Effects of Oxytocin in the Medial Prefrontal Cortex: Anxiety, Maternal Care, and Maternal Aggression

THESIS

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Abstract

The neuropeptide oxytocin (OT) regulates anxiety, maternal care, and maternal aggression. The neural circuitry underlying OT's behavioral effects have yet to be fully elucidated but may include the mPFC, a brain region that contains OT-sensitive neurons, expresses OT receptors (OTR), and receives long-range axonal projections from OT-producing neurons in the hypothalamus. To test this possibility we performed two sets experiments. In the first set, we examined anxiety-like behavior after OT, or the closely related neuropeptide vasopressin, was infused into mPFC of virgin male and female rats. In the second set of experiments, an oxytocin receptor antagonist was infused into the mPFC of postpartum female rats and maternal care, maternal aggression as well as anxiety-like behavior were evaluated. Our results show that OT, but not vasopressin, reduced anxiety-like behavior in both virgin males and females. In addition, blocking OTR in the mPFC of postpartum females increased anxiety, impaired maternal behavior and enhanced maternal aggression. Overall, these results suggest that the mPFC is a site where OT acts to modulate maternal care and maternal aggression during the postpartum period as well as anxiety-related behavior regardless of sex or reproductive status.

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1.1: Oxytocin: storage, release, and signaling

Oxytocin (OT) is a nonapeptide hormone best known for its role in lactation and parturition. The word "oxytocin" was coined from the Greek words meaning "quick birth" after its uterine-contracting properties were discovered (Dale, 1906). Shortly thereafter, the milk ejection property of OT was described (Ott, 1910). OT is present not only in the hypothalamic neurohypophyseal system as a hormone, but also in other areas of the central nervous system (CNS) where it exerts widespread neuromodulatory effects on behavior.

OT was the first peptide hormone to have its structure determined and the first to be chemically synthesized in biologically active form (Du Vigneaud et al., 1953). OT is composed of nine amino acids (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH_2) with a sulfur bridge between the two cysteines. The structure of OT is very similar to another nonapeptide, arginine vasopressin (AVP), which differs from OT by only two amino acids. In the vertebrate brain, OT and AVP are synthesized in separate neuronal populations in the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei. As

such, expression of AVP and OT in the PVN and SON occurs in strictly separate neuronal populations (Gainer, 2012).

OT is stored in secretory vesicles called large dense-core vesicles (LDCV) along with its respective carrier proteins called neurophysins. These proteins are synthesized in the cell body as part of a precursor protein that also contains the OT sequence. Significant processing of this prohormone takes place in the LDCV that contains the enzymes for post-translational processing during its transport to the axon terminal (Stoop, 2012). Thus, OT is synthesized as a non-glycosylated protein which undergoes endoproteolytic cleavage by aminopeptidases. The product of this cleavage is converted to the final nonapeptide, OT (Burbach et al., 1995; Ebstein et al., 2012). As a result, release at the nerve endings includes the hormone, the carrier proteins, and residual bits of precursor (Stoop, 2012). The LDCV containing OT are distributed not only in the nerve endings but also in the soma, dendrites, and axonal varicosities, and are released by calcium (Ca^{2+})-dependent exocytosis in vivo and in vitro by physiological and pharmacological stimuli (Benarroch, 2013). OT can also undergo somatodendritic release; this requires high-frequency stimulation and involves both Ca^{2+} influx and mobilization of intracellular Ca^{2+} stores (Tobin et al., 2011).

Neuroanatomical and immunocytochemical studies have shown that the PVN and the SON of the hypothalamus contain the cell bodies of two kinds of oxytonergic neurons, the magnocellular neurons and the parvocellular neurons. The magnocellular PVN or SON neurons synthesizing OT send their projections to the posterior pituitary, from which the peptide is released into the blood circulation where OT acts as a hormone

and plays a major role in parturition and lactation (Poulain and Wakerley, 1982; Benarroch, 2013). In the PVN and SON, OT is released from the soma and dendrites, thus acting as an autocrine signal which controls activity of the magnocellular neurons themselves. The axons of the parvocellular neurons are widely distributed throughout the CNS including the amygdala, septum, hippocampus, ventral tegmental area, medial preoptic area, bed nucleus of the stria terminalis (BNST), frontal cortex, brainstem, pons, medulla, and spinal cord (Gimpl and Fahrenholz, 2001). At the axonal level, OT may be released at presynaptic terminals and at nonterminal axonal varicosities and can thus act in synaptic manner or in a paracrine manner and diffuse to targets via the extracellular fluid. It is important to keep in mind that in the CNS OT acts as a neuromodulator. As such, OT is co-released with other classical neurotransmitters such as glutamate and GABA at higher frequencies of stimulation (Ludwig and Leng, 2006).

In addition to peripheral release, microdialysis studies have shown that social stimuli, including sexual behavior and stress, trigger OT release via diffusion or targeted release from magnocellular neurons within the CNS (Landgraf and Neumann, 2004). Local release from dendrites and subsequent diffusion of OT have been proposed to be an important route of action, however it is unclear how OT spreads after release, to where it diffuses, how quickly, and at what concentrations. Alternatively, because axonal fibers containing OT have been found in a large number of brain areas (Buijs et al., 1978; Sofroniew, 1983; Tobin et al., 2011; Knobloch et al., 2012), it is possible that OT from the hypothalamus arrives at various brain regions by axonal release from OT containing fibers specifically targeting brain areas expressing OT receptors (OTR). Recently,

Knobloch (2012) and colleagues revealed that an extensive network of OT fibers from the hypothalamus target various brain regions including the medial prefrontal cortex (mPFC). Thus, centrally, OT participates in classical synaptic transmission via long-range axonal projections as well as volume transmission by diffusion to nearby or remote receptors via the extracellular fluid (de Vries et al., 2012; Stoop, 2012; Neumann and Landgraf, 2012). This allows for OT to communicate with neurons and modulate different brain structures in a multimodal manner, both through a "wired," axonal , fast, and focal manner as well as an "unwired," diffusive, slow, and global fashion (Landgraf and Neumann, 2004).

OT has a short half-life of about 20 min in cerebrospinal fluid (CSF) (Ludwig and Leng, 2006) and much shorter in blood (only 1-2 min) (Gimpl and Fahrenholz, 2001). OT concentrations in the extracellular fluid of the SON are calculated to be >100-1,000 fold higher than the basal OT concentration in plasma. Plasma OT does not readily cross the blood-brain barrier, however, if pharmacological doses are administered peripherally, the exogenous neuropeptide may reach the brain parenchyma in minute, but functionally significant amounts (Landgraf and Neumann, 2004). Centrally released OT is degraded by aminopeptidases within brain tissue and then enters the CSF, where it is cleared into the circulation. Interestingly, the aminopeptidases, can produce shorter peptides from OT that in some cases may have biological effects (Ludwig and Leng, 2006). These shorter peptides have been shown to facilitate avoidance behavior of rats at concentrations 1000x smaller than AVP, although their direct efficiency as neuromodulators is much smaller

than AVP (Burbach et al., 1995). Moreover, these shorter OT fragments could cross the blood-brain barrier more easily.

1.2 Oxytocin receptors

OT receptors (OTRs) are present in the smooth muscle of the uterus and myoepithelial cells of the mammary gland. OTRs are also abundant in the central nervous system (CNS) on both neurons and astrocytes (Zingg and Laporte, 2003). The distribution of OTR expression within the CNS of numerous species has been examined using *in situ* hybridization (Yoshimura et al., 1993; Ostrowski, 1998), transgenic mouse models (Gould and Zingg, 2003), and receptor autoradiography and these have shown widespread distribution of OTR throughout the brain. In rodents, OTRs are prominent in the olfactory bulb, central (CEA) and lateral (LA) amygdala, CA1 region of the hippocampus, frontal cortex, ventromedial hypothalamus, BNST, nucleus accumbens, VTA, autonomic nuclei of the brainstem, and dorsal horn (Insel et al., 1991; Gimpl and Fahrenholz, 2001; Benarroch, 2013). In the adult rat, there are no major differences in receptor distribution between male and female brains (Gimpl and Fahrenholz, 2001). It is important to note that the distribution of OTRs in the human brain differs slightly from that of rodents (Tribollet et al., 1997) and have been detected in the basal nucleus of Meynert, vertical limb of the diagonal band of Broca, ventral part of the lateral septal nucleus, preoptic/anterior hypothalamic area, posterior hypothalamic area, globus pallidus, and ventral pallidum (Tribollet et al., 1997). Interestingly, the distribution

pattern of OT binding sites is markedly different from that of binding sites for AVP; whenever both are present in the same area, binding sites for OT and AVP are located in different regions of that particular area (Gimpl and Fahrenholz, 2001). The OTR is relatively unselective with only about a 10-fold higher affinity of the receptor for OT than for AVP (Tribollet et al., 1997; Smeltzer et al., 2006). Thus, AVP can act as a partial agonist on the OTR. However, to elicit the same response as induced by OT, ~100-fold higher concentrations of AVP would be necessary (Gimpl and Fahrenholz, 2001).

OT is currently known to have only one receptor isoform. This receptor belongs to the rhodopsin-type (class I) G protein- coupled receptor (GPCR) family. These GPCRs are coupled to various intracellular signaling cascades and activation of these cascades brings about biochemical and transcriptional changes that account for immediate and long-term neuromodulatory effects. Specifically, the OT receptor is coupled to three different G proteins, $G_{q/11}$, $G_{i/o}$, and G_s (van den Burg and Neumann, 2011; Stoop, 2012). Signaling via G_q (most common) leads to activation of the phospholipase-Cβ (PLC β) cascade which stimulates Ca^{2+} release from intracellular stores via inositol triphosphate production (IP3) and then activates diacyl glycerol (DAG). G_q also stimulates the activity of two mitogen-activated protein kinase (MAPK) cascades, leading to phosphorylation of extracellular signal-regulated kinase 1/2 (ERK 1/2 ; Zhong et al., 2003), or the related MAPK p38 (Devost et al., 2008). This pathway underlies uterine smooth muscle cell contraction (Alberi et al., 1997), and in neurons can inhibit inward rectifying currents (Gravati et al., 2010). In neurons, OT can also activate inward rectifying currents through $G_{i/o}$ protein, thus modulating neuronal excitability (Gravati et

al., 2010). Further, G_i signaling mobilizes intracellular Ca^{2+} stores independent of IP3, and only activates p38 (Hoare et al., 1999). In addition to G_q and G_i signaling, OT can activate adenylate cyclase via G_s and activate cyclic adenosine monophosphate (Campbell and Macqueen, 2004) production (Stoop, 2012). It is possible that these various signaling pathways are differentially expressed in neuronal versus peripheral tissues. Importantly, endogenous OT is able to bind and activate any OTR, regardless of what G protein it is coupled to (Stoop, 2012). Via these mechanisms, OT may indirectly affect excitatory or inhibitory synaptic transmission.

When receptors are persistently stimulated with agonists, they desensitize. Desensitization is a two-step process which consists of phosphorylation and subsequent arrestin binding. First, the receptor uncouples from G proteins and undergoes either endocytosis, internalization, or sequestration. OTRs are phosphorylated by G protein coupled receptor kinase-2, then bind beta-arrestin and are endocytosed via clathrin-coated vesicles. After internalization, they do not recycle back to the cell surface (Gimpl and Fahrenholz, 2001). This internalization is thought to underlie the rapid desensitization that may occur upon OTR activation (Smith et al., 2006b). Interestingly, whereas endogenous OT can activate OTRs regardless to which G protein they are coupled, specific agonists and antagonists may exhibit differential affinity to OTRs, depending on the specific G protein to which they are coupled and therefore not cause such internalization (Stoop, 2012).

1.3 Sex differences

While there are no differences in distribution between male and female brains (Benarroch, 2013), it should be noted that OT and OTR expression is usually higher in females (Zingg and Laporte, 2003; Carter, 2007). The effects OT on behavior and physiology are strongly dependent on steroid hormones and gender differences in OT release and OTR expression between brains of different sexes have been reported (Haussler et al., 1990; Insel et al., 1991; Tribollet et al., 1997; Carter, 2007). This could be due to higher estrogen levels and higher expression of estrogen receptor in females versus males. Indeed, estrogens upregulate the OTR expression, OT release from the hypothalamic neurons, and OT binding in the amygdala (Young et al., 1998). Additionally, in female rats, OTR expression increases in a number of brain areas just prior to parturition (Meddle et al., 2007) accompanied by an increase in gonadal hormones, particularly estrogen (Rosenblatt et al., 1988).

1.4 Functions of oxytocin

OT is widely known for its endocrine effects and the role it plays in parturition and lactation (Pedersen et al., 2006). OT is also commonly regarded as a stress hormone – it is released in response to a variety of stressful conditions (Neumann et al., 2000a; Heinrichs et al., 2001, Onaka et al., 2012). However, oxytocin also buffers stress hormone effects on the brain and body (Neumann, 2002) – it reduces blood pressure

(Petersson et al., 1996; Petersson et al., 1999), attenuates the HPA axis response to stress including the release of stress hormones such as ACTH and glucortcortiods (Windle et al., 1997), and dampens stress-induced neuronal activation (Windle et al., 2004). Similarly, lactating females exhibit a reduction in the HPA response to stress which has been attributed to suckling-induced increases in OT release (Neumann et al., 2000a; Heinrichs et al., 2001; Lonstein, 2005; Febo et al., 2009).

Behaviorally, OT plays a prominent role in a wide range of social functions that, in rodents, includes maternal care (Pedersen et al., 2006; Bosch and Neumann, 2012), sexual behavior (Bale et al., 2001), and pair boding (Lee et al., 2009; Norman et al., 2010). Similarly, a large number of human studies have also demonstrated the pro-social effects of OT including trust, social support, social cognition, and emotion recognition (Heinrichs and Domes, 2008; Meyer-Lindenberg et al., 2011; Bethlehem et al., 2013). In both rodents and humans, OT has been further implicated in the regulation of anxiety (Neumann et al., 2000b; Neumann et al., 2000a; Waldherr and Neumann, 2007; Slattery and Neumann, 2010; Neumann and Landgraf, 2012) and aggression (Caughey et al., 2011; Bosch and Neumann, 2012; Malik et al., 2012; Campbell and Hausmann, 2013). Anxiety, maternal behavior, and aggression will be the main focus of this thesis.

Anxiety and oxytocin

There are a wide variety of anxiety disorders and collectively, they are among the most common mental disorders experienced by Americans (NIMH) affecting about

about 40 million of American adults (about 18%) in a given year (Kessler et al., 2006). Among adults in the U.S., anxiety disorders have a lifetime prevalence rate of nearly 30% (Kessler et al., 2006). Additionally, women are 60% more likely than men to experience an anxiety disorder over their lifetime (Kessler et al., 2006). Anxiety disorders last at least six months and commonly occur along with other mental or physical illnesses, including major depression, postpartum depression, and social phobia, each of which can exacerbate anxiety symptoms (NIMH). If not treated, anxiety disorders can worsen and become excessive such that the afflicted individual may have difficulty controlling it to the point that their day-to-day living is negatively affected. Numerous studies in rodents have shown that OT reduces anxiety (Neumann et al., 2000b; Neumann et al., 2000a; Waldherr and Neumann, 2007; Slattery and Neumann, 2010; Neumann and Landgraf, 2012). Peripheral or central administration of OT to male and female rats reduces anxiety-related behavior in the elevated plus maze (EPM), open field (OF), and other related tests measuring inherent anxiety (Bale et al., 2001; Ring et al., 2006; Figueira et al., 2008; Ayers et al., 2011). These anxiolytic effects of OT have been localized to actions within the PVN of males and central amygdala of females (Bale et al., 2001; Knobloch et al., 2012). Likewise, intranasal administration of OT to humans has been shown to decrease anxiety which neuroimaging studies suggest may be due to the actions of OT within the amygala (Meyer-Lindenberg et al., 2011).

In the rodent literature, OT has also emerged as a neuropeptide that promotes social approach behavior and helps to overcome avoidance. As such, behavioral tests in rodents for anxiety that measure social approach behavior, such as the social interaction

(SI) test (Lapiz-Bluhm et al., 2008), have shown that ICV OT administration reduces anxiety in social situations and thus, increases the amount of time spent in social interaction with a conspecific (Norman et al., 2010; Lukas et al., 2011). Similarly, in humans, OT is recognized as a social neuropeptide and exogenous intranasal OT administration mediates social cognition and increases trust, perhaps secondary to effects on anxiety (Kosfeld et al., 2005; Heinrichs and Domes, 2008, Meyer-Lindenberg et al., 2011). The brain regions where OT acts to influence social anxiety have yet to be identified but at least one study in rodents suggests that the amygdala is not involved even though this area is strongly implicated in both social and OT-mediated actions (Lukas et al., 2011). Nonetheless, these findings suggest that OT plays a major role in the attenuation of anxiety as well as anxiety that presents in a social context.

Maternal care, maternal aggression, and postpartum anxiety: link to oxytocin

Maternal behavior is a very complex behavioral pattern whose primary role is to ensure survival of the offspring. In rodents, maternal care is characterized by nest building, retrieving pups back to the nest, nursing, and licking and grooming of the pups (Numan and Woodside, 2010). At the time of parturition, profound hormonal changes occur in the mother and many are associated with the onset and maintenance of maternal behavior, among them OT. OT acts as a neurohormone in the periphery and is critical for female reproductive functions such as induction of labor and milk ejection (Bosch and Neurmann, 2012). Suckling stimulates the release of OT simultaneously into the

bloodstream and the CNS (Neumann et al., 1993). Thus, during parturition and the postpartum period, there is not only an increase of peripheral OT, but also central OT. In addition, the postpartum period is accompanied by an increase in OT release and of OTR expression in various brain regions including the PVN, SON, septum, hippocampus, medial preoptic area (MPOA), prefrontal cortex (PFC), olfactory bulb (OB), and the bed nucleus of the stria terminalis (BNST) (Landgraf et al., 1991; Landgraf et al., 1992; D'Cunha et al., 2011; Bosch and Neumann, 2012).

The central activation of the OT system at parturition plays a critical role in the onset and maintenance of maternal behavior. Pedersen and Prange (1979) were the first to show that ICV injection of OT induces maternal behavior in estrogen treated virgin female rats. Since then, numerous studies have implicated OT as a mediator of maternal care (Nelson and Panksepp, 1998; Shahrokh et al., 2010; Bosch and Neumann, 2012). For example, it's been shown that lesions of the PVN and SON reduce or block the onset of maternal care (van Leengoed et al., 1987; Insel and Harbaugh, 1989; Neumann et al., 1993; Pedersen et al., 1994). Similarly, maternal care is impaired following intracerebroventricular (ICV) or site-specific administration of OTR antagonist (OTR-A) into regions implicated in maternal care such as the MPOA (Numan and Woodside, 2010), the mPFC (Febo et al., 2010), the amygdala (Numan et al., 2010), and the VTA (Shahrokh, et al., 2010). Once maternal care is established postpartum, ICV OT administration does not further enhance ongoing maternal care (Fahrbach et al., 1985), whereas ICV OTR-A lowers the display of maternal behaviors (Pedersen and Boccia, 2003).

Other studies have investigated the importance OT for maternal care by utilizing OT and OTR knockout mice. These studies have shown that OT knockouts have normal parturition, unaltered maternal behavior, but an inability to eject milk (resulting in loss of pups) (Nishimori et al., 1996; Young et al., 1996), although subtle deficits in maternal behavior were found in later studies (Pedersen et al., 2006). Similarly, OTR knockout lines exhibit subtle maternal care deficits and increased pup mortality, even conditional OTR knockouts in which the OTR is lacking only in regions of the forebrain and are thus able to nurse (Takayanagi et al., 2005; Macbeth and Luine, 2010). Recent work also indicates that a less efficient variant of the *Oxtr* gene in human mothers is correlated with lower levels of maternal responsiveness (Bakermans-Kranenburg and van Ijzendoorn, 2008).

In addition to expressing maternal behavior, postpartum females exhibit maternal aggression, an offensive behavior characterized by attacks, pins, and threats on an intruder introduced into the home cage (Johns et al., 1994). Maternal aggression is generally considered to be adaptive because it serves to protect the offspring and ensure their survival during the most vulnerable period of their lives. However, in cases when postpartum females become highly aggressive it may be threatening to the pups because it leaves them vulnerable and unprotected allowing them to be injured (Johns et al., 1994; Pedersen et al., 1995; Lubin et al., 2003).

The relationship between OT and maternal aggression is controversial. Because the onset of maternal aggression occurs during the postpartum period, which is also related to the increase of OT, it is reasonable to predict that OT would increase maternal

aggressive behavior. Consistent with this are studies demonstrating a reduction in maternal aggression following PVN lesions (Consiglio and Lucion, 1996) but an increase following elevation of OT in the PVN (Bosch et al., 2005). However, other work contradicts these findings and instead show that potentiated aggressive behavior during the postpartum period correlates with *decreased* levels of OT. For example, lesions of the PVN (Giovenardi et al., 1998), blockade of OT activity in the PVN (Giovenardi et al., 1998) or OTR-A infused into the amygdala (Lubin et al., 2003) all increase maternal aggression in postpartum females. An inverse relationship between OT and maternal aggression has also been observed following OT infusion into the amygdala which was shown to decrease maternal aggressive behavior (Consiglio et al., 2005; Caughey et al., 2011). Indeed, maternal aggression has been inversely correlated to OT levels in the amygdala (Johns et al., 1994, 1998; Elliot et al., 2001; Neumann et al., 2000c). Together these results indicate that OT could have an inhibitory effect on maternal aggression.

In addition to the expression of maternal care and maternal aggression, another behavioral adaptation to emerge postpartum is reduced anxiety (Lonstein, 2005; Macbeth and Luine, 2010). In postpartum rodents, this attenuation of anxiety has been observed in many experimental paradigms. For example, postpartum rats are more active in an open field (Curley et al., 2012), enter the open arms of an elevated plus-maze more often and for longer durations of time (Neumann et al., 2000a; Lonstein, 2007; Figueira et al., 2008), and are less anxious in a light/dark choice test (Miller at al., 2011; Jurek et al., 2012). Similar to rodents, anxiolyisis is also observed in nursing mothers (Heinrichs et

al., 2001; Lonstein, 2007; Macbeth and Luine, 2010) and has been suggested to be important for the display of adequate maternal care.

Attenuated anxiety during the postpartum period results, at least in part, from OT. For example, chronic ICV administration of OT decreases anxiety of lactating rats (Bosch and Neumann, 2008; Windle et al., 1997). Conversely, infusion of a selective OTR-A increases anxiety in postpartum dams without altering anxiety in virgin females suggesting that the postpartum increase in OT and OTR is essential for the reduction of anxiety that is typically observed in the postpartum period (Neumann et al., 2000a; Bosch and Neumann, 2008). Additionally, site specific administration of OTR-A to the ventromedial periaqueductal gray region (PAG; Figueira et al., 2008), PVN (Jurek et al., 2012), or amygdala (Bosch et al., 2005) of postpartum rats effectively increased anxiety but had no effect on anxiety in diestrus virgins. This differential effect of OTR antagonism in virgin versus postpartum females likely reflects the ability of elevated circulating ovarian hormones to upregulate OT and OTR expression in many brain regions implicated in maternal behavior as well as anxiety.

It should be noted that there are numerous complex interactions among OT, maternal care, maternal aggression, and anxiety and the brain regions that regulate these behaviors. These interactions will be discussed further in the following chapters.

The medial prefrontal cortex

The brain regions where OT acts to modulate anxiety and OT-induced maternal behavior/aggression have yet to be fully elucidated. As noted above, studies have pointed to the PVN of males (Blume et al., 2008), the amygdala of females (Bale et al., 2001), and the PAG, PVN, and amygdala of postpartum females (Bosch et al., 2005; Figueira et al., 2008; Jurek et al., 2012) as potential sites where OT may mediate anxiety-related behaviors. OT's actions in promoting maternal care, have been localized to regions such as the PVN, amygdala, mPOA, nucleus accumbens, and VTA (Insel and Harbaugh, 1989; Neumann and Landgraf, 1993; Pedersen et al., 1994; Numan and Woodside, 2010; Shahrokh et al., 2010) while OT's actions in the lateral septum, amygdala, BNST, MPOA, and PVN (Giovenardi et al., 1997; Lubin et al., 2003; Johns et al., 2004; Consiglio et al., 2005; Caughey et al., 2011) influence maternal aggression. However, many of these sites are likely to be part of a network which includes the mPFC.

Human studies have suggested that basolateral amygdala (BLA) is responsible for promoting anxiety, however, amygdalar activity is modulated by the top-down governance of the mPFC (Rauch et al., 1997; Ressler and Mayberg, 2007). Additionally, lesion studies in rodents have shown that the mPFC plays a role in regulating anxiety-like behavior, such that lesions of this region result in decreased anxiety (Lacroix et al., 2000; Shah and Treit, 2003; Vertes, 2004; Shah et al., 2004). Specifically, Stern and colleagues (2010), have implicated activity in the PL region of the mPFC as an area that modulates anxiety-like behavior in the EPM. Moreover, social interaction, has also been implicated

as a behavior that is also regulated by the mPFC (Gonzalez et al., 2000) such that lesions of the mPFC resulted in increased time spent with an unknown stimulus rat in the SI test.

There are very few studies that have examined the role of the PFC in aggression. Much of the research that does exist has implicated the ventral orbital or ventral medial PFC in the modulation of aggression (Morrison et al., 2013; Stein et al., 2013). However, in the mPFC, it has been observed that maternal aggression leads to increased expression of Fos levels (Gammie et al., 2004; Wang et al., 2012). Thus, these results may implicate the mPFC in the modulation of aggressive behavior during the postpartum period.

There are also several recent studies that point to a role of the mPFC in maternal care (Hernandez-Gonzalez et al., 2005; Afonso et al., 2007; Febo et al., 2009; Febo et al., 2010). For example, the mPFC of postpartum females has been shown to be activated by offspring contact (Febo, 2012). Furthermore, inactivation or lesions of the mPFC result in pup retrieval deficits (Afonso et al., 2007; Febo et al., 2010). These effects occur whether the manipulations are performed pre-pregnancy or postpartum suggesting that the mPFC may be involved in the development of maternal behaviors (Afonso et al., 2007) but also play an active part in ongoing maternal behaviors after they have arisen during the early postpartum period (Febo et al., 2010). In addition to the links above, the mPFC contains OT-sensitive neurons (Ninan, 2011), expresses OT receptors (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). Taken together, these findings suggest that the mPFC may be a target where OT acts to diminish anxiety and modulate behaviors such as maternal care and maternal aggression.

1.5 Experiments

To investigate the potential role of OT in the mPFC in relation to anxiety and maternal care and maternal aggression, we performed two separate experiments. In the first experiment, OT was delivered to the mPFC and anxiety-like behavior assessed in two exploratory tests, the EPM and the OF, as well as in the SI test which examines social behavioral responses (Lapiz-Bluhm et al., 2008). Furthermore, males and females were tested to evaluate whether the mPFC is a common site of action for the anxiolytic effects of OT in both sexes. The specificity of OT was assessed by administering the closely related neuropeptide, AVP in the mPFC. In the second experiment, we examined maternal care, anxiety, and maternal aggression following blockade of OTR in the mPFC of postpartum female rats.

Chapter 2: Oxytocin in the medial prefrontal cortex reduces anxiety in virgin females and males

2.1 Introduction

Oxytocin (OT) is 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). In addition to 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 (Sofreniew, 1983; Knobloch et al., 2012). Within the brain, OT acts as a neurotransmitter/neuromodulator and is known to play a role in parental care (Bosch and Neumann, 2012), sexual behavior (Bale et al., 2001), affiliation (Lim and Young, 2006) and other prosocial behaviors (Lukas et al., 2011; Benarroch, 2013). In addition, numerous studies have demonstrated that OT is an important regulator of anxiety (Neumann and Landgraf, 2012). For example, OT knockouts present with an anxious phenotype (Mantella et al., 2003). Moreover, in rodents, OT administered peripherally or centrally has anxiolytic effects in both males (Uvnas-Moberg et al., 1994; Ring et al., 2006; Waldherr and Neumann, 2007; Yoshida et

al., 2009; Ayers et al., 2011; Mak et al., 2012) and females (McCarthy et al., 1996; Bale et al., 2001; Slattery and Neumann, 2010) and is directly involved in anxiolysis during the postpartum period (Bosch and Neumann, 2012) as well as after mating (Waldherr and Neumann, 2007). Similarly in humans, exogenous intranasal administration of OT has been shown to attenuate anxiety in healthy and clinical populations (Heinrichs et al., 2003; Guastella et al., 2010; de Oliveira et al., 2012).

The specific brain regions where OT acts to modulate anxiety remain to be fully elucidated. Previous work has implicated the PVN of males (Blume et al., 2008) and amygdala of females (Bale et al., 2001) 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 studies have shown that the mPFC plays a role in regulating anxiety-like behavior (Lacroix et al., 2000; Vertes, 2004). The mPFC also contains OT-sensitive neurons (Ninan, 2011), expresses OT receptors (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). Taken together, these findings suggest that the mPFC may be a target where OT acts to diminish anxiety. To investigate this possibility, OT was delivered into the mPFC and anxiety-like behavior assessed in two exploratory tests, the elevated plus maze (EPM) and the open field (OF), as well as in the social interaction (SI) test which examines social behavioral responses (Lapiz-Bluhm et al., 2008). Furthermore, males and females were tested to evaluate whether the mPFC is a common site of action for the anxiolytic effects of OT in

both sexes. Lastly, the specificity of OT was assessed by administering the closely related neuropeptide, arginine vasopressin (AVP) in the mPFC.

2.2 Methods

Animals

Age-matched adult (9-12 weeks of age) female (225-250g) and male (300-350g) Sprague-Dawley rats from Taconic (Germantown, NY) were used. All 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 virgin females that had normal 4-5 day estrous cycles were used.

Surgical procedures

After approximately 7 days 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: -3.2 mm) (Paxinos and Watson, 1998). The cannula were secured by stainless steel screws and dental cement. A bilateral stainless steel obdurator (0.35 mm diameter; Plastics One) extending 0.5 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 days before behavioral testing.

mPFC 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 obduators were then removed and 28-gauge bilateral injection cannulas extending 0.5 mm beyond the tip of the guide cannula into the PL cortex were inserted into the guide. The injection cannulas

were left in place for 3 min then removed and the obdurator replaced. On the day of testing (during diestrus for females and 1 week post-surgery for males), 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) at a rate of 0.3 µl/min and were approximately 3 min in duration. The injector was left in place for an additional 1 min.

Anxiety-like behavior

Anxiety-like behavior was evaluated using three well validated models - the EPM, the OF and SI test (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 x 10 cm), facing a junction between an open and closed arm and allowed to explore for 5 min under bright light conditions. 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 x 100) were used as measures of anxiety-like behavior. 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. Locomotor activity was assessed using the number of closed arm entries.

For the OF test, a 60 x 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 and divided the arena into outer (60 x 60 cm) and inner (40 x 40 cm) areas. During a 5 min test under bright light conditions, the percentage of time spent in the center of the arena (time spent in center/total time x 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 x 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 experimental and an unfamiliar age, weight (+/- 10 g), and gender matched 'stimulus' rat were placed in opposite corners of the OF arena described above, facing away from each other. Stimulus rats were used a maximum of two times, and were never used twice in the same day. During a 5 min test under bright light conditions, the time spent in active social behavior (i.e. communal grooming, sniffing, approaching, following, climbing on or under) initiated by the experimental rat was scored. The time the experimental rat spent interacting with the stimulus rat was used as a measure of anxiety - increased anxiety is reflected by a decrease in social interaction time (File, 1980).

All behavioral tests were performed during diestrus in virgin females to control for fluctuations in anxiety due to hormone changes. They were also done 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 mPFC reduces anxiety, female rats in diestrus (n = 8-11/group) received infusions of 0.1µg/1µl of OT (Sigma, St. Louis, MO) (Lukas et al., 2011), 1.0µg/1µl OT (Bale et al., 2001), or 1µl of saline vehicle in the mPFC and anxiety-like behavior assessed in the EPM and SI test. To test the specificity of OT, separate groups of females (n = 8-11/group) received infusions (1.0µg/1µl) of the closely related neuropeptide, vasopressin (AVP; Sigma), OT (1.0µg/1µl), or saline vehicle (1µl) in the mPFC and anxiety-like behavior assessed in the EPM and OF or the SI test. Lastly, to determine whether the effects of OT in the mPFC were sex dependent, male rats (n = 7-9/group) received infusions of 1.0µg/µl OT, 1.0µg/1µl AVP, or saline vehicle (1µl) in the mPFC followed by testing in the EPM and the OF or SI test. In all cases, testing for anxiety-related behavior was done 15 min after infusions. The two tests were done 5 min apart and the order of tests was counterbalanced among rats.

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 sections were collected throughout the area of the cannula implant and stained with 0.2% cresyl violet for verification of correct placement (Fig. 1). Those animals with cannula placements outside of the PL region of the mPFC were excluded from the study.

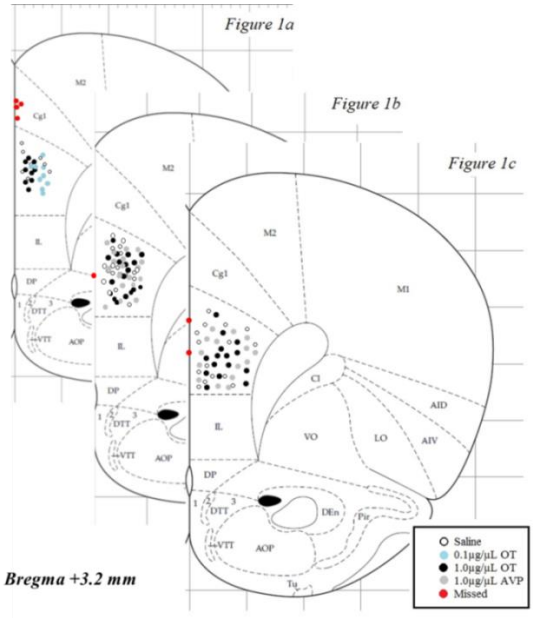

Figure 1. Schematic representation of mPFC infusion sites. Cannula tip placements were in the prelimbic region (PL) of the mPFC (AP: +3.2 mm, ML: ±0.5 mm, DV: -3.2 mm). Each dot indicates an individual subject. Infusions were bilateral but are represented unilaterally (a) Cannula placements for diestrus females receiving an infusion of 0.1µg/1µl OT, 1.0µg/1 µl OT, or saline. Animals with missed cannula placements in Cg1 were excluded from analysis. (b) Cannula placements for diestrus females receiving an infusion of 1.0µg/1 µl OT, 1.0µg/1 µl AVP, or saline. (c) Cannula placements for males receiving an infusion of 1.0µg/1 µl OT, 1.0µg/1 µl AVP, or saline. Adapted from Paxinos and Watson, 1998.

Statistical analysis

All statistical analyses were performed using Graphpad Prism software version 5.01 (La Jolla, CA). Behavioral data were analyzed separately in males and females using one-way analysis of variance (ANOVA) followed by post-hoc analysis using the Newman-Keuls Multiple Comparison test. Graphs display means ± SEM. P values < 0.05 were regarded as statistically significant.

2.3 Results

Experiment 1: OT infused into the mPFC reduces anxiety-like behavior in females

In the EPM, the higher 1.0µg/µl dose of OT infused into the prelimbic region of the mPFC decreased anxiety as compared to the lower 0.1µg/µl dose of OT and saline controls (Fig. 2). Specifically, females infused with the higher OT dose spent a greater percentage of time in the open arms ($F_{2,23} = 6.30$, $p < 0.05$; Fig. 2a) and made a greater number of entries into the open arms ($F_{2,23} = 4.97$, $p < 0.05$; Fig. 2b). Locomotor activity, as measured by the number of closed arm entries, was not altered ($F_{2,23} = 0.95$, $p > 0.05$; Fig. 2c). In the SI test, the higher dose of OT was also effective in reducing anxiety as demonstrated by a greater amount of time spent interacting with an unknown stimulus rat ($F_{2,23} = 7.74$, $p < 0.05$; Fig. 2d).

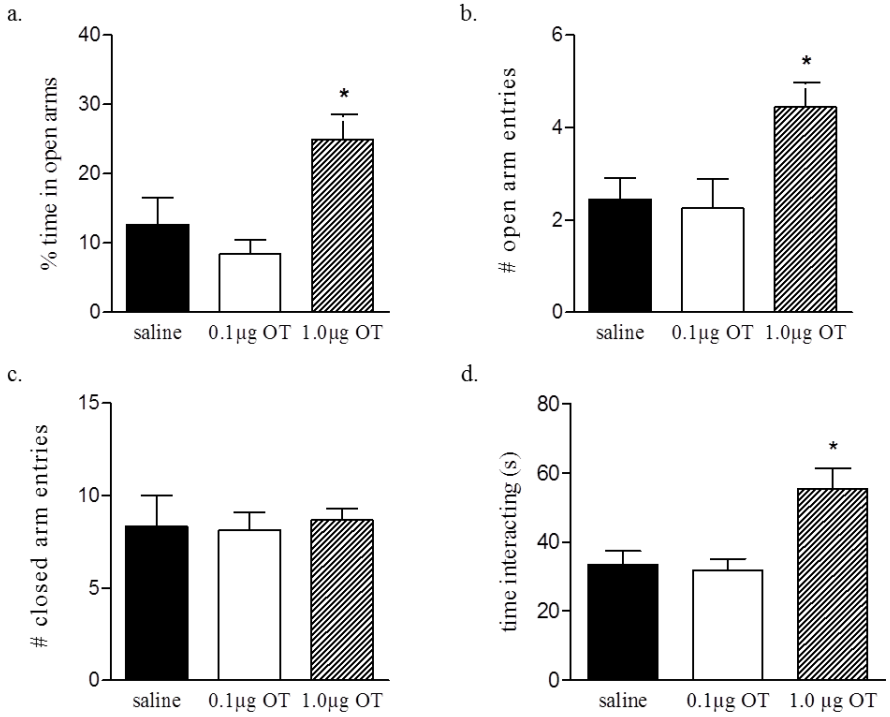

Figure 2. Female rats in diestrus receiving 1.0µg/1µl OT in the mPFC (a) spent more time in the open arms of the EPM and (b) had more entries into the open arms as compared to females receiving 0.1µg/1µl OT or saline, which did not differ from one another. (c) Locomotor activity (number of closed arm entries) was not altered by either dose of OT. (d) The higher dose of OT increased the time spent interacting with an unfamiliar stimulus rat in the SI test. Bars represent mean ± SEM; **P* < 0.05.

Experiment 2: AVP infused into the mPFC does not alter anxiety-like behavior in females

Females receiving OT, but not AVP, in the mPFC displayed reduced anxiety in the EPM and SI tests. Specifically, OT increased the percentage of time spent in the open arms ($F_{2,52} = 17.92$, $p < 0.05$; Fig. 3a) and the number of open arm entries ($F_{2,52} = 19.56$; $p < 0.05$; Fig. 3b) in the EPM as compared to saline or an equivalent dose of AVP. In contrast to the anxiolytic effects of OT, AVP (1.0µg/ µl) in the mPFC had no effect on anxiety-like behavior in the EPM as demonstrated by a similar percentage of time spent in the open arms ($p > 0.05$; Fig. 3a) and a similar number of open arm entries ($p > 0.05$; Fig. 3b) as saline treated animals. Locomotor activity in the EPM was not altered by OT or AVP ($F_{2,52} = 0.2242$, $p > 0.05$; Fig. 3c). In the SI test, females infused with OT spent a significantly greater amount of time interacting with an unknown stimulus rat than those infused with AVP or saline ($F_{2,21} = 7.85$, $p < 0.05$; Fig. 3d), which did not differ from one another. However, in the in the OF, neither OT nor AVP altered anxiety-like behavior as measured by the percentage of time spent in the center of the field ($F_{2,28} = 1.43$, $p > 0.05$; Fig. 3e) or percentage of center gridlines crossed ($F_{2,28} = 3.27$, $p = 0.05$; Fig. 3f). Locomotor activity in the OF test was not affected (Saline: 154.0 ± 6.96, AVP: 173.7 ± 12.23, OT: 144.0 ± 7.64; $F_{2, 28} = 0.09$, $p > 0.05$).

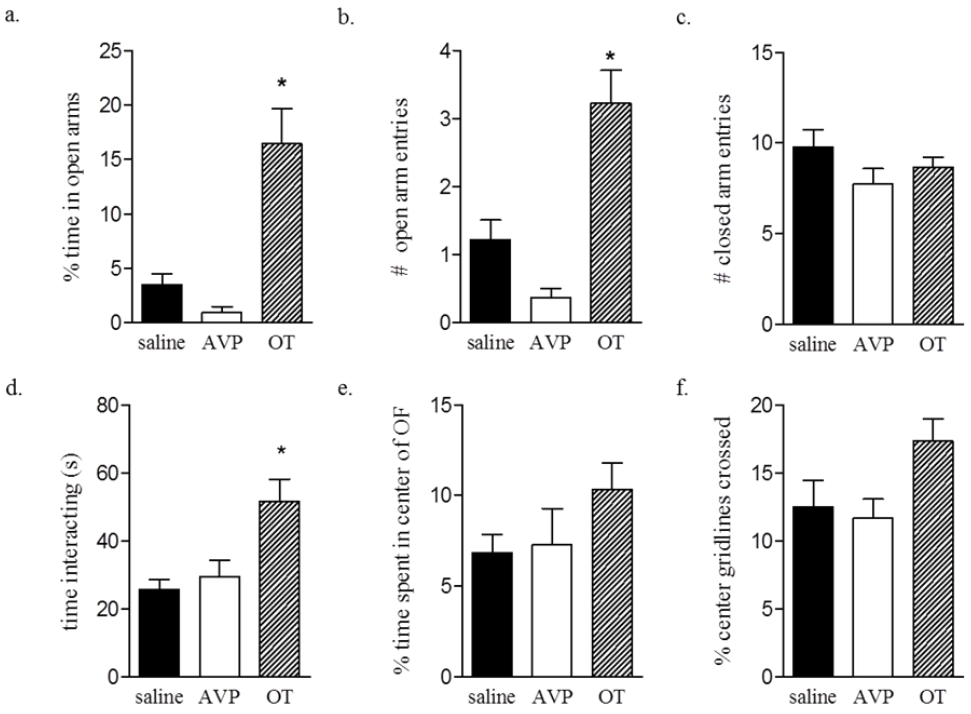

Figure 3. In contrast to the anxiolytic effects of OT (1.0µg/1µl) , AVP (1.0µg/1µl) in the mPFC had no effect on anxiety-related behavior in diestrus females as demonstrated by (a) a similar percentage of time spent in the open arms and (b) a similar number of open arm entries in the EPM. (c) Locomotor activity (number of closed arm entries) was not altered by either AVP or OT. (d) In the social interaction test, OT (1.0µg/1µl) treated females in diestrus spent more time engaging in social interaction than did those treated with AVP treated or saline. Neither OT nor AVP had an effect in the OF test when measuring the percentage of time spent in the center of the field (e) or percentage of center gridlines crossed (f). Bars represent mean ± SEM; **P* < 0.05.

Experiment 3: OT, but not AVP, infused into the mPFC reduces anxiety in males

There was a similar anxiolytic action of OT in the mPFC of males. In the EPM, males infused with same dose of OT shown to be effective in females (1.0µg/ µl) spent a greater percentage of time in the open arms ($F_{2,42} = 5.56$, $p < 0.05$; Fig. 4a) and had a greater number of open arm entries ($F_{2,42} = 6.42$, $p < 0.05$; Fig. 4b), but locomotor activity was not altered ($F_{2, 42} = 2.42$, $p > 0.05$; Fig. 4c). In the SI test, males infused with OT also

spent a significantly greater percentage of time interacting with an unknown stimulus rat ($F_{2,21} = 5.22$, $p < 0.05$; Fig. 4d). However, when males were tested in the OF, OT did not alter anxiety-like behavior (Percentage of time spent in the center of the field: $F_{2,18} = 1.29$, $p > 0.05$; Fig. 4e, Percentage of center gridlines crossed: $F_{2,18} = 0.55$, $p > 0.05$; Fig. 4f) or locomotor activity (Saline: 129.4 ± 14.09, AVP: 150.7 ± 7.65, OT: 148.4 ± 16.71; $F_{2, 28} = 0.09$, $p > 0.05$). In all tests, AVP-treated males did not differ from those infused with saline indicating that AVP had no effect on anxiety-like behavior in males.

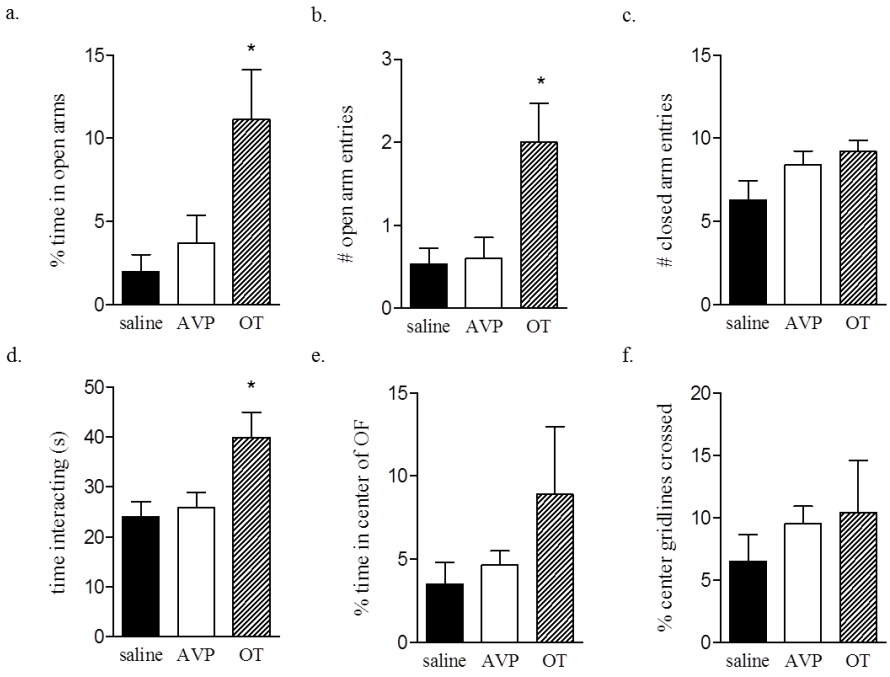

Figure 4. In males, OT (1.0µg/1µl) was anxiolytic, as demonstrated by (a) an increase in the percentage of time spent in the open arms and (b) a greater number of open arm entries in the EPM. (c) Locomotor activity (number of closed arm entries) was not altered by either vasopressin or OT. (d) OT (1.0µg/1µl) treated males also spent more time engaging in social interactions than did those treated with AVP treated or saline. Neither OT nor AVP altered anxiety-like behavior in the OF measured by (e) time spent in the center of the open field and (f) ratio of inside gridlines crossed. Bars represent mean ± SEM; *$P < 0.05$.

2.4 Discussion

Here we show that OT infused into mPFC reduces anxiety-like behavior in both male and female rats. In contrast to the anxiolytic effects of OT, an equivalent dose of AVP infused into the mPFC had no effect on anxiety-like behavior. Together, these findings identify the mPFC as a component of the neural circuitry underlying OT's effects on anxiety in both males and females.

Numerous studies have explored the anxiety modulating effects of OT using models of anxiety-like behavior that feature exploration and utilize anxiogenic stimuli of open spaces including the EPM, OF, elevated zero maze and light-dark box (Rotzinger et al., 2010). In general, these studies have demonstrated that OT is anxiolytic in both males and females when delivered centrally or peripherally (Uvnas-Moberg et al., 1994; Bale et al., 2001; Ring et al., 2006; Waldherr and Neumann, 2007; Blume et al., 2008; Yoshida et al., 2009; Ayers et al., 2011; Mak et al., 2012). Here, we confirm these findings and show that OT, when centrally administered at a higher dose, reduces anxiety-like behavior in the EPM regardless of sex. However, in the OF, the anxiolytic actions of OT were undetectable. Although the EPM and OF both have an exploratory component, the EPM is considered a more sensitive test of anxiety (Hilakivi and Lister, 1990) and behavior in one test does not always predict behavior in the other (Bale et al., 2001; Bhatnagar et al., 2004). It is also possible that the inconsistencies in the OF may be related to variations in the testing conditions known to influence OF behavior (Lapiz-

Bluhm et al., 2008) or differential sensitivity of the OF to OT which may require different doses than those used here for an anxiolytic effect to be revealed.

While the SI test is regarded as another model of anxiety, its emphasis on social behavioral responses distinguishes it from other tests of anxiety such as the EPM and OF (Rotzinger et al., 2010). Oxytocin is strongly implicated in the control of social behavior (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 in 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 mPFC in the regulation of social interaction (Gonzalez et al., 2000) but are also in agreement with other work demonstrating that social approach and preference behavior depends on endogenous OT (Lukas et al., 2011). Thus, combined with the data in the EPM, it appears that OT exerts an anxiolytic effect in both non-social and social contexts.

Investigation into specific sites in the brain where OT acts to reduce anxiety-like behaviors has been limited to a few studies which have demonstrated an involvement of the PVN in virgin males (Blume et al., 2008), the amygdala of virgin females (Bale et al., 2001) and the periaqueductal gray in postpartum females (Figueira et al., 2008). Here we extend this prior work and show that in both sexes, the anxiolytic action of OT can be localized to the mPFC. The mPFC has been implicated in anxiety regulation (Lacroix et al., 2000; Vertes, 2004) - inactivation or excitotoxic lesions of the mPFC attenuate anxiety (Lacroix et al., 2000; Shah and Treit, 2003). When combined with the

observations that the mPFC also contains OT-sensitive neurons (Ninan, 2011), expresses OT receptors (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), the mPFC is a likely target for the control of anxiety by OT. It is important to note that in the rodent brain, the mPFC consists of 3 subregions, 2 of which have distinct roles in regulating fear and anxiety-like behavior - the infralimbic (IL) (Vidal-Gonzalez et al., 2006; Peters et al., 2009) and PL (Hoover and Vertes, 2007; Stern et al., 2010) regions, respectively. The anterior cingulate (Cg1) is the third region and does not seem to have any discernible effects on fear and anxiety (Vidal-Gonzalez et al., 2006; Peters et al., 2009; Maroun, 2012). Our results specifically localize the anxiolytic actions of OT to the PL region. In the absence of OT, the PL region of the mPFC promotes fear and anxiety through direct glutamatergic projections to the basolateral nucleus of the amygdala (BLA). The BLA in turn sends glutamatergic projections to the central nucleus of the amygdala (CEA) and the bed nucleus of the stria terminalis (BNST), structures which have distinct roles in fear and anxiety-related behavior (Fig. 5a). Consistent with this, stimulation of the PL increases anxiety-like behavior while PL lesions or inhibition of the PL cortex diminishes anxiety-like behavior (Vidal-Gonzalez et al., 2006; Stern et al., 2010). How then might OT in the mPFC reduce anxiety? Recent studies have shown that OT suppresses glutamatergic transmission in the mPFC (Ninan, 2011; Qi et al., 2012) and can also increase the release of GABA (Qi et al., 2012). By decreasing glutamate and increasing GABA in the PL region of the mPFC, OT would

decrease excitation of the glutamatergic projections from the mPFC to the BNST, thus resulting in a decrease in anxiety-like behavior (Fig. 5b).

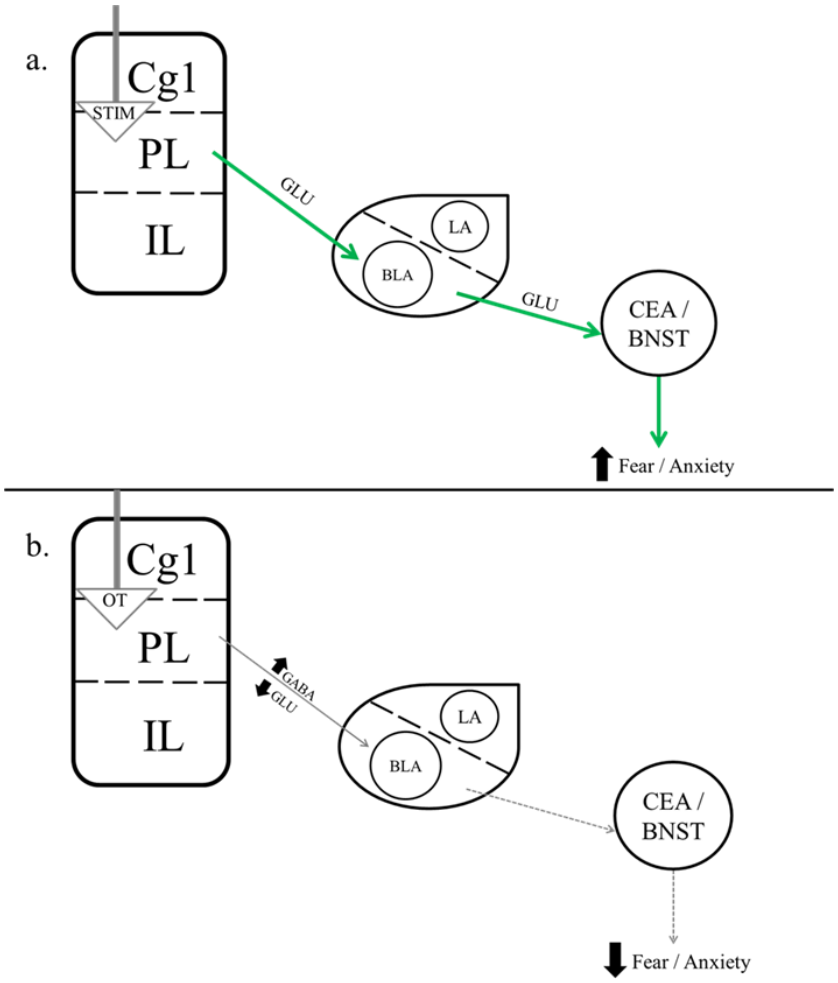

Figure 5. Diagram depicting mPFC connections to the amygdala. (a) The PL region sends glutamatergic projections to the BLA, which then sends glutamatergic projections to the CEA. Stimulation of the PL region results in an increase in fear and anxiety-like behavior. (b) When OT is infused into the PL region of the mPFC, levels of GABA are increased (Qi et al., 2012), while glutamatergic transmission is suppressed (Ninan, 2011). This results in an overall decrease in excitation of the amygdala, resulting in a decrease in fear and anxiety. Cg1= anterior cingulate, PL= prelimbic, IL= infralimbic, LA= lateral amygdala, BLA= basolateral amygdala, CEA= central amygdala, BNST= bed nucleus of the stria terminalis.

Whether OT exerts similar anxiolytic actions in the other regions of the mPFC has yet to be determined. The IL cortex projects to GABAergic intercalated cells (ITC) which have been shown to exert inhibitory control over CEA output neurons. As a result, stimulation of the IL inhibits fear responses via the ITC cell groups (Peters et al., 2009). However, when anxiety rather than fear is assessed, a different pattern of results emerges such that enhanced excitability of the IL region produces anxiety-like behavior (Bi et al., 2013). Thus, the IL region may play a differential role in the regulation of conditioned fear versus anxiety. Given these differences, the subregional specificity of OT in the mPFC warrants further investigation but may be a mechanism that could contribute to recent observations that OT may also be anxiogenic under certain conditions (Guzmán et al., 2013).

In numerous studies, AVP has been shown to have anxiogenic properties (McCarthy et al., 1996; Engin and Treit, 2008), presumably through its actions on the V1a receptor subtype (Frank and Landgraf, 2008) which is the most abundant receptor subtype in the CNS (Stoop, 2012). However, the compound used in this experiment was an agonist at the V2 receptor subtype, a less commonly studied subtype in the modulation of anxiety. Thus, our results demonstrating no significant effect of AVP on anxiety may be related to the receptor subtype that was targeted.

Overall, the results of this study implicate the PL region of the mPFC as a site where 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 provide a potential therapeutic role for OT as an agent for management of disorders of social behavior and anxiety.

Chapter 3: Oxytocin in the postpartum mPFC: effects on maternal care, maternal aggression, and anxiety

3.1 Introduction

The postpartum period is a time of dramatic behavioral changes for all mammalian species. In rodents, females that were previously unresponsive or infanticidal towards pups will engage in an elaborate repertoire of caregiving activities after parturition that includes nest building, nursing and/or crouching over pups, retrieving pups to the nest, body/genital licking of the pups and heightened aggression towards conspecifics in order to protect their young (Rosenblatt, 1967; Erskine et al., 1978; Rosenblatt et al., 1994; Numan, 2006; Numan et al., 2010). In addition, postpartum females show changes in their emotional state characterized by attenuated levels of anxiety (Neumann et al., 2000a; Lonstein, 2005; Figueira et al., 2008; Macbeth and Luine, 2010).

Each of the behavioral changes that emerge postpartum is mediated by a vast array of neurochemicals, including oxytocin (OT). OT is neurohormone synthesized in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus. In postpartum rats, suckling simultaneously stimulates the release of oxytocin from the pituitary into the bloodstream as well as into the CNS (Neumann et al., 1993). In the

periphery, OT enhances smooth muscle contractility for milk ejection while the behavioral effects of OT are mediated by OT release in the CNS. However, both the central and peripheral actions of OT are transduced by a single isoform of the oxytocin receptor (OTR). During the peripartum period, expression of the OTR increases not only on mammary contractile cells but also within various regions of the brain including the lateral septum, medial preoptic area (MPOA), central nucleus of the amygdala (CEA), the ventromedial nucleus of the hypothalamus (VMH), the nucleus accumbens, and the ventral tegmental area (Landgraf et al., 1991; Landgraf et al., 1992; Pedersen et al., 1994; Young et al., 1997; Meddle et al., 2007; Shahrokh et al., 2010, D'Cunha et al., 2011; Bosch and Neumann, 2012). Not surprisingly, many of these brain regions have been implicated as sites where OT regulates maternal care, maternal aggression, and postpartum anxiety. For example, key areas involved in OT-induced maternal behavior include the MPOA (Pedersen et al., 1994), ventral tegmental area (VTA; Shahrokh et al., 2010), and the olfactory bulb (OB) (D'Cunha et al., 2011) while OT has been shown to influence maternal aggression through its effects in the PVN (Giovenardi et al., 1998, Bosch et al., 2005), bed nucleus of the stria terminalis (BNST; Consiglio et al., 2005), and CEA (Lubin et al., 2003; Bosch et al., 2005; Consiglio et al., 2005). Although less studied, the postpartum-associated reduction in anxiety has been attributed to OT acting within the midbrain periaqueductal gray (PAG; Figueira et al., 2008), PVN (Jurek et al., 2012), and the amygdala (Bosch et al., 2005). Thus, OT acts on a widespread network of brain regions to influence postpartum behaviors.

Another component of this network may include the medial prefrontal cortex (mPFC). In addition to expressing OT receptors (Liu et al., 2005; Smeltzer et al., 2006), the mPFC contains OT-sensitive neurons (Ninan, 2011), and receives long-range axonal projections from OT producing neurons in the hypothalamus (Sofroniew, 1983; Knobloch et al., 2012). Behaviorally, the mPFC has been implicated in the regulation of anxiety (Shah and Treit, 2003; Stern et al., 2010), aggression (Gammie et al., 2004), and maternal care (Afonso et al., 2007; Febo et al., 2010; Pereira and Morrell, 2011; Febo, 2012; Zhao and Li, 2012). Taken together, these findings suggest that the mPFC may be a common target where OT acts to induce maternal behavior, modulate maternal aggression, and regulate anxiety during the postpartum period. To investigate this possibility, a highly specific OTR antagonist (OTR-A) was delivered into the mPFC of postpartum rats and these various behaviors were assessed.

3.2 Methods

Animals

Age matched adult (9-12 weeks of age) virgin (225-250 g) and timed pregnant [gestation day (GD) 14] female Sprague-Dawley rats from Taconic (Germantown, NY) were used. All 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.

For postpartum females, the day of birth was designated as postpartum day 0 (PD0) and on PD1 each litter was culled to 5 male and 5 female pups. In virgin females, stages of estrous were monitored 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 virgin females that had normal 4-5 day estrous cycles were used.

Surgical procedures

Rats were anesthetized with a 2-4% isoflurane gas/air mixture and aligned on the 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; Febo et al., 2010; Paxinos and Watson, 1998). The cannula were secured by stainless steel screws and dental cement. A bilateral stainless steel obdurator (0.35 mm diameter; Plastics One) extending 0.5 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. Surgeries were performed on pregnant rats on GD16-17. Following surgery, rats were allowed to recover for at least 7 days before behavioral testing.

mPFC infusions

On days 2 and 4 post-surgery, all rats were habituated to the handling and infusion procedures. During habituation, rats were removed from their home cage and handled for approximately 3 min while being lightly restrained in a terrycloth towel. The obduators were then removed and 28-gauge bilateral injection cannulas extending 0.5 mm beyond the tip of the guide cannula into the PL cortex were inserted into the guide. The injection cannulas were left in place for 3 min then removed and the obdurator replaced. On testing days (during diestrus for virgin females and on PD 3, 5, and 7 for postpartum rats), 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) and were approximately 3 min in duration at an infusion rate of 0.3 µl/min. The injector was left in place for an additional 1 min**.**

Experimental design

To investigate the effects of OTR-A in the mPFC, virgin female rats (n = 7-9/group) and postpartum rats (n = 7-9/group) received infusions of the highly specific OTR-A, desGly-NH_2-d$(CH_2)_5$[D-Tyr^2,Thr^4]OVT (courtesy of Dr. Maurice Manning, University of Toledo) or saline. Either 0.1µg/1µl of OTR-A (Figueira et al., 2008), 0.5µg/1µl OTR-A (Lubin et al., 2003), or 1µl of saline vehicle was infused into the mPFC and maternal behavior, anxiety-like behavior, and maternal aggression were assessed in mothers. Only anxiety-like behavior was assessed in virgin females and this was done during diestrus in order to control for fluctuations in anxiety across the estrous cycle. In all cases, testing for these behaviors was done between 900-1200 hr in bright light conditions, 20 min after infusions. Maternal behavior was tested on PD3, anxiety-like behavior on PD5, and maternal aggression on PD7. The two anxiety tests were done 5 min apart in the same day and the order of these tests was counterbalanced among rats.

Maternal behavior

On PD3, dams were infused with either OTR-A or saline and returned to their home cage. Before the infusion, their litters were removed and placed in a separate cage on a heating pad. After a 20 min habituation to the testing room, pups were reintroduced into the home cage in a scattered manner in the corner diagonally opposite to the nest. A

30 min video recording began immediately following the return of the pups to the cage. During maternal observations, the following latencies and durations (when appropriate) of behaviors were recorded: pup retrieval (pulling stray pups by the scruff back to the nest), nest building (collecting bedding to create a nest), exploratory behavior (exploration of the cage), rearing on hind legs (standing on hind legs), and pup directed behaviors which included the following: licking (anogenital or body licking of pups), pup contact (dam is in close contact with pups, but not nursing, sniffing, or licking), active (nursing while maintaining an arched posture), and passive (nursing while on side) nursing. This 30 min testing procedure typically induces dams to retrieve and lick pups; however, because the OTR-A may result in atypical caregiving, other maternal behaviors were assessed as well.

Anxiety-like behavior

Both postpartum females (on PD5) and virgin females (in diestrus) were tested for anxiety behavior. Anxiety-like behavior was evaluated using two well validated models-the EPM, and the OF tests (Lapiz-Bluhm et al., 2008). 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 (10x10 cm), facing a junction between an open and closed arm and allowed to explore for 5 min under bright light conditions. 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 x 100) were used as measures of anxiety-like behavior. 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. Locomotor activity was assessed using the number of closed arm entries.

For the OF test, a 60x60 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 and divided the arena into outer (60 x 60 cm) and inner (40 x 40 cm) areas. During a 5 min test under bright light conditions, the percentage of time spent in the center of the arena (time (s) spent in center divided by total time (300 s)) as well as the percentage of gridlines crossed in the center of the arena (number of center gridlines crossed divided by total number of gridlines crossed during the testing period) were used as measures of anxiety-like behavior. An increase in the percentage of time or percentage of gridlines crossed in the center of the arena correlate with lower anxiety. After infusion of OTR-A or saline and a 20 min habituation period to the testing room, rats were tested in the EPM and OF tests. Tests were counterbalanced among all subjects.

Maternal aggression

The intensity of maternal aggression has been found to dramatically change over the peripartum period, with its first appearance on the day before parturition. Maternal aggression intensity then falls immediately after parturition, and increases again during postpartum days 4-7- this is typically the time period in which maternal aggression has

been tested in previous studies (Caughey et al., 2011). Thus, in this study each dam was assessed for aggressive behavior using the maternal defense test (Lubin et al., 2003) on PD7.

Each dam was infused with either OTR-A or saline and replaced in her home cage with her pups in the testing room. After a 20 min habituation period, a weight matched (+/- 10 g) intruder female was introduced into the home cage. Each intruder was used for no more than 2 aggression tests, and none were used twice in the same day. Video recording began as soon as the intruder was placed in the cage and continued for 10 min. The following frequencies, latencies, and duration (when appropriate) of behaviors were assessed: contact with intruder (included following or sniffing of the intruder), bites (dam bites any part of the intruder's body), pins (dam pins and holds down intruder to the floor of the cage), and attacks (dam lunges quickly at intruder). One test session was immediately discontinued because the intruder and some pups were wounded. Data from this animal were not included in the analysis.

All behavioral tests were digitally recorded and videos were later scored blind by a trained observer using BEST Collection and BEST Analysis software (Education Consulting Inc., Hobe Sound, FL).

Histology

After the completion of aggression 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 sections were collected throughout the area of the cannula implant and stained with 0.2% cresyl violet for verification of correct placement (Fig. 6). Those animals with cannula placements outside of the PL region of the mPFC were excluded from the study.

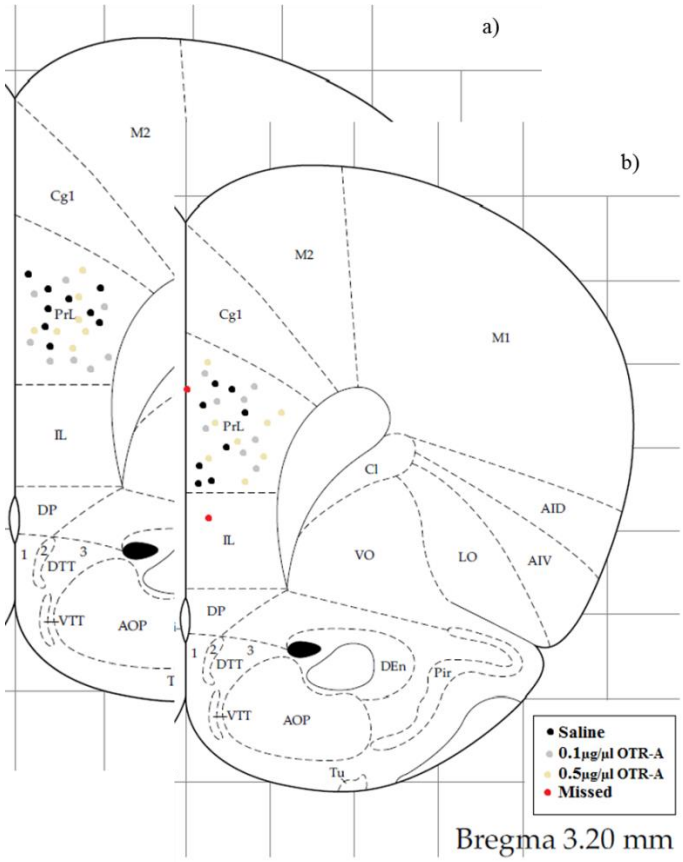

Figure 6. Schematic representation of mPFC infusion sites. Cannula tip placements were in the prelimbic region (PL) of the mPFC (AP: +3.2 mm, ML: ±0.5 mm, DV: -3.2 mm). Each dot indicates an individual subject. Infusions were bilateral but are represented unilaterally (a) Cannula placements for postpartum females receiving an infusion of 0.1µg/1µl OTR-A, 0.5µg/1µl OTR-A, or saline. Animals with missed cannula placements in IL were excluded from analysis. (b) Cannula placements for diestrus females receiving an infusion of 0.1µg/1µl OTR-A, 0.5µg/1µl OTR-A, or saline. Adapted from Paxinos and Watson, 1998.

Statistical analysis

All statistical analyses were performed using Graphpad Prism software version 5.01 (La Jolla, CA). Behavioral data in the maternal behavior and maternal aggression tests were analyzed separately in postpartum females using one-way analysis of variance (ANOVA) followed by post-hoc analysis using the Newman-Keuls Multiple Comparison test. Anxiety-like behavior was analyzed with a 2 x 3 ANOVA with reproductive state (postpartum or virgin) and infusion type (saline, 0.1 µg/1µl OTR-A, or 0.5 µg/1µl OTR-A) as factors followed by post-hoc analysis using the Bonferroni post-test. Graphs display means ± SEM. *P* values < 0.05 were regarded as statistically significant.

3.3 Results

Blocking OTR in the mPFC of postpartum rats alters maternal care

Blocking OTR in the prelimbic region of the mPFC in postpartum females impaired pup retrieval behavior. Specifically, the lower OTR-A dose (0.1 µg/µl) increased the latency to retrieve the first pup ($F_{2, 20} = 3.74$, $p < 0.05$, Fig. 7a) and decreased the percentage of pups retrieved ($F_{2, 20} = 4.71$, $p < 0.05$, Fig. 7b) as compared to the higher 0.5µg/µl dose of OTR-A and saline controls. There was a trend for the dams infused with the 0.1µg/µl dose of OTR-A to also have an increased latency to retrieve

their last pup (p=0.07) as compared to the higher OTR-A dose and saline controls (data not shown). Additionally, OTR blockade decreased the amount of time postpartum females spent engaged in in pup-directed behaviors, although this was found only for the lower dose of the OTR-A ($F_{2, 20} = 8.98$, $p < 0.05$, Fig. 7c). However, dams administered both the low and high dose of the OTR-A spent a greater amount of time exploring the home cage ($F_{2, 20} = 4.60$, $p < 0.05$, Fig. 7d) when compared to saline controls suggesting the effects of OTR antagonism on maternal behavior is unrelated to its effects on locomotor activity. Nest building and latency to nest build were not affected by either dose of OTR-A (p's > 0.05).

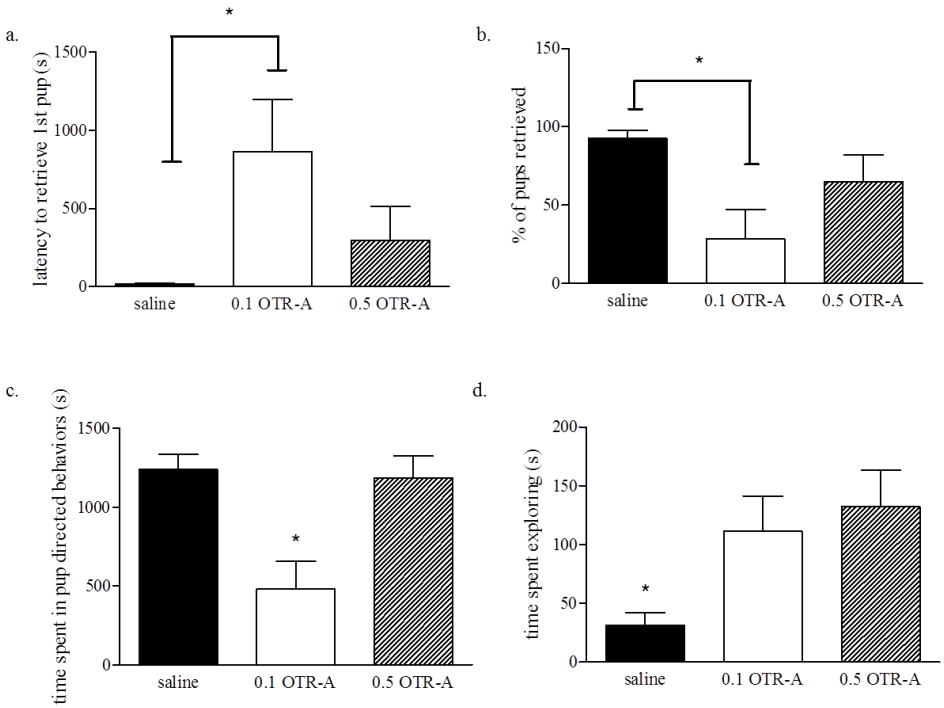

Figure 7. Blocking OTR in the mPFC impairs maternal behavior. Postpartum females receiving the lower 0.1µg/µl dose of the antagonist (OTR-A) (a) took longer to retrieve the first pup and retrieved fewer pups (b) as compared to the higher 0.5µg/µl dose of OTR-A and saline controls. The lower dose of the OTR-A also (d) decreased decrease the amount of time spent in pup directed behaviors. However, dams administered both the low and high dose of OTR-A spent (c) a significantly greater amount of time exploring the home cage compared to saline controls. Bars represent mean ± SEM; **P* < 0.05.

Blocking OTR in the mPFC of postpartum rats increases maternal aggression

Blocking OTR in the prelimbic region of the mPFC increased maternal aggression in postpartum females. Dams infused with both the low (0.1 µg/µl) and high (0.5 µg/µl) dose of the OTR-A displayed a significant decrease in the latency to attack the intruder ($F_{2, 20} = 5.38$, $p < 0.05$, Fig. 8a) and a greater number of intruder attacks ($F_{2, 20} = 7.69$, $p < 0.05$, Fig. 8b) as compared to saline controls. Additionally, dams infused with the higher dose of OTR-A had a greater number ($F_{2, 20} = 5.51$, $P < 0.05$, Fig. 8c) and duration ($F_{2, 20}$

= 3.62, P < 0.05, Fig. 8d) of pins as compared to dams infused with saline. There was a trend for the higher dose of the OTR-A to increase the time spent in contact with the intruder (p= 0.07; data not shown) but the number of intruder bites was not significant (p > 0.05; data not shown).

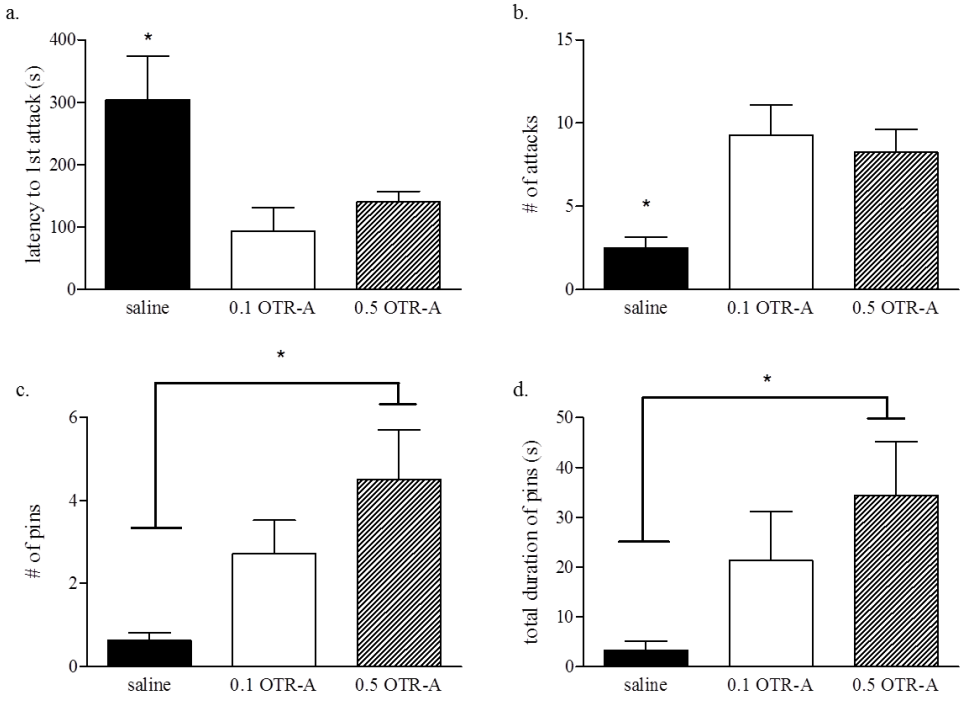

Figure 8. Blocking OTR in the mPFC enhances maternal aggression. Dams infused with both the low 0.1 µg/µl and high 0.5 µg/µl OTR-A dose displayed (a) a significant decrease in latency to attack the intruder and (b) a greater number of intruder attacks as compared to saline controls. Dams infused with the higher dose of OTR-A also exhibited (c) a greater number and (d) duration of pins. Bars represent mean ± SEM; *$P < 0.05$.

Attenuated anxiety during the postpartum period is prevented by blocking OTR in the mPFC

Blocking OTR in the mPFC prevented the postpartum attenuation of anxiety in the EPM. There was a significant main effect of reproductive state on the percentage of time that females spent in the open arms of the EPM (Fig. 9a), with postpartum females greater than diestrus virgins ($F_{1, 40} = 15.07$, $p < 0.0005$) thus indicating lower anxiety in postpartum females. There was also a significant main effect of infusion type ($F_{1, 40} = 6.97$, $p < 0.005$) and a significant reproductive state by infusion type interaction ($F_{2, 40} = 4.5$, $p < 0.05$). Post hoc analysis revealed that for postpartum rats, the group receiving the 0.5 µg/µl dose of the OTR-A spent less time in the open arms compared to both the saline and 0.1 µg/µl OTR-A groups (p's < 0.01), which were similar to each other. None of the virgin groups significantly differed from each other.

The number of entries that were made into the open arms of the EPM (Fig. 9b) showed a significant main effect of reproductive state with postpartum females making more open arm entries than diestrus virgins ($F_{1, 40} = 15.21$, $p < 0.0005$) again indicating lower anxiety in postpartum females. There was also a significant main effect of infusion type ($F_{2,40} = 6.08$, $p < 0.005$) and a marginally significant reproductive state by infusion type interaction ($F_{2, 40} = 3.11$, $p = 0.06$). Post hoc analysis revealed that for postpartum rats, the group receiving the 0.5 µg/µl dose of the OTR-A made fewer open arm entries compared to both the saline and.1 µg/µl OTR-A groups (p's < 0.01), which were similar to each other. None of the virgin groups significantly differed from each other.

The number of closed arm entries in the EPM (Fig. 9c) did not show significant main effects of reproductive state ($F_{1, 40} = 1.96$, p = .017), infusion type ($F_{2, 40} = 1.17$, p = 0.32) or a significant reproductive state by infusion type interaction ($F_{2, 40} = 0.47$, p = 0.63) indicating that locomotor activity was not affected. In contrast to the EPM, there were no significant main effects or interactions for any behaviors measured in the open field (Fig. 9d-f).

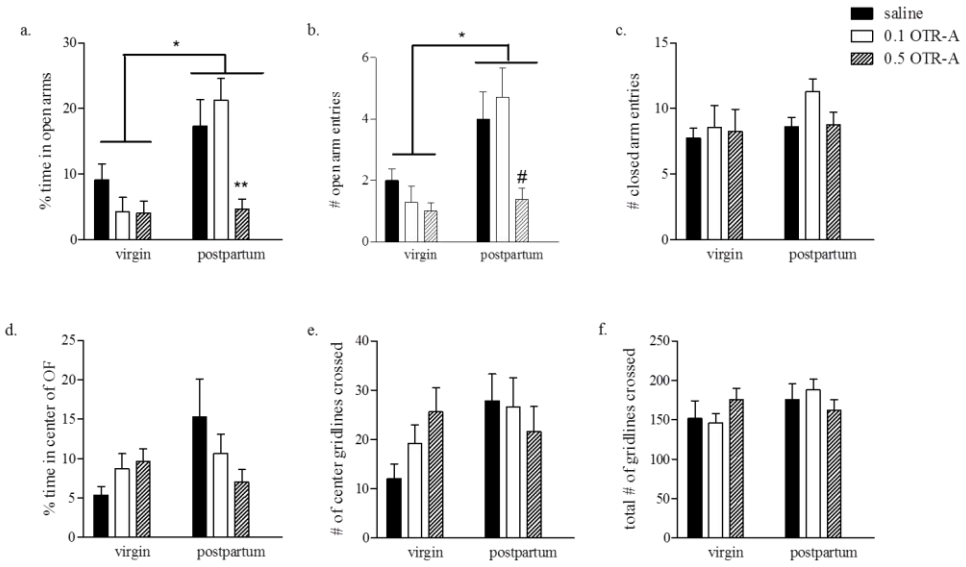

Figure 9. Blocking OTR in the mPFC at high doses enhances postpartum anxiety, but has no effect on anxiety in virgin females. Dams infused with saline or the low dose of the OTR-A antagonist in the PL region of the mPFC (a) spent a greater percentage of time in the open arms and (b) made more open arm entries as compared to virgins. In contrast, dams receiving the high 0.5 µg/µl dose of OTR-A displayed (a) a decrease in the percentage of time spent in the open arms and (b) made fewer open arm entries as compared to saline or low dose infusion dams. Locomotor activity, as measured by (c) the number of closed arm entries, was not altered. None of the virgin groups differed significantly from one another (a, b, c). In the OF, administration of OTR-A did not alter anxiety-like behavior in virgin or postpartum females as demonstrated by a similar (d) percentage of time spent in the center of the field and (e) a similar number of center gridlines crossed. Locomotor activity (f) was not affected in the OF test as measured by the total number of gridlines crossed. Bars represent mean ± SEM; * $P < 0.05$, virgin versus postpartum , ** $P < 0.05$, postpartum 0.5 µg/µl vs postpartum 0.1 µg/µl and postpartum saline , # $P = 0.06$, postpartum 0.5 µg/µl vs postpartum 0.1 µg/µl and postpartum saline.

3.4 Discussion

The present work shows that OT receptor activity within the PL region of the mPFC modulates maternal care, maternal aggression, and anxiety-like behavior during the early postpartum period. We observed that infusion of a highly specific OTR-A into mPFC of postpartum females impaired pup retrieval as well as reduced the display of pup-directed behaviors. Blockade of OT within the mPFC was also sufficient to increase maternal aggressive behavior and to prevent the reduction in anxiety typically observed during the postpartum period. Together, these findings identify the mPFC as a common brain site for the regulation of numerous postpartum-related behaviors by OT.

OT has long been examined for its role in regulating maternal care behaviors. In general, OT has been shown to be critical for the induction and maintenance of maternal behavior (Pedersen and Prange, 1979; van Leengoed et al., 1987; Insel, 1990; Pedersen et al., 1994; Neumann et al., 2000a; Smith and Merrill, 2006a; Pedersen et al., 2006; Shahrokh et al., 2010; Bosch and Neumann, 2012). There are numerous brain sites where OT acts to influence maternal care including the MPOA (Pedersen et al., 1994), ventral tegmental area (VTA; Shahrokh et al., 2010), PAG (Figueira et al., 2008), and the olfactory bulb (D'Cunha et al., 2011). The mPFC sends projections to each of these regions (Febo et al., 2010, Numan and Woodside, 2010, Pereira and Morrell, 2011) and has itself been shown to regulate some aspects of maternal behavior such as pup retrieval (Afonso et al., 2007; Febo et al., 2010). Thus, the present findings extend this prior work and identify the mPFC as another component of the "maternal circuit" (Numan and

Woodside, 2010) where OT activity can influence pup retrieval. However, unlike prior studies we also found that mothers infused with lower levels of OTR-A spent less time engaging in pup-directed behaviors that included licking, pup contact, and active and passive nursing. This discrepancy may reflect the importance of OT in these behaviors (Farbach et al., 1985; Nishimori et al., 1996; Nelson and Panksepp, 1998; Lopatina et al., 2012) or may be the result of methodological differences in the way in which the maternal behavior test was done.

Enhanced aggressive behavior toward an intruder is part of the complex pattern of maternal behavior and it too was affected when OTR activity in the postpartum mPFC was blocked. Specifically, postpartum females treated with the OTR-A displayed a reduction in the latency to attack the intruder, an increased number of intruder attacks, and pinned the intruder more often and for longer durations of time. These results suggest an inhibitory effect of OT on the aggressive behavior of postpartum females consistent with a number of previous findings showing an inverse relationship between OT and maternal aggression (Giovenardi et al, 1998; Lubin et al., 2003; Johns et al., 1994; Consiglio et al., 2005; but see Bosch et al., 2005 and Caughey et al., 2011). Furthermore, they implicate the mPFC, in addition to the PVN (Giovenardi et al., 1998), BNST (Consiglio et al., 2005), and amygdala (Lubin et al., 2003) as a brain site for OT's actions on maternal aggression. These results may seem rather surprising since OT is considered a neuropeptide that initiates maternal care behaviors. However, it is important to consider that while maternal care is a relationship between the mother and the pups, maternal aggression is an activity directed towards an adult intruder (Giovenardi et al., 1998).

Moreover, while maternal care implies affiliation and pair bonding, aggressive behaviors tend to disperse to the members of a group. Therefore, the nature of the two behaviors is different and it would not be surprising that the relationship between maternal care and maternal aggression and OT would be different (Giovenardi et al., 1998).

The postpartum period has repeatedly been shown to be a time that is associated with anxiolysis (Neumann et al., 2000a; Boccia and Pedersen, 2001; Bosch et al., 2005; Lonstein, 2007; Figueira et al., 2008). We again support these findings here by showing that lactating rats spend a greater percentage of time in the open arms of an elevated plus maze and make more entries into the open arms as compared to diestrus virgins. Although OT has frequently been examined for its role in modulating anxiety (McCarthy et al., 1996; Windle et al., 1997; Neumann et al., 2000b; Bale et al., 2001; Waldherr and Neumann, 2007; Lonstein, 2007; Figueira et al., 2008; Neumann and Landgraf, 2012), investigation into specific sites in the brain where OT acts to reduce anxiety-like behaviors in postpartum females has been limited. The PAG (Figueira et al., 2008), PVN (Jurek et al., 2012), and amygdala (Bosch et al., 2005) are the only regions that have been implicated in the OT-mediated regulation of anxiety in postpartum females. All of these regions are regulated by the mPFC (Peters et al., 2009; Numan and Woodside, 2010), express OTR (Zingg et al., 2003; Bosch et al., 2005; Lonstein, 2007, Figueira et al., 2008), have OT projections (Knobloch et al., 2012), and are sensitive to mediation by OT (Bosch et al., 2005; Figueira et al., 2008). Thus, our findings add to existing data by identifying the mPFC as another brain region mediating changes in anxiety-like behavior during the postpartum period.

In contrast to postpartum females, OTR-A infused into the mPFC of diestrus virgins did not affect the percentage of time or number of entries into the open arms of the EPM. These results are in line with previous findings demonstrating that OTR blockade decreases the percentage of time spent in open-arms by postpartum, but not virgin female rats (Neumann et al., 2000b; Neumann et al., 2000a; Figueira et al., 2008). The differential effects of OTR antagonism in lactating and virgin females likely reflects reproductive differences in OT release and OT receptor expression in many brain regions (Windle et al., 1997; Neumann et al., 2000a; Bosch et al., 2007; Macbeth and Luine, 2010). While postpartum females exhibit elevated OT receptor expression and/or peptide release, virgin females do not. Therefore, it is reasonable that OT manipulations within the mPFC only impacted anxiety-like behavior in postpartum females.

In the OF, the anxiogenic actions of OTR-A were undetectable in both lactating and virgin females. Although the EPM and OF both have an exploratory component, the EPM is considered a more sensitive test of anxiety (Hilakivi and Lister, 1990) and behavior in one test does not always predict behavior in the other (Bale et al., 2001; Bhatnagar et al., 2004). It is also possible that the inconsistencies in the OF may be related to variations in the testing conditions known to influence OF behavior (Lapiz-Bluhm et al., 2008) or differential sensitivity of the OF to the OTR-A which may require different doses than those used here for an anxiogenic effect to be revealed.

The effects of the OTR-A on the various postpartum behaviors measured were largely dose specific. The effects of OTR blockade on maternal care occurred when a 0.1µg/µl dose, but not a 0.5µg/µl dose, of OTR-A was used. The ability of the lower, but

not higher dose, of the OTR-A to modify maternal behavior is consistent with the dose-dependent but often nonlinear effect found for increased doses of neuropeptides or their antagonists on behavior (Landgraf and Neumann, 2004; Figueira et al., 2008; Miller et al., 2010; Numan and Woodside, 2010; D'Cunha et al., 2011). Such nonlinear effects may be due to many factors including a refractory state of receptors at higher doses (Neumann et al., 2000b; Landgraf and Neumann, 2004). However, this seems unlikely as the higher dose of the OTR-A seemed to have more widespread effects on maternal aggression and exclusively impacted anxiety-like behavior. Another possibility is that the dose-dependent effects of the OTR-A may be related to the fact that maternal care is considered an affiliative behavior whereas anxiety and aggression are fear/defensive behaviors. Since the nature of the behaviors we examined is different and involves different neural circuitries (Lonstein and Gammie, 2002; Gammie, 2005; Bosch and Neumann, 2012), they may be differentially sensitive to OTR blockade in the mPFC.

The interactions among anxiety, maternal care, and maternal aggression are complex. Since the postpartum period has the potential to be physically and psychologically distressing, it has been suggested that attenuated anxiety may be required for adequate display of maternal care (Lonstein, 2007). In humans, increased postpartum anxiety is associated with delayed physical growth (Barnett and Parker, 1986), impaired cognitive and social development (Galler et al., 2000), irregularities in mother-child interactions and attachment (Woodruff-Borden et al., 2002), and increased propensity for anxiety in the child (Hirschfeld et al., 1997). Similarly, anxiety-like behavior in postpartum rats has been shown to impair maternal care which in turn can have dramatic

effects on the later physiology and behavior of the offspring (Boccia and Pedersen, 2001; Lonstein, 2005; Bosch and Neumann, 2008). Although some studies have failed to support the link between increased anxiety-like behavior and deficient maternal behavior (Bosch, 2011; Curley et al., 2012), our findings show that anxiety is increased and maternal care is impaired following OTR-A in the mPFC thus supporting the possibility that decreased anxiety during the postpartum period may be critical in the display of maternal care. Similarly, decreased aggression may also be important for displaying proper maternal care (Caughey et al., 2011), and like maternal care, it has been suggested that heightened aggression may depend on a reduction anxiety such that a less anxious rat will be less hesitant to attack a potentially threatening and normally fear-evoking stimulus. Although the hypothesis that heightened aggression during lactation requires a concomitant reduction in fear and anxiety is logical, there are numerous examples where such a simple association does not exist (Maestripieri and D'Amato, 1991; Lonstein et al., 1998; Parmigiani et al., 1999; Boccia and Pedersen; 2001; Bosch et al., 2005) including the present results showing both high levels of anxiety and high levels of aggression in the postpartum females administered the OTR-A. Thus, even though reduced anxiety is often found in lactating rodents, in many cases it is neither sufficient nor necessary for their heightened aggression (Lonstein, 2005).

A large body of work over many years has identified an extensive network of brain sites underlying maternal care and maternal aggression (Lonstein and Gammie, 2002; Gammie, 2005, Numan and Woodside, 2010, Bosch and Neumann, 2012). However, in each case there has been a lack of overlap between brain regions that

modulate maternal care behaviors and those that modulate maternal aggression (Gammie, 2005). These results may be the first that indicate the mPFC as a region that modulates both of these behaviors as well as postpartum anxiety and provide new insights into the neural circuitry underlying the behavioral effects of OT during the postpartum period.

Chapter 4: Conclusion

Oxytocin has long been known for the role it plays in parturition and lactation. Not only does it act as a hormone in the periphery, but it also acts as a neuromodulator in the CNS where it can affect behaviors such as anxiety, sociability, maternal care, and maternal aggression (Pedersen et al., 1994; Neumann et al., 2000a; Lubin et al., 2003; Heinrichs and Domes, 2008; Meyer-Lindenberg et al., 2011; Mak et al., 2012). However, sites within the CNS where OT acts to modulate these behaviors are not fully understood.

Lesion studies have shown that the mPFC plays a role in regulating anxiety-like behavior (Lacroix et al., 2000; Vertes, 2004), aggression (Gammie et al., 2004; Wang et al., 2012) as well as maternal care (Afonso et al., 2007; Febo et al., 2010). The mPFC also contains OT-sensitive neurons (Ninan, 2011) and expresses OT receptors (Liu et al., 2005; Smeltzer et al., 2006). In addition, an elegant study by Knobloch and colleagues (2012) has recently shown that OT neurons of the hypothalamus have long range axonal projections to many regions of the brain, including the mPFC. Taken together, these findings suggested that the mPFC may be a region that overlaps in the modulation of all three behaviors.

In our experiments, we examined: 1) how OT in the mPFC affects anxiety-like behavior in virgin males and females (Chapter 2) and 2) how OTR blockade in the mPFC

impacts maternal care, maternal aggression, and anxiety-like behavior during the postpartum period (Chapter 3). Our results show that OT, but not the closely related neuropeptide vasopressin, reduces anxiety-like behavior in both virgin males and females. In addition, blocking OTR in the mPFC of postpartum females increases anxiety, impairs maternal behavior, and enhances maternal aggression. Overall, these results suggest that the mPFC is a site where OT acts to modulate maternal care and maternal aggression during the postpartum period as well as anxiety-related behavior regardless of sex or reproductive status and provide new insights into the neural circuitry underlying the effects of OT.

The PFC has been implicated in mood disorders, including anxiety. Among adults in the U.S., anxiety disorders have a lifetime prevalence rate of nearly 30% (Kessler et al., 2006). Additionally, the postpartum period is a time of enhanced susceptibility to anxiety disorders which afflict about 15% of new mothers (Lonstein, 2007) and have been associated with poor maternal care or increased aggression towards others. Because abnormalities in OT have been implicated in these conditions (Hoge et al., 2008; Stuebe et al., 2013), identifying the mPFC as a target for OT's actions may provide critical information about the brain regions that may be affected. Furthermore, this work may provide insight into the neural circuitry underlying some of the behavioral effects found in humans after intranasal administration of OT for the treatment of mood disorders including anxiety (Labuschange et al., 2010), as well as disorders that cause deficits in sociability including autism (Bakermans-Kranenburg and van Ijzendoorn, 2013) and schizophrenia (Meyer-Lindengerg et al., 2011).

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