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Fos expression is increased in oxytocin neurons of female rats with a sexually conditioned mate preference for an individual male rat

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ABSTRACT

Evidence suggests an important role of Pavlovian learning in sexual partner selection. Female rats that experience paced copulation with a male scented with a neutral odor selectively solicit and receive ejaculations from the scented male relative to an unscented male. Exposure to the conditioned odor alone induces Fos protein in regions of the brain associated with sexual excitation. Here we tested whether female rats can be conditioned to show a sexual preference for an unscented male rat of the same strain. Female Long-Evans rats were given 10 copulatory trials with either a one-hole pacing divider or a four-hole pacing divider in a unilevel chamber with the same conspecific male (n = 16). Females were then given an open-field partner preference test with the paired male versus a novel male. After two reconditioning trials females were exposed to the partner or a novel male to induce Fos expression. Females that paced with the one-hole divider received the first ejaculation and more ejaculations overall from the paired compared to novel male. Fos immunoreactivity within oxytocin neurons in the PVN, mPOA, and VMH was increased in females with a preference that were exposed to the paired male. These data indicate that female rats can form selective sexual preferences for an individual conspecific and that their formation depends on the type of pacing during conditioning. These findings further suggest the involvement of oxytocin in the display of conditioned preferences. Thus, early copulatory experience appears to determine the mating strategy used by female rats.

1. Introduction

In natural and semi-natural environments rats are generally considered promiscuous copulators. For example, within a group mating situation female and male rats change sexual partners regularly (McClintock, 1984). In contrast, monogamous prairie voles form long lasting breeding pairs and close social bonds (Getz et al., 1981). Pair-bonded prairie voles care for young offspring together, and are aggressive toward unfamiliar conspecifics, a behavior termed mate guarding (Getz et al., 1981; Carter et al., 1995). However, despite evidence of promiscuous copulation in rats, females exercise selectivity in their choice of male sexual partners. In the rat, female choice has an important influence on mating success such that a female can determine from which male it receives an ejaculation (McClintock and Adler, 1978), and females compete to receive the ejaculation of a dominant male. Indeed, in a group mating situation, dominant females have been observed to intercept a preferred male immediately prior to his ejaculation, and to take ejaculations selectively from a preferred male (McClintock, 1984). Copulatory experience and learning can also affect female sexual partner choices. For example, female rats can form sexual preferences for partner cues (e.g., odor, strain) and places associated with sexual reinforcement (Coria-Avila et al., 2005; Coria-Avila et al., 2006). This suggests that neural systems for sexual preferences based on novelty and familiarity exist and are modifiable by experience (Pfaus et al., 2012).

Sexually conditioned preferences depend on the pairing of a salient cue with sexually reinforcing sexual simulation (Paredes and Alonso, 1997; Coria-Avila et al., 2005; Coria-Avila et al., 2006). For female rats, the ability to pace the initiation rate at which they receive penile intromission (and thus both clitoral and cervical stimulation) is reinforcing. When female rats are allowed to pace their copulatory interaction with males, both place and partner preferences can be formed (Paredes and Alonso, 1997; Jenkins and Becker, 2003; Coria-Avila et al., 2005), indicating that paced sexual stimulation is rewarding. Neutral odor cues, such as almond or lemon scent, or strain cues, such as those that exist between albino and pigmented strains, have been used to

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condition sexual partner preferences (Coria-Avila et al., 2005; Coria-Avila et al., 2006). Females are given repeated, multiejaculatory paced copulations with either a scented male or one of a specific strain. When females are given the choice on a final test to copulate with a male bearing the pacing-related cue and a novel male, females solicit the pacing-related male more, and often choose from which male it receives ejaculations (Coria-Avila et al., 2005; Coria-Avila et al., 2006). Thus, the cues that were present during the early experience of paced copulation come to guide female sexual partner choice.

Although much has been learned about the neurobiological basis of pair bonds in the prairie vole, the mechanisms by which sexual preferences for familiar cues are formed in the female rat are not fully understood. Pacing-related odor cues have been shown to increase Fos immunoreactivity (Fos-IR), a marker of neuronal activation, in the piriform cortex (PirCtx), the Nucleus Accumbens (NAc), the medial preoptic area (mPOA), the paraventricular nucleus of the hypothalamus (PVN), and the ventral tegmental area (VTA; Coria-Avila and Pfaus, 2007). In contrast, when females were conditioned with a particular strain of rat, cues from a male of that strain increased Fos-IR in the PirCtx, mPOA, the corticomedial amygdala (CoA), VTA, and the ventromedial hypothalamus (VMH). Thus, although both pacing-related odor and strain cues activate some brain areas in common, the sensory nature of the pacing-related cue appears to differentially activate other brain areas. Importantly, both odor and strain cues increased Fos in areas that have also been shown to be activated during partner preference formation in mated female prairie voles, i.e. the mPOA, NAc, and CoA (Curtis and Wang, 2003).

Previous studies that conditioned partner preferences in female rats have used either salient odor cues or strain cues during conditioning (Coria-Avila et al., 2005; Coria-Avila et al., 2006). Female prairie voles form preferences for individual conspecific males (Getz et al., 1981) and female rats are also capable of forming such preferences. Holley et al. (2014) gave ovariectomized hormonally-primed sexually naïve female rats either 10 paced or non-paced copulatory trials with one particular male conspecific each. Females were given a test in an open field with their paired male in the presence of a sexually receptive intruder female. Females that received paced copulation with the same conspecific male showed three characteristic mate-guarding behaviors in the presence of the intruder female: hovering next to the male and presenting a pre-lordosis crouch, blocking access of the approaching intruder female by imposing themselves between it and the male, and agonistic mounting of the intruder female if it made an active solicitation (headwise orientation and runaway) of the male. Females that did not pace copulations during training did not display these behaviors. Moreover, paced females solicited and received more ejaculations from the pacing-associated males compared to non-paced females. Mate guarding has typically been associated with monogamous animals, such as the prairie vole. However, these findings indicate that female rats can be conditioned to mate guard conspecific males that are associated with paced copulation.

Mate-guarding females exposed to their conspecific males had increased Fos-IR in a number of brain regions, including the paraventricular nucleus (PVN), and the supraoptic nucleus (SON) of the hypothalamus. Both the PVN and SON contain large populations of parvocellular and magnocellular neurons that synthesize oxytocin (OT) or vasopressin (AVP) and send their projections to the rest of the brain or to the posterior pituitary, respectively (Swanson and Sawchenko, 1983). These neuropeptides have been shown to regulate pair bonding in prairie voles (Cho et al., 1999). Interestingly, Holley et al. (2015) pre-treated females with OT, AVP, or saline, during their first paced copulatory conditioning trial. Four days later, females were then given an open-field test with an intruder female and either the familiar male or a novel male. OT-treated females increased their hovering and presenting behaviors, whereas AVP-treated females increased their blocking behaviors, compared to saline-treated females. Females presented with a novel male competed with the intruder female as has been described previously (McClintock, 1984). Additionally, Fos-IR was increased in OT and AVP neurons in the PVN and SON of mate-guarding females. Thus, central OT or AVP transmission may facilitate different aspects of mate guarding, with OT facilitating the formation of sexually conditioned preferences and affiliation, and AVP affecting the actual mate-guarding behavior of the female.

In previous studies of sexually-conditioned partner preferences in female rats (Coria-Avila et al., 2005; Coria-Avila et al., 2006), preference was assessed in a large open field with two males, one that had the cue (i.e. odor or strain) associated with pacing, and a novel male. In the absence of an externally applied odor like almond scent, it is not clear which cues guide female approach, solicitation, and selective choice of ejaculation. The strain of a male might be signalled by strain differences in certain pheromonal cues, such as major histocompatibility complexes (MHCs), the visible pigmentation of the male, and/or differences in ultrasonic vocalisation. Although the conditioned mate guarding studies of Holley et al. (2014, 2015) demonstrate that females can differentiate between two unscented males of the same strain, it is unclear whether females express this only in the presence of a competitor female, or whether they could show this preference in a copulatory choice test between two males of the same strain. Therefore, the aim of the current study was to assess whether female rats can be conditioned to prefer a conspecific male associated with paced copulation over a novel conspecific of the same strain. Additionally, we also examined whether sexually conditioned females show increased Fos-IR in OT neurons in areas previously associated with selective copulatory behaviors in the female rat.

2. Methods

2.1. Animals

Thirty-two Long Evans female rats weighing 175–200 g purchased from Charles River were used in this experiment (Charles River, St-Constant, Qc, Canada). Females were sexually naïve. Thirty-two sexually vigorous Long-Evans males were used as sexual stimuli. The male rats were used in a previous experiment. All animals were housed in the Animal Care Facility at Concordia University at a constant temperature and humidity and given ad libitum access to standard laboratory chow (Charles River #5075, Montreal, Canada) and water. Female rats were paired-housed in Plexiglass shoebox cages and maintained on a 12 h:12 h reverse light cycle (8:00 pm lights on). All procedures had ethical approval from the Concordia University Animal Research Ethics Committee.

2.2. Ovariectomy

Females were bilaterally ovariectomized to allow for the control of sexual receptivity with exogenous steroid hormone administration. Females were anaesthetized with a 4:3 mixture of ketamine hydrochloride (100 mg/ml; Ketaset, Wyeth Cananda) and xylazine hydrochloride (20 mg/ml; Rompum, Bayer Healthcare). Females were injected intraperitoneally with the ketamine: xylazine mixture at a dosage of 1 ml/kg. Ovaries were removed through a lower-lumbar incision. Following surgeries, females were subcutaneously injected with 0.1 ml Penicillin G (PenG, antibiotic), and given a non-steroidal anti-inflammatory drug, Ketoprofen (Anafen 100 mg/ml, Boehringer Ingelheim) at a dosage of 0.03 ml. Ketoprofen was administered at the same dosage for the next 3 days to provide analgesia to the female rats. Females were given one week to recover in their home cage before testing began.

2.3. Conditioning apparatus

Females were sexually conditioned in Plexiglass unilevel pacing chambers (Height 38 cm × Width 60 cm × Length 38 cm). Chambers

contained a metal wire-mesh grid as a floor covered with a layer of woodchip bedding. Each conditioning chamber included a divider with either one hole or four holes (4 cm × 4 cm) large enough that the female could pass through but too small for the male to enter. The inclusion of the divider gave female rats the ability to pace copulation in the chamber with either the one-hole or four-hole divider, as has been done previously (Ismail et al., 2009).

2.4. Conditioning procedure

Sexually naïve females were randomly assigned to paced copulation with either the one-hole or four-hole pacing condition ($n = 16$ per pacing group). Each female rat was paired with a specific male sexual partner. Females only copulated with the paired male during sexual conditioning trials. Forty-eight hours prior to conditioning trials, females were subcutaneously injected with estradiol benzoate (EB) (10 μg in 0.1 ml sesame oil), followed by progesterone (P) (500 μg in 0.1 ml sesame oil) 4 h before sexual conditioning. Hormonal priming with this dosage of EB and P has previously been shown to induce sexual receptivity (Jones et al., 2013). All conditioning trials were held during the middle third of the rats' dark circadian phase (2:00 pm to 4:00 pm). Female rats underwent ten sexual conditioning trials with the same male rat. All conditioning trials lasted 30 min and occurred once every four days for 10 trials. Before the commencement of each conditioning trial males were habituated to the conditioning chamber for 5 min. Reagent grade sesame oil was purchased from Sigma Aldrich (Sigma Aldrich, Canada, Lot # MKBR2026V). Both EB and P were supplied by Steraloids INC (Newport, RI, USA).

2.5. Partner preference test

After ten sexual conditioning trials female rats were given a sexual partner preference test. In this test females could copulate freely with the paired male from the prior conditioning trials or a novel male. The partner preference test was held in an open field apparatus (123 cm × 123 cm × 46 cm) with woodchip bedding covering the floor. The paired and novel males were tethered to opposite corners of the open field via a 30 cm metal spring attached to a small jacket as described in Coria-Avila et al. (2005). The tethered males had a roaming range of approximately 45 cm. Females were placed into a neutral corner of the open field and the partner preference test lasted 30 min. All trials were recorded with ceiling-mounted Sony Handicam and scored by a researcher who was blinded to the male type (paired or novel) and pacing condition.

The frequency of solicitations, hops and darts, and visits (entering the roaming space of the male) made toward the paired male and novel male by each female was recorded. The number of ejaculations the female received from each male was scored, as well as the female's choice of male from which the female received the first ejaculation in the copulatory series. The time each female spent in each of the male's roaming space was also recorded.

2.6. Tissue preparation

Females were given two reconditioning trials after the partner preference test. This was done to re-establish the association between the paired male and paced copulation because females also copulated with males that were not the paired male rat during the preference test. The reconditioning trials were conducted identically to the trials prior to the preference test.

To induce Fos protein, female rats from both pacing conditions were exposed to either the paired male rat or a novel male rat in the unilevel pacing chamber, $n = 5$ per group × 2 (1-hole vs 4-hole), × 2 (Paired vs Novel). Physical interaction was restricted by the inclusion of a wire mesh in place of the one/four-hole divider, allowing olfaction and auditory stimulation from the male. Females did not copulate as we aimed to induce Fos protein expression to a male associated with prior pacing. Exposure to a novel male was used as a control to compare Fos expression to a pacing associated- vs non-associated male. After 1 h of exposure to a male, females rested for 15 min. Females were injected with an overdose of sodium pentobarbitol (120 mg/kg; Euthanyl) and perfused intracardially with 250 ml of phosphate buffered solution followed by 250 ml of 4% paraformaldehyde. The brains were removed and post-fixed in 4% paraformaldehyde for 4 h, after which the brains were dehydrated in a 30% sucrose solution for 48 h. Brains were then wrapped in aluminium foil and flash frozen on dry ice and stored at −80 °C until sectioning. Brains were coronally sectioned at a thickness of 30 μm using a Leica microtome. Five brains from each condition were sectioned and slices from the mPOA (B 0.00 to −1.32), PVN (B-1.08 to −1.80), SON (B-0.48 to −1.72), VTA (B-5.28 to −6.84), VMH (B-2.28 to −3.12) were selected for immunohistochemistry. The Paxinos and Watson (2006) rat brain atlas was used to identify brain regions. All sections were co-labelled for oxytocin and Fos protein expression.

2.7. Immunohistochemistry and quantification

Sections were washed in fresh 0.9% tris-buffered saline (TBS; 22.27 mmol Trizma Hydrochloride and 1.651 mmol Trizma Base in 0.9% Saline), and quenched in 30% H_20_2 and TBS for 30 min at room temperature. Sections were then pre-blocked with 3% Normal Goat Serum (NGS) in 0.2% Triton-TBS for 2 h at room temperature. Tissue sections were incubated with the primary polyclonal anti-rat Fos antibody made in rabbit (1:20,000, Synaptic Systems, 226 003) with 3% NGS in 0.05% Triton-TBS for 72 h at 4 °c. The sections were then incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories Canada, Burlington, ON; 1:200) in 3% NGS and 0.2% Triton TBS for 1 h at 4 °C. Sections were then incubated sequentially in 0.05% Triton-TBS with 3% NGS and avidin-biotinylated-peroxidase complex (Vectastain *ELITE*® ABC KIT, Vector Laboratories Canada; diluted 1:55) for 2 h at 4 °c. Between each incubation sections were washed three times in cold TBS for 5 min.

The sections were stained using a 3,3′-diaminobenzidine (DAB) to react the peroxidase, and nickel chloride to turn the nuclear reaction product blue-black. Sections were first washed in 50 mM Tris for 10 min, followed by DAB in 50 mM Tris (0.5 mg/ml) at pH 7.6 for 10 min. Finally, 3% H_20_2 (0.1 ml per 100 ml of DAB solution) was added with 8% nickel chloride (400 μl per 100 ml of DAB/Tris buffer/H_20_2) and the sections were washed in the solution for 10 min. The reaction was stopped by rinsing in cold TBS. To double label cells for cytoplasmic OT, the above steps were repeated, with the exception of the quenching and pre-blocking phase. Sections were incubated in a rabbit anti-rat oxytocin antibody (AB911; Millipore Sigma, 1:10,000) for 72 h. During the DAB reaction, nickel chloride was not added which gave a reddish-brown stain to cytoplasmic OT-IR.

Sections were mounted onto gel-coated slides. Mounted sections were dehydrated with a 1-min wash in nanopure distilled water, then 10-min each in 75% ethanol, 90% ethanol, and 99% ethanol, followed by 2 h in xylenes. The slides were then coverslipped using Permount (Fisher Scientific, SP15-500).

Photomicrographs of all brain regions of interest were captured using an Olympus light microscope at 20× magnification using Q-Capture Pro software. On average, 3 to 5 bilateral sections of each brain region of interest per rat were included in the analyses. Fos IR cells were identified by the dark-brown/black nuclear stain, and OT IR cells identified by the reddish-brown cytoplasmic stain (Fig. 3). When the black Fos IR was found within an OT IR cell, it was counted as a co-labelled Fos/OT cell (see Fig. 3). ImageJ was used to identify OT IR cells. However, all identified OT-IR cells with Fos IR was counted manually by a researcher blinded to the conditions. Cell counts are reported as the number colabelled OT- and Fos-IR cells/mm^2.

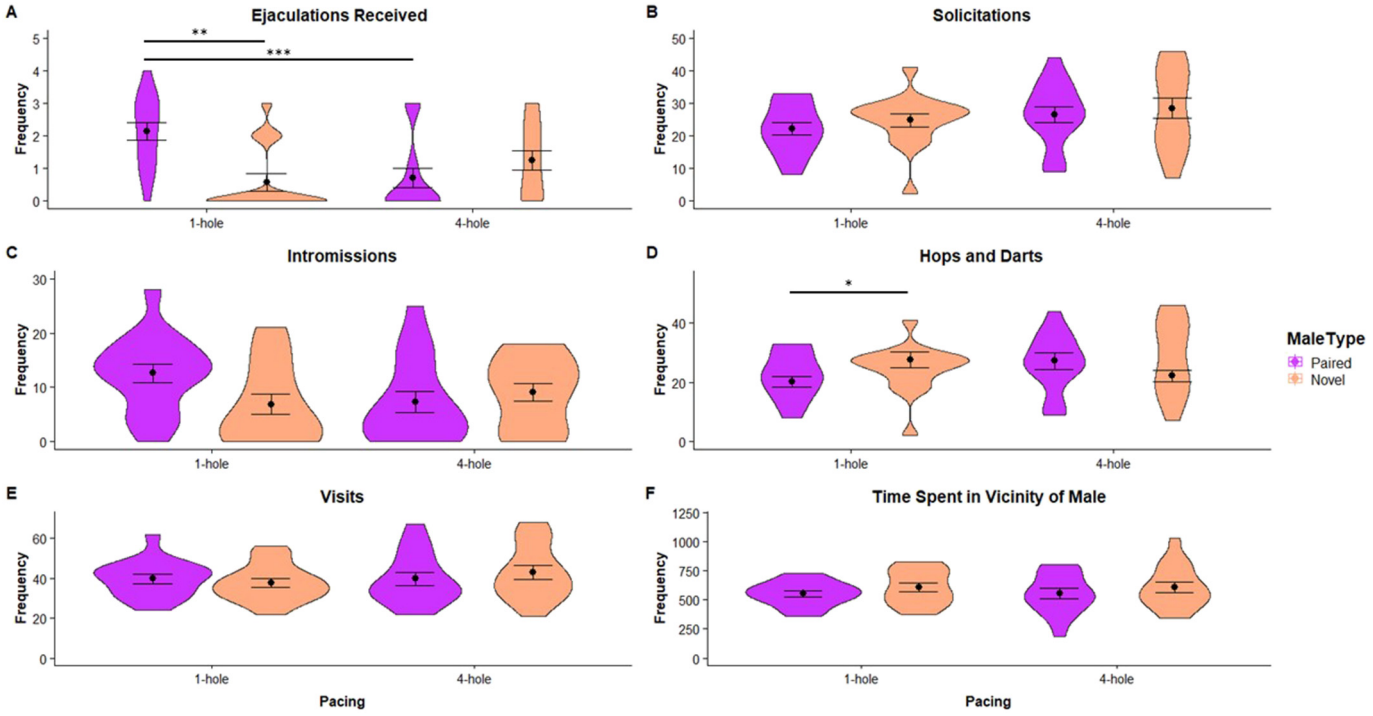

Fig. 1. The distribution of data for each group is represented using violin plots. Black dots and bars represent the mean and standard error for each group. A) Displays the number of ejaculations received by a female from a paired and novel male. B) Displays the frequency of solicitations made by females toward the paired and novel males across pacing conditions. C) The number of intromissions received by each female for the paired and novel male in both pacing conditions. D) The number of hops and darts made by females toward a paired and novel male. E) No difference in visits, entries to a males' (paired and novel) roaming, ranges were observed. F) Across both pacing conditions, females did not spend time with the paired male over the novel male. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .005$.

2.8. Data analysis

Data were analysed with R software Version 3.5.1 (R Development Core Team, 2018) through R Studio Version 1.1.456 (RStudio Team, 2016). All data and analysis scripts can be accessed at Open Science Framework, https://osf.io/ty9ma/. Video recordings from open field partner preference tests were scored by a researcher blind to both the pacing group of the female and the male to which the female directed her sexual behaviors. The female's choice of male for first ejaculation was calculated and analysed using a χ^2 test through base R (R Development Core Team, 2018). *p*-Values from χ^2 tests were generated with a Monte Carlo simulation with 20,000 iterations, hence, as with all simulated *p*-values, degrees of freedom are not reported for these tests. All other behavioral data were analysed using mixed between-within 2×2 analysis of variance (ANOVAs). Each female's behavior toward the paired or novel male was treated as a within subject condition, a factor referred to as male type. The pacing group was a between-subjects condition in the mixed ANOVAs, with two levels, one-hole and four-hole. ANOVAs were conducted using the Analysis of Factorial Experiments (afex) package (Singmann et al., 2018). Levene's test, to assess the assumption of homogeneous variances was conducted with the car package (Fox and Weisberg, 2011). The assumption of homogeneous variances was not met for the number of solicitations but was met for all other ANOVA models.

Estimated marginal means for the ANOVA models were calculated using the emmeans: Estimated Marginal Means, aka Least-Squares Means package (Lenth et al., 2018). Planned comparisons were used to compare the means of behaviors made toward each male within each pacing condition, and also to contrast behaviors made to the paired male and the novel male across pacing conditions (1-hole vs 4-hole). The comparisons were as follows: 1) 1-hole: Paired vs Novel. 2) 4-hole: Paired vs Novel. 3) Paired Male: 1-hole vs 4-hole. 4) Novel male: 1-hole vs 4-hole. Multiple comparisons were adjusted for using the Holm correction (Aickin and Gensler, 1996). The same comparisons were used to compare Fos/OT-IR across groups. Generalised η^2 was calculated as an effect size and reported for all significant F-tests. Hedge's G_{avg} was used as an effect size for the planned comparisons, of within-subjects conditions. Hedge's G is reported as an effect sizes for between-subjects comparisons. Both Hedge's G_{avg} and Hedge's G were calculated with the supplementary materials of Lakens (2013). All data were visualised using the package ggplot2: Elegant Graphics for Data Analysis (Wickham, 2016).

3. Results

3.1. Open-field partner preference test

Overall, females did not demonstrate increased appetitive sexual or social behaviors (i.e. time spent or visits) toward the male they were conditioned with over the novel male. However, females that paced with the one-hole divider during conditioning trials selectively received the paired males' ejaculations first and more frequently.

Females in the one-hole pacing condition received the first ejaculation significantly more often from the paired male compared to the novel male, $\chi^2 = 6.25$, $p = .019$. Moreover, 11 of 16 females only received ejaculations from the paired male. In the four-hole pacing condition, four females did not receive ejaculations during the partner preference test despite receiving intromissions and soliciting males. Therefore, the data from 12 females were used to compare the choice of first ejaculation in the four-hole pacing condition. Females did not receive the first ejaculation from the paired male more than the novel male.

There was a significant interaction between pacing (1- vs 4-hole) and male type (paired vs novel) for the number of ejaculations received, $F(1, 30) = 9.86$, $p = .004$, $\eta^2_G = 0.19$. Planned comparisons revealed that females in the one-hole pacing condition, but not four-hole pacing condition, received significantly more ejaculations from the paired male ($M = 2.125$, $SD = 1.09$) than from the novel male ($M = 0.56$,

SD = 1.03), $t(30) = 3.26$, $p = .008$, $G_{avg} = 0.90$. It was also found that one-hole conditioned females received significantly more ejaculations from the paired male than four-hole conditioned females ($M = 0.69$, *SD* = 1.2), $t(50.14) = 3.61$, $p = .002$, $G = 1.22$ (Fig. 1A).

No significant interaction, or main effect of pacing or male type were found for solicitations (Fig. 1B). There was a significant interaction between pacing and male type on the frequency of visits to either male, $F(1, 30) = 7.82$, $p = .009$. However, the effect size was small for this interaction $\eta^2_G = 0.01$. Planned comparisons of the interaction with Holm adjustment revealed no mean group differences, (Fig. 1E). A similar result was found for hops and darts. A significant interaction between pacing condition and male type was found, $F(1, 30) = 10.05$, $p = .004$, $\eta^2_G = 0.11$. The planned comparisons found that females conditioned with the one-hole pacing divider made more hops and darts toward the novel male ($M = 27.625$, *SD* = 10.31) compared to the paired male ($M = 20.125$, *SD* = 7.43), $t(30) = -2.663$, $p = .0493$, $G_{avg} = 0.79$ (Fig. 1D).

The mixed ANOVA found no interaction or main effects on the frequency of intromissions received by females (Fig. 1C). There was also no significant interaction or main effects on the amount of time spent by females with each male (Fig. 1F).

3.2. Fos and oxytocin co-localised immunoreactive cell counts

As females from both pacing groups were only exposed to either a paired male or a novel male to induce Fos protein, the reported omnibus tests are 2 (Paired/Novel Male) × 2 (1-hole/4-hole) factorial ANOVAs.

Overall, it was found that Fos/OT-IR was increased in the PVN in females conditioned using one-hole pacing divider and that were exposed to a paired male. Additionally, one-hole paced females had increased Fos/OT-IR in the mPOA and VMH when exposed to the paired male but not in females conditioned with the four-hole divider or those exposed to a novel male.

There was a significant interaction between male type and pacing on Fos/OT-IR in the PVN, $F(1, 16) = 11.87$, $p = .003$, $\eta^2_G = 0.43$. There were main effects of both pacing ($F(1,16) = 7.13$, $p = .02$, $\eta^2_G = 0.31$) and male exposure on Fos/OT-IR in the PVN ($F(1, 16) = 17.23$, $p = .0008$, $\eta^2_G = 0.52$). Planned comparisons revealed that females in the one-hole pacing condition had significantly increased Fos/OT-IR when exposed to the paired male ($M = 17.06$, *SD* = 4.11) compared to females exposed to the novel male ($M = 5.51$, *SD* = 3.53), $t(16) = 5.37$, $p = .0002$, G = 2.72. Additionally, females in the one-hole condition exposed to the paired male had significant increased OT/Fos-IR compared to four-hole condition females exposed to the paired male ($M = 7.76$, *SD* = 3.74), $t(16) = 4.325$, $p = .0016$, G = 2.13 (Fig. 2A).

There was a significant interaction between pacing condition and male exposure on the number of Fos/OT-IR cells in the mPOA, $F(1, 16) = 5.73$, $p = .03$, $\eta^2_G = 0.26$. The ANOVA also revealed a significant main effect of pacing on the number of Fos/OT-IR neurons in the mPOA, $F(1, 16) = 11.49$, $p = .004$, $\eta^2_G = 0.48$. Planned comparisons revealed that females conditioned with the one-hole divider exposed to the paired male displayed significantly increased Fos/OT-IR cells ($M = 19.14$, *SD* = 6.70) compared to females conditioned with the four-hole pacing divider and exposed to a paired male ($M = 6.22$, *SD* = 3.09), $t(16) = 4.09$, $p = .0034$, $G = 2.24$ (Fig. 2D).

There was a significant interaction between pacing condition and male exposure on Fos/OT-IR cells within the VMH, $F(1, 16) = 4.55$, $p = .048$, $\eta^2_G = 0.22$. There was a significant main effect of pacing on Fos/OT-IR in the VMH, $F(1, 16) = 5.09$, $p = .038$, $\eta^2_G = 0.24$. The planned comparisons revealed that there were significantly more Fos/OT-IR cells in the VMH in one-hole conditioned females exposed to the paired male ($M = 20.09$, *SD* = 10.71) compared to females in the four-hole condition exposed to the paired male ($M = 5.03$, *SD* = 1.71), $t(16) = 3.104$, $p = .027$, $G = 1.77$ (Fig. 2E). No other significant effects on OT/Fos IR cell densities were found in the VTA and SON (Fig. 2B and C). See Fig. 4 for representative photomicrographs of each brain region across all comparison groups.

4. Discussion

The present study was designed to examine whether female rats could be sexually conditioned to prefer one male of the same strain, relative to another male, without the addition of odors or somatosensory cues as conditioned stimuli. Female rats made preferential and selective sexual behaviors toward an individual male rat of the same strain associated with their experience of pacing conditions during conditioning trials. It was found that in the one-hole pacing condition, females were significantly more likely to choose the paired male for their first and subsequent ejaculations compared to the novel male. Females in the four-hole pacing condition did not display such a preference. Furthermore, there was a significant increase in Fos IR within OT IR neurons in the PVN, the mPOA, and the VMH, of females with a conditioned preference for the paired male relative to females in the other conditioning groups. Taken together, these data add to previous studies (e.g., Holley et al., 2015) suggesting that under certain pacing conditions (i.e. with a one-hole pacing divider) females can be conditioned to prefer an unscented but familiar individual male over another unscented novel male. Furthermore, the presentation of the preferred male activates significantly more OT neurons than does the presentation of a novel male.

Females that paced with the four-hole divider during conditioning trials did not form a preference for the pacing-associated male. This is an interesting finding given that previous studies have conditioned preferential and selective sexual behaviors with the four-hole divider (Coria-Avila et al., 2005; Coria-Avila et al., 2006; Holley et al., 2014; Holley et al., 2015). Moreover, the four-hole pacing condition was associated with less Fos/OT IR in the mPOA irrespective of the male to which females were exposed. Males used in the current study had prior sexual experience and were sexually vigorous during conditioning trials. However, Ismail et al. (2009) suggest that the return latencies to the male's compartment are typically longer than with one-hole dividers compare to four-hole dividers (Ismail et al., 2009). Because a male can block a female's return to the other side of the pacing chamber when a one-hole divider is used, females can more easily pace with the four-hole divider. However, it appears that females pacing with the one-hole divider compensate by increasing the interval between intromissions. This resembles the proposal by McClintock and Adler (1978) that females regulate the number and timing of intromissions to maximise the likelihood of pregnancy.

Longer intervals between penile intromissions prior to ejaculation facilitates pregnancy (Edmonds et al., 1972). Both paced copulation and paced experimenter-administered artificial vaginocervical stimulation (VCS) or clitoral stimulation (CLS) are rewarding, as indicated by the induction of a conditioned place preference (Jenkins and Becker, 2003; Parada et al., 2010; Paredes and Alonso, 1997). Furthermore, paced copulation and both VCS and CLS induce a progestational state that facilitates successful pregnancy and hastens estrous termination (Cibrian-Llanderal et al., 2010; Erskine et al., 1989; Georgescu et al., 2012; Erskine et al., 2004; Pfaus et al., 2000). The rewarding and reproductive effects of paced copulation are thus inextricably linked. In the present study, we demonstrated that female rats can be conditioned to prefer an individual male rat paired with one-hole divider pacing. The reproductive and rewarding effects of paced copulation with the one-hole divider may drive the observed mate preference. Thus, Pavlovian preferences may depend not just on the reinforcing aspects of pacing, but also its pro-reproductive effects.

In semi-naturalistic and other environments, female rats pace by running away from males, imposing a distance of centimetres or even meters between themselves and a male, exiting into, and then returning from, burrow systems, or jumping on rocks or other obstacles that the male cannot get to them from (McClintock and Adler, 1978;

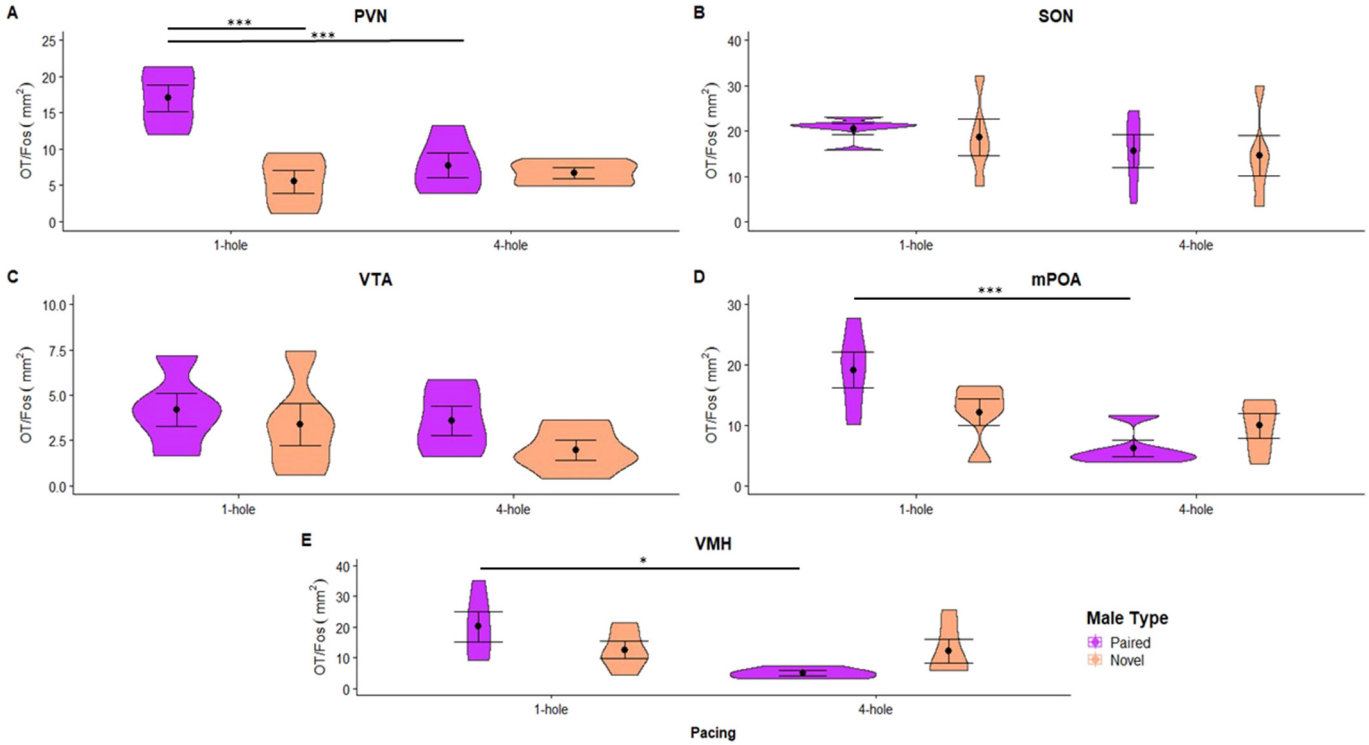

Fig. 2. Displays violin plots of co-labelled Fos/OT IR cell per mm^2 across groups. Black dots represent means and black bars indicate standard error of the mean ($n = 5$). A) Fos/OT IR in the PVN was highest in females that were exposed to the paired male and conditioned with the one-hole pacing divider. One-hole conditioned females exposed to the paired male had significantly more Fos/OT IR compared to one-hole females exposed to a novel male, and four-hole conditioned females exposed to paired males. B) No differences in Fos/OT IR were found in the SON. C) Exposure to the paired male in one-hole paced females did not induce more Fos/OT IR in the VTA. D) In the mPOA females that paced with the one-hole had significantly more Fos/OT IR than females that paced with the four-hole divider. Additionally, Fos/OT IR was significantly greater in the one-hole females exposed to the paired male compared to four-hole females. E) In the VMH, females in one-hole condition exposed to the paired male showed significantly greater Fos/OT IR compared to four-hole conditioned females exposed to the paired males. $*p < .05$, $**p < .01$, $***p < .005$.

McClintock, 1984; Pfaus et al., 1999). Even though pacing in the current study is non-naturalistic, the pacing conditions we imposed allowed females to control their interaction with a male. This ability serves the same function as pacing in naturalistic environments. In naturalistic mating encounters, females act in ways that increase the likelihood of being impregnated by a dominant male (McClintock et al., 1982). This requires the ability to recognise males that are dominant or subordinate and to adapt sexual behaviors toward dominant males. As dominance in male rats is related to the age of the male (MacDonald et al., 1995), female rats may use a flexible strategy for mate choice that is shaped by proximal factors. The data from the current study suggest that prior rewarding copulation with a specific male may be another proximal factor that shapes female mate choice. In naturalistic environments the copulatory patterns of specific males may also differ in reward value. The current study suggests Pavlovian associations formed during mating for different patterns of copulation may guide future mate choice.

We demonstrated that the pacing conditions during a female's early sexual experiences are an important determinant of whether it exhibits a future mate preference. Previous partner conditioning studies have

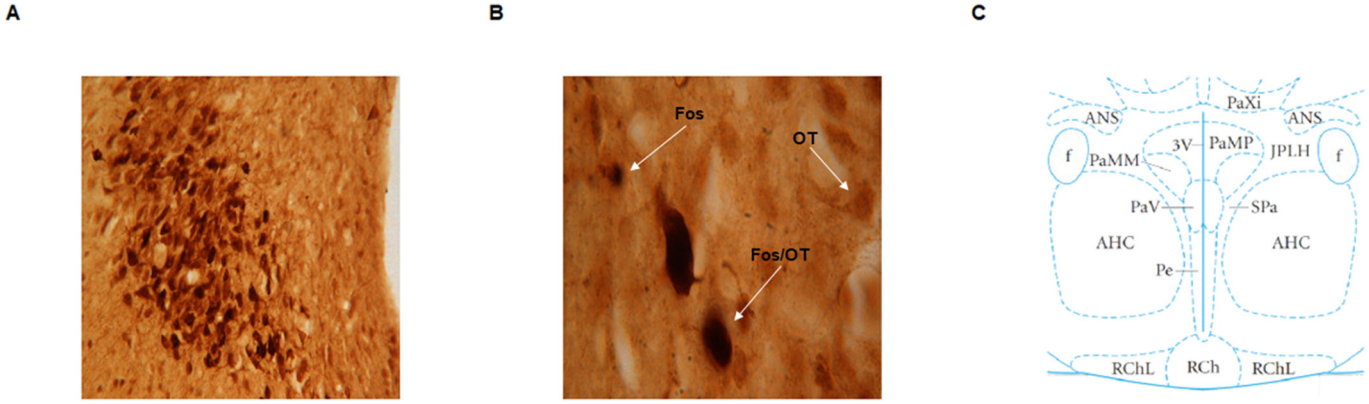

Fig. 3. Photomicrographs demonstrating Fos/OT-IR in the PVN. A) A photomicrograph (×20) of the PVN. B) Demonstrates a Fos-IR cell, identified by a round black stain. Fos/OT-IR is demonstrated by the round black stain within a dark brown cytoplasmic stain. OT-IR without Fos is shown as a dark brown stain with no black stain. C) A plate from the Paxinos and Watson brain atlas demonstrating one area in which the photomicrogrpahs were captured. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

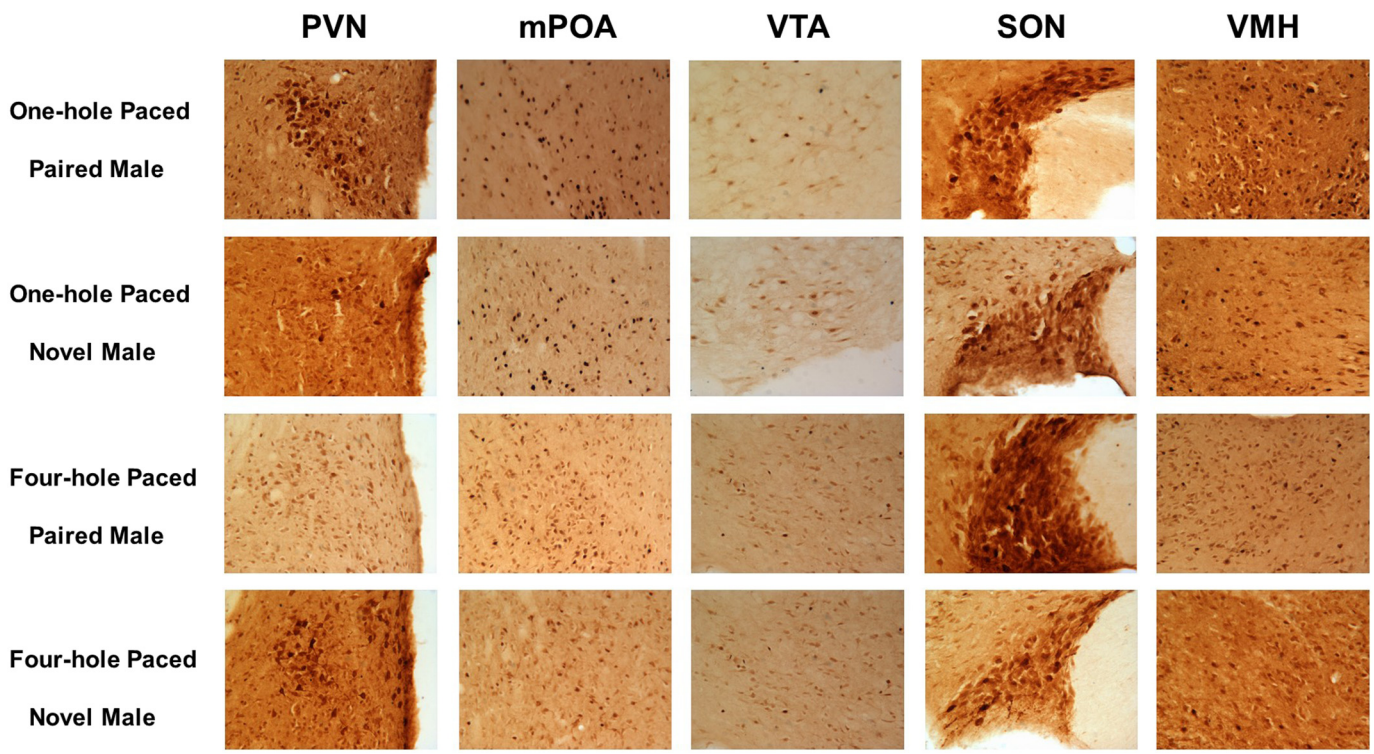

Fig. 4. displays representative photomicrographs of Fos/OT-IR in each brain region across all experimental conditions.

given females copulatory experience with scented males in a unilevel pacing chamber with a four-hole divider versus unscented males in the same chamber with no divider (e.g., Coria-Avila et al., 2005; Coria-Avila et al., 2006). Paired females (e.g., odor + pacing) displayed a preference to solicit and receive ejaculations from the scented versus unscented male. This conditioned sexual partner preference thus encompassed both appetitive and consummatory aspects of sexual behavior, and subsequent presentation of the odor alone activated significantly more Fos-labelled neurons in the PVN and SON (Coria-Avila and Pfaus, 2007). Another set of studies established that female rats display mate-guarding behaviors when given their first experiences of paced copulation in pacing chambers with a four-hole divider with a specific individual male. Females were subsequently presented with either their familiar male or a novel male along with a sexually receptive female conspecific in a large open field (e.g., Holley et al., 2014; Holley et al., 2015). Paired females presented with the familiar male displayed mate guarding behaviors, whereas females presented with a novel male, and unpaired females (for whom the male was not associated with pacing), did not display these behaviors. As in the present study, paired females presented with their familiar male had increased numbers of double labelled Fos/OT neurons in the PVN and SON relative to unpaired females.

These data suggest that the intensity of pacing paired with various external cues forms a hierarchy of reward value resulting in the display of preference behaviors. First, only the one-hole pacing experience induced a preference to receive ejaculations from the unscented familiar male. This is reminiscent of the results of Ismail et al. (2009) who found that the formation of a conditioned ejaculatory preference in male rats for a familiar, almond-scented female occurred only if their early copulatory experiences were in unilevel pacing chambers with a one-hole, but not four-hole, divider. Second, four-hole pacing conditions are sufficient, relative to no pacing (removal of the divider), to induce an either preference for a male scented with a neutral odor, such as almond or lemon, or mate guarding of an unscented male. Finally, although pacing is rewarding to female rats as assessed by the induction of a conditioned place preference (Jenkins and Becker, 2003; Paredes and Alonso, 1997; Paredes and Vazquez, 1999), a place preference can be conditioned in female rats with non-paced copulation (Meerts and Clark, 2007; Meerts and Clark, 2009) so long as a sufficient number of intromissions and ejaculations is delivered by the same male.

Within the framework of a hierarchy, it is possible that different types of rewarding stimulation come together to induce a place and/or partner preference. Artificial VCS or CLS activates Fos in regions of the mPOA and medial amygdala, respectively, but not in the PVN or SON (Parada et al., 2010; Pfaus et al., 1993). In contrast, nonpaced copulation with a male activated low amounts of Fos in the PVN, but not in the SON, whereas paced copulation in a bilevel chamber activated Fos moderately in OT neurons in the PVN and to a small but significant extent in the SON (Flanagan et al., 1993; Pfaus and Heeb, 1997). Paced copulation with scented males with the four-hole divider produced a partner preference to solicit and receive ejaculations selectively from the scented male. This condition also activated Fos to a much larger extent in both the PVN and SON, and such activation could be induced by presentation of the conditioned odor alone following conditioning (Coria-Avila and Pfaus, 2007). Although a four-hole pacing chamber is sufficient to induce conditioned mate-guarding for an unscented familiar male, in the present study only the one-hole divider was sufficient to induce a preference to receive ejaculations.

Numerous studies across different species have suggested that central OT transmission is involved in the formation of pair bonding and affiliative behaviors (Insel and Shapiro, 1992; Cushing and Carter, 2000; Liu and Wang, 2003; Ross et al., 2009; Holley et al., 2015). In rats, oxytocin facilitates lordosis when infused into the mPOA (Caldwell et al., 1989). Furthermore, the ovarian steroids estradiol and progesterone, which are high during periods of sexual receptivity in the female rat, regulate the density of oxytocin receptors (OTRs) in the VMH and the mPOA (Schumacher et al., 1990; Coirini et al., 1991; Caldwell et al., 1994). In the current study, sexual receptivity during conditioning, partner preference testing, and partner exposure for Fos induction, was induced with estradiol benzoate and progesterone. Thus, there should have been induced a high density of OTRs in both areas. If preferred partners paired with paced copulation induces increased OT

transmission in both areas, this may facilitate lordosis in the presence of that partner which would have the effect of facilitating the rate at which the male intromits and ejaculates. Neurons in the mPOA of female rodents are also highly responsive to male cues (McHenry et al., 2017) Increased activation of the mPOA from the OT-projections of the PVN may drive sexual motivation toward the one-hole pacing-associated male (Xiao et al., 2017).

Although female rats have been described as promiscuous in their choice of copulatory partners, McClintock et al., (1982) demonstrated that in semi-natural environments females exercise selectivity during mating. Females compete more to receive the ejaculation of dominant males, and dominant females typically are more successful than subordinate females in this competition. In contrast, prairie voles are described as socially and parentally "monogamous", with both males and females showing a preference for the first individual they have sex with (Getz et al., 1981). Yet, many litters are sired by multiple males (Solomon et al., 2004). Indeed, whether pair-bonded prairie voles demonstrate similar sexual partner preferences to conditioned rats has not yet been studied. Given that female rats can show preferences for odor, strain, and individual conspecifics, the use of a Pavlovian conditioning procedure shows that more animals than would be predicted to be dominant made a selective mate choice. This suggests that females can use both either promiscuous or monogamous-like mating strategies, and the strategy used is modulated by social hierarchy and/or copulatory experience as we have shown (Coria-Avila et al., 2005; Coria-Avila et al., 2006; Holley et al., 2014; Holley et al., 2015). Females showing a Coolidge effect demonstrate an innate preference for novel partners (Ventura-Aquino et al., 2016). Pavlovian partner conditioning demonstrates that this innate preference is malleable by experience with copulatory reward.

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