Published in final edited form as:
Psychoneuroendocrinology. 2014 July ; 45: 31–42. doi:10.1016/j.psyneuen.2014.03.009.

Oxytocin in the prelimbic medial prefrontal cortex reduces anxiety-like behavior in female and male rats

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Abstract

The neuropeptide oxytocin (OT) has anxiolytic effects in rodents and humans. However, the specific brain regions where OT acts to regulate anxiety requires further investigation. The medial prefrontal cortex (mPFC) has been shown to play a role in the modulation of anxiety-related behavior. In addition, the mPFC contains OT-sensitive neurons, expresses OT receptors, and receives long range axonal projections from OT-producing neurons in the hypothalamus, suggesting that the mPFC may be a target where OT acts to diminish anxiety. To investigate this possibility, females rats were administered OT bilaterally into the prelimbic (PL) region of the mPFC and anxiety-like behavior assessed. In addition, to determine if the effects of OT on anxiety-like behavior are sex dependent and to evaluate the specificity of OT, male and female anxiety-like behavior was tested following delivery of either OT or the closely related neuropeptide arginine vasopressin (AVP) into the PL mPFC. Finally, the importance of endogenous OT in the regulation of anxiety-like behavior was examined in male and female rats that received PL infusions of an OT receptor antagonist (OTR-A). Overall, even though males and females showed some differences in their baseline levels of anxiety-like behavior, OT in the PL region of the mPFC decreased anxiety regardless of sex. In contrast, neither AVP nor an OTR-A affected anxiety-like behavior in males or females. Together, these findings suggest that although endogenous OT in the PL region of the mPFC does not influence anxiety, the PL mPFC is a site where exogenous OT may act to attenuate anxiety-related behavior independent of sex.

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Conflict of interest

The authors have no conflict of interest to declare.

Contributors

Sara Sabihi: study design, performing experiments, data analysis, manuscript preparation.
Nicole E. Durosko: performing experiments, data analysis.
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Keywords

medial prefrontal cortex; anxiety; prelimbic; oxytocin; vasopressin; sex difference

Introduction

Oxytocin (OT) is a nonapeptide synthesized within the hypothalamic paraventricular (PVN) and supraoptic nuclei. OT neurons of the hypothalamus project to the posterior pituitary and secrete OT into the bloodstream, where its peripheral actions are critical to the processes of lactation and parturition (Gimpl and Fahrenholz, 2001). Besides peripheral release, OT also reaches many regions of the forebrain either through diffusion following dendritic release (Ludwig and Leng, 2006) or via axonal projections from OT synthesizing neurons of the PVN (Sofroniew, 1983; Knobloch et al., 2012). Within the brain, OT acts as a neurotransmitter/neuromodulator and is known to play a role in numerous social functions of female rodents including maternal care (Bosch and Neumann, 2012), sexual receptivity (Bale et al., 2001), pair bonding (Lim and Young, 2006), as well as social recognition and social memory (Engelmann et al., 1998). Although sexual dimorphisms in the brain OT system exist (De Vries, 2004; Smeltzer et al., 2006; Carter, 2007; Dumais et al., 2013), the prosocial effects of OT are not limited to females and also occur in male rodents where brain OT is similarly important for the regulation of sexual behavior (Argiolas and Melis, 2004), social preference (Lukas et al., 2011), and social cognition (Popik and van Ree, 1991). Like rodents, OT has been shown to have a facilitatory influence on various aspects of human social behavior (Heinrichs et al., 2009; McCall and Singer, 2012).

In addition to its effects on sociability, OT is an important regulator of anxiety (Neumann and Landgraf, 2012). For example, OT knockouts present with an anxious phenotype indicating an involvement of endogenous OT (Mantella et al., 2003). Endogenous oxytocin is also directly involved in anxiolysis during the postpartum period (Bosch and Neumann, 2012) as well as in males after mating (Waldherr and Neumann, 2007). Moreover, in rats and mice, OT administered peripherally or centrally attenuates anxiety (Uvnas-Moberg et al., 1994; McCarthy et al., 1996; Windle et al., 1997; Bale et al., 2001; Ring et al., 2006; Blume et al., 2008; Yoshida et al., 2009; Ayers et al., 2011; Mak et al., 2012). The anxiolytic effects of OT are also evident in humans where intranasal administration of OT has been shown to suppress anxiety responses in healthy and clinical populations (Heinrichs et al., 2003; Guastella et al., 2010; de Oliveira et al., 2012). In general, the ability of exogenous OT to reduce anxiety appears to occur regardless of sex (Neumann, 2008) although some sex-specific effects have been reported in rodents (Slattery and Neumann, 2010) and humans (Weisman et al., 2013).

The brain regions where OT acts to modulate anxiety remain to be fully elucidated. Previous work has implicated the PVN of males (Waldherr and Neumann, 2007; Blume et al., 2008) and amygdala of females (Bale et al., 2001; Neumann, 2002) as sites mediating the anxiolytic actions of OT. However, these areas are likely to be part of a widespread network that may also include the medial prefrontal cortex (mPFC). Lesion, inactivation, and molecular approaches have shown that the prelimbic (PL) subregion of the mPFC plays a

role in regulating anxiety-like behavior as assessed in a variety of rodent behavioral paradigms including the elevated plus maze (EPM), open field (OF), and social interaction (SI) test (Maaswinkel et al., 1996; Gonzalez et al., 2000; Lacroix et al., 2000; Sullivan and Gratton, 2002; Shah and Treit, 2003; Shah et al., 2004; Resstel et al., 2008; Stack et al., 2010; Stern et al., 2010). The mPFC also contains OT-sensitive neurons (Ninan, 2011), abundantly expresses OT receptors (Insel and Shapiro, 1992; Gould and Zingg, 2003; Liu et al., 2005; Smeltzer et al., 2006), and may receive long range axonal projections from OT producing neurons in the hypothalamus (Sofroniew, 1983; Knobloch et al., 2012). Taken together, these findings suggest that the mPFC may be a target where OT acts to diminish anxiety. To investigate this possibility, OT was administered bilaterally into the PL region of the mPFC of female rats and its effects on anxiety-like behavior assessed. In addition, to determine if the effects of OT on anxiety-like behavior are sex dependent and to evaluate the specificity of OT, male and female anxiety-like behavior was tested following delivery of either OT or the closely related neuropeptide arginine vasopressin (AVP) into the PL mPFC. Finally, the importance of endogenous OT in the regulation of anxiety-like behavior was evaluated in male and female rats in which the OT receptor was blocked with an OT receptor antagonist (OTR-A).

Methods

Animals

Age-matched adult (9–12 weeks of age) virgin female (225–250g) and male (300–350g) Sprague-Dawley rats from Taconic (Germantown, NY) were used. Rats were housed individually in a temperature and humidity controlled room and maintained on a 12h/12h light/dark cycle (lights on at 0600 hr) with access to food and water ad libitum. All procedures were conducted in accordance with The Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by The Ohio State University Institutional Animal Care and Use Committee.

Throughout the experiment, stages of estrous were monitored in all females through daily vaginal swabs. Samples of cells were obtained with a sterile cotton swab saturated in 0.9% saline and applied to a glass slide. After drying, slides were stained with 1% aqueous Toluidine Blue and cell types characterized under 10X magnification (Everett, 1989). Only those females that had normal 4–5 d estrous cycles were used.

Surgical procedures

After approximately 7 d of acclimation to the colony, rats were anesthetized with a 2–4% isoflurane gas/air mixture and aligned on a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). Body temperature was maintained throughout the surgery with a warming pad. Bilateral cannula guides (pedestal mounted 22-gauge stainless steel tubes with 1.5 mm separation and cut 3.5 mm below the pedestal; Plastics One, Roanoke, VA) were secured in a stereotaxic holder and lowered into the prelimbic region (PL) of the mPFC (AP: + 3.2 mm, ML: ± 0.75 mm, DV: − 3.2mm) (Paxinos and Watson, 1998). The cannulae were secured by stainless steel screws and dental cement. A bilateral stainless steel obturator (0.35 mm diameter; Plastics One) extending 0.2 mm beyond the tip of the guide cannula was placed

into the guide cannula after surgeries. The scalp was closed around the protruding portion of the cannula with sutures. Rats were allowed to recover for at least 7 d before behavioral testing.

Central Infusions

On days 3 and 5 post-surgery, rats were habituated to the handling and infusion procedures. During habituation, rats were removed from their home cage and handled for 3 min while being lightly restrained in a terrycloth towel. The obturators were then removed and a 28-gauge bilateral injection cannula extending 0.2 mm beyond the tip of the guide cannula into the PL cortex was inserted into the guide. The injection cannulas were left in place for 3 min then removed and the obturator replaced. On the day of testing, rats underwent the same procedure as described above except that an injection cannula attached to a 1 μl Hamilton Syringe via PE-10 tubing was inserted into the guide cannula. Infusions were made using a Harvard Apparatus Pico Plus Elite infusion pump (Holliston, MA) which delivered a 1.0 μl volume over 3 min. The injector was left in place for an additional 1 min before withdrawal.

Anxiety-like Behavior

Anxiety-like behavior was evaluated using three well validated models - the EPM, the OF and SI test (Prut and Belzung, 2003; Lapiz-Bluhm et al., 2008; Rotzinger et al., 2010). The EPM consisted of a cross-shaped platform (height: 50 cm) with four arms (width: 10 cm, length: 50 cm), two of which were enclosed by walls 50 cm in height. Rats were placed in the center of the platform (10 × 10 cm), facing a junction between an open and closed arm and allowed to explore for 5 min. The number of entries into the open arms and the percentage of time spent in the open arms (time in open arms/time in open and closed arms×100) were used as measures of anxiety-like behavior (Pellow et al., 1985; Cruz et al., 1994; Lapiz-Bluhm et al., 2008). An increase in the percentage of time spent in the open arms and a greater number of open arm entries are indicative of reduced anxiety. Closed arm entries were used as a measure of locomotion independent of anxiety (Pellow et al., 1985; Cruz et al., 1994; Lapiz-Bluhm et al., 2008). The EPM measures of anxiety and locomotion that we analyzed are consistent with numerous other studies investigating anxiety like-behavior including those manipulating OT (Mantella et al., 2003; Waldherr and Neumann, 2007; Mak et al., 2012).

For the OF test, a 60 × 60 cm Plexiglas arena with walls 40 cm high was used. The floor of the arena was covered with gridlines which allowed for measurement of locomotion. The gridlines were spaced 10 cm apart yielding a total of 36 10 × 10 cm squares. The inner area was considered the central 16 squares which covered a 40 × 40 cm area. Rats were placed in the center of the open field and during a 5 min test, the percentage of time spent in the center of the arena (time spent in center/total time × 100) as well as the percentage of gridlines crossed in the center of the arena (number of center gridlines crossed/total number of gridlines crossed × 100) were used as measures of anxiety-like behavior. An increase in the percentage of time or percentage of center gridlines crossed correlate with lower anxiety. Locomotor activity was assessed using the total number of gridlines crossed.

In the SI test, an age, weight (+/− 10 g), and gender matched 'stimulus' rat which was unfamiliar to the test subject was placed in the corner of the OF arena described above, opposite from the corner in which the test rat was placed. Stimulus rats were used a maximum of two times and were never used twice in the same day. The assignment of a stimulus rat to a test rat was random and not restricted to a particular drug or dose of drug. During a 5 min test, the time spent in active social behavior (i.e. communal grooming, sniffing, approaching, following, climbing on or under the stimulus rat) initiated by the test rat was scored. The time the experimental rat spent interacting with the stimulus rat was used as a measure of anxiety-like behavior. Increased anxiety is reflected by a decrease in social interaction time (File, 1980).

Beginning on day 7 post-surgery, females underwent behavioral testing upon entering their first diestrus in order to control for fluctuations in anxiety due to hormonal changes across the estrous cycle. Although social anxiety is not affected by the estrous cycle (Stack et al., 2010), other measures of anxiety-like behavior as assessed in the EPM and OF are such that during the stage of proestrus females exhibit a reduction in anxiety while during estrus and diestrus anxiety is relatively stable (Mora et al., 1996; Marcondes et al., 2001; Walf and Frye, 2007). Studies examining factors which regulate anxiety-like behavior in females also commonly test during diestrus (De Almeida et al., 1998; Marcondes et al., 2001; Hiroi and Neumaier, 2006; Figueira et al., 2008). Males were tested 1 week post-surgery.

All behavioral tests, except those performed in red light (~12 lux), were performed under lighting conditions of ~550 lux within the same time range each day (approximately 0900–1300h), which is sufficiently separated from light-dark transitions (lights on at 0600h, lights off at 1800h) to avoid any potential diurnal variations in exploratory behavior (Lapiz-Bluhm et al., 2008). Tests were digitally recorded and later scored blind by a trained observer using BEST Collection and BEST Analysis software (Education Consulting Inc., Hobe Sound, FL).

Experimental Design

To investigate whether OT in the PL region of the mPFC reduces anxiety, three separate experiments were performed. In the first experiment, female rats in diestrus received infusions of synthetic OT (cat# O6379; Sigma, St. Louis, MO) dissolved in saline at a dose of 0.1μg/1μl (n = 8) or 1.0μg/1μl (n = 9) (Bale et al., 2001; Ring et al., 2006; Blume et al., 2008; Ayers et al., 2011). Control rats received a 1 μl infusion of saline (n = 9). Anxiety-like behavior was evaluated using the EPM and SI test.

In the second experiment, we evaluated the specificity of OT as well as whether the effects of OT on anxiety-like behavior are sex dependent. Separate groups of female and male rats received infusions of 1.0μg/1μl OT (n = 18 female; n = 14 male), 1.0μg/1μl of the closely related neuropeptide, arginine vasopressin (AVP; cat# V9879; Sigma, St. Louis, MO) (n = 19 female; n = 15 male), or 1μl of saline vehicle (n = 18 female; n = 15 male) in the PL region of the mPFC. Previous studies that have looked at the effects of AVP versus OT on anxiety-like behavior have used a similar dose of each (Lee et al., 2007; Blume et al., 2008). All subjects in the second experiment underwent testing in the EPM; approximately half of the animals in each group were then tested in the OF (males: OT n = 6, AVP n = 7, saline n

= 7; females: OT n = 10, AVP n = 11, saline n = 10) and the remainder were tested in the SI test (males: OT n = 8, AVP n = 8, saline n = 8; females: OT n = 8, AVP n = 8, saline n = 8). Because both the SI and OF tests were conducted in the same arena, animals were split between tasks in order to avoid any confounding effects such as habituation to the arena. In both the first and second experiments, testing for anxiety-related behavior was done 15 min after infusions. The two tests of anxiety-like behavior (EPM and either SI or OF) were done 5 min apart and the order of the two tests was counterbalanced among rats.

In the third experiment, the importance of endogenous OT in the regulation of anxiety-like behavior was evaluated using an OTR antagonist (OTR-A; $d(CH_2)_5^1$,Tyr(Me)^2, Thr^4,Orn^8,des-Gly-NH_2^9)OVT; cat# H-2098; Bachem, Torrance, CA). Separate groups of male and female rats were infused with 0.1μg/1μl OTR-A (n = 6 female; n = 5 male) (Lukas et al., 2011) or 1μl saline vehicle (n = 6 female; n = 6 male) in the PL region of the mPFC and were tested in both the EPM and SI tests. All rats underwent testing 20 min after OTR-A infusions (Ring et al., 2006; Nyuyki et al., 2011). Tests were done 5 min apart and the order of the two tests was counterbalanced among rats.

Because baseline levels of anxiety in the EPM were high in experiment 3, an additional groups of rats were infused with 0.1μg/1μl OTR-A (n = 6 female; n = 6 male) or 1μl saline vehicle (n = 6 female; n = 6 male) in the PL region of the mPFC. These groups were tested 20 min later in the EPM under red light conditions in order to evaluate whether OTR-A would alter anxiety if baseline levels were decreased.

Histology

After the completion of anxiety testing, rats were overdosed with Euthasol and transcardially perfused with 4% paraformaldehyde. Brains were removed, postfixed for 24 hr and then sectioned on a Vibratome. 40-μm thick coronal sections were collected throughout the area of the cannula implant and stained with 0.2% cresyl violet for verification of correct placement (Fig. 1). Examination under high magnification (40X) revealed limited to no damage at the tip of the cannula. Those animals with cannula placements outside of the PL region of the mPFC (four in Cg1, three in the ventricles) were excluded from the study. These missed cannula placements included both males and females from different drug and dosage groups. As such, statistical analyses could not be completed in order to examine behavioral effects of OT outside of the PL region. Additionally, because the PL cannula placements were not homogenous with this region, behavioral analysis of medial/lateral and dorsal/ventral PL cannula placements were performed for each experiment but revealed no differences. Thus, all cannula placements within a drug type or dose were grouped together regardless of location within the PL region.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software version 5.01 (La Jolla, CA). For the first experiment, anxiety-like behavioral data were analyzed using a one-way analysis of variance (ANOVA). Anxiety-like data from the second and third experiments were analyzed using a 2 X 3 ANOVA or 2 X 2 ANOVA with sex (male or female) and infusion type (experiment 2: saline, AVP or OT; experiment 3: saline or OTR-

A) as factors. Percentage of time spent in the open arms of the EPM for rats tested under red light conditions was combined for males and females because of high variability. These data were compared to combined male and female data from experiment 3 and analyzed using a 2 × 2 ANOVA with lighting condition (bright light or red light) and infusion type (saline or OTR-A) as factors. Grubb's test was used to detect statically significant outliers, two of which were removed from the second experiment. Statistical significance for main effects and interactions were indicated by p values less than 0.05 and when significance was found, they were followed by Tukey's HSD post hoc comparison test.

Results

OT infused into the PL region of the mPFC reduces anxiety-like behavior in females

In the EPM, there was a significant effect of dose on the percentage of time spent in the open arms ($F_{2,23} = 6.30$, $p < 0.05$) and the number of open arm entries ($F_{2,23} = 4.97$, $p < 0.05$). Post hoc analysis of dose revealed that females infused with the higher 1.0μg/1μl dose of OT in the PL region of the mPFC spent a greater percentage of time in the open arms ($p < 0.05$; Fig. 2a) and made a greater number of entries into the open arms ($p < 0.05$; Fig. 2b) as compared to the lower 0.1μg/μl dose of OT ($p < 0.05$) and saline controls ($p < 0.05$) which did not differ from one another ($p > 0.05$), indicating a decrease in anxiety-like behavior. Locomotor activity, as measured by the number of closed arm entries, was not altered by OT ($F_{2,23} = 0.05$, $p > 0.05$; Fig. 2c).

In the SI test, there was also a significant effect of dose ($F_{2,23} = 7.74$, $p < 0.05$; Fig. 2d). Post hoc analysis showed that the higher 1.0μg/1μl dose of OT was effective in reducing anxiety as demonstrated by a greater amount of time spent interacting with an unknown stimulus rat compared with both the lower 0.1μg/μl dose of OT ($p < 0.05$) and saline ($p < 0.05$) which did not differ ($p > 0.05$).

OT, but not AVP, infused into the PL region of the mPFC reduces anxiety-like behavior in females and males

For percentage of time spent in the open arms of the EPM (Fig. 3a), there was a significant main effect of infusion type ($F_{2,93} = 18.70$, $p < 0.05$) but no main effect of sex ($F_{1,93} = 1.18$, $p > 0.05$) and no interaction between infusion type and sex ($F_{2,93} = 2.66$, $p > 0.05$). Post hoc analysis of infusion type showed that overall, animals infused with 1.0μg/μl OT into the PL region of the mPFC were less anxious as demonstrated by a greater percentage of time in the open arms than those infused with saline ($p < 0.05$) and those infused with AVP ($p < 0.05$), which did not differ from one another ($p > 0.05$). For number of open arm entries in the EPM (Fig. 3b), there was also a significant main effect of infusion type ($F_{2,93} = 21.22$, $p < 0.05$) with post hoc analysis revealing a greater number of open arm entries in animals receiving OT infusion into the PL region of the mPFC as compared to saline ($p < 0.05$) and AVP ($p < 0.05$), which did not differ ($p > 0.05$), again indicating an anxiolytic effect of OT. There was also a significant main effect of sex ($F_{1,93} = 4.99$, $p < 0.05$) on the number of open arm entries with females entering the open arms more than males. The interaction between infusion type and sex on the number of open arm entries was not significant ($F_{2,93} = 2.90$, $p > 0.05$). The number of entries into the closed arms (Fig. 3c), an indicator of

locomotor activity, showed no significant effects of infusion type ($F_{2,93} = 0.68$, $p > 0.05$) or sex ($F_{2,93} = 1.14$, $p > 0.05$) and no interaction between these factors ($F_{2,93} = 3.67$, $p > 0.05$).

In the SI test (Fig. 3d), there was a main effect of infusion type ($F_{2,42} = 13.05$, $p < 0.05$) but no main effect of sex ($F_{1,42} = 2.41$, $p > 0.05$) and no interaction between infusion type and sex ($F_{1,42} = 0.72$, $p > 0.05$) in time spent interacting with an unknown stimulus rat. Post hoc analysis of infusion type showed that overall, animals infused with OT spent a greater amount of time interacting with an unknown stimulus rat than those infused with AVP ($p < 0.05$) or saline ($p < 0.05$), which did not differ from one another ($p > 0.05$).

In the OF, there was no main effect of infusion type on the percentage of time spent in the center of the OF ($F_{2,45} = 1.50$, $p > 0.05$; Fig. 3e) or in the percentage of center gridlines crossed ($F_{2,45} = 1.03$, $p > 0.05$; Fig. 3f). However, there was a main effect of sex on both measures such that females spent more time in the center of the OF ($F_{2,45} = 7.21$, $p < 0.05$) and crossed more center gridlines ($F_{2,45} =15.96$, $p < 0.05$) than males. No interaction between infusion type and sex was found for either percentage of time spent in the center of the OF ($F_{2,45} = 0.34$, $p > 0.05$) or in the percentage of center gridlines crossed ($F_{2,45} = 2.57$, $p > 0.05$). The total number of gridlines crossed (data not shown), an indicator of locomotor activity, showed no significant effects of infusion type ($F_{2,45} = 1.76$, $p > 0.05$) or sex ($F_{2,45} = 2.86$, $p > 0.05$) and no interaction between these factors ($F_{2,45} = 1.35$, $p > 0.05$).

OTR-A infused into the PL region of the mPFC does not alter anxiety-like behavior in females or males

In the EPM, there were no significant main effects for infusion type ($F_{1,19} = 0.62$, $p > 0.05$) or sex ($F_{1,19} = 0.002$, $p > 0.05$) and no infusion type by sex interaction ($F_{1,19} = 0.09$, $p > 0.05$) in the percentage of time spent in the open arms (Fig. 4a). Similarly, for number of open arm entries (Fig. 4b) there was no significant main effect for infusion type ($F_{1,19} = 0.009$, $p > 0.05$) or sex ($F_{1,19} =0.41$, $p > 0.05$) and no infusion type by sex interaction ($F_{1,19} = 0.02$, $p > 0.05$). Locomotor activity, as measured by the number of closed arm entries in the EPM, was not altered (p's > 0.05; Fig. 4c). Even when baseline anxiety levels in the EPM were reduced by testing in red light conditions, OTR-A in the PL mPFC had no effect (Fig. 5). Indeed, statistical analysis confirmed that for the percentage of time spent in open arms, there was a significant main effect of lighting condition ($F_{1,43} = 7.61$, $p < 0.01$) but no main effect of infusion type ($F_{1,43} = 0.91$, $p > 0.05$) and no interaction between infusion type and lighting condition ($F_{1,43} = 0.25$, $p > 0.05$).

In the SI test (Fig. 4d), there was no significant main effects for either infusion type ($F_{1,19} = 0.02$, $p > 0.05$) or sex ($F_{1,19} = 1.03$, $p > 0.05$) and no infusion type by sex interaction ($F_{1,19} = 0.11$, $p > 0.05$) in the amount of time spent interacting with an unknown stimulus rat.

Discussion

Here we show that although males and females exhibit some differences in their baseline levels of anxiety, OT infused into the PL region of the mPFC reduces anxiety-like behavior regardless of sex. In contrast, neither administration of AVP nor an OTR-A into the PL mPFC affected anxiety-like behavior in males or females. Together, these findings suggest

that although endogenous OT in the PL region of the mPFC does not influence anxiety-like behavior, the PL mPFC is a component of the neural circuitry where exogenous OT may act to attenuate anxiety.

Investigation of specific brain sites underlying the anxiolytic action of OT have been limited to a few studies which have demonstrated an involvement of the PVN in males (Waldherr and Neumann, 2007; Blume et al., 2008), the amygdala of females (Bale et al., 2001; Neumann, 2002) and the periaqueductal gray in postpartum females (Figueira et al., 2008). We focused on the mPFC because of its role in anxiety regulation (Maaswinkel et al., 1996; Gonzalez et al., 2000; Lacroix et al., 2000; Sullivan and Gratton, 2002; Shah and Treit, 2003; Shah et al., 2004; Resstel et al., 2008; Stack et al., 2010; Stern et al., 2010). In addition, the mPFC contains OT-sensitive neurons (Ninan, 2011), expresses OT receptors (Insel and Shapiro, 1992; Gould and Zingg, 2003; Liu et al., 2005; Smeltzer et al., 2006) and receives long range axonal projections from OT producing neurons in the hypothalamus (Sofroniew, 1983; Knobloch et al., 2012). Together, these observations render the mPFC a likely target for the control of anxiety by OT. Indeed, our results show that anxiety-like behavior is attenuated by OT in the PL mPFC and thus extends prior studies by identifying another component of the neural circuitry underlying OT’s effects on anxiety.

In the rodent brain, the mPFC consists of 3 subregions - the infralimbic cortex (IL), PL cortex, and anterior cingulate cortex (Cg1) (Heidbreder and Groenewegen, 2003). In this study, we targeted the PL mPFC which when stimulated increases anxiety-like behavior but when inhibited or lesioned diminishes anxiety-like behavior (Maaswinkel et al., 1996; Sullivan and Gratton, 2002; Shah and Treit, 2003; Shah et al., 2004; Stern et al., 2010; Bi et al., 2010; but see Jinks and McGregor, 1997; Covington et al., 2010; Stack et al., 2010). Thus, PL mPFC typically promotes anxiety and is thought to do so via indirect glutamatergic projections to central nucleus of the amygdala (CeA) and bed nucleus of the stria terminalis (BNST), key structures in the expression of anxiety-like behavior (Maaswinkel et al., 1996; Lacroix et al., 2000; Stern et al., 2010). How might OT in the PL mPFC reduce anxiety? Recent studies have shown that OT suppresses glutamatergic transmission in the mPFC (Qi et al., 2009; Ninan, 2011; Qi et al., 2012) and can also increase the release of GABA (Qi et al., 2012). Consequently, OT would presumably decrease excitation of the glutamatergic projections from the PL mPFC to the CeA/BNST, thus resulting in a decrease in anxiety-like behavior. This hypothesis remains to be tested as does the question of whether OT in the IL and/or Cg1 region of the mPFC would have a similar anxiolytic effect. In this regard, it is important to consider that the various subregions of the mPFC show different patterns of connectivity with subcortical and cortical structures and thus differentially contribute to a variety of physiological and behavioral processes (Vertes, 2004; Radley et al., 2006; Hoover and Vertes, 2007; Hamani et al., 2010; Pereira and Morrell, 2011; Sierra-Mercado et al., 2011). However, regional differences within the mPFC in mediating anxiety-like behavior have not been consistently shown (Cg 1: Bissiere et al., 2008; Kim et al., 2011; IL: Jinks and McGregor, 1997; Sullivan and Gratton, 2002; Bi et al., 2013) which may indicate that OT's effects on anxiety would not be subregion specific. Additionally, a further possibility remains that there exist subtypes of anxiety-like behaviors, differentially supported by the mPFC regions in question (Maroun, 2012).

Numerous studies have demonstrated sex differences in emotionally-linked behaviors of rats. Generally, female rats exhibit less anxiety-like behavior than do their male conspecifics (Johnston and File, 1991; Stack et al., 2010). While we found no sex difference in the social interaction test, our data show that females had a greater number of open arm entries in the EPM (but only in experiment 2), a greater percentage of time in the center of the OF, as well as a greater percentage of inside gridlines crossed in the OF and therefore at least partially support prior findings. However, while males and females showed some differences in their baseline levels of anxiety, OT when administered into the PL mPFC attenuated anxiety regardless of sex. This finding is in line with prior work demonstrating that exogenous OT is anxiolytic in both males and females when delivered peripherally or centrally (Uvnas-Moberg et al., 1994; McCarthy et al., 1996; Windle et al., 1997; Bale et al., 2001; Ring et al., 2006; Blume et al., 2008; Yoshida et al., 2009; Ayers et al., 2011; Mak et al., 2012) but is the first to implicate the PL region of the mPFC as a site of action for OT's effects on anxiety. Thus, although females have higher OTR binding densities in the mPFC (Smeltzer et al., 2006), they do not respond differentially to mPFC OT administration.

It is worth noting that the anxiolytic effects of PL OT reported here were evident in the EPM and SI test, but not the OF. Although both the EPM and OF are models of anxiety-like behavior that feature exploration and utilize anxiogenic stimuli of open spaces, behavior in one test does not always predict behavior in the other (Bale et al., 2001; Bhatnagar et al., 2004) possibly because OF measures have been suggested to relate to a wide range of emotional behavior and physiological correlates of emotion, which may not be specific to anxiety (Royce, 1977). As a result, the OF may be differentially sensitive to PL OT and require different doses than those used here for an anxiolytic effect to be revealed. Moreover, while the SI test is regarded as another model of anxiety, its emphasis is on social behavioral responses (Lapiz-Bluhm et al., 2008; Rotzinger et al., 2010). OT is strongly implicated in the control of social behavior (Choleris et al., 2008; Hoge et al., 2008; Lukas et al., 2011; Neumann and Landgraf, 2012; Benarroch, 2013) and thus may also regulate anxiety in a social context. Indeed, the present results reveal that, independent of sex, OT delivered to the PL region of the mPFC promotes a significant increase in social interaction time with an unknown stimulus rat, thereby reflecting decreased social anxiety. These findings not only confirm the involvement of the PL region of the mPFC in the regulation of social interaction (Gonzalez et al., 2000; Stack et al., 2010) but are also in agreement with other work demonstrating that social approach and preference behavior depends on OT (Lukas et al., 2011). Thus, combined with the data in the EPM, it appears that OT in the PL mPFC exerts an anxiolytic effect in both non-social and social contexts.

In contrast to exogenous OT, endogenous OT in the PL mPFC does not appear to be directly involved in regulating anxiety-like behavior since administration of an OTR-A was without effect, at least at the dosage we tested. However, baseline anxiety levels were high in the EPM and thus could have precluded the ability to detect an effect of the OTR-A. Even under red-light testing conditions which increased the percentage of time spent in the open arms, OTR-A did not alter anxiety-like behavior. Prior studies have similarly shown no effect on anxiety behavior following acute ICV OTR-A administration in male or virgin female rats (Neumann et al., 2000; Neumann, 2002; Slattery and Neumann, 2010). This does not eliminate the possibility that endogenous PL OT could be involved in regulating anxiety

during times of elevated OT system activity. Indeed, ICV administration of an OTR-A during the peripartum period (Neumann at al., 2000) or in males after mating (Waldherr and Neumann, 2007) blocks the anxiolytic properties of high endogenous OT. Future studies blocking OTR in the PL mPFC during times when the OT system is upregulated will be necessary to address this issue.

AVP is also a well-known regulator of anxiety but its effects are often opposite to that of OT and thus act to increase anxiety (Neumann and Landgraf, 2012). However, we found that AVP in the PL mPFC did not significantly impact anxiety-like behavior. Although this suggests that the PL mPFC is not a site of action for the anxiogenic actions of AVP, anxiety levels in controls were already high which may have interfered with the ability to detect an anxiogenic effect of AVP. It is also possible that the inability to uncover a change in anxiety-like behavior following AVP administration may be related to the 1 μ g dosage used which may or may not have the same efficacy as a 1 μ g dose of OT. Nonetheless, while our results suggest that the anxiolytic effects of PL OT are neuropeptide specific, it is important to keep in mind that OT and AVP show structural similarities, and cross-reactivity at the receptor level has been described (Postina et al., 1998). OT does indeed have a moderate to strong affinity for the AVP 1A (V1aR) receptor (Hicks et al., 2012) and can act as a partial agonist at this site (Chini et al., 1996). Recent studies have shown that some of the prosocial effects of OT may be mediated by the V1aR rather than the OTR (Sala et al., 2011; Ramos et al., 2013). Therefore, exogenous OT may potentially be acting at the V1aR to reduce anxiety. Although unlikely given that AVP did not elicit the same behavioral response as OT, it may also be the case that an anxiogenic action of AVP at V1b receptors (Neumann and Landgraf, 2012) counteracts the prosocial action at V1aR. Determining which specific receptors are responsible for the anxiolytic effect of exogenous OT is an important question for future studies.

Although our findings support the conclusion that a 1.0 μ g dose of OT delivered into the PL mPFC reduces anxiety there are at least two caveats. First, studies examining the effects of acute centrally administered OT on anxiety-like behavior have not found a consistently effective dose with some reporting anxiolytic effects with 0.1 μ g OT or less (Blume et al., 2008; Lukas et al., 2011) while here and elsewhere (Bale et al., 2001; Ring et al., 2006; Ayers et al., 2011) the effective dose was 1.0 μ g. However, in our first experiment, the cannula placements for each dose were heterogeneous. Thus, where the lower 0.1 μ g dose seems ineffective, target rather than dosage may be the issue. This observation, however, does not detract from the evidence for an effect at the higher of the two doses. In addition, the placements in the subsequent experiment were more dispersed and the effect of OT on anxiety similar. Second, we did not determine the diffusion of OT from the PL mPFC to adjacent structures. Consequently, leakage into the ventricles (Ring et al., 2006; Ayers et al., 2011; Lukas et al., 2011) or other regions of the mPFC may mediate the observed effects of OT on anxiety. To address this issue, we performed a behavioral analysis of medial/lateral and dorsal/ventral PL cannula placements and found no differences lending support to the conclusion that OT specifically in the PL mPFC is anxiolytic.

In humans, an abundance of studies using intranasal administration confirm that OT reduces anxiety in both healthy and clinical populations (Heinrichs et al., 2003; Guastella et al.,

2010; de Oliveira et al., 2012). These anxiolytic effects of OT are mediated by the amygdala possibly through connectivity with the mPFC (Neumann and Landgraf, 2012; Sripada et al., 2013). However, OT has also been shown to be anxiogenic depending on certain contextual cues and interindividual factors (Bartz et al., 2011; Tabak et al., 2011; Grillon et al., 2012; Striepens et al., 2012; Kanat et al., 2013). A recent study in mice suggests that OT's anxiogenic action may involve the lateral septum (Guzman et al., 2013), which like the amygdala receives input from the mPFC (Sheehan et al., 2004). Together, these emerging data suggest that rather than exerting a unidirectional influence on anxiety, the oxytocin system has a modulatory role possibly through its effects on the mPFC.

Overall, the results of this study implicate the PL region of the mPFC as a site where exogenous OT acts to mediate anxiety-like behavior in male and female rats. These observations provide new insights into the neural circuitry underlying the anxiolytic effects of OT and may be relevant to understanding the potential therapeutic role for OT as an agent for the management of anxiety disorders.

Acknowledgments

We thank Joshua Fedder for his assistance with statistical analyses.

Role of funding source

This work was supported by a grant from the National Institute of Mental Health (R0084148) to BL.

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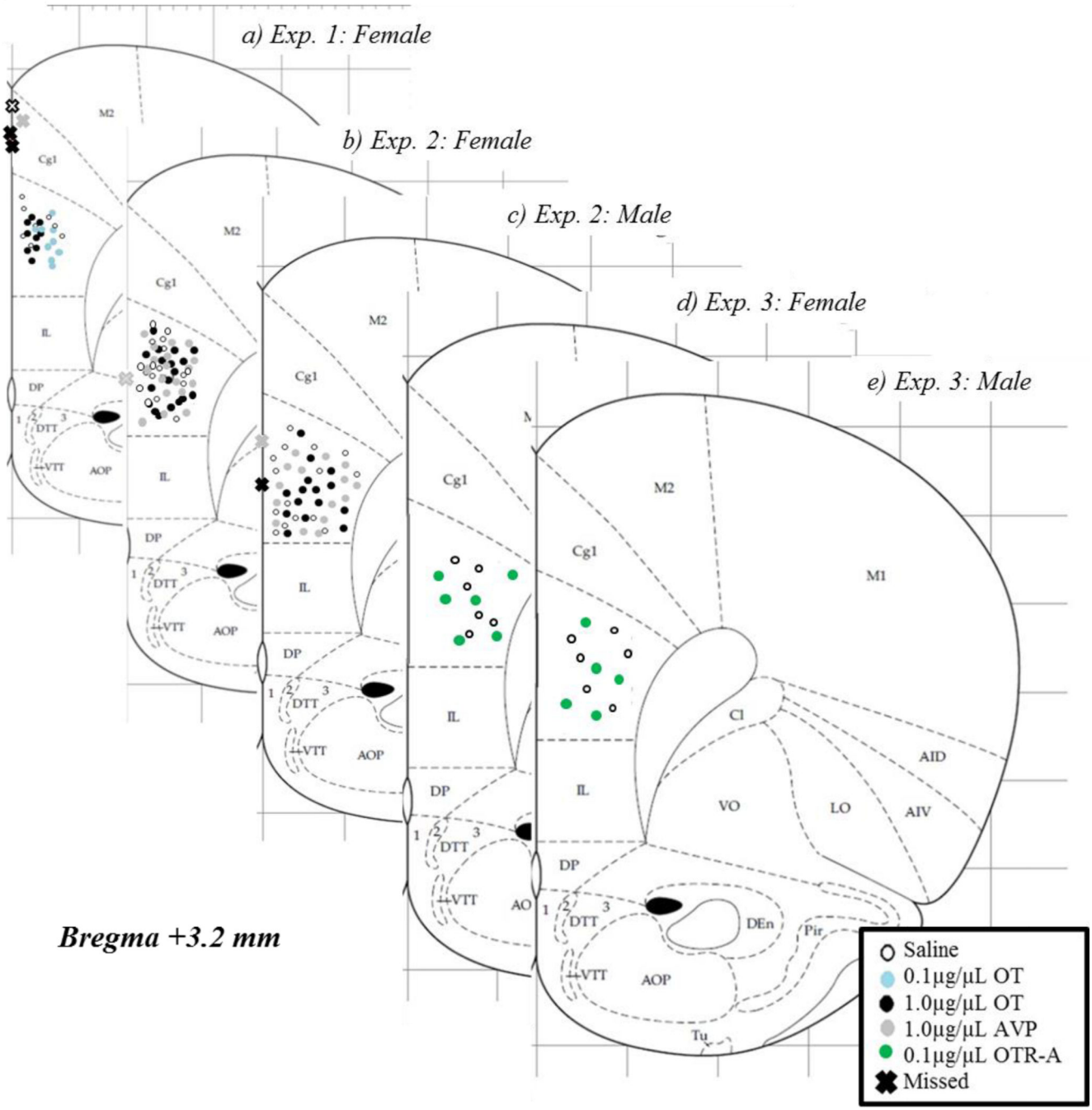

Figure 1. Schematic representation of mPFC infusion sites

Cannula tip placements were in the prelimbic region (PL) of the mPFC (AP: +3.2 mm, ML: ±0.5 mm, DV: −3.2 mm). Each dot indicates an individual subject. Infusions were bilateral but are represented unilaterally. (a) Cannula placements for experiment 1. Females received an infusion of 0.1µg/1µl OT, 1.0µg/1 µl OT, or saline. (b, c) Cannula placements for experiment 2. Females (b) and males (c) received an infusion of 1.0µg/1 µl OT, 1.0µg/1 µl AVP, or saline. (d, e) Cannula placements for experiment 3. Females (d) and males (e) received an infusion of 0.1µg/1µl OTR-A or saline. Animals with missed cannula

placements in Cg1 or the ventricles are marked with an “x” in the color of their respective group and were excluded from analysis. Adapted from Paxinos and Watson, 1998.

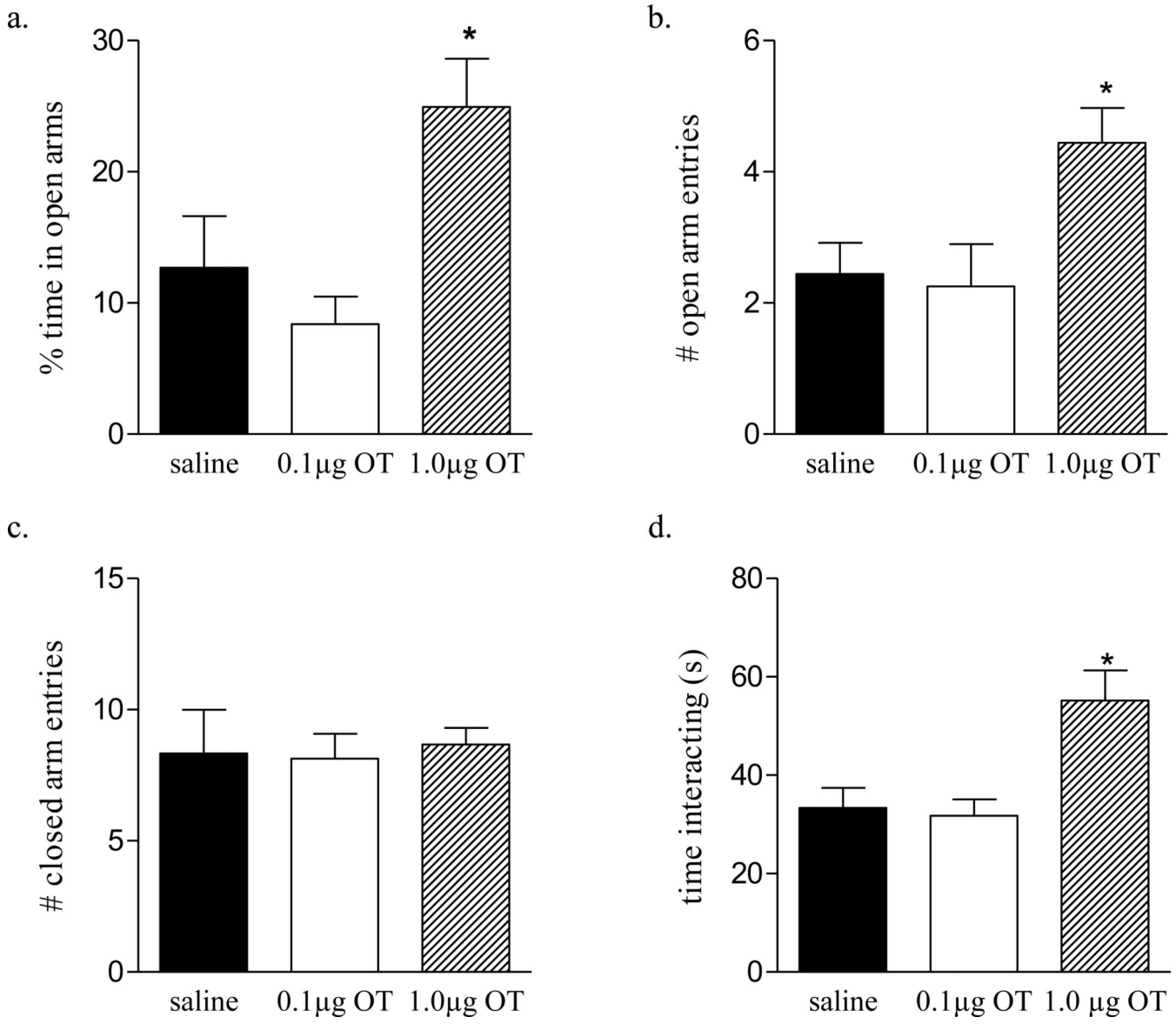

Figure 2. OT in the PL mPFC is anxiolytic in females

Females who received 1.0µg/1µl OT in the PL mPFC spent more time in the open arms of the EPM (a) made more entries into the open arms (b) as compared to females receiving 0.1µg/1µl OT or saline. Locomotor activity (number of closed arm entries) was not altered by either dose of OT (c). The higher 1.0µg/1µl dose of OT also increased the time spent interacting with an unfamiliar stimulus rat in the SI test (d). Bars represent mean ± SEM; *p < 0.05.

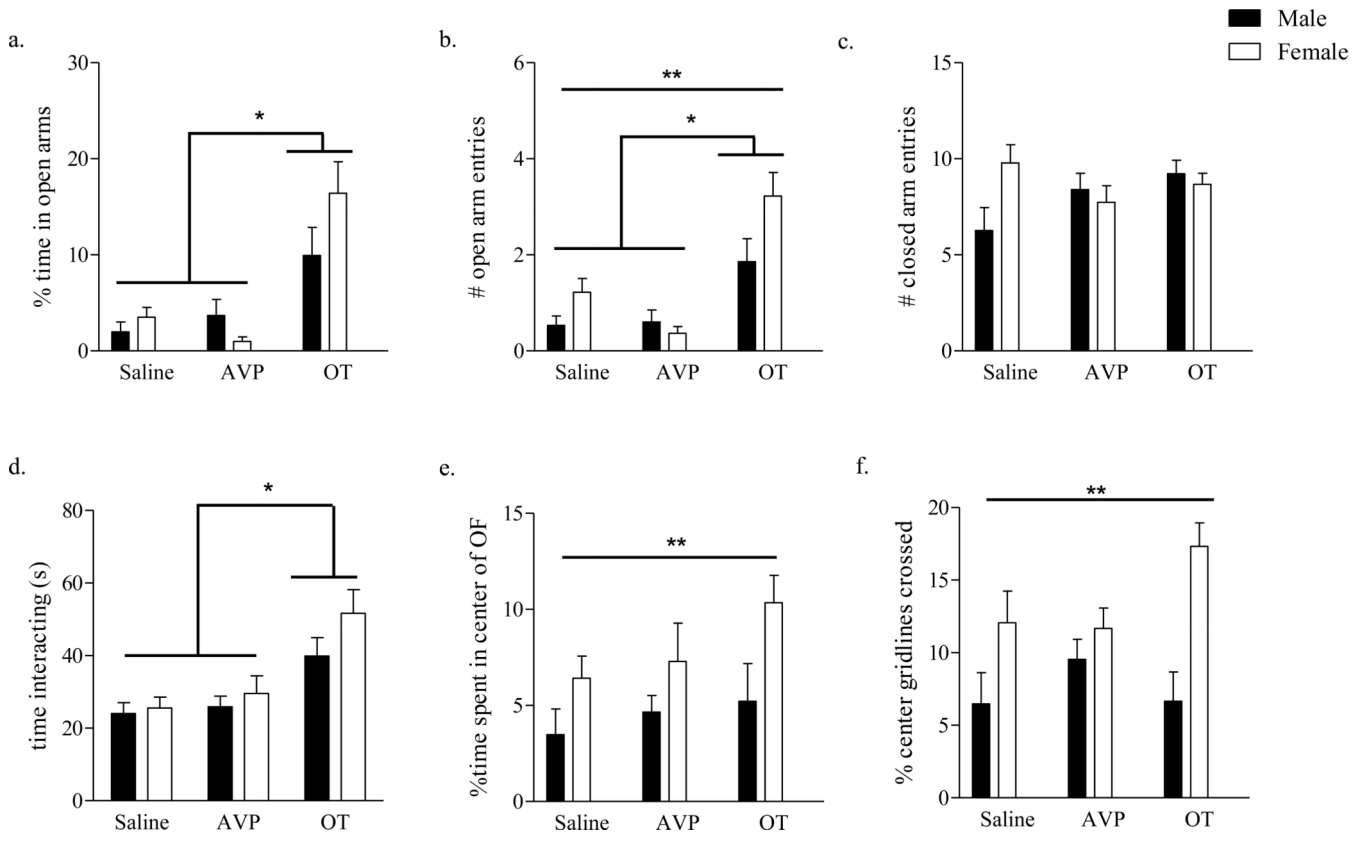

Figure 3. OT, not AVP, in the PL mPFC attenuates anxiety-like behavior in males and females

In the EPM, males and females infused with 1.0μg/μl OT into the PL region of the mPFC spent a greater percentage of time in the open arms (a) and made more open arm entries (b) than rats infused with saline or AVP. Females also entered the open arms of the EPM more than males (b). Locomotor activity (closed arm entries) was not altered by sex or infusion type (c). In the SI test, both males and females infused with OT spent a greater amount of time interacting with an unknown stimulus rat than those infused with AVP or saline (d). In the OF, females spent a greater percentage of time spent in the center of the OF (e) and crossed more center gridlines (f) than males. Bars represent mean ± SEM; *$p < 0.05$, main effect of dose; **$p < 0.05$, main effect of sex.

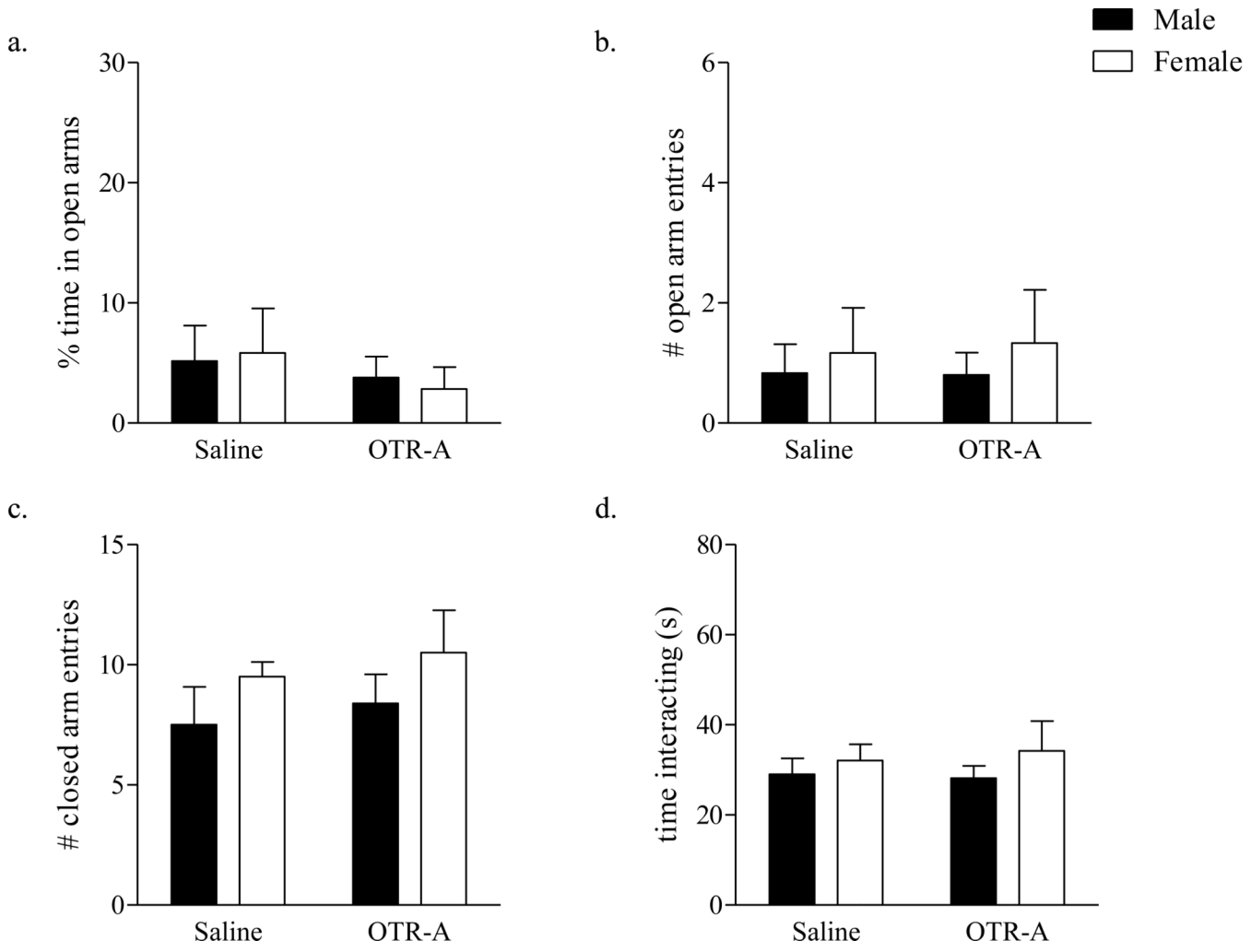

Figure 4. OTR-A in the PL mPFC has no effect on anxiety-like behavior in males or females

In both males and females, blockade of OTR in the PL mPFC did not alter the percentage of time spent in the open arms (a) or the number of open arm entries (b) in the EPM. Locomotor activity in the EPM (number of closed arm entries) was unaffected by sex or infusion type (c). OTR-A in the PL region of the mPFC also had no effect on the amount of time spent interacting with an unknown stimulus rat in the SI test (d). Bars represent mean ± SEM.

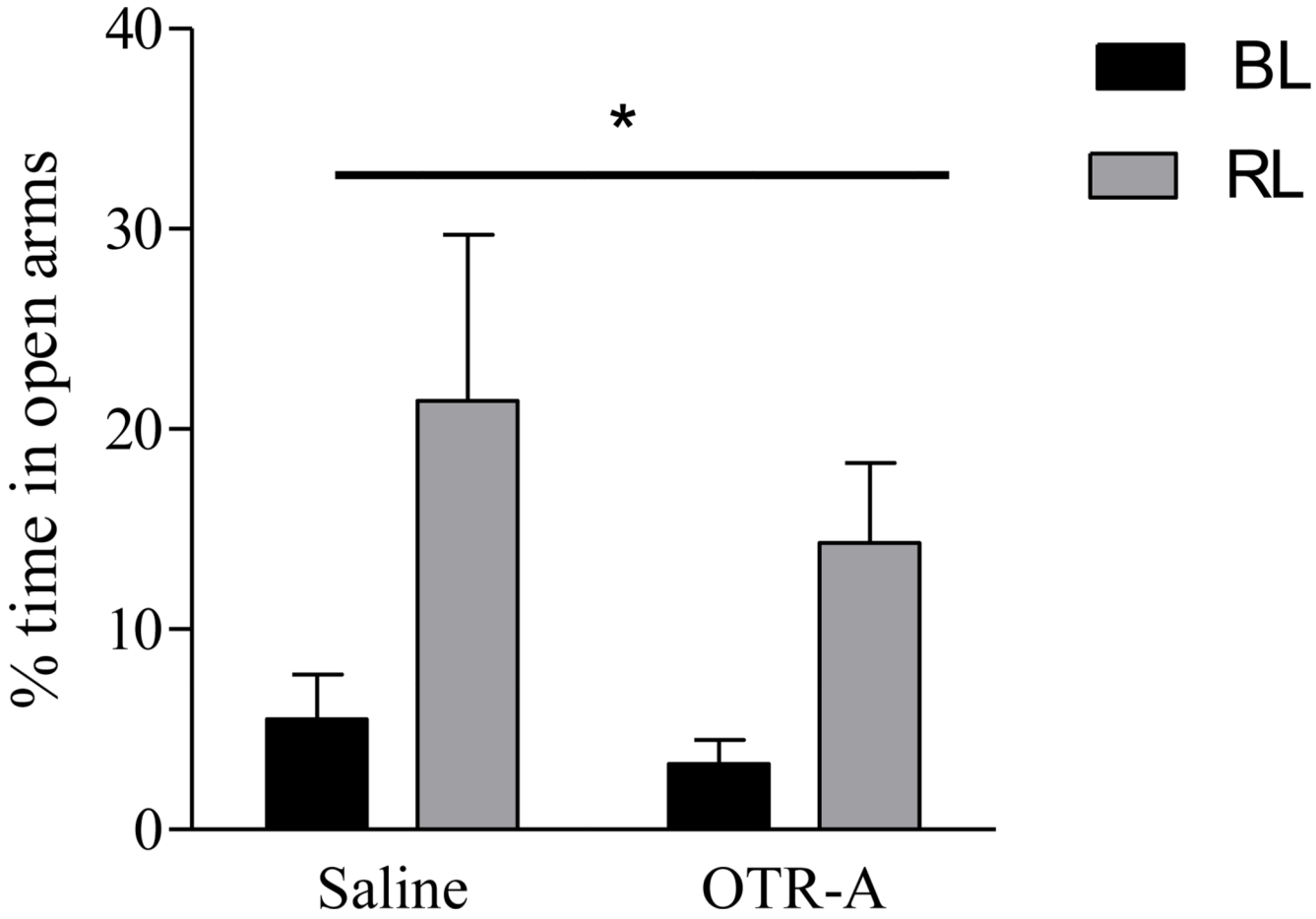

Figure 5. OTR-A in the PL mPFC does not alter anxiety-like behavior when tested in red light conditions

Baseline anxiety levels in the EPM, as measured by the percentage of time spent in the open arms, was increased by testing in red light. Under these conditions, OTR-A in the PL mPFC had no effect. Bars represent mean ± SEM; *$p < 0.05$, main effect of lighting condition.