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Involvement of the oxytocin system in the bed nucleus of the stria terminalis in the sex-specific regulation of social recognition

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ABSTRACT

Sex differences in the oxytocin (OT) system in the brain may explain why OT often regulates social behaviors in sex-specific ways. However, a link between sex differences in the OT system and sex-specific regulation of social behavior has not been tested. Here, we determined whether sex differences in the OT receptor (OTR) or in OT release in the posterior bed nucleus of the stria terminalis (pBNST) mediates sex-specific regulation of social recognition in rats. We recently showed that, compared to female rats, male rats have a three-fold higher OTR binding density in the pBNST, a sexually dimorphic area implicated in the regulation of social behaviors. We now demonstrate that OTR antagonist (5 ng/0.5 μl/side) administration into the pBNST impairs social recognition in both sexes, while OT (100 pg/0.5 μl/side) administration into the pBNST prolongs the duration of social recognition in males only. These effects seem specific to social recognition, as neither treatment altered total social investigation time in either sex. Moreover, baseline OT release in the pBNST, as measured with in vivo microdialysis, did not differ between the sexes. However, males showed higher OT release in the pBNST during social recognition compared to females. These findings suggest a sex-specific role of the OT system in the pBNST in the regulation of social recognition.

1. Introduction

The neuropeptide oxytocin (OT) is synthesized mainly in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus (Buijs, 1978; Sofroniew, 1983), and regulates a wide variety of social behaviors in rodents and humans (Veenema and Neumann, 2008; Heinrichs et al., 2009; Ross and Young, 2009; Goodson and Thompson, 2010; Guastella and MacLeod, 2012). Importantly, OT has been shown to regulate some of these behaviors in sex-specific ways (reviewed in Dumais and Veenema, 2015, 2016). This may be due to sex differences in the OT system in the brain. Although OT mRNA expression is similar in the PVN and SON of male and female rats (Dumais et al., 2013), the OT receptor (OTR) is highly sexually dimorphic, with male rats showing higher OTR binding densities in many forebrain regions compared to female rats (Uhl-Bronner et al., 2005; Dumais et al., 2013).

The most robust sex difference in OTR binding density in the rat brain is found in the posterior bed nucleus of the stria terminalis (pBNST), in which males have a three-fold higher OTR binding density compared to females (Dumais et al., 2013). The pBNST has extensive connections with areas involved in social information processing (most notably the accessory olfactory bulb and medial amygdala; Scalia and Winans, 1975; Weller and Smith, 1982; Gu et al., 2003; Dong and Swanson, 2004) and is part of the social decision-making network (O'Connell and Hofmann, 2011). Indeed, the pBNST plays an essential role in transmitting chemosensory social information and modulating olfactory-guided social behaviors (Petrulis, 2013). For example, neuronal activation is increased in the pBNST in male Mandarin voles (He et al., 2014) and in female rats (Hosokawa and Chiba, 2007) in response to opposite-sex odors, and lesioning the pBNST impairs opposite-sex odor preference in male hamsters (Been and Petrulis, 2010). In addition, blocking OTR in the pBNST reduced male odor-induced vaginal marking in female hamsters (Martinez et al., 2010), suggesting a role for the OTR in the pBNST in social odor processing and/or olfactory-guided social behaviors. To the best of our knowledge, there are no comparative studies on the role of OTR in the pBNST in males and females.

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We hypothesized that the sex difference in OTR binding density in the pBNST is implicated in the sex-specific regulation of social behavior. To test this, we determined the effects of acute pharmacological manipulations of the OT system in the pBNST on social investigation (reflecting the motivation to approach a conspecific for the assessment of social cues) and social recognition (the ability to discriminate between familiar and unfamiliar conspecifics) in adult male and female rats. Social investigation and social recognition were chosen because these behaviors are modulated by the OT system (Gabor et al., 2012; Lukas et al., 2013; Dumais et al., 2013), require the processing of chemosensory social information, and can be tested with neutral social stimuli (i.e., juvenile rats), allowing focus on social odor processing without interference of sexual or aggressive behaviors.

We further hypothesized that the sex difference in OTR binding density in the pBNST corresponds with a sex difference in local OT release. Higher OTR binding density, as seen in males (Dumais et al., 2013), could be associated with higher OT release or could be a compensatory mechanism for lower OT release. To determine the relationship between sex differences in OTR binding density and OT release in the pBNST, we used in vivo microdialysis to measure extracellular OT release in the pBNST of male and female rats under baseline conditions and during exposure to the social recognition test.

2. Methods

2.1. Animals

Wistar rats were obtained from Charles River at 8–9 weeks of age (Wilmington, MA) and maintained on a 12 h light/dark cycle, lights on at 0700 h, and food and water were available ad libitum. Subjects were adult male and female rats housed in same-sex pairs in standard rat cages ($26.7 \times 48.3 \times 20.3$ cm) unless otherwise mentioned, and were given at least one week to acclimate to our facilities. Stimulus male and female rats were 22 days at arrival, were housed four per cage, and were used at 25–30 days of age. All experiments were conducted in accordance with the guidelines of the NIH and approved by the Boston College Institutional Animal Care and Use Committee (IACUC).

2.2. Stereotaxic surgery

2.2.1. Cannulation

After daily handling for one week to familiarize them with the injection procedure, experimental rats were anesthetized using isoflurane and mounted on a stereotaxic frame. A heating pad was used to regulate body temperature of rats while anesthetized. Guide cannulae (22 gauge; Plastics One, Roanoke, VA) were implanted bilaterally 2 mm dorsal to the pBNST (0.8 caudal to bregma, 1.5 and −1.5 lateral to midline, and 4.8 ventral to the skull surface; according to Paxinos and Watson, 1998). Guide cannulae were fixed to the skull with four stainless steel screws and acrylic glue and closed with dummy cannulae (26 gauge; Plastics One, Roanoke, VA). After surgery, rats were individually housed in standard rat cages ($26.7 \times 48.3 \times 20.3$ cm). Behavioral testing was performed 3 and 5 days after surgery.

2.2.2. Microdialysis probe placement

A separate set of rats was used for in vivo measurement of extracellular OT release. Handling and surgical procedures were similar to the procedures described above except for the placement of microdialysis probes instead of cannulae. Microdialysis probes (BrainLink, the Netherlands) were implanted unilaterally into the pBNST (0.8 caudal to bregma, −1.5 lateral to midline, and 7.0 ventral to the skull surface). Two inch pieces of polyethylene tubing were

fixed to the ends of the microdialysis probes in order for attachment to the microinfusion pumps and eppendorf tubes for sample collection. After surgery, rats were individually housed in standard rat cages ($26.7 \times 48.3 \times 20.3$ cm). Microdialysis and behavioral testing were performed 2 days after surgery.

2.3. Behavioral testing

2.3.1. Social investigation test

To test for social investigation, the time rats spent investigating an unfamiliar same-sex juvenile rat was measured according to Dumais et al. (2013). A juvenile rat was used in order to assess general social approach of the experimental rat toward a social stimulus that does not elicit aggressive or sexual behaviors. Indeed, no aggressive or mounting behaviors were observed during the social investigation test. A juvenile rat was placed into the experimental rat's home cage for 4 min, and time spent investigating the juvenile was measured. Testing was performed during the light phase between 1200 h and 1700 h. Behaviors were video recorded and analyzed using JWatcher (http://www.jwatcher.ucla.edu) by an experimenter blind to treatment groups. Behavior was considered social investigation when the experimental rat was actively sniffing the juvenile, including sniffing the anogenital and head/neck regions.

2.3.2. Social recognition test

Social recognition was measured using the social discrimination paradigm, according to Veenema et al. (2012) and adapted from Engelmann et al. (1995). This paradigm consists of two trials. In the first trial (T1), the experimental rat is exposed in its home cage to an unfamiliar same-sex juvenile for 4 min (T1 is the same as the social investigation test described above). After a preset interval, the experimental rat undergoes a second trial (T2) in which the rat is exposed in its home cage to the same (familiar) juvenile along with an unfamiliar same-sex juvenile for 4 min (see Movie 1 in Supplementary material). To allow the experimenter to distinguish between the two juveniles, juveniles were marked on their backs with either red or black permanent marker 1 h prior to testing. The color of the marker was counterbalanced between novel and familiar juveniles. Using this social discrimination paradigm, previous studies found that adult rats show social recognition after a 1 h interval, but not after a 3 h interval (Veenema et al., 2012; Bernal-Mondragon et al., 2013; Lee et al., 2014). We hypothesized that OTR blockade may impair social recognition, while OT administration may prolong social recognition. Therefore, we used a 1 h interval for the OTR antagonist experiment, and a 3 h interval for the OT experiment. Testing was performed during the light phase between 1200 h and 1700 h. Behaviors were video recorded, and time spent investigating the juvenile rats was measured using JWatcher by an experimenter blind to treatment groups. As expected, no aggressive or mounting behaviors of the experimental rats toward the juvenile rats were observed during the test. The percentage of time investigating the novel juvenile (time investigating novel juvenile/time investigating familiar + novel juvenile × 100) during T2 was calculated as the measure of social recognition. The time spent investigating one juvenile in T1 and two juveniles in T2 was calculated as the measure of total social investigation time (in seconds) in T1 and T2, respectively.

2.4. Experimental procedures

2.4.1. Experiment 1: effect of OTR manipulations in the BNST on social investigation and social recognition

The effects of the OTR antagonist desGly-NH$_2$,d(CH$_2$)$_5$-[Tyr(Me)^2,Thr^4]OVT (Manning et al., 2008; 5 ng/0.5 μl/side) and synthetic OT (Sigma; 100 pg/0.5 μl/side) were each compared to
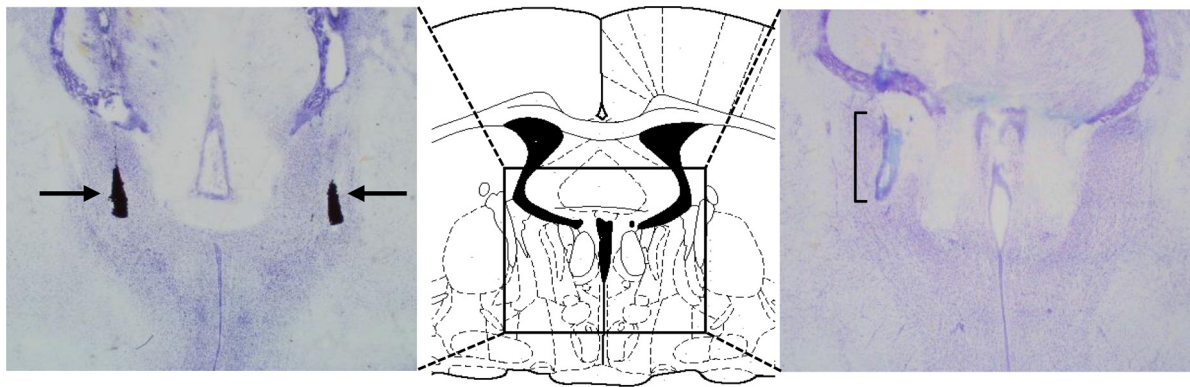
Fig. 1. Schematic drawing of the posterior bed nucleus of the stria terminalis (pBNST; bregma −0.80 mm; adapted from Paxinos and Watson, 1998) and representative enlargements of photomicrographs of Nissl-stained coronal sections of the rat brain indicating with arrows the bilateral microinjection locations in the pBNST using charcoal as marker (left) and indicating with a bracket the location and extend of the semipermeable membrane of a microdialysis probe placement unilaterally in the pBNST (right). Correct placements were considered at bregma −0.80 mm ± 0.2 mm using the brain atlas by Paxinos and Watson (1998).

their own vehicle (Ringer's solution, 0.5 μl/side) control group. Drug doses were based on prior microinjection studies in rats in which these doses were effective in altering diverse social behaviors (Guzman et al., 2013, 2014; Bredewold et al., 2014; Laszlo et al. 2015). Injection systems were composed of polyethylene tubing connected to an injector cannula which extended 2 mm beyond the guide cannula. Injections were made using a 10 μl Hamilton syringe (Hamilton, Reno, NV) and was kept in place for 30 s following injection to allow for tissue uptake. Following the last behavioral test, rats were killed with CO_2, a small amount of charcoal was injected into the guide cannulae, and proper cannulae placement was verified histologically on Nissl-stained coronal brain sections (see Fig. 1). Rats that did not have proper cannulae placement were removed from analysis (OTR antagonist experiment: males = 4, females = 8; OT experiment: males = 4, females = 6). For the OTR blockade experiment, 2 males in the vehicle group were excluded after the social investigation test due to dental cement becoming loose. For the OT experiment, 2 males and 3 females in the vehicle group and 1 female in the OT injection group were excluded after the social investigation test due to clogging of the guide cannulae. The final number of rats per group is indicated below.

2.4.1.1. Experiment 1a: social investigation. To determine the effects of OTR blockade in the pBNST on social investigation, subjects were injected with either Ringer's solution (males = 15, females = 16) or the OTR antagonist (males = 10, females = 13). To determine the effects of OT administration into the pBNST on social investigation, subjects were injected with either Ringer's solution (males = 9, females = 8) or OT (males = 7, females = 9). Rats were injected 20 min before the start of the social investigation test.

2.4.1.2. Experiment 1b: social recognition. Rats were exposed to the social discrimination test two days after the social investigation test, and received the same treatment. The time window of two days was chosen to allow the drugs to leave the system (OTR antagonists and OT have a half-life of approx. 20 min; Mens et al., 1983; Goodwin et al., 1995; Ludwig and Leng, 2006) and to prevent any residual effects of drug injections during the social investigation test on social recognition. To determine the effects of OTR blockade in the pBNST on social recognition (1 h interval), subjects were injected with either Ringer's solution (males = 13, females = 16) or OTR antagonist (males = 10, females = 13) in each side. To determine the effects of exogenous OT in the pBNST on social recognition (3 h interval), subjects were injected with either Ringer's solution (males = 7, females = 5) or OT (males = 7, females = 8) in each side.

Rats were injected immediately after exposure to the first stimulus juvenile.

2.4.2. Experiment 2: OT release in the BNST during social recognition

OT release in the pBNST was measured in a separate group of male and female rats exposed to the social discrimination test (1 h interval). Two to three days before microdialysis probe implantation, rats were exposed to the social discrimination paradigm to habituate them to the behavioral test. One day after probe implantation, rats were habituated to the sampling procedure for 1 h. Two days after probe implantation, microdialysis probes were connected via polyethylene tubing to Hamilton syringes mounted on a microinfusion pump. Rats were perfused with Ringer's solution (with 0.25% BSA; 3 μl/min) for 2 h to establish an equilibrium between the inside and outside of the microdialysis membrane. Five consecutive 30-min dialysates were then collected in 0.5 ml eppendorf tubes containing 0.1 M HCl to inhibit protein degradation. Dialysate 1 was collected before the first juvenile exposure (baseline), dialysate 2 started with the 4-min exposure to the first stimulus juvenile (T1), dialysate 3 started 30 min after T1, dialysate 4 started with the 4-min exposure to the same and novel stimulus juveniles (T2), and dialysate 5 started 30 min after T2. Dialysates were immediately frozen on dry ice, and stored at −45 °C until quantification. OT content was measured using radioimmunoassay (RIAgnostics, Munich, Germany). Following the last behavioral test, rats were killed with CO_2, and proper probe placement was verified histologically on Nissl-stained coronal brain sections (see Fig. 1). Rats that did not have proper probe placement were removed from analysis (males = 6, females = 4). Six rats (males = 1, females = 5) were also excluded due to technical issues with dialysate sampling. This resulted in a final number of 19 males and 16 females.

2.5. Estrus phase measurement

To control for effects of estrus cycle, estrus phase was determined via vaginal smears (according to Dumais et al., 2013) taken from each female immediately following each behavioral test. Using a pipette and a small amount of distilled water, vaginal secretions were taken and assessed for estrus cycle phase via cell characteristics according to Goldman et al. (2007). Females were categorized as being in proestrus/estrus (cells characteristic of proestrus and estrus phases in which females show higher levels of estradiol and progesterone), or non-estrus (cells characteristic of diestrus and metestrus in which females show lower levels of estradiol and progesterone). Number of females in estrus or nonestrus
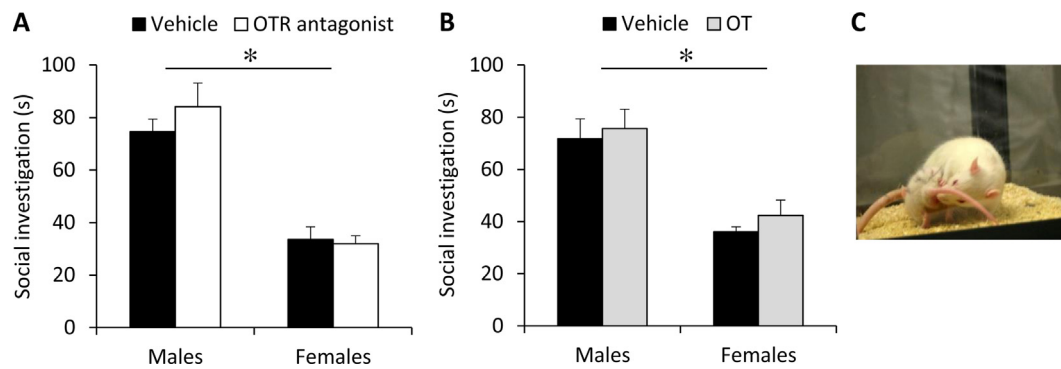
Fig. 2. There are no effects of oxytocin (OT) receptor (OTR) antagonist (A) or OT (B) injections into the posterior bed nucleus of the stria terminalis (pBNST) on social investigation in male and female adult rats. However, there is a main effect of sex in both (A) and (B), in which females show lower social investigation compared to males. Social investigation, depicted in (C), represents the total time spent investigating a novel, same-sex juvenile rat placed in the home cage of the experimental rat for a 4-min period. Bars indicate mean + SEM; *$p < 0.001$, two-way ANOVA.

for each experiment were as follows: social investigation (OTR blockade experiment, vehicle: non-estrus = 9, estrus = 7, OTR antagonist: non-estrus = 8, estrus = 5; OT injection experiment, vehicle: non-estrus = 4, estrus = 4, OT injection: non-estrus = 7, estrus = 2) and social recognition (OTR blockade experiment, vehicle: non-estrus = 13, estrus = 3, OTR antagonist: non-estrus = 9, estrus = 4; OT injection experiment, vehicle: non-estrus = 4, estrus = 1, OT injection: non-estrus = 5, estrus = 3).

2.6. Statistical analysis

For experiment 1, social investigation was analyzed using two-way ANOVA (treatment × sex) with social investigation time (in seconds) as the dependent factor. Social recognition was analyzed using two-tailed one-sample *T*-tests, with the percent novel investigation for each group tested against 50% (chance level) and Bonferroni correction for multiple comparisons (corrected alpha, $p = 0.0125$). To determine differences between groups, social recognition was analyzed using two-way ANOVA (treatment × sex) with percent novel investigation as the dependent factor. Because of main effects of treatment and sex, two-tailed independent sample *T*-tests were run separately in males and females to test for treatment effects by sex (OTR antagonist and OT) and separately by treatment to test for sex effects by treatment (OT). The effects of estrus phase on social investigation time and on social recognition were analyzed using one-way ANOVAs (treatment) with estrus phase as covariate.

For experiment 2, rats were assigned into two groups based on their ability to show social recognition. Rats were considered to show social recognition if the percent novel investigation was higher than the average percent novel investigation for each sex (males: >57% $n = 9$; females: >62% $n = 9$), and rats were considered to not show social recognition if the percent novel investigation was below the average percent novel investigation for each sex (males: <57% $n = 10$, females: <62% $n = 7$). Four males and 4 females were excluded from the study because they showed higher than 57% investigation of the familiar juvenile.

Effects of sex and social recognition ability on percent novel investigation and total investigation time were measured using two-way ANOVAs. To confirm a significant difference in social recognition ability in each sex, two-tailed independent sample *T*-tests were run separately in males and females to test for effects of social recognition ability. Effects of sex and social recognition ability on baseline OT concentration (pg/dialysate) were analyzed using the non-parametric Kruskal–Wallis test. OT release was then converted into percent change from baseline OT release for each rat. Effects of sex and social recognition ability on the percentage

of OT release was analyzed using a three-way ANOVA for repeated measures (dialysate × sex × social recognition). When main effects were found, differences between groups were analyzed with an LSD post hoc test. Effect of estrus phase on OT release was tested using a two-way ANOVA for repeated measures (dialysate × social recognition) with estrus phase as covariate. Data are presented as mean + SEM, and significance was set at $p < 0.05$.

3. Results

### 3.1. Experiment 1: effect of OTR manipulations in the pBNST on social investigation and social recognition

3.1.1. Experiment 1a: social investigation

OTR blockade did not alter social investigation time in either sex (treatment: $F_{(1,50)} = 0.91$, $p = 0.35$; sex × treatment: $F_{(1,50)} = 1.40$, $p = 0.24$). Likewise, OT administration did not alter social investigation time in either sex (treatment: $F_{(1,29)} = 0.67$, $p = 0.42$; sex × treatment: $F_{(1,29)} = 0.03$, $p = 0.85$). However, a sex effect was found in both experiments (OTR antagonist: $F_{(1,50)} = 85.6$, $p < 0.0001$; OT: $F_{(1,29)} = 30.8$, $p < 0.001$), in which males spent more time investigating the social stimulus (Fig. 2A and B). There was no effect of estrus phase on social investigation for either the OTR antagonist experiment ($F_{(1,26)} = 1.08$, $p = 0.31$) or the OT experiment ($F_{(1,14)} = 0.05$, $p = 0.83$).

3.1.2. Experiment 1b: social recognition

Two days after the social investigation test, and using the same drug treatment, rats were exposed to the social recognition test. OTR blockade impaired social recognition in both sexes. In contrast to vehicle-treated rats (males: $t_{(12)} = 3.01$, $p < 0.0125$; females: ($t_{(15)} = 2.91$, $p < 0.0125$; Bonferroni corrected p value), OTR antagonist-treated rats did not investigate the novel rat more than chance level (males: $t_{(9)} = -1.52$, $p = 0.16$; females: $t_{(12)} = -0.75$, $p = 0.47$, Fig. 3A), revealing a lack of social discrimination after OTR blockade. As a result, the percent novel investigation was significantly lower in OTR antagonist-treated rats compared to vehicle-treated rats ($F_{(1,48)} = 15.5$, $p < 0.0001$). Independent samples *T*-tests showed that OTR blockade decreased the percent novel investigation in both males ($t_{(21)} = 3.20$, $p < 0.01$) and females ($t_{(27)} = 2.41$, $p < 0.05$; Fig. 3A). No main effects of sex ($F_{(1,48)} = 0.01$, $p = 0.93$) or treatment × sex ($F_{(1,48)} = 0.40$, $p = 0.53$) were found for social recognition, nor did estrus phase have an effect on social recognition ($F_{(1,26)} = 0.96$, $p = 0.34$).

There was no difference in social investigation time between vehicle- and OTR antagonist-treated rats during T1 (vehicle-treated males: 71.3 ± 5.0 s, vehicle-treated females: 24.1 ± 2.7 s,
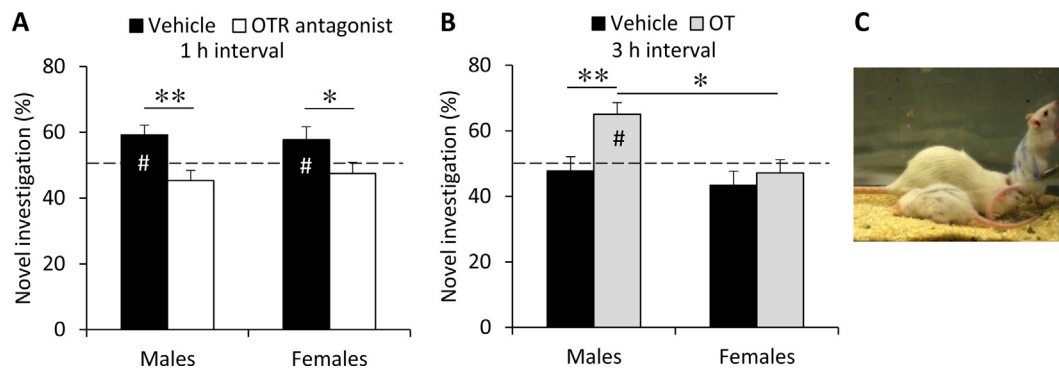
Fig. 3. Role of the posterior bed nucleus fo the stria terminalis (pBNST)- oxytocin (OT) system in social recognition. (A) OT receptor (OTR) antagonist injected into the pBNST impaired social recognition in both male and female adult rats. (B) OT injected into the pBNST improved social recognition in males, but not in females. Social recognition is expressed as the percentage of time spent investigating a novel same-sex juvenile rat (time investigating novel rat/total time investigating novel + familiar rat × 100). Social recognition was tested after an interval of 1 h (A) or 3 h (B). The picture in (C) shows an experimental rat exposed to a novel and a familiar juvenile rat and investigating one of the juvenile rats. Bars indicate mean + SEM; #: significantly different from chance level ($p < 0.05$, one sample T-test); *$p < 0.05$, **$p < 0.01$, two-way ANOVA followed by T-test.

OTR antagonist-treated males: 69.8 ± 7.0 s, OTR antagonist-treated females: 28.1 ± 3.3 s; treatment: $F_{(1,48)} = 0.08$, $p = 0.78$; sex × treatment: $F_{(1,48)} = 0.40$, $p = 0.53$; two-way ANOVA) and T2 (vehicle-treated males: 72.7 ± 6.8 s, vehicle-treated females: 22.2 ± 3.7 s, OTR antagonist-treated males: 68.3 ± 7.7 s, OTR antagonist-treated females: 24.7 ± 2.0 s; treatment: $F_{(1,48)} = 0.01$, $p = 0.96$; sex × treatment: $F_{(1,48)} = 0.24$, $p = 0.62$; two-way ANOVA). However, confirming the sex difference in experiment 1a, males spent more time investigating the juveniles during both T1 ($F_{(1,48)} = 102$, $p < 0.00001$) and T2 ($F_{(1,48)} = 69.3$, $p < 0.00001$). Estrus phase did not have an effect on social investigation time (T1: $F_{(1,26)} = 0.96$, $p = 0.34$; T2: $F_{(1,26)} = 0.01$, $p = 0.93$).

OT treatment prolonged the duration of social recognition in males, but not in females. In detail, OT-treated males investigated the novel juvenile more than chance level ($t_{(6)} = 4.14$, $p < 0.0125$) while vehicle-treated males ($t_{(6)} = -0.52$, $p = 0.62$), vehicle-treated females ($t_{(4)} = -1.53$, $p = 0.20$) and OT-treated females ($t_{(7)} = -0.71$, $p = 0.50$) did not (Fig. 3B). While there was no sex × treatment interaction ($F_{(1,23)} = 2.60$, $p = 0.12$), there were main effects of treatment ($F_{(1,23)} = 6.35$, $p < 0.05$) and sex ($F_{(1,23)} = 7.10$, $p < 0.05$). To determine whether the effects of OT on social recognition were sex-specific, the data were tested separately by sex and treatment using independent samples T-tests. These tests indicated that OT-treated males spent more time investigating the novel juvenile compared to vehicle-treated males ($t_{(12)} = -3.06$, $p < 0.01$) and compared to OT-treated females ($t_{(13)} = 3.25$, $p < 0.01$; Fig. 3B). OT-treated females did not investigate the novel juvenile more than vehicle-treated females ($t_{(11)} = -0.61$, $p = 0.55$) nor was there a difference in percent novel investigation between vehicle treated male and female rats ($t_{(10)} = 0.70$, $p = 0.50$). Finally, estrus phase did not have an effect on social recognition ($F_{(1,10)} = 0.68$, $p = 0.43$).

There was no difference in social investigation time between vehicle- and OT-treated rats during T1 (vehicle-treated males: 53.6 ± 2.5 s, vehicle-treated females: 41.7 ± 8.7 s, OT-treated males: 64.3 ± 7.6 s, OT-treated females: 33.1 ± 7.4 s; treatment: $F_{(1,23)} = 4.03$, $p = 0.06$; sex × treatment: $F_{(1,23)} = 0.55$, $p = 0.47$; two-way ANOVA) and T2 (vehicle-treated males: 48.4 ± 5.2 s, vehicle-treated females: 36.5 ± 6.0 s, OT-treated males: 71.1 ± 7.0 s, OT-treated females: 38.4 ± 7.4 s; treatment: $F_{(1,23)} = 0.02$, $p = 0.88$; sex × treatment: $F_{(1,23)} = 2.00$, $p = 0.17$; two-way ANOVA). However, confirming the sex difference in experiment 1a and in the OTR experiment, males showed longer social investigation times during both T1 ($F_{(1,23)} = 10.1$, $p < 0.01$) and T2 ($F_{(1,23)} = 4.76$, $p < 0.05$). Estrus phase did not have an effect on social investigation time (T1: $F_{(1,10)} = 0.99$, $p = 0.35$; T2: $F_{(1,10)} = 0.31$, $p = 0.59$).

3.2. Experiment 2: OT release in the BNST during social recognition

Rats were grouped based on their social recognition ability (see statistics section for selection criteria), and behavioral data for each group is shown in Fig. 4A–C. As expected, the percent novel investigation was significantly different from chance level for rats that showed social recognition (males: $t_{(8)} = 5.19$, $p < 0.0125$; females: $t_{(8)} = 8.26$, $p < 0.0125$; Bonferroni corrected p value), but not for rats that did not show social recognition (males: $t_{(9)} = 1.81$, $p = 0.11$; females: $t_{(6)} = 0.35$, $p = 0.74$; Fig 4A). This was also reflected by a main effect of social recognition ability ($F_{(1,31)} = 76.1$, $p < 0.0001$, two-way ANOVA) and a lack of main effects for sex ($F_{(1,31)} = 3.63$, $p = 0.07$) and sex × social recognition ability ($F_{(1,31)} = 3.52$, $p = 0.07$). Independent samples T-tests were then run separately by sex, which confirmed that the percent novel investigation was higher in males ($t_{(17)} = 5.33$, $p < 0.0001$) and females ($t_{(14)} = 6.813$, $p < 0.00001$) that showed social recognition compared to those that did not (Fig. 4A).

In agreement with the sex difference in experiments 1a and 1b and irrespective of social recognition ability (T1, social recognition ability: $F_{(1,31)} = 0.09$, $p = 0.76$; social recognition ability × sex: $F_{(1,31)} = 0.09$, $p = 0.77$. T2, social recognition ability: $F_{(1,31)} = 0.61$, $p = 0.44$; social recognition ability × sex: $F_{(1,31)} = 0.13$, $p = 0.72$), males showed higher social investigation compared to females during both T1 ($F_{(1,31)} = 14.8$, $p < 0.001$, two-way ANOVA; Fig. 4B) and T2 ($F_{(1,31)} = 6.67$, $p < 0.05$, two-way ANOVA; Fig. 4C).

There was no difference in baseline OT concentration in the pBNST between males and females that did and did not show social recognition ($x^2_{(3)} = 3.87$, $p = 0.28$; Fig. 4D). Effect of social recognition ability was also not significant when analyzing baseline OT concentration independently by sex (males: $x^2_{(1)} = 0.43$, $p = 0.51$; females: $x^2_{(1)} = 3.05$, $p = 0.08$).

While there were no main effects of dialysate ($F_{(4,120)} = 1.19$, $p = 0.32$), sex ($F_{(1,30)} = 0.13$, $p = 0.72$), or social recognition ($F_{(1,30)} = 0.93$, $p = 0.34$) on the percentage of OT release, a significant dialysate × sex × social recognition interaction effect was found ($F_{(4,120)} = 2.81$, $p < 0.05$). *Post hoc* testing revealed a sex difference in the percentage of OT release in rats that showed social recognition. Here, males show a significant increase in the percentage of OT release during T1 ($p < 0.05$ vs dialysate 1 and 3) and a trend toward an increase in the percentage of OT release during T2 ($p = 0.072$ vs dialysate 1), while females do not show a change in the percentage of OT release during T1 ($p = 0.42$ vs dialysate 1; $p = 0.64$ vs dialysate 3) or T2 ($p = 0.29$ vs dialysate 1; Fig. 4E). This sex difference is further reflected by males showing
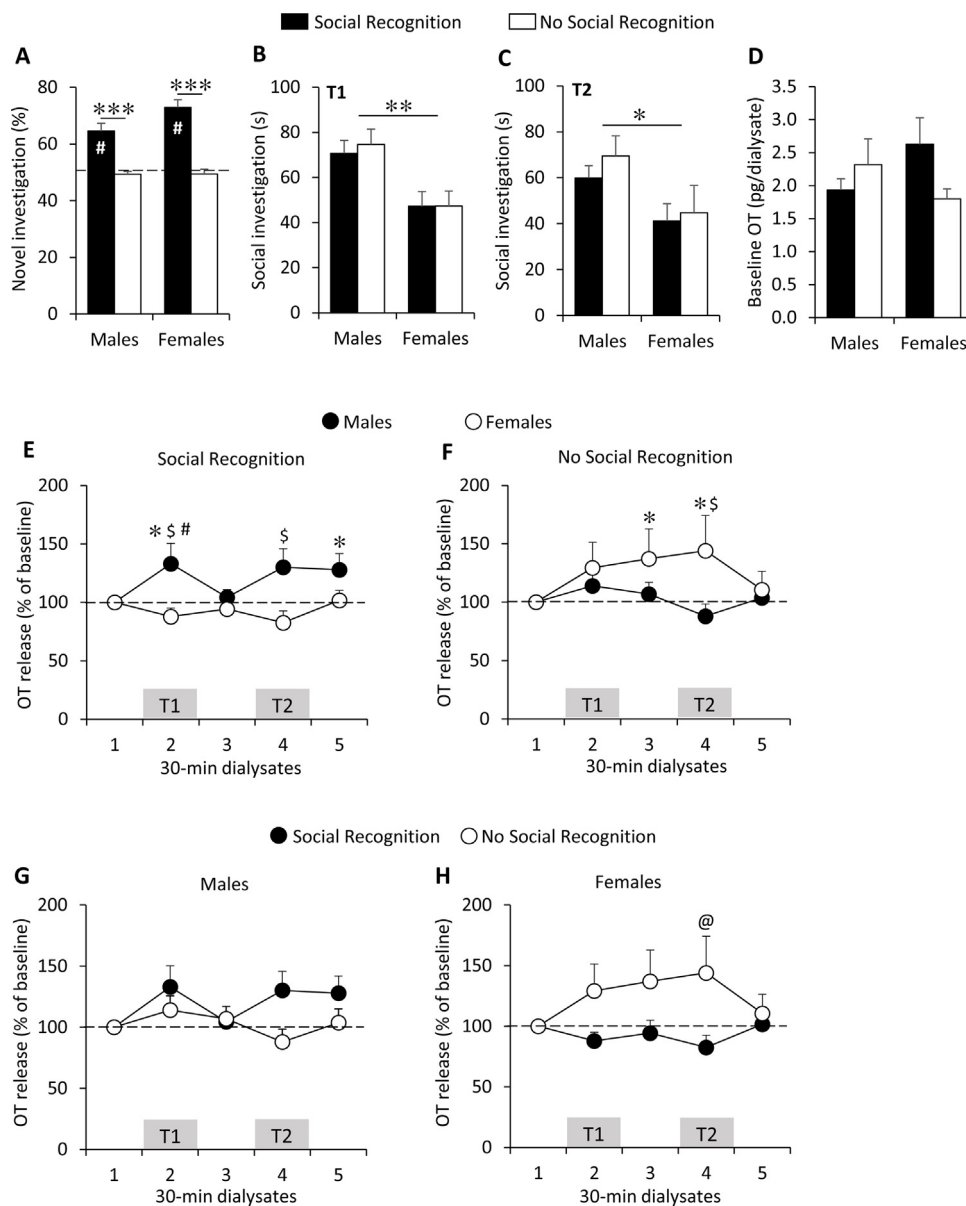
Fig. 4. Sex-specific oxytocin (OT) release in the posterior bed nucleus of the stria terminalis (pBNST) as a function of social recognition ability. (A) Males and females that showed social recognition have a significantly higher percent novel investigation compared to males and females that did not show social recognition (see statistics section for selection criteria). Independent of social recognition ability, males show significantly higher total social investigation time during T1 (B) and during T2 (C) compared to females. (D) There is no effect of sex or social recognition ability on baseline OT concentrations in the pBNST. (E) The percentage of OT release in the pBNST of rats that showed social recognition is significantly higher during T1 versus dialysate 1 and 3 and during dialysate 5 versus dialysate 1 in males only. The percentage of OT release in the pBNST of rats that showed social recognition is also significantly higher in males than in females during T1 and T2. (F) In females that did not show social recognition, the percentage of OT release is higher during T2 and during dialysate 3 versus dialysate 1. The percentage of OT release in the pBNST of rats that did not show social recognition is higher in females than in males during T2. (G) The percentage of OT release in the pBNST is not different between males that showed social recognition and males that did not show social recognition. (H) The percentage of OT release is higher in females that did not show social recognition compared to females that showed social recognition during T2. OT release is expressed as percent change from baseline OT concentrations. Data are expressed as mean + SEM. A–D: **$p < 0.001$, *$p < 0.05$, two-way ANOVA, ***$p < 0.0001$, two-way ANOVA, followed by T-test, #: significantly different from chance level ($p < 0.05$, one sample T-test). E-H: *$p < 0.05$ versus dialysate 1, #$p < 0.05$ versus dialysate 3, $$p < 0.05$ versus other sex, @$p < 0.05$ versus females that show social recognition, three-way ANOVA for repeated measures followed by LSD post hoc test; T1, 4-min exposure to first juvenile; T2, 4-min exposure to previously-exposed juvenile along with a novel juvenile.

higher OT release compared to females during both T1 ($p < 0.05$) and T2 ($p < 0.05$; Fig. 4E). In addition, the percentage of OT release was higher in dialysate 5 compared to dialysate 1 ($p < 0.05$) in males that showed social recognition (Fig. 4E).

In rats that did not show social recognition, females showed an increase in the percentage of OT release during T1 (trend: $p = 0.089$ vs dialysate 1), T2 ($p < 0.05$ vs dialysate 1), and dialysate 3 ($p < 0.05$ vs dialysate 1), while males did not (T1 vs dialysate 1: $p = 0.38$; T2 vs dialysate 1: $p = 0.67$; Fig. 4F). This sex difference was further

reflected by higher OT release in females compared to males during T2 ($p < 0.05$; Fig. 4F).

There was no difference in the percentage of OT release in males that showed social recognition compared to males that did not show social recognition (Fig. 4G). However, females that showed social recognition had lower OT release during T2 compared to females that did not show social recognition ($p < 0.05$; Fig. 4H). No effects of estrus phase were found for the percentage OT release (estrus phase: $F_{(1,13)} = 0.28$, $p = 0.61$; dialysate × estrus phase: $F_{(4,52)} = 1.2$, $p = 0.34$).

4. Discussion

We demonstrated that endogenous activation of the OTR in the pBNST is necessary for social recognition in both males and females and that exogenous OT facilitates social recognition in males only. Specifically, OTR antagonist (5 ng/0.5 μl/side) injected into the pBNST impaired social recognition in both sexes, while OT (100 pg/0.5 μl/side) injected into the pBNST prolonged the duration of social recognition in males, but not females, to 3 h. We speculate that the higher OTR binding density in the pBNST of males compared to females (Dumais et al., 2013) may explain the sex-specific facilitating effects of exogenous OT on social recognition. The higher percentage of OT release in the pBNST of males versus females during successful social recognition further indicates the sex-specific involvement of OT in the pBNST in social recognition. Furthermore, our current microdialysis findings along with our previous receptor autoradiography findings (Dumais et al., 2013) demonstrate that the sex difference in OTR binding density in the pBNST is not associated with a sex difference in extracellular OT concentration in the pBNST. To the best of our knowledge, this is the first report exploring the concomitant dynamics between OTR binding density and OT release in a single brain region.

OT and OTR are well known to play a role in the regulation of social recognition in rats and mice (reviewed in Gabor et al., 2012). For example, male OTR knockout mice (Takayanagi et al., 2005; Lee et al., 2008; Macbeth et al., 2009), male OT knockout mice (Ferguson et al., 2000; Macbeth et al., 2009) and female OT knockout mice (Choleris et al., 2003, 2006) show impaired social recognition. In rats, intracerebroventricular administration of an OTR antagonist impaired social recognition in both males (Lukas et al., 2013) and females (Engelmann et al., 1998). Moreover, brain-region specific regulation of social recognition by the OTR has been found in which social recognition was impaired after OTR blockade in the medial amygdala in male mice and rats (Ferguson et al., 2001; Lukas et al., 2013), in the lateral septum (Lukas et al., 2013) and ventral hippocampus (van Wimersma Greidanus and Maigret, 1996) in male rats, and in the olfactory bulbs in female rats (Larrazolo-Lopez et al., 2008). Our results confirm the involvement of the OT system in social recognition, and add the pBNST as a region important in OTR-mediated regulation of social recognition in both male and female rats.

A role for the OTR in the pBNST in social recognition complements previous findings demonstrating a role for the OTR in the pBNST in male odor-induced vaginal marking in female hamsters (Martinez et al., 2010). Interestingly, our study used juvenile conspecifics as social stimuli, suggesting that the pBNST may be involved in the processing of social odors outside the context of sociosexual behaviors. The processing of social cues could be mediated via direct projections from the accessory olfactory bulb to the pBNST (Halpern, 1987; Guillamon and Segovia, 1997). On the other hand, the pBNST has extensive connections with areas involved in learning, memory, and motivation (i.e., medial amygdala, hippocampus, hypothalamus, midbrain; Krettek and Price, 1978; Georges and Aston-Jones, 2002; Gu et al., 2003; Dong and Swanson, 2004; Krüger et al., 2015). This may indicate the potential of the pBNST to modulate the learning and/or motivational aspects associated with social recognition. Future research is needed to determine whether OTR activation in the pBNST regulates social recognition by modulating the processing of social cues, or by modulating higher order memory and learning processes.

Importantly, the effects of OT system manipulations in the pBNST appear specific to social recognition, as the same dose of OTR antagonist or OT injected into the pBNST did not alter social investigation time in either sex (as tested in the social investigation test and in T2 of the social recognition test). This was surprising given that both the OT system and the pBNST are known to modulate social investigation. For example, chronic intracerebroventricular OT administration in adult male rats (Witt et al., 1992) and acute intranasal OT administration in adult male mice (Huang et al., 2014) increased social investigation of adult females, while intracerebroventricular OTR antagonist injections in adult male rats and mice decreased the investigation time toward adult males (Lukas et al., 2011). In addition, excitotoxic lesions of the pBNST in male hamsters reduced the investigation time toward female hamsters (Been and Petrulis, 2010). However, stimuli in the above-mentioned studies were adults (i.e., highly salient social stimuli), while our study used juveniles (neutral and less salient social stimuli and thus avoiding aggression or sexual behaviors). We therefore cannot exclude that the OT system in the pBNST is involved in the investigation of more salient social stimuli. Overall, our data indicate that the OT system in the pBNST facilitates social recognition without affecting general social approach to investigate neutral social stimuli.

We also provide evidence for the sex-specific involvement of the OT system in the pBNST in social recognition. OT injected bilaterally into the pBNST (100 pg/0.5 μl/side) prolonged social recognition to 3 h in adult male rats, but failed to do so in adult female rats. This seems in line with previous studies demonstrating that intracerebroventricular administration of OT (1 ng) prolonged the duration of social recognition in adult male rats (Benelli et al., 1995), but not in adult female rats (Engelmann et al., 1998). It should be noted that it is unclear whether higher doses of OT would prolong social recognition in female rats. Dose dependent effects of OT administration on social recognition have been found in adult male rats, with sometimes higher doses impairing social recognition. However, these effects were seen after either subcutaneous or intracerebroventricular administration of OT (Popik et al., 1996, 1992a; Dantzer et al., 1987; Benelli et al., 1995; Popik and Vetulani, 1991). In contrast, studies using locally administered OT at different doses have reported only facilitating effects (i.e., medial preoptic area, Popik and van Ree, 1991; lateral septum, Popik et al., 1992b). Therefore, the facilitating effect of OT in the pBNST on social recognition in male rats is in line with the effects of OT in other brain regions in males. Moreover, females have much lower OTR binding density in the pBNST compared to males (Dumais et al., 2013), making it less likely that a higher dose of OT were to have an effect in females. Together, this indicates that male rats are more sensitive to the effects of the same dose of OT on social recognition and that the pBNST is a critical component of this effect.

A limitation of the current OTR manipulation study is that rats were not drug naïve during the social recognition test (i.e., the rats received the same drug treatment 2 days prior during the social interest test). However, the half-life of OTR antagonists and of OT is approximately 20 min (Mens et al., 1983; Goodwin et al., 1995; Ludwig and Leng, 2006). Therefore, a washout of 2 days would likely allow for the OTR antagonist and OT to leave the system, making it less likely that the effects of OT or OTR antagonist on social recognition are due to residual drug effects.

Furthermore, recent studies have shown that OT can mediate its behavioral effects via the vasopressin V1a receptor (Schorscher-Petcu et al., 2010; Sala et al., 2011; Ramos et al., 2013; Qiu et al., 2014; Song et al., 2014). Indeed, there is cross-reactivity between OT and vasopressin and their receptors (Manning et al., 2012). However, V1a receptor binding density is virtually absent in the area of the pBNST where OTR binding is very dense (Dumais and Veenema, 2015), making it less likely that the effects of OT on social recognition are mediated through the vasopressin V1a receptor.

Importantly, our in vivo microdialysis data also reveal a sex-specific involvement of OT in the pBNST in successful social recognition. In detail, males, but not females, showed an increase in the percentage of OT release in the pBNST during T1 ($p < 0.05$; exposure to an unfamiliar juvenile) and T2 ($p = 0.072$; exposure to the

same T1 juvenile along with a novel juvenile) compared to baseline OT release. As a result, the percentage of OT release in the pBNST was higher during both T1 and T2 in males than in females that showed social recognition. We further found that males investigated juveniles longer than females did, suggesting that the sex difference in percentage OT release in the pBNST could be associated with the sex difference in social investigation. However, as discussed above, OTR blockade in the pBNST did not alter social investigation in either sex. We therefore propose that higher OT release in males versus females during T1 and T2 of the social recognition test does not play a role in the sex difference to investigate neutral social stimuli, but rather is part of a sex-specific mechanism underlying social recognition.

Interestingly, females that did not show social recognition had higher OT release during T2 compared to baseline OT release. Further, females that did not show social recognition had higher OT release during T2 compared to females that showed social recognition and males that did not show social recognition. These findings, although highly surprising, may provide a rationale as to why exogenous OT (and thus, increasing OT levels) in the pBNST failed to improve social recognition after a 3 h interval in females. Yet, OTR blockade impaired social recognition in females, suggesting that a baseline level of OT release in the pBNST is still necessary for the appropriate expression of social recognition at the 1 h interval, possibly by maintaining baseline neuronal activity. Although it is yet unclear whether the increase in OT release in males and females predetermines their social recognition ability or is an effect of social stimuli exposure, findings from both OT injections and OT release suggest that higher OT signaling in the pBNST facilitates social recognition in males, but not in females.

A limitation of the current microdialysis study is the relatively long dialysate sampling period (30 min) as compared to the short stimulus exposure period (4 min). The 30-min sampling period is required due to detection limits of the radioimmunoassay, but this could have obscured a possible significant rise in OT release during T2 in males that show social recognition and during T1 in females that did not show social recognition.

Although extracellular OT release has been measured in various brain regions of male (Engelmann et al., 1999; Ebner et al., 2000; Waldherr and Neumann, 2007) and female (Nyuyki et al., 2011; Neumann et al., 1993; Bosch et al., 2010, 2004) rats, we are the first to compare OT release patterns between males and females in the same study. Our data show that extracellular OT concentrations in the pBNST are similar between sexes. This is in line with the absence of a sex difference in OT mRNA expression in the rat PVN and SON (Dumais et al., 2013) of which the PVN is a likely source of OT release in the pBNST (Knobloch et al., 2012). This also suggests that, at least for the pBNST, a sex difference in static OTR binding density (Dumais et al., 2013) does not correspond with a sex difference in static OT release. Instead, the higher OT release in the pBNST seen in males versus females during successful social recognition may indicate that higher OTR binding density in males serves to accommodate stimulus-induced dynamic increases in local OT release. Similarly, the higher OTR binding density in males may allow exogenous OT injections to have a greater effect in males than in females by binding to unoccupied OTRs in the pBNST of males, while endogenous OT may have already saturated all OTR binding sites in the pBNST of females.

Interestingly, the pBNST is a highly sexually dimorphic structure in volume, cell number, neurochemical expression, and neurocircuitry. For example, the pBNST is larger and contains more cells in males than in females in rats, mice, guinea pigs, and humans (Hines et al., 1985; Guillamon et al., 1988; Del Abril et al., 1987; Hines et al., 1992; Allen and Gorski, 1990; Chung et al., 2002; Forger et al., 2004). In rats, males show higher vasopressin (de Vries and Miller, 1998; Miller et al., 1989), estrogen receptor-alpha (Kelly

et al., 2013), substance P (Malsbury and McKay, 1987), and cholecystokinin (Micevych et al., 1988) immunoreactivity in the pBNST. Moreover, male rats have denser projections of the pBNST to several hypothalamic areas than female rats (Gu et al., 2003). Along with higher OTR binding (Dumais et al., 2013), all these parameters are higher or denser in males than in females. This suggests that the pBNST in males is programmed to respond differently to incoming social olfactory information from afferent brain regions such as the accessory olfactory bulb and medial amygdala (Halpern, 1987; Guillamon and Segovia, 1997) and/or may convey such information differently onto downstream projection areas that mediate behavioral responses. This may allow for the pBNST to promote male and female-specific expression of social behaviors, including male copulatory behavior (Emery and Sach, 1976; Claro et al., 1995), male aggressive behavior (Patil and Brid, 2010; Calcagnoli et al., 2014; Masugi-Tokita et al., 2015), and maternal behavior (Numan and Numan, 1996). Our findings are the first to suggest that the processing of social cues by the pBNST-OT system is different in males and females and may contribute to sex-specific modulation of social recognition.

5. Conclusions

In summary, our results show that the OT system in the pBNST is implicated in sex-specific regulation of social recognition in adult rats. We discussed that this could be due to higher OTR binding density in the pBNST of male versus female rats as was previously reported (Dumais et al., 2013) in combination with higher endogenous OT release in the pBNST of male versus female rats during successful social recognition.

Contributions

Authors Kelly M. Dumais and Alexa H. Veenema designed the study and wrote the manuscript. Author Kelly Dumais performed all experiments and analyzed the data. Authors Andrea G. Alonso, Marisa A. Immormino and Remco Bredewold assisted in the experiments. Author Remco Bredewold edited the manuscript. All authors have approved the final manuscript.

Appendix A. Supplementary dat

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2015.11.007.

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