Involvement of the oxytocin system in sex-specific regulation of social behavior and sex-specific brain activation

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INVOLVEMENT OF THE OXYTOCIN SYSTEM IN SEX-SPECIFIC REGULATION OF SOCIAL BEHAVIOR AND SEX-SPECIFIC BRAIN ACTIVATION

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The poorly understood, but robust sex differences in prevalence, symptom severity, and treatment responses of many psychiatric disorders characterized by social dysfunction signifies the importance of understanding the neurobiological mechanisms underlying sex differences in the regulation of social behaviors. One potential system involved is the oxytocin (OT) system. OT is an evolutionarily conserved neuropeptide that has been implicated in the regulation of a variety of social behaviors in rodents and humans. This thesis aims to clarify the role of OT in sex-specific regulation of social behavior and brain function in rats. Study 1 characterized sex differences in the OT system in the brain, and found that males show higher OT receptor (OTR) binding densities in several forebrain regions compared to females. Studies 2 and 3 then determined the relevance of these sex differences in OTR binding densities for the sex-specific regulation of social behavior using pharmacological manipulations of the OTR and *in vivo* measurement of OT release. Study 2 focused on the function of the OT system in the posterior bed nucleus of the stria terminalis (BNSTp), because this region showed the largest sex difference in OTR binding density, and is part of the core social behavior network. Results show that endogenous OT in the BNSTp is important for social recognition in both sexes, but that exogenous OT facilitated social recognition in males only. Furthermore, social recognition in males, but not in females, was associated with higher endogenous OT release in BNSTp. This study is the first to provide a link between sex differences in OTR binding density and OT release with sex-specific regulation of social recognition by OT. Study 3 focused on amygdala subregions because

these regions were found to show sex-specific correlations of OTR binding density with social interest. Results show that the OT system modulates social interest in the central amygdala (CeA), but not the medial amygdala, in sex-specific ways, with activation of the OTR in the CeA facilitating social interest in males, but not in females. These results provide evidence that the CeA is a brain region involved in the sex-specific processing of social stimuli by the OT system. Finally, Study 4 examined whether sex differences in OTR binding densities in forebrain regions lead to sex-specific brain activation in response to OT. Functional magnetic resonance imaging was used to examine blood oxygen level-dependent (BOLD) activation in awake male and female rats following central or peripheral administration of OT. Central OT administration induced sex differences in BOLD activation in numerous brain regions (including several regions with denser OTR binding in males), in which males showed predominantly higher activation compared to females. Peripheral OT administration also induced sex differences in BOLD activation, but in fewer brain regions and in different brain regions compared to central OT, indicating that the pattern and the magnitude of sex differences in neural activation induced by OT strongly depend on the route of administration. Together, outcomes of this thesis provide novel insight into the sexual dimorphic structure and function of the OT system in rats, and highlights the fact that research seeking a full understanding of the role of the OT system in behavioral and brain responses is incomplete without the inclusion of both sexes. These results may be informative given the increasing popularity of the use of OT as a potential therapeutic agent in the treatment of social dysfunction in sex-biased psychiatric disorders.

Keywords: Oxytocin, oxytocin receptor, sex differences, social interest, social recognition, bed nucleus of the stria terminalis, amygdala, fMRI

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I. General Introduction

People are highly motivated to form social relationships. In a sense, we are hard-wired to seek connections with others (Cacioppo and Patrick, 2008; Crosier et al., 2012; Gilbert, 2015). Indeed, across social species, sociality is an extremely diverse yet integral feature of life (Goodson et al., 2012; Anacker and Beery, 2013; Kelly and Ophir, 2015). From gregariousness in birds and monogamous pair-bonding in prairie voles to the mother-infant bond in humans, social relationships are exhibited across the animal kingdom and are imperative for survival and reproduction. The importance of these social interactions becomes even more apparent with the evidence showing disturbances in both emotional and physiological well-being when these social relationships are impaired (Umberson and Montez, 2010). Unfortunately, impairments in social functioning are often key features in neuropsychiatric disorders, and can be debilitating for people living with these disorders. Therefore, to understand the neural mechanisms underlying social dysfunction, it is imperative to understand the neural regulation of social behavior.

Importantly, many psychiatric disorders characterized by social dysfunction display sex differences in prevalence, symptom severity and treatment responses. For example, autism spectrum disorders are four to eight times more common in boys than in girls (Fombonne, 2003; Knickmeyer and Baron-Cohen, 2006; Beaudet, 2012), schizophrenia is twice as common in men than in women (Nicole et al., 1992), and borderline personality disorder is three times more common in women than in men (Widiger & Trull, 1993). The vulnerability of one sex to particular neuropsychiatric disorders is overwhelmingly apparent, yet poorly understood. Sex differences in the neural systems that regulate social behavior may contribute to these sex biases in psychiatric disorders, making it imperative to understand sex differences in the brain and the neurobiological mechanisms regulating social behavior in both males and females. Unfortunately, a major barrier in the field of

neuroscience research is that most studies on social behavior are being conducted using only males as subjects (Zucker & Beery, 2010). Therefore, the overall aim of my research is to elucidate the neurobiological mechanisms regulating social behavior in both sexes.

In recent years, research in social neuroscience has characterized the key neural circuitry and essential neurochemical regulators of social behavior. Though the complexity of social living varies across species, oxytocin (OT) has been found to be a common neural substrate which regulates various social behaviors in both sexes and across species, including rodents and humans (Heinrichs et al., 2009; Ross and Young, 2009; Goodson and Thompson, 2010; Anacker and Beery, 2013; Hammock, 2015; Dumais and Veenema, 2016). OT is a highly conserved nine amino acid neuropeptide, which acts as both a hormone in the periphery via the hypothalamic-neurohypophysial system, and as a neuromodulator in the central nervous system. OT-producing magnocellular neurons of the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus project to the posterior pituitary, where it is released into the general circulation as a hormone. Moreover, OT synthesized in parvocellular neurons of the PVN projects centrally (Buijs, 1978; Buijs and Swaab, 1979; Sofroniew, 1980; Sofroniew, 1983) where it can modulate the activation of many brain regions via binding to the widely distributed OT receptor (OTR; Gimpl and Fahrenholz, 2001). Only recently was it found that magnocellular OT neurons of the SON also project to a variety of forebrain regions (Knobloch et al., 2012).

Remarkably, OT neurons in the PVN and SON as well as their fiber projections have been characterized using males only, females only, or sex of subjects was not specified (Buijs, 1978; Buijs and Swaab, 1979; Sofroniew, 1980; Sofroniew, 1983; Knobloch et al., 2012). Furthermore, most studies that quantify OTR binding in the rat brain have also been performed in one sex only (Freund-Mercier et al., 1987; deKloet et al., 1985; Insel, 1986; Elands et al., 1988; Tribollet et al., 1988, 1989; Shapiro and Insel, 1989). Indeed,

investigation into sex differences in OTR binding density is limited, with studies that include both males and females either using a non-specific ligand for the OTR or making only few direct comparisons between males and females (Tribollet et al., 1990; Uhl-Bronner et al., 2005). Therefore, a thorough analysis of OTR binding sex differences is still to be determined, and is a major aim of Study 1. Across species, the OTR is highly expressed in brain regions involved in the regulation of social behavior (Tribollet et al., 1990; Ophir et al., 2012; Zheng et al., 2013). Therefore, knowledge of sex differences in the expression of both OT and OTR is an important step towards understanding the role of OT in sex-specific regulation of social behavior.

OT is well established as a key neuromodulator of social behavior, however its history in the scientific literature presents challenges in understanding the role of OT in social behavior and in brain function in both sexes. The classically known function of OT in mammals is its regulation of the maternal response, in which peripheral OT is important in the induction of uterine contractions during parturition and milk ejection during lactation (Fuchs and Poblete, 1970; Belin et al., 1984). Since the late 1970's, researchers have extensively investigated the role of OT in the maternal brain, finding that OT is important in the parturient female via the regulation of both active and passive forms of maternal behavior, such as nesting, retrieving pups, and licking and grooming of pups (Pedersen and Prange, 1979). It wasn't until the late 1980's and early 1990's that OT's role in other social behaviors, such as social bonding and social recognition, started being explored. Ironically, when the role of OT in these other forms of social behavior started being investigated, males, rather than females, were predominantly used as subjects, and OT was found to be a key regulator of social approach and social recognition in male rats (Dantzer et al., 1987; Popik and vanRee, 1991, Popik et al., 1992; Witt et al., 1992, Benelii et al., 1995, Arletti et al., 1995). With these studies highlighting the role of OT in male social behavior, this classically

known “maternal hormone” is now seen as an important regulator of both male and female social behavior. Unfortunately, there have been few studies which directly compare the role of OT in social behavior in males and females, which is a key objective of the present thesis.

The investigation of sex differences in OT system parameters and function is especially important given the increasing popularity of OT for use as a therapeutic agent in humans. Indeed, since the development of the non-invasive intranasal application of OT, there has been an influx of research into the role of OT in humans. This research has strongly implicated OT as an important modulator of human social behavior and cognition (Donaldson and Young, 2008; Heinrichs et al., 2009; Guastella and MacLeod; 2012), as well as a therapeutic agent for social dysfunction in various sex-biased neuropsychiatric disorders, including autism spectrum disorder, schizophrenia, depression, and anxiety disorders (Guastella et al., 2010; Meyer-Lindenberg et al., 2011; Cochran et al., 2013). Though only a few studies have compared the effects of OT administration on social behavior and neural activity in men and women, these few studies have shed light on sex-specific actions of OT in humans (for review, see Dumais and Veenema, 2015, 2016). Therefore, a better understanding of whether there are sex-specific actions of OT on behavioral and brain function is imperative if we are to use OT as a potential therapeutic agent in the treatment of social dysfunction.

Aims: Our overall aim is to further understand sex-specific regulation of social behavior and brain function by OT. The specific aims of this dissertation are three-fold. First, we aimed to characterize the OT system in adult male and female rats to determine potential sex differences in OT mRNA and in OTR binding densities in the brain (Study 1). Because our results from Study 1 showed robust sex differences in OTR binding densities, our second aim was to determine how sex differences in OTR are linked to sex-specific regulation of social behavior (Study 2 and 3). Finally, given the robust sex differences in OTR, our third

aim was to determine whether OT activates neural systems differently in males and females (Study 4).

Our experimental designs use multidisciplinary approaches, including behavioral, molecular, and neuroimaging techniques to best address our aims. In Study 1, I used *in situ* hybridization and receptor autoradiography to determine potential sex differences in OT mRNA and OTR binding densities, respectively, in adult male and female rats. I also determined whether different hormonal states of females (estrus phase, reproductive status) modulate OTR binding densities, and whether OTR binding densities correlate with social interest. Next, I used behavioral, pharmacological, and microdialysis techniques to explore the functional significance of sex differences in OTR binding densities (Study 2) and of sex-specific correlations of OTR binding densities and social interest (Study 3) that were found in Study 1. Utilizing rats' spontaneous motivation to investigate conspecifics, and their innate drive to investigate novel over familiar conspecifics, we employed the well-established social interest (Thor, 1980; Johnson and File, 1991) and social discrimination (Engelmann et al., 1995) paradigms to investigate sex-specific regulation of social behavior by OT. These studies aim to clarify the role of OT in sex-specific regulation of social behavior, and to determine a potential functional link between sex differences in OTR binding with sex-specific regulation of social behavior by OT.

Finally, to understand the neural mechanisms underlying sex-specific regulation of social behavior by OT, it is also important to map sex differences in the neural circuits that are modulated by OT. Indeed, sex differences in OTR binding densities found in Study 1 suggest that OT may modulate neural activation differently in male and female rats. We employed state-of-the-art functional magnetic resonance imaging (fMRI) in awake male and female rats to address this aim. fMRI allows mapping of real-time neuronal activation, and serves as a useful complementary neuroscience technique that provides adequate spatial and

temporal resolution to observe patterns of whole brain neuronal activity in less than a minute (Ferris et al., 2006). Importantly, studies using animal models provide a certain design flexibility that is not always present with human research (i.e., central administration of OT). This research is the first to use fMRI to determine OT-mediated sex differences in brain activation patterns in rats, and our outcomes will provide novel insights into how OT modulates neuronal activation in sex-specific ways.

The rationale for this dissertation research is that outcomes will advance our understanding of the neurobiological mechanisms underlying sex differences in social behavior. This may provide a first step in gaining insight into potential mechanisms underlying sex differences in healthy and impaired social behaviors that may underlie sex biases in neuropsychiatric disorders characterized by social dysfunction. Insight into the role of OT in the regulation of social behavior may further provide insight into the appropriate use of OT as a therapeutic agent, with sex as a major factor for consideration.

II. Study 1: Sex differences in oxytocin receptor binding in forebrain regions: correlations with social interest in brain region- and sex- specific ways*

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Abstract: Social interest reflects the motivation to approach a conspecific for the assessment of social cues and is measured in rats by the amount of time spent investigating conspecifics. Virgin female rats show lower social interest towards unfamiliar juvenile conspecifics than virgin male rats. We hypothesized that the neuropeptide oxytocin (OT) may modulate sex differences in social interest because of the involvement of OT in pro-social behaviors. We determined whether there are sex differences in OT system parameters in the brain and whether these parameters would correlate with social interest. We also determined if estrus phase or maternal experience would alter low social interest and whether this would correlate with changes in OT system parameters. Our results show that regardless of estrus phase, females have significantly lower OT receptor (OTR) binding densities than males in the majority of forebrain regions analyzed, including the nucleus accumbens, caudate putamen, lateral septum, bed nucleus of the stria terminalis, medial amygdala, and ventromedial hypothalamus. Interestingly, male social interest correlated positively with OTR binding densities in the medial amygdala, while female social interest correlated negatively with OTR binding densities in the central amygdala. Proestrus/estrus females showed similar social interest to non-estrus females despite increased OTR binding densities in several forebrain areas. Maternal experience had no immediate or long-lasting effects on social interest or OT brain parameters except for higher OTR binding in the medial amygdala in primiparous

females. Together, these findings demonstrate that there are robust sex differences in OTR binding densities in multiple forebrain regions of rats and that OTR binding densities correlate with social interest in brain region- and sex-specific ways.

Introduction

Social interest provides the motivation to approach a conspecific for the assessment of social cues which will, in turn, facilitate appropriate behavioral responses. Social interest therefore provides the initial step for the expression of a wide range of social behaviors. Sex differences in social interest have been consistently reported in rats and mice. In detail, virgin females show less social interest than virgin males as indicated by their lower levels of social investigation toward juvenile conspecifics (Holmes et al., 2011; Johnson & File, 1991; Tejada & Rissman, 2012; Thor, 1980; Thor et al., 1988). The mechanisms underlying this sex difference, however, are not well understood.

We hypothesized that such sex differences might be mediated by sex differences in parameters and/or function of the oxytocin (OT) system. OT is mainly synthesized in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus and can modulate the activation of many brain regions via binding to the widely distributed OT receptor (OTR; Gimpl & Fahrenholz, 2001). OT promotes pro-social behaviors that require social interest, such as affiliative behavior, maternal behavior, pair-bonding, and social recognition in rodents and humans (Donaldson & Young, 2008; Ferguson et al., 2002; Insel et al., 1997; Ross & Young, 2009; Striepens et al., 2011; Veenema & Neumann, 2008). OT modulates these pro-social behaviors by acting on core components of the "social behavioral network" (Goodson, 2005; Newman, 1999) such as the medial preoptic area (MPOA), lateral septum (LS), bed nucleus of the stria terminalis, (BNST), and medial amygdala (MeA). OT also plays a key role in the modulation of fear and anxiety in rodents and humans especially

via activation of OTR in the central amygdala (CeA; Knobloch et al., 2012; Labuschagne et al., 2010), which may influence levels of social interest.

Furthermore, behavioral and neuronal effects of OT are often sex-specific. For example, intracerebroventricular administration of OT promotes pair-bonding in female prairie voles, but not in male prairie voles (Insel and Hulihan, 1995). In addition, blocking OTR on the first day of life altered activation of the CeA in response to novel heterosexual pairing in adult female, but not male, prairie voles (Kramer et al., 2006). In humans, intranasally applied OT improved kinship recognition in women only, and competition recognition in men only (Fischer-Shofty et al., 2012). Moreover, intranasally applied OT reduces amygdala activity in men, but increases amygdala activity in women when viewing emotional facial expressions (Domes et al., 2007, 2010). These findings suggest that the OT system functions differently in males versus females, possibly via sex differences in OT brain parameters. However, a systematic analysis of sex differences in OT brain parameters, and linking such parameters to sex differences in social behavior, such as social interest, is lacking thus far.

Social interest in females may also be regulated by sex hormone effects on the OT system. For example, ovariectomized females given estrogen treatment showed increased OTR mRNA expression in brain areas involved in social and sexual behaviors, such as the ventromedial hypothalamus (VMH), MeA, and hippocampal CA1 region (Bale et al., 1995b, 2001; Quinones-Jenab et al., 1997). This up regulation of the OT system via estrogen may therefore enhance general social approach behaviors, and thus increase social interest.

In addition, pregnancy and parturition induce alterations of the OT system in the maternal rat brain. Specifically, OT facilitates the onset of maternal behaviors by acting at critical areas in the brain, modulating the switch from avoidance to approach behaviors toward pup stimuli (Numan & Stolzenberg, 2009). Nulliparous female rats (virgin females)

avoid pups, while primiparous female rats (females who have experienced one episode of pregnancy and parturition) readily approach and take care of pups immediately post-parturition (Numan et al., 2006; Numan & Insel, 2003). Therefore, we speculate that dynamic changes in the OT system in females due to pregnancy may have the potential to facilitate, more broadly, approach behaviors toward neutral social stimuli.

In the present study, we investigated sex differences in OT mRNA expression in the PVN and SON, and OTR binding densities in forebrain regions. In addition, we investigated whether OTR binding densities correlated with social interest scores. We further investigated effects of estrus phase and maternal experience on social interest, OT mRNA expression, and OTR binding densities. Because OT facilitates pro-social behaviors and females show lower social interest than males, we hypothesized that females would show lower levels of OTR binding densities than males in brain areas involved in approach behaviors. We also hypothesized that OTR binding densities in specific brain regions would positively correlate with social interest. Because females in estrus show increased activation of the OT system, we hypothesized that they would exhibit higher social interest than non-estrus females, and that this would correlate with increased OTR mRNA expression and/or OTR binding densities. Finally, we hypothesized that an increase in approach behaviors towards pups in primiparous females would generalize to an increase in approach behaviors towards juveniles, and that this would correlate with increased OT mRNA expression and/or OTR binding densities.

Methods

Animals. Wistar rats were obtained from Charles River (Raleigh, NC) and maintained on a 12 h light/dark cycle, lights on at 07:00am, 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 x 48.3 x 20.3cm) 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. Same-sex stimulus rats were used for all behavioral testing. All experiments were conducted in accordance with the guidelines of the NIH and approved by the Boston College Institutional Animal Care and Use Committee.

Behavioral Testing. *Social Investigation Test*. To test for social interest, we measured how long subjects spent investigating a stimulus juvenile using the social investigation test, adapted from Thor (1980). A juvenile was used in order to assess general social approach toward neutral stimuli that do not elicit threatening or sexual behavior. A juvenile was placed into the subject's home cage for 4 min, and time spent investigating the juvenile was measured. Testing was performed during the light phase between 12:00 h and 15:00 h. All behaviors were recorded and analyzed using JWatcher (http://www.jwatcher.ucla.edu) by an experimenter blind to treatment groups. Behavior was considered social investigation when the subject was actively sniffing the juvenile, including sniffing the back, neck, face, and anogenital area.

OT *in situ* hybridization. Two days after the last social investigation test, subjects were anesthetized under CO_2 and decapitated. Brains were removed and quickly frozen in methylbutane on dry ice, and stored at -80°C. Brains were cut into 16-µm coronal sections on a cryostat and mounted on slides. Slides were processed and hybridized according to Veenema et al. (2006). Briefly, slides were fixed in 4% paraformaldehyde, rinsed twice in PBS, dipped in distilled H_2O, and immersed in acetic anhydride + triethanolamine. Slides were then rinsed in 1X SSC (sodium chloride-sodium citrate) and dehydrated in a series of ethanol rinses and delipidated in chloroform. Slides were then incubated with a prehybridization buffer (4/5 hybridization buffer + 1/5 tRNA) for 2 hours at 50°C. Slides were then washed again in sodium chloride citrate and ethanol, and hybridized in

hybridization buffer with a ^{35}S-labeled oligonucleotide (5'CTCGGAGAAGGCAGACTCAGGGTCGCAGGCGGGGTCGGTGCGGCAGCC-3') overnight at 50°C in an incubation chamber. After the incubation, slides were washed three times in heated 1X SSC with shaking, and a fourth time shaking, allowing to cool to room temperature. The last wash steps were with ammonium acetate mixed with ethanol at 50% and 85%, then 100% ethanol. Slides were then air dried and exposed to film (Kodak) for three hours. The optical density of OT mRNA was measured as arbitrary units using ImageJ (NIH, http://rsb.info.nih.gov/ij/). Regions of interest included the PVN and SON. Each measurement was subtracted by tissue background, and OT mRNA expression was calculated by taking the mean of 2-4 bilateral brain section measurements per region of interest per rat. Brain tissue of males and females of Exp. 1-3 were run simultaneously to be able to compare groups.

OTR autoradiography. Additional sections of the brains (removed as described above) were processed for receptor autoradiography. The receptor autoradiography procedure was performed according to Lukas et al. (2010) using [^{125}I]-Ornithine Vasotocin Analog (d(CH_2)$_5$[Tyr(Me)^2,Thr^4,Orn^8,[^{125}I]Tyr^9-NH_2]-OVTA; Perkin Elmer, USA) as tracer. Briefly, the slides were thawed and dried at room temperature. Slides were then fixed in 0.1% paraformaldehyde and washed two times in Tris buffer (pH 7.4). The slides were then exposed to tracer buffer (Tris + 10mM $MgCl_2$, 0.1% BSA, and tracer) for 60 min, and then washed four times in Tris + $MgCl_2$. The slides were then dipped in distilled water, dried, and exposed to film (Kodak) for 7 days. The optical density of OTR binding was measured using ImageJ (NIH, http://rsb.info.nih.gov/ij/). Regions of interest included: agranular insular cortex (AIP), NACC, caudate putamen (CPU), LS, BNST, MPOA, PVN, CeA, MeA, hippocampus (CA1 region), and VMH. Each measurement was subtracted by tissue background, and receptor densities were calculated by taking the mean of 4-6 (depending on

the region being analyzed) bilateral brain section measurements per region of interest per rat. The data was converted to dpm/mg (disintegrations per minute/milligram tissue) using a [^{125}I] standard microscale (American Radiolabeled Chemicals Inc, St. Louis, MO). Brain tissue of males and females of Exp. 1-3 were run simultaneously to be able to compare groups.

Experimental Procedures

Experiment 1: Sex differences in the OT system and correlations of OTR binding with social interest

Males (n=12) and females (n=28) were housed in same-sex pairs, and were single-housed 24 h before testing. Each subject was tested four times (Tests 1-4) in the social investigation test, at least one week apart, in order to get a representative average social interest score. Two days after the last social investigation test, brains were collected and processed for OT *in situ* hybridization and OTR autoradiography as described above.

Experiment 2: Effect of estrus cycle phase on social interest and the OT system

Females (n=28) were single-housed 24 h before testing and tested one time in the social investigation test, and brains were removed 2 days later as described above for OT mRNA and OTR processing. Estrus cycle phase was assessed via vaginal smears taken from each animal immediately following behavioral testing and at the time of brain removal. Using a pipette and a small amount of distilled water, a sample of vaginal secretions were taken and put onto a glass slide. Using a microscope, estrus cycle phase was determined via cell characteristics based on Goldman et al. (2007). Because there was no statistical difference in OT mRNA expression or OTR binding densities between estrus and proestrus females or between diestrus and metestrus females, subjects were categorized as in proestrus/estrus (n=13; cells characteristic of proestrus and estrus phases in which females show higher levels of estradiol and progesterone), or non-estrus (n=15; cells characteristic of diestrus and metestrus in which females show lower levels of estradiol and progesterone).

Experiment 3: Effect of maternal experience on social interest and the OT system

Virgin females were mated (primiparous group, n=16) by putting one male in a cage with two females for five days. Following parturition, all litters were culled to 8 pups (4 males and 4 females), and weaned after 21 days. Nulliparous females (n=12) were similarly housed to primiparous females according to the following schedule: primiparous females were single-housed during the last week of pregnancy, during lactation, and for 24 hours before all behavioral tests.

Each animal was exposed to the social investigation test four times during four different testing periods to assess both short- and long-term effects of maternal experience on social interest. Each testing period included subjects from both the primiparous and nulliparous groups. Females were tested when they were all virgins, 10 days after the last day of mating ("pregnancy"), 3 days after weaning the litters ("3d post wean"), and 3 weeks after weaning the litters ("3 wks post wean"). Timing of these tests was chosen in order to determine if changes in social interest corresponded with a particular time-point during pregnancy or post parturition. We did not test females during the lactation stage because females showed high levels of maternal aggression toward the stimulus juveniles. Two days after the last behavioral test, brains were collected and processed for OT *in situ* hybridization and OTR autoradiography as described above.

In the first set of animals, we found a significant difference in social interest at 3 days post weaning in which primiparous females showed lower social interest compared to nulliparous females. To determine if this difference was an effect of the experimental conditions, or an effect of repeated testing, we tested an additional group of nulliparous (n=12) and primiparous (n=12) females four times starting at the time point at which the

difference was found (3 days post weaning), and continued to test them at 3, 7, and 10 weeks post weaning.

Statistical Analysis. Analyses for social investigation times (calculated as seconds) for experiments 1 and 3 were performed using one-way ANOVA with repeated measures (Experiment 1: Sex is the between-subject factor and social investigation test is the within-subject factor; Experiment 3: Maternal experience is the between-subject factor and social investigation test is the within-subject factor). Bonferroni post-hoc tests were used when appropriate. One-way ANOVAs were used to test for differences in OT mRNA expression and OTR binding densities between males and females, between non-estrus and proestrus/estrus females, and between nulliparous and primiparous females. A bivariate correlation and a curve estimate regression analysis were used to test correlations between social investigation scores and OTR densities in areas of interest. Significance was set at $p<0.05$.

Results

Experiment 1: Sex differences in the OT system and correlations of OTR binding with social interest

Females showed consistently lower social investigation times compared to males (main effect of sex: $F_{(1,38)}= 169$, $p<0.001$, Fig. 1.1). No differences in OT mRNA expression in either the PVN or SON were found between males and females (PVN: males = 147 ± 4.1, females = 152 ± 3.3; SON: males= 149 ± 3.0, females=155 ± 2.5). In contrast, females showed lower OTR binding densities than males in 9 out of 15 forebrain regions analyzed (Figs. 1.2 and 1.3). In detail, compared to males, females showed significantly lower OTR binding densities in the AIP ($F_{(1,34)}=7.49$, $p<0.05$), posterior NAc (NACCp; $F_{(1,34)}=5.90$, $p<0.05$,), dorsal CPU (CPUd; $F_{(1,38)}=55.1$, $p<0.001$), intermediate LS (LSi; $F_{(1,36)}=48.4$, $p<0.001$), posterior BNST (BNSTp; $F_{(1,34)}=219$, $p<0.001$,), MPOA ($F_{(1,35)}=5.64$, $p<0.05$),

VMH ($F_{(1,36)}$=109, p<0.001), MeA ($F_{(1,37)}$=67.6, p<0.001), and CA1 region ($F_{(1,37)}$=8.51, p<0.01; Fig. 1.2). Females showed higher OTR binding density only in the medial part of the CPU (CPUm; $F_{(1,37)}$=23.7, p<0.001; Fig. 1.2). No sex differences in OTR binding density were found in the anterior NACC (NACCa), ventral LS (LSv), PVN, dorsal lateral BNST (BNSTdl), and CeA (Fig. 1.2).

We further found that social investigation scores in males correlate positively with OTR binding densities in the MeA (p<0.05; Fig. 1.4). In females, social investigation scores correlate positively with OTR binding densities in the CPUm (p<0.001) and CA1 region (p<0.05), and correlate negatively with OTR binding densities in the CeA (p<0.05; Fig. 1.4).

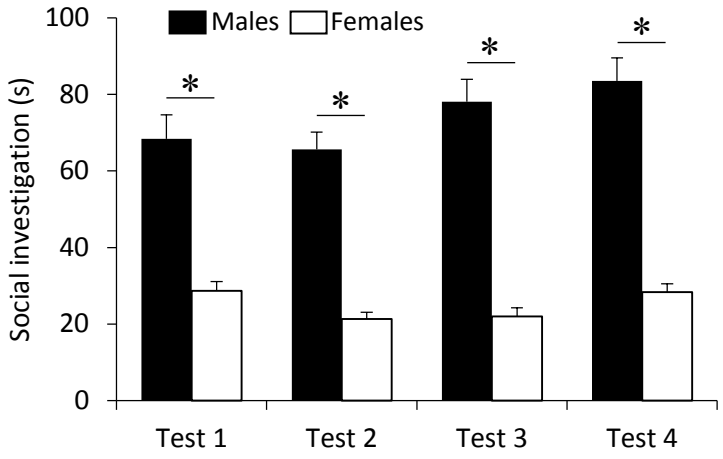

Fig. 1.1 Social investigation scores of male and female rats. Each subject was tested four times in the social investigation test at least one week apart. Females show significantly less investigation of an unfamiliar juvenile rat compared to males (main effect of sex: $F_{(1,38)}$= 169, p<0.001), which was consistent across the four tests (*=p<0.001; Test 1 $F_{(1,38)}$=52.7; Test 2 $F_{(1,39)}$ =123; Test 3 $F_{(1,39)}$=119; Test 4$_{(1,39)}$ F=115). Bars indicate means + SEM.

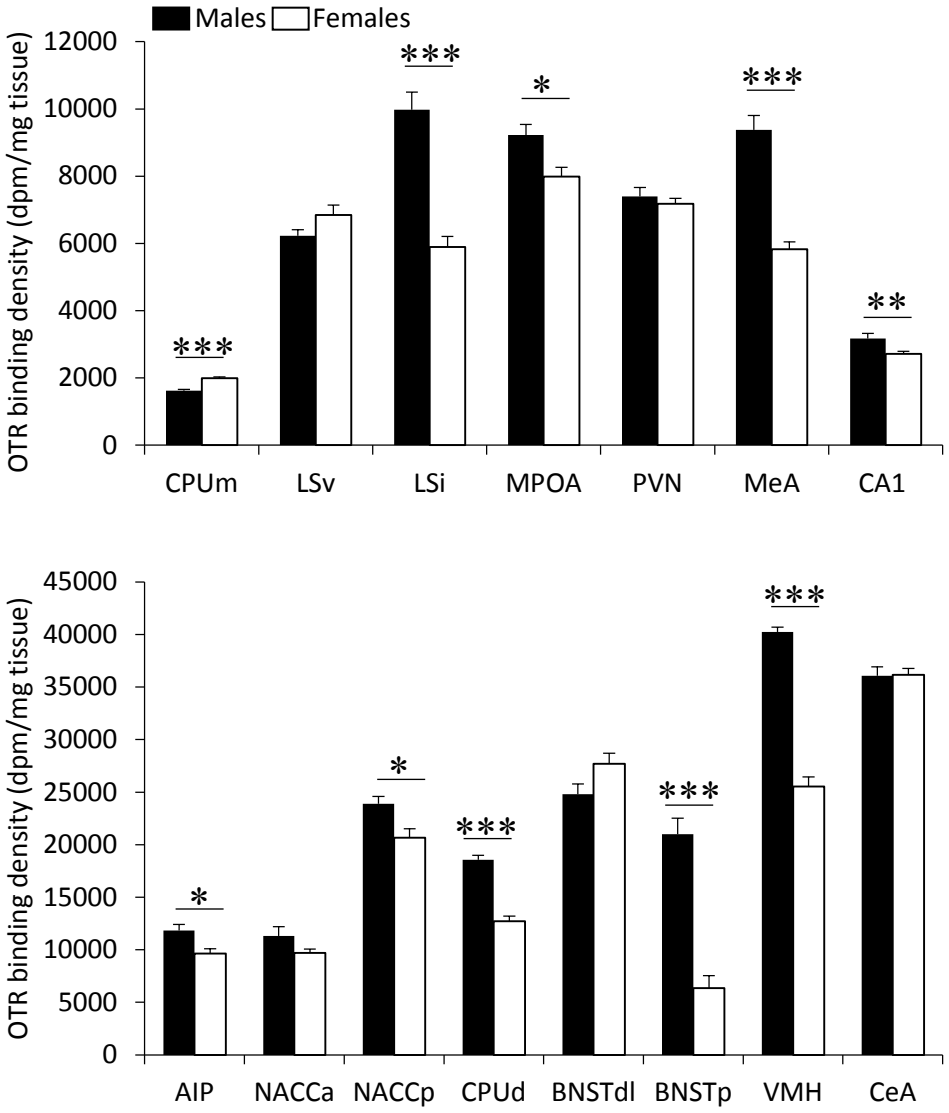

Fig. 1.2 OTR binding densities in forebrain regions of male and female rats. Females have lower OTR binding densities than males in 9 out of 15 forebrain regions analyzed, while females have a higher OTR binding density in 1 (CPUm) out of 15 forebrain regions analyzed. Bars indicate means + SEM. *= $p<0.05$, **=$p<0.01$, ***=$p<0.001$, one-way ANOVA.

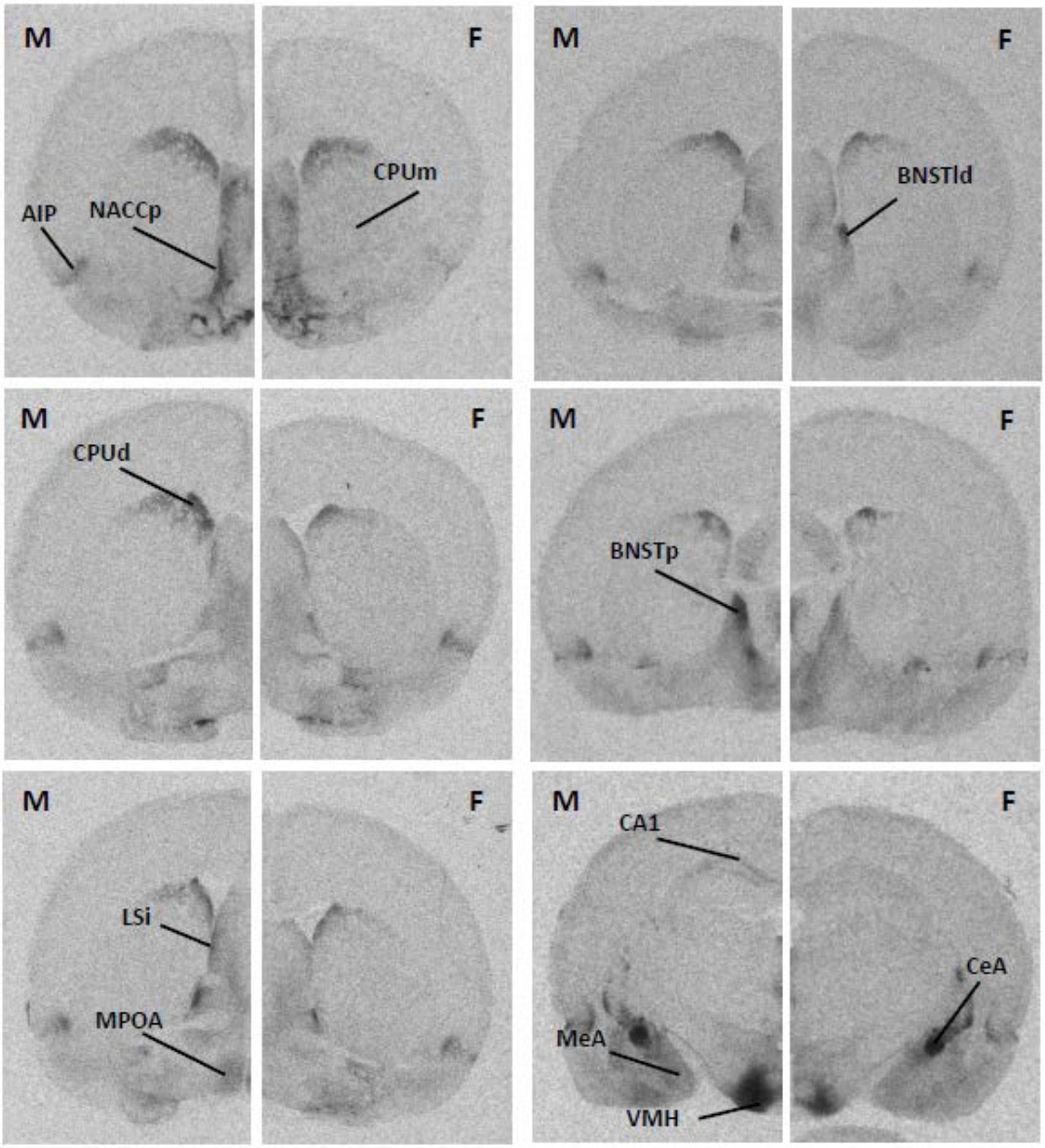

Fig. 1.3 Representative coronal sections showing OTR binding densities in forebrain areas of male (M) and female (F) rats. Compared to males, females have higher OTR binding density in the CPUm, while having lower OTR binding densities in the AIP, NACCp, CPUd, LSi, MPOA, BNSTp, CA1, MeA, and VMH. Males and females show similar OTR binding densities in the CeA and BNSTld.

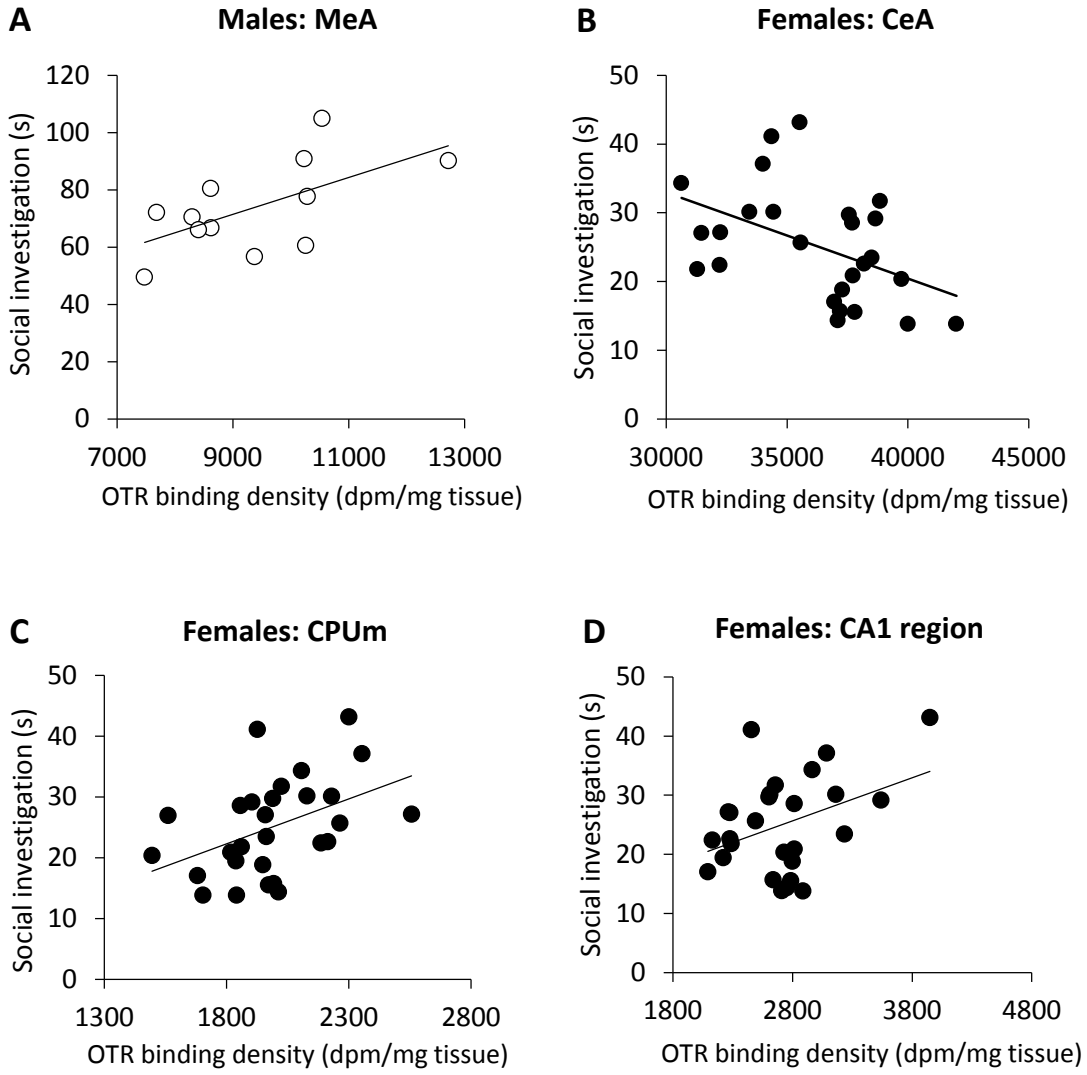

Fig. 1.4 Correlations of OTR binding densities and social investigation scores. (A) In males, social investigation scores positively correlate with OTR binding density in the MeA ($p<0.05$, $r=0.61$; A). (B-D) In females, social investigation scores negatively correlate with OTR binding density in the CeA ($p<0.05$, $r=0.46$; B), and positively correlate with OTR binding density in the CPUm ($p<0.05$, $r=0.44$; C), and the CA1 region ($p<0.05$, $r=0.39$; D).

Experiment 2: Effect of estrus cycle phase on social interest and the OT system

Estrus cycle phase had no effect on social interest: Non-estrus and proestrus/estrus females showed similar social investigation times (Fig. 1.5A). Also, no differences in OT mRNA expression in either the PVN or SON were found between non-estrus and proestrus/estrus females (PVN: non-estrus = 153 ± 4.2, estrus = 151 ± 5.3; SON: non-estrus= 155 ± 4.0, estrus= 155 ± 3.0).

However, proestrus/estrus females showed higher OTR binding densities than non-estrus females in the NACC (anterior plus posterior NACC; $F_{(1,22)}$=9.83, $p<0.01$), BNSTp ($F_{(1,21)}$=9.13, $p<0.05$), MPOA ($F_{(1,26)}$=8.58, $p<0.01$), VMH ($F_{(1,24)}$=6.83, $p<0.05$), and approaches significance in the MeA ($F_{(1,25)}$=4.20, p=0.051; Fig. 1.5B; see Table 1.1 for values of the areas that are not statistically significant). Notably, despite higher OTR binding densities in specific brain areas of proestrus/estrus females, OTR binding densities were still lower when compared to those in males, except for the MPOA.

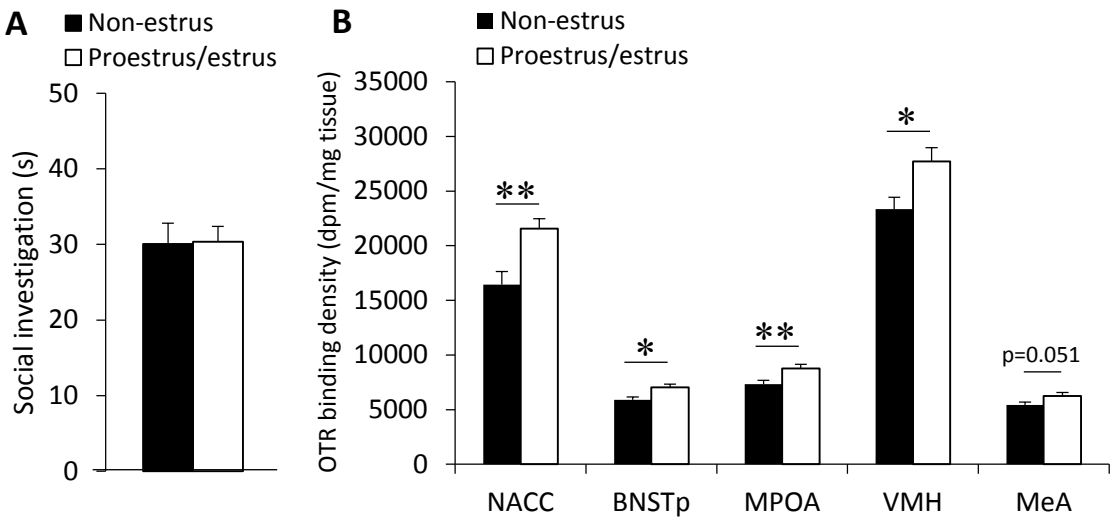

Fig. 1.5 Social investigation scores and OTR binding densities in non-estrus and proestrus/estrus females. (A) Social investigation scores were not different between non-estrus and estrus females. (B) Estrus females have higher OTR densities in the NACC, BNSTp, MPOA, and VMH, and show a trend toward significance in the MeA, compared to non-estrus females. Bars indicate means + SEM. *=p<0.05, **=p<0.01, one-way ANOVA.

OTR binding densities	Non-estrus	Proestrus/estrus	Estrus effect	Cohen's *d*
Agranular insular cortex	9283 ± 785	10038 ± 507	$F_{(1,22)} = 0.60$, p = 0.45	-0.335
Dorsal caudate putamen	12508 ± 736	12942 ± 631	$F_{(1,26)} = 0.19$, p = 0.66	-0.169
Medial caudate putamen	2030 ± 74	1941 ± 46	$F_{(1,26)} = 0.97$, p = 0.34	0.394
Ventral lateral septum	6588 ± 282	7130 ± 529	$F_{(1,25)} = 0.85$, p = 0.37	-0.365
Intermediate lateral septum	5785 ± 441	5917 ± 469	$F_{(1,25)} = 0.003$, p = 0.96	-0.021
Dorsolateral BNST	26080 ± 1169	29468 ± 1555	$F_{(1,23)} = 3.66$, p = 0.07	-0.785
Paraventricular nucleus	7048 ± 230	7297 ± 227	$F_{(1,21)} = 0.59$, p = 0.45	-0.321
Central amygdala	35962 ± 921	36379 ± 763	$F_{(1,24)} = 0.12$, p = 0.73	-0.137
Hippocampal CA1 region	2740 ± 131	2683 ± 104	$F_{(1,25)} = 0.12$, p = 0.74	0.132

Table 1.1 Forebrain areas of non-estrus and proestrus/estrus female rats in which no estrus phase difference was found for OTR binding densities (dpm/mg tissue). Data indicate means ± SEM.

Experiment 3: Effect of maternal experience on social interest and the OT system

A significant test x maternal experience interaction effect was found for social interest ($F_{2,52}$=4.87, $p<0.05$). Post-hoc comparisons revealed a significant difference in social interest at 3 days post-weaning, with primiparous females investigating less than nulliparous females ($p<0.05$, Fig. 1.6A). However, this effect could not be confirmed in an additional set of primiparous and nulliparous females (Fig. 1.6B). It is therefore unlikely that maternal experience induces a consistent change in social interest.

No differences in OT mRNA expression in either the PVN or SON were found between nulliparous and primiparous females (PVN: nulliparous = 154 ± 5.1, primiparous= 151 ± 4.4; SON: nulliparous= 154 ± 2.6, primiparous 156 ±4.0). Except for higher OTR binding density in the MeA in primiparous females ($F_{(1,26)}$= 5.6, $p<0.05$), no long-term effect of pregnancy and lactation was found for OTR binding densities in any of the other forebrain regions analyzed (data not shown).

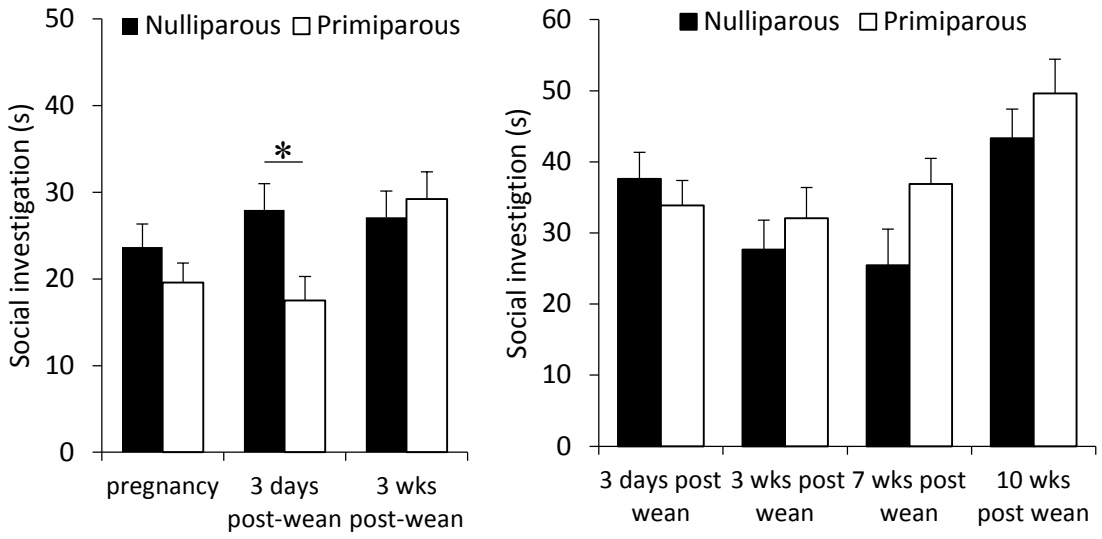

Fig. 1.6 No consistent effect of maternal experience on social investigation scores. Note that the difference in social investigation between nulliparous and primiparous females at 3 days post weaning (A) could not be confirmed in a second group of nulliparous and primiparous females (B). Bars indicate means + SEM; *=$p<0.05$, one-way ANOVA followed by Bonferroni post-hoc test.

Discussion

Our results reveal that female rats show significantly lower OTR binding densities than male rats in the majority of forebrain regions analyzed. Importantly, despite higher OTR binding densities in the NACC, BNSTp, MPOA, VMH, and MeA in proestrus/estrus versus non-estrus females, females still showed significantly lower OTR binding densities in the BNSTp, VMH, and MeA than males. We further confirmed that females show lower social interest than males and demonstrated that estrus phase and maternal experience failed to enhance the social interest of females towards juvenile rats. Interestingly, OTR binding densities correlated with social interest scores in specific subregions of the amygdala in sex-specific ways; social interest scores in males correlated positively with OTR binding densities in the MeA, while social interest scores in females correlated negatively with OTR binding densities in the CeA.

Sex differences in OTR binding

Female rats show significantly lower OTR binding densities than males in 9 out of 15 forebrain regions analyzed including the NACCp, CPUd, LSi, BNSTp, MPOA, AIP, hippocampal CA1 region, MeA, and VMH. These sex differences are maintained even when females are in estrus, except for OTR binding densities in the NACC and MPOA. Lower OTR binding densities in females could be a compensatory response to higher OT peptide expression and/or release. Female mice indeed show higher numbers of OT-immunoreactive neurons in the PVN and higher hypothalamic OT content than male mice (Haussler et al., 1990). However, we did not find a sex difference in OT mRNA expression in the PVN or SON. To the best of our knowledge, sex differences in rat hypothalamic OT mRNA or peptide expression have not been reported before. Despite the lack of a sex difference in OT mRNA expression, it is still possible that there are sex differences in OT peptide expression and/or OT release due to e.g., sex differences in posttranslational mechanisms or release

mechanisms, respectively. Therefore, the functional significance of OTR binding densities may depend on release characteristics in males and females during specific social behaviors. Future investigations are required to determine potential sex differences in OT peptide expression and OT release.

It has been suggested that OT may have a greater influence in the expression of pro-social behaviors in females than in males (reviewed in Cushing & Kramer, 2005), making it surprising that female rats have lower OTR binding densities than males in so many forebrain regions. However, there may be species-specific effects on OTR binding densities. For example, a lack of sex differences in OTR binding densities in the LS, BNST, MeA and CeA was reported in prairie voles (Bales et al., 2007) except that females showed higher OTR binding densities in the medial prefrontal cortex compared to males (Smeltzer et al., 2006). These and our current findings indicate the need for studies comparing OT parameters in males and females across diverse species to understand the role of the OT system in sex-specific expression and/or regulation of social behaviors.

Our finding of a sex difference in OTR binding density in the VMH agrees with other studies in rats showing a similar sex difference in OTR mRNA expression (Bale & Dorsa, 1995) and OTR binding density (Uhl-Bronner et al., 2005). However, another study reported no sex difference in OTR binding density in the VMH (Tribollet et al., 1990). This could be due to the use of different ligands across studies: [^{3}H]OT in the study by Tribollet et al. (1990) versus a highly specific [^{125}I]-OTR antagonist in our study and in the study by Uhl-Bronner et al. (2005). To our knowledge, we are the first to report sex differences in OTR binding densities in intact (i.e., non-gonadectomized) males and females in the NACCp, CPUd, LSi, BNSTp, MPOA, AIP, hippocampal CA1 region, and MeA.

It is possible that the sex differences in OTR binding densities in some of these regions may be due to sex differences in the organizational and/or activational effects of

gonadal steroids. Indeed, females treated neonatally with testosterone show higher levels of OTR binding densities in the VMH, BNSTp, and MeA (Uhl-Bronner et al., 2005), but it's unclear if it is to the level of males in these regions. Gonadectomy of adult rats decreased OTR binding in areas such as the VMH, BNSTdl, and CPUd in both males and females (Tribollet et al., 1990), suggesting that testosterone's metabolite estradiol is important for the expression of OTR in these brain regions. Interestingly, gonadectomy also decreased social investigation in males (Thor, 1980). Therefore, sex hormone differences in males and females may explain some of the sex differences in OTR densities, but not all. Indeed, we find sex differences in OTR binding densities in multiple areas of the forebrain that may not be sensitive to gonadal steroids, such as the NACCp and hippocampal CA1 region (Tribollet et al., 1990).

Correlations of OTR binding and social interest

The function of the observed sex differences in OTR binding densities in specific forebrain regions is unclear. It is possible that higher OTR binding density in males compared to females is involved in modulating male-typical behaviors. For example, individual differences in OTR binding densities in forebrain regions correlated with individual differences in mating tactics in male prairie voles (Ophir et al., 2012). We confirmed that males show higher social interest compared to females (Johnson & File, 1991; Tejada & Rissman, 2012; Thor, 1980) and speculate that higher levels of OTR in males may modulate higher levels of social interest. In support, we showed for the first time that OTR binding densities in specific brain regions correlate with social interest in rats. These brain regions may be implicated in a neural circuitry regulating low (females) versus high (males) social interest.

Our results show that male social interest positively correlates with OTR binding densities in the MeA. Likewise, social investigation time toward a stimulus male positively

correlated with OTR mRNA expression in the MeA in male mice (Murakami et al., 2011).These findings suggest that OTR activation in the MeA may facilitate increased social approach behaviors, including social interest. Furthermore, social investigation scores in females correlate negatively with OTR densities in the CeA, and positively with OTR densities in the CPUm and hippocampal CA1 region. Because OT in the CeA reduces anxiety-related behaviors (Bale et al., 2001; Huber et al., 2005; Knobloch et al. 2012), perhaps higher OT action in the CeA may decrease the need to investigate novelty, and thus decrease social interest. Whether the correlation between OTR binding densities in these brain regions and high versus low social interest in male and female rats, respectively is causal remains to be determined.

A reason for the specificity of these correlations of OTR binding densities and social interest to a particular sex is unclear. It is likely that a similar core social behavior network (Goodson & Kabelik, 2009; Newman, 1999) is activated in both males and females in response to social stimuli, but specific nodes of the network may be differentially regulating high social interest in males (MeA), and low social interest in females (CeA/CPUm/CA1 region).

Estrus effects on social interest and OTR binding

We did not find higher OT mRNA expression in the PVN or SON of females in proestrus/estrus, which contradicts Van Tol et al. (1988). However, females in proestrus/estrus showed increases in OTR binding densities in the NACC, BNSTp, MPOA, VMH, , and MeA, areas associated with the regulation of social and affiliative behaviors and/or motivation and reward (Daniels & Flanagan, 2000; Ferguson et al., 2001; Fibiger & Phillips, 1986; Koob, 1992; Numan & Stolzenberg, 2009; Ophir et al., 2012). The estrus-induced increase in OTR binding density in the VMH and MeA is in line with other studies reporting higher OTR mRNA expression in these areas in estrogen-treated versus non

estrogen-treated ovariectomized females (Bale et al., 1995a; Quinones-Jenab et al., 1996). Bale et al. (1995a) also found an increase in OTR mRNA in the VMH in estrus females compared to non-estrus females. However, we are the first to report increased OTR binding densities in the MeA, NACC, BNSTp, and MPOA due to natural hormonal fluctuations using intact (i.e., non-ovariectomized) females. Contrary to our hypothesis, females in proestrus/estrus did not show increased social investigation. This suggests that estrus-induced increases in OTR binding densities do not play a role in mediating enhanced general interest of female rats toward social stimuli. Instead, the enhanced OTR binding densities in proestrus/estrus females may regulate more specific sexual behaviors, motivational behaviors, or other social approach behaviors not examined here. Indeed, OT administration in the VMH or MPOA of estrogen-treated ovariectomized females enhanced lordosis behavior (Bale et al., 2001; Caldwell et al., 1990).The functional significance of increases in OTR binding densities in the NACC, BNSTp, and MeA of proestrus/estrus females is in need of further research.

Maternal experience effects on social interest and OTR binding

Maternal experience did not enhance social interest indicating that an increased interest toward pups in primiparous females does not generalize to an increase in interest toward juveniles. Our results further indicate that increases in OT synthesis and OTR expression in specific brain regions observed in late pregnancy, around parturition and in early lactation (Bealer et al., 2006; Van Tol et al., 1988; Young et al., 1997) are not long-lasting (i.e., post-lactation), except for OTR binding density in the MeA. This latter finding should be confirmed as others reported either a temporal increase in OTR mRNA in the MeA around parturition (Meddle et al., 2007) or no change in OTR binding in the MeA across the peripartum period (Caughey et. al., 2011).

Even so, long-term changes due to maternal experience have been observed, including a faster approach to unfamiliar pups post-lactation (Bridges, 1975; Bridges, 1977). This consolidation of maternal responsiveness suggests permanent, most likely epigenetic, modifications in neural circuits which involve OT and dopamine systems (Numan & Stolzenberg, 2009; Stolzenberg et al., 2012). Our results suggest that such increased maternal responsiveness is specific to pups rather than any social stimulus.

Conclusion

In conclusion, our results show that regardless of estrus phase or maternal experience, females have significantly lower OTR binding densities than males in several forebrain regions involved in social and affiliative behaviors. These sex differences in OTR binding densities may be implicated in a neural circuitry regulating low (females) versus high (males) social interest. The sex-specific correlations of OTR binding densities in distinct amygdala subregions with social interest suggest that sex differences in the OT system may modulate sex differences in social interest via different mechanisms and pathways. This knowledge could provide insights into social dysfunction (such as low social interest) and sex-biases commonly seen in disorders such as depression and autism.

III. Study 2: Involvement of the oxytocin system in the bed nucleus of the stria terminalis in the sex-specific regulation of social recognition *

Published Manuscript: Dumais, K.M., Alonso, A.G., Immormino, M.A., Bredewold, R., & Veenema, A.H. (2016). Involvement of the oxytocin system in the bed nucleus of the stria terminalis in the sex-specific regulation of social recognition. Psychoneuroendocrinology 64, 79-88.

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 (BNSTp) 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 BNSTp, 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 BNSTp impairs social recognition in both sexes, while OT (100 pg/0.5 µl/side) administration into the BNSTp 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 BNSTp, as measured with *in vivo* microdialysis, did not differ between the sexes. However, males showed higher OT release in the BNSTp during social recognition compared to females. These findings suggest a sex-specific role of the OT system in the BNSTp in the regulation of social recognition.

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; Dumais and Veenema, 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 (BNSTp), in which males have a three-fold higher OTR binding density compared to females (Dumais et al., 2013). The BNSTp 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 BNSTp 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 BNSTp 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 BNSTp impairs opposite-sex odor preference in male hamsters (Been and Petrulis, 2010). In addition, blocking OTR in the BNSTp reduced male odor-induced vaginal marking in female hamsters (Martinez et al.,

2010), suggesting a role for the OTR in the BNSTp 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 BNSTp in males and females.

We hypothesized that the sex difference in OTR binding density in the BNSTp 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 BNSTp 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 BNSTp 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 BNSTp, we used *in vivo* microdialysis to measure extracellular OT release in the BNSTp of male and female rats under baseline conditions and during exposure to the social recognition test.

Methods

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 x 48.3 x 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).

Stereotaxic surgery

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 BNSTp (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 x 48.3 x 20.3 cm). Behavioral testing was performed 3 and 5 days after surgery.

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 BNSTp (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 x 48.3 x 20.3 cm). Microdialysis and behavioral testing were performed 2 days after surgery.

Behavioral Testing

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.

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. 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 towards 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 x 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.

Experimental Procedures

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 (5 ng/0.5 µl/side) and synthetic OT (Sigma; 100 pg/0.5 µl/side) were each compared to 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; Guzman et al., 2014; Bredewold et al., 2014; László et al., 2015). Injection systems were composed of polyethylene tubing connected to an injector cannula which extended 2mm 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 $C0_2$, 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. 2.1). 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.

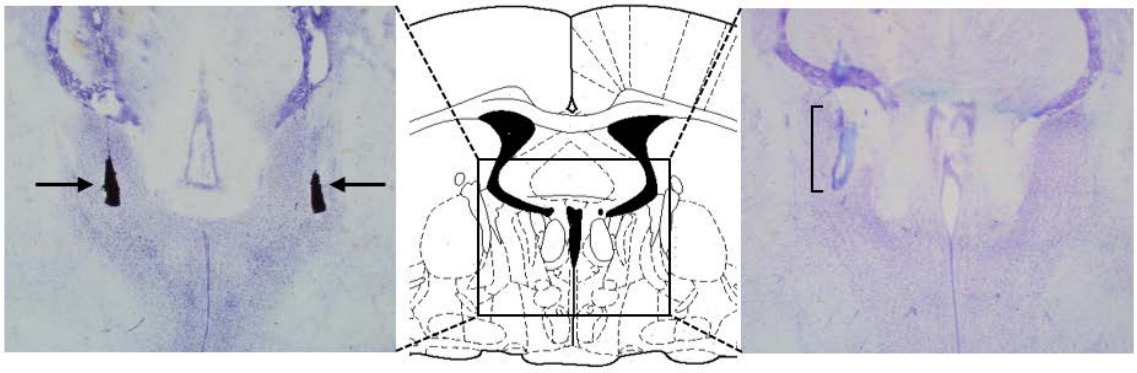

Fig. 2.1 Representative cannula and microdialysis probe placement from an adult male rat. Schematic drawing of the BNSTp (bregma -0.80 mm; adapted from Paxinos and Watson, 1998) and representative enlargements of photomicrographs of Nissl-stained coronal sections of the rat brain indicating with arrows the bilateral microinjection locations in the BNSTp 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 BNSTp (right). Correct placements were considered at bregma -0.80 mm ± 0.2 mm using the brain atlas by Paxinos and Watson (1998).

Experiment 1a: Social investigation

To determine the effects of OTR blockade in the BNSTp 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 BNSTp 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.

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 BNSTp 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 BNSTp 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.

Experiment 2: OT release in the BNST during social recognition

OT release in the BNSTp 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; 3µl/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 0.1M 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 $C0_2$, and proper probe placement was verified histologically on Nissl-stained coronal brain sections (see Fig. 2.1). 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 technical issues with dialysate sampling. This resulted in a final number of 19 males and 16 females.

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 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).

Statistical Analysis. For experiment 1, social investigation was analyzed using two-way ANOVA (treatment x 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 x 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 x sex x 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 x social recognition) with estrus phase as covariate. Data are presented as mean + SEM, and significance was set at $p<0.05$.

Results

Experiment 1: Effect of OTR manipulations in the BNSTp on social investigation and social recognition

Experiment 1a: Social investigation

OTR blockade did not alter social investigation time in either sex (treatment effect: $F_{(1,50)}=0.91$, $p=0.35$; sex x treatment effect: $F_{(1,50)}=1.40$, $p=0.24$). Likewise, OT administration did not alter social investigation time in either sex (treatment: $F_{(1,29)}=0.67$, $p=0.42$; sex x 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. 2.2A, 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 ($F_{(1,14)}=0.05$, $p=0.83$).

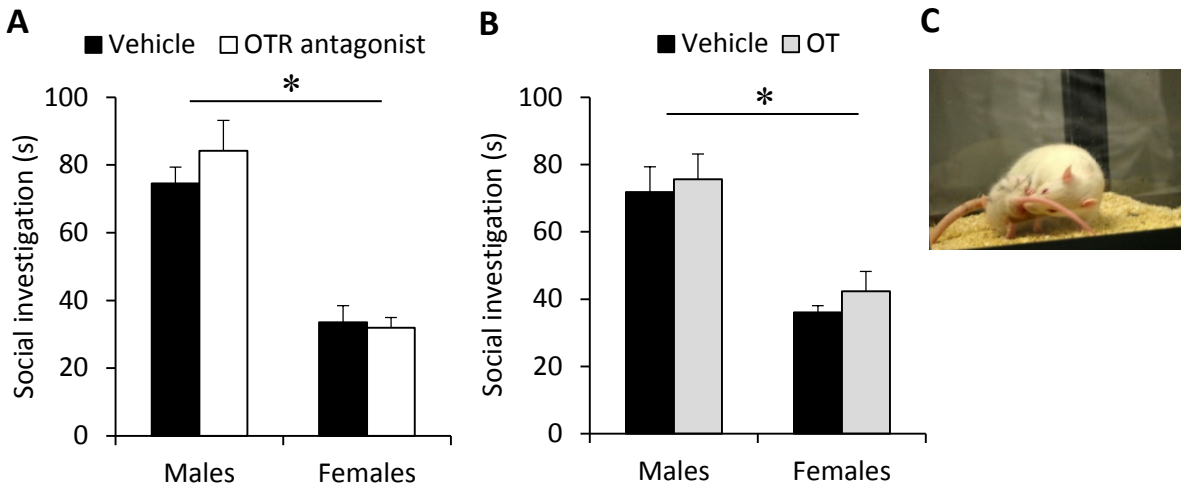

Fig. 2.2 Effect of OTR manipulations in the BNSTp on social investigation. There are no effects of OTR antagonist (A) or OT (B) injections into the BNSTp on social investigation in male and female adult rats. However, there is a main effect of sex in both (A) and (B), in which females show lower social 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).

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; Bonferroni corrected p value); females: $t_{(15)}$=2.91, p<0.0125), 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. 2.3A), 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. 2.3A). No main effects of sex ($F_{(1,48)}$=0.01, p=0.93) or treatment x 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 sec, vehicle-treated females: 24.1 ± 2.7 sec, OTR antagonist-treated males: 69.8 ± 7.0 sec, OTR antagonist-treated females: 28.1 ± 3.3 sec; treatment: $F_{(1,48)}$=0.08, p=0.78; sex x treatment: $F_{(1,48)}$=0.40, p=0.53; two-way ANOVA) and T2 (vehicle-treated males: 72.7 ± 6.8 sec, vehicle-treated females: 22.2 ± 3.7 sec, OTR antagonist-treated males: 68.3 ± 7.7 sec, OTR antagonist-treated females: 24.7 ± 2.0 sec; treatment: $F_{(1,48)}$=0.01, p=0.96; sex x 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. 2.3B). While there was no sex x 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. 2.3B). 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 sec, vehicle-treated females: 41.7 ± 8.7 sec, OT -treated males: 64.3 ± 7.6 sec, OT -treated females: 33.1 ± 7.4 sec; treatment: $F_{(1,23)}$=4.03, p=0.06; sex x treatment: $F_{(1,23)}$=0.55, p=0.47; two-way ANOVA) and T2 (vehicle-treated males: 48.4 ± 5.2 sec, vehicle-treated females: 36.5 ± 6.0 sec, OT-treated males: 71.1 ± 7.0 sec, OT-treated females: 38.4 ± 7.4 sec; treatment: $F_{(1,23)}$=0.02, p=0.88; sex x 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).

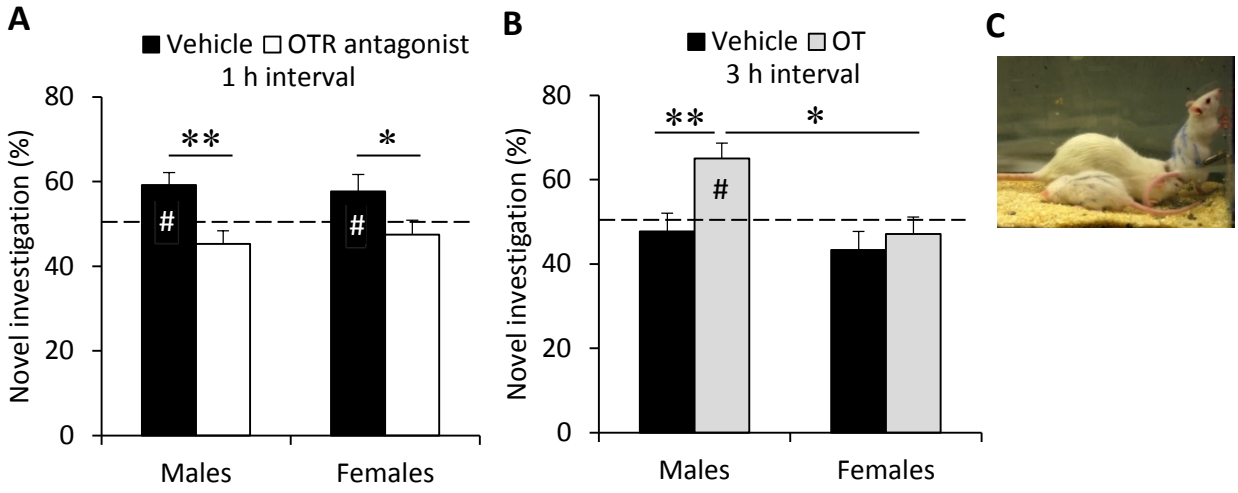

Fig. 2.3 Effect of OTR manipulations in the BNSTp on social recognition. (A) OTR antagonist injected into the BNSTp impaired social recognition in both male and female adult rats. (B) OT injected into the BNSTp improved social recognition in males, but not in females. Social recognition is expressed as the percentage of time spent investigating a novel same-sex juvenile rat (time investigating novel rat/total time investigating novel + familiar rat x 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.

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. 2.4A-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 2.4A). 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 x 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. 2.4A).

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 x sex: $F_{(1,31)}$=0.09, p=0.77. T2, social recognition ability: $F_{(1,31)}$=0.61, p=0.44; social recognition ability x 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. 2.4B) and T2 ($F_{(1,31)}$=6.67, p<0.05, two-way ANOVA; Fig. 2.4C).

There was no difference in baseline OT concentration in the BNSTp between males and females that did and did not show social recognition ($x^2_{(3)}$=3.87, p=0.28; Fig. 2.4D). 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 x sex x social recognition interaction effect was found ($F_{(4,120)}$=2.81, p<0.05). *Post hoc* testing revealed a sex difference in 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. 2.4E). This sex difference is further reflected by males showing higher OT release compared to females during both T1 (p<0.05) and T2 (p<0.05; Fig. 2.4E). 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. 2.4E).

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. 2.4F). This sex difference was further reflected by higher OT release in females compared to males during T2 (p<0.05; Fig. 2.4F).

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. 2.4G). 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. 2.4H). No effects of estrus phase were found for the percentage OT release (estrus phase: $F_{(1,13)}$=0.28, p=0.61; dialysate x estrus phase: $F_{(4,52)}$=1.2, p=0.34).

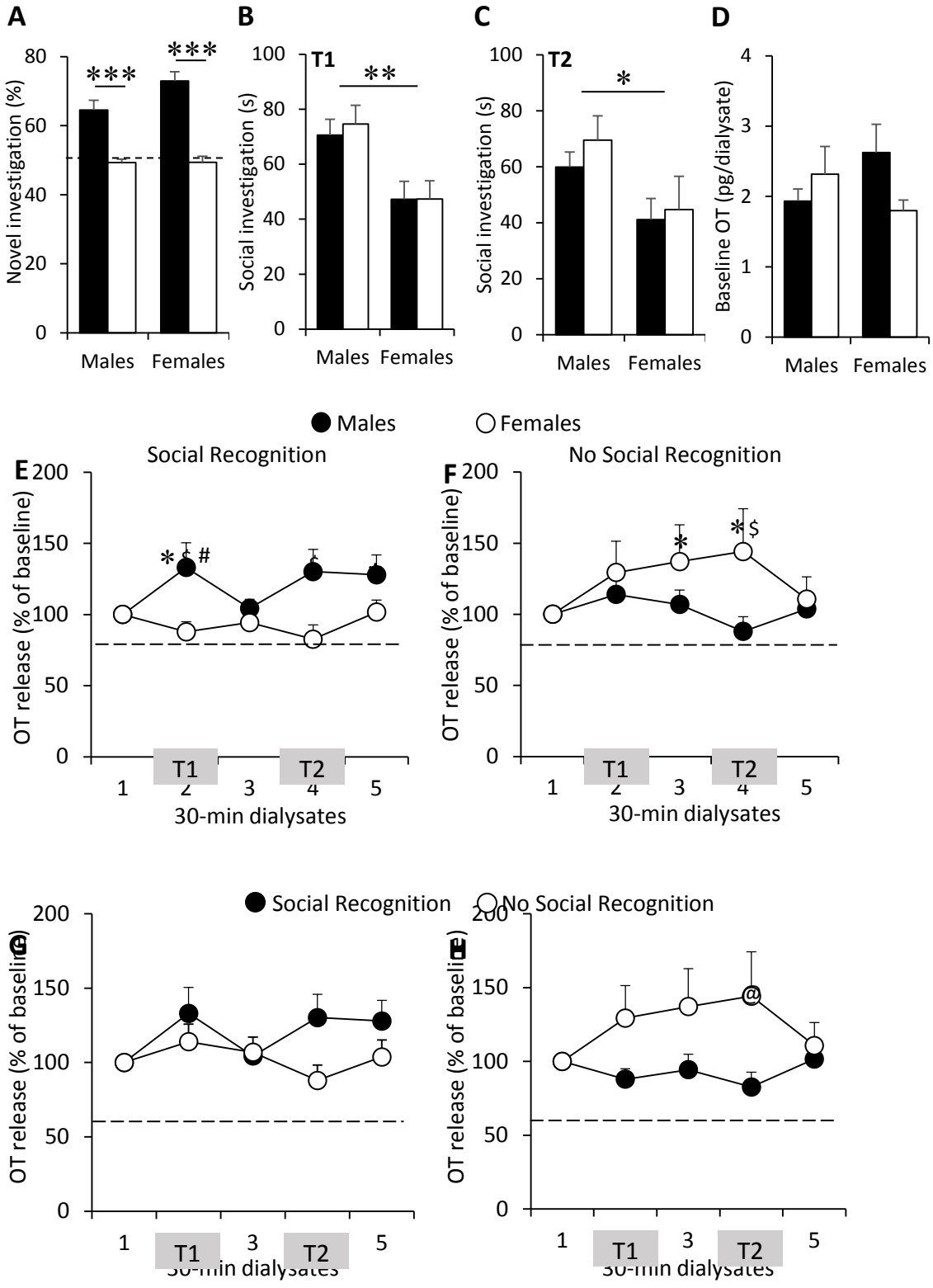
A
B
C
D
Novel investigation (%)
Social investigation (s)
Social investigation (s)
Baseline OT (pg/dialysate)
T1
T2
Males
Females
Social Recognition
No Social Recognition
OT release (% of baseline)
30-min dialysates
T1
T2
Social Recognition
No Social Recognition

Fig. 2.4 Sex-specific OT release in the BNSTp 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 BNSTp. (E) The percentage of OT release in the BNSTp 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 BNSTp 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 BNSTp 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 BNSTp 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: **$p<0.001$, *<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.

Discussion

We demonstrated that endogenous activation of the OTR in the BNSTp 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 BNSTp impaired social recognition in both sexes, while OT (100 pg/0.5 µl/side) injected into the BNSTp prolonged the duration of social recognition in males, but not females, to 3 h. We speculate that the higher OTR binding density in the BNSTp 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 BNSTp of males versus females during successful social recognition further indicates the sex-specific involvement of OT in the BNSTp 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 BNSTp is not associated with a sex difference in extracellular OT concentration in the BNSTp. 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; Choleris et al, 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 BNSTp as a region important in OTR-mediated regulation of social recognition in both male and female rats.

A role for the OTR in the BNSTp in social recognition complements previous findings demonstrating a role for the OTR in the BNSTp 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 BNSTp 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 BNSTp (Halpern, 1987; Guillamon and Segovia, 1997). On the other hand, the BNSTp 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 BNSTp to modulate the learning and/or motivational aspects associated with social recognition. Future research is needed to determine whether OTR activation in the BNSTp 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 BNSTp appear specific to social recognition, as the same dose of OTR antagonist or OT injected into the BNSTp 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 BNSTp 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 towards adult males (Lukas et al., 2011). In addition, excitotoxic lesions of the BNSTp in male hamsters reduced the investigation time towards 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 BNSTp is involved in the investigation of more salient social stimuli. Overall, our data indicate that the OT system in the BNSTp 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 BNSTp in social recognition. OT injected bilaterally into the BNSTp (100pg/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; Popik et al., 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 vanRee, 1991; lateral septum, Popik et al., 1992b). Therefore, the facilitating effect of OT in the BNSTp 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 BNSTp 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 BNSTp 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 BNSTp 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 BNSTp in successful social recognition. In detail, males, but not females, showed an increase in the percentage of OT release in the BNSTp 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 BNSTp 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 BNSTp could be associated with the sex difference in social investigation. However, as discussed above, OTR blockade in the BNSTp 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 BNSTp 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 BNSTp 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 BNSTp 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; Bosch et al., 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 BNSTp 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 BNSTp (Knobloch et al., 2012). This also suggests that, at least for the BNSTp, 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 BNSTp seen in males versus females during successful social recognition test 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 BNSTp of males, while endogenous OT may have already saturated all OTR binding sites in the BNSTp of females.

Interestingly, the BNSTp is a highly sexually dimorphic structure in volume, cell number, neurochemical expression, and neurocircuitry. For example, the BNSTp 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 (Miceevych et al., 1988) immunoreactivity in the BNSTp. Moreover, male rats have denser projections of the BNSTp 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 BNSTp 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 BNSTp 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 BNSTp-OT system is different in males and females and may contribute to sex-specific modulation of social recognition.

Conclusion

In summary, our results show that the OT system in the BNSTp 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 BNSTp of male versus female rats as was previously reported (Dumais et al., 2013) in combination with higher endogenous OT release in the BNSTp of male versus female rats during successful social recognition.

IV. Study 3: Involvement of the oxytocin system in amygdala subregions in the sex-specific regulation of social interest*

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Abstract: We previously found that oxytocin (OT) receptor (OTR) binding density in the medial amygdala (MeA) correlated positively with social interest (i.e., the motivation to investigate a conspecific) in male rats, while OTR binding density in the central amygdala (CeA) correlated negatively with social interest in female rats. Here, we determined the causal involvement of OTR in the MeA and CeA in the sex-specific regulation of social interest in adult rats by injecting an OTR antagonist (5 ng/0.5 µl/side) or OT (100 pg/0.5 µl/side) before the social interest test (4-min same-sex juvenile exposure). OTR blockade in the CeA decreased social interest in males but not females, while all other treatments had no behavioral effect. Next, we examined potential sex differences in endogenous OT release in the CeA. In vivo microdialysis revealed similar extracellular OT release at baseline and during social interest in males and females. Interestingly, CeA-OT release correlated positively with social investigation time in females. Subsequent division of rats into those showing high or low social interest revealed that CeA-OT release decreased during social interest in females that expressed low compared to high social interest. Overall, we show that the previously observed sex-specific correlations of OTR binding density in the MeA and CeA with social interest do not predict a sex-specific role of OTR in these regions. Instead, despite a lack of sex difference in CeA-OT release, the OTR in the CeA plays a causal role in the regulation of social interest in males, but not females.

Introduction

Social interest reflects the motivation to investigate a conspecific for the assessment of social cues which will, in turn, facilitate context-appropriate social behavior responses. Social interest can therefore be seen as an initial step in mediating the subsequent expression of a wide range of social behaviors, such as aggression, mating, and parental care. Interestingly, there are sex differences in the expression of social interest in both rats and mice. In both species, adult males, compared to adult females, show higher levels of social investigation toward juvenile conspecifics (Thor, 1980; Johnson and File, 1991; Bluthe and Dantzer, 1990; Dumais et al., 2013, 2016). However, the neural mechanisms underlying this sex difference in social interest have not been assessed.

A key candidate for the sex-specific regulation of social interest is the oxytocin (OT) system. OT is primarily synthesized in the paraventricular nucleus and supraoptic nucleus of the hypothalamus. Upon central release, OT modulates the activation of many brain regions via binding to the widely distributed OT receptor (OTR; Gimpl and Fahrenholz, 2001). Importantly, the OT system regulates various social behaviors in humans and rodents (Ross and Young, 2009; Veenema and Neumann, 2008; Guastella and MacLeod, 2012), often in sex-specific ways (for review see Dumais and Veenema, 2015, 2016). The amygdala is of particular interest because it has been shown to be a core region of sex-specific activation by OT. For example, human fMRI studies have shown that exogenous OT modulates amygdala activation in response to social stimuli differently in men compared to women (Domes et al., 2007; Domes et al., 2010; Rilling et al., 2012; Rilling et al., 2013). Furthermore, correlational studies in rodents suggesting that the OT system in subregions of the amygdala, namely the medial amygdala (MeA) and central amygdala (CeA), plays a differential role in mediating male versus female social interest. In detail, male mice showing high levels of social investigation have higher OTR mRNA expression in the MeA compared to males showing

low levels of social investigation (Murakami et al., 2011). In rats, males have higher OTR binding density in the MeA compared to females, and OTR binding density in the MeA correlates positively with social investigation time in males, but not females (Dumais et al., 2013). In contrast, OTR binding density in the CeA does not show a sex difference, but correlates negatively with social investigation time in females, but not in males (Dumais et al., 2013). Together, this suggests that OTR activation in the MeA facilitates social investigation in males, while OTR activation in the CeA decreases social investigation in females.

Previous studies that have implicated the MeA and CeA in OT-mediated social behaviors further support the hypothesis that these amygdala subregions may be involved in modulating social interest. For example, OT in the MeA is known to regulate behaviors such as social approach and social recognition (Arakawa et al., 2010; Lukas et al., 2013). OT in the CeA, while more commonly known for its role in fear and anxiety (Bale et al., 2001; Viviani et al., 2011; Knobloch et al., 2012), has been implicated in maternal and intermale aggression (Bosch et al., 2005; Consiglio et al., 2005; Calcagnoli et al., 2015). However, comparisons between males and females regarding the role of OT in these amygdala subregions in the regulation of social behavior is very limited.

In the current study, we aimed to determine the role of the OT system in the regulation of social interest in male and female rats, with the hypothesis that the sex difference in social interest is mediated by OT acting on OTR in the MeA of males and acting on OTR in the CeA of females. Therefore, we determined the effects of acute pharmacological OTR blockade and OT administration in the MeA and CeA on social investigation time in adult male and female rats. Because these pharmacological manipulations showed a sex-specific effect of OTR blockade in the CeA on social investigation time, we further aimed to determine potential sex differences in extracellular

OT release in the CeA under baseline conditions and during exposure to the social interest test using *in vivo* microdialysis.

Methods

Animals. Male and female Wistar rats were obtained from Charles River Laboratories (Raleigh, NC) at 8-9 weeks of age for experimental rats and at 22 days of age for stimulus rats. Rats were maintained on a 12 h light/dark cycle, lights on at 0700 h, and food and water were available *ad libitum*. Experimental rats were housed in same-sex pairs in standard rat cages (26.7 x 48.3 x 20.3 cm) unless otherwise mentioned, and were given at least one week to acclimate to our facilities. Stimulus rats were housed in same-sex groups of 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).

Stereotaxic surgery

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 MeA (2.8 mm caudal to bregma, 3.3 and -3.3 mm lateral to midline, and 7.3 mm ventral to the skull surface) or CeA (2.5 mm caudal to bregma, 4.2 and -4.2 mm lateral to midline, and 5.9 mm 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, and behavioral testing was performed 3-4 days after surgery.

Microdialysis probe placement. A separate cohort of rats was used for *in vivo* measurement of extracellular OT release in the CeA. 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 CeA (2.5 mm caudal to bregma, -4.2 mm lateral to midline, and 8.9 mm ventral to the skull surface). Two inch pieces of polyethylene tubing were fixed to the ends of the microdialysis probes to allow for attachment to the microinfusion pumps and eppendorf tubes for sample collection. After surgery, rats were individually housed in standard rat cages. Microdialysis and behavioral testing were performed 2 days after surgery. This short postoperative recovery period is necessary for optimal detection of neuropeptides in microdialysates of chronically implanted probes (Horn and Engelmann, 2001). We have demonstrated that prior surgery and ongoing microdialysis had no effect on social investigation time in rats (Dumais et al., 2016).

Behavioral Testing

Social Interest Test. To test for social interest, 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 conspecific that does not elicit aggressive or sexual behaviors. A juvenile rat was placed into the experimental rat's home cage for 4 min, and the time spent investigating the juvenile was measured. Testing was performed during the light phase between 1200 h and 1700 h. Behavior was 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.

Elevated Plus Maze Test. To determine whether the effects of OTR blockade on social interest were secondary due to generalized effects on anxiety-related behavior, the same rats under the same drug condition were tested for anxiety using the elevated plus maze 2 days after the social interest test. The maze consists of two opposing open (50 x 10 cm) and two opposing closed (50 x 10 cm) plastic arms with a 10 x 10 cm central area. The maze is elevated to 90 cm above the floor. Each experimental rat was placed on the central area of the maze facing a closed arm, and was allowed to explore the maze for 5 min. The maze was washed thoroughly with diluted detergent between each test. Testing was performed during the light phase between 900 h and 1200 h. Behavior was video recorded and time spent on the open arms, closed arms, and central area were measured, along with entries made into the open and closed arms, using JWatcher by an experimenter blind to treatment groups. Rats were considered to be on a particular maze arm when both forepaws and shoulders of the rat were on the respective arm of the elevated plus maze. The percentage of time spent on the open arms ([time on open arms/ (time on open arms + time on closed arms)] x 100) and the percentage of open arm entries ([open arm entries/ (open arm entries + closed arm entries)] x 100] were used as parameters of anxiety-like behavior. The total number of arm entries (open arm entries + closed arm entries) was calculated as a measure of locomotor activity.

Drug Injections and Procedures. The effects of the selective 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) administration were each compared to a vehicle (Ringer's solution, 0.5 µl/side) control group. Concentrations for the OTR antagonist and OT were based on prior research in which these doses were effective in altering diverse social behaviors (Guzmán et al., 2013, 2014; Bredewold et al., 2014; László et al., 2016; Dumais et al., 2016). Injection systems were composed of polyethylene tubing connected to an injector cannula which extended 2mm beyond the guide cannula. Injections were made

using a 10 µl Hamilton syringe (Hamilton, Reno, NV) which was kept in place for 30 sec 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 cannulae placement was examined histologically on 30 µm thick Nissl-stained coronal brain sections (see Fig. 3.1A, B). Cannulae placements were considered correct if they fell within the neuroanatomical borders of the MeA at bregma -2.80 ± 0.2 mm or the CeA at bregma -2.5 ± 0.2 mm. Rats with incorrect placements were removed from analysis.

Microdialysis Procedures. One day after probe implantation, rats were habituated to the sampling procedure for 1 h. The following day, microdialysis probes were connected via polyethylene tubing to 2.5 mm Hamilton syringes mounted on a microinfusion pump. Rats were perfused with Ringer's solution (with 0.25% BSA; 3µl/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 10 µl 0.1M HCl to inhibit protein degradation. Dialysates 1 and 2 were taken under baseline conditions, dialysate 3 started concurrently with the 4-min social interest test, and dialysates 4 and 5 were taken thereafter. Dialysates were immediately frozen on dry ice, and stored at -45°C until quantification. OT content was measured using radioimmunoassay (RIAgnostics, Munich, Germany). Rats were killed with CO_2 and proper probe placement was verified histologically on 30 µm thick Nissl-stained coronal brain sections (see Fig. 3.1B). Probe placements were considered correct if they fell within the neuroanatomical borders of the CeA at bregma -2.5 ± 0.2 mm. Rats with incorrect probe placements were removed from analysis.

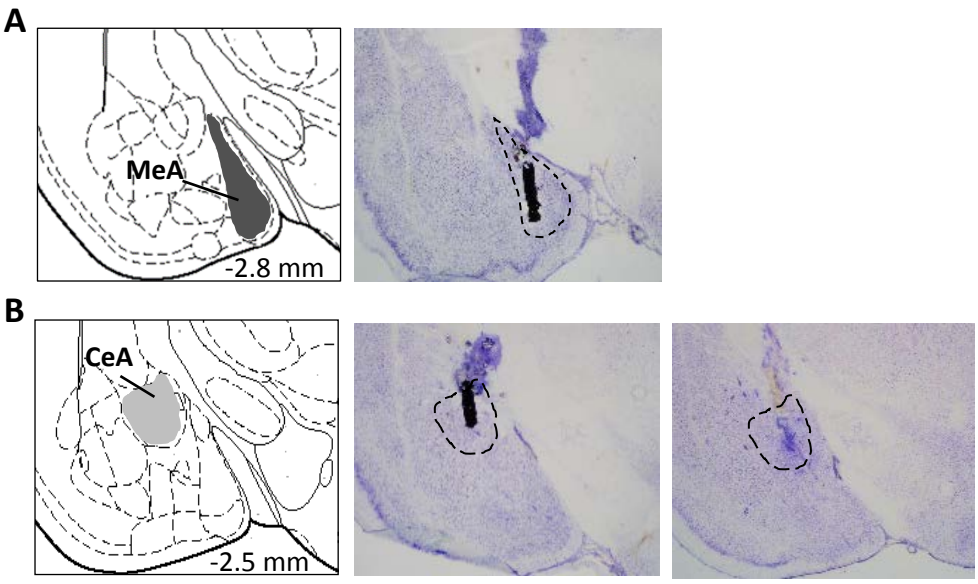


Fig. 3.1 Cannula and probe placement in amygdala subregions. (A) Atlas template indicating the location of the medial amygdala (MeA; dark gray; bregma -2.8 mm; adapted from Paxinos and Watson, 1998; left) and representative photomicrograph of a Nissl-stained coronal section of the rat brain with a representative microinjection location shown by charcoal (right). (B) Atlas template indicating the location of the central amygdala (CeA; light gray; bregma -2.5 mm; adapted from Paxinos and Watson, 1998; left), and representative photomicrographs of Nissl-stained coronal sections of the rat brain with a representative microinjection location shown by charcoal (middle) and the location of the semipermeable membrane of a microdialysis probe (right). Correct placements were considered at bregma -2.80 mm ± 0.2 mm for the MeA and at bregma -2.5 ± 0.2 mm for the CeA according to the brain atlas by Paxinos and Watson (1998).

Experimental Design

Experiment 1: Effects of pharmacological manipulations of the OT system in the MeA on social interest

To determine the effects of OTR blockade in the MeA on social interest, rats received bilateral injections of either Ringer's solution (males: n=9, females: n=14) or the OTR antagonist (males: n=5, females: n=9). To determine the effects of OT administration in the MeA on social interest, rats received bilateral injections of either Ringer's solution (males: n=12, females: n=9) or OT (males: n=6, females: n=7). Rats were injected 20 min before the start of the social interest test.

Experiment 2: Effects of pharmacological manipulations of the OT system in the CeA on social interest and anxiety-related behavior

Experiment 2a: Social interest: To determine the effects of OTR blockade in the CeA on social interest, rats received bilateral injections of either Ringer's solution (males: n=7, females: n=7) or the OTR antagonist (males: n=6, females: n=7). To determine the effects of OT administration into the CeA on social interest, rats received bilateral injections of either Ringer's solution (males: n=12, females: n=10) or OT (males: n=9, females: n=6). Rats were injected 20 min before the start of the social interest test.

Experiment 2b: Elevated plus maze: To determine whether effects of OTR blockade in the CeA on social interest are due to effects on general anxiety, the same rats were tested on the elevated plus maze two days after the social interest test. Rats were bilaterally injected with the same drug treatment 20 min before the start of the elevated plus maze test.

Experiment 3: Extracellular OT release in the CeA

Because of the sex-specific effect of OTR blockade in the CeA on social interest, we used *in vivo* microdialysis to measure extracellular OT release in the CeA of a separate

cohort of adult male (n=8) and female (n=8) rats under baseline conditions and during exposure to the social interest test.

Estrus phase measurement. Although previously we found no effect of estrus phase on social interest (Dumais et al., 2013), to control for any effects of estrus cycle in the present study, estrus phase was determined via vaginal smears (according to Dumais et al., 2013) taken from each female immediately following each behavioral test. Estrus cycle phase was determined via cell characteristics based on Goldman et al. (2007). Females were categorized as being in 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).

Statistical Analysis. For experiments 1 and 2, effect of treatment on social interest (investigation time in seconds), anxiety-related behavior (percent time on open arms, percentage of open arm entries), and locomotor activity (total number of arm entries) were each analyzed using two-way ANOVAs (treatment x sex). When appropriate, Bonferroni post hoc tests were used to examine the nature of interaction effects. For experiment 3, the effects of sex on social investigation time and on baseline OT concentration (pg/dialysate; average of dialysates 1 and 2) were analyzed using one-way ANOVAs. For all 5 dialysate time points, OT release was converted into percent change from the average of the two baseline samples for each rat, and these percentages were analyzed using a two-way ANOVA for repeated measures (sex x dialysate). A bivariate correlation and a curve estimate regression analysis were used to determine correlations of social investigation time with the percentage of OT release during exposure to the social interest test (i.e., dialysate 3). Because of the outcomes of the correlation analysis, we subsequently grouped rats into those that showed low social interest (social investigation time lower than the average; males: < 83 seconds, n=4; females: < 48 seconds, n=4), and those that showed high social interest (social

investigation time greater than the average; males: > 83 seconds, n=4; females: > 48 seconds, n=4). Social investigation time was then analyzed using two-way ANOVA (social interest x sex). To confirm a significant difference in social investigation time according to social interest group in each sex, one-way ANOVAs were run separately per sex. Furthermore, the percentage of OT release was analyzed using a three-way ANOVA for repeated measures (social interest x sex x dialysate). When appropriate, LSD post hoc tests were used to examine the nature of interaction effects. Finally, because of low numbers of females in estrus and non-estrus groups, effects of estrus phase on social interest, anxiety-related behavior, and locomotor activity in Experiments 1 and 2 were analyzed separately for each treatment by one-way ANOVAs. Only those treatments with at least an n=3 females in estrus and in non-estrus were included in the statistical analysis. Effect of estrus phase on the percentage of OT release in Experiment 3 was analyzed using a one-way ANOVA for repeated measures with estrus phase as covariate. None of these analyses showed an effect of estrus phase. Data are presented as mean + SEM, and significance was set at $p<0.05$.

Results

Experiment 1: Effects of pharmacological manipulations of the OT system in the MeA on social interest

A significant main effect of sex was found for social investigation in both OTR manipulation experiments in the MeA (OTR antagonist: $F_{(1,33)}=18.0$, $p<0.001$; OT: $F_{(1,30)}=24.8$, $p<0.0001$), in which males showed higher social investigation time compared to females (Fig. 3.2). However, OTR manipulations in the MeA did not alter social investigation time in either sex (OTR antagonist: treatment, $F_{(1,33)}=1.54$, $p=0.22$; sex x treatment, $F_{(1,33)}=0.13$, $p=0.73$; OT: treatment, $F_{(1,30)}=2.27$, $p=0.14$; sex x treatment, $F_{(1,30)}=2.68$, $p=0.11$; Fig. 3.2).

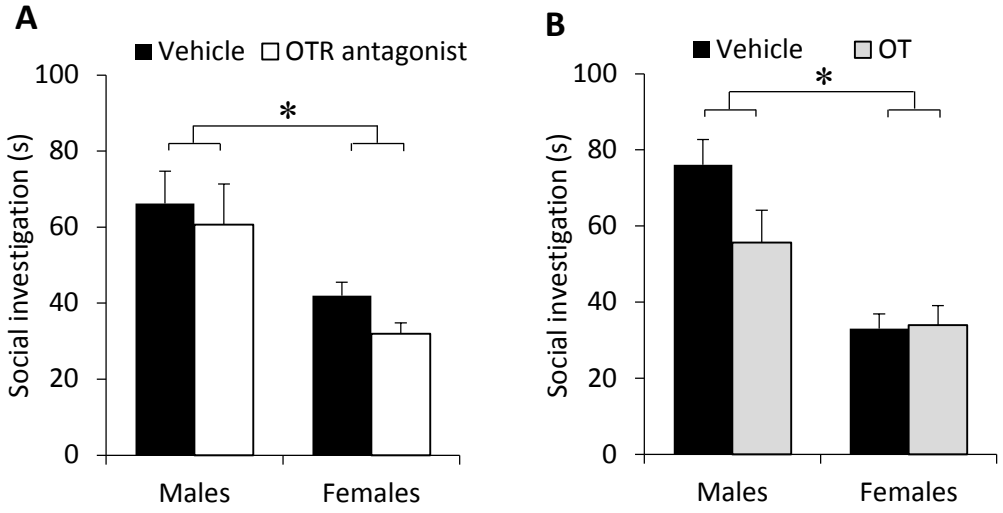


Fig. 3.2 OTR manipulations in the medial amygdala do not affect social interest in either sex. OTR antagonist (A) or OT (B) injections did not alter social investigation time in male and female adult rats. However, males showed higher social investigation time compared to females (A and B). Social investigation time represents the total investigation time in seconds (s) toward a novel, same-sex juvenile rat placed in the home cage of the experimental rat for a 4-min period. Data are presented as mean + SEM; * $p<0.01$, two-way ANOVA.

Experiment 2: Effects of pharmacological manipulations of the OT system in the CeA on social interest and anxiety-related behavior

Experiment 2a: Social interest: OTR blockade in the CeA decreased social investigation time in males, but not females (Fig. 3.3A). This is reflected by a significant main effect of treatment ($F_{(1,23)}$=5.89, p<0.05) and sex x treatment ($F_{(1,23)}$=6.25, p<0.05), with *post hoc* tests indicating that OTR antagonist-treated males showed lower social investigation time compared to vehicle-treated males (p<0.01; Bonferroni *post hoc* test), while the two female groups exhibited similar social investigation times (p=0.96; Bonferroni *post hoc* test). Furthermore, a main effect of sex was found for social interest, in which males showed higher social investigation time compared to females ($F_{(1,23)}$=34.8, p<0.00001), and this was true for vehicle-treated rats (p<0.00001, Bonferroni *post hoc* test) and OTR antagonist-treated rats (p<0.05, Bonferroni *post hoc* test). OT injections did not alter social investigation time in either sex (treatment: $F_{(1,33)}$=0.23, p=0.64; sex x treatment: $F_{(1,33)}$=0.0000002, p=0.99; Fig. 3.3B), but again, a main effect of sex was found ($F_{(1,33)}$=19.1, p<0.001) in which males showed higher social investigation time compared to females (Fig. 3.3B).

Experiment 2b: Elevated plus maze: Because the OT system in the CeA is known to play a role in anxiety (Bale et al., 2001; László et al., 2016), we determined whether the effects of OTR blockade in the CeA on social investigation time in males were secondary due to effects on anxiety-related behavior. There was no effects of sex or OTR antagonist treatment on the percentage of time spent on the open arms (sex: $F_{(1,23)}$=0.05, p=0.83, treatment: $F_{(1,23)}$=1.95, p=0.18, sex x treatment: $F_{(1,23)}$=0.27, p=0.61; Fig. 3.3C), or the percentage of open arm entries (vehicle-treated males=45.4 ± 2.8 %, vehicle-treated females= 46.9 ± 3.8 %, OTR antagonist-treated males= 47.1 ± 2.7 %, OTR antagonist- treated females= 46.7 ± 2.5 %; sex: $F_{(1,23)}$=0.04, p=0.85; treatment: $F_{(1,23)}$=1.07, p=0.31; sex x treatment: $F_{(1,23)}$=2.63, p=0.12). Furthermore, there was no effect of sex or OTR blockade on

locomotor activity, as reflected by the total number of arm entries (vehicle-treated males=24.7 ± 1.8, vehicle-treated females= 27.1 ± 1.9, OTR antagonist-treated males= 27.7 ± 2.7, OTR antagonist-treated females= 25.4 ± 1.5; sex: $F_{(1,23)}$=0.002, p=0.96; treatment: $F_{(1,23)}$=0.10, p=0.76; sex x treatment: $F_{(1,23)}$=1.41, p=0.25).

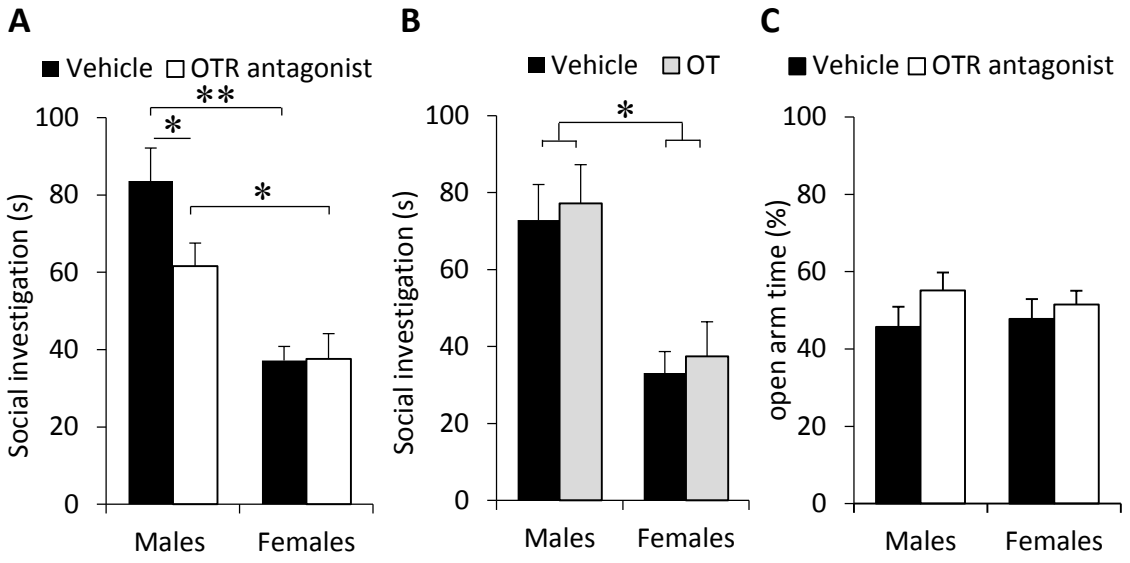


Fig. 3.3 The OTR in the central amygdala (CeA) modulates social interest in sex-specific ways, without an effect on anxiety-related behavior. (A) OTR antagonist injections reduced social investigation time in male, but not female, adult rats. (B) OT injections had no effect on social investigation time in either sex. Males showed higher social investigation time compared to females (A and B). (C) OTR antagonist injections in the CeA had no effect on the percentage of time spent on the open arms of the elevated plus maze in either males or females. Social investigation time represents the total investigation time in seconds (s) toward a novel, same-sex juvenile rat placed in the home cage of the experimental rat for a 4-min period. Open arm time is the percentage of time spent on the open arms during a 5-min exposure to the elevated plus maze. Data are presented as mean + SEM; * $p<0.01$, ** $p<0.00001$, two-way ANOVA followed by Bonferroni post hoc test.

Experiment 3: Extracellular OT release in the CeA

The observed sex-specific effect of OTR blockade in the CeA on social investigation time may suggest sex differences in the endogenous activation of OTR during exposure to the social interest test. Therefore, we determined potential sex differences in endogenous OT release in the CeA under baseline conditions and during exposure to the social interest test. Consistent with the sex difference found for social interest in experiments 1 and 2, males showed higher social investigation time compared to females during ongoing microdialysis ($F_{(1,14)}$=19.7, p<0.001; Fig. 3.4A). However, there was no sex difference in baseline extracellular OT concentrations in the CeA ($F_{(1,14)}$=0.0001, p=0.99; Fig. 3.4B), nor was there an effect of sex or dialysate on the percent change of OT release from baseline during exposure to the social interest test (sex: $F_{(1,14)}$=0.15, p=0.71; dialysate: $F_{(4,56)}$=1.11, p=0.36; sex x dialysate: $F_{(4,56)}$=0.23, p=0.92; Fig. 3.4C). Correlation analysis revealed a trend toward a negative correlation between social investigation time and the percentage of OT release during the social interest test in males ($r_{(8)}$=-0.68, p=0.064; Fig. 3.4D). In females, there was a significant positive correlation between social investigation time and the percentage of OT release during the social interest test($r_{(8)}$=0.78, p<0.05; Fig. 3.4E).

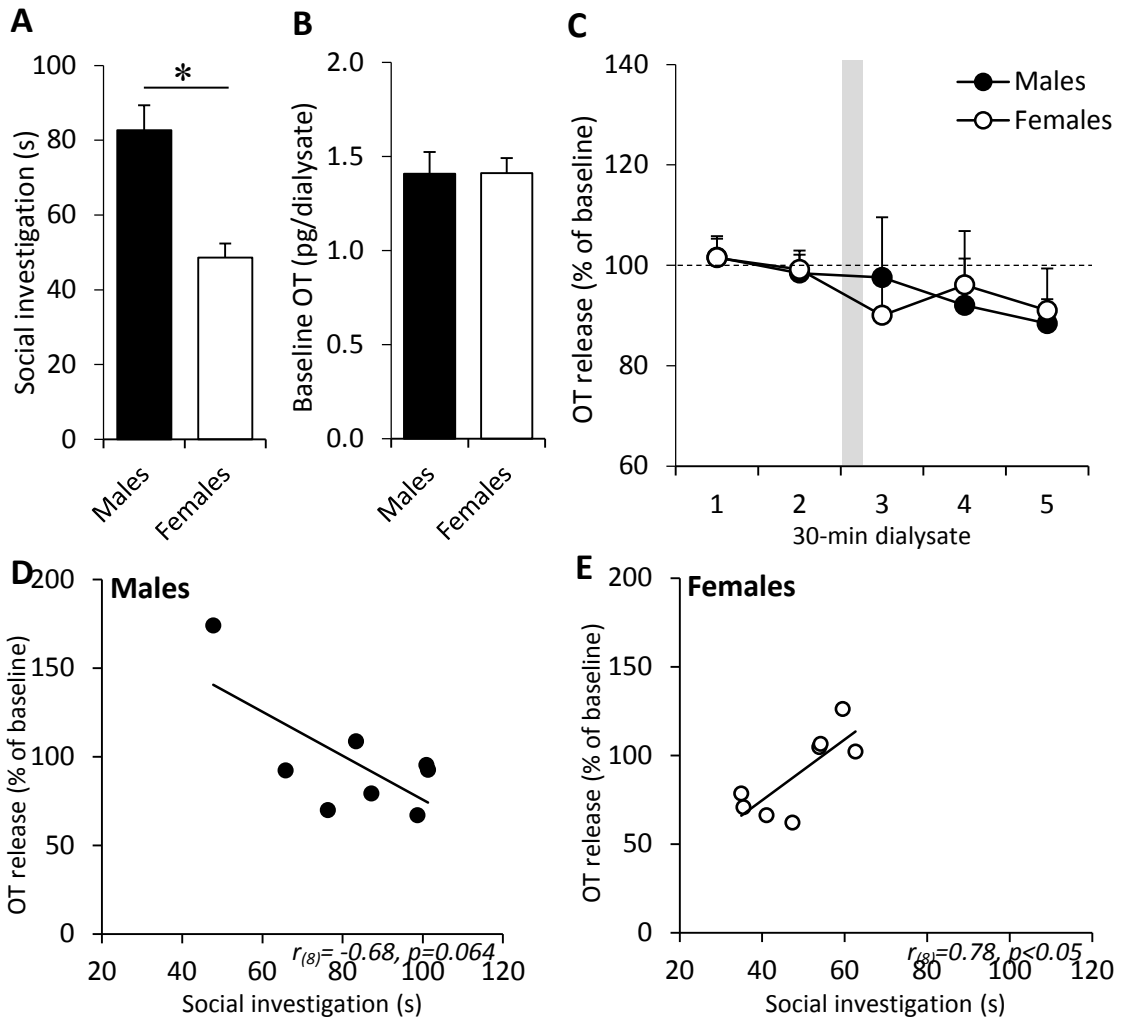


Fig. 3.4 Extracellular OT release in the central amygdala (CeA) of male and female rats exposed to the social interest test. (A) Male rats showed higher social investigation time compared to female rats in the social interest test during ongoing microdialysis. (B) Baseline extracellular OT concentrations in the CeA is similar between males and females. (C) The percentage of OT release in the CeA did not change in either sex when rats were exposed to the social interest test (dialysate 3). (D) In males, there was a trend toward a negative correlation between social investigation time and the percentage of OT release during the social interest test ($p=0.064$). (E) In females, social investigation time correlated positively with the percentage of OT release in the CeA during the social interest test ($p<0.05$). Social investigation time represents the total investigation time in seconds (s) toward a novel, same-sex juvenile rat placed in the home cage of the experimental rat for a 4-min period. The gray bar in (C) indicates the timing of the social interest test during sampling of the third microdialysate. Data are presented as mean + SEM; * $p<0.001$.

We subsequently divided rats into high and low social interest groups (based on average social investigation times within each sex), and confirmed significant differences between high and low social interest groups ($F_{(1,12)}$=32.4, p<0.001, two-way ANOVA) within both sexes (males: $F_{(1,6)}$=15.4, p<0.01; females: $F_{(1,6)}$=24.6, p<0.01; one-way ANOVAs; Fig 3.5A). There was no effect of social interest on baseline OT concentrations in the CeA (social interest: $F_{(1,12)}$=0.05, p=0.83; social interest x sex: $F_{(1,12)}$=1.10, p=0.32, two-way ANOVA; Fig 3.5B). However, a dialysate x social interest x sex interaction effect was found for the percentage of OT release ($F_{(4,48)}$=3.30, p<0.05). In detail, the percentage of OT release during the social interest test (dialysate 3) in females with low social interest was lower compared to baseline (dialysate 1, 2, and 4; p<0.05, LSD post hoc) and compared to the percentage of OT release during the social interest test of females with high social interest (p<0.05; LSD *post hoc* test) and males with low social interest (p<0.05; LSD *post hoc* test; Fig. 3.5C).

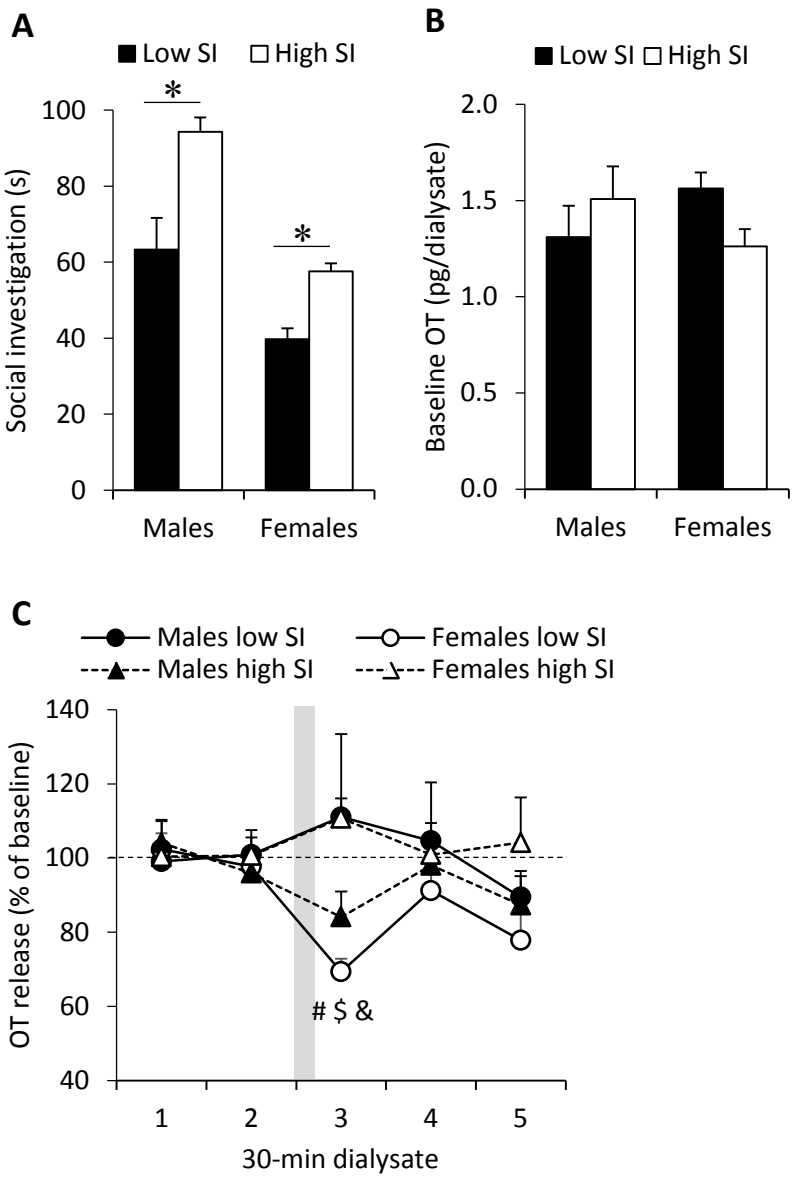


Fig. 3.5 Extracellular OT release in the CeA of male and female rats showing high or low social interest (SI). (A) Male and female rats were divided into high and low SI based on the average social investigation time within each sex. (B) Baseline extracellular OT concentrations were similar between males and females of both low and high SI groups. (C) The percentage of OT release during the social interest test (dialysate 3) in low SI female rats was lower compared to the percentage OT release in dialysate 1, 2, and 4 of low SI female rats, dialysate 3 of high SI female rats, and dialysate 3 of high SI male rats. Social investigation time represents the total investigation time in seconds (s) toward a novel, same-sex juvenile rat placed in the home cage of the experimental rat for a 4-min period. The gray bar indicates the timing of the social interest test during sampling of the third microdialysate. Data are presented as mean + SEM; *$p<0.05$, two-way ANOVA followed by one-way ANOVA; # $p<0.05$, versus dialysate 1, 2, and 4; $ $p<0.05$, versus dialysate 3 of high SI females; & $p<0.05$, versus dialysate 3 of low SI males; three-way ANOVA for repeated measures followed by LSD post hoc test.

Discussion

We previously observed correlations of OTR binding density in the MeA and CeA with social interest in male and female rats, respectively. This led us to hypothesize that activation of the OTR in the MeA would mediate male social interest, while activation of the OTR in the CeA would mediate female social interest. Contrary to our hypotheses, pharmacological manipulations of MeA-OTR or CeA-OTR had no effect on social interest in males or females, respectively. However, we found that OTR antagonist (5 ng/0.5 µl/side) injected into the CeA decreased social investigation time in males. We then determined whether this sex-specific effect of CeA-OTR blockade on social interest could be explained by a sex difference in endogenous CeA-OT release. However, no sex difference was found in CeA-OT release under baseline conditions or during exposure to the social interest test. Moreover, there was no change in CeA-OT release during exposure to the social interest test in either sex. Yet, CeA-OT release in females correlated positively with social investigation time. Subsequent grouping of rats in high and low social interest groups revealed that females with low social interest showed a decrease in OT release in the CeA during the social interest test. Based on these findings, we conclude that the OTR in the CeA plays a causal role in the acute modulation of social interest in males, but not in females. We discuss below the possibility that baseline CeA-OT release is sufficient to facilitate social interest in males and that the decrease in CeA-OT release observed in females with low social interest is not a cause, but a consequence, of social stimulus exposure.

Causal involvement of the OT system in the CeA in social interest in males but not in females

We previously showed that OTR binding density in the CeA negatively correlates with social investigation time in female, but not male, rats (Dumais et al., 2013). This suggested a role for the OTR in the CeA in regulating social investigation in females. However, in the current study, neither OTR antagonist (5 ng/0.5 µl/side) nor OT (100 pg/0.5

μl/side) administration into the CeA altered social investigation time in females. In contrast, OTR blockade in the CeA decreased social investigation time in males. This may suggest that, during the social interest test, OTR activation is higher in males than in females to promote social investigation. Therefore, we tested the hypothesis that CeA-OT release is higher in males than in females during social interest. However, males and females showed similar levels of extracellular OT release in the CeA under baseline conditions and during exposure to the social interest test. Moreover, there was no change in extracellular OT release during the social interest test compared to baseline in either sex. It is possible that the relatively short (4-min) duration of the social stimulus exposure compared to the relatively long (30-min) dialysate sampling period (which is required due to detection limits of the radioimmunoassay) could have obscured a possible significant rise in OT release during social investigation. This may also explain why, in two other similarly designed microdialysis studies, OTR blockade in the lateral septum of male rats (Lukas et al., 2013) or OTR blockade in the posterior bed nucleus of the stria terminalis of female rats (Dumais et al., 2016) impaired social recognition without a corresponding change in local extracellular OT release. Alternatively, the absence of a change in OT release may indicate that baseline OT release is sufficient to facilitate social investigation in males. In this scenario, baseline OT release could have a neuromodulatory role in the regulation of social behavior by e.g., altering the actions of fast-acting neurotransmitters (Joëls, 2000). Whether this indeed explains a role for baseline OT release in facilitating social interest in males remains to be tested.

Our finding that OTR blockade in the CeA has a sex-specific effect on social behavior regardless of OTR binding density (Dumais et al., 2013) and OT release (current study) in the CeA being similar between males and females offers a new level of complexity to the mechanisms by which OT may regulate social behavior in sex-specific ways. How,

then, is OT acting in sex-specific ways in the CeA? One mechanism may be through a sex difference in OTR-expressing neurons in the CeA. Male rats have fewer GABAergic neurons in the CeA than female rats (Stefanova, 1998) and GABAergic neurons in the CeA express OTR (Huber et al., 2005). This could produce a sex difference in the percentage of OTR-expressing neurons that are GABA-ergic (potentially being lower in males compared to females) which may cause a sex difference in CeA output in response to OTR inhibition, a hypothesis that would require further testing. The sex-specific effect of OTR blockade could also be mediated by sex differences in relative projections from the CeA, such as sex differences in the density of efferents from the CeA, or sex differences in social stimulus-induced activation of specific CeA projections. This would require systematic comparative analysis of CeA efferents in males and females, because current CeA tracing studies have not included a comparison between sexes (Wallace et al., 1992; Petrovich and Swanson, 1997; Zahm et al., 1999; Dong et al., 2001; Petrovich et al., 2001).

Importantly, we showed that OTR blockade in the CeA did not affect anxiety-related behavior in male or female rats as measured on the elevated plus maze. This lack of effect on anxiety is despite the well-known role of the OT system in the CeA in the modulation of both fear and anxiety-related behaviors. For example, an increase in OT activity in the CeA has been found to decrease freezing in fear-conditioned male (Viviani et al., 2011; Lahoud and Maroun, 2013) and female (Knobloch et al., 2012) rats. Furthermore, OT administration in the CeA increased time spent on the open arm on the elevated plus maze in male rats (László et al., 2016) and in the middle quadrant in the open field test in female rats (Bale et al., 2001). However, our finding is consistent with László et al (2016), reporting a null effect of OTR antagonist injections in the CeA on anxiety-related behavior using the elevated plus maze. Therefore, although exogenously administered OT in the CeA may reduce anxiety-related behavior, blockade of endogenous OT release via OTR antagonist has not been found to

affect anxiety-related behavior. This suggests that the sex-specific effect of OTR blockade in the CeA on social interest is not due to an effect on general anxiety.

The role of OT in the CeA in females: Could a change in OT release be a consequence of social stimuli exposure?

We recently reported a negative correlation between OTR binding density in the CeA and social investigation time in female rats (Dumais et al., 2013). We now find a positive correlation between the percentage of OT release in the CeA and social investigation time. Furthermore, females with low social interest showed a decrease in OT release during exposure to the social interest test. These opposite correlation patterns and the decrease in OT release are puzzling, especially because neither OTR antagonist (5 ng/0.5 µl/side) nor OT (100 pg/0.5 µl/side) administration into the CeA altered social interest in females. We therefore suggest the possibility that the decrease in OT release in the CeA of females is a consequence rather than a cause of low social investigation time. Indeed, previous studies provide evidence that exposure to a social stimulus can be associated with a change in OT release that does not seem to have an immediate effect on the behavior tested. For example, OT release in the lateral septum increased during retrieval of social memory in male rats, but blocking the actions of OT had no effect on the retrieval of social memory (Lukas et al., 2013). Likewise, OT release in the bed nucleus of the stria terminalis increased in male rats during exposure to a juvenile, but blocking the actions of OT had no effect on the behavior towards the juvenile (Dumais et al., 2016). In these studies, and in our present study, it could be that the change in OT release brings about changes in neural processes that will serve future behaviors. Support for this comes from studies with prairie voles, in which females given an OTR antagonist immediately prior to exposure to a male showed normal mating behavior, but impaired partner preference formation when tested 14 or 24 hours later (Insel and Hulihan, 1995), suggesting that OT activity during mating is critical for the subsequent

formation of later partner preference. It would be of interest to determine whether the decrease in OT release in the CeA of females with low social interest has effects on subsequent behaviors by, e.g., affecting social memory for the exposed juvenile.

OTR in the MeA may not be involved in social interest toward juvenile conspecifics

We recently showed that OTR binding densities in the MeA are higher in male rats compared to female rats, and that OTR binding density in the MeA correlates positively with social investigation time in males (Dumais et al., 2013). In addition, OTR mRNA expression in the MeA correlates positively with social investigation time in male mice (Murakami et al., 2011). These findings suggested a role for the OTR in the MeA in mediating social investigation in males. However, in the current study, neither OTR antagonist (5 ng/0.5 µl/side) nor OT (100 pg/0.5 µl/side) administration into the MeA altered social investigation time in either sex. Although the same dose of OTR antagonist and OT were effective in altering social behavior when administered into the CeA (present study) or the bed nucleus of the stria terminalis (Dumais et al., 2016), respectively, we cannot exclude that higher drug doses could be effective in altering social interest. Alternatively, this lack of effect could be due to the low social salience of juvenile conspecifics. In support, OTR antagonist administered into the MeA of adult male rats was found to reduce the investigation of soiled bedding from unfamiliar adult male conspecifics (Arakawa et al., 2010), but did not affect investigation toward juvenile or ovariectomized female rats (Lukas et al., 2013). Furthermore, extracellular recordings revealed that the MeA responds to social stimuli in both male and female mice, but stronger to opposite-sex stimuli compared to same-sex-stimuli (Bergan et al., 2014). Finally, MeA regulation of social recognition also depends on the salience of the social stimulus, as OTR blockade in the MeA impaired recognition of an ovariectomized female in male mice (Ferguson et al., 2001) and rats (Lukas et al., 2013), but not the recognition of a juvenile conspecific (Lukas et al., 2013). Taken into account the

difference in social stimuli (i.e., adult male, adult ovariectomized female, and juvenile conspecific) an interesting pattern emerges, such that the OT system in the MeA facilitates social interest and social recognition depending on the salience of the social stimulus. This hypothesis requires further testing.

Relevance of comparing males and females in studying the role of the amygdala-OT system in behavioral regulation

Given the key role of the OT system in the CeA in mediating incentive learning and emotion processing (Balleine and Killcross, 2006; Ledoux, 2000), fear and anxiety responses (Bale et al., 2001; Viviani et al., 2011; Knobloch et al., 2012; Lahoud and Maroun, 2013; László et al., 2016), and aggression in rodents (Lubin et al., 2003; Bosch et al., 2005; Consiglio et al., 2005; Calcagnoli et al., 2015), it is surprising that, to the best of our knowledge, we are the first to compare the role of the OT system in the CeA between males and females. This comparison is important given that human studies demonstrated that OT modulates amygdala function differently in men and women. Specifically, it was found that intranasal OT administration altered amygdala activation in opposite ways in men and women toward socially-relevant stimuli, such as exposure to fearful faces and during cooperative social interactions (Domes et al., 2007, 2010; Rilling et al., 2012, 2013). Importantly, the idea that the amygdala may be a site for sex-specific action of OT could be of relevance for the treatment of sex-biased psychiatric disorders of social dysfunction. Indeed, altered amygdala functioning is a main characteristic of autism spectrum, social anxiety, and borderline personality disorders (Baron-Cohen et al., 2000; Kleinhans et al., 2015; Kim et al., 2015; Evans et al., 2008; Goldin et al., 2009; Bruhl et al., 2014; Donegan et al., 2003; Herpertz et al., 2001) and has been found to be normalized in response to intranasal OT (Domes et al., 2013; Bertsch et al., 2013; Labuschagne et al., 2010). Therefore, it is

imperative to further understand the sex-specific role of the OT system in the amygdala and its subregions in the regulation of social behavior in both rodents and humans.

Conclusion

Sex differences in the effects of the OT system on various social behaviors are well known (for review, see Dumais and Veenema, 2015, 2016). Our current finding adds to this growing body of literature showing that the OT system in the CeA of rats modulates social interest in sex-specific ways. This sex-specific modulation of social interest by the CeA-OT system is found despite there being no sex differences in OTR binding density or extracellular OT release in the CeA. Further research is required to investigate the underlying mechanisms. Overall, we show that in rodents, much like humans, the amygdala may is a key brain region involved in the sex-specific processing of social stimuli by the OT system.

V. Study 4: Sex differences in neural activation following oxytocin administration in awake rats

** Manuscript in prep: Dumais, K.M., Kulkarni, P., Ferris, C.F. & Veenema, A.H. Sex differences in neural activation following oxytocin administration in awake rats.*

Abstract: The evolutionarily conserved neuropeptide oxytocin (OT) often regulates social behavior in sex-specific ways in rodents and humans. The OT system in the rat brain is sexually dimorphic, with males showing higher OT receptor (OTR) binding densities compared to females in several forebrain regions. However, little is known about potential sexually dimorphic effects of OT on brain function. We investigated neural activation patterns in response to central or peripheral OT administration in adult male and female rats. Functional magnetic resonance imaging was used to examine blood oxygen level-dependent (BOLD) signal intensity changes in the brains of awake rats within 20 min after intracerebroventricular (ICV; 1µg/5µl) or intraperitoneal (IP; 0.1mg/kg) administration of OT. Following ICV OT administration, sex difference in BOLD activation were observed in 26 brain regions, with 20 regions showing higher activation in males, and 6 regions showing higher activation in females. Among these were 11 regions dense in OT receptors (OTR), including the nucleus accumbens and insular cortex which showed higher activation in males, and the lateral and central amygdala which showed higher activation in females. Interestingly, compared to ICV OT, IP OT injections activated fewer brain regions dense in OTR (5 brain regions) and elicited fewer sex differences in brain activation (12 brain regions). Sex differences in activation in response to IP OT were also in different brain regions than those showing sex differences in response to ICV OT. Overall, these results indicate that exogenous OT modulates neural activation differently in males and females, and

that the pattern and the magnitude of sex differences in BOLD activation depends on the route of administration. Because OT has been found to have promising effects on improving social deficits in patients with sex-biased neuropsychiatric disorders, this knowledge may be informative when using OT as a therapeutic agent in both men and women.

Introduction

Oxytocin (OT) is an evolutionarily conserved neuropeptide synthesized mainly in the paraventricular nucleus and supraoptic nucleus of the hypothalamus (Buijs, 1978; Sofroniew, 1980). OT acts through the OT receptor (OTR) both centrally upon release from axonal projections throughout the brain, and systemically upon release from the posterior pituitary (Buijs and Swaab, 1979; Sofroniew, 1980; Gimpl and Fahrenholz, 2001; Knobloch et al., 2012). In addition to its well-known function as a hormone modulating uterine contractions during birth and milk ejection during lactation (Fuchs and Poblete, 1970; Belin et al., 1984), OT regulates a wide variety of social behaviors in rodents and humans (reviewed in Veenema and Neumann, 2008; Ross and Young, 2009; Guastella and MacLeod, 2012). Importantly, OT often regulates social behaviors in sex-specific ways (reviewed in Dumais and Veenema, 2015, 2016). For example, intracerebroventricular (ICV) injections of OT enhanced social recognition (Benelli et al., 1995) and reversed social defeat-induced social avoidance (Lukas et al., 2011) in male rats, but did not affect either behavior in female rats (Engelmann et al., 1998; Lukas and Neumann, 2014). These results suggest that central OT may modulate brain regions important for social behavior in sex-specific ways. However, no study has directly compared the effects of OT on neural activity between male and female rats.

We recently used functional magnetic resonance imaging (fMRI) to measure blood oxygen level dependent (BOLD) contrast to examine neural activity in awake adult male rats, and found robust neural activation in response to OT administration. Specifically, ICV

injections of OT activated numerous forebrain regions, such as the bed nucleus of the stria terminalis, lateral septum, ventral medial striatum, and nucleus accumbens (Ferris et al., 2015). Interestingly, many of these brain regions activated by central OT in males (bed nucleus of the stria terminalis, lateral septum, nucleus accumbens) show higher OTR binding density in male compared to female rats (Dumais et al., 2013). These sex differences in OTR binding densities in the rat brain suggest the potential for OT to modulate brain activity differently in males versus females.

The use of OT in humans is becoming increasingly popular, especially given its suggested therapeutic role in the treatment of social dysfunction in a range of psychiatric disorders (Andari et al. 2010; Guastella et al., 2010; Woolley et al., 2014). Peripheral OT administration, including intranasal OT administration, is the most commonly used route of administration in human studies, and has been found to have a variety of effects on neural activation, social cognition and behavior (Hollander et al., 2003, 2007; Guastella and MacLeod, 2012; Guastella et al., 2013). However, only a few studies have compared the effects of exogenous OT on BOLD activation in men and women. Of these few studies, it was found that intranasal OT decreased amygdala activation in men, but increased amygdala activation in women, in response to fearful faces (Domes et al., 2007; Domes et al., 2010). Furthermore, intranasal OT increased activation in the amygdala and caudate nucleus in men, but decreased or had no effect in these regions respectively, in women during cooperative social interaction (Rilling et al., 2013). The likelihood that intranasal OT modulates neural activation differently in males and females may have important implications for the potential therapeutic use of OT in humans.

In the current study, we examined BOLD activation in the brains of awake male and female rats in response to ICV OT or intraperitoneal (IP) OT administration. The robust sex differences in OTR binding density in the rat brain (Dumais et al., 2013) led us to

hypothesize that ICV OT administration will elicit sex differences in neural activation. It is unknown whether peripheral OT administration is directly activating OT-sensitive neural circuits or acting indirectly via peripheral mechanisms to modulate brain activity and cognition (Quintana et al., 2015). Because peripheral administration is the most commonly used route of administration in human studies, we also examined neural activation patterns in male and female rats following IP OT to determine whether peripheral OT may also elicit sex differences in neural activation patterns.

Methods

Animals. Adult male (300-325g) and female (280-300g) Sprague Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were maintained on a 12 h light/dark cycle, lights on at 0800 h, and food and water were available *ad libitum*. Rats were housed in standard Plexiglas cages in same-sex pairs unless otherwise mentioned, and were given at least one week to acclimate to the facilities at Northeastern University. All experiments were conducted in accordance with the guidelines of the NIH and approved by the Boston College and Northeastern University Institutional Animal Care and Use Committee (IACUC).

Stereotaxic surgery. For ICV injections, rats were cannulated with custom-designed cannulas created for compatibility with MRI (Plastics One, Roanoke, VA). Cannulations were performed 1-2 days after the last acclimation session (as described below). 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 were implanted unilaterally 1.5 mm dorsal to the lateral ventricle (0.5 caudal to bregma, -1.5 lateral to midline, and 3.0 ventral to the skull surface) according to Paxinos and Watson (2007). Guide cannulae were fixed to the skull with four plastic screws and acrylic glue and closed with

dummy cannulae (Plastics One, Roanoke, VA). After surgery, rats were individually housed and scanning was performed 3 and 5 days after surgery. Fig. 4.1 shows an example of correct cannula placement into the lateral ventricle, and only scans with cannula placements correctly aimed at the lateral ventricle were used in analysis.

Oxytocin Preparation and Administration. The vehicle used for control injections and for dissolving OT (Sigma, St. Louis, MO, USA) was Ringer's solution for ICV injections and saline (0.9% NaCl) for IP injections. Separate cohorts of rats were used for ICV and IP injections. Each rat was scanned during two imaging sessions, two days apart, receiving either vehicle or OT (1 μg/5 μl ICV, 0.1 mg/kg IP,) in counterbalanced order. Doses of OT were chosen because they were effective in eliciting BOLD responses in adult male rats (Ferris et al., 2015) and in lactating female rats (Febo et al., 2005). Vehicle and OT injections were delivered remotely during the imaging session. In detail, for ICV injections, polyethylene tubing was connected to an injector cannula which extended 1.5 mm beyond the guide cannula and extended approximately 30 cm in length in order to attach to a 10 μl Hamilton syringe. For IP injections, polyethylene tubing was positioned in the peritoneal cavity, and extended approximately 30 cm to attach to a 10 ml syringe.

Acclimation for awake imaging. To reduce the stress associated with awake imaging, each rat was acclimated to the restraining system (head holder and body restrainer) and to prerecorded magnetic resonance pulse sequence sounds using procedures outlined previously (King et al., 2005). Acclimation sessions were run once a day for a total of four days prior to the imaging session. Rats were briefly anesthetized with 2-3% isoflurane to secure the head into a padded head holder. The forepaws were secured with surgical tape, and the body was placed into a body tube to restrict overall movement. When fully conscious, the restrained rat was placed into a black opaque box ("mock scanner") for 30 min with a tape-recording of the MRI pulse sequence to simulate the sounds of the imaging session. This

acclimation procedure has been previously found to reduce respiration, heart rate, motor movements, and plasma corticosterone levels when comparing the first and last acclimation periods (King et al., 2005).

MRI procedures. Rats were briefly anesthetized with 2-3% isoflurane, and were positioned into a quadrature transmit/receive radiofrequency coil built into a rat head holder and restraining system (Animal Imaging Research, Holden, MA, USA). For a video of the full rat preparation for imaging, see www.youtube.com/watch?v=JQX1wgOV3K4. Experiments were conducted using a Bruker Biospec 7.0 T/20-cm USR horizontal magnet (Bruker, Billerica, MA, USA), and a 20-G/cm magnetic field gradient insert (inner diameter, 12 cm; 120 μs rise time). The scanner is controlled by Bruker Paravision 5.0, which automatically finds the basic frequency, shims, power requirements for 90° and 180° pulses, and sets the receiver gain. Imaging sessions included an anatomical scan followed by a functional scan. The anatomical images were collected using the RARE pulse sequence [22 slices; 1.1mm; field of view (FOV) 3.0 cm; matrix size 256 X 256; repetition time (TR) 2.5 s; echo time (TE) 12 ms; NEX 2; 3 min acquisition time]. The functional images were collected using a multi-slice half-Fourier acquisition single-shot turbo spin echo (HASTE) pulse sequence. A single functional scanning session acquired 22 slices, 1.1 mm thick, every 6.0 s (TR), using a TE of 48 ms, FOV 3.0 cm, matrix size 96 X 96, and NEX 1. This was repeated 250 times (250 acquisitions) for a total scanning time of 25 min. These parameters provided complete coverage of the brain from olfactory bulbs to brainstem, with an in-plane pixel resolution of 312 μm^2. The functional scanning session was continuous, with a 5 min baseline (50 acquisitions), followed by a 20 min treatment period (200 acquisitions). Acute vehicle or OT injections were given remotely immediately following the 5 min baseline while the rat was in the scanner (see Fig. 4.1 for functional imaging paradigm).

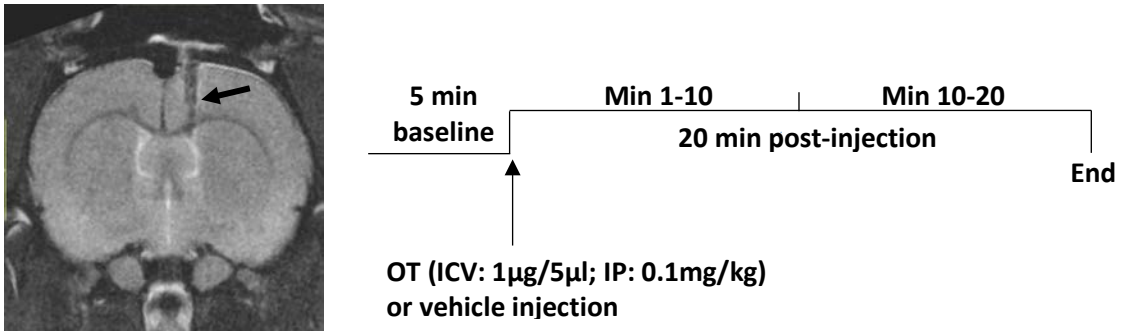


Fig 4.1 Intracerebroventricular cannula placement and functional imaging paradigm. Anatomical image depicting a coronal section of the brain of an adult male rat showing the location of the guide cannula with injector directed at the lateral ventricle with a black arrow (left). Diagram showing the paradigm used for functional imaging (right). Functional imaging sessions consisted of a 25 minute scan, in which an acute injection of OT or vehicle was administered after a 5 min baseline. ICV, intracerebroventricular; IP, intraperitoneal.

The neural responses to OT were evaluated using the BOLD response based on the principals of Ogowa et al (1993), which describe the BOLD signal as an indirect measure of neuronal activity via a change in local oxygenated cerebral blood flow. Increased blood flow of oxygenated hemoglobin increases magnetic resonance signal (Ogawa et al., 1990), and because enhanced brain activity is accompanied by local increases in blood flow, the magnetic resonance signal represents an indirect measure of neuronal activity (Fox & Raichle 1986; Sokoloff, 2008). Positive BOLD signal represents an increase in oxygenated blood when compared to baseline, while negative BOLD signal represents a decrease in oxygenated blood. Sustained negative BOLD may be attributed to a decrease in blood flow caused by neural inhibition, or alternatively, a redistribution of blood flow into neighboring regions of high activity, termed "vascular steal" (Kim and Ogawa, 2012).

Data analysis. Full details of the MRI data analysis have been previously reported in Ferris et al. (2008). Data analysis involved 1) co-registration of scans to a 3D rat brain atlas

with clearly delineated regions of interest (ROIs), and 2) statistical analysis to determine BOLD signal change in response to OT compared to baseline in each ROI. Motion artifact was determined by analysis of voxel displacement and spikes of raw data activity over time. Original group sizes were 14-18 rats per group. Final group sizes after prescreening for motion were as follows: ICV OT: vehicle-treated males= 9, vehicle-treated females=14, OT-treated males=10, OT-treated females=11; IP OT: vehicle-treated males=12, vehicle-treated females=11, OT-treated males=9, OT-treated females= 9.

Functional imaging data were localized to precise 3D volumes of interest using a segmented 3D MRI rat brain atlas (Ekam Solutions, Boston, MA, USA). These 3D volumes of interest are delineated into 171 distinct ROIs. Full details of the process of aligning functional scans to the segmented rat brain atlas have been described previously (Ferris et al., 2005). Briefly, individual functional scans were preprocessed using SPM8's co-registrational code (quality: 0.97; smoothing: 0.35 mm; and separation: 0.5 mm) and smoothed using Gaussian smoothing with a FWHM of 0.8 mm. Preprocessed functional files were then analyzed using Medical Image Analysis and Visualization software (MIVA). Using this interactive interface, functional scans were aligned and registered to the segmented 3D MRI rat brain atlas. Registration included translating, rotating, and scaling each subject's anatomy to align within each slice of the atlas.

For voxel-based analysis, the percent change in BOLD signal for each independent voxel was averaged for all subjects. A baseline threshold of 2% BOLD change was used to account for normal fluctuations of BOLD signal in the rat brain under the awake condition (Brevard et al., 2003). Statistical t tests were performed on each voxel of each subject within their original coordinate system. Voxel locations are then translated into ROIs within the atlas. A composite image of the brain representing the average of all subjects was constructed for each group, allowing us to look at each ROI separately to determine the BOLD change

and the number of activated voxels in each ROI. The t-tests used in analysis were performed using a 95% confidence level, two-tailed distributions, and heteroscedastic variance assumptions. Because of the multiple t-tests performed, a false-positive detection controlling mechanism was introduced into the analysis (Genovese et al., 2002), which ensured that the false-positive detection rate is below our confidence level of 5% (Ferris et al., 2005). Volume of activation was determined by comparing the average signal intensity in each voxel within each ROI of the first 5 min of baseline (acquisitions 1-50) to min 5-15 (acquisitions 51-150) and to min 15-25 (acquisitions 151-250). This resulted in BOLD activation summaries for each ROI during the first 10 min post injection (acquisitions 51-150, referred to as Min 1-10) and the second 10 min post injection (acquisitions 151-250, referred to as Min 10-20). The non-parametric Kruskal-Wallis test was used to compare the volume of activation in each ROI across experimental groups and for each time period (Min 1-10, Min 10-20). Estrus phase was determined via vaginal smears (according to Dumais et al., 2013) taken from each female immediately following each scanning session. However, because the number of estrus or nonestrus females in some of the experimental groups were too small (ICV injections, vehicle-treated: non-estrus=7, estrus=7, OT-treated: non-estrus=9, estrus=2; IP injections, vehicle-treated: non-estrus=9, estrus=2, OT-treated: non-estrus=6, estrus=3), estrus and non-estrus females given the same treatment were grouped together for analysis. Activation was considered to be different between experimental groups when comparisons yielded p values less than 0.05.

Results

BOLD activation in response to ICV OT administration

ICV OT induces activation in many brain regions dense in OTR

ICV OT activated 11 brain regions known to be dense in OTR (Fig. 4.2, highlighted in gray; Freund-Mercier et al., 1987; Tribollet et al., 1988; Dumais et al., 2013), six of which show sex differences in OTR binding densities (Fig. 4.2, bold text; Dumais et al., 2013; Smith et al., 2016). These are the insular cortex, nucleus accumbens shell, paraventricular hypothalamic nucleus, and ventromedial hypothalamus, which show higher activation in OT-treated males compared to vehicle-treated males, and the perirhinal cortex and medial preoptic area, which show higher activation in OT-treated females compared to vehicle-treated females. All of these brain regions show higher OTR binding density in males except for the perirhinal cortex, which shows higher OTR binding density in females (Dumais et al., 2013; Smith et al., 2016). Notably, ICV OT induced sustained activation of the insular cortex and nucleus accumbens shell in males, as activation was higher in these two regions compared to vehicle during Min 1-10 and Min 10-20. The other five brain regions activated by OT, but that do not show a sex difference in OTR binding density (Smith et al., 2016), are the ventral subiculum, lateral hypothalamus, and anterior olfactory nucleus, which show higher activation in OT-treated males compared to vehicle-treated males, and the basal amygdala and nucleus accumbens core which show higher activation in OT-treated females compared to vehicle-treated females.

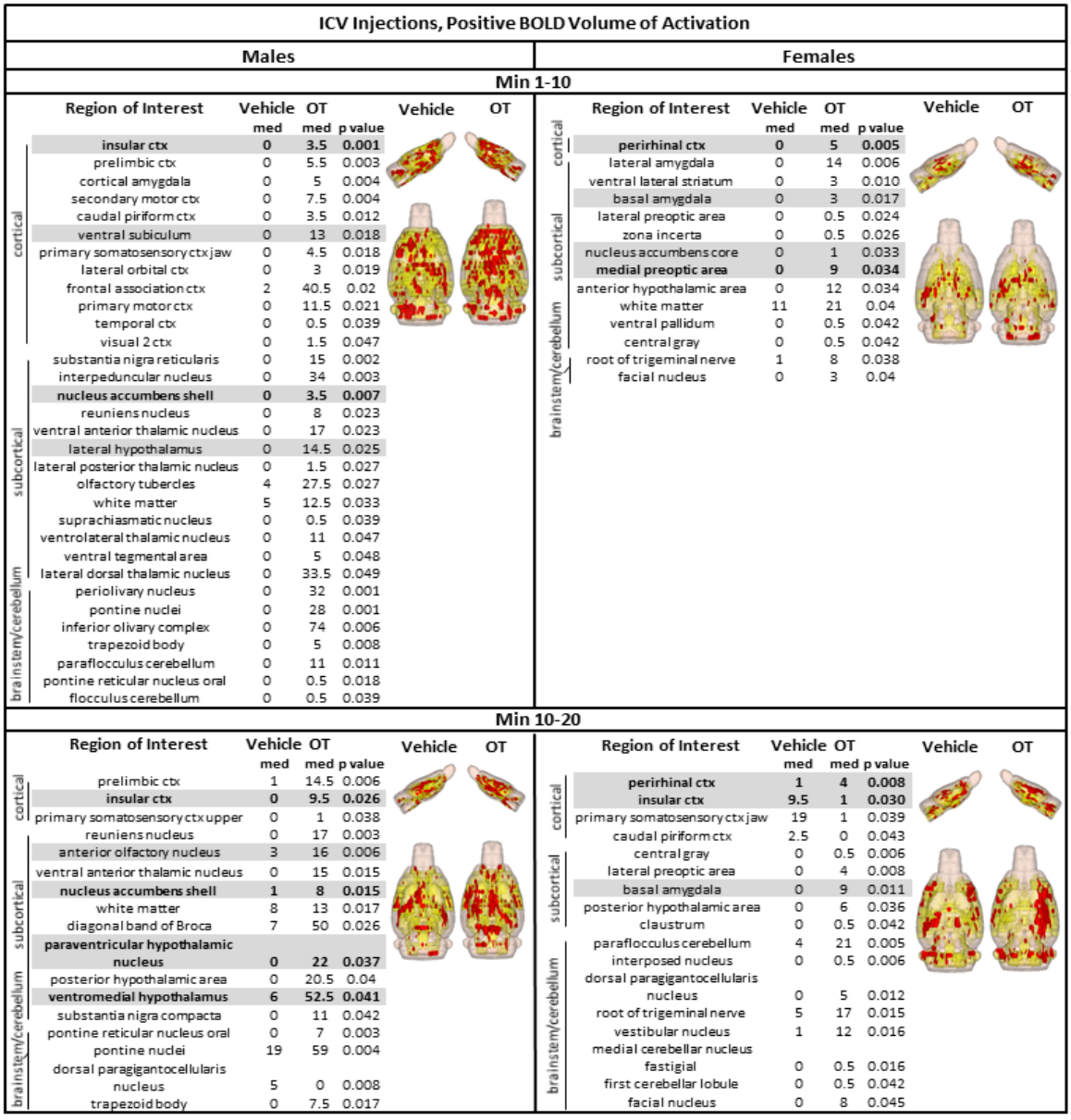


Fig 4.2 ICV OT activates many brain regions in both sexes, but more so in male than in female rats. Listed are brain regions that show treatment-induced differences in positive BOLD activation ($p<0.05$) for both males and females, with the corresponding median (med) number of voxels showing positive BOLD activation during either the first 10 minutes (Min 1-10) or last 10 minutes (Min 10-20) post vehicle or OT injection. Treatment groups are compared within each sex, and brain regions are clustered into cortical, subcortical, and brainstem/cerebellum regions. The 3D color model shows the location of the listed brain regions in yellow. Positive BOLD responses for each voxel are averaged across subjects within each experimental group, and in red is the composite average of voxels that show a significant increase in positive BOLD. Brain regions highlighted in gray in the text indicate regions that are known to show a high density of OTR (Freund-Mercier et al., 1987; Tribollet et al., 1988; Dumais et al., 2013). Brain regions that are bold are regions that are known to show sex differences in OTR binding densities (Dumais et al., 2013; Smith et al., 2016).

ICV OT induces robust activation throughout the brain, and more so in males than in females

Compared to vehicle, ICV OT induced positive BOLD activation in more brain regions in males (41 brain regions) than in females (26 brain regions; Fig. 4.2). Most noticeable are a higher number of cortical (during Min 1-10) and subcortical (during Min 1-10 and 10-20) regions showing BOLD activation in ICV OT-treated males.

Furthermore, compared to vehicle, ICV OT induced negative BOLD activation in fewer brain regions in males (7 brain regions) than in females (21 brain regions; Supplementary Table 4.1). Most noticeable was ICV OT-induced negative BOLD activation in females in the striatum, lateral amygdala, central amygdala, nucleus accumbens core, and lateral septum.

ICV OT induces robust sex differences in neural activation

A direct comparison between the sexes revealed that ICV OT induced significant sex differences in positive BOLD activation in 26 brain regions (Fig. 4.3). Compared to OT-treated females, OT-treated males showed higher positive BOLD activation in 20 distinct brain regions (13 brain regions during Min 1-10 and 20 brain regions during Min 10-20). Notably, compared to OT-treated females, OT-treated males showed higher positive BOLD activation in the nucleus accumbens shell, insular cortex, olfactory bulb (external plexiform layer and granular cell layer), olfactory tubercles, suprachiasmatic nucleus, and caudal piriform cortex during both Min 1-10 and Min 10-20. Compared to OT-treated males, OT-treated females showed higher positive BOLD activation in 6 distinct brain regions (3 brain regions during Min 1-10 and 4 brain regions during Min 10-20). In detail, OT-treated females showed higher activation compared to OT-treated males in the lateral amygdala during both Min 1-10 and 10-20, in the central amygdala and ventral lateral striatum during Min 1-10,

and in the dorsal paragigantocellularis nucleus, hippocampal CA2 region, and vestibular nucleus during Min 10-20.

Importantly, brain regions that show ICV OT-induced sex differences in positive BOLD activation are specific to OT-treated rats, as these sex differences were not seen in vehicle-treated rats, with two exceptions. Specifically, the insular cortex shows lower positive BOLD activation in vehicle-treated males compared to vehicle-treated females, but higher positive BOLD activation in OT-treated males compared to OT-treated females (Fig. 4.3; Fig. 4.4; Supplementary Table 4.2). The vestibular nucleus shows higher positive BOLD activation in vehicle-treated males compared to vehicle-treated females (Supplementary Table 4.2), but lower positive BOLD activation in OT-treated males compared to OT-treated females (Fig. 4.3).

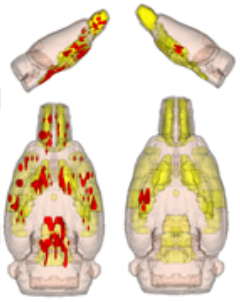
Sex differences in negative BOLD activation after ICV OT administration are found in 7 brain regions, namely the pineal gland (higher in males) and ventral lateral striatum (higher in females) during Min 1-10, and the olfactory bulb (external plexiform layer, glomerular layer, and granular layer), anterior lobe pituitary, and reticular nucleus (all higher in females) during Min 10-20 (Supplementary Table 4.3). Negative BOLD sex difference are specific to OT-treated rats, except for the anterior lobe pituitary (higher in vehicle-treated females) and reticular nucleus (higher in vehicle-treated males) which also show sex differences in vehicle-treated rats (Supplementary Table 4.2).

Sex Differences in Positive BOLD Volume of Activation in Response to ICV OT

Males > Females

Min 1-10

	Region of Interest	OT Males med	OT Females med	p value
olfactory bulb	granular cell layer	4.5	0	0.028
cortical	caudal piriform ctx	3.5	0	0.04
cortical	**insular ctx**	**3.5**	**1**	**0.05**
subcortical	interpeduncular nucleus	34	0	0.009
subcortical	external plexiform layer	11.5	3	0.011
subcortical	suprachiasmatic nucleus	0.5	0	0.023
subcortical	olfactory tubercles	27.5	1	0.032
subcortical	**nucleus accumbens shell**	**3.5**	**0**	**0.033**
subcortical	ventral pallidum	2	0	0.048
pituitary	anterior lobe pituitary	30.5	6	0.04
brainstem	pontine nuclei	28	2	<0.001
brainstem	periolivary nucleus	32	0	0.023
brainstem	trapezoid body	5	0	0.044

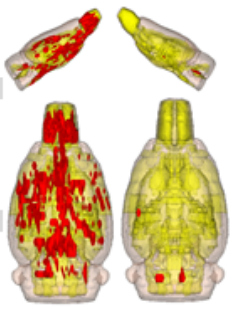


Females > Males

	Region of Interest	OT Males med	OT Females med	p value
subcortical	lateral amygdala	0	14	0.007
subcortical	central amygdala	0	7	0.025
subcortical	ventral lateral striatum	0	3	0.04

Min 10-20

	Region of Interest	OT Males med	OT Females med	p value
olfactory bulb	granular cell layer	10.5	2	0.004
olfactory bulb	external plexiform layer	22.5	12	0.012
olfactory bulb	glomerular layer	35.5	13	0.032
cortical	**insular ctx**	**9.5**	**1**	**0.015**
cortical	caudal piriform ctx	9	0	0.015
cortical	infralimbic ctx	29	3	0.048
subcortical	olfactory tubercles	44	15	0.003
subcortical	suprachiasmatic nucleus	50	0	0.009
subcortical	**nucleus accumbens shell**	**8**	**0**	**0.01**
subcortical	reuniens nucleus	17	0	0.011
subcortical	substantia nigra compacta	11	0	0.019
subcortical	white matter	13	11	0.036
subcortical	diagonal band of Broca	50	13	0.037
subcortical	interpeduncular nucleus	53	5	0.026
pituitary	anterior lobe pituitary	62.5	17	0.022
brainstem	pontine nuclei	59	9	0.001
brainstem	trapezoid body	7.5	0	0.002
brainstem	pontine reticular nucleus oral	7	0	0.002
brainstem	periolivary nucleus	43.5	0	0.048



	Region of Interest	OT Males med	OT Females med	p value
subcortical / cortical	CA2 hippocampus	0	14	0.029
subcortical / cortical	lateral amygdala	0	8	0.043
brainstem	dorsal paragigantocellularis nucleus	0	5	0.008
brainstem	vestibular nucleus	4	12	0.047

Fig 4.3 ICV OT induces robust sex differences in activation. Listed are brain regions showing sex differences in positive BOLD activation following ICV OT ($p<0.05$), with the corresponding median (med) number of voxels showing positive BOLD activation during either the first 10 minutes (Min 1-10) or last 10 minutes (Min 10-20). In the left column are brain regions that show higher activation in OT-treated males compared to OT-treated females, and in the right column are brain regions that show higher activation in OT-treated females compared to OT-treated males. Brain regions are clustered into olfactory bulb, cortical, subcortical, pituitary, and brainstem/cerebellum regions. The 3D color model shows

the location of the listed brain regions within each time point in yellow. Positive BOLD responses for each voxel is averaged across subjects within each experimental group, and in red is the composite average of voxels that show a significant increase in positive BOLD in regions that show higher activation in males (OT Males) and in regions that show higher activation in females (OT Females). Brain regions highlighted in gray in the text indicate regions that are known to show a high density of OTR (Freund-Mercier et al., 1987; Tribollet et al., 1988; Dumais et al., 2013). Brain regions that are bold are regions that are known to show sex differences in OTR binding densities (Dumais et al., 2013; Smith et al., 2016).

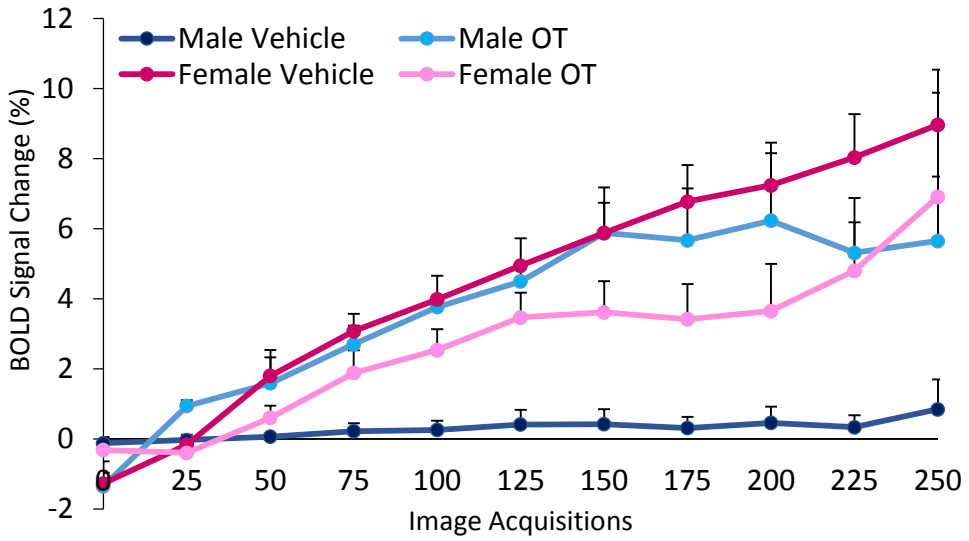


Fig 4.4 BOLD activation in the insular cortex varies with sex and treatment. Shown are the time-course plots for the change in BOLD signal in the insular cortex following injection (arrow) of OT or vehicle in male and female rats into the lateral ventricle. Vehicle-treated males show lower positive BOLD activation compared to vehicle-treated females and compared to OT-treated males. Vehicle-treated females show higher positive BOLD activation compared to OT treated females. Data represent mean + SEM.

BOLD activation in response to IP OT administration

IP OT induces activation in few brain regions dense in OTR

IP OT activated 5 brain regions known to be dense in OTR (Fig. 4.5, highlighted in gray; Freund-Mercier et al., 1987; Tribollet et al., 1988; Dumais et al., 2013), of which 3 brain regions show a sex difference in OTR binding densities (bold text; Dumais et al., 2013). In detail, compared to vehicle-treated males, OT-treated males showed higher positive BOLD activation in the nucleus accumbens shell, medial amygdala, and perirhinal cortex. The nucleus accumbens shell and medial amygdala show higher OTR binding density in males, while the perirhinal cortex shows higher OTR binding density in females (Dumais et al., 2013; Smith et al., 2016). Compared to vehicle-treated females, OT-treated females showed higher positive BOLD activation in the anterior olfactory nucleus, and dorsal subiculum, regions that so not show a sex difference in OTR binding density (Smith et al., 2016).

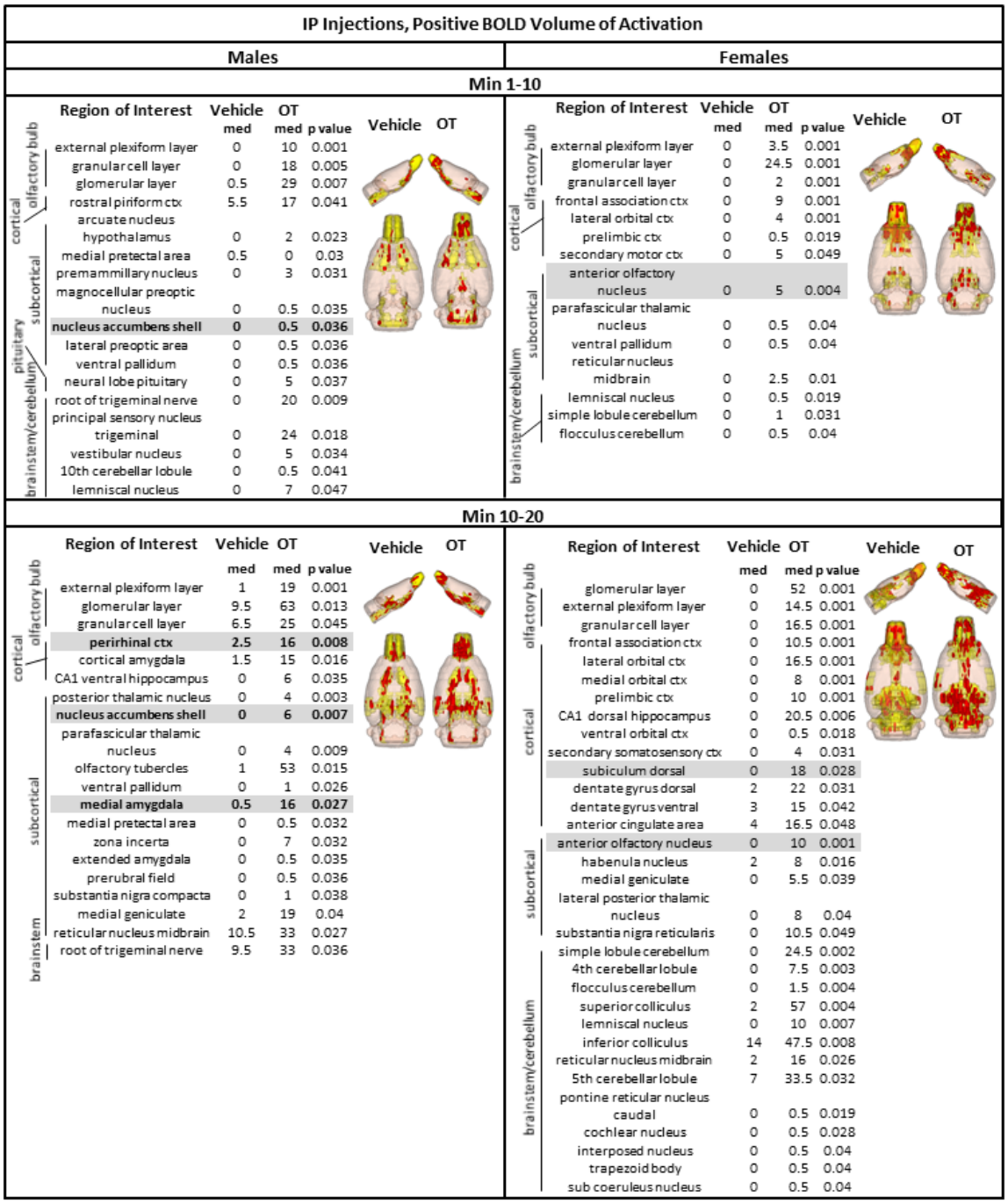


Figure 4.5 IP OT activates many brain regions in both sexes. Listed are brain regions that show treatment-induced differences in positive BOLD activation ($p<0.05$) for both males and females, with the corresponding median (med) number of voxels showing positive BOLD activation during either the first 10 minutes (Min 1-10) or last 10 minutes (Min 10-20) post vehicle or OT injection. Treatment groups are compared within each sex, and brain regions are clustered into olfactory bulb, cortical, subcortical, pituitary, and brainstem/cerebellum regions. The 3D color model shows the location of the listed brain regions in yellow. Positive BOLD responses for each voxel are averaged across subjects within each experimental group, and in red is the composite average of voxels that show a significant increase in

positive BOLD. Brain regions highlighted in gray in the text indicate regions that are known to show a high density of OTR (Freund-Mercier et al., 1987; Tribollet et al., 1988; Dumais et al., 2013). Brain regions that are bold are regions that are known to show sex differences in OTR binding densities (Dumais et al., 2013; Smith et al., 2016).

IP OT induces robust activation throughout the brain in both sexes

Compared to vehicle, IP OT induced positive BOLD activation in 31 brain regions in males and 35 brain regions in females (Fig. 4.5). Several of these brain regions were located within the olfactory system (Martinez-Marcos, 2009). In detail, compared to vehicle, IP OT induced positive BOLD activation in all three layers of the olfactory bulb (external plexiform layer, glomerular layer, and granular layer) during Min 1-10 and Min 10-20 in both sexes. In addition, compared to vehicle-treated males, OT-treated males showed higher positive BOLD activation in the rostral piriform cortex during Min 1-10 and in the olfactory tubercles and cortical amygdala during Min 10-20. Compared to vehicle-treated females, OT-treated females showed higher positive BOLD activation in the anterior olfactory nucleus during Min 1-10 and Min 10-20.

In addition, several brain regions within the cerebellum and brainstem showed positive BOLD activation in males and females in response to IP OT. Specifically, compared to vehicle-treated males, OT-treated males showed higher positive BOLD activation in the root of trigeminal, principal sensory nucleus trigeminal, vestibular nucleus, tenth cerebellar lobule, lemniscal nucleus, and reticular nucleus midbrain. Compared to vehicle-treated females, OT-treated females showed higher positive BOLD activation in the reticular nucleus midbrain, lemniscal nucleus, simple lobule cerebellum, and flocculus cerebellum, fourth cerebellar lobule, and superior colliculus. In addition, compared to vehicle-treated females, OT-treated females showed robust activation of cortical regions, especially during Min 10-20, a pattern of activation that was not evident in males.

Finally, compared to vehicle, IP OT induced negative BOLD activation in 18 brain regions in males and 23 brain regions in females (Supplementary Table 4.4). Most noticeable was prolonged IP OT-induced negative BOLD activation in both sexes in brain regions within the olfactory system (i.e, the external plexiform layer, glomerular layer, and granular cell layer).

IP OT causes limited sex differences in neural activation

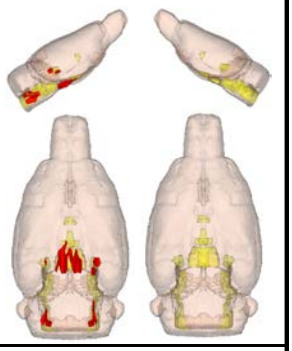
There were 12 distinct brain regions that show sex differences in positive BOLD activation in response to IP OT (7 during Min 1-10 and 5 during Min 10-20), with all 12 regions showing higher activation in OT-treated males compared to OT-treated females (Fig. 4.6). In detail, OT-treated males showed higher activation compared to OT-treated females in the arcuate nucleus, medial geniculate, premammilary nucleus, root of trigeminal nerve, triangular septal nucleus, neural lobe pituitary, and anterior lobe pituitary during Min 1-10, and in the cortical amygdala, medial amygdala, olfactory tubercles, magnocellular preoptic nucleus, and paraflocculus cerebellum during Min 10-20. Brain regions that show sex differences in positive BOLD activation in response to IP OT are specific to OT-treated rats, as these sex differences were not seen in vehicle-treated rats (Supplementary Table 4.5), except for the medial geniculate and the paraflocculus cerebellum which show higher activation in males compared to females irrespective of treatment.

Sex differences in negative BOLD activation after IP OT administration are found in the 7^{th}, 8^{th}, and 9^{th} cerebellar lobules and the basal amygdala (all higher in females) during Min 10-20 (Supplementary Table 4.3). Negative BOLD sex differences are specific to OT-treated rats, as these sex differences were not seen in vehicle-treated rats (Supplementary Table 4.5).

Sex Differences in Positive BOLD Volume of Activation in Response to IP OT

Min 1-10

	Region of Interest	OT Male med	OT Female med	p value
subcortical	arcuate nucleus hypothalamus	2	0	0.013
subcortical	medial geniculate	10	0	0.028
subcortical	premammillary nucleus	3	0	0.030
subcortical	triangular septal nucleus	0.5	0	0.036
pituitary	neural lobe pituitary	5	0	0.045
pituitary	anterior lobe pituitary	33	1	0.048
brainstem	root of trigeminal nerve	20	0	0.032



Min 10-20

	Region of Interest	OT Male med	OT Female med	p value
cortical	cortical amygdala	15	1	0.007
subcortical	olfactory tubercles	53	10.5	0.019
subcortical	**medial amygdala**	16	3	0.034
subcortical	magnocellular preoptic nucleus	1	0	0.042
cerebellum	paraflocculus cerebellum	64	25.5	0.047

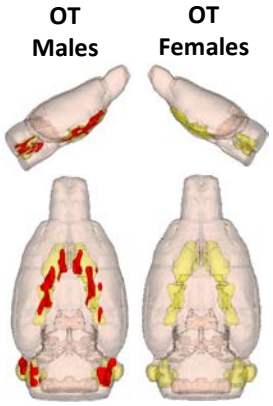


Figure 4.6 IP OT induces few sex differences in activation. Listed are brain regions showing sex differences in positive BOLD activation following IP OT ($p<0.05$), with the corresponding median (med) number of voxels showing positive BOLD activation during either the first 10 minutes (Min 1-10) or last 10 minutes (Min 10-20). All brain regions that show sex differences show higher activation in males compared to females, and all brain regions are clustered into cortical, subcortical, brainstem, and cerebellum regions. The 3D color model shows the location of the listed brain regions within each time point in yellow. Positive BOLD responses for each voxel is averaged across subjects within each experimental group, and in red is the composite average of voxels that show a significant increase in positive BOLD in regions that show higher activation in males (OT Males) and in regions that show higher activation in females (OT Females). The brain region highlighted in gray (medial amygdala) is known to show a high density of OTR (Freund-Mercier et al., 1987; Tribollet et al., 1988; Dumais et al., 2013), and to show a sex differences in OTR binding density (Dumais et al., 2013; Smith et al., 2016).

Discussion

The current study is the first to compare BOLD activation patterns between male and female rats in response to OT administration. This revealed that ICV OT activated more brain regions with dense OTR binding compared to IP OT (11 versus 5 brain regions). Furthermore, ICV OT induced sex differences in more (26 versus 12 brain regions) and in distinct (overlap in only 2 brain regions) brain regions compared to IP OT. Interestingly, of the sex differences observed in response to OT, males showed higher positive BOLD activation than females in nearly all brain regions following ICV OT (i.e., 20 out of 26 brain regions) and in all brain regions following IP OT (12 brain regions). Overall, these results show that exogenous OT induces robust sex differences in neural activation, but that the pattern and the magnitude of sex differences in neural activation strongly depend on the route of administration. Below we discuss how and why OT may produce ICV-, IP-, and sex-specific neural activation patterns, including their potential behavioral implications.

Sex-specific BOLD activation patterns following ICV OT

Our results confirm and extend previous findings in male rats showing robust ICV OT-induced BOLD activation in brain regions with high OTR expression (Ferris et al., 2015). Specifically, we confirmed the ICV OT-induced neural activation in the nucleus accumbens shell, olfactory tubercles, and subiculum, but also found additional activation in the anterior olfactory nucleus, insular cortex, paraventricular hypothalamus, and ventromedial hypothalamus. The BOLD activation found in the anterior olfactory nucleus and paraventricular hypothalamus is supported by data showing Fos activation in these regions in male rats following ICV OT injections (Ludwig et al., 2013). Interestingly, OT-treated males showed activation in more (7 versus 4) and in different brain regions that were dense in OTR than OT-treated females. Three of these brain regions activated by ICV OT in males but not

in females (i.e., insular cortex, paraventricular hypothalamus, ventromedial hypothalamus) show higher OTR binding density in males compared to females (Dumais et al., 2013; Smith et al., 2016). One of the brain regions activated by ICV OT in females but not males (perirhinal cortex) shows higher OTR binding density in females compared to males (Smith et al., 2016). These sex differences in OTR binding densities may, in part, explain the sex-specific activation patterns following ICV OT.

Sex-specific activation in response to ICV OT was also found in brain regions that do not show sex differences in OTR binding densities (i.e., ventral subiculum, lateral hypothalamus, basal amygdala; Smith et al., 2016). These regions may show sex-specific activation due to sex differences in affinity of OT to OTR, or via different modes of action of the OTR between the sexes (i.e., direct post-synaptic excitation or indirect modulation of synaptic inputs upon OT binding; Raggenbass, 2001; Joels, 2000). These hypotheses for sex-specific modulation of neural activity by OT have not been tested and are in need of further investigation. Interestingly, we previously found that OT administration into the central amygdala, which shows sex-specific BOLD activation (current study) but not a sex difference in OTR binding density (Dumais et al., 2013; Smith et al., 2016), modulates social investigation in sex-specific ways (Dumais et al., under review). Therefore, the ability of OT to modulate behavior differently in males and females in a region that does not show a sex difference in OTR binding density may lend support to the finding that OT modulates neural activation in sex-specific ways in this region. Finally, sex-specific activation in response to ICV OT was found in brain regions lacking dense OTR binding (Freund-Mercier et al., 1987; Tribollet et al., 1988; Dumais et al., 2013; Smith et al., 2016). Sex-specific activation of these brain regions may be due to indirect activation by other brain regions that were sex-specifically activated. For instance, the ventromedial hypothalamus, which shows a sex difference in OTR binding density (Dumais et al., 2013; Smith et al., 2016) and sex-specific

BOLD activation, projects to the prelimbic cortex and ventral tegmental area (Canteras et al., 1994), which show sex-specific BOLD activation but do not show dense OTR binding (Dumais et al., 2013; Smith et al., 2016). In summary, while some sex-specific activation patterns may be modulated by sex differences in OTR binding densities, it is likely that additional mechanisms contribute to the sex-specific activation profiles elicited by central OT administration.

Sex differences following ICV OT: Males show higher positive BOLD activation in olfactory and reward processing regions

Males showed higher positive BOLD activation than females in nearly all brain regions after ICV OT (20 out of 26 brain regions). A number of these brain regions include those involved in social olfactory processing (olfactory bulb layers, olfactory tubercles, caudal piriform cortex) and in the regulation of reward and motivated behaviors (nucleus accumbens shell, ventral pallidum). Most of these regions do not show sex differences in OTR binding (piriform cortex, ventral pallidum) or sex differences in OTR binding have not been examined (olfactory bulb layers, olfactory tubercles). However, in the nucleus accumbens shell, males show higher OTR binding density compared to females (Dumais et al., 2013). Interestingly, sex-specific activation of the nucleus accumbens was also found in humans, in which intranasal OT administration increased activation in the nucleus accumbens in men but not in women, in response to reciprocated cooperation (Rilling et al., 2013). Given the range of behavioral implications of these sex-specific neural activation patterns, it would be of interest to investigate whether ICV OT may produce sex-specific expression of social behaviors that may require olfactory and reward processing brain regions. Interestingly, ICV OT has been found to facilitate social recognition and to increase social approach toward stimuli that are typically avoided (conspecifics that previously defeated them in a social-defeat paradigm) in male but not female rats (Benelli et al., 1995;

Engelmann et al., 1998; Lukas et al., 2011; Lukas and Neumann, 2014). It would be interesting to examine whether brain regions that show higher BOLD activation in males compared to females following ICV OT may be causally involved in these behaviors that are facilitated by ICV OT in males but not in females.

Sex differences following ICV OT: Females show higher positive BOLD activation in brain regions involved in maternal behavior and in the amygdala

We are the first to examine ICV-OT induced BOLD activation in virgin female rats, as previous reports of ICV OT-induced BOLD activation has been in lactating rats only (Febo et al., 2005, 2009). Interestingly, virgin females showed higher activation in response to OT compared to vehicle-treated females and compared to OT-treated males in brain regions known to be important for OT-mediated maternal behavior, including the basal amygdala, lateral amygdala, central amygdala, and medial preoptic area (Pedersen et al., 1994; Bosch et al., 2005; Numan et al., 2010). In rats, pups are raised by the dam only (Lonstein and Fleming, 2002). Our findings indicate that brain regions which control maternal behavior are already more sensitive to OT in virgin females than in males.

The ICV OT-induced sex-specific BOLD activation of the amygdala seems to correspond with findings in humans. Here, intranasal administration of OT increased fear-induced amygdala activation in women, but decreased fear-induced amygdala activation in men (Domes et al., 2007; Domes et al., 2010). The opposite effect was found in response to positive social interactions, in which intranasal OT decreased amygdala activation in women, but increased amygdala activation in men, during cooperative social exchanges (Rilling et al., 2012; Rilling et al., 2013). Given these findings in humans, it would be interesting to examine whether the direction of sex-specific activation in the amygdala in response to ICV OT in rats depends on exposure to negative or positive social stimuli. Together, these data support the idea that, across species, the amygdala may be a site for sex-specific activation by

OT, and highlights the need to consider factors such as sex and social context when using OT in humans. Indeed, this may have important implications for the use of OT in the treatment of social dysfunction in neuropsychiatric disorders, as intranasal OT has been found to modulate amygdala activation in both men and women with sex-biased psychiatric disorders, such as autism spectrum disorder, social anxiety, and borderline personality disorder (Labuschagne et al., 2010; Domes et al., 2013; Bertsch et al., 2013).

Positive BOLD activation in the insular cortex depends on sex and ICV OT treatment

We observed an interesting pattern of positive BOLD activation in the insular cortex, in which vehicle-treated males showed lower activation compared to vehicle-treated females, while OT-treated males showed higher activation compared to OT-treated females. The insular cortex plays an important role in linking interoceptive information processing with emotional/social salience, by integrating homeostatic, visceral, and nociceptive inputs with emotional/social cues in mice, rats, monkeys, and humans (Craig, 2002; Rodgers et al., 2008; Gogolla et al., 2014; Jezzini et al., 2015), in which to generate a unified self-awareness (Craig, 2002, 2003; Critchley et al., 2004). Indeed, the insular cortex has been widely implicated in emotion perception, social cognition, and in coordinating a sense of "self" (Karnath and Baier, 2010; Menon and Uddin, 2010; Decety, 2011). Interestingly, sex differences have been found in the structure and function of the insular cortex in rats, in which females have greater dendritic branching in the insular cortex (Kolb and Stewart, 1991) and higher Fos expression in response to conditioned cocaine reinstatement (Zhou et al., 2014) compared to males. Sex differences were also found in humans, in which women showed higher insular cortex activity in response to negative images compared to men (Andreano et al., 2014). It is tempting to speculate that the higher BOLD activation in the insular cortex of females compared to males may reflect sex differences in response to a negative or stressful stimulus, i.e., the imaging procedure.

The switch in insular cortex activation in response to ICV OT with higher BOLD activation in males than in females suggest an opposite role for OT by enhancing BOLD activation in males but dampening BOLD activation in females. The higher OTR binding density in the insular cortex in male rats compared to female rats (Dumais et al., 2013), may, in part, contribute to this sex-specific effect. Interestingly, intranasal OT induced higher activation in the insular cortex in men compared to women during reciprocated cooperation (Rilling et al., 2013). This may suggest a similar sex-specific function of OT acting on the insular cortex (directly or indirectly) in rats and humans. Furthermore, it has been suggested that OT within the insular cortex encodes the saliency of interoceptive signals, and a dysregulation of the OT system may explain emotion and social "self" deficits characteristic of autism, a male-biased social disorder (Quattrocki and Friston, 2014). We propose that male and female rats could be used as a model system to study the mechanisms by which OT modulates insula-mediated emotional and social functioning in sex-specific ways, which, in turn, may inform studies in healthy and autistic humans.

BOLD activation following IP OT is distinct from BOLD activation following ICV OT

The activation patterns elicited by IP OT were very different than those elicited by ICV OT. Compared to ICV OT, IP OT induced activation of fewer brain regions that are known to be dense in OTR (5 versus 11 brain regions) and induced fewer sex differences in BOLD activation (12 versus 26 brain regions). Moreover, IP OT induced sex differences in BOLD activation in different brain regions than ICV OT. Together, this strongly suggests that ICV OT and IP OT induce their effects on the brain via different mechanisms. Indeed, because peripheral OT cannot readily pass the blood brain barrier (Mens et al., 1983; Ermisch et al., 1985), it is unlikely that a substantial amount of IP OT gained direct access to the brain to activate central OTR. Interestingly, changes in social behavior in male rats are seen 30-40

min after IP OT administration (Kent et al., 2015; Bowen et al., 2011; Suraev et al., 2014; Ramos et al., 2013). While the precise route remains to be elucidated, IP OT rapidly modulates brain activity that in turn can affect behavioral responses. The most robust patterns of activation induced by IP OT, as well as the potential mechanisms of activation by IP OT, are discussed below.

Possible mechanisms by which IP OT induces BOLD activation in the brain

We confirmed previous results showing IP OT-induced BOLD activation in cerebellum and brainstem regions in male rats (Ferris et al., 2015), and now demonstrate for the first time that IP OT in adult female rats also induced BOLD activation in cerebellum and brainstem regions. Brainstem activation in response to IP OT has also been found using Fos as a marker for neuronal activation in male rats and mice (Maejima et al., 2011; Morton et al., 2012; Iwasaki et al., 2015). OTR is widely expressed in peripheral systems involved in autonomic function (i.e., reproductive system, kidney, heart, vascular smooth muscle, thymus; Gimpl and Fahrenholz, 2001). These peripheral systems provide direct input to the cerebellum and brainstem via the vagus nerve (Newman and Paul, 1966, Rubia and Phelps, 1970; Menetrey and De Pommery, 1991). Moreover, IP OT-induced Fos activation of the brainstem in male mice was blocked after vagotomy (Iwasaki et al, 2015). Together, this strongly suggests a possible peripheral mechanism that may explain IP OT activation of cerebellum and brainstem regions. Given the role of the cerebellum and brainstem in autonomic nervous system regulation (i.e., heart rate, blood pressure, respiration, piloerection; Snider and Maiti, 1976; Haines et al., 1984; Cavdar et al., 2001), we propose that activation of the cerebellum and brainstem via a peripheral OTR-mediated route may serve to transmit viscero-sensory information from the body to guide autonomic responses, and in a more real-world setting, behavioral responses.

We confirmed previous results showing increased BOLD activation in the olfactory bulb following IP OT in male rats (Ferris et al., 2015), and we now demonstrate that IP OT in adult female rats also induced BOLD activation in the olfactory bulb. This activation in both sexes is of particular interest given the importance of olfactory bulb activation for a range of social behaviors in both male and female rats, including social recognition (Yu et al., 1996; Larrazolo-Lopez et al., 2008; Dluzen et al., 1998; Dluzen et al., 2000). The exact mechanisms by which IP OT increases BOLD activation in the olfactory bulb is at present unclear. IP OT may have gained direct access to the olfactory bulb and activated OTR in the olfactory bulb (Ferris et al., 2015) via bypassing the blood brain barrier at the level of the nasal epithelium (Mathison et al., 1998). On the other hand, the olfactory bulb receives direct input from the cerebellum (de Olmos et al., 1978; Matsutani and Yamamoto, 2008), and thus, IP OT-induced BOLD activation in the olfactory bulb may have been caused by IP OT-induced activation of the cerebellum.

Sex-specific BOLD activation patterns following IP OT

A number of regions showing sex-specific activation following IP OT injections were within the cerebellum and brainstem. If these regions are activated by IP OT via autonomic nervous system input (as discussed above), then this may suggest differences in autonomic regulation of cerebellum and brainstem activity between males and females. This could be mediated by sex differences in peripheral OTR, but unfortunately, no studies to date have compared peripheral OTR expression between the sexes. However, the testis and ovaries, which both express the OTR (Gimpl and Fahrenholz, 2001), have bi-directional, multisynaptic connections to the brainstem (and forebrain) via the vagus nerve (Ortega-Villalobos et al., 1990; Lee et al., 2002), and these connections are sexually dimorphic. Specifically, fibers projecting to particular brain regions (i.e., to the insular cortex, parasympathetic nucleus), are more dense coming from the testis than from the ovaries

(Gerendai et al., 1998, 2002; Gerendai, 2004). The sex-specific innervation of the brain by OTR-expressing reproductive organs may provide a mechanism by which IP OT induces sex differences in BOLD activation. Interestingly, sex differences in IP OT-induced BOLD activation were also found in subregions of the amygdala and hypothalamus. These regions have also been shown to receive direct afferents from the spinal cord (Menetrey and De Pommery, 1991), which may provide a mechanism for peripheral OT-mediated activation of these regions. However, whether these projections differ between males and females is unknown. Given the importance of the amygdala and hypothalamus for a range of social behaviors, sex differences in activation of these brain regions may suggest sexually dimorphic actions of IP OT on behavior. Because direct comparisons of the effect of IP OT on behavior between males and females is lacking, this would be an interesting area for future research.

Limitations and considerations

When imaging awake animals, there is concern for the stress associated with head and body restraint and the noise from the gradient coil. To address these issues, protocols have been made to acclimate rats to the imaging procedure by habituating the rat to the head and body restraining systems and the noise associated with scanning (described in Methods). This acclimation has been found to significantly reduce the stress associated with awake imaging. Specifically, rats show significant decreases in body temperature, motor movement, heart rate, and plasma corticosterone levels when comparing the first to the last acclimation days (King et al., 2005). However, while these signs of autonomic arousal and stress are reduced with acclimation, they are not eliminated entirely. Therefore, the OT-induced changes in BOLD activation may have occurred against a backdrop of heightened arousal and stress. In addition, OT injections were given in the absence of social stimuli. Therefore,

OT-induced activation patterns may be different when given in conjunction with various social stimuli. Finally, the exogenous OT-induced activation patterns in the current study may not represent endogenous OT-induced activation patterns. Determining neural activation patterns following techniques to induce endogenous OT release would be an important avenue for future research.

Conclusion

The underrepresentation of females in neuroscience research studies (Zucker and Beery, 2010) raises an important limitation in understanding the role of OT in the regulation of behavior and neural responses in both sexes. Importantly, the growing popularity of exogenous OT as a potential therapeutic agent in the treatment of a range of neuropsychiatric disorders highlights the need to understand how OT modulates the brain and behavior in both males and females. The present work demonstrates that exogenous OT administration activates the rat brain in sex-specific ways, albeit more so after central than after peripheral OT administration. A full understanding of the functional implications of these sex-specific neural activation patterns is still to be determined. However, these robust sex differences in neural activation patterns in rats may provide incentive for comprehensive investigation into possible OT-induced sex differences in neural activation in other species, including humans.

Supplementary Material: Study 4

ICV Injections, Negative BOLD Volume of Activation

Min 1-10

Males

Region of Interest	Vehicle med	OT med	p value
10th cerebellar lobule	0	36	0.003
flocculus cerebellum	0	27	0.003
superior colliculus	0	11	0.012
dorsal paragigantocellularis nucleus	0	2.5	0.018
pineal gland	0	100	0.029
cochlear nucleus	0	7.5	0.038
vestibular nucleus	0	9	0.042

Females

None significant

Min 10-20

Males

Region of Interest	Vehicle med	OT med	p value
flocculus cerebellum	0	61	<0.001
cochlear nucleus	0	18	0.012
10th cerebellar lobule	0	37.5	0.025
medial cerebellar nucleus fastigial	0	11	0.029
vestibular nucleus	4	43.5	0.04
central gray	0	24.5	0.046

Females

Region of Interest	Vehicle med	OT med	p value
reticular nucleus	0	12	<0.001
dorsal medial striatum	0	17	0.001
White Matter	4	29	0.005
ventral medial striatum	0	6	0.006
dorsal lateral striatum	0	5	0.007
lateral amygdaloid nucleus	0	26	0.009
accumbens core	0	1	0.021
lateral geniculate	0	24	0.022
visual 1 ctx	4	18	0.024
lateral septal nucleus	2	9	0.027
ventral lateral striatum	0	4	0.029
primary somatosensory ctx jaw	0	26	0.034
external plexiform layer	7	23	0.037
central amygdaloid nucleus	4	18	0.043
intercalated amygdaloid nucleus	0	0	0.049

Supplementary Table 4.1 Listed are brain regions that show ICV treatment-induced differences in negative BOLD activation ($p<0.05$) for both males and females, with the corresponding median (med) number of voxels showing negative BOLD activation during either the first 10 minutes (Min 1-10) or last 10 minutes (Min 10-20) post vehicle or OT injection. Treatment groups are compared within each sex, and all brain regions are ranked in order of their significance.

Sex differences in response to ICV vehicle						
Min 1-10				Min 10-20		
Positive BOLD Volume of Activation						
Region of Interest	Vehicle Male med	Vehicle Female med	p value	Region of Interest	Vehicle Male med	Vehicle Female med	p value
primary somatosensory ctx jaw	0	6	0.001	central gray	0	0	0.008
insular ctx	0	5	0.005	primary somatosensory ctx jaw	0	19	0.001
primary motor ctx	0	6	0.01	primary somatosensory ctx upper lip	0	1.5	0.013
lateral posterior thalamic nucleus	0	2.5	0.011	dorsal paragigantocellularis nucleus	5	0	0.014
ventrolateral thalamic nucleus	0	13	0.011	primary somatosensory ctx forelimb	0	0.5	0.015
lateral dorsal thalamic nucleus	0	39	0.015	cochlear nucleus	8	0	0.015
habenula nucleus	0	19.5	0.017	root of trigeminal nerve	18	5	0.016
intercalated amygdala	0	0	0.024	primary motor ctx	3	6.5	0.019
				first cerebellar lobule	0	0	0.024
				substantia innominata	0	0	0.024
				insular ctx	0	9.5	0.026
				ventral anterior thalamic nucleus	0	8	0.027
				ventrolateral thalamic nucleus	0	9	0.037
				lateral orbital ctx	0	6.5	0.047
				vestibular nucleus	8	1	0.049
Negative BOLD Volume of Activation						
Region of Interest	Vehicle Male med	Vehicle Female med	p value	Region of Interest	Vehicle Male med	Vehicle Female med	p value
dorsal medial striatum	10	0	0.004	dorsal medial striatum	13	0	0.001
cochlear nucleus	0	10.5	0.027	cochlear nucleus	0	32	0.009
rostral piriform ctx	1	5.5	0.045	anterior lobe pituitary	0	13.5	0.016
				flocculus cerebellum	0	13.5	0.020
				reticular nucleus	6	0	0.021
				precuniform nucleus	0	0	0.024
				facial nucleus	0	13	0.038
				ventral posteriomedial thalamic nucleus	2	0	0.04

Supplementary Table 4.2 Listed are brain regions that show sex differences in response to ICV vehicle ($p<0.05$) for both positive and negative BOLD activation, with the corresponding median (med) number of voxels showing BOLD activation during either the first 10 minutes (Min 1-10) or last 10 minutes (Min 10-20) in males and females. All brain regions are ranked in order of their significance.

Sex differences in negative BOLD volume of activation in response to OT

ICV

Min 1-10

Males > Females: Region of Interest	OT Males med	OT Females med	p value
pineal gland	100	0	0.006

Females > Males: Region of Interest	OT Males med	OT Females med	p value
ventral lateral striatum	0	0.5	0.039

Min 10-20

None significantly different

Females > Males: Region of Interest	OT Males med	OT Females med	p value
external plexiform layer	9	23	0.006
anterior lobe pituitary	3	28	0.024
glomerular layer	10.5	18	0.024
granular cell layer	6	10	0.026
reticular nucleus	1	12	0.043

IP

Min 1-10

None significantly different

None significantly different

Min 10-20

None significantly different

Females > Males: Region of Interest	OT Male med	OT Female med	p value
7th cerebellar lobule	0	4	0.02
8th cerebellar lobule	0	12.5	0.027
9th cerebellar lobule	0	12	0.032
basal amygdaloid nucleus	0	11	0.037

Supplementary Table 4.3 Listed are brain regions that show sex differences in negative BOLD activation ($p<0.05$) in response to ICV OT and IP OT, with the corresponding median (med) number of voxels showing BOLD activation during either the first 10 minutes (Min 1-10) or last 10 minutes (Min 10-20) in males and females. All brain regions are ranked in order of their significance.

IP Injections, Negative BOLD Volume of Activation							
Min 1-10				Min 10-20			
Males							
Region of Interest	Vehicle med	OT med	p value	Region of Interest	Vehicle med	OT med	p value
lateral dorsal thalamic nucleus	0	0.5	0.013	external plexiform layer	0	27	0.006
dorsal medial striatum	0	6	0.016	dorsal medial striatum	0	40	0.016
primary somatosensory ctx forelimb	0	2	0.016	CA3 dorsal hippocampus	0	28	0.017
primary somatosensory ctx upper lip	0	7	0.016	primary somatosensory ctx forelimb	0.5	22	0.018
CA1 dorsal hippocampus	0	14	0.028	granular cell layer	0	51	0.036
white matter	0	9	0.033	reticular nucleus	0	10	0.037
simple lobule cerebellum	0	10	0.035				
secondary somatosensory ctx	0	1	0.040				
primary somatosensory ctx barrel field	0	8	0.042				
Females							
Region of Interest	Vehicle med	OT med	p value	Region of Interest	Vehicle med	OT med	p value
frontal association ctx	0	4.5	<0.001	external plexiform layer	0	24.5	<0.001
glomerular layer	0	13	0.001	glomerular layer	0	42.5	<0.001
external plexiform layer	0	6.5	0.001	granular cell layer	0	25.5	<0.001
granular cell layer	0	3.5	0.004	frontal association ctx	0	12	0.001
anterior olfactory nucleus	0	4	0.009	anterior olfactory nucleus	0	10	0.001
lateral orbital ctx	0	1	0.009	pontine reticular nucleus oral	0	2	0.001
supramammillary nucleus	2	0	0.011	lateral orbital ctx	0	5.5	0.001
lateral hypothalamus	7	0	0.012	CA1 ventral hippocampus	0	11.5	0.007
medial orbital ctx	0	0.5	0.040	lateral amygdaloid nucleus	0	5	0.008
prelimbic ctx	0	0.5	0.040	prelimbic ctx	0	4.5	0.009
				parietal ctx	0	9.5	0.011
				pineal gland	3	0	0.014
				anterior thalamic nuclei	0	1.5	0.017
				medial orbital ctx	0	0	0.019
				basal amygdaloid nucleus	0	11	0.023
				primary somatosensory ctx shoulder	0	2	0.023
				reticular nucleus	0	4	0.025
				ventral orbital ctx	0	0.5	0.040
				dorsal paragigantocellularis nucleus	0	3	0.046
				dorsal lateral striatum	0	9	0.049

Supplementary Table 4.4 Listed are brain regions that show IP treatment-induced differences in negative BOLD activation ($p<0.05$) for both males and females, with the corresponding median (med) number of voxels showing negative BOLD activation during either the first 10 minutes (Min 1-10) or last 10 minutes (Min 10-20) post vehicle or OT injection. Treatment groups are compared within each sex, and all brain regions are ranked in order of their significance.

Sex differences in response to IP vehicle							
Min 1-10				Min 10-20			
Positive BOLD Volume of Activation							
Region of Interest	Vehicle Male med	Vehicle Female med	p value	Region of Interest	Vehicle Male med	Vehicle Female med	p value
flocculus cerebellum	1	0	0.009	8th cerebellar lobule	0	33	0.002
medial pretectal area	0.5	0	0.018	flocculus cerebellum	1.5	0	0.009
CA1 ventral hippocampus	0	1	0.026	simple lobule cerebellum	12.5	0	0.011
basal amygdala	0	1	0.033	10th cerebellar lobule	0	19	0.012
ninth cerebellar lobule	0	0.5	0.038	crus 2 of ansiform lobule	7.5	58	0.013
medial geniculate	0.5	0	0.040	paraflocculus cerebellum	40.5	9	0.015
periaqueductal gray thalamus	0.5	0	0.041	9th cerebellar lobule	0	28	0.016
reticular nucleus midbrain	1	0	0.045	CA1 dorsal hippocampus	14	0	0.018
				medial pretectal area	0.5	0	0.019
				paramedian lobule	4.5	48	0.024
				auditory ctx	3.5	0	0.025
				habenula nucleus	10	2	0.035
				infralimbic ctx	7.5	0	0.036
				7th cerebellar lobule	0	14	0.037
				interposed nucleus	0.5	0	0.040
				pontine reticular nucleus caudal	0.5	0	0.040
				ventral medial striatum	0	1	0.046
				CA1 ventral hippocampus	0	3	0.046
Negative BOLD Volume of Activation							
Region of Interest	Vehicle Male med	Vehicle Female med	p value	Region of Interest	Vehicle Male med	Vehicle Female med	p value
fourth cerebellar lobule	0	11	0.005	pontine reticular nucleus oral	0	1	0.004
retrosplenial rostral ctx	2	39	0.024	medial amygdaloid nucleus	11	1	0.012
medial amygdala	0	3	0.029	paraflocculus cerebellum	74	13	0.026
white matter	0	16	0.030	medial cerebellar nucleus fastigial	2	0	0.031
CA1 dorsal hippocampus	0	11	0.030	supraoptic nucleus hypothalamus	0.5	0	0.045
dentate gyrus dorsal	0	2	0.030				
anterior cingulate area	0	4	0.034				
periaqueductal gray thalamus	0	10	0.036				
subiculum dorsal	0	5	0.038				
medial geniculate	0	4	0.038				
paraflocculus cerebellum	4	26	0.049				

Supplementary Table 4.5 Listed are brain regions that show sex differences in response to IP vehicle ($p<0.05$) for both positive and negative BOLD activation, with the corresponding median (med) number of voxels showing BOLD activation during either the first 10 minutes (Min 1-10) or last 10 minutes (Min 10-20) in males and females. All brain regions are ranked in order of their significance.

VI. General Discussion

The focus of my thesis has been to investigate sex differences in the brain oxytocin (OT) system, and how these sex differences may regulate social behavior and neural activation in sex-specific ways. We demonstrated that the OT receptor (OTR) in the rat brain is highly sexually dimorphic, with males showing higher OTR binding densities than females in a variety of forebrain regions (Study 1; Dumais et al., 2013). Furthermore, we found a functional link between a sex difference in OTR binding in the posterior bed nucleus of the stria terminalis (BNSTp) and sex-specific regulation of social recognition by OT (Study 2; Dumais et al., 2016). Interestingly, we also found that the OT system modulates social interest in sex-specific ways in the central amygdala (CeA), a region that does not show a sex difference in OTR binding (Study 3; Dumais et al., 2016, under review). Finally, we showed that exogenous OT induced robust sex differences in neural activation, and that the pattern and the magnitude of these sex differences strongly depend on the route of administration (central versus peripheral; Study 4). Together, these results show the complex ability of OT to regulate behavioral and brain responses in sex-specific ways.

It is important to consider whether sex differences in OTR binding densities found in rats (Study 1) are unique to rats, or whether they are also found in other mammalian species, including humans. This species comparison will allow for the formulation of a framework in which the functional relevance of sex differences, or lack thereof, in the OT system can then be tested. Therefore, I discuss below whether similar patterns of sex differences in the OT system exist across species, and I will argue that the functional relevance of sex differences in the OT system can only be understood by taking into account the social organization of a given species. I will further discuss how the sex-specific role of the OT system in the BNSTp and CeA in the regulation of social recognition and social interest, respectively (Study 2 and 3), updates our existing knowledge of the neural network that is currently known to underlie

social behavior. Finally, I discuss the implications of sex-specific modulation of brain activation by exogenous OT (Study 4), and how these results may inform clinical health sectors about using OT as a therapeutic agent in both men and women.

Sex differences in the OT system in rats: How does this compare to other species?

In order to better understand the functional role of the OT system in both males and females, it is important to understand whether sex differences in the OT system are found across species, or are species-specific. Indeed, generalizing the idea of a "sexually dimorphic OT system" to all species may be damaging to scientific progression if this were not the case. When comparing across species, the lack of sex differences in OT mRNA expression in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus in virgin rats (Study 1; Dumais et al., 2013) is consistent with the lack of sex differences in OT-immunoreactive neurons in the PVN and SON in prairie, pine, meadow, and montane voles (Wang et al., 1996), naked mole rats (Rosen et al., 2008), long tailed hamsters (Xu et al., 2010), and non-human primates (macaques: Caffé et al., 1989; marmosets: Wang et al., 1997). Similarly, in humans, there are no sex differences in the number of OT neurons in the PVN (Wierda et al., 1991) or in the size of OT neurons in the PVN and SON (Fliers et al., 1985; Ishunina and Swaab, 1999). However, the lack of sex differences in OT mRNA in rats (Study 1; Dumais et al., 2013) is in contrast to some other species, in which females have higher OT-immunoreactive neurons in the PVN and SON compared to males in CD mice (Häussler et al., 1990), mandarin voles (Qiao et al., 2014), and Brandt's voles (PVN only; Xu et al., 2010). Together, these studies show that sex differences in OT synthesis is species-specific, but that there is a lack of sex differences in OT synthesis in the brain in the majority of species analyzed.

Sex differences in OTR binding densities are also species-specific. Study 1 found that, compared to female rats, male rats have higher OTR binding densities in 9 out of 15 forebrain regions analyzed (Dumais et al., 2013). This male dominated pattern in OTR binding density is also seen in deer mice (*P. maniculatus* and *P. californicus*), in which males showed higher OTR binding densities compared to females in 8 out of 20 brain regions analyzed, including the olfactory bulb, cingulate cortex, dorsal lateral septum (LS), and BNST (lateral, medial anterior, and medial posterior parts; Insel et al., 1991). Furthermore, higher OTR binding density in males compared to females in the medial amygdala (MeA) and hippocampal CA1 region in Study 1 (Dumais et al., 2013) is consistent with higher OTR mRNA expression in the MeA in male mandarin voles (Cao et al., 2013) and higher OTR binding density in the MeA and hippocampal CA1 region in male S. xerampelinus singing mice (Campbell et al., 2009) compared to females. However, in other species, females display higher OTR binding densities than males. For example, compared to males, females showed higher OTR binding densities in the medial prefrontal cortex in prairie and pine voles (Smeltzer et al., 2006) and in the ventromedial hypothalamus (VMH) in ICR mice (Tribollet et al., 2002). Further, limited or no sex differences in OTR binding densities have been found in C57Bl/6J mice (Hammock and Levitt, 2013), solitary golden hamsters (Dubois-Dauphin et al., 1992), and prairie voles (Bales et al., 2007a; except for the medial prefrontal cortex in Smeltzer et al., 2006). Only one study thus far has addressed potential sex differences in OTR in the human brain and reported no sex differences in OTR binding densities, but they had a very low number of female subjects (n=4; Loup et al., 1991). Unfortunately, there are still large gaps in our knowledge of sex differences in OTR binding densities across species, as most studies only analyzed sex differences in OTR in a few brain regions (mice [Hammock and Levitt; 2013]; voles [Smeltzer et al., 2006; Bales et al., 2007a; Cao et al., 2013]; hamsters [Dubois-Dauphin et al., 1992]). Therefore, additional and more comprehensive studies would

be helpful in providing a more complete understanding of sex differences, or lack thereof, in OTR binding densities across species.

Do sex differences in the OT system relate to species-specific social organizations?

Collectively, these findings show that there is evidence for both the presence and absence of sex differences in the OT system, depending on the species and the specific OT system parameters (OTR binding, OT mRNA, OT-immunoreactivity) being investigated. This raises the question, what does this variability in sex-specific expression of the OT system mean? We propose that the presence or absence of sex differences in the brain OT system depends largely on the social organization of that particular species. Social organization encompasses the organization of social relations within a group, including mating systems (polygamy, monogamy, promiscuity), parental systems (biparental care, maternal care), and sociality systems (the degree to which individuals live in social groups, ranging from solitary to eusocial). Accordingly, we would predict that species in which males and females *differ* in at least one aspect of social organization (e.g., polygamous species, which show sex differences in mating and parental systems; Lande & Arnold, 1985) will show more sex differences in the OT system compared to species in which males and females exhibit very *similar* social organizations (e.g., monogamous species, which show few sex differences in mating and parental systems; Kleiman, 1977). In support of these predictions, sex differences in OT and/or OTR have been found in polygamous rodent species such as CD mice (OT-ir in PVN and SON; Häussler et al., 1990), P. maniculatus (OTR binding; Insel et al., 1991), Sprague Dawley rats (OTR binding; Uhl-Bronner et al., 2005), Wistar rats (OTR binding; Dumais et al., 2013), and Chinese striped hamsters (OT-ir; Wang et al., 2013), while a lack of sex differences in OT system parameters has been found in monogamous rodent

species, such as P. californicus mice (OTR binding; Insel et al., 1991) and prairie and pine voles (OT-ir in PVN and SON and OTR; Wang et al., 1996; Bales et al., 2007).

However, in contrast to these predictions, polygamous species such as meadow and montane voles do not show sex differences in OT system parameters (OT-ir in PVN and SON and OTR; Wang et al., 1996). This highlights the need to not only focus on one or two aspects of the social organization (in this case mating and parental systems), but to consider *all* aspects of the social organization (including sociality systems). For example, polygamous species can be primarily solitary (such as meadow and montane voles, and hamsters; Madison, 1980; Webster and Brooks, 1981; Gattermann et al., 2001) or can be living in more complex social groups (such as rats and some mouse species; Brett, 1991; Manning et al., 1992). The concept of integrating mating systems with sociality systems has been elegantly proposed by Ophir (2011) and Kelly and Ophir (2015) in order to provide a framework that may help us understand species-specific neuropeptide control of social behavior across vertebrate taxa. We believe that a similar approach is necessary to understand the functional significance of sex differences, or a lack thereof, in the OT system, as the roles of males and females may differ according to their specific sociality system. For example, a difference in the expression of territoriality may be larger between the sexes in highly complex social groups (only males may show territoriality) compared to less complex social groups (both sexes need to show territoriality). In addition, it will also be important to consider factors such as season and social density, as these might influence changes in the social organization and OT parameters differently in males than in females (Beery and Zucker, 2010; Anacker and Beery, 2013). Furthermore, it will be important to consider social context and the way we house laboratory animals, as this may or may not diverge from the natural social organization of a given species. Therefore, it is important to keep in mind that neural systems (i.e., the OT system) that represent ethologically relevant behavior in wild animals may have been altered

in laboratory animals (Kelly and Ophir, 2015; Baumans & Van Loo, 2013). Finally, we should consider that sex differences in the OT system may not always function to induce sex differences in behavior. De Vries (2004) proposed a dual-function hypothesis of sex differences, in which sex differences in the brain may function to either cause sex differences in behavior, or may function to prevent sex differences in behavior. The latter may be important when compensating for other sex differences in physiological conditions (i.e. hormone levels). This hypothesis may have consequences for how we should interpret the function of the presence or absence of sex differences in the OT system. Translating this to our proposal, the dual-function hypothesis would suggest that males and females with a similar social organization may actually show sex differences in OT parameters in order for them to show similar behaviors. Together, taking into account the social organization of a given species may be an important step towards understanding why certain species show sex differences in the brain OT system while other species do not.

Sex-specific roles of the OT system in the BNSTp in social recognition: New insights into the neural network regulating social recognition

Social recognition describes the ability to recognize individual conspecifics, and is important for a range of social behaviors, such as the establishment and maintenance of social hierarchies, parent-offspring bonding, mating and sexual behaviors, and aggressive behaviors. Therefore, social recognition is imperative for the appropriate expression of a wide range of social behaviors important for survival and successful reproduction. In the preceding chapters, we have shown evidence for the involvement of the OT system in the BNSTp in the sex-specific regulation of social recognition (Study 2; Dumais et al., 2016). Below, we describe how this data provides new insights into the network of brain regions involved in regulating social recognition (Wacker and Ludwig, 2012; Gabor et al., 2012;

Choleris et al., 2009). We start by describing the initial detection of the olfactory cue by the vomeronasal system, and how this social olfactory information is transmitted to brain regions that are known to regulate social recognition. We then describe how the OT system acts in a number of these brain regions to regulate social recognition, and how our BNSTp data (Study 2; Dumais et al., 2016) adds to our understanding of the neural circuitry involving OT-mediated social recognition in both sexes.

Neural circuitry underlying social information processing

Detection of the social olfactory cue is the first step in the process underlying a wide variety of social behaviors. The initial detection of social odors are via volatile odor detection by the main olfactory epithelium which is relayed to the main olfactory bulb, and non-volatile odor detection by the vomeronasal organ which is relayed to the accessory olfactory bulb (Johnston, 1998; Baum and Bakker, 2013). These two detection mechanisms make up the start of two distinct olfactory pathways, the main olfactory pathway and the accessory olfactory pathway, respectively (Johnston, 1998; Baum and Bakker, 2013). Recent evidence however suggests that the main olfactory system can contribute to the ability of the accessory olfactory system to generate responses to biologically relevant cues via convergence of these two systems in the MeA (Martinez-Garcia et al., 2009; Guthman and Vera, 2016). While both the main and accessory olfactory systems have been shown to be involved in mediating neuroendocrine and behavioral responses to social cues across a variety of species (Johnston, 1998), the accessory olfactory system has been shown to be the main modulator of social recognition processing in rats (Noack et al., 2010). Indeed, the social recognition paradigm employed in Study 2 measures a rat's ability to discriminate between individual conspecifics, and this type of individual recognition is in part modulated by chemical signals (i.e. pheromones) which are mainly detected by the vomeronasal organ (Dulac and Torello, 2003; Johnston, 1998). After detection of these chemical signals which make up a conspecific's

"olfactory signature", the neurons of the vomeronasal organ, located in the nasal cavity, gain direct access to the brain by penetrating the cribriform plate and terminating in the glomerular layer of the accessory olfactory bulb (Johnston, 1998). Through this process, the initial detection of the social olfactory cue is made.

For the ability to recognize and then appropriately respond to conspecifics, the neural circuitry underlying social information processing must include not only systems necessary to perceive social cues, but systems to link these cues to emotion, motivation, and adaptive behavior (Camats-Perna and Engelmann, 2015). This includes the BNSTp and MeA, which receive direct projections from the accessory olfactory bulb, and have hence been described as the "vomeronasal amygdala" (Davis et al., 1978; deOlmos et al., 1978; Scalia and Winans, 1975; Winans and Scalia, 1970; Halpern, 1987). The BNSTp and MeA then project to hypothalamic nuclei (medial preoptic area [MPOA], VMH, and ventral premammillary nucleus) for motivational output behavior, which have been described as the tertiary projections of the accessory olfactory bulb (Kevetter and Winans, 1981; Halpern and Martinez-Marcos, 2003, Kretteck and Price, 1977, 1978). More recently, other projections of the BNSTp and MeA have been described as being part of the accessory olfactory pathway, such as the LS, ventral tegmental area, and entorhinal cortex (Baum and Bakker, 2013; Camats-Perna and Engelmann, 2015). Importantly, many of these regions within the accessory olfactory system have been found to be involved in the regulation of social recognition. Of these brain regions, the olfactory bulb, MeA, BNSTp, LS, MPOA, and hippocampus have all been found to regulate social recognition in rodents (Popik et al., 1992; Van Wimersma Greidanus and Maigret, 1996; Dluzen et al., 1998; Kogan et al., 2000; Ferguson et al., 2001; Larrazolo-Lopez et al., 2008; Veenema et al., 2012; Lukas et al., 2013; Pena et al., 2014; Dumais et al., 2016).

OT-mediated regulation of social recognition

A simplified model outlining the brain regions involved in social recognition has been proposed previously (Bielsky and Young, 2004; Baum and Bakker, 2013; Camats-Perna and Engelmann, 2015). I herein propose a revised version of this model in which to highlight the unique role of the OT system in the regulation of social recognition in each of these brain regions, and where in this network sexual dimorphisms within the OT system are found (Fig 5.1). Within this model, the olfactory bulb, MeA, BNSTp, LS, MPOA, and hippocampus are regions that regulate social recognition via an OT-mediated mechanism. The olfactory bulb is at the start of this circuitry, and is where OT has been found to modulate social recognition in both male and female rats. Specifically, in males, social recognition was facilitated after OT injection into the olfactory bulb (Dluzen et al., 1998). In females, vaginocervical stimulated-OT release in the olfactory bulb enhanced social recognition, while OTR blockade in the olfactory bulb blocked the enhancement of social recognition in vaginocervical stimulated females (Larrazolo-Lopez et al., 2008). Regions downstream from the olfactory bulb have also been implicated in OT system-mediated social recognition, though most of these studies only used males as subjects. In detail, in males, social recognition was impaired after OTR blockade in the MeA in rats and mice (Lukas et al., 2013; Ferguson et al., 2001), in the LS in rats (Lukas et al., 2013), and in the hippocampus in rats (Van Wimersma-Greidanus and Maigret, 1996), and facilitated after OT injection in the MPOA in rats (Popik and van Ree, 1991). The only region other than the olfactory bulb where OT system-mediated social recognition was also investigated in females was the MeA, in which it was found that OTR gene expression in the MeA is required for social recognition in female mice (Choleris et al., 2007). Taken together, the OT system has been found to modulate social recognition in the olfactory bulb in both male and female rats, and in the MeA in male rats and female mice.

However, further investigation is required to understand more fully the role of OT in the MeA, LS, MPOA, and hippocampus for social recognition in female rats.

Importantly, Study 2 (Dumais et al., 2016) now adds the BNSTp as a region important for OT system-mediated regulation of social recognition in both male and female rats, in which OTR blockade impaired social recognition in both sexes. We also found that the OT system in the BNSTp regulates social recognition in sex-specific ways. Here, OT administration in the BNSTp enhanced social recognition in males but not females. Furthermore, endogenous OT release within the BNSTp was higher in males compared to females during successful social recognition (Study 2; Dumais et al., 2016). We can hypothesize that the higher OTR binding density in males compared to females (Study 1; Dumais et al., 2013) may allow for the sex-specific facilitating effects of exogenous OT on social recognition. Further, higher OTR binding in males may serve to accommodate the stimulus-induced increase in endogenous OT release, in males, but not females, during successful social recognition. Interestingly, the BNSTp is also sexually dimorphic in cell volume and number (Hines et al., 1985; Guillamon et al., 1988; Del Abril et al., 1987; Hines et al., 1992), axonal projections (Gu et al., 2003), and neuropeptide density (De Vries and Miller, 1998; Miller et al., 1989; Kelly et al., 2013; Malsbury and McKay, 1987; Miceevych et al., 1988), all being greater in males compared to females. The sexually dimorphic nature of the BNSTp therefore makes it well suited to play a role in male- typical and female-typical responses to social stimuli. Indeed, the BNSTp has been found to modulate male-specific behaviors such as male copulatory behavior (Emery and Sach, 1976; Claro et al., 1995) and inter-male aggression (Patil and Brid, 2010; Calcagnoli et al., 2014; Masugi-Tokita et al., 2015), and female-specific behavior such as maternal behavior (Numan and Numan, 1996; Consiglio et al., 2005). Though social recognition is important for both males and females, our findings are the first to suggest that the OT system within the BNSTp responds

differently during social recognition in males and females. This suggests that, even though the expression of social recognition is similar, the neural mechanisms to produce this behavior are different in males and females.

Above I discussed the brain regions in which OT has been found to modulate social recognition in rodents (see Fig 5.1). Though a number of brain regions have been found to modulate social recognition in males, only studies that investigated the role of the olfactory bulb, MeA, and BNSTp for OT-mediated social recognition included females as subjects. Even so, only investigation of one brain region (BNSTp; Study 2) for the role of OT for social recognition directly compared males and females. Interestingly, all brain regions within the proposed model network that show OT system-mediated social recognition, show higher OTR binding densities in males compared to females (BNSTp, MeA, LS, MPOA, hippocampus; Study 1; Dumais et al., 2013). One exception is the olfactory bulb, where sex differences in OTR binding have not been measured. This suggests that the BNSTp may not be the only region that shows sex-specific regulation of social recognition by OT, at least in rats. Therefore, it would be interesting to determine whether the MeA, LS, MPOA, and hippocampus may also show sex-specific regulation of social recognition by OT in rats. This would provide potential additional links between sex differences in the OTR system that were found in Study 1 (Dumais et al., 2013) with sex-specific regulation of social behavior, which is required to gain a better understanding of the role of OTR in both sexes.

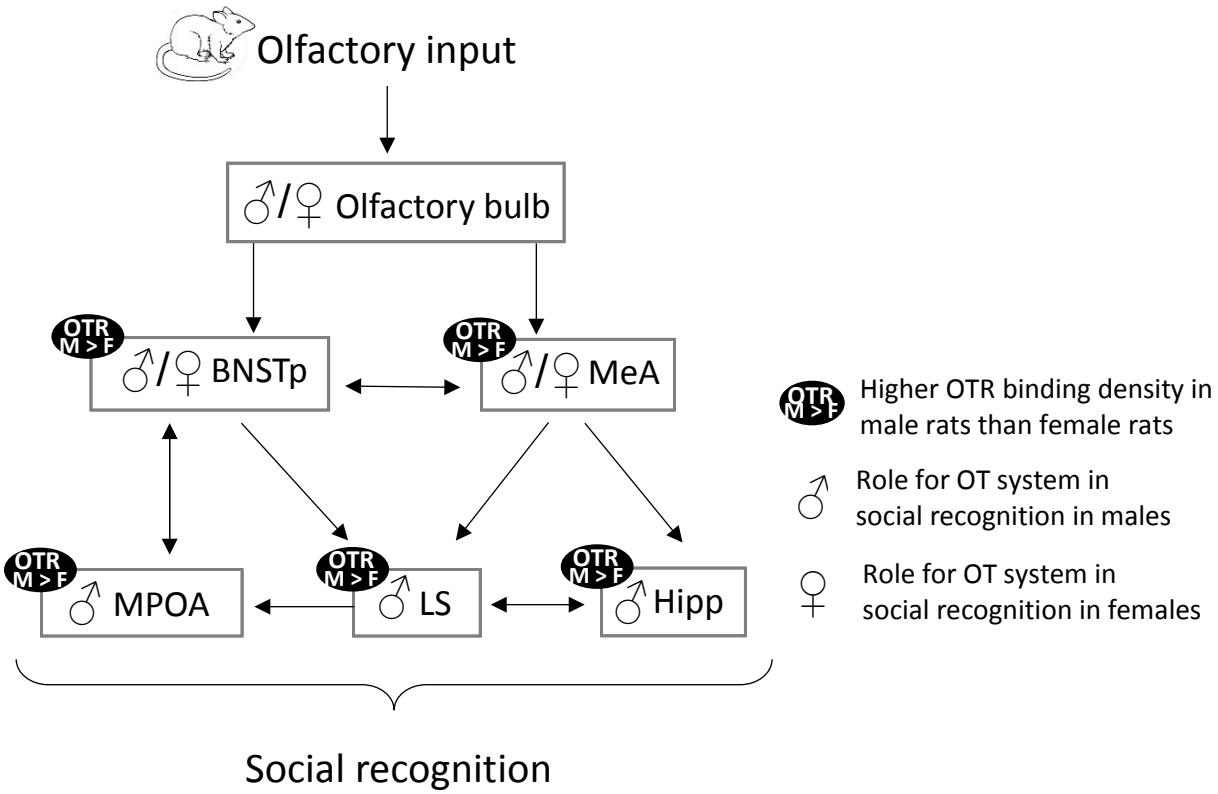


Fig. 5.1 Simplified model circuit showing brain regions that regulate social recognition via an OT system-mediated mechanism in rodents. See text in General Discussion for references. A male or female symbol indicates a brain region in which OT modulates social recognition in that sex. Note that most brain regions in which OT modulates social recognition show higher OTR binding density in male compared to female rats (Dumais et al., 2013), with the olfactory bulb containing OTR but a comparison between males and females is lacking. BNSTp, posterior bed nucleus of the stria terminalis; LS, lateral septum; MeA, medial amygdala; MPOA, medial preoptic area; Hipp, hippocampus.

Sex-specific role of the OT system in the CeA in social interest: New insight into the neural network regulating social interest

Social interest reflects the motivation to approach a conspecific for the assessment of social cues which will, in turn, facilitate appropriate behavioral responses. In our paradigm, we assess social interest by measuring the amount of time an adult rat spends investigating a juvenile rat. Knowledge of the neural mechanisms that regulate social interest as measured using juvenile conspecifics is valuable for assessing neutral social approach behaviors, unbiased with sexual and aggressive motivation. This paradigm therefore provides a platform for assessing the motivational drive for social interaction that does not depend on evolutionary gain. Interestingly, sex differences in the expression of social interest in rats has been widely documented (Johnson & File, 1991; Thor, 1980; Thor et al., 1988; Dumais et al., 2013; Dumais et al., 2016), yet there has been limited investigation into the neural mechanisms underlying this sex difference.

Indeed, we are the first to investigate the role of the OT system in regulating social interest in both male and female rats using juvenile stimuli. Interestingly, we found that the CeA regulates social interest in sex-specific ways, with the OTR in the CeA playing a causal role in the regulation of social interest in males but not females (Study 3; Dumais et al., 2016, under review). Moreover, females expressing low social interest showed a decrease in endogenous OT release in the CeA during the social interest test compared to females expressing high social interest. However, because OTR manipulations did not causally affect social interest in females, this suggests that the change in OT release in the CeA may be a consequence, and not a cause, of low social interest. The amygdala in general is well known for guiding attention toward socially relevant stimuli (Adolphs, 2009, 2010), though investigation of the role for the CeA specifically in social behavior in rats has been limited to its role in maternal and inter-male aggression (Bosch et al., 2005; Consiglio et al., 2005;

Calcagnoli et al., 2015). We are the first to report a role of the CeA for social interest toward juvenile conspecifics, suggesting that the OT system in the CeA may be involved in the general motivation to seek social contact.

Interestingly, in studies where the OT system was found to modulate social recognition by acting on the MeA (Lukas et al., 2013), BNSTp (Study 2, Dumais et al., 2016), and LS (Lukas et al., 2013), it did not affect social interest (i.e., total social investigation time; Study 2; Dumais et al., 2016; Lukas et al., 2013). Study 3 (Dumais et al., 2016, under review) also confirmed the lack of involvement of the OT system in the MeA for social interest. This may suggest a somewhat unique role of the OT system in the CeA in modulating social interest towards juvenile stimuli. However, the MeA and the BNSTp have been implicated in the regulation of social interest toward *adult* conspecifics, which may represent more salient social stimuli compared to juvenile conspecifics. For example, OTR antagonist administered into the MeA of adult male rats was found to reduce the investigation of soiled bedding from unfamiliar adult male conspecifics (Arakawa et al., 2010). Also, extracellular recordings revealed that the MeA responds to adult conspecifics in both male and female mice, but stronger to opposite-sex stimuli compared to same-sex-stimuli (Bergan et al., 2014). Furthermore, c-fos expression in the BNSTp increased in response to opposite-sex adult odors in rats (Hosokawa and Chiba, 2007), and excitotoxic lesions of the BNSTp decreased investigation toward opposite-sex adults in hamsters (Been and Petrulis, 2010). Taken into account the difference in social stimuli (i.e., juvenile versus adult conspecific) we can hypothesize that the MeA and BNSTp may facilitate social interest depending on the salience of the social stimulus.

In conclusion, knowledge about the neural circuitry underlying social interest is limited. This may be complicated by studies using either juvenile or adult conspecifics as social stimuli. A juvenile represents a more neutral stimulus, permitting an unadulterated

measure of social interest, while an adult represents a more salient stimulus, but may activate brain regions involved in sexual or aggressive processes. A comparison between the neural networks underlying social interest toward juvenile conspecifics versus adult conspecifics may help in unravelling the neural network underlying social motivation toward social stimuli that vary in salience. This may have translational value to humans because social interest toward a range of social stimuli is lower in disorders like autism spectrum disorders and schizophrenia (Mahoney et al., 2014; Sarkar et al., 2015).

Sex-specific neural activation following different routes of OT administration: possible implications for the use of OT in humans

Because OT is a key regulator of social behavior in humans (Guastella and MacLeod, 2012; Kanat et al., 2014; Carter, 2014), a role of the OT system in the etiology of neuropsychiatric disorders characterized by social dysfunction has been an important area of research. For example, compared to healthy controls, plasma OT levels were found to be lower in patients with autism (Modahl et al., 1998; Green et al., 2001), schizophrenia (Jobst et al., 2014), and major depression (Frasch et al., 1995). Links have also been made between variants in the OTR gene and autism and Asperger syndrome (Wu et al., 2005; Jacob et al., 2007; Lerer et al., 2008; Yrigollen et al., 2008; Campbell et al., 2011; Di Napoli et al., 2014), schizophrenia (Souza et al., 2010; Teltsh et al., 2012; Montag et al., 2013), and major depression (Costa et al., 2009; Myers et al., 2014). Since the discovery of the non-invasive intranasal delivery of OT, the potential role of OT as a therapeutic agent to restore social dysfunction in neuropsychiatric disorders has gained increased attention. For example, intranasal OT has been found to ameliorate some of the symptoms and/or abnormal neural activation patterns in patients with autism or Asperger's syndrome (Hollander et al., 2003; Andari et al., 2010; Guastella et al., 2010; Anagnostou et al., 2012; Kosaka et al., 2012;

Domes et al., 2013), schizophrenia (Feifel et al., 2010, 2012; Modabbernia et al., 2013; Gibson et al., 2014; Woolley et al., 2014; Shin et al., 2015), generalized anxiety disorder (Guastella et al., 2009; Labuschagne et al., 2010), and borderline personality disorder (Bertsch et al., 2013). In PubMed, there are currently 393 publication results of a search for "oxytocin and autism" alone. Therefore, the studies above are certainly not all-inclusive, and are for the purpose of highlighting the involvement and the potential therapeutic role of OT in a multitude of psychiatric disorders. Unfortunately, most studies linking the OT system to psychiatric disorder etiology or to effective treatment outcomes only include one sex, or do not analyze results according to sex. This limits our understanding of whether the OT system may be a liable treatment option for both sexes, and highlights the need for more comparative studies to assess the therapeutic potential of OT in both men and women.

An important step in this endeavor is to understand more fully the role of OT on social behavior and neural activation in healthy men and women. However, only a few studies have compared the effects of exogenous OT on social behavior and neural activation between men and women. Furthermore, of these studies, only a few behaviors and a limited amount of brain regions were investigated (for review, see Dumais and Veenema, 2015, 2016). For example, intranasal OT impaired recognition memory of neutral and happy faces in men, but not women (Herzmann et al., 2013), and lowered rates of cooperation in a computer task following deception in women, but not men (Rilling et al., 2012, Rilling et al., 2013). Moreover, intranasal OT increased activation in the amygdala, nucleus accumbens, and caudate nucleus in men, but decreased or had no effect in these regions in women during cooperative social interaction (Rilling et al., 2013). Also, it was found that intranasal OT decreased amygdala activation in men, but increased amygdala activation in women, in response to fearful faces (Domes et al., 2007; Domes et al., 2010). Though research is limited, these studies highlight the ability of OT to modulate activation in sex-specific ways

in brain regions that are known to be important for the regulation of emotion and cognition. This may have important implications for the role of OT in modulating these processes in patients that show impaired social behaviors. Therefore, a thorough comparative analysis of the effects of exogenous OT on whole-brain neural activation between the sexes is necessary.

To address this need, we investigated neural activation in response to OT administration in rats using fMRI. Given the evolutionarily conserved nature of OT, findings of the role of OT in rodents can be informative for the role of OT in humans. Further, rodent fMRI provides great spatial and temporal resolution to observe patterns of whole brain neuronal activity, and provides a certain design flexibility that is not always present with human research. To this end, we were able to investigate sex-specific neural activation in response to OT administered directly into the brain as well as OT administered peripherally (the most common route of administration in humans; Study 4). We found that central OT administration induced sex differences in BOLD activation in numerous brain regions (26 brain regions), many of which are implicated in emotion regulation and social cognition (i.e., amygdala, ventral striatum, hippocampus). Peripheral OT administration also induced sex differences in BOLD activation, but in fewer (12 brain regions) and in different brain regions compared to central OT. These results demonstrate robust sex-specific brain activation following OT, but also show that the pattern and the magnitude of sex differences in neural activation induced by OT strongly depend on the route of administration.

Interestingly, our data in rats suggest that sex-specific neural activation patterns in humans may depend on whether intranasal OT delivery directly enters the brain or only enters the periphery. Indeed, because OT cannot readily pass the blood brain barrier, it is still unknown whether intranasal OT exerts its effects on the brain via central or peripheral mechanisms. Some studies suggest that intranasal OT directly enters the brain based on increased OT levels in the brain and in cerebrospinal fluid following intranasal OT. For

example, using vasopressin, a neuropeptide with very similar structure as OT, one study showed an increase in vasopressin in cerebrospinal fluid within 10 min after intranasal vasopressin administration in humans (Born et al., 2002). In addition, intranasal OT increased OT levels in cerebrospinal fluid in rhesus macaques at 15-30 min post administration (Freeman et al., 2016) and in brain microdialysates in rats and mice 30-60 min post administration (Neumann et al., 2013). On the other hand, some studies suggest that intranasal OT also may enter the periphery, with intranasal OT increasing OT levels in plasma 30 min post administration (Gossen et al., 2012) and in saliva as soon as 15 min post administration (Weisman et al., 2012; van Ijzendoorn et al., 2012; Daughters et al, 2015) in humans. However, with the possibility that peripheral OT administration may induce endogenous OT release from the hypothalamus (as suggested in mouse models; Zhang and Cai, 2011), or the possibility for OT in the brain to induce OT release into the bloodstream (Ludwig and Leng, 2006), these studies do not provide conclusive evidence of the target sites of intranasally administered OT. This question may be better answered with the use of radioactively labeled OT to determine the exact destinations of intranasally applied OT. In summary, with the abundance of evidence showing changes in behavior and neural activity following intranasal OT in humans, it is undeniable that intranasal OT is able to exert both behavioral and cognitive effects mediated by the brain. However, whether these effects are modulated by exogenous OT acting directly on the brain or indirectly via peripheral feedback to the brain is still debated (Evans et al., 2014; Quintana et al., 2015; Leng and Ludwig, 2016).

In addition to considering factors such as sex and route of administration when investigating the potential therapeutic role of OT in humans, long-term effects and adverse side effects of chronic OT administration also have to be considered in order to assess the safety of clinical use of OT. Indeed, research in rodents and humans has uncovered potential

precautions that should be considered when using chronic OT as a therapeutic agent. For example, chronic intranasal OT caused long-term impairments in partner preference formation in male prairie voles (Bales et al., 2013), and decreased social interactions toward females in male mice (Huang et al., 2014). Furthermore, intranasal OT was found to hinder trust and cooperation in patients with borderline personality disorder (Bartz et al., 2011a; Ebert et al., 2013). Therefore, taking into account sex (male versus female), mechanism of action (peripheral versus central), length of administration (acute versus chronic), and individual differences (patients with psychiatric disorders versus healthy populations), it is clear that we need to understand more fully the role of intranasal OT on human cognition and behavior before use in clinical populations (for further review, see Bartz et al., 2011b; Guastella et al., 2012; Weisman and Feldman, 2013).

Future directions and conclusions

My research has contributed valuable new information regarding sex differences in both the structure (sex differences in OTR binding densities) and function (sex-specific action of OT on behavior and neural activation) of the OT system. However, I believe that more research is required to gain a better understanding of how the OT system regulates the brain and behavior differently in males and females. First of all, we have provided evidence for the sex-specific role of OT in the regulation of social recognition and social interest in the BNSTp and CeA, respectively. It would also be important to investigate whether OTR in these brain regions may regulate other social behaviors in sex-specific ways, and whether sex differences in OTR binding densities in other brain regions known to regulate social behavior (i.e., VMH, LS, MPOA) may also be implicated in sex-specific regulation of social behavior. This research would be informative for gaining a more comprehensive understanding of the functional significance of sex differences in the OTR system. Furthermore, we showed that

exogenous OT induces robust sex differences in neural activation, but it would also be informative to investigate how the endogenous OT system modulates brain activation, which could be accomplished by using techniques such as chemogenetics. Further, because intranasal OT is the most common route of administration in humans, it would be informative to determine neural activation in rats following intranasal OT administration. If intranasal OT is able to directly access the brain, we would predict that intranasal OT would activate many of the same brain areas and induce many of the same sex differences as ICV OT. On the other hand, if intranasal OT is not entering the brain, we would predict that intranasal OT would induce activation patterns similar to those induced by IP OT. This future aim may provide further insight as to whether intranasal OT in humans may be activating the brain via direct or indirect mechanisms. Together, these future directions would provide a deeper understanding of the role of OT in sex-specific regulation of social behavior and neural activation, which may be informative when considering the use of OT in both men and women to treat social dysfunction.

The poorly understood but robust sex differences in prevalence, symptom severity, and treatment responses of many psychiatric disorders characterized by social dysfunction signifies the importance of understanding the neurobiological mechanisms underlying sex differences in social behaviors, which may underlie vulnerability or resistance to the development of social dysfunction. The lack of research investigating the neurobiological mechanisms underlying social behavior in both sexes (Zucker and Beery, 2010) has impeded scientific progress toward a better understanding of these sex biases. In the present work, we have examined sex differences in the OT system, and how these sex differences may regulate social behavior and brain function differently in males and females. Our data showing sexually dimorphic OTR binding densities (Study 1) and sex-specific effects of OT on behavior (Study 2 and 3) and brain function (Study 4), has significantly advanced our

understanding of the sexually dimorphic function of the OT system, and indicates that only with inclusion of both males and females in research studies can we fully understand the role of OT in social behavior and in brain function.

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