fos-IR mean count than the SAL control group, $t(8) = 2.505$, $p = 0.037$, $d = 1.585$.

Infusions of NAL or SAL to VTA: behavioral analyses As shown in Fig. 2, males in the SAL group chose to ejaculate first more with the ScF, whereas males in the NAL group clearly preferred more the UnScF. A 2 × 2 χ^2 between groups showed a statistically marginally different pattern to choose different females to ejaculate first with, $\chi^2(1) = 1.818$, $p = 0.178$, $\varphi = 0.201$.

The analyses of the remaining variables (i.e., mounts, intromissions, ejaculation latency, or the mean of ejaculation) found no interactions between the factors, as well as no differences in the χ^2 1 × 2 analyses.

Infusions of NAL or SAL to VTA: Fos-IR analyses As shown in Fig. 3, when analyzing, the VTA of males infused with NAL into the VTA showed a lower Fos-IR mean count than the SAL control group, $t(8) = 3.237$, $p = 0.014$, $d = 2.172$, whereas no differences were found in their mPOA, $t(8) = 0.289$, $p = 0.780$, $d = 0.178$.

Somatosensory conditioning

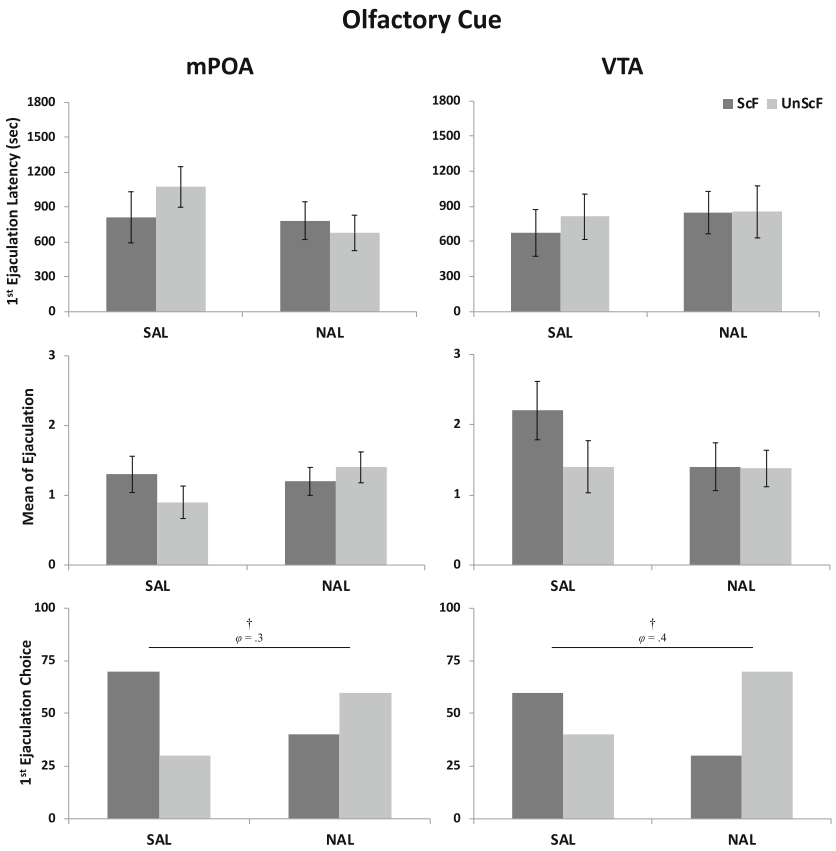
Infusions of NAL or SAL to the mPOA: behavioral analyses Figure 3 shows the scores for the different copulatory behaviors by female and by brain area for both groups during the open-field test.

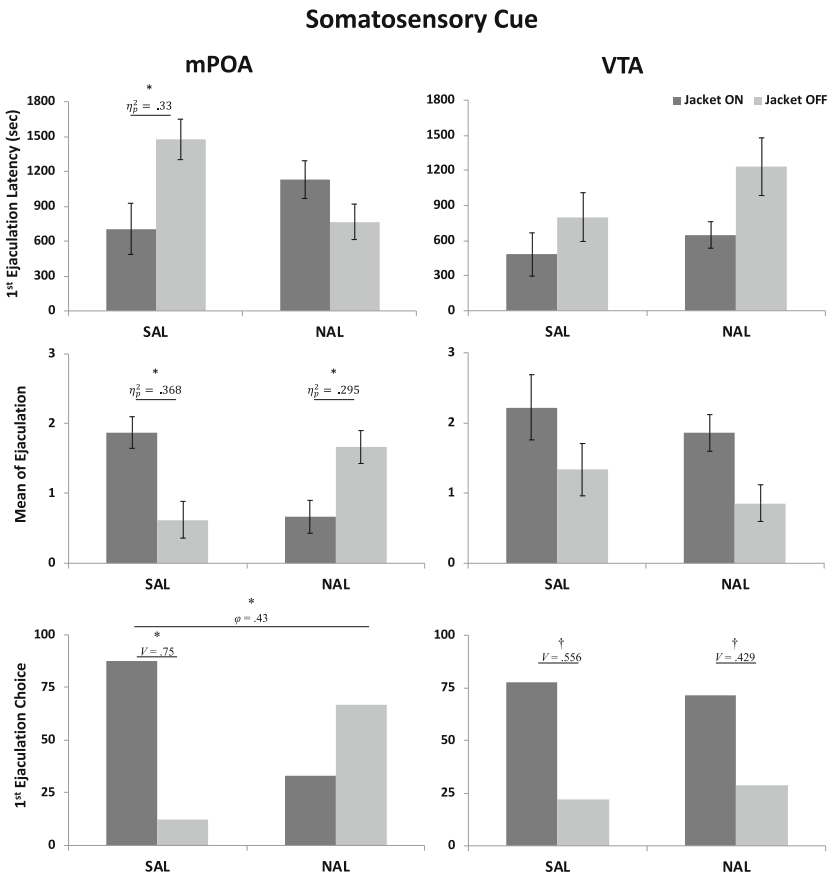
Males in the SAL group ejaculated first faster with the unjacketed females, whereas the opposite was true for males in the NAL group, $F(1,15) = 8.478$, $p = 0.011$, $\eta_p^2 = 0.361$. Bonferroni post hoc analyses revealed that males in the SAL displayed a statistically significantly lower mean first ejaculation latency for the jacketed female ($M = 706.13$, $SD = 463.593$) than for the unjacketed one ($M = 1129.11$, $SD = 617.017$, $p = 0.016$, $\eta_p^2 = 0.33$), whereas males in the NAL group had the opposite pattern of first ejaculation latency, yet did not reach statistically significant differences, $p = 0.194$, $\eta_p^2 = 0.11$. Males in the SAL group ejaculated more with the jacketed female whereas males in the NAL group ejaculated more with the unjacketed female, $F(1,15) = 14.959$, $p = 0.002$, $\eta_p^2 = 0.499$. Bonferroni post hoc analyses revealed that males in the SAL displayed a statistically significantly higher mean of ejaculation towards the jacketed female ($M = 1.88$, $SD = 0.641$) than for the unjacketed one ($M = 0.67$, $SD = 0.707$, $p = 0.01$, $\eta_p^2 = 0.368$), whereas males in the NAL group displayed a statistically significantly higher mean of ejaculation towards the unjacketed female ($M = 1.67$, $SD = 0.707$) than for the jacketed one ($M = 0.63$, $SD = 0.744$, $p = 0.024$, $\eta_p^2 = 0.295$).

Finally, the 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 opposite was true for males in the NAL group. Indeed, males in the SAL group significantly preferred to ejaculate first more with the jacketed female, $\chi^2(1) = 4.5$, $p = 0.034$, $V = 0.75$. Furthermore, a 2 × 2 χ^2 revealed a statistically significantly different pattern of preference to ejaculate first for both groups, $\chi^2(1) = 5.13$, $p = 0.024$, $\varphi = 0.43$.

Infusions of NAL or SAL to the mPOA: Fos-IR analyses As depicted in Fig. 3, when analyzing, the mPOA of males infused with NAL into the mPOA showed a marginally lower, yet of large magnitude, Fos-IR mean count than the SAL control group, where the SAL group ($M = 206.58$, $SD = 70.21$) had a higher Fos-IR count than the NAL group ($M = 136.53$, $SD = 17.53$, $t(9) = 2.157$, $p = 0.059$, $d = 1.313$). When analyzing, the VTA of males infused with NAL into the mPOA showed a marginally lower, also of large magnitude, Fos-IR mean count than the SAL control group, where the SAL group ($M = 28.33$, $SD = 7.5$) had a higher Fos-IR count than the NAL group ($M = 17.3$, $SD = 11.46$, $t(9) = 1.925$, $p = 0.086$, $d = 1.165$).

Infusions of NAL or SAL to the VTA: behavioral analyses As shown Fig. 2, the χ^2 analysis for the percentage of first ejaculation choice shows that both groups have a similar pattern of preference to ejaculate first more with the jacketed female, $\chi^2(1) = 0.085$, $p = 0.771$, $\varphi = 0.073$. The analyses of the remaining variables (i.e., mounts, intromissions, ejaculation latency, or the mean of ejaculation) found no interactions between the factors, as well as no differences in the 1 × 2 χ^2 analyses.


Olfactory Cue
mPOA
VTA
ScF
UnScF
1st Ejaculation Latency (sec)
Mean of Ejaculation
1st Ejaculation Choice
SAL
NAL
† φ = .3
† φ = .4


Somatosensory Cue
mPOA
VTA
Jacket ON
Jacket OFF
1st Ejaculation Latency (sec)
Mean of Ejaculation
1st Ejaculation Choice
SAL
NAL
* η²p = .33
* η²p = .368
* η²p = .295
* φ = .43
* V = .75
† V = .556
† V = .429

◀ **Fig. 2** Mean of 1st ejaculation choice, mean of ejaculation, and 1st ejaculation choice per brain area (columns), group, and experimental cue during the open-field test. †$p < 0.01$; *$p < 0.05$. V = Cramer's V, η_p^2 = partial eta square, φ = phi

Infusions of NAL or SAL to the VTA: Fos-IR analyses As depicted in Fig. 3, when analyzing, the mPOA of males infused with NAL into the VTA showed no difference in their Fos-IR mean count than the SAL control group. An independent sample *t* test analysis revealed there were no statistically significant differences in the Fos-IR count between the SAL

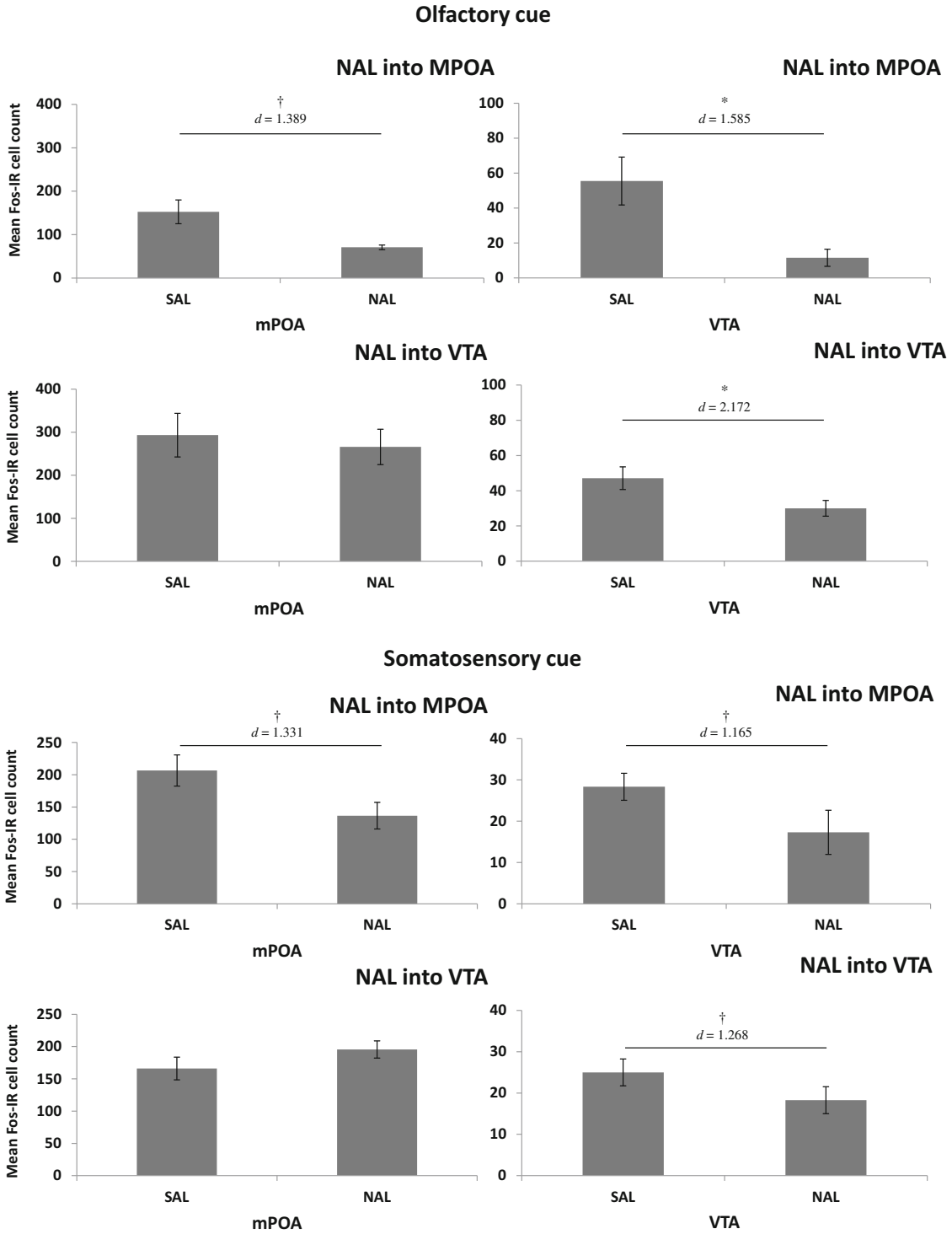


Fig. 3 Fos-IR-positive cells summary by brain area, group, and experimental cue. †$p < 0.01$; *$p < 0.05$. *d* = Cohen's *d*

group ($M = 166.07$, $SD = 55.91$) and the NAL group ($M = 195.5$, $SD = 35.9$, $t(8) = 0.991$, $p = 0.351$, $d = 0.626$).

When analyzing, the VTA of males infused with NAL into the VTA showed a marginally lower, yet of large magnitude, Fos-IR mean count than the SAL control group, where the SAL group ($M = 25$, $SD = 6.01$) had a higher Fos-IR count than the NAL group ($M = 18.27$, $SD = 4.5$, $t(8) = 2.005$, $p = 0.08$, $d = 1.268$).

Discussion

The present study evaluated the role of opioid receptor antagonism in the mPOA and VTA of male rats trained to associate either an olfactory cue (almond) or somatosensory cue (rodent jacket) with their post-ejaculatory reward state. Males given SAL during training showed significant CEP for the odor or jacket, whereas males given NAL during training showed a differential response depending on the site of infusion: NAL infusions to the mPOA during training shifted the preference to the novel (unscented or unjacketed) female, whereas NAL infusions to the VTA abolished CEP altogether. Subsequent detection of Fos protein induced in these regions by the odor alone showed that prior experience with NAL infusions to the mPOA suppressed Fos in both the mPOA and VTA compared to males infused with saline, whereas prior experience with NAL infusions to the VTA suppressed Fos in the VTA alone.

Copulatory behaviors and particularly ejaculation increase whole brain opioid content and receptor internalization in the caudate, hypothalamus, and midbrain of male rats (Szechtman et al. 1981; Arletti et al. 1997; Rodríguez-Manzo et al. 2002; Coolen et al. 2004). Ågmo and Berenfeld (1990) showed that male rats who associated their post-ejaculatory period with a particular side of a three-compartment chamber later on spend more time in it over other sides of that chamber just like males who were only injected with morphine, an effect that was reversed with NAL (Ågmo and Berenfeld 1990). Furthermore, copulation to ejaculation has also been used as an unconditional stimulus to train a neutral stimulus as a predictor of sexual reward, which ultimately leads to a CPP or CEP (Tenk et al. 2009; Kippin and Pfaus 2001). This trained preference shifts towards the unfamiliar female in males who are injected systemically with NAL using an olfactory (Kippin and Pfaus 2001) or somatosensory cue (Quintana et al. 2019b). Together, these data indicate that blockade of opioid receptors results in a shift of male preference to cues not associated with the antagonist state, further reinforcing the notion that opioid receptor antagonism induces an aversive state (Parker and Rennie 1992).

Opioid receptors exist in high density in the mPOA and VTA (Simerly et al. 1988). Copulation to ejaculation increases μ-receptor internalization in the mPOA (Coolen et al. 2004) and VTA (Balfour et al. 2006), effects that are prevented by injections of NAL before copulation. Although both μ- and δ-receptor internalization occur in the VTA as a consequence of copulation to ejaculation (Garduño-Gutiérrez et al. 2013), only μ-receptor internalization was correlated with the amount of sexual activity. Infusions of μ agonists like morphine or morphiceptin to the mPOA inhibit male rat sexual behavior (Band and Hull 1990; Matuszewich and Dornan 1992; Matuszewich et al. 1995), and infusions of NAL to the mPOA, but not the NAc, disrupt a sexually conditioned place preference in male rats (Ågmo and Gómez 1993). Likewise, infusions of β-endorphin into mPOA impaired sexual behavior in male rats (van Furth et al. 1995a, b) and produced a refractory-like state (Hughes et al. 1987, 1990) NAL treatment prevented this impairment. Taken together with the present findings, opioid activation of μ receptors in the mPOA appears to produce a sexual reward state.

The VTA is the origin of the mesocorticolimbic DA system which plays a major role in motivated behaviors (Fibiger and Phillips 1988; Le Moal and Simon 1991; Blackburn et al. 1992; Wise 2005; Hull and Rodriguez-Manzo 2009) through the control of the attention towards reward-related stimuli and their incentive salience (Balfour et al. 2004; Berridge 2007). In contrast to their effects in the mPOA, infusions of μ-receptor antagonists to the VTA either facilitate sexual behavior in sexually naïve males (Mitchell and Stewart 1990) or have no effect in sexually experienced males (van Furth and van Ree 1996). This suggests that the sensitization of DA systems as male rats acquire sexual experience (Fiorino and Phillips 1999a, b) may be mediated by opioid turnover in general, and binding to μ receptors in particular (van Ree et al. 2000; Balfour et al. 2004). Moreover, connections from the mPOA to the VTA (Brackett et al. 1986; Edwards and Einhorn 1986) are responsible for drug reward-related locomotion (Tobiansky et al. 2013; Will et al. 2016) and modulate the sexual reward value of a sexually receptive female (Lyilikci et al. 2017). Indeed, DA release patterns in the mPOA and NAc are virtually identical (Blackburn et al. 1992), and this activation increases the incentive value of the reward-related cue (Berridge 2007; Pfaus et al. 2012). Thus, we suggest that opioid activation in the mPOA underlies both refractoriness and the sexual reward state, whereas opioid activation in the VTA modulates the incentive value of the reward-related cue, regardless of its sensory modality.

A differential modulation of Fos induction by the cues following NAL infusions to the mPOA versus the VTA is in accordance with the previous literature. Connections from the mPOA to the VTA have been established in both male and female rats (Brackett et al. 1986; Edwards and Einhorn 1986; Graham et al. in press), and Edwards and Einhorn (1986) suggested that outputs from the mPOA to the midbrain in male rats (including both dorsolateral tegmentum and VTA) modulated the sexual reward value of a sexually receptive female. The origin of these connections was localized mainly in the

rostral portion of the mPOA, traced back to the mesolimbic DA system, and shown to be mainly GABAergic and also sensitive to DA (i.e., co-localization of DA receptors, Tobiansky et al. 2013). Another study in males that mapped the GABAergic and glutamatergic projection from hypothalamic nuclei to the VTA through retrograde labelling showed that nearly 24% of the projecting neurons were found in the preoptic area, and nearly 77% in the anterior tuberal and mammillary areas of the hypothalamus (Kallo et al. 2015). Furthermore, estradiol benzoate has also shown to regulate GABA cell containing D1-like DA receptors in the mPOA projecting into the VTA of female rats, which indicates a modulating effect on the expression of DA receptors that ultimately regulates copulatory and proceptive behaviors in the female rat (Graham et al. in press). Considering that other studies have shown the role of the mPOA in the execution of copulatory behaviors (Hull and Rodriguez-Manzo 2009), these differences in the control of appetitive vs. consummatory mPOA-VTA connections likely depend on subregions of the mPOA. For example, rostral regions appear to modulate appetitive behaviors, whereas more caudal regions appear to control consummatory behaviors in the male rat (Balthazart and Ball 2007). Ultimately, these findings support the idea that the mPOA modulates the mesolimbic DA system by controlling the activation of dopamine neurons in the VTA, which in modulate the incentive value of the reward-related cue (Fields and Margolis 2015; Micevych and Meisel 2017; Wise 2009), as both the behavioral and Fos-IR outcomes of the present study suggest.

A crucial difference between the olfactory and somatosensory cue was found in the behavioral analyses, yet not through the Fos-IR. On the one hand, male rats micro-infused with NAL into their VTA while being conditioned to prefer females with the jacket still displayed a CEP towards the jacketed females, just like males micro-infused with SAL. In contrast, micro-infusion of NAL into the mPOA disrupted the CEP relative to males infused with SAL to the mPOA. On the other hand, while using the same training conditions with the olfactory cue, males micro-infused with NAL into either the VTA or mPOA did not display a CEP for the scented females, unlike their respective SAL control groups. It was expected that this would have resulted in a different pattern of Fos activation. However, the pattern of Fos-IR results between the two cues was exactly the same, where males in the NAL groups yielded a lower Fos-IR count when infused either into the mPOA or VTA compared to males infused with SAL, but also a lower Fos-IR count in the VTA when NAL was infused into the mPOA, yet not vice versa. Watabe-Uchida et al. (2012) delineated a whole-brain map of the direct excitatory inputs to the midbrain DA neurons, VTA, and the substantia nigra past compacta. This work demonstrated that, among several other brain regions, the somatosensory and motor cortex send direct inputs (i.e., higher density and number of neurons) mainly to the SNc. Thus, the fact that males micro-infused with NAL into their VTA still displayed a CEP towards the jacketed female suggests that this cue may require input from other brain areas (e.g., somatosensory cortex). Opioid receptors are located in somatosensory cortex in different quantities depending on the subtype of receptor (Mansour et al. 1988, 1994). Particularly, the pattern of receptor binding in the neocortex of the rat brain demonstrated that both μ and δ receptor are more prevalent than κ receptors, while their precise distributions differ greatly among the different layers. The μ-subtype receptor appeared most prominent in layers I and III/IV of the frontal, parietal, and temporal cortex, whereas δ receptors tend to be diffusely localized in layers II, III, V, and VI (Mansour et al. 1994). Therefore, to disrupt the development of a CEP based on an olfactory cue, micro-injections of NAL into either the mPOA or VTA are necessary and sufficient, while for a somatosensory cue, the disruption of a CEP by NAL micro-infusions is sufficient and necessary in the mPOA, while in the VTA is not, suggesting the involvement of other brain regions.

A possible alternative explanation to account for the disruption of the CEP by NAL is a hindering of familiarity and not reward. In previous studies using conditioned place preference (CPP), NAL not only disrupts, but also shifts the preference towards the opposite side of the chamber (e.g., Lett et al. 2001). To test this hypothesis properly, CPP boxes would need a middle "neutral" compartment that is not associated with any training condition. Thus, a CPP disrupted by NAL based on reward should result in a higher time spent in the opposite side, whereas a disruption based on familiarity should result in a more evenly distributed time between the opposite side and the middle compartment of the chamber. Many drug reward studies use a ratio between the time spent in the drug-associated side over the time spent in the other side of the chamber, assessing only the time spent in the "opposite" compartment (e.g., Hasenöhrl et al. 1991; Spyraki et al. 1985; Trujillo et al. 1991). We note that NAL disrupted a copulatory reward-based CPP in male rats (Mehrara and Baum 1990), and animals in that study spent very little time in the middle compartment during the test phase. It is thus most likely that NAL disrupts preference by disrupting reward rather than familiarity. Indeed, males injected with NAL throughout the development of a CEP based on either an odor (Ismail et al. 2009) or a jacket cue (Quintana et al. 2019b) also displayed a preference for females without the cue during the preference test rather than no preference for either female.

In summary, opioid antagonism in the mPOA appears to produce a state of non-reward, whereas in the VTA, such antagonism produces a state in which the odor has no incentive properties. Thus, opioids modulate CEP by multiple pathways that convey both the incentive value and valence of the reward-related odor CS, regardless of the neutral cue used, but depending where in the brain they act.

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Compliance with ethical standards

The authors declare that all animal procedures conformed to the guidelines of the Canadian Council for Animal Care. All procedures were approved by the Concordia University Animal Research Ethics Committee.

Conflict of interest The authors declare they have no conflicts of interest.

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