THE ROLE OF OXYTOCIN RECEPTORS IN THE DORSOMEDIAL TEGMENTUM IN POSTPARTUM SOCIOEMOTIONAL BEHAVIORS

By

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ABSTRACT

THE ROLE OF OXYTOCIN RECEPTORS IN THE DORSOMEDIAL TEGMENTUM IN POSTPARTUM SOCIOEMOTIONAL BEHAVIORS

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Oxytocin signaling is well known to positively influence maternal caregiving behaviors. Therefore, oxytocin receptor (OTR) expression has been characterized in many sites of the brain across female reproductive states. However, almost all characterization of OTRs across reproduction has focused on forebrain sites, even though there are known OTRs in the midbrain dorsal raphe (DR) and periaqueductal gray (PAG) (i.e., the dorsomedial tegmentum). Ignoring these sites is surprising, given that these sites are part of the neurobiological network underlying postpartum behaviors (see background review in Chapter One). To begin to understand the role of OTRs in the dorsomedial tegmentum on postpartum behavior, the experiments in Chapter Two of this dissertation measured autoradiographic binding of the selective OTR antagonist, d(CH2)5–8-ornithine-vasotocin, across four different female reproductive states, diestrous virgins (DV), pregnancy day 10, day of parturition, and postpartum day 7 (PPD 7). OTR binding in the rostral DR and the lateral PAG were higher in recently-parturient dams compared to DV females, but these levels were returned to DV levels by PPD 7. Additionally, there was increased oxytocin-immunoreactive (ir) fiber length in the DR and PAGvl in PPD 7 dams compared to either DV or recently-parturient dams. Given the heterogenous populations of cells in the DR, expression of OTRs on three of the most abundant neuronal phenotypes in the DR, serotonin, dopamine, and GABA, were analyzed in groups of DV and recently-parturient dams. There were more dopaminergic

and serotonergic neurons containing OTR immunoreactivity in the rostral DR of recently-parturient dams compared to DV, whereas the number of glutamic acid decarboxylase-OTR colocalized cells was lower in the rostral DR of recently-parturient dams compared to DV. Overall, these data suggest that specific regions of the midbrain PAG are more sensitive to oxytocin signaling around parturition, with dopaminergic and serotonergic neurons accounting for some of that increased sensitivity. Given these changes in dorsomedial tegmentum OTR expression across the early postpartum period, their potential role in postpartum socioemotional behaviors was directly examined. An adeno-associated virus promoting the expression of shRNA against OTR mRNA was created to establish a long-term knock down of OTR expression in the dorsomedial tegmentum (Chapter Three). On pregnancy day 8, females received site-specific infusion of either OTR shRNA vector or a scrambled control vector. Following parturition, dams' socioemotional behaviors (i.e. caregiving, aggressive, anxiety-like, and depressive-like behaviors) were observed. OTR knockdown (OTRKD) in the dorsomedial tegmentum lead to higher rates of infanticide, less kyphotic nursing (i.e., nursing in an upright erect posture), and more non-pup directed behaviors. There were no effects of OTRKD on dams' retrieval performance. OTRKD in the dorsomedial tegmentum also increased postpartum aggression, decreased postpartum anxiety, and increased depressive-like behaviors. Finally, OTRKD in the dorsomedial tegmentum decreased serotonin-ir fiber length in the primary somatosensory cortex (S1). Overall, OTR expression in the dorsomedial tegmentum is sensitive to female reproductive state and modifies numerous postpartum behaviors. It may do so by affecting the S1 plasticity necessary to optimize maternal tactile sensitivity to offspring (discussed in Chapter Four).

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KEY TO ABBREVIATIONS

5,7-DHT	5,7-Dihydroxytryptamine
5-HT	serotonin
5-HIAA	5-Hydroxyindoleacetic acid
AAV	Adeno-associated virus
aq	cerebral aqueduct
BNST	bed nucleus of the stria terminalis
BOLD	Blood-oxygen-level dependent imaging
cDNA	complementary DNA
CeA	central nucleus of the amygdala
cm	centimeter
DAB	3,3'-Diaminobenzidine
DTN	dorsal tegmental nucleus
DR	dorsal raphe nucleus
DV	diestrous virgin
GAD	glutamic acid decarboxylase
GFP	green fluorescent protein
hr	hour
I.C.V.	intracerebral ventricular
IP	intraperitoneal
ir	immunoreactivity
kg	kilogram

LDT	laterodorsal tegmental nucleus
lOFC	lateral orbitofrontal cortex
M1	primary motor cortex
mg	milligram
min	minute
μl	microliter
μm	micrometer
mPOA	medial preoptic area
NAc	nucleus accumbens
NDS	normal donkey serum
NGS	normal goat serum
OT	oxytocin
OTR	oxytocin receptor
OTRKD	oxytocin receptor knockdown
PAG	periaqueductal gray
PAGd	dorsal periaqueductal gray
PAGdl	dorsolateral periaqueductal gray
PAGl	lateral periaqueductal gray
PAGvl	ventrolateral periaqueductal gray
Part	recently-parturient dam
PBS	phosphate-buffered saline
pCBA	chicken beta-actin promotor/cytomegalovirus enhancer promotor hybrid
PCPA	para-chlorophenylalanine

PCR	polymerase chain reaction
PMT	photomultiplier tube
PPD	postpartum day
Preg	pregnancy
PVN	paraventricular nucleus
RF	rhinal fissure
s	second
S1	primary somatosensory cortex
SEM	standard error of the mean
shRNA	short hairpin RNA
SSRI	selective serotonin reuptake inhibitor
TH	tyrosine hydroxylase
TPH	tryptophan hydroxylase
VTA	ventral tegmental area

CHAPTER 1: INTRODUCTION

Historical perspective on the role of oxytocin in maternal behaviors

The neuropeptide, oxytocin, is a nonapeptide mainly produced by cells in the paraventricular and supraoptic nuclei of the hypothalamus (Gimpl and Fahrenholz, 2001; Jurek and Neumann, 2018; Mohr et al., 1988; Swaab et al., 1975; Vandesande and Dierickx, 1975). Oxytocin has historically been connected to the processes involved with the physiology and behavior of motherhood and, consistent with this, one of the earliest functional roles for oxytocin described by Sir Henry Dale in 1906 was the ability of oxytocin to induce the uterine contractions necessary for parturition (Dale, 1906). Indeed, this ability of oxytocin to induce parturition is where the name oxytocin is derived, Greek for *oxys* and *toketos*, meaning “quick birth” (Kamm et al., 1928). Oxytocin’s role in maternal physiology was extended soon thereafter, as oxytocin was found to induce milk letdown in cows (Ott and Scott, 1910) and sheep (Sharpey-Schafer and Mackenzie, 1911). Given oxytocin’s role in the physiology of mothering, it is surprising that it wasn’t until 1968 that evidence for a role of oxytocin in modifying postpartum behavior was suggested by Peter Klopfer. Klopfer found that in goats, immediately replacing a doe’s kid with an adoptive kid at parturition could induce caregiving towards the adoptive kid (Klopfer and Klopfer, 1968); this normally does not occur in goats because they form a singular selective bond with their own offspring (Collias, 1956; Hersher et al., 1963). Klopfer postulated that the oxytocin released during parturition in response to distension of the cervix/vagina was important in rapidly inducing caregiving to any present offspring (Fitzpatrick, 1961; Folley and Knaggs, 1965; Fuchs, 1964; Klopfer and Klopfer, 1968; Klopfer, 1971). This

parsimonious idea associating oxytocin with caregiving then sparked five decades of investigation into the behavioral role of oxytocin in nearly all aspects of maternal behavior.

Oxytocin and maternal caregiving

Oxytocin and maternal caregiving in rats

In most species of mammals, maternal caregiving is important for offspring survival and includes a stereotyped repertoire of behaviors. In many small rodents, these behaviors include nest building (building a walled area to safely contain pups and help them thermoregulate), retrieving displaced pups to the nest, licking the pups (to clean them, facilitate urination and defecation, and prepare them for nursing), and nursing the pups (during which the transfer of milk occurs) (Bridges, 2015; Lonstein et al., 2014; Rosenblatt, 1969). In laboratory rats, all these caregiving behaviors have a rapid onset during very late pregnancy into parturition and require a change in the mother's response to offspring - from avoidance to offspring approach (Bridges, 2015; Numan and Insel, 2003; Rosenblatt and Mayer, 1995; Wiesner and Sheard, 1933). In fact, virgin female rats and numerous other mammals will usually avoid the aversive sensory cues emitted by pups (Fleming and Luebke, 1981; Rosenblatt, 1967), while late-pregnant females begin to approach pups (Slotnick et al., 1973), and finally postpartum dams show highly motivated caregiving (Fleming et al., 1994a; Hansen, 1994; Lee et al., 1999; Pereira et al., 2005). Given that the first experiment to suggest a specific role of oxytocin in maternal caregiving was published in 1968 (Klopfer and Klopfer, 1968), it was surprising that it took over a decade for the first paper to report a positive relationship between oxytocin and mothering to be published (Pedersen and Prange, 1979). This delay was likely due to

experimental approaches that only manipulated peripheral oxytocin. For example, infusing oxytocin intravenously in virgin female rats had been found to have no effect on the maintenance of caregiving (Rosenblatt, 1969), and deafferentation of the neurohypophysis in late pregnancy did not affect pup retrievals or nursing behavior (Herrenkohl and Rosenberg, 1974). However, once it was clear that oxytocin doesn't cross the blood-brain-barrier and that there are central oxytocin projections in addition to projections to the neurohypophysis (Buijs, 1978; Sofroniew, 1983a), a role for central oxytocin in maternal behavior was quickly assessed. It was found that oxytocin injected intracerebral ventricularly (I.C.V) could induce the full range of maternal behaviors in ~40% of the virgin females tested within hours after infusion (Pedersen and Prange, 1979). Furthermore, when analyzing the estrous state of the females that did display caregiving, almost all were in high estradiol phases of their cycles (i.e. late diestrous, proestrous, and estrous), suggesting a role for estradiol-priming in these pro-maternal effects of oxytocin. Estrogen had been previously shown to induce caregiving in and of itself (Moltz et al., 1970; Siegel and Rosenblatt, 1975a, 1975b, 1978), so to address whether estrogens were necessary for oxytocin to induce caregiving, a follow-up experiment was conducted in which female rats were subcutaneously injected with estradiol benzoate 48 hours before I.C.V. oxytocin infusion. It was found that this estrogen priming increased the percentage of virgin females displaying full maternal behavior from ~40% to ~85%. This was the first work to demonstrate the key role of estradiol for oxytocin's ability to promote caregiving, and now it's well known that estradiol is one of the most potent upregulators of central OTRs (Bale and Dorsa, 1997; Champagne et al., 2001).

Following this work, many others became interested in the role of oxytocin in caregiving behaviors, and while a number of studies found that I.C.V. oxytocin could facilitate caregiving in estrogen-primed virgin female rats (Fahrbach et al., 1984, 1986; Pedersen et al., 1982), there were other groups who were unable to replicate this finding (Bolwerk and Swanson, 1984; Fahrbach et al., 1984; Rubin et al., 1983; Wamboldt and Insel, 1987). These discrepancies were probably due to multiple factors, including the testing paradigm and the strain of rat used. For example, if donor pups were given to a virgin female rat in their homecage, some labs found that oxytocin was unable to induce caregiving (suggesting that pre-test stress was relevant for the effects of oxytocin) (Bolwerk and Swanson, 1984; Rubin et al., 1983), while I.C.V. infusion of oxytocin after a 1 - 2 hour habituation to a new testing cage was able to induce caregiving (Fahrbach et al., 1984, 1986; Pedersen et al., 1982; Pedersen and Prange, 1979). Additionally, all virgin female rats that displayed mothering in response to oxytocin were Sprague Dawley rats from the Zivic Miller laboratories (a well-known laboratory animal vendor at the time) (Fahrbach et al., 1984, 1986; Pedersen et al., 1982; Pedersen and Prange, 1979), which were known to be infected with pulmonary pathogens that might have rendered then partially anosmic (Pedersen et al., 1992; Wamboldt and Insel, 1987). Given that maternal behavior in virgin rats is under inhibitory control by pup odors, such that lesions of the olfactory bulb or chemically inducing anosmia with zinc sulfate can help facilitate caregiving (Fleming and Rosenblatt, 1974a, 1974b), oxytocin was proposed to be able to induce maternal behavior in virgin female rats only under anosmic conditions when they were already primed to respond to pups (Fahrbach et al., 1984; Fahrbach et al., 1985; Pedersen et al., 1982; Pedersen and Prange, 1979; Wamboldt and Insel, 1987). It should

be noted, however, that while oxytocin's ability to induce maternal behavior might only occur under certain conditions, oxytocin is still a component of estrogen-induced caregiving, as I.C.V. infusion of an anti-oxytocin antiserum decreases the percentage of estrogen-primed virgin females displaying full maternal behavior within two hours of infusion (from ~83% to ~38%) (Pedersen et al., 1985), and I.C.V infusion of an OTR antagonist, d(CH2)5–8-ornithine-vasotocin, increases the latency for estrogen-primed virgin female rats to display full maternal behavior (Fahrbach et al., 1985).

While the role of oxytocin in inducing caregiving behavior in virgin female rats is complicated, the role of oxytocin in mothering in pregnant and peripartum females is much clearer. Electrolytically lesioning the paraventricular nucleus (PVN) on pregnancy day 15 decreases later postpartum pup retrieval, nursing, and nestbuilding (Insel and Harbaugh, 1989; however, see Numan and Corodimas, 1985), while kainic acid lesions of the PVN on PPD 2 only impair pup retrieval (Olazabal and Ferreira, 1997). The behavioral effects of lesioning the PVN are regulated, at least in part, by the loss of oxytocinergic cells as central infusion of an anti-oxytocin antiserum or an OTR antagonist in pregnancy-terminated rats (a model used for rapidly inducing maternal caregiving (Rosenblatt and Siegel, 1975)) or recently parturient dams also block the onset of maternal behavior (Fahrbach et al., 1985; Van Leengoed et al., 1987). This suggests that oxytocin has a key role in initiating maternal behavior under natural conditions.

Oxytocin also modulates maternal caregiving during the postpartum period, as I.C.V. infusion of an OTR antagonist decreases pup licking, kyphotic nursing, and the amount of time dams spend interacting with their litter (Bosch and Neumann, 2008; Pedersen and Boccia, 2003). Furthermore, infusing an OTR antagonist I.C.V. in

postpartum dams that naturally display high levels of pup licking decreases their pup licking to similar levels as dams that naturally display low levels of pup licking (Champagne et al., 2001). Therefore, oxytocin is most relevant for inducing caregiving in reproducing female rats (Fahrbach et al., 1985; Insel and Harbaugh, 1989; Van Leengoed et al., 1987), but thereafter only somewhat modulates caregiving (Bosch and Neumann, 2008; Champagne et al., 2001; Olazabal and Ferreira, 1997; Pedersen and Boccia, 2003). Importantly, there is a ceiling effect on how oxytocin evokes caregiving, as I.C.V. infusion of oxytocin cannot further enhance caregiving in most postpartum animals once the behavior is established (Fahrbach et al., 1985). However, in animals that are specifically bred to display less caregiving behaviors, oxytocin increases kyphotic nursing (i.e. nursing in an upright arch-backed posture) (Bosch and Neumann, 2008).

Oxytocin and maternal caregiving in mice

In contrast to laboratory rats, the role of oxytocin for caregiving in laboratory mice is less clear. A null mutation of the oxytocin gene in postpartum mice completely abolishes milk letdown without impairing pup retrieval (Nishimori et al., 1996), but in virgin mice this null mutation decreases pup licking and retrieval in response to foster pups (Pedersen et al., 2006). Additionally, behavioral deficits after oxytocin's absence might only be observed under stressful conditions, as oxytocin knockout dams under food and water restriction have higher rates of infanticide (100%) compared to wildtype mice (16%) under the same conditions (Ragnauth et al., 2005).

The relative lack of behavioral effects seen in oxytocin knockout mice might be, in part, related to compensatory mechanisms. Indeed, I.C.V. infusion of the oxytocin-related

neuropeptide, vasopressin, induces the full range of maternal behaviors in 55% of hormonally-primed virgin female rats (Pedersen et al., 1982), and increases kyphotic nursing in postpartum rats (Bosch and Neumann, 2008). Therefore, vasopressin might substitute for oxytocin in these mice. Vasopressin might achieve this by binding to OTRs (Chini and Manning, 2007; Manning et al., 2012), as a null mutation of the OTR gene does decrease time spent nursing in kyphosis and increase pup retrieval latencies in postpartum mice (Takayanagi et al., 2005). However, decreased caregiving behavior in OTR knockout mice are not consistently found in the literature, and others have found that OTR knockout mice display normal maternal behavior (Macbeth et al., 2010; Rich et al., 2014; Yoshihara et al., 2017), even if 67% of them have dead pups in the cage (either through infanticide or lack of caregiving) compared to 20% of wild type controls (Rich et al., 2014). This increased pup mortality appears to be related to oxytocin's role in behavior, as forebrain-specific OTR knockout (which allows postpartum mice to let down milk) increases pup mortality to 40% compared to 10% for wildtype controls (Macbeth et al., 2010).

In addition to compensatory mechanisms by other neuropeptides, the lack of a robust effect of genetic manipulations of the oxytocin system (the ligand or receptor) on postpartum behavior is probably largely due to using mice as the model species. Laboratory mice of many strains are often spontaneously maternal (Jakubowski and Terkel, 1982; Mann et al., 1983; McCarthy and Vom Saal, 1985), which limits the possible positive influences of oxytocin on caregiving. Interestingly, in strains where infanticide is high, such as wild mice, I.C.V. infusion of oxytocin decreases infanticide (McCarthy, 1990). That is, oxytocin is able to push female mice that are prone to be infanticidal to a

maternal state (Macbeth et al., 2010; McCarthy, 1990; Ragnauth et al., 2005; Rich et al., 2014), but does not appear to be particularly important in the onset of caregiving behavior in mice that already show a high baseline of spontaneous caregiving, or modulate it after the behavior has already been initiated (Macbeth et al., 2010; Pedersen et al., 2006; Rich et al., 2014). This all-or-none aspect of oxytocin's role in maternal behavior in mice, therefore, makes rats and other species that are unlikely to spontaneously show positive responses to pups much better models for understanding the more nuanced role of oxytocin in caregiving.

Oxytocin and maternal caregiving in sheep

Oxytocin plays a particularly prominent role in the induction of caregiving behavior in sheep. While many species will display caregiving immediately or after a short habituation period (Jakubowski and Terkel, 1982; Mann et al., 1983; McCarthy and Vom Saal, 1985; Rosenblatt, 1967), non-reproductive ewes will typically reject lambs, which involves headbutting and withdrawing from the lamb (Collias, 1956; Hersher et al., 1963). Immediately following parturition, however, ewes will begin to care for offspring (Collias, 1956; Hersher et al., 1963). Oxytocin is centrally released during parturition in response to vaginal distension (Flint et al., 1975; Kendrick et al., 1988a; Kendrick et al., 1992; Kendrick et al., 1988b; Kendrick et al., 1986; Levy et al., 1995), and mimicking parturition by artificially stimulating the vaginas of non-pregnant, ovarian hormone-primed ewes rapidly induces caregiving of a foster lamb (Keverne et al., 1983). The rapid induction of caregiving after vaginal stimulation is likely caused by simultaneous peripheral and central oxytocin release, as I.C.V. infusion of oxytocin also facilitates maternal licking and

nursing of lambs by estrogen-primed ewes (Kendrick et al., 1987; Keverne and Kendrick, 1992; Levy et al., 1992). Conversely, parturient ewes given peridural anesthesia, which prevents oxytocin release (Levy et al., 1992), have more rejection of their lamb (Krehbiel et al., 1987).

Oxytocin and maternal caregiving in humans

Oxytocin is also often positively associated with maternal caregiving behaviors in humans. Plasma oxytocin levels during the third trimester of pregnancy are positively correlated with gazing at the infant, positive affect, affectionate touch with the infant, and motherese ("baby" talk) vocalizations following parturition (Feldman et al., 2007). Similarly, increases in plasma oxytocin levels across pregnancy are positively associated with mother-fetal attachment scores, indicative of positive representations/affect towards the arriving newborn (Levine et al., 2007). These studies suggest a role for oxytocin in the prepartum organization of the maternal brain.

Postpartum, plasma oxytocin levels across the first six months are also positively correlated with caregiving (Feldman et al., 2007; Gordon et al., 2010), mother-infant synchrony ("episodes where mothers and infants coordinate their positive social engagement") (Atzil et al., 2011), and levels of affectionate touch between the mother with her infant (Feldman et al., 2010). Furthermore, intranasal oxytocin administration decreases activation of the maternal insula in response to infant crying, which might help to mitigate negative emotionality to her infant (Riem et al., 2011), while it increases connectivity between the amygdala and the orbitofrontal cortex, anterior cingulate cortex, and hippocampus, which might function to increase positive emotionality caused by infant

laughter (Riem et al., 2012). Intranasal oxytocin in human mothers also enhances activation of the mesolimbic system (Atzil et al., 2011) and decreases activation of negative emotion-related structures, such as the insula (Riem et al., 2011; Riem et al., 2012). This corresponds to what is thought to occur in rats, in which caregiving requires both an increase in pup approach and a decrease in pup avoidance (Bridges, 2015; Numan and Insel, 2003; Rosenblatt and Mayer, 1995; Wiesner and Sheard, 1933). Interestingly, one study found that oxytocin release is higher in human mothers who are less sensitive to their infant's needs (Elmadih et al., 2014), suggesting that overly high levels of oxytocin might interfere with caregiving. One major caveat to all studies of oxytocin in human mothering is that plasma oxytocin levels, which is invariably measured, are not a proxy for neural levels (Kagerbauer et al., 2013). Cerebral spinal fluid and plasma oxytocin levels are often not correlated with each other (Altemus et al., 2004; Amico et al., 1990; Amico et al., 1983; Takeda et al., 1985), and plasma oxytocin does not cross into the brain in appreciable amounts (i.e., estimated ~1% of plasma oxytocin crosses into the brain) (Ermisch et al., 1985a; Ermisch et al., 1985b; Mens et al., 1983). Therefore, while plasma oxytocin is positively related to caregiving in humans, human studies should be interpreted with caution if the goal is to understand central control of caregiving.

Central location of oxytocin's positive role on maternal caregiving

Specific sites in the brain where oxytocin acts to positively influence caregiving have been widely studied. Two of the best-studied areas in the control of maternal caregiving are the medial preoptic area (mPOA) (Lee et al., 1999; Numan, 1974; Numan

et al., 1988; Numan et al., 1977; Numan and Stolzenberg, 2009; Pereira and Morrell, 2009) and the ventral tegmental area (VTA) (Gaffori and Le Moal, 1979; Hansen et al., 1991; Numan and Smith, 1984; Numan et al., 2009). Both the mPOA (Fleming et al., 1994b; Lonstein et al., 1997; Luckman, 1995; Numan and Sheehan, 1997) and VTA (Febo et al., 2005) of postpartum rats are activated during dam-pup interactions, with the mPOA showing increased expression of the immediate-early gene product Fos (Fleming et al., 1994b; Lonstein et al., 1997; Luckman, 1995; Numan and Numan, 1995, 1997; Numan and Sheehan, 1997), and the mPOA and VTA showing increased functional MRI BOLD signal in response to suckling (Febo et al., 2005). Interestingly, this enhanced BOLD signal in both sites is at least partly driven by oxytocin as I.C.V. infusion of an OTR antagonist prevents the suckling-induced BOLD signal (Febo et al., 2005). OTR mRNA and autoradiographic binding are higher in the mPOA and VTA of postpartum rats when compared to virgins (Bosch et al., 2010; Caldwell et al., 1994; Caughey et al., 2011; Meddle et al., 2007; Pedersen et al., 1994), and inhibiting these receptors by site-specific administration of an OTR antagonist delays the onset of maternal caregiving in recently-parturient laboratory rats (Pedersen et al., 1994). Similarly, infusing an OTR antagonist into the mPOA during the first 5 days postpartum decreases time spent kyphotic nursing by over 50% (Bosch and Neumann, 2012). OTR expression in the mPOA is also related to natural differences in caregiving, with high kyphotic nursing/pup licking dams having higher OTR binding in their mPOA compared to low kyphotic nursing/pup licking dams (Champagne et al., 2001; Francis et al., 2000), and the differences in behavior can be eliminated by I.C.V. infusion of an OTR antagonist (Champagne et al., 2001). Therefore, the mPOA and VTA are critical sites where oxytocin can act to positively influence

caregiving behavior. However, many brain sites are involved in the expression of maternal caregiving behaviors (Bridges, 2015; Lonstein et al., 2014; Rosenblatt, 1969) and express OTRs (Yoshimura et al., 1993), suggesting that many yet unexplored brain sites probably underlie oxytocin's facilitation of mothering.

Summary

Overall, while the role of oxytocin in inducing caregiving in virgin rats only occurs under specific experimental conditions, in periparturient rats, oxytocin is a key promoter of the onset of maternal behavior and also modifies caregiving once established. Additionally, rats are an excellent model to study oxytocin's role in mothering, as the spontaneously maternal/infanticidal aspects of caregiving in mice precludes detailed analyses of oxytocin's role in caregiving in many strains. Finally, a handful of brain sites expressing OTRs have been studied for where oxytocin positively influences maternal behavior, but, there are probably many more sites that have, so far, been neglected. Studying such sites is critical for understanding how oxytocin influences caregiving and might provide insights into the discrepancies in the behavioral effects of global versus localized oxytocin manipulations on mothering.

Oxytocin and postpartum aggression

While the relationship between oxytocin and caregiving has been widely studied, the relationship between oxytocin and postpartum aggression has received much less attention. During the postpartum period, dams display aggression towards conspecifics, presumably with the goal of protecting the offspring from infanticide (Agrell et al., 1998;

Wolff, 1985, 1993). Postpartum aggression in laboratory rats peaks during the first week of lactation and diminishes as weaning approaches (Caughey et al., 2011; Erskine et al., 1980b; Flannelly and Flannelly, 1987). Lesions of the PVN and its oxytocin cells have been reported to either decrease postpartum aggression (Consiglio and Lucion, 1996), increase postpartum aggression (Giovenardi et al., 1998), or have no effect on it (Olazabal and Ferreira, 1997). Discrepancies between these studies might be, in part, due to the type and extent of the lesions performed, with electrolytic lesions that damage the entire PVN and fibers of passage decreasing postpartum aggression (Consiglio and Lucion, 1996), but ibotenic acid lesions that spare the magnocellular oxytocin neurons increasing postpartum aggression (Giovenardi et al., 1998). Similar inconsistencies have also been found when examining the specific role of oxytocin in postpartum aggression, as infusion of oxytocin mRNA antisense into the PVN of postpartum rats increases postpartum aggression (Giovenardi et al., 1998), but neither I.C.V. infusion of oxytocin nor an OTR antagonist change maternal aggression in postpartum laboratory rats (Neumann, 2001; Neumann et al., 2001). These mixed results indicate that oxytocin acts in many brain sites to affect maternal aggression. In support, infusion of oxytocin into the central nucleus of the amygdala (CeA) increases maternal aggression towards an intruder male (Ferris et al., 1992; however, see Lubin et al., 2003), and endogenous oxytocin release in the CeA is positively correlated to aggressive behaviors in dams (Bosch et al., 2005). On the other hand, infusion of oxytocin into the bed nucleus of the stria terminalis (BNST) (Consiglio et al., 2005) or the medial prefrontal cortex (mPFC) (Sabihi et al., 2014a) decreases maternal aggression.

Oxytocin and postpartum anxiety-like behavior

During the early postpartum period, female rats display especially low levels of anxiety-like behaviors. Dams spend more time in the center of an open field (Fleming and Luebke, 1981; Toufexis et al., 1999) and in the open arms of an elevated plus maze (Kellogg and Barrett, 1999; Lonstein, 2005; Yang et al., 2015), display less freezing in response to an acoustic startle stimulus (Hård and Hansen, 1985; Toufexis et al., 1999), and show decreased burying of an electrified probe (Picazo and Fernandez-Guasti, 1993) compared to virgins. These low levels of anxiety are thought to help facilitate the acceptance of pups that underlies maternal caregiving (Fleming and Luebke, 1981; Hård and Hansen, 1985; Lonstein, 2005, 2007).

Numerous neurochemicals likely act together to reduce postpartum anxiety (Lonstein, 2007), one of which is oxytocin. In nulliparous rats and mice, oxytocin is a potent anxiolytic (Amico et al., 2004; Bale et al., 2001; Francis et al., 2000; Jurek and Neumann, 2018; Mantella et al., 2003; McCarthy et al., 1996; Sabihi et al., 2014b; Waldherr and Neumann, 2007; Windle et al., 1997; Windle et al., 2006). It is not surprising, then, that oxytocin is also anxiolytic in reproductive females (Figueira et al., 2008; Neumann et al., 1999; Sabihi et al., 2014a). I.C.V. infusion of an OTR antagonist increases dams' anxiety-like behaviors on an elevated plus maze (Neumann et al., 1999). Similarly, infusion of an OTR antagonist into the medial prefrontal cortex (mPFC) (Sabihi et al., 2014a) or ventrolateral periaqueductal grey (PAGvl) (Figueira et al., 2008) increases dams' anxiety-like behavior, while dams selectively bred to express high anxiety levels have more oxytocin release into the CeA (area intimately involved in fear

and possibly anxiety (Walker et al., 2003)) in response to a social stressor (Bosch et al., 2005).

In women, low plasma oxytocin is associated with higher levels of postpartum anxiety (Nissen et al., 1998; Uvnäs-Mobcrg et al., 1990), while total plasma oxytocin released during a nursing bout is inversely related to anxiety (Stuebe et al., 2013). Postpartum anxiety in women is also reduced by infant holding even without breastfeeding and milk letdown (Heinrichs et al., 2001), which suggests a central action of oxytocin in reducing postpartum anxiety. Higher plasma levels of serum prolyl endopeptidase, which enzymatically degrades serum peptides (including oxytocin), is associated with higher anxiety in postpartum women (Maes et al., 2000). However, as stated earlier, a major caveat to the study of oxytocin in human maternal anxiety, is that plasma levels of oxytocin are not an accurate reflection of central levels of oxytocin (Altemus et al., 2004; Amico et al., 1990; Amico et al., 1983; Kagerbauer et al., 2013; Takeda et al., 1985). In any case, studies demonstrate that oxytocin decreases anxiety in both rodent and human mothers and does so by acting in a site-specific manner.

Serotonin, dorsal raphe, and maternal behaviors

Most work investigating the neurobiological underpinnings of maternal behaviors has focused on steroid hormones and neuropeptides (Bridges, 2015; Lonstein et al., 2014; Numan and Insel, 2006), with considerably less work examining the role of classic neurotransmitters, especially serotonin. Serotonin is a strong candidate for further study of the regulation of maternal behavior because serotonin metabolism is higher in several pro-maternal brain sites in postpartum female rats compared to prepartum or virgin

females (Lonstein et al., 2003; Smith et al., 2013). Furthermore, blockade of serotonin 2A/2C receptors decreases dams retrieval performance, pup licking, and nursing, and this effect can be prevented with the serotonin 2A/2C agonist, dimethoxy-4-iodoamphetamine (Li et al., 2004; Zhao and Li, 2009; Zhao and Li, 2010). Global activation of either serotonin 2C (Chen et al., 2014; Wu et al., 2016) or serotonin 2A receptors (Gao et al., 2018; Wu et al., 2018), with selective agonists (i.e., MK212 and TCB-2, respectively) also interferes with caregiving. These results should be interpreted carefully, because the drugs were administered intraperitoneally and globally affect serotonin 2C and 2A receptors, although they do suggest a complex interplay of serotonin receptors in facilitating and inhibiting maternal caregiving in rats.

The source of serotonin relevant for maternal behaviors is likely the midbrain dorsal raphe (DR), which accounts for a vast majority (~80%) of the forebrain-projecting serotonergic fibers (Hensler et al., 1994; Lowry et al., 2008; Steinbusch, 1981). The DR projects to many pro-maternal forebrain sites, such as the mPOA, the BNST, and the nucleus accumbens (NAc) (Azmitia and Segal, 1978; McDevitt et al., 2014; Muzerelle et al., 2016; Vertes, 1991). Parturient and lactating rats have higher Fos expression in the DR when compared to diestrus virgins (Lin et al., 1998; however, see Lonstein and Stern, 1997a), and the firing rates of DR serotonin neurons is 63% higher in lactating than in nulliparous females (Klink et al., 2002). These DR serotonergic neurons are important for caregiving behaviors, as lesioning them increases the incidence of pup mortality and affects the types of nursing postures displayed across lactation (Barofsky et al., 1983; Holschbach et al., 2018). Furthermore, a null mutation of the *Pet-1* gene (transcription factor that determines a serotonergic cell type fate) or tryptophan hydroxylase 2 (the rate-

limiting enzyme in serotonin synthesis), results in high rates of pup death in mice, which was apparently caused by decreased kyphotic nursing, nest building, and pup retrieval/huddling together rather than lactational deficits (Angoa-Pérez et al., 2014; Lerch-Haner et al., 2008).

Serotonin is also critical for postpartum aggression. Selective lesions of the serotonergic neurons in the DR of postpartum rats reduces the frequency of attacks and the duration of attacks by 40% and 69%, respectively, when compared to control dams (Holschbach et al., 2018). Similarly, activating serotonin autoreceptors with an I.P. injection of the serotonin 1A receptor agonist, 8-OH-DPAT decreases the frequency of attacks (De Almeida and Lucion, 1994; Ferreira et al., 2000; however, see Da Veiga et al., 2011). On the other hand, treatment with the selective serotonin reuptake inhibitor (SSRI), fluoxetine, which increases synaptic serotonin concentrations, increases maternal aggression (Johns et al., 2005). The behavioral effects of serotonin on maternal aggression are site-specific as injecting the serotonin receptor 1A agonist, 8-OH-DPAT, or the serotonin 2A/2C agonist, α-methyl-5-hydroxytryptamine, into the CeA or medial septum also increases maternal aggression (Almeida et al., 2006; De Almeida and Lucion, 1997), whereas injecting these compounds into the medial amygdala or dorsal PAG decreases postpartum aggression (De Almeida et al., 2005; De Almeida and Lucion, 1997).

Anxiety, in general, is positively influenced by serotonin. Lesions of the serotonergic raphe nuclei (Andrade and Graeff, 2001; Briley et al., 1990; File et al., 1979), or depletion of tryptophan hydroxylase with para-chlorophenylalanin (PCPA) (Geller and Blum, 1970), reduce anxiety-like behavior in male rodents. Similarly, activating

autoreceptors on serotonergic neurons in the DR increases time they spend in the light side of a light/dark box (Higgins et al., 1988; Schreiber and De Vry, 1993; Sena et al., 2003). Given the large literature linking serotonin with anxiety in nulliparous animals (Gordon and Hen, 2004; Żmudzka et al., 2018), there is surprisingly little research investigating the role of serotonin in postpartum anxiety. Similar to other behaviors, it depends on the brain sites/receptors on which serotonin is acting. For example, activating serotonin 2C receptors in the basolateral amygdala increases anxiety-like behaviors in male laboratory rats (Anderson et al., 2002; Campbell and Merchant, 2003), while activating serotonin 2A receptors in the basolateral amygdala decreases it (Maisonnette et al., 2000; Zangrossi and Graeff, 1994).

It has been postulated that anxiety-like behavior might be under the control of different subpopulations of DR serotonergic neurons (Lowry and Hale, 2010). There is already evidence for subpopulations of serotonin neurons being involved in specific behaviors, as only ~25% of DR serotonin neurons respond to oral movements, such as chewing and grooming (Fornal et al., 1996), and distinct subpopulations of DR serotonergic neurons respond to motor and sensory tasks (Ranade and Mainen, 2009). In reproductive females, subpopulations of serotonergic neurons might be regulated by ovarian hormones, as only some serotonergic neurons express estrogen and progestin receptors (Alves et al., 1998; Nomura et al., 2005; VanderHorst et al., 2005). Estrogen-treatment in ovariectomized female rats increases serotonin synthesis and concomitantly reduces anxiety-like behavior (Hiroi et al., 2011), while ovariectomy prevents the anxiolytic actions of SSRIs and estrogen replacement restores it (Charoenphandhu et al., 2011). Estrogens also increase the expression of tryptophan hydroxylase in the DR of

ovariectomized virgin rats (Charoenphandhu et al., 2011; Donner and Handa, 2009; Hiroi and Neumaier, 2009), and both estrogens and progesterone decrease serotonin 1A receptor mRNA and binding (Hiroi and Neumaier, 2009; Lu and Bethea, 2002; Pecins-Thompson and Bethea, 1999), serotonin transporter mRNA (Pecins-Thompson et al., 1998), and serotonin's degradation enzyme monoamine oxidase A (Gundlah et al., 2002; Smith et al., 2004) in the DR of ovariectomized rhesus macaques and mice. Therefore, ovarian hormones present in reproductive rats likely upregulate the serotonergic system.

Overall, the serotonergic DR is critical for the display of postpartum behavior. Given that the DR expresses OTRs in rats (Yoshimura et al., 1993), oxytocin might regulate serotonergic output from the DR in reproductive female rats. Therefore, the DR is a strong candidate for further studying oxytocin's role in regulating postpartum behaviors.

Interactions between oxytocin and serotonin

In recent years there has been a growing literature suggesting that oxytocin and serotonin interact to influence socioemotional behaviors. In male mice, serotonergic fibers originating from the DR project to the NAc and express OTRs that facilitate local serotonin release (Dölen et al., 2013). Conversely, oxytocin administered either I.C.V. or intranasally can decrease serotonin release in the amygdala of rhesus macaques (Arthur et al., 2017) and the amygdala and orbitofrontal cortex of humans (Mottolese et al., 2014). In both rhesus macaques and humans, oxytocin-mediated reduction of serotonin release in the amygdala and orbitofrontal cortex was associated with decreased serotonin 1A receptor displacement binding in those sites, suggesting that oxytocin-mediated

decreases in serotonin release might increase activation of these affect-associated regions (Arthur et al., 2017; Mottolese et al., 2014). OTRs expressed on serotonergic neurons are functionally important, as oxytocin infused into the raphe nuclei increases intra-raphe serotonin release and decreases anxiety-like behaviors in male mice (Yoshida et al., 2009). Knocking out OTR expression specifically on serotonergic neurons (including oxytocin receptors expressed in the DR and median raphe) does not affect maternal behavior in postpartum female mice (Pagani et al., 2015). However, as stated earlier, oxytocin is not a potent stimulator of maternal behavior in most strains of mice, anyway (Macbeth et al., 2010; Pedersen et al., 2006; Rich et al., 2014). Additionally, their mice were of the C57BL/6J strain, which are spontaneously maternal and might have precluded any effects of OTR knockout (Jakubowski and Terkel, 1982; Mann et al., 1983). Furthermore, OTR expression on serotonergic neurons of the raphe nuclei differs between rats and mice, with mice expressing OTRs on serotonergic cells in both the DR and median raphe (Yoshida et al., 2009), while rats only express OTRs in the DR (Yoshimura et al., 1993). Therefore, examining the relationship between oxytocin signaling in the DR and postpartum behaviors in laboratory rats would better elucidate oxytocin's role in maternal caregiving and associated behaviors. Finally, it should be noted that there is a reciprocal relationship between oxytocin and serotonin, such that oxytocin can influence serotonin release (Arthur et al., 2017; Dölen et al., 2013; Mottolese et al., 2014; Yoshida et al., 2009) and serotonin can regulate oxytocin release (Bagdy and Kalogeras, 1993; Emiliano et al., 2007; Osei-Owusu et al., 2005). Given that serotonin and oxytocin can both work to promote social behavior (Kiser et al., 2012; Marlin

and Froemke, 2017), oxytocin and serotonin might function together to promote maternal behavior.

Ventrolateral periaqueductal gray and maternal behavior

The midbrain periaqueductal gray (PAG) is involved in multiple physiological and behavioral functions, including pain processing, sexual behavior, defensive behaviors, and blood pressure (Bandler and Keay, 1996; Benarroch, 2012). There is a dichotomy of function between the ventrolateral (PAGvl) and the lateral and dorsolateral subregions of the PAG, with the PAGvl enhancing passive coping/immobility, hypotension, and hypoventilation, while the lateral and dorsolateral PAG enhance active coping/fight-or-flight, hypertension, and hyperventilation (Benarroch, 2012). Given the PAGvl's role in immobility and passive coping, is it not surprising that electrolytic lesions of the PAGvl greatly decreases kyphotic nursing (defined by motor quiescence; Stern, 1996) and anxiety-like behaviors, while increasing aggression towards an intruder male (Lonstein et al., 1998; Lonstein and Stern, 1997a). The behavioral effects of PAGvl lesions are likely mediated by disrupted somatosensory stimulation from the pups, as somatosensory inputs synapse onto the PAG (Clement et al., 1996; Yezierski, 1991), and suckling stimulation from pups induces Fos expression uniquely in the PAGvl (Lonstein and Stern, 1997a, 1997b). Oxytocin might mediate postpartum behavior by interacting with the PAGvl, as stimulation of the ventrum of male rats increases oxytocin release in the PAGvl (Lund et al., 2002), and oxytocin dose-dependently increases the percentage of PAGvl cells that are electrophysiologically active and the number of spikes in those neurons (Ogawa et al., 1992). In the only study to examine oxytocin signaling's role in the PAGvl

on postpartum behavior, infusion of an OTR antagonist into the PAGvl increased anxiety-like behaviors (Figueira et al., 2008). Therefore, oxytocin signaling in the PAGvl might also affect other maternal behaviors in the postpartum period, such as kyphotic nursing, postpartum aggression and anxiety (Lonstein et al., 1998; Lonstein and Stern, 1997a).

Overview of dissertation chapters

In a number of mammalian species, oxytocin is a key regulator of maternal caregiving, maternal aggression, and anxiety. Given that the serotonergic DR expresses OTRs, oxytocin might be a pertinent neurotransmitter in regulating the DR during reproduction. The serotonin system is upregulated in postpartum females, and blocking serotonin receptors interferes with caregiving, while serotonin receptor agonists that might overactivate the serotonergic system also interfere with caregiving. Consequently, it is likely that there is tight regulation, potentially by oxytocin, of the serotonergic DR in reproductive females that helps facilitate caregiving. Therefore, I hypothesized that oxytocin signaling in the DR is vital for the onset and display of a suite of maternal behaviors in laboratory rats.

To begin testing this hypothesis, Chapter Two of this dissertation investigated how the oxytocin system in the midbrain DR and neighboring PAG change across reproduction, with a focus on the expression of OTRs on serotonergic, GABAergic, and dopaminergic neurons in the DR and PAG. Given that the oxytocinergic system is known to be upregulated in many forebrain sites during the peripartum period, I hypothesized that numerous aspects of the oxytocin system would similarly be upregulated in the DR and PAGvl (i.e., dorsomedial tegmentum) of peripartum females. Consistent with this

hypothesis, I did find that OTR expression in the DR and oxytocin fiber length in the DR and PAGvl were upregulated in peripartum females. Therefore, in Chapter Three the behavioral consequences of this upregulation were studied by injecting a viral construct expressing shRNA targeting the OTR mRNA (which knocks down OTR expression) into the dorsomedial tegmentum. A suite of maternal behaviors were then examined across the early postpartum period. Given that oxytocin regulates caregiving, postpartum aggression, and anxiety-like behaviors, I hypothesized that knocking down OTR expression in the dorsomedial tegmentum would affect caregiving, postpartum aggression, and anxiety. Finally, the implications of my results are discussed in Chapter Four.

CHAPTER 2: OXYTOCIN SIGNALING IN THE MIDBRAIN DORSAL RAPHE AND PERIAQUEDUCTAL GRAY ACROSS REPRODUCTIVE STATE; POTENTIAL INFLUENCE ON SEROTONERGIC, GABAERGIC, AND DOPAMINERGIC NEURONS

ABSTRACT

Oxytocin signaling is well known to positively influence maternal caregiving behaviors. Not surprisingly, then, expression of its receptor has been characterized in many sites of the brain across female reproductive states. However, almost all characterization of OTRs across reproduction have focused on the forebrain, even though there are OTRs in midbrain sites such as the DR and PAG. Ignoring these sites is surprising, given that the DR and PAG influence postpartum behaviors. To investigate whether there were reproductive state differences in OTR expression in the DR and PAG, I used a selective radiolabeled OTR antagonist to measure autoradiographic binding across four different female reproductive states: diestrous virgins (DV), pregnancy day 10, day of parturition, and postpartum day 7 (PPD 7). I found that OTR binding in the rostral subregion of the DR and the lateral subregion of the PAG were 145% higher in parturient dams compared to DV, and that the high levels dropped to DV levels again by PPD 7. In a follow-up experiment, I analyzed oxytocin-immunoreactive fiber length in these sites across three female reproductive states (DV, parturient dams, and PPD 7) and found higher oxytocin-immunoreactive fiber length in both the DR and PAGvl in PPD 7 dams compared to the DV and recently-parturient dams. Finally, given the heterogenous chemical nature of DR cells, I examined the expression of OTRs on three of its the most abundant neuronal phenotypes - serotonin, GABA and dopamine - in DV and recently-parturient dams. Using double-labeled immunohistochemistry and *in situ* hybridization, I found that in the rostral subregion of the DR there were more serotonergic

and dopaminergic neurons containing OTR immunoreactivity in recently-parturient dams compared to DV females. Additionally, there were regionally-specific changes in GABA neurons expressing OTRs, with fewer GABAergic neurons expressing OTRs in the rostral DR, and more GABAergic neurons expressing OTRs in the caudal DR of recently-parturient dams compared to DV females. There were no differences between dams and virgins in OTR/neurochemical colocalization in any other DR subregion. Overall, these data suggest that specific subregions of the midbrain DR and PAG are more sensitive to oxytocin around the time of parturition, with dopaminergic and serotonergic neurons accounting for at least a portion of those neurons with increased sensitivity. Furthermore, these results suggest a potential role for oxytocin signaling in these sites in the numerous behavioral changes occurring across the early postpartum period that promote successful mothering.

Introduction

Central activity of the neuropeptide, oxytocin, influences the expression of nearly all maternal behaviors in laboratory rats. Oxytocin acts along with estradiol and progesterone to help induce the onset of caregiving (Fahrbach et al., 1984; Fahrbach et al., 1985; Pedersen et al., 1982; Pedersen and Prange, 1979); modifies caregiving once initiated (Bosch and Neumann, 2008; Champagne et al., 2001; Pedersen and Boccia, 2003); increases postpartum aggression under certain circumstances (Bosch et al., 2005; Ferris et al., 1992; Sabihi et al., 2014a); and decreases postpartum anxiety (Figueira et al., 2008; Neumann et al., 1999; Sabihi et al., 2014a). Given the overall importance of oxytocin to mothering, the neural substrates for its behavioral effects have often been studied. For instance, OTR action in the mPOA facilitates caregiving behaviors (Bosch and Neumann, 2012; Champagne et al., 2001; Pedersen et al., 1994; Shahrokh et al., 2010), in the CeA increases maternal aggression (Bosch et al., 2005; Ferris et al., 1992), and in the mPFC mitigates postpartum anxiety (Sabihi et al., 2014a). In these areas of the brain, OTR expression changes across female reproductive state, with its expression often highest around the time of parturition (Bosch et al., 2010; Caldwell et al., 1994; Caughey et al., 2011; Insel, 1986, 1990; Meddle et al., 2007; Pedersen et al., 1994).

However, nearly all studies characterizing changes in the expression of OTRs across reproduction have focused on forebrain sites, even though there are OTRs in the midbrain, including in the DR and neighboring PAG (Yoshida et al., 2009; Yoshimura et al., 1993). The lack of research investigating oxytocin's role in influencing caregiving when acting in these sites is surprising given that the DR and PAG are otherwise known to influence postpartum behaviors (Barofsky et al., 1983; Holschbach et al., 2018;

Lonstein et al., 1998; Lonstein and Stern, 1997a). For instance, lesioning the serotonin neurons within the DR increases the incidence of pup mortality, affects the patterning of nursing across lactation, and decreases postpartum aggression (Barofsky et al., 1983; Holschbach et al., 2018). Lesioning the PAGvl decreases kyphotic nursing and anxiety-like behavior, but increases postpartum aggression (Lonstein et al., 1998; Lonstein and Stern, 1997a). Therefore, these midbrain sites are prime targets for where oxytocin might exert it effects on mothering behaviors.

A first step toward understanding what role oxytocin signaling in the DR and PAG might have on maternal behavior is to understand whether sensitivity to oxytocin signaling in these sites change across reproductive state. Therefore, I examined OTR autoradiographic binding at four timepoints across female reproduction (i.e., DV, on pregnancy day 10, within 3 hours of parturition, and on PPD 7). I hypothesized that OTR autoradiographic binding in the DR and PAG would change across female reproductive state to render these sites more or less sensitive to oxytocin and its effects on maternal behaviors. Specifically, I predicted that, similar to other brain sites that positively influence postpartum behavior (Bosch et al., 2010; Caldwell et al., 1994; Caughey et al., 2011; Insel, 1986, 1990; Meddle et al., 2007; Pedersen et al., 1994), OTR binding would be higher in recently-parturient dams compared to all other timepoints.

Studying levels of OTR binding across female reproductive state will help us to understand the potential role of oxytocin in the DR and PAG in postpartum behavior, but it is also crucial to understand how other aspects of the oxytocin system in the DR and PAG might change across reproduction. Oxytocin fibers project to the DR and PAG (Lund et al., 2002; Sofroniew, 1983b), and release oxytocin in response to stroking-like

somatosensory stimulation (Lund et al., 2002). Therefore, I also measured the length of oxytocin-immunoreactive fibers within the DR and PAG across reproductive state (i.e., females sacrificed as DV, within 3 hours of parturition, or on PPD 7). Oxytocin fiber length is an indicator of the capacity for local oxytocin release. For example, lactating rodents have higher oxytocin-immunoreactive fiber density than virgin females in many sites of the forebrain (Caldwell et al., 1987; Jirikowski et al., 1989; Knobloch et al., 2012), which is positively associated with oxytocin released in those sites (Neumann and Landgraf, 1989; Neumann et al., 1993). I hypothesized that oxytocin-immunoreactive fiber length in the DR and PAG would change across female reproductive state. Specifically, I predicted that all postpartum dams (recently-parturient and PPD 7 dams) would have more oxytocin-immunoreactive fibers in the DR and PAG compared to DV.

Finally, although there are OTRs present in the DR (Yoshida et al., 2009; Yoshimura et al., 1993), there is no information about which neuronal phenotypes express them in laboratory rats. The DR contains nearly 15,000 serotonergic neurons in rats (Descarries et al., 1982; Lowry et al., 2008; Vertes and Crane, 1997), so one would expect that many of its serotonin cells would express OTRs. However, only ~35% of DR neurons are serotonergic (Descarries et al., 1982). In laboratory mice, only around one third of the neurons in the DR expressing OTRs are serotonergic (Yoshida et al., 2009), suggesting that OTRs must also be expressed on other neuronal populations in the DR. The second largest population of cells in the rat DR are GABAergic, which accounts for ~15% of all neurons (~2,700 cells) (Belin et al., 1979; Ford et al., 1995; Higgins et al., 1988; Nanopoulos et al., 1982; Reichling and Basbaum, 1990). These GABA neurons are mostly inhibitory interneurons (Bagdy et al., 2000; Baraban and Aghajanian, 1980; Belin

et al., 1979; Boothman and Sharp, 2005), and OTRs expressed on these cells might regulate tonic inhibition of serotonergic release. Finally, in addition to its serotonergic and GABAergic populations, the rat DR also contains a large, but often ignored, population of dopamine neurons (~1000 cells) (Ferré and Artigas, 1993; Matthews et al., 2016; Stratford and Wirtshafter, 1990). These dopaminergic neurons are generally considered a caudal extension of the A10 dopamine group (i.e., VTA) and project to a number of pro-maternal brain sites, including the mPOA (Miller and Lonstein, 2009), NAc (Stratford and Wirtshafter, 1990), and BNST (Hasue and Shammah-Lagnado, 2002; Meloni et al., 2006), which all respond to dopamine with a facilitation of maternal behaviors (Keer and Stern, 1999; Miller and Lonstein, 2005). These DR dopamine neurons have some similarities to the A10 group (Flores et al., 2006), but also may have some more unique functions, including contributing to wakefulness (Cho et al., 2017; Lu et al., 2006) and responding to social isolation (Matthews et al., 2016). These more unique functions could be essential for patterns in maternal caregiving (Ader and Grota, 1970; Grota and Ader, 1969; Melanie et al., 1988), especially the changes in arousal state involved in nursing (Keer and Stern, 1999; Stern and Taylor, 1991; Voloschin and Tramezzani, 1979), and the dams' motivation to be in contact with her pups rather than spend time alone (Numan, 2006).

In this chapter, I hypothesize that OTR autoradiographic binding in the DR and PAG will change across female reproductive state to render these sites more or less sensitive to oxytocin and its effects on maternal behaviors. Specifically, I predict that, similar to other brain sites that positively influence postpartum behavior, OTR binding would be higher in recently-parturient dams compared to all other timepoints. I also hypothesize that OTR expression in serotonergic, GABAergic, and dopaminergic neurons

in the DR and PAG will change across have female reproductive state. I predict that the percentage of serotonergic and dopaminergic neurons expressing OTRs will be higher in recently-parturient dams compared to DV, and that the percentage of GABAergic neurons expressing OTRs will be lower in recently-parturient dams. The pattern of OTR expression on serotonergic, GABAergic, and dopaminergic neurons will provide clues as to how the balance of OTR influences in the DR might change across female reproductive state and might also modify maternal behaviors. Overall, greater expression of OTRs and oxytocin-immunoreactive fibers in the DR could suggest increased oxytocinergic control over the DR in postpartum dams, with implications for their behavior.

Materials and methods

Subjects

Subjects were female Long-Evans rats descended from rats purchased from Harlan Laboratories (Indianapolis, IN), born and raised in the Lonstein breeding colony at Michigan State University. Females were housed with 2 or 3 same-sex littermates in clear polypropylene cages (48 cm x 28 cm x 16 cm) containing wood chip bedding, with Food (Tekland rat chow, Indianapolis, IN) and water available *ad libitum*, the room was maintained on a 12:12 light/dark cycle (lights on at 0700 hr) with temperature at 22 ± 1 °C. After reaching 65 days of age, estrous cycles were monitored daily by vaginal smearing. On a day of proestrus, subjects used for the reproductive groups were placed overnight with a sexually-experienced male from the colony. Pregnancy was confirmed the next day by the presence of semen in a vaginal smear or the presence of a vaginal plug. Subjects were then housed with 1-2 other pregnant females until 5-7 days before

expected parturition, after which they were singly-housed. Soon after parturition litters were culled to contain 8 pups (4 males, 4 females). All procedures were performed in accordance with the principles of the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Michigan State University.

Tissue collection and perfusion

In Experiments 1 and 4, on a scheduled day of sacrifice (Experiment 1: day of diestrous (DV), pregnancy day 10, Part - within 3 hours of birthing the last pup, and postpartum day 7 (PPD 7); *n* = 5/group: Experiment 4; DV, Part; *n* = 3/group) separate groups of females were weighed, rendered unconscious with CO_2, and rapidly decapitated. Brains were removed from the skull, flash frozen with isopentane, and stored at -80°C until sectioning.

In Experiment 2, separate groups of animals were anesthetized with sodium pentobarbital either as DV, Part, or on PPD 7 (*n* = 5/group). In Experiment 3a, separate groups of animals were anesthetized with sodium pentobarbital either as DV or Part (*n* = 5/group). In Experiment 3b, separate groups of animals were anesthetized with sodium pentobarbital either as DV, Part, PPD 7, or PPD 18 (*n* = 6/group). These subjects were all perfused transcardially with saline followed by 4% paraformaldehyde, the brains extracted, postfixed overnight, and submerged in 30%. Tissue was cut into 40-μm sections in four series and stored in cryoprotectant at -20ºC until immunohistochemical processing.

Experiment 1: Oxytocin receptor autoradiography

Brains were cut coronally into 15-µm sections and slide mounted in 4 series using a cryostat (Leica CM1950, Nussloch, Germany) to obtain 16 matching sections containing the DR (-7.3 to -8.7 mm from bregma), 14 matching sections including PAGvl (-7.1 to -8.3 mm from bregma), 24 matching sections including the dorsal PAG (PAGd) and dorsolateral PAG (PAGdl; -6.3 to -8.5 mm from bregma), and 20 matching sections including the lateral PAG (lPAG; -6.5 to -8.5 mm from bregma). Following sectioning, slides were stored at -80 °C. On a day of autoradiography, slides were removed from -80 °C and allowed to thaw at room temperature for ~15 mins. Slides were then incubated for 2 mins in 1% paraformaldehyde, followed by two rinses for 10 mins each in 50 mM Tris-HCl buffer. Slides were then incubated for 1 hour in 50 mM Tris-HCl buffer containing 0.1% bovine serum albumin and 50 pM of the radioactive tracer ornithine vasotocin analog, [125I]-OVTA (Perkin-Elmer, MA; Cat. # NEX254050UC). Slides were then rinsed twice in ice-cold 50 mM Tris-HCl buffer for 5 mins, followed by a final single wash in ice-cold water. Slides were then allowed to dry overnight at 4 °C. Slides were then placed against Kodak BioMaxMR film, and based on preliminary work were exposed for 7 days and developed using a Kodak X-OMAT 1000A Processor (Kodak, Rochester, NY). Optical density for each section was analyzed using Imagej software (NIH, Bethesda, MD). For all areas of interest, mean density per region was calculated by subtracting background signal from the density of the region of interest. For final analysis, data from two adjacent brain sections were combined into a single data point for each animal. The rostral DR was from two sections between -7.3 to -7.6 mm from bregma; the medial DR

was from four sections between -7.7 to -8.3 mm from bregma; and the caudal DR from 2 sections between -8.5 to -8.7 mm from bregma as defined by (C. A. Lowry et al., 2008).

Experiment 2: Oxytocin fiber immunohistochemistry

Seven matched brain sections containing the DR (-7.1 to -8.9 mm from bregma) and four matched sections containing the PAGvl (-7.3 to -8.3 mm from bregma) were selected for analysis. All rinses were done in TBS. Sections were incubated in 0.1% sodium borohydride for 10 min, followed by a 10-min incubation in a 1% H_2O2 in 0.3% triton-TBS solution. Sections were then blocked in a solution containing 20% NGS and 0.3% Triton-X for 60 min at room temperature. Sections were then incubated in a triton-TBS solution contain 2% NGS and a mouse anti-oxytocin polyclonal antiserum (MAB5296, Millipore, Burlington, MA; 1:300), for 72 hrs at 4 °C. Tissue was then incubated in biotinylated goat anti-mouse secondary antiserum (BA-2000; Vector Labs, Burlingame, CA; 1:500) in a triton-TBS solution containing 2% NGS for 1 hr at room temperature. The sections were then incubated in ABC (PK 6100, Vectors Labs, Burlingame, CA) for 1 hr at room temperature. oxytocin-ir was then visualized using Vector-SG (SK-4700; Vector labs. Burlingame, CA). Sections were mounted onto glass microscope slides and coverslipped. The length of oxytocin-ir fibers in the entire area of the brain structures was traced on each section by experimenter's blind to the subjects' experimental condition under 200X magnification using a Nikon Eclipse E600 light microscope. The length of oxytocin-ir fibers traced across sections per site was used for data analyses. Additionally, the DR was also divided into its rostral (two sections between -7.1 to -7.3 mm from bregma); medial (three sections between -7.6 to -8.3 mm from

bregma); and caudal (two sections between -8.5 to -8.9 mm from bregma) subregions for follow-up analyses.

Experiment 3a: Oxytocin receptor and tyrosine hydroxylase or tryptophan hydroxylase double-label immunohistochemistry

In Experiment 3a, double labelled immunohistochemistry was used to examine the percentage of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) immunoreactive cells in the DR that also express OTR immunoreactivity. All rinses were done in PBS. On a day of immunohistochemistry, slides were removed from -80 °C, and allowed to reach room temperature (~15 mins). Slides to be used for TH-OTR immunohistochemistry were then blocked in a 0.2% triton-PBS solution containing 5% NDS for 3 hrs at room temperature. Slides were then incubated in purified rabbit anti-OTR primary antiserum (Gift Drs. Robert Froemke and Moses Chao: 1:200) and mouse anti-TH primary antiserum (MAB5986, Millipore, Burlington, MA; 1:2000) for 48 hrs at 4 °C. Slides were then incubated in donkey anti-mouse Alexafluor 647 (A21448, Fisher Scientific, Pittsburgh, PA; 1:500) and donkey anti-rabbit Alexafluor 488 (A21206, Fisher Scientific, Pittsburgh, PA; 1:500) secondary antisera for 2 hrs, followed by coverslipping using fluoromount G (0100-01, Southern Biotech, Birmingham, AL).

Slides to be used for TPH-OTR immunohistochemistry were blocked in 5% NDS in PBS for 1 hr at room temperature. Following blocking, slides were incubated in sheep anti-TPH primary antiserum (T857501VL, Sigma-Aldrich, St. Louis, MO; 1:1200) overnight at room temperature. The following day, slides were blocked for 3 hrs in a 0.2% triton-PBS solution containing 5% NDS. Slides were then incubated in purified rabbit anti-

OTR primary antiserum (Gift Drs. Robert Froemke and Moses Chao: 1:200) for 48 hrs at 4 °C. Finally, slides were incubated in donkey anti-sheep Alexafluor 647 (A21448, Fisher Scientific, Pittsburgh, PA; 1:500) and donkey anti-rabbit Alexafluor 488 (A21206, Fisher Scientific, Pittsburgh, PA 1:500) secondary antisera for 90 mins, followed by coverslipping using fluoromount G (0100-01, Southern Biotech, Birmingham, AL).

Sections were analyzed with a Nikon C2 confocal microscope, using 488 nm (OTR immunofluorescence) and 647 nm (TH/TPH immunofluorescence) lasers and band pass emission filters for wavelength selection. High-resolution confocal fluorescence was collected through a single, variable pinhole aperture and recorded using three high-sensitivity photomultiplier (PMT) detectors. Z-stack images were collected at 1.64 µm intervals through three sections containing the rostral DR (i.e., the location of the TH-ir neurons) and seven sections containing the entire DR (i.e., the location of the TPH-ir neurons) per animal. Colocalization with of OTR-ir with TH/TPH-ir was determined via 3D reconstruction using a Z-stack orthogonal viewer.

Experiment 3b: Tyrosine hydroxylase immunohistochemistry

In Experiment 3a, there was a marginally significant increase in TH-ir neurons in the DR of parturient dams compared to DV ($p = 0.051$). To more carefully analyze if the number of TH-ir neurons is higher in postpartum females, brains from a larger sample of females were run through single-label, non-fluorescence immunohistochemistry to quantify the number of TH-ir neurons in the DR across female reproductive states. Five instead of three matched sections per subject containing the DR (-7.0 to -7.8 mm from bregma) were selected for analysis. Additionally, to confirm that changes in TH-ir neuron

number were specific to the DR, the number of TH-ir cells was also analyzed in the VTA. Three matched sections per subject containing the VTA (-5.65 to -6.06 mm from bregma) were selected for analysis. Immunohistochemistry was conducted using methods previously reported in detail elsewhere (Miller and Lonstein, 2009). All rinses were done in 0.1 M TBS. Briefly, sections were incubated in 0.1% sodium borohydride for 15 min, followed by a 10 min incubation in 1% hydrogen peroxide diluted in 0.3% triton-X TBS. Tissue was then blocked in a solution contain 20% NGS in 0.3% triton-X TBS for 1 hr at room temperature. Sections were then incubated in a triton-TBS solution contain 2% NGS and a mouse anti-TH polyclonal antiserum (AB5986; Millipore, Burlington, MA; 1:2000), for ~16 hr at room temperature, then in a biotinylated goat anti-mouse secondary antiserum (BA-9200; Vector Labs, Burlingame, CA; 1:500) for 1 hr at room temperature. Sections were incubated in ABC solution (PK 6100, Vectors Labs, Burlingame, CA) for 1 hr at room temperature, TH-ir visualized using Vector-SG (SK-4700; Vector Labs, Burlingame, CA), mounted, and the slides coverslipped. The number of TH-ir cells in the DR (complete visual area) was counted on each section by experimenters' blind to the subjects' experimental condition under 200X magnification using a Nikon Eclipse E600 light microscope. Given the very low background staining (see Fig 6), somata with any visible TH-ir were included in the quantification. The summed number of TH-ir cells counted in all sections was used for data analyses. In addition to the number of TH-ir cells, the percentage of total DR area covered by TH-ir pixels (using a standardized light level and threshold for optic density across sections and subjects with NIS Elements software) was also analyzed.

Experiment 4: In situ hybridization analysis of oxytocin receptor and tyrosine hydroxylase, tryptophan hydroxylase, or glutamic acid decarboxylase mRNA in the DR

Brains were cut coronally into 20-µm sections using a cryostat (Leica CM1950, Nussloch, Germany), into 16-series through the DR. This resulted in 7 sections through the DR (2 sections in the series containing the rostral subregion, 3 sections containing the medial subregion, and 2 sections containing the caudal subregion of the dorsal raphe). Following sectioning, slides were stored in -80 °C until RNAscope® processing. On a day of RNAscope® processing to detect OTR and TH, TPH, or glutamic acid decarboxylase (GAD) colocalized hybridization, slides were removed from -80 °C and immediately immersed in 4% paraformaldehyde pre-chilled to 4°C for 15 min. The tissue was then dehydrated through serial ethanol solutions, 50%, 70%, 100%, 100%, for 5 min each at room temperature. Following dehydration, slides were allowed to air dry for 5 min, followed by creating a hydrophobic barrier with the Immedge™ hydrophobic barrier pen (repeated 2 – 4 times). After the barrier was dry (~1 min), slides were incubated with ~5 drops of RNAscope protease IV (Cat. 322340) for 30 min at room temperature. Immediately following incubation, slides were rinsed twice with 1 X PBS by slight agitation. In preparation for hybridization, the probes were warmed for 10 min at 40 °C in a water bath, and then allowed to cool to room temperature. Probes were then mixed for their respective runs: Run 1#) involved GAD (Cat. 316401), OTR (Cat. 483671), and TPH (Cat. 316411); Run #2) involved TH (314651) and OTR. Excess liquid was removed from the slides and ~4-5 drops of the mixed probes were added to the slides. The slides were then placed on the HybEZ™ humidity control tray and into the HybEZ™ oven for 2 hrs at 40 °C. After every incubation, slides were rinsed 2 times for 2 minutes at room in wash

buffer. Slides were incubated in ~4-5 drops of Amp 1-FL for 30 min at 40 °C in the HybEZ™ oven. The slides were then incubated in Amp 2-FL for 15 min at 40 °C. Following rinses, the slides were then incubated in Amp 3-FL for 30 min at 40 °C, followed by an incubation in Amp 4-FL for 15 min at 40 °C. Slides were then briefly incubated with DAPI (~ 30 sec), followed by ~2 – 3 drops of Prolong Gold Antifade Mountant (P36930, Thermo Fisher Scientific, Waltham, MA) and coverslipped. Sections were analyzed with a Nikon C2 confocal microscope, using 488 nm (GAD/TH fluorescence), 568 nm (OTR fluorescence), and 647 nm (TPH fluorescence) lasers and band pass emission filters for wavelength selection. High-resolution confocal fluorescence was collected through a single, variable pinhole aperture and recorded using three high-sensitivity PMT detectors. Z-stack images were collected at 1.64 µm intervals through two sections containing the rostral dorsal raphe (for colocalization of TH/OTR) and seven sections containing the entire dorsal raphe (for colocalization of TPH/OTR and GAD/OTR) per animal. Colocalization with of OTR fluorescence with TH/TPH/GAD fluorescence was determined via 3D reconstruction using a Z-stack orthogonal viewer.

Statistical analyses

Optical density of OTR binding (Experiment 1) and oxytocin-ir fiber length (Experiment 2) were analyzed with mixed-design repeated-measures ANOVAs (with Greenhouse-Geisser correction) involving rostrocaudal levels as the repeated measure and reproductive state as the between-subject variable. The number of TH-ir neurons in the DR across reproduction (Experiment 3b), were analyzed with one-way ANOVAs. In cases of statistical significance, LSD post-hoc tests were conducted to compare across

rostrocaudal level and between groups. For the immunohistochemical (Experiment 3a) and *in situ* hybridization (Experiment 4) colocalization studies involving two groups, Student's *t*-tests were run. All data were found to be normally distributed with no significant outliers using Dixon-Q extreme outlier tests. Partial η^2 (η^2_p) are reported as measures of effect sizes from the ANOVAs and Cohen's *d* are reported as measures of effects sizes from the pairwise comparisons. $p < 0.05$ was considered statistically significant.

Results

Experiment 1: Oxytocin receptor autoradiography

OTR binding in the rostral DR was 145% and 157% higher in recently-parturient dams compared to DV females and PPD 7 dams, respectively ($F_{(3, 16)} = 6.13$, $p = 0.01$, $\eta^2_p = 0.54$; Fig 1C). OTR binding did not differ among groups in the medial ($F_{(3, 16)} = 0.61$, $p = 0.62$, $\eta^2_p = 0.10$; Fig 1D) or caudal subregions ($F_{(3, 16)} = 1.97$, $p = 0.16$, $\eta^2_p = 0.27$; Fig 1E) of the DR. OTR binding in the DR collapsed across all rostrocaudal levels and subregions was unaffected by reproductive state (Main effect of group: $F_{(3, 16)} = 1.90$, $p = 0.17$, $\eta^2_p = 0.26$; Fig 1B), but did significantly decrease across rostrocaudal level (Main effect of level: $F_{(7)} = 13.34$, $p < 0.001$, $\eta^2_p = 0.46$; Fig 1B). There were no significant interactions between reproductive state and rostrocaudal level on OTR binding (Table 1).

In the PAG, there was a significant main effect of reproductive state, such that pregnant females and recently-parturient dams had 61% and 76% more OTR binding compared to DV, respectively ($F_{(3, 16)} = 3.78$, $p = 0.03$, $\eta^2_p = 0.42$; Fig 2A). When examined by subregion, OTR binding in the PAGl was significantly 89% and 122% higher in

pregnant and recently-parturient dams compared to DV ($F_{(3, 16)}$ = 3.55, p = 0.04, η^2_p = 0.40; Fig 2D), while no other subregion differed by reproductive state (Table 1). There were significant effects of rostrocaudal level for OTR binding in the PAGd ($F_{(11)}$ = 10.54, $p < 0.001$, η^2_p = 0.40; Fig 2B), PAGdl ($F_{(11)}$ = 11.95, $p < 0.001$, η^2_p = 0.43; Fig 2C) and the PAGvl ($F_{(7)}$ = 3.03, p = 0.04, η^2_p = 0.16; Fig 2E), such that OTR binding gradually declined across level for the PAGd and PAGdl, but was highest at mid-rostrocaudal levels compared to either end in the PAGvl. There were no significant interactions between reproductive state and PAG level for any subregion on OTR binding (Table 1).

Experiment 2: Oxytocin fiber immunoreactivity

Total oxytocin-ir fiber length in the DR was ~50% higher in PPD 7 females when compared to DV and recently-parturient dams ($F_{(2,14)}$ = 4.55, p = 0.04, η^2_p = 0.45; Fig 3B). Oxytocin-ir fiber length in the DR also changed across its rostrocaudal level, being lower more caudally ($F_{(6)}$ = 35.64, $p < 0.001$, η^2_p = 0.76; Fig 3B).

When examining subregions of the DR, oxytocin-ir fiber length in the medial DR was 41% and 76% higher at PPD 7 when compared to DV and recently-parturient dams, respectively ($F_{(2,14)}$ = 6.51, p = 0.01, η^2_p = 0.54). There was also an interaction between total oxytocin-ir fiber length and rostrocaudal level of the DR, such that DV had the lowest oxytocin-ir fiber length in the rostral and medial subregions, but the highest in the caudal subregion ($F_{(4,22)}$ = 3.36, $p < 0.05$, η^2_p = 0.38; Fig 3B). There were no differences among reproductive state groups on oxytocin-ir fiber length in either the rostral or caudal subregions of the DR (Table 2).

Oxytocin-ir fiber length in the PAGvl was 88% and 50% higher in PPD 7 dams compared to DV and recently parturient dams, respectively ($F_{(2,11)}$ = 8.04, p < 0.01, η^2_p = 0.59; Fig 3C). There were no significant effects of group or rostrocaudal level on oxytocin-ir fiber length in the PAGl ($F_{(2,14)}$ = 3.33, p = 0.07, η^2_p = 0.38; Fig: 3D). There were few if any oxytocin-ir fibers in the PAGdl or PAGd in females from any reproductive state, so these subregions were not analyzed.

Experiment 3a: Oxytocin receptor and tyrosine hydroxylase or tryptophan hydroxylase dual-label immunohistochemistry

Across all groups ~50% of all TH-ir cells also expressed OTR-ir. The number and percentage of TH-ir cells in the DR expressing OTR-ir was 135% and 23% higher, respectively, in recently-parturient dams when compared to DV (number: $t_{(5)}$ = 5.76, p = 0.02, d = 4.65; percentage: $t_{(5)}$ = 6.29, p < 0.01, d = 4.47; Fig 4C). There were no effects of reproductive state on the number of OTR-ir cells ($t_{(5)}$ = 1.25, p = 0.30, d =1.11), nor the percentage of OTR-ir cells expressing TH-ir ($t_{(5)}$ = 2.46, p = 0.09, d = 2.10). There was, however, a trend for recently-parturient dams to have more TH-ir cells when compared to DV ($t_{(5)}$ = 3.89, p = 0.051, d = 3.15) (Table 3).

A slightly larger percentage of TPH-ir cells contained OTR-ir (~55% across all groups). In contrast to the TH-ir cells, the number and percentage of TPH-ir cells expressing OTR-ir was not significantly affected by reproductive state (number: $t_{(6)}$ = 0.93, p = 0.41, d = 0.66; percentage: $t_{(6)}$ = 1.91, p = 0.11, d = 1.36), nor were the total numbers of single plus double-labeled TPH-ir, OTR-ir cells, or the percentage of OTR-ir cells expressing TPH-ir (Table 4). However, the percentage of TPH-ir cells expressing OTR-ir

was 27% higher in the rostral subregion of the DR of recently-parturient dams compared to DV ($t_{(6)}$ = 2.60, p = 0.04, d = 1.85; Fig 4C). There were no effects of reproduction on TPH-ir cells expressing OTR-ir in any other DR subregions (Table 4).

Experiment 3b: Tyrosine hydroxylase immunohistochemistry

Consistent with the trend found in Experiment 3a, single-label immunohistochemistry and light microscopy revealed that there was significantly more TH-ir cells in the DR in the recently-parturient dams when compared to all other groups ($F_{(3, 18)}$ = 4.04, p = 0.02, η^2_p = 0.40; Fig 6C). There were no effects of reproductive state, however, on the percentage of total DR area covered by TH-ir (although it was highest in recently-parturient dams) ($F_{(3, 18)}$ = 1.14, p = 0.36, η^2_p = 0.16; Fig 6D). The greater number of TH-ir cells in the recently-parturient dams was specific, as there was no effect of female reproductive state on the number of TH-ir neurons in the VTA ($F_{(3,16)}$ = 0.51, p = 0.68, η^2_p = 0.09; data not shown).

Experiment 4: In situ hybridization analysis of oxytocin receptor and tyrosine hydroxylase, tryptophan hydroxylase, or glutamic acid decarboxylase mRNA in the DR

The percentage of TH mRNA-containing cells in the DR that also express OTR mRNA was not affected by reproductive state ($t_{(4)}$ = 1.47, p = 0.24, d = 1.50 Fig 7B), nor were the number of TH mRNA or TH mRNA-containing cell in the DR that also express OTR mRNA (Table 5). The percentage of TPH mRNA-containing cells in the DR that also expressed OTR mRNA was not affected by reproductive state ($t_{(4)}$ = 1.94, p = 0.13, d = 1.57; Fig 8B); this was also the case for each subregion (Table 5). There was not a

significant effect of reproduction on the percentage of GAD mRNA-containing cells that also expressed OTR mRNA ($t_{(4)}$ = 1.19, p = 0.30, d = 0.95; Fig 8C). However, the percentage of GAD mRNA-containing cells that also expressed OTR mRNA was 44% lower specifically in the rostral subregion of the DR ($t_{(4)}$ = 4.29, p = 0.01, d = 3.48; Fig 8D) and 67% higher in the caudal subregion of the DR ($t_{(4)}$ = 2.87, p < 0.05, d = 2.34; Fig 8F) when compared to DV females. There were no significant effects of reproductive state on the percentage of GAD mRNA-containing cells that also expressed OTR mRNA in the medial subregion of the DR ($t_{(4)}$ = 1.07, p = 0.35, d = 0.87; Fig 8E).

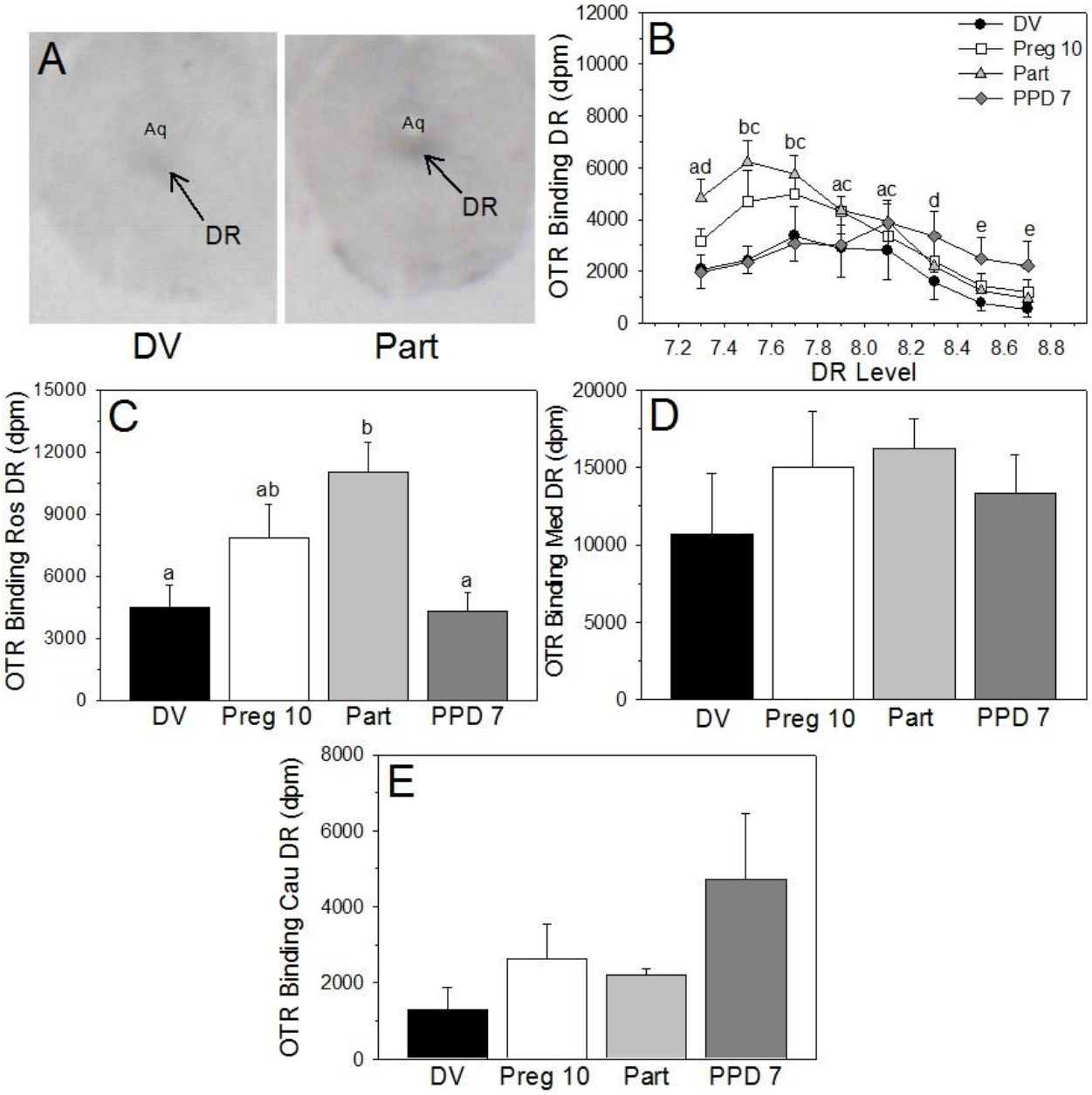

Figure 1: OTR binding in the dorsal raphe across female reproductive state. A) Representative radiograms of the dorsomedial tegmentum of a diestrous virgin (DV) and a recently parturient dam (Part). (B) OTR binding (Mean ± SEM) across rostrocaudal level of the dorsal raphe of female rats sacrificed as DV, on pregnancy day 10 (Preg 10), soon after Part, or on postpartum day 7 (PPD 7). Different letters above the lines indicates significant differences across level, $p < 0.05$. OTR binding (Mean ± SEM) in the rostral (C) medial (D) and caudal (E) subregion of the DR of female rats sacrificed as DV, Preg 10, Part, or PPD 7. Different letters above bars indicates significant difference between groups in the optical density of OTR binding, $p < 0.05$.

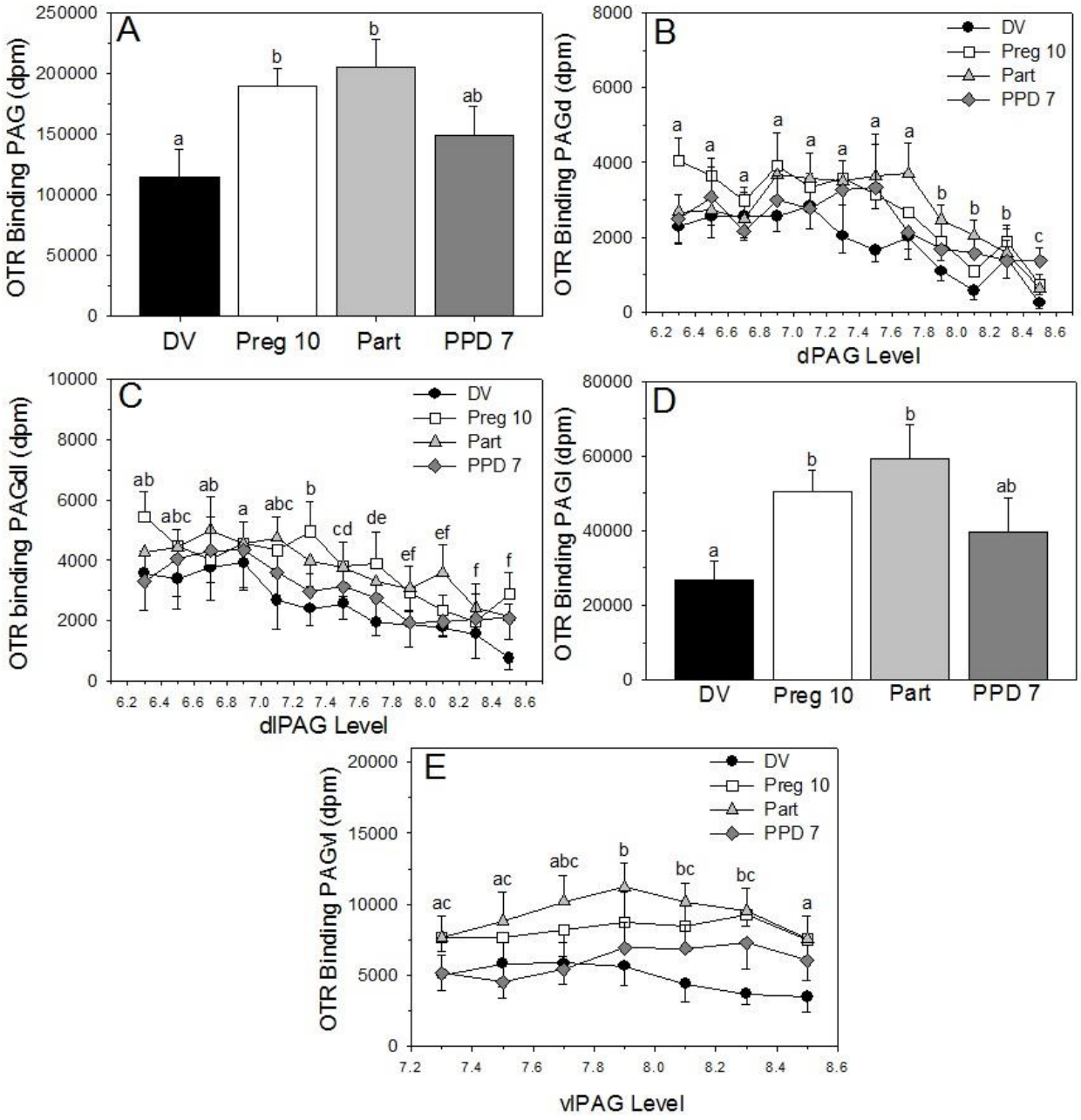

Figure 2: OTR binding in the periaqueductal gray across female reproductive state. OTR binding (Mean ± SEM) in the periaqueductal gray (PAG) (A) and lateral PAG (D) of female rats sacrificed as diestrous virgins (DV), on pregnancy day 10 (Preg 10), soon after parturition (Part), or on postpartum day 7 (PPD 7). Different letters above bars indicates significant difference between groups in levels of OTR binding, $p < 0.05$. OTR binding (Mean ± SEM) across rostrocaudal level of the dorsal PAG (B), dorsolateral PAG (C), and ventrolateral PAG (E) of female rats sacrificed as DV, Preg 10, Part, or PPD 7. Different letters above the lines indicates significant differences across rostrocaudal level, $p < 0.05$.

Region	Diestrous Virgins (M ± SEM)	Pregnancy day 10 (M ± SEM)	Parturition (M ± SEM)	Postpartum day 7 (M ± SEM)	Level (*F; p; $\eta^2_{p)}$*	Group (*F; p; $\eta^2_{p)}$*	Interaction (*F; p; $\eta^2_{p)}$*
Total DR	16486 ± 4849	25543 ±4297	29485 ±2761	22335 ±3738	13.34; <0.001*; 0.46	1.90; 0.17; 0.26	2.11; 0.06; 0.20
Rostral DR	4515 ± 1089[a]	7880 ± 1620[ab]	11069 ± 1411[b]	4306 ± 941[a]	5.39; 0.01^; 0.25	6.13; 0.01; 0.54	1.21; 0.32; 0.19
Medial DR	10667 ± 3982	15042 ± 3540	16220 ±1879	13325 ± 2486	4.57; <0.01*; 0.22	0.34; 0.80; 0.06	0.95; 0.50; 0.15
Caudal DR	1304 ± 582	2621 ± 925	2195 ± 177	4704 ± 1731	2.10; 0.11; 0.12	0.63; 0.61; 0.11	2.71; 0.02; 0.34
Total PAG	105470 ± 20577[a]	169747 ± 12736[b]	185191 ± 19653[b]	133364 ± 20274[ab]	N/A	3.74; 0.03; 0.41	N/A
PAGvl	26740 ± 6281	40764 ± 8382	48001 ± 7403	28876 ± 6706	3.03; 0.04[#]; 0.16	2.39; 0.11; 0.31	1.21; 0.31; 0.19
PAGl	26690 ± 5212[a]	50546 ± 5566[b]	59265 ± 9036[b]	39778 ± 9105[ab]	2.33; 0.08; 0.13	3.55; 0.04; 0.40	1.22; 0.30; 0.19
PAGdl	30209 ± 6791	45561 ± 6890	45231 ± 8118	36489 ± 8273	11.95; <0.01*; 0.43	0.97; 0.43; 0.15	0.69; 0.79; 0.12
PAGd	21830 ± 4221	32877 ± 2621	32694 ± 2054	28221 ± 4866	10.54; <0.01*; 0.41	2.05; 0.15; 0.28	0.74; 0.71; 0.12
Total DR+PAG	121956 ± 24795[a]	195290 ± 16014[b]	214675 ± 21166[b]	155698 ± 22484[ab]	N/A	3.75; 0.03; 0.41	N/A

Table 1: OTR binding in the dorsal raphe and periaqueductal gray across female reproductive state. Levels of autoradiographic OTR binding (Mean ± SEM) in the dorsal raphe and periaqueductal gray of female rats sacrificed as diestrous virgins, on pregnancy day 10, 3 hours after parturition, or on postpartum day 7. ^ indicates increasing OTR binding across rostrocaudal level. *indicates decreasing OTR binding across rostrocaudal level. # indicates inverted U-shaped-pattern of OTR binding across rostrocaudal level. Different letters indicate differences between groups in level OTR binding, $p < 0.05$.

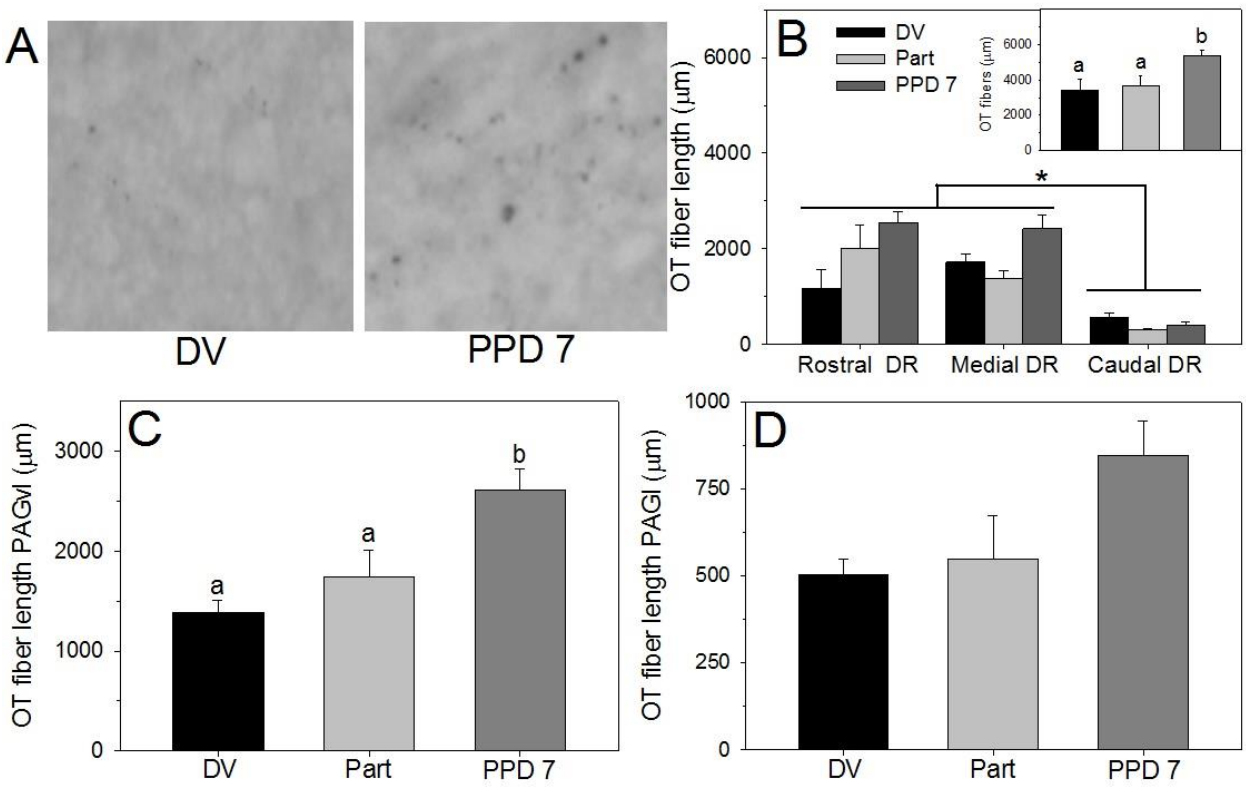

Figure 3: Oxytocin-immunoreactive fiber length in the dorsal raphe and periaqueductal gray across female reproductive state. A) Representative photomicrograph of oxytocin-immunoreactive fibers the dorsal raphe (DR). B) Oxytocin-immunoreactive fiber length (Mean ± SEM) in the rostral, medial, and caudal levels of the dorsal raphe of female rats sacrificed as diestrous virgins (DV), soon after parturition (Part), or on postpartum day 7 (PPD 7). Oxytocin-immunoreactive fiber length (Mean ± SEM) in the (C) ventrolateral PAG or (D) lateral PAG of female rats sacrificed as DV, Part, or PPD 7. * indicates significant difference across rostrocaudal level, $p < 0.05$. Different letters above bars indicates significant difference between groups in oxytocin-immunoreactive fiber length, $p < 0.05$.

Region	Diestrous Virgins (M ± SEM)	Parturition (M ± SEM)	Postpartum day 7 (M ± SEM)	Group (*F; p;* $\eta^2_{p)}$
Total DR	3442 ± 582[a]	3680 ± 560[a]	5369 ± 342[b]	4.55; 0.04; 0.45
Rostral DR	1169 ± 396	2006 ± 484	2540 ± 233	3.02; 0.09; 0.35
Medial DR	1723 ± 155[a]	1376 ± 160[a]	2424 ± 282[b]	6.51; 0.01; 0.54
Caudal DR	551 ± 94	297 ± 36	404 ± 66	3.58; 0.06; 0.39
Total PAG	1891 ± 113[a]	2291 ± 363[a]	3456 ± 296[b]	7.47; <0.01; 0.58
PAGvl	1389 ± 122[a]	1744 ± 264[a]	2612 ± 216[b]	8.04; <0.01; 0.59
PAGl	503 ± 46	547 ± 126	845 ± 100	3.33; 0.07; 0.38
Total DR+PAG	5334 ± 470[a]	5970 ± 887[a]	8825 ± 593[b]	7.05; 0.01; 0.56

Table 2: Oxytocin fiber length in the dorsal raphe and periaqueductal gray across female reproductive state. Oxytocin-immunoreactive fiber length (Mean ± SEM) in the dorsal raphe and periaqueductal gray of female rats sacrificed as diestrous virgins, 3 hours after parturition, or on postpartum day 7. Different letters indicate group differences in oxytocin fiber length, $p < 0.05$.

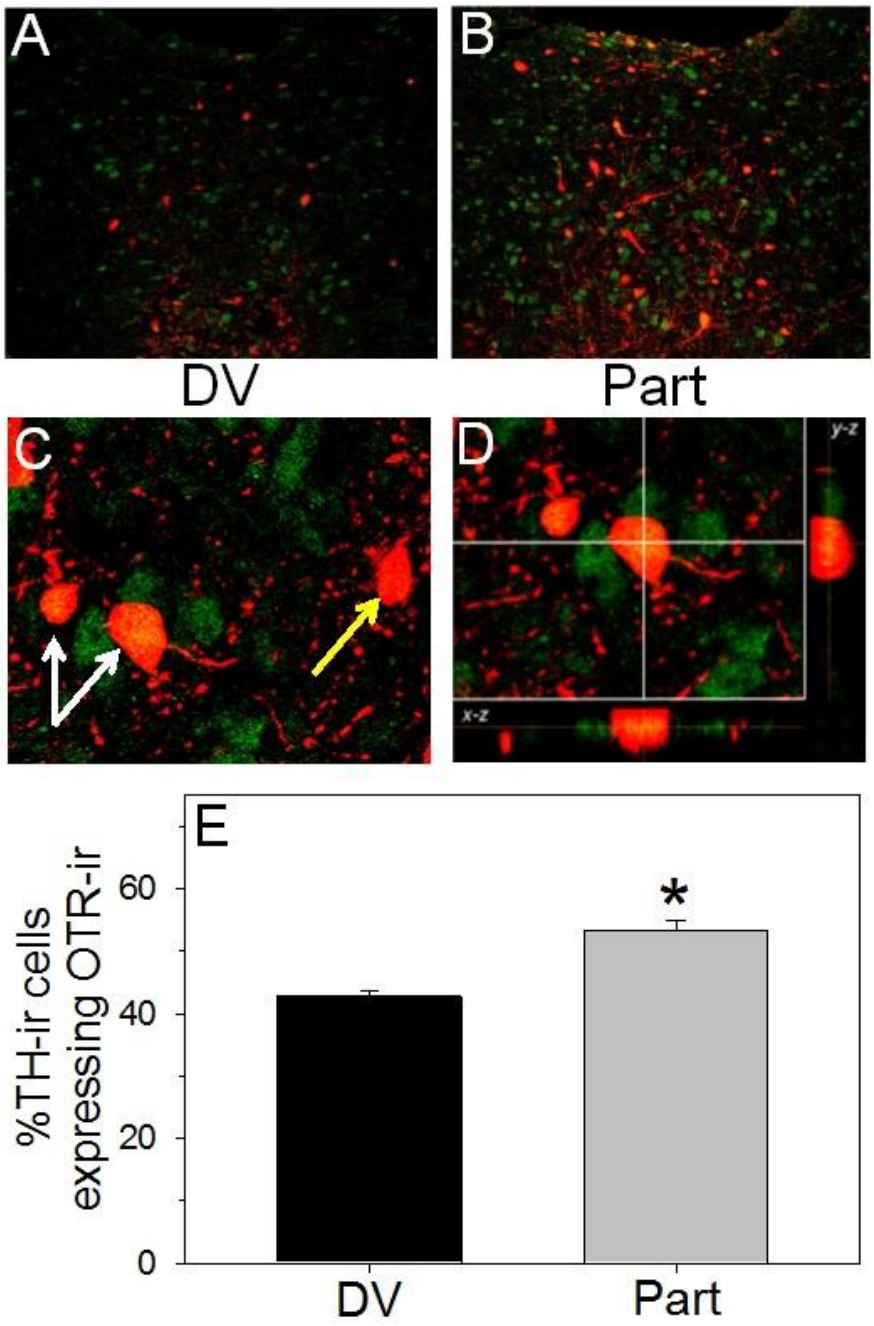

Figure 4: The percentage of tyrosine hydroxylase-immunoreactive neurons expressing OTR immunoreactivity across female reproductive state. Representative photomicrographs of the dorsal raphe of (A) diestrous virgins (DV) and (B) recently parturient dams (Part). (C) Photomicrograph showing colocalized tyrosine hydroxylase (red) and OTR (green) immunoreactivity, indicated by white arrows. Yellow arrow indicates a tyrosine hydroxylase-immunoreactive neuron not expressing OTR immunoreactivity. (D) Orthogonal view showing colocalization of tyrosine hydroxylase and OTR-immunoreactivity. (E) Percentage of tyrosine hydroxylase-immunoreactive neurons expressing OTR immunoreactivity (Mean ± SEM) of female rats sacrificed as DV and Part. * indicates statistically significant between group, $p < 0.05$.

	Diestrous Virgins (M ± SEM)	Parturition (M ± SEM)	Group ($t_{(5)}$; p; d)
# TH-ir cells	170 ± 9	326 ± 39	3.89; 0.051; 3.15
# OTR-ir cells	878 ± 132	1155 ± 190	1.25; 0.30; 1.11
# TH-ir cells expressing OTR-ir	73 ± 4	172 ± 17	5.76; 0.02*; 4.65
%TH-ir cells expressing OTR-ir	43 ± 1.5	53 ± 3.0	6.29; <0.01*; 4.47
% OTR-ir cells expressing TH-ir	9.3 ± 1.6	16.9 ± 3.0	2.46; 0.09; 2.10

Table 3: Number of tyrosine hydroxylase-immunoreactive neurons expressing oxytocin receptor immunoreactivity across female reproductive state. Number of tyrosine hydroxylase-immunoreactive neurons expressing OTR-immunoreactivity (Mean ± SEM) of female rats sacrificed as diestrus virgins or 3 hours after parturition. * indicates statistically significant group difference, $p < 0.05$.

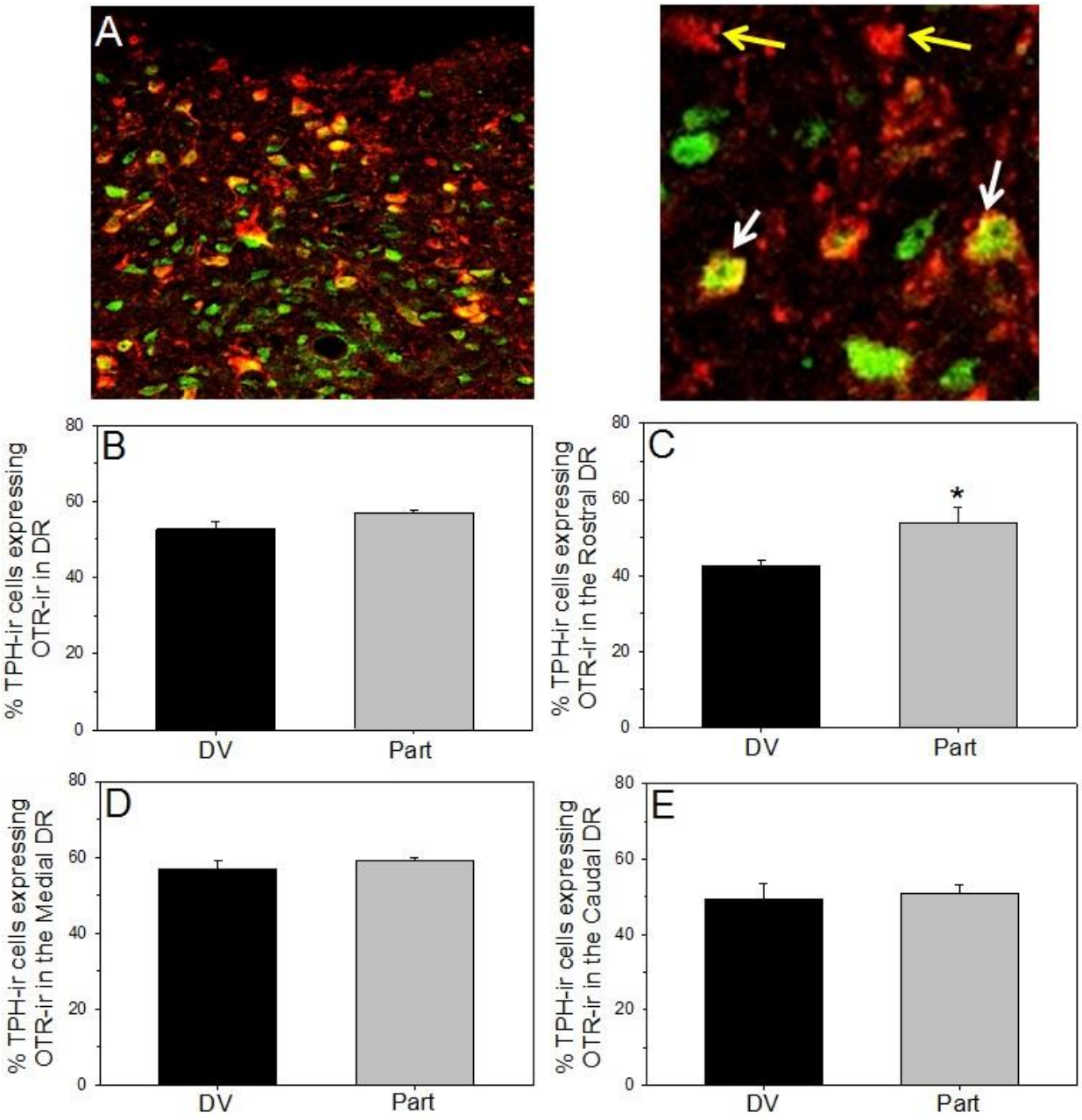

Figure 5: The percentage of tryptophan hydroxylase-immunoreactive neurons expressing oxytocin receptor-immunoreactivity is higher in recently parturient dams in the rostral dorsal raphe. A) Representative photomicrograph of the dorsal raphe (left). Photomicrograph showing colocalized tryptophan hydroxylase (red) and OTR (green) immunoreactivity, indicated by white arrows. Yellow arrow indicates a tyrosine hydroxylase-immunoreactive neuron not expressing oxytocin receptor-immunoreactivity (right). Percentage of tryptophan hydroxylase-immunoreactive neurons expressing oxytocin receptor-immunoreactivity (Mean ± SEM) of female rats sacrificed as diestrus virgins or soon after parturition in the (B) entire dorsal raphe area examined, (C) Rostral dorsal raphe, (D) Medial dorsal raphe, and (E) Caudal dorsal raphe. * indicates statistically significant difference between group, $p < 0.05$.

	Diestrous Virgins (M ± SEM)	Parturition (M ± SEM)	Group ($t_{(6)}$; p; d)
Total DR			
# TPH-ir cells	912 ± 22	975 ± 140	0.44; 0.69; 0.31
# OTR-ir cells	1258 ± 37	1439 ± 143	1.22; 0.27; 0.86
# TPH-ir cells expressing OTR-ir	480 ± 25	556 ± 77	0.93; 0.41; 0.66
%TPH-ir cells expressing OTR-ir	52.6 ± 2.1	57.2 ± 1.8	1.91; 0.11; 1.36
% OTR-ir cells expressing TPH-ir	38.3 ± 2.3	38.2 ± 1.8	0.03; 0.98; 0.02
Rostral DR			
# TPH-ir cells	241 ± 17	231 ± 36	0.26; 0.80; 0.18
# OTR-ir cells	326 ± 32	403 ± 50	1.30; 0.24; 0.92
# TPH-ir cells expressing OTR-ir	102 ± 8	127 ± 27	0.88; 0.42; 0.61
%TPH-ir cells expressing OTR-ir	42.3 ± 1.9	53.8 ± 4.0	2.60; 0.04*; 1.85
% OTR-ir cells expressing TPH-ir	31.7 ± 1.9	31.2 ± 4.0	0.12; 0.91; 0.08
Medial DR			
# TPH-ir cells	584 ± 38	628 ± 84	0.49; 0.64; 0.34
# OTR-ir cells	815 ± 72	862 ± 73	0.46; 0.66; 0.33
# TPH-ir cells expressing OTR-ir	334 ± 29	369 ± 45	0.65; 0.54; 0.46
%TPH-ir cells expressing OTR-ir	57.0 ± 2.3	59.0 ± 0.8	0.82; 0.45; 0.60
% OTR-ir cells expressing TPH-ir	41.5 ± 3.8	42.5 ± 2.7	0.23; 0.83; 0.15
Dorsomedial DR			
# TPH-ir cells	179 ± 20	176 ± 30	0.09; 0.93; 0.06
# OTR-ir cells	334 ± 31	352 ± 37	0.39; 0.71; 0.27
# TPH-ir cells expressing OTR-ir	109 ± 16	103 ± 18	0.23; 0.83; 0.18
%TPH-ir cells expressing OTR-ir	60.3 ± 2.8	58.5 ± 0.5	0.63; 0.57; 0.45

Table 4: Number of tryptophan hydroxylase-immunoreactive neurons expressing oxytocin receptor immunoreactivity across female reproductive state. Number of tryptophan hydroxylase-immunoreactive neurons expressing OTR-immunoreactivity (Mean ± SEM) in female rats sacrificed as diestrus virgins or 3 hours after parturition. * indicates statistically significant group difference, $p < 0.05$.

Table 4 (cont'd)

% OTR-ir cells expressing TPH-ir	32.7 ± 3.7	28.8 ± 2.5	0.89; 0.41; 0.62
Ventromedial DR			
# TPH-ir cells	246 ± 15	299 ± 41	1.20; 0.28; 0.86
# OTR-ir cells	317 ± 24	350 ± 25	0.95; 0.38; 0.67
# TPH-ir cells expressing OTR-ir	135 ± 14	174 ± 23	1.47; 0.19; 1.03
%TPH-ir cells expressing OTR-ir	54.5 ± 2.7	58.5 ± 0.8	1.40; 0.21; 1.00
% OTR-ir cells expressing TPH-ir	42.6 ± 3.2	49.7 ± 5.3	1.15; 0.29; 0.82
Lateral Wings DR			
# TPH-ir cells	159 ± 9	154 ± 16	0.25; 0.81; 0.19
# OTR-ir cells	392 ± 29	396 ± 22	0.09; 0.93; 0.08
# TPH-ir cells expressing OTR-ir	91 ± 11	92 ± 8	0.11; 0.92; 0.05
%TPH-ir cells expressing OTR-ir	56.5 ± 4.2	60.3 ± 3.2	0.73; 0.50; 0.51
% OTR-ir cells expressing TPH-ir	23.2 ± 2.8	23.3 ± 2.1	0.03; 0.98; 0.02
Caudal DR			
# TPH-ir cells	88 ±12	154 ± 29	2.10; 0.08; 1.47
# OTR-ir cells	117 ± 24	232 ± 49	2.10; 0.08; 1.49
# TPH-ir cells expressing OTR-ir	44 ± 9	79 ± 17	1.81; 0.12; 1.29
%TPH-ir cells expressing OTR-ir	49.4 ± 4.2	50.9 ± 2.2	0.31; 0.77; 0.22
% OTR-ir cells expressing TPH-ir	39.3 ± 5.6	33.9 ± 1.3	0.94; 0.38; 0.61

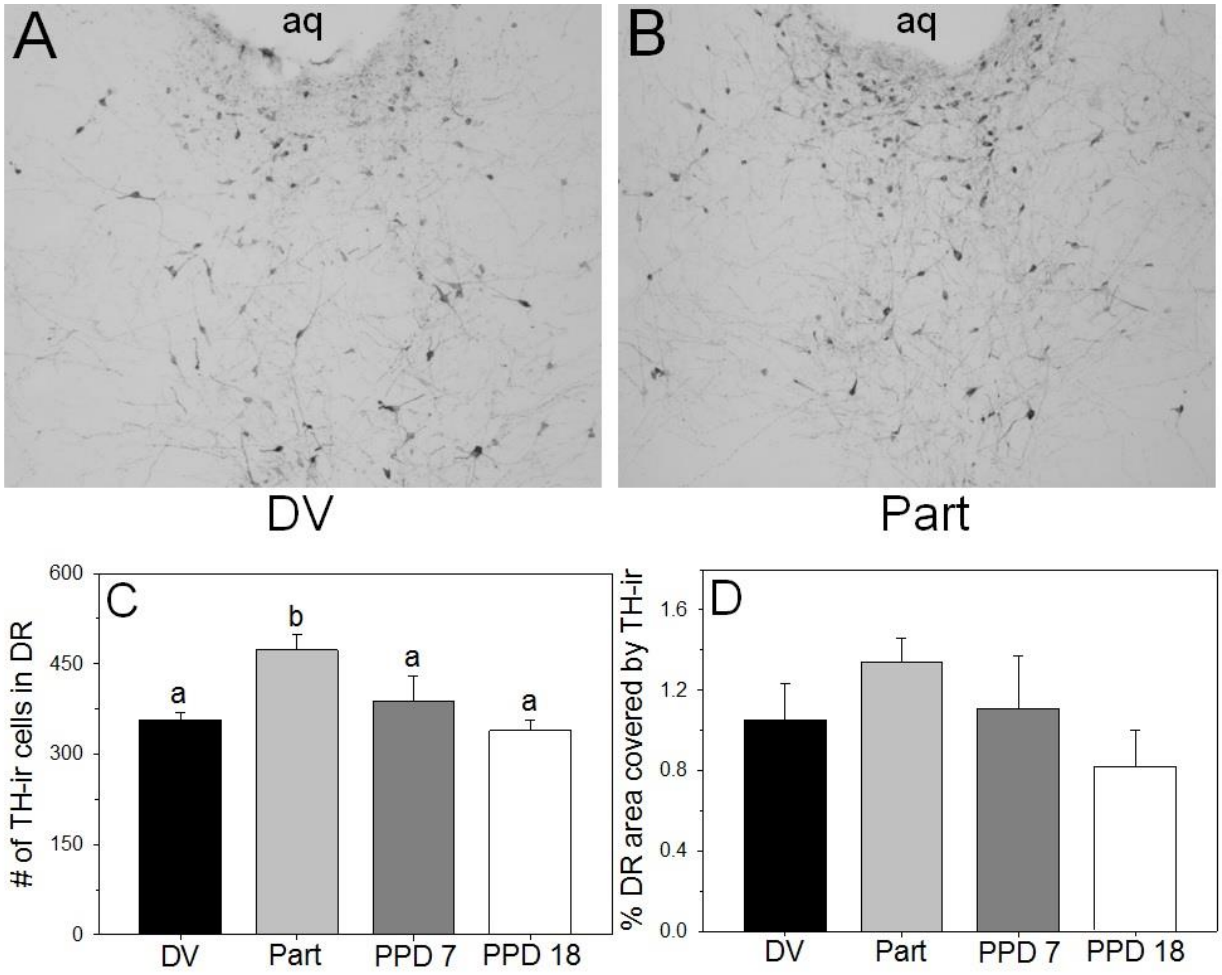

Figure 6: Number of tyrosine hydroxylase immunoreactive neurons in the dorsal raphe across female reproductive state. Representative photomicrographs of the dorsal raphe of a (A) diestrous virgin (DV) and a (B) recently parturient dam (Part). C) Number of tyrosine hydroxylase immunoreactive neurons (Mean ± SEM) in the dorsal raphe of female rats sacrificed as DV, Part, or on postpartum day (PPD) 7, or PPD 18. D) Percentage of the dorsal raphe area covered by tyrosine hydroxylase immunoreactivity (Mean ± SEM) of female rats sacrificed as DV, Part, PPD 7, or PPD 18. Different letters above bars indicates significant difference between groups, $p < 0.05$.

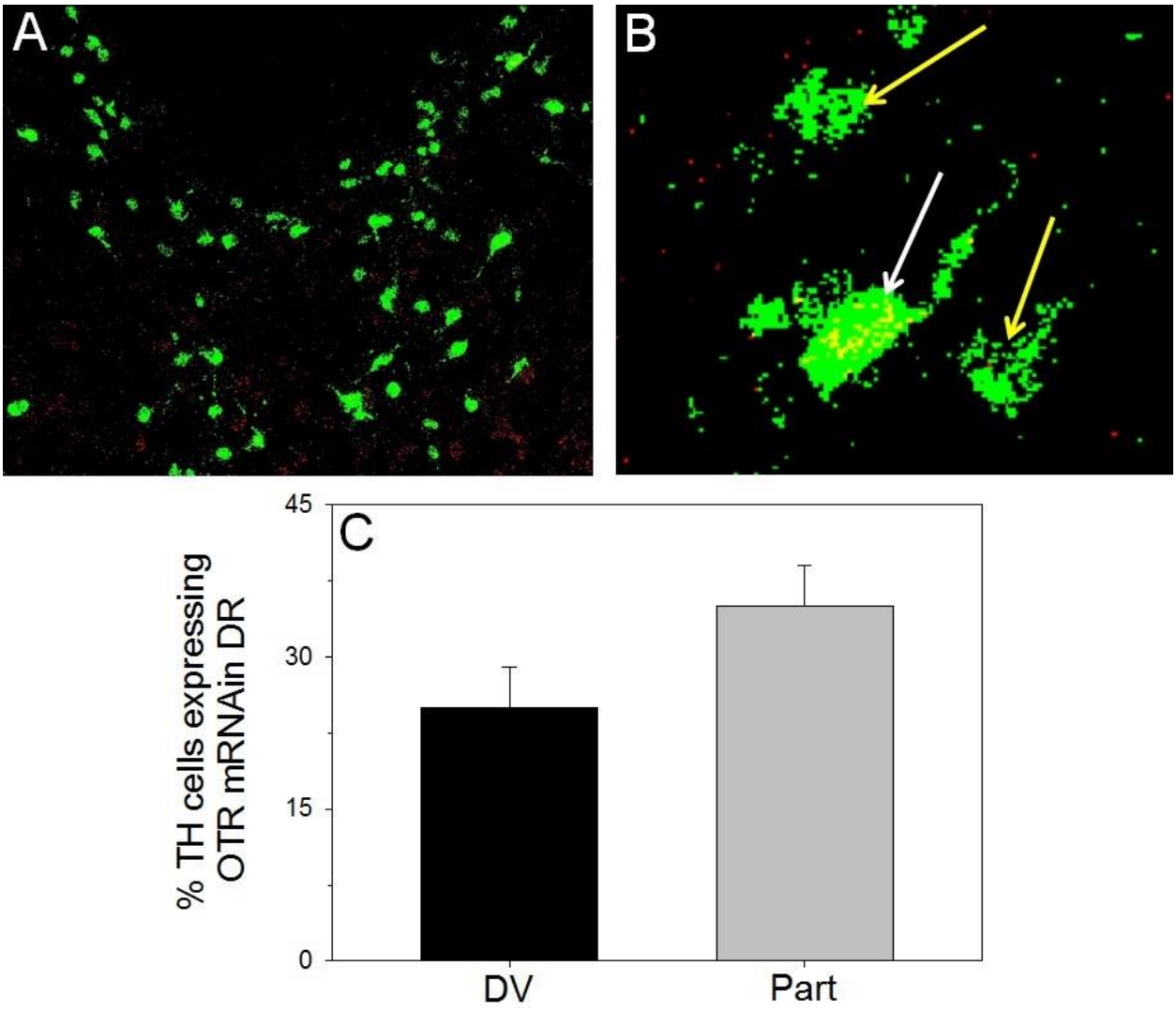

Figure 7: The percentage of tyrosine hydroxylase mRNA-expressing cells that also express OTR mRNA across female reproductive state. A) Representative photomicrograph of the dorsal raphe (tyrosine hydroxylase (green) OTR (red)). B) Photomicrograph showing colocalized tyrosine hydroxylase (green) and OTR (red) hybridization, indicated by white arrows. Yellow arrow indicates a tyrosine hydroxylase-hybridized neuron not expressing OTR hybridization. C) Percentage of tyrosine hydroxylase mRNA-expressing cells that also express OTR mRNA (Mean ± SEM) in the dorsal raphe of female rats sacrificed as diestrous virgins (DV) or soon after parturition (Part).

	Diestrous Virgins (M ± SEM)	Parturition (M ± SEM)	Group ($t_{(4)}$; p; d)
# TH cells	86 ± 3	112 ± 15	1.67; 0.33; 1.68
# TH cells expressing OTR	22 ± 5	40 ± 10	1.91; 0.15; 1.67
%TPH cells expressing OTR	25 ± 4	35 ± 4	1.47; 0.24; 1.50

Table 5: Number of tyrosine hydroxylase mRNA-expressing cells that also express OTR mRNA across female reproductive state. Number of tyrosine hydroxylase mRNA-expressing cells also expressing OTR mRNA (Mean ± SEM) of female rats sacrificed as diestrous virgins or 3 hours after parturition.

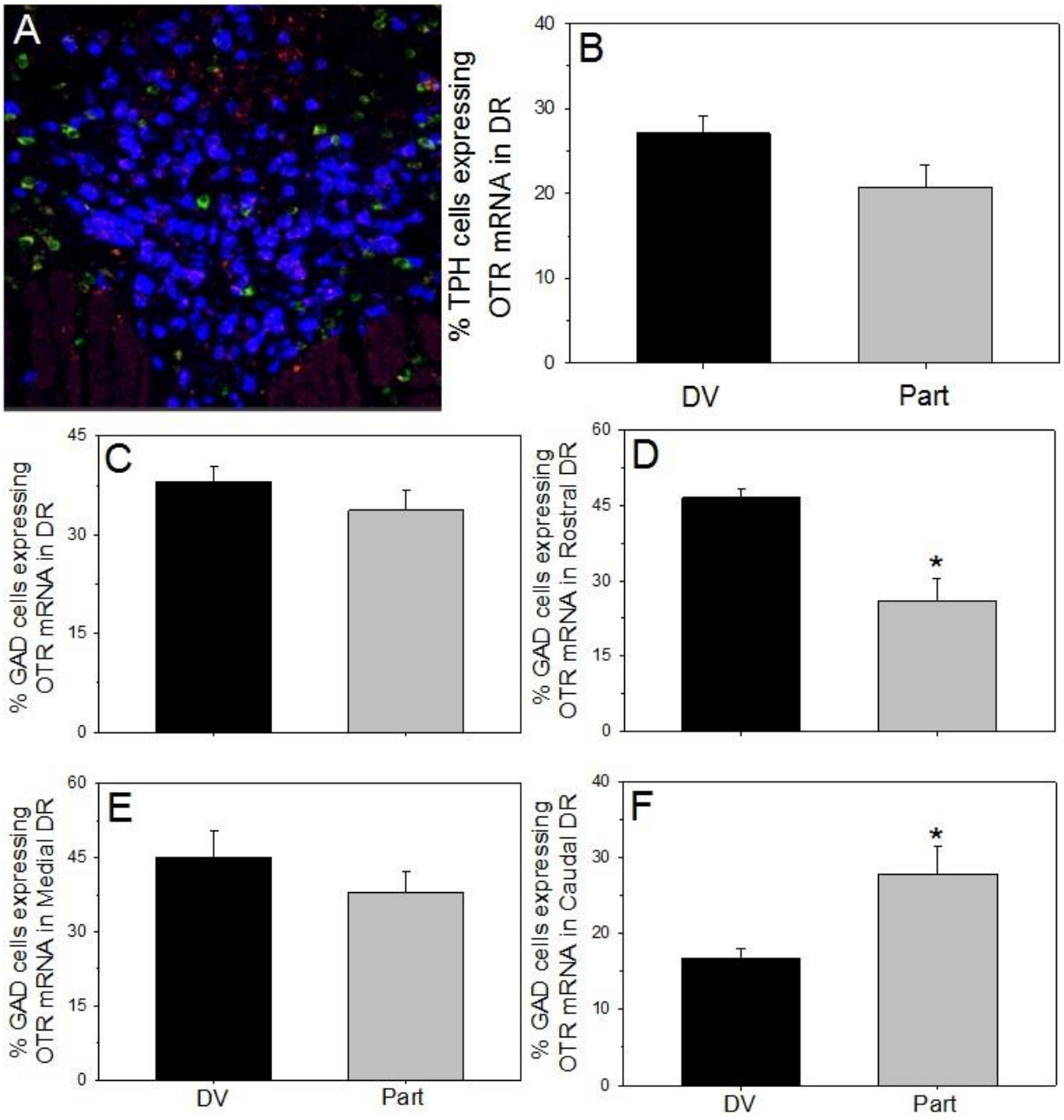

Figure 8: The percentage of glutamic acid decarboxylase mRNA-expressing cells that also express OTR mRNA in the rostral dorsal raphe across female reproductive state. A) Representative photomicrographs of the dorsal raphe. B) Percentage of tryptophan hydroxylase mRNA-expressing cells that also express OTR mRNA (Mean ± SEM) in the dorsal raphe of female rats sacrificed as diestrous virgins (DV) or soon after parturition (Part). Percentage of glutamic acid decarboxylase mRNA-expressing cells that also express OTR mRNA (Mean ± SEM) in the (C) dorsal raphe, (D) rostral dorsal raphe, (E) medial dorsal raphe, or (F) caudal dorsal raphe of female rats sacrificed as DV or Part. * indicates statistically significant difference between groups, $p < 0.05$.

	Diestrous Virgins (M ± SEM)	Parturition (M ± SEM)	Group ($t_{(4)}$; p; d)
Total DR			
# TPH cells	951 ± 135	842 ± 100	0.65; 0.55; 0.53
# GAD cells	830 ± 64	620 ± 43	2.71; 0.053; 2.23
# TPH cells expressing OTR	262 ± 51	179 ± 44	1.23; 0.29; 1.02
# GAD cells expressing OTR	318 ± 38	211 ± 32	2.15; 0.10; 1.76
%TPH cells expressing OTR	27.1 ± 2.1	20.7 ± 2.6	1.94; 0.13; 1.57
% GAD cell expressing OTR	38.1 ± 2.2	33.7 ± 3.0	1.19; 0.30; 0.95
Rostral DR			
# TPH cells	220 ± 61	71 ± 22	2.30; 0.08; 1.89
# GAD cells	199 ± 23	129 ± 4	2.97; 0.09; 2.43
# TPH cells expressing OTR	74 ± 36	19 ± 7	1.53; 0.26; 1.23
# GAD cells expressing OTR	92 ± 7	34 ± 7	6.18; <0.01*; 5.04
%TPH cells expressing OTR	30.3 ± 6.2	25.6 ± 3.5	0.68; 0.54; 0.55
% GAD cells expressing OTR	46.5 ± 1.9	26.1 ± 4.4	4.29; 0.01*; 3.48
Medial DR			
# TPH cells	639 ± 74	650 ± 58	0.12; 0.91; 0.10
# GAD cells	430 ± 72	385 ± 24	0.59; 0.59; 0.48
# TPH cells expressing OTR	164 ± 25	134 ± 33	0.72; 0.51; 0.59
# GAD cells expressing OTR	193 ± 37	148 ± 24	1.04; 0.36; 0.85
%TPH cells expressing OTR	25.6 ± 2.4	20.2 ± 3.3	1.31; 0.26; 1.09
%GAD cells expressing OTR	45.1 ± 5.2	38.0 ± 4.1	1.07; 0.35; 0.87
Caudal DR			
# TPH cells	93 ± 9	121 ± 39	0.71; 0.52; 0.58
# GAD cells	201 ± 4	106 ± 16	5.67; 0.01*; 4.65
# TPH cells expressing OTR	23 ± 6	26 ± 7	0.28; 0.79; 0.26
# GAD cells expressing OTR	34 ± 4	30 ± 5	0.63; 0.57; 0.52
%TPH cells expressing OTR	25.1 ± 5.5	21.9 ± 2.5	0.52; 0.63; 0.43
% OTR cells expressing TPH	16.7 ± 1.4	27.9 ± 3.7	2.87; <0.05*; 2.34

Table 6: Number of tryptophan hydroxylase and glutamic acid decarboxylase mRNA-expressing cells also expressing oxytocin receptor mRNA across female reproductive state. Number of tryptophan hydroxylase and glutamic acid decarboxylase mRNA-expressing cells also expressing OTR mRNA (Mean ± SEM) of female rats sacrificed as diestrous virgins or 3 hours after parturition. * indicates statistically significant difference between groups, $p < 0.05$.

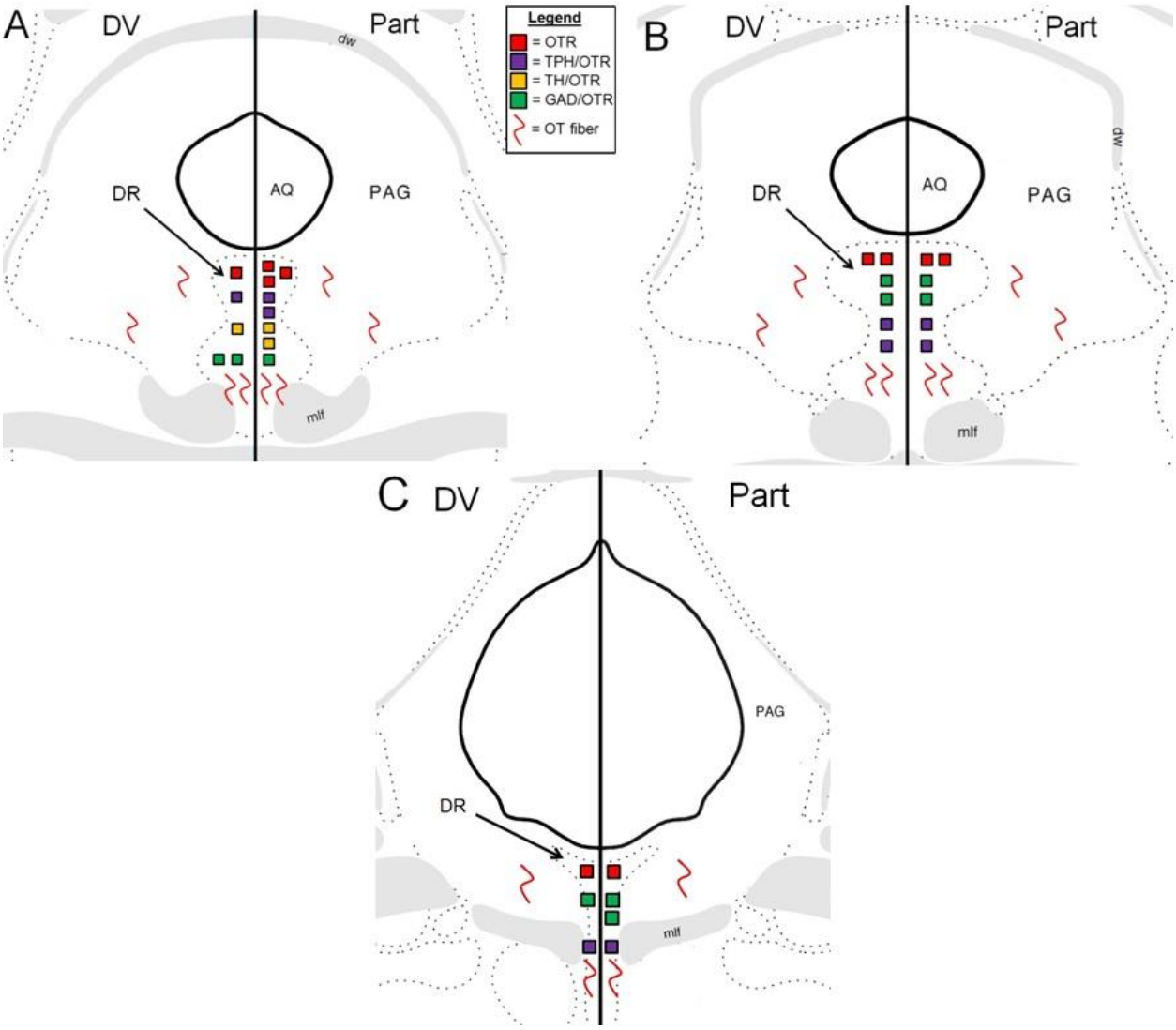

Figure 9: Schematic summarizing the major findings from Chapter 2. Diestrous virgins (DV) are shown on the left of each panel and recently parturient dams (Part) on the right. Levels of OTR binding (red), TPH/OTR (purple), TH/OTR (yellow), GAD/OTR (green), and oxytocin fibers (red lines) are depicted in three rostrocaudal levels of the dorsal raphe (A-C). Modified images from (Swanson, 2004).

Discussion

Oxytocin is an important neuropeptide for the expression of postpartum behaviors. The DR and PAG regulate caregiving behaviors and express OTRs (Barofsky et al., 1983; Holschbach et al., 2018; Lonstein et al., 1998; Lonstein and Stern, 1997a; Yoshida et al., 2009; Yoshimura et al., 1993), but how their OTR expression changes across female reproduction has never been analyzed. Other sites of the brain that positively influence maternal behavior have higher OTR expression during the postpartum period (Bosch et al., 2010; Caldwell et al., 1994; Caughey et al., 2011; Insel, 1986, 1990; Meddle et al., 2007; Pedersen et al., 1994) and are positively associated with maternal behavior (Champagne et al., 2001; Francis et al., 2000; Pedersen et al., 1994). Therefore, I hypothesized that OTR expression in the DR and PAG would be higher in postpartum females compared to DV and possibly also higher than pregnant and later-postpartum groups. I also hypothesized that reproduction would affect OTR expression in a variety of phenotypes of DR cells. I found that: 1) OTR binding in the rostral subregion of the DR was higher in recently parturient dams compared to DV and PPD 7 dams; 2) oxytocin-ir fiber length was higher in the DR and PAGvl of PPD 7 dams compared to DV females and recently-parturient dams; 3) the percentage of both serotonergic and dopaminergic neurons expressing OTRs was highest in the rostral DR of recently-parturient dams compared to DV females; 4) the number of TH-ir neurons in the DR was higher in recently-parturient dams compared to DV females, PPD 7, and PPD 18 dams; and 5) the percentage of GAD mRNA expressing neurons also expressing OTR mRNA was lower in the rostral DR, and higher in the caudal DR of recently-parturient dams compared to DV females.

In partial support of my overall hypothesis, I found 2.5-fold more OTR binding in the rostral subregion of the DR in recently-parturient rats when compared to DV females and PPD 7 dams. This suggests that the rostral DR has increased sensitivity to oxytocin around the time of parturition. Given that other sites of the brain that facilitate the rapid onset of maternal behavior at parturition also have increased OTR expression at parturition (Bosch et al., 2010; Caldwell et al., 1994; Caughey et al., 2011; Meddle et al., 2007; Pedersen et al., 1994), oxytocin acting on the DR might be relevant for the transition to motherhood at parturition.

The higher OTR binding in the rostral DR of recently-parturient dams is likely driven by the high circulating estrogens present at this time (Bridges, 2015; Hansen et al., 1983; Smith and Neill, 1977; Taya and Greenwald, 1982). The OTR gene has an estrogen-response element in its promoter that is responsive to estrogen receptor α (Bale and Dorsa, 1997; Young et al., 1998). In other brain sites, such as the mPOA, there are positive relationships among estrogen receptor α, OTR expression, and the onset of maternal caregiving (Champagne et al., 2001). Estrogen receptor α is expressed in the DR, albeit at low levels (Mitra et al., 2003; Nomura et al., 2005; Shughrue et al., 1997; VanderHorst et al., 2005), where it might increase the expression of OTRs at parturition. Interestingly, in rats, estrogen receptor expression is largely localized to the rostral regions of the DR (Alves et al., 1998), where I also found the highest levels of OTR binding and the most pronounced effect of female reproductive state on OTR binding. Of note, estrogen receptor α is present on both serotonergic and non-serotonergic neurons in the DR (Alves et al., 1998; Nomura et al., 2005; VanderHorst et al., 2005), suggesting that multiple cellular phenotypes likely contribute to the increased OTR binding in the rostral

DR. Consistent with this idea, Experiment 3a revealed that parturient dams had a higher percentage of both serotonergic and dopaminergic neurons expressing OTRs when compared to DV females. It should be noted that the mRNA data from Experiment 4 did not match the immunohistochemical data from Experiment 3a. However, mRNA and protein oftentimes do not match (Maier et al., 2009; Tian et al., 2004), which has also been found for OTR mRNA and protein (Adan et al., 1995; Phaneuf et al., 1997; Yoshimura et al., 1993). Finally, in addition to estrogen's positive influence on OTR expression, estrogen itself can change OTRs from a low-affinity binding state to a high-affinity binding state (Caldwell et al., 1994). Therefore, estrogens likely increase OTR binding in the rostral DR of recently-parturient rats by enhancing OTR expression and increasing OTR binding affinity.

The high percentage of serotonergic neurons expressing OTRs in the rostral DR of parturient dams likely has functional consequences for the peripartum female. These serotonergic neurons preferentially project to cortical regions (Coffield et al., 1992; Kazakov et al., 1993; Kirifides et al., 2001; Waterhouse et al., 1986a), while the more caudal serotonergic neurons of the DR project to subcortical regions (Köhler and Steinbusch, 1982; Krout et al., 2002; Morin and Meyer-Bernstein, 1999). This suggests that oxytocin might have increased control over serotonin release in the cortex during the peripartum period. Because only 45% of the serotonergic neurons express OTRs, oxytocin might control specific serotonergic subcircuits. For example, separate populations of serotonergic neurons in the DR are responsible for motor and sensory control (Fornal et al., 1996; Ranade and Mainen, 2009). Given the results presented here, future studies would benefit from tracing the projections of OTR-expressing serotonergic

neurons, which might help provide further details into the role of oxytocin signaling in the DR. Overall, the high OTR binding in the rostral DR appears to be driven, in part, through increased OTR expression on serotonergic neurons.

There are other cell phenotypes in the rostral DR. It contains a large (~ 1000 neurons) population of dopaminergic neurons (Ochi and Shimizu, 1978; Stratford and Wirtshafter, 1990). I found that just under half (43%) of the TH-ir neurons in the rostral DR expressed OTRs in DV females, but recently-parturient rats had 23% more dual-labeled cells, suggesting that dopamine cells in the DR are also more sensitive to oxytocin around the time of parturition. I also found that the total number of TH-ir neurons was higher in recently-parturient dams when compared to all other females; this was specific to the rostral DR because there was no effect of reproductive state on the number of TH-ir neurons in the VTA. This is the first study to demonstrate an increase in the number of putative dopaminergic neurons in any site in the brain across female reproduction. The possibility arises that these dopaminergic neurons are newly-born, as there is neurogenesis in the DR of female rats, and the number of newborn DR cells that survive is higher during the early postpartum period and lower during the late postpartum period (Holschbach and Lonstein, 2017). In fact, this pattern of cell survival partly matches the increase in TH-ir neurons at parturition and decline thereafter that I found in my study. Importantly, though, studies in mice that use fluorescently-tagged TH find that only 67% (McDevitt et al., 2014) and 74% (Dougalis et al., 2012) of their yellow fluorescent protein-expressing cells in the DR are colabeled with TH-ir. This finding suggests that approximately 30% of dopamine neurons in the DR do not express TH levels high enough for immunohistochemical detection under basal conditions. Consistent with this idea,

when comparing the average number of TH-ir neurons in my DV females (i.e., 357) to recently-parturient dams (i.e., 473), recently-parturient dams have 32% more TH-ir neurons compared to DV females. Given that I found a 23% increase in the percentage of TH-ir neurons expressing OTRs in the DR concurrent with a 32% increase in the number of TH-ir neurons, there are 135% more TH-ir neurons expressing OTRs at parturition compared to what is found in DV females. I conclude that the increased OTR binding in the rostral DR at parturition is driven not only by higher OTR expression on serotonin cells, but also, in part, through increased OTR expression on dopaminergic neurons.

Similar to the reproductive state change in OTR binding, the greater number of TH-ir neurons in recently-parturient dams is likely driven by the high circulating estrogens around parturition (Bridges, 2015; Hansen et al., 1983; Smith and Neill, 1977; Taya and Greenwald, 1982). Administration of estradiol via subcutaneous injections of estradiol benzoate or by implanting estradiol-filled subcutaneous capsules can increase the expression of TH in catecholamine-producing sites (Beattie et al., 1972; Serova et al., 2002; Serova et al., 2004), including in the VTA (Serova et al., 2004), and is estrogen receptor α dependent (Maharjan et al., 2005). As mentioned earlier, estrogen receptor expression is localized to the rostral areas of the DR (Alves et al., 1998), where the dopaminergic neurons are located (Descarries et al., 1986; Lowry et al., 2008; Stratford and Wirtshafter, 1990), and many dopaminergic neurons in the DR express estrogen receptor α (VanderHorst et al., 2005). This suggests that the high estrogens during the peripartum period might underlie the increased number of dopaminergic neurons at that time. Given this result, it would be interesting to see if manipulations that artificially

increase circulating estrogens also increase the number of dopaminergic neurons in the DR. Additionally, OTRs might increase TH translation by activating CaMKII and PKC (Daubner et al., 2011; Vyas et al., 1990). Given that estrogens can increase the transcription of OTRs (Bale and Dorsa, 1997; Young et al., 1998), estrogens might increase the expression of TH through two separate mechanisms.

The higher number of dopaminergic neurons and the percentage of these neurons expressing OTRs at parturition is likely imperative for peripartum changes in behavior. These dopamine neurons increase their firing in response to a social stimulus following isolation, and optogenetic activation of these neurons increase social investigation (Matthews et al., 2016). Conversely, photoinhibition of these neurons decreases social investigation following social isolation (Matthews et al., 2016). Unlike the VTA, these dopaminergic neurons are not reward signaling, as optogenetic activation of them increased place avoidance (Matthews et al., 2016). In addition to enhancing motivation for social contact, these dopamine neurons in the DR are also active during wakefulness, increase wakefulness when optogenetically stimulated, and lesioning them increases the time spent sleeping (Cho et al., 2017; Lu et al., 2006). Enhanced wakefulness and a corresponding decrease in sleep states is seen at parturition (Branchey and Branchey, 1970; Kimura et al., 1996). Thereafter, the decline in the number of dopaminergic neurons following the peripartum period might be necessary for nursing behavior and milk ejection, which in rats requires dams to be in a slow-wave sleep state (Benedetto et al., 2017; Lincoln et al., 1980; Voloschin and Tramezzani, 1979). Therefore, oxytocin activation of dopaminergic neurons in the rostral DR might be involved in both encouraging the dams

to recontact the litter following separation and changes in arousal states involved in nursing.

There were subregion-specific differences among female groups in the percentage of GAD mRNA-containing cells that also expressed OTR mRNA; in the rostral DR/PAGvl, the percentage of GAD mRNA-containing cells that also expressed OTR mRNA was lower in parturient dams compared with DV, while in the caudal DR/PAGvl, that percentage was higher in parturient dams compared with DV females. These GABAergic neurons are largely inhibitory interneurons that regulate serotonergic cells in DR (Boothman and Sharp, 2005; Day et al., 2004; Jolas and Aghajanian, 1997; Roche et al., 2003). OTR activation on these GABA interneurons would presumably inhibit nearby DR serotonin. Given that the serotonergic neurons of the rostral DR preferentially project to cortical regions (Coffield et al., 1992; Kazakov et al., 1993; Kirifides et al., 2001; Waterhouse et al., 1986a), while the more caudal serotonergic neurons project to subcortical regions (Köhler and Steinbusch, 1982; Krout et al., 2002; Morin and Meyer-Bernstein, 1999), my results collectively suggest that oxytocin released into the DR of parturient dams would be predicted to increase serotonin release in cortical areas and decrease serotonin release in subcortical areas.

Oxytocin-ir fiber length was also higher in the DR and PAGvl of PPD 7 dams compared with DV and recently parturient dams. Oxytocin-ir fiber length is an indicator of the capacity for local oxytocin release. For example, lactating rodents have higher oxytocin-ir fiber density than DV females in many sites of the forebrain (Caldwell et al., 1987; Jirikowski et al., 1989; Knobloch et al., 2012), and this is positively associated with the levels of oxytocin released in those sites (Neumann and Landgraf, 1989; Neumann

et al., 1993). This suggests an increased capacity for oxytocin release into the DR and PAGvl of PPD 7 females. Given the pattern of higher OTR binding in the rostral DR of recently-parturient females, along with greater TPH/OTR and TH/OTR, but less GAD/OTR, increased release of serotonin and dopamine from the rostral DR in postpartum dams would be the expected consequence. With regards to the PAG, given that the PAGvl enhances passive coping/immobility, while the PAGl and PAGdl enhance active coping/fight-or-flight (Bandler and Keay, 1996; Benarroch, 2012), oxytocin released into the midbrain PAG might bias the PAG to a passive coping/immobility phenotype. Given that there were longer oxytocin-ir fibers in the DR/PAGvl, it would be valuable to monitor oxytocin release *in vivo* using microdialysis to confirm increased oxytocin release into the DR/PAGvl of postpartum dams.

Finally, OTR binding was higher in the PAGl of pregnancy day 10 and parturient females compared to DV and PPD 7 females. This increase in OTR binding may be important in the analgesia required for parturition. A meta-analysis of oxytocin's effects on pain suggest that oxytocin is a potent analgesic (Rash et al., 2014). I.C.V. infusion of oxytocin increased pain tolerance thresholds (Arletti et al., 1993; Yang et al., 2007), and infusion of an anti-oxytocin serum or an OTR antagonist I.C.V. decreased pain tolerance thresholds in rats (Yang et al., 2011; Yang et al., 2007). These antinociceptive effects of central oxytocin are in part mediated by the PAG, as painful stimuli increase oxytocin release into the PAG (Yang et al., 2011), which then acts to increase pain thresholds (Ge et al., 2002; Yang et al., 2011). This is consistent with the increased pain threshold seen in pregnant and parturient females (Cogan and Spinnato, 1986; Gintzler, 1980; Ohel et al., 2007; Toniolo et al., 1987; Whipple et al., 1990).

In sum, numerous aspects of the oxytocin system in some subregions of the DR and PAG are upregulated in postpartum female rats (Figure 9). Given the higher OTR binding in their rostral DR, along with a greater percentage of serotonergic and dopaminergic neurons expressing OTRs in the face of a lower percentage of GABAergic neurons expressing OTRs there, one could predict increased serotonin and dopamine release from the rostral DR in postpartum dams in response to either their naturally high endogenous or administration of exogenous oxytocin. The serotonergic neurons of the rostral DR preferentially projects to cortical regions (Coffield et al., 1992; Kazakov et al., 1993; Kirifides et al., 2001; Waterhouse et al., 1986a), suggesting that oxytocin's effects may especially increase serotonin release into the cortex. Such changes in the pattern of serotonin and dopamine release in the postpartum brain might be critical for the transition to motherhood that occurs in the peripartum period.

CHAPTER 3: EFFECTS OF OXYTOCIN RECEPTOR KNOCKDOWN IN THE DORSOMEDIAL TEGMENTUM ON POSTPARTUM SOCIOEMOTIONAL BEHAVIORS

ABSTRACT

Oxytocin often facilitates numerous maternal behaviors, but this depends on the brain site where oxytocin is acting. In Chapter Two, I found that aspects of the oxytocin system in the dorsomedial tegmentum (i.e., DR and PAG) are upregulated in postpartum rats. Given that these sites are already known to influence maternal behaviors, oxytocin might be involved by acting within this midbrain region. To investigate the role of OTRs in the dorsomedial tegmentum on postpartum behaviors, I injected an adeno-associated virus promoting the expression of shRNA targeting OTR mRNA on pregnancy day 8. Starting the day following parturition I observed the dams' undisturbed maternal behavior, maternal motivation using pup retrieval tests, anxiety-like behavior in an elevated plus maze and light-dark box, postpartum aggression in a resident-intruder paradigm, and depressive-like behaviors with saccharin preference and forced swim tests. I found that shRNA-mediated knockdown of OTRs in the dorsomedial tegmentum increased infanticide, decreased nursing, and increased non-pup directed behaviors. These effects were not due to decreased maternal motivation as dams' rapid retrieval of pups was unaffected. OTR knockdown also decreased dams' anxiety-like behaviors in an elevated plus maze, increased postpartum aggression, and increased the percentage of time spent floating in the forced swim test. An analysis of serotonin fibers in the forebrain revealed lower fiber length in OTR-knockdown dams in the face and trunk regions of the primary somatosensory cortex (S1) and higher fiber length in the lateral orbitofrontal cortex (lOFC) and mPOA. Serotonin fiber length in the S1 was positively associated with time in the

nest and kyphotic nursing and negatively associated with postpartum aggression. Conversely, serotonin fiber length in the lOFC was positively associated with postpartum aggression, as well as time spent in the open arms. Therefore, OTR signaling in the dorsomedial tegmentum is critical for the normal display of numerous postpartum behaviors. Given that serotonin influences somatosensory processing and tactile stimulation from the pups regulate all maternal behaviors, implications of dorsomedial tegmental OTR signaling for how mothers sense tactile inputs from their offspring are discussed.

Introduction

Oxytocin positively influences caregiving (Champagne et al., 2001; Fahrbach et al., 1984, 1986; Fahrbach et al., 1985; Pedersen et al., 1982; Pedersen and Boccia, 2003; Pedersen and Prange, 1979) and postpartum aggression (Bosch et al., 2005; Ferris et al., 1992; Sabihi et al., 2014a), while decreasing postpartum anxiety (Figueira et al., 2008; Neumann et al., 1999; Sabihi et al., 2014a). Oxytocin does this by acting in the mPOA to facilitate caregiving activities (Bosch and Neumann, 2012; Champagne et al., 2001; Pedersen et al., 1994; Shahrokh et al., 2010), in the CeA for maternal aggression (Bosch et al., 2005; Ferris et al., 1992), and in the mPFC for postpartum anxiety (Sabihi et al., 2014a). However, there is much less information about the role of OTRs outside forebrain sites in postpartum behavior. Two sites likely important for OTR regulation of postpartum behavior are the midbrain DR and PAGvl. Both sites express OTRs (Yoshida et al., 2009; Yoshimura et al., 1993), and are already known to be critical for numerous postpartum behaviors (Barofsky et al., 1983; Holschbach et al., 2018; Lonstein et al., 1998; Lonstein and Stern, 1997a). For example, lesioning the serotonin neurons within the DR increases pup mortality, affects the patterning of nursing across lactation, and decreases postpartum aggression (Barofsky et al., 1983; Holschbach et al., 2018). Lesioning the PAGvl decreases kyphotic nursing and anxiety-like behavior, while increasing postpartum aggression (Lonstein et al., 1998; Lonstein and Stern, 1997a).

In Chapter Two, OTR expression and oxytocin-ir fiber length in the DR and PAGvl were found to be upregulated in postpartum rats. OTR binding was higher in the rostral DR in recently parturient dams when compared to DV, suggesting increased sensitivity to oxytocin during the peripartum period. This was found to be driven, in part, by a greater

percentage and number of serotonin and dopamine neurons expressing OTRs. Additionally, oxytocin-ir fiber length in the DR and PAGvl was greater in PPD 7 dams, suggesting a higher capacity for oxytocin release into the dorsomedial tegmentum at that time. However, the behavioral consequences of OTR expression in the dorsomedial tegmentum in mother rats is unknown. To address this, an AAV producing shRNA targeting OTR mRNA was injected into the dorsomedial tegmentum on pregnancy day 8, before any of the reproductive-state changes I found occur. Maternal caregiving, retrieval, aggression, anxiety and depressive-like behaviors were then observed following parturition (Figure 10). I hypothesized that OTR knockdown would affect multiple aspects of postpartum behavior. I predict that knocking down OTRs in the dorsomedial tegmentum would generally interfere with most maternal caregiving behaviors.

Regarding aggression, oxytocin has variable effects on postpartum aggression depending on the site where it acts (Bosch et al., 2005; Consiglio et al., 2005; Ferris et al., 1992; Giovenardi et al., 1998; Sabihi et al., 2014a). Given that the DR is a major serotonergic nucleus, and that serotonin from the DR is positively related to postpartum aggression (De Almeida and Lucion, 1994; Ferreira et al., 2000; Holschbach et al., 2018; Johns et al., 2005), I predict that knocking down OTR expression in the dorsomedial tegmentum likely decreases its serotonin output and will decrease postpartum aggression. Oxytocin is anxiolytic in postpartum rats (Figueira et al., 2008; Neumann et al., 1999; Sabihi et al., 2014a), so I predict that OTR knockdown in the dorsomedial tegmentum will increase dams' anxiety-like behaviors. Finally, oxytocin has antidepressant effects (Arletti et al., 1995; Arletti and Bertolini, 1987; Nowakowska et al., 2002; Ring et al., 2010; however, see Slattery and Neumann, 2010), so I predict that

knocking down OTR expression in the dorsomedial tegmentum will increase depressive-like behaviors.

Somatosensory inputs that mothers receive from their offspring is essential for the expression of all postpartum behaviors; preventing mothers from touching their pups eventually causes all peripartum behavioral modifications to return to a pre-mating state (Erskine et al., 1978; Erskine et al., 1980b; Lonstein, 2005; Miller et al., 2011; Morgan et al., 1992; Stern and Johnson, 1990; Svare and Gandelman, 1973). This may depend on oxytocin/serotonin-induced plasticity in brain sites involved in somatosensation. The primary somatosensory cortex (S1) and lateral orbitofrontal cortex (lOFC) are crucial for sensing and providing affective information about somatosensory cues (Francis et al., 1999; McGlone et al., 2012; Rule et al., 2002; Trotter et al., 2016). DR serotonergic neurons project to the S1 and lOFC (Kirifides et al., 2001; Linley et al., 2013; Waterhouse et al., 1986a), where serotonin has an inhibitory effect (Dugué et al., 2014; Maia and Cano-Colino, 2015; Trotter et al., 2016; Waterhouse et al., 1986b). Serotonin in the lOFC helps rodents switch between tasks (Boulougouris et al., 2008; Boulougouris and Robbins, 2010; Hatcher et al., 2005; McLean et al., 2009). Therefore, changes in serotonin signaling in the S1 and lOFC could reflect changes in serotonin necessary for dams' ability to detect pup cues and switch their display of various postpartum behaviors. To begin exploring this possibility, serotonin-ir fiber length was measured in the S1 and lOFC.

Materials and methods

Subjects

Subjects were female Long-Evans rats descended from rats purchased from Harlan Laboratories (Indianapolis, IN), born and raised in the Lonstein breeding colony at Michigan State University. Females were housed with 2 or 3 same-sex littermates in clear polypropylene cages (48 cm x 28 cm x 16 cm) containing wood chip bedding, with food (Tekland rat chow, Indianapolis, IN) and water available *ad libitum*, the room was maintained on a 12:12 light/dark cycle (lights on at 0700 hr). Females' estrous cycles were monitored daily by vaginal smearing. On a day of proestrus, subjects used for the pregnant and postpartum groups were placed overnight with a sexually-experienced male from our colony. Pregnancy was confirmed the next day by semen in a vaginal smear or by the presence of a vaginal plug. Subjects were then housed with 1-2 other pregnant females until stereotaxic surgeries on pregnancy day 8, after which they were singly housed. All procedures were performed in accordance with the principles of the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Michigan State University.

Creation of viral construct

A collection of candidate shRNAs against OTR mRNA (NCBI reference sequence: NM_012871.3) were amplified using real-time PCR. These shRNAs were designed to ensure target specificity. The shRNAs were then cloned into a specialized adeno-associated virus (AAV) genome under control of the H1 promotor. After preparing the completed shRNA constructs, we examined each for the efficacy to knockdown OTR

expression *in vitro* using a dual-luciferase (Firefly-Renilla) assay system, and our best candidate shRNA led to a ~79% knockdown of OTR mRNA (Figure 11B). The chosen OTR shRNA sequence was CGGTGAAGATGACCTTCAT. The completed vector genome was then packaged into an AAV 9 capsid, which has been shown to be have high transduction in the DR (Vincent et al., 2014). In addition to the OTR shRNA vector, a control shRNA was produced with the OTR-specific shRNA replaced with a scrambled shRNA containing the same nucleotide composition as the OTR shRNA. Both AAV genomes contain a GFP reporter gene under the control of the chicken beta-actin promotor/cytomegalovirus enhancer promotor hybrid (pCBA).

Stereotaxic injections

On pregnancy day 8, female rats were weighed and anesthetized with ketamine (90 mg/kg IP; Butler, Dublin, OH) and xylazine (8 mg/kg IP; Butler, Dublin, OH) and placed in a stereotaxic apparatus. The scalp was retracted, and a hole was drilled into the skull above the dorsal raphe (DR; A/P = 7.8 mm, M/L = 0.0 mm from bregma). 1 µL of OTR-shRNA or the scrambled control vector solution was slowly injected (~0.5 µL / 5 mins) into the DR through a Neuros Hamilton syringe at 6.7 mm ventral from the skull. The needle remained in the DR for 10 min and then slowly retracted. The scalp was closed with surgical staples. Subjects received postoperative care including twice-daily subcutaneous injections of buprenorphine (0.015 mg/kg) for 1 day after surgery and then left undisturbed until parturition. On the day of parturition (PPD 1), litters were culled to 8 pups per subject (4 males and 4 females).

In vivo determination of oxytocin receptor knockdown

Before any behavior studies were conducted, level of oxytocin receptor knockdown was determined *in vivo*, with the goal of reaching at least 50% knockdown (Khvorova et al., 2003; Reynolds et al., 2004; Ui-Tei et al., 2004). Within 3 hrs of parturition, the OTRKD- and Scramble-injected dams used to determine level of oxytocin receptor knockdown (n = 6/group) were weighed, rendered unconscious with CO_2 and rapidly decapitated. Brains were removed from the skull, flash frozen with isopentane, and stored at -80 °C until sectioning. Brains were cut coronally into 300-µm-thick sections using a cryostat (Leica CM1950, Nussloch, Germany) to obtain 3 sections that included the DR (-7.3 to -8.3 mm from bregma). The DR was punched from the sections using a 1-mm-diameter micropuncher (Harris Micropunch, Hatfield, PA) to examine OTR mRNA levels. The tissue was homogenized in RLT buffer (74134, Qiagen, Valencia, CA) containing β-mercaptoethanol by pulsed sonication for 20 sec at 20% amplitude (Fisher Scientific, Pittsburgh, PA). mRNAs were then extracted using the RNeasy Plus Mini Kit (74134, Qiagen, Valencia, CA) per the manufacturer's instructions. The extracted mRNAs were quantified using a Gene Quant 100 spectrophotometer (General Electric, Marlborough, MA) by measuring the 260 nm absorbance values. 100 ng of mRNAs were then converted to cDNA using a high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA) per the manufacturer's instructions. After conversion to cDNA, samples were stored at -20 °C until being analyzed with real time RT-PCR.

OTR mRNA was run in triplicate and included cDNA, primers, and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a 25-µL reaction. All primers were from Integrated DNA Technologies (Coralville, Iowa). An ABI PRISM 7000

Sequence Detection System (Applied Biosystems, Foster City, CA) was used for quantification, with the following settings: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. A dissociation curve was run for each sample to ensure that only a single product was transcribed. To analyze changes in OTR mRNA, two transcripts were run: OTR (200 nM primers: Forward- TGC TCG TTA CCT GAA GGG C; Reverse- TCT TGC TGA CAC TCG TCT CG) and our control gene, HPRT-1 (200 nM primers: Forward- GAA ATG TCT GTT GCT GCG TCC; Reverse – GCC TAC AGG CTC ATA GTG CAA). Additionally, because the vasopressin V1a receptor gene has high homology with the oxytocin receptor gene, PCR was also run for V1a receptor to confirm specificity of the oxytocin receptor viral construct (200nM primers: Forward – GTG GTC GTC TTG GGT ACA TGC; reverse – CTT CAC AGT GCG GAT CTT GGC). PCR products of each primer set were sequenced at the RTSF Genomics Core at Michigan State University to confirm specificity. During quantification, a no-template control was run alongside the samples to ensure that no primer-dimer amplification had occurred. In addition, mRNA samples that were not run through the reverse transcription kit were run simultaneously to ensure no gDNA contamination. Amplification efficiencies were calculated for each primer set, and each was within the accepted range (1.90- 2.10) to use the $\Delta\Delta$CT method to calculate fold change between groups, with OTR and V1a normalized to HPRT-1 (Schmittgen and Livak, 2008).

Undisturbed maternal behavior observations

Dams' behavior in the homecage under undisturbed conditions was recorded 3 times daily (0900, 1300, and 1500 hr; Fig 10) for 30 min each on postpartum days 2 – 8.

These timepoints were chosen to ensure that we would be able to observe the maternal caregiving behaviors of interest, given that dams spend much more time interacting with their litters during the light photophase compared to during the dark photophase (when dams are more likely to forage, explore, and sleep away from the nest) (Ader and Grota, 1970; Grota and Ader, 1969). Spot checks were made every 30 s and the maternal behaviors recorded included pup licking, nest building, hovering over the pups in the nest, and nursing the litter in three distinct postures (i.e., kyphosis/crouched, supine/on side, and prone/flat). The four in-nest postures were analyzed individually as well as in two broader categories – those involving erect postures (hovering over and kyphosis/crouched) and those involving passive posture (supine and prone) (Rees et al., 2004). Non-maternal behaviors including sleeping away from the pups, exploring the home cage, self-grooming, and eating or drinking were also recorded. Additionally, quality of the dams' nests were scored during each undisturbed observation on a scale of 0-3 (0 = no nest, 1 = poor nest, 2 = partial nest, 3 = complete nest with high walls). Frequencies of each behavior (i.e., the total number of instances of each behavior) were totaled for each day and those totals were used for data analyses. The multiple observers established an inter-rater reliability of >90% before beginning data collection.

Retrieval testing

Dams rarely retrieve pups during early-postpartum observations under undisturbed conditions (Brewster and Leon, 1980); therefore, litters were experimentally removed from the nest to induce retrieval behavior from the dams as an indicator of maternal motivation under mildly stressful conditions. On PPDs 2, 4, and 6, immediately

follow undisturbed maternal behavior observations, litters were removed from their homecage and placed in an incubator set to nest temperature (34 °C) for 15 min. Following separation, the pups were scattered in the homecage on the opposite side of the nest site. Latencies for the dams to retrieve each pup and hover over all of the pups in the nest were recorded. Any subject that failed to retrieve all 8 of their pups to the nest site within 5 min had their pups placed into the nest by the experimenter and received a retrieval latency of 600s for each pup that wasn't retrieved. Maternal behaviors were then observed and recorded by spot checks every 30 s for an additional 10 min. Given that there were no main effects of postpartum day nor any interactions between postpartum day and any behaviors, the behavioral results from the three retrieval days were averaged together for final analyses.

Elevated plus maze

On PPD 3, following undisturbed maternal behavior observations (~1600- 1700 hr), dams were brought in their homecage to a nearby behavior testing room containing an elevated plus-maze. The room was illuminated by one 100W light bulb. The elevated plus-maze was elevated 50 cm from the floor and made of black plastic with four arms emerging from a 10 X 10 cm center square. Arms were 10 cm wide by 50 cm long, two of which had 40-cm high walls, 2 of which had no walls. Approximate illumination on the open arms was 28 lux, and approximate illumination on the closed arms was 2 lux (Miller et al., 2011). At the start of testing, each subject was removed from their homecage and placed in the middle square facing one of the open arms and released. Their homecage containing the pups was then removed from the testing room. Behavior was recorded

from a camera hanging above the testing apparatus for 10 min. The elevated plus-maze was cleaned with 70% ethanol between subjects. The recording of the behavior was scored with a computerized data acquisition system that allowed recording the time spent in the open arms and closed arms, and the frequency of each. An entry into an arm was coded when the dam placed her head and both paws into an arm, while time spent in the center square was recorded as time spent in neither arm.

Light-dark box

On PPD 5, following undisturbed maternal behavior observations (~1600-1700 hr), dams were brought in their homecage to a nearby behavior testing room containing the light-dark box. The light-dark box was made of white and black opaque Plexiglas chambers (20 X 30 X 30 cm light chamber, 30 X 30 X 30 cm dark chamber) connected by a 10 X 10 cm door in the middle of the wall. At the start of testing animals were placed in the middle of the light chamber facing away from the door and released. Behavior was recorded from a camera hanging above the testing apparatus for 10 min. Ambient light in the light side of the chamber was 624 lux, while the ambient light in the dark side was 3 lux (Miller et al., 2011). After testing, females were removed from the light-dark box and returned to the homecage and the colony room. The light-dark box was cleaned with 70% ethanol between each subject. Females' latency to enter the dark chamber was scored, total time spent, entries, and nose pokes into the light chamber was then scored using the recording and a computerized data acquisition system. Rears while in the light chamber were also scored.

Maternal aggression

On PPD 7, following undisturbed maternal behavior observations, dams were brought in their homecage to a nearby behavior testing room. An experimentally naïve, post-pubertal male (50-60 days old) smaller in size than the dam was then placed into the dams' homecage with the litter present. The dam's behavior was recorded for 10 min, and the frequencies of and time spent performing frontal attacks and lateral attacks were scored and analyzed. The total time spent attacking the male was also analyzed. Immediately following the 10-min test, males were removed from the cage and sacrificed by CO_2 asphyxiation. Females were then returned to the colony room.

Saccharin preference test

A saccharin habituation period began in the morning of PPD 8 (~0800 hr), when a two water bottles (one containing 0.1% saccharin and another water) was placed in the dams' homecage after being weighed. Approximately 8 hours later, the position of the two bottles were weighed again, and their positions switched to avoid confounds related to side preference. Testing on the morning of PPD 9 (~0800 hr) involved both bottles being weighed, and then removed from the cage along with all food for 4 hr. Following the 4 hr food and water deprivation, food and both water bottles were returned to the homecage and pups removed from the homecage (to limit the potential distraction of having the pups in the cage). Following a 1 hr testing period, the bottles were removed and weighed, and the regular water bottle returned to the cage (Fernandez et al., 2014; Green et al., 2009). The change in bottle weight between pre- and post-1 hr testing was used for data analyses.

Forced swim test

Following undisturbed behavior observation on PPD 8, dams were brought in their homecage to a nearby behavior testing room containing a 50 cm X 20 cm Plexiglas cylinder 40 cm full of 24ºC water for pre-exposure to the testing procedure. The dams were placed into the cylinders and behavior recorded for 15 min. Following the 15-min period, dams were removed from the cylinder, towel-dried, and placed into a new cage containing a prewarmed heating pad until dry. Once dry, dams were returned to their homecages and the colony room. The next day, 1 hr after saccharin preference testing the dams were brought in their homecage back to the behavior testing room. The dams were then placed into the cylinder and recorded for 10 min. Following the 10 min test, dams were removed from the cylinder, towel-dried, and placed into a new cage containing a prewarmed heating pad until dry. Once dry, dams were placed in the homecage until sacrifice that same evening. Females' behavior was then scored from the digital recording with a computerized data acquisition system (Soloman coder). The percentages of time swimming and floating were coded and analyzed. Importantly, while forced swim testing was conducted after numerous other behaviors tests, the forced swim test has been previously shown to be unaffected by repeated handling (Platt and Stone, 1982).

Sacrifice, perfusion, and brain extraction

Following forced-swim testing on PPD 9, subjects were perfused transcardially with saline followed by 4% paraformaldehyde, the brains extracted, postfixed overnight, and submerged in 30% sucrose. Brains were cut into 40-μm sections in three series and stored at -20 ºC in a sucrose-based cryoprotectant until processing. One full series was

later processed to determine viral injection sites and another for serotonin-immunoreactive fiber analysis in the forebrain.

GFP immunohistochemistry for injection localization

All rinses were in PBST. On a day of IHC, sections were rinsed in PBST and then blocked in a 0.1% triton-PBS solution containing 2% NDS for 1 hr at room temperature. Sections were then incubated in purified rabbit anti-GFP primary antiserum (A6455; Thermo-Scientific, Waltham, MA; 1:10,000) for 24 hrs at 4 °C. Finally, tissue was incubated in donkey anti-rabbit Alexafluor 488 secondary antisera (A21206, Fisher Scientific, Pittsburgh, PA; 1:500) for 2 hrs at room temperature, followed by slide mounting and coverslipping using fluoromount G. The entirety of the dorsomedial tegmentum was scanned for GFP-ir cells under 40X magnification using a Nikon Eclipse E600 light microscope. Any subjects that had GFP-ir cells outside the dorsomedial tegmentum were removed from analyses.

Serotonin immunohistochemistry

To help verify that shRNA infusion did not produce gross impairments in DR cell health, three matched sections per subject containing the DR (-7.5 to -8.4 mm from bregma) were selected for immunohistochemical analysis of the number of serotonin-ir cells. Additionally, to determine the effects of OTR knockdown on serotonin-ir fiber length in forebrain sites relevant to maternal behavior, one section containing each of the S1 (-2.85 mm from bregma), the motor cortex (M1; -0.0 mm from bregma), the mPOA (-0.51 mm from bregma), and the lOFC (+3.2 mm from bregma) were analyzed (n = 6/group).

Serotonin-ir fiber length has often been used as a reflection of the capacity for serotonin release, with manipulations that decrease central serotonin levels decreasing serotonin-ir fiber length and serotonin turnover (Edwards et al., 1986; Hritcu et al., 2007; Tohyama et al., 1988; Wallace et al., 1982), while manipulations that increase central serotonin levels increase serotonin-ir fiber and serotonin release (Bel and Artigas, 1995; Celada and Artigas, 1993; Nielsen et al., 2006). Importantly, though, this occurs without changing serotonin transporter-ir fiber levels (Dewar et al., 1992; Graham et al., 1987; Nielsen et al., 2006), suggesting that serotonin-ir fiber length is not necessarily a measure of serotonin fiber length, but of the capacity for serotonin release.

Immunohistochemistry was conducted using methods previously reported in detail elsewhere (Holschbach et al., 2018). All rinses were in 0.1 M PBS containing 0.1% triton-X. Briefly, sections were incubated in 0.1% sodium borohydride for 10 min, followed by a 10 min incubation in 0.5% hydrogen peroxide diluted in 0.1% triton-X PBS. Tissue was then blocked in a solution contain 20% NGS in 0.1% triton-X PBS for 1 hr at room temperature. Sections were then incubated in a triton-X-PBS solution contain 2% NGS and a rabbit anti-serotonin polyclonal antiserum (NT-102; Protos Biotech Corp, New York, NY; 1:7500), for 72 hrs at 4 ºC, then in a biotinylated goat anti-rabbit secondary antiserum (BA-9200; Vector Labs, Burlingame, CA; 1:500) for 1 hr at room temperature. Sections were incubated in ABC solution (PK 6100, Vectors Labs, Burlingame, CA) for 1 hr at room temperature, serotonin-ir visualized using DAB, mounted, and the slides coverslipped. The number of serotonin-ir cells in the DR (complete visual area) was counted on each section by experimenters' blind to the subjects' experimental condition under 100X magnification using a Nikon Eclipse E600 light microscope. Given the very low

background staining (Figure 13), somata with any visible serotonin immunoreactivity were included in the quantification. The summed number of serotonin-ir cells counted in all sections for each subject was used for data analyses. The length of serotonin-ir fibers in the trunk and barrel cortex regions of the somatosensory cortex, and in the entire area of the motor cortex, mPOA, and lOFC on the sections, were traced bilaterally by experimenter's blind to the subjects' experimental condition under 200X magnification using a Nikon Eclipse E600 light microscope. The length of serotonin-ir fibers traced per site for each subject was used for data analyses.

Statistical analyses

Undisturbed maternal behavior was analyzed using mixed-design repeated-measures ANOVAs (with Greenhouse-Geisser correction) involving time (i.e., postpartum day) as the repeated measure and experimental condition (i.e., scrambled or oxytocin receptor shRNA) as the between-subject variable. Data for all between-subjects variables were normally distributed. Analysis of infanticide occurrence was done using Fisher's exact test. Two dams completely cannibalized their litters and the replacement litters given to them, so were removed from all behavioral analyses other than infanticide. Another three dams killed 1 - 3 pups in their litters, but after replacement with foster pups, these dams were able to maintain the litter and remained in the study. Retrieval, aggressive, anxiety-like, and depressive-like behaviors were analyzed using Student's *t*-tests. In cases of unequal variances, Welch's *t*-tests were used. Partial η^2 (η^2_p) are reported as measures of effect sizes from the ANOVAs, Cohen's *d* are reported as

measures of effect sizes from the pairwise comparisons, and ϕ is reported as a measure of effect size for Fisher's exact test. $p < 0.05$ was considered statistically significant.

Results

Confirmation of viral-mediated oxytocin receptor knockdown

The shRNA vector produced a 79 ± 0.2 % knockdown in OTR mRNA *in vitro*, while the scrambled vector had no effect on OTR mRNA compared to a control vector (Fig 11B). When tested *in vivo*, the shRNA vector construct injected during pregnancy produced a 58 ± 4 % knockdown in OTR mRNA when animals were examined within 3 hrs after parturition (Fig 11C). There were no effects of shRNA viral injection on V1a receptor expression ($t_{(10)} = 1.63$, $p = 0.16$; data not shown).

Confirmation of injection sites with GFP

In all cases (16 shRNA injected dams), GFP was found in the dorsomedial tegmentum (i.e., DR and PAGvl). In cases used for analyses, GFP was never found as far rostrally to be in the Edinger-Westphal nucleus or laterocaudally to be in the locus coeruleus. Caudally, GFP in the 16 dams did spread to the caudal DR, dorsomedial tegmental nucleus, and laterodorsal tegmental nucleus (Fig 12). Four of the 16 shRNA-injected dams were removed for final analysis. In two cases there was minimal GFP in the dorsomedial tegmentum, and GFP had spread to the PAGdl and PAGd. In two other cases, GFP was in the dorsomedial tegmentum but spread rostrally to the Edinger-Westphal nucleus and ventrally to the VTA.

Number of serotonin neurons in dorsal raphe

There was no effect of OTR knockdown on the number of serotonin-ir neurons in the DR as a whole ($t_{(10)} = 0.46$, $p = 0.65$, $d = 0.27$; Fig 13B), or in any of its subregions (Table 7).

Maternal and litter health

OTR-knockdown dams gained over 2% of their body weight over the testing period, while scrambled controls lost -0.2% of their body weight ($t_{(18)} = 2.43$, $p = 0.03$, $d = 1.06$; Fig 14A). There were no effects of maternal OTR knockdown on daily litter weight gains ($t_{(18)} = 1.12$, $p = 0.28$, $d = 0.49$; Fig 14B).

Maternal behavior

Almost half (5/12) of the OTR-knockdown dams committed infanticide compared to none of the controls ($p = 0.04$, 95% CI [1.06, 2.77], $\phi = 0.50$; Fig 15A). Incidence of infanticide occurred on PPDs 2 and 3 only. OTR-knockdown dams were in the nest ($F_{(1,18)} = 17.56$, $p = <0.01$, $\eta^2_p = 0.49$; Fig 15B), nursed in any position ($F_{(1,18)} = 15.15$, $p = <0.01$, $\eta^2_p = 0.46$; Fig 15C), and nursed specifically in a kyphotic posture ($F_{(1, 18)} = 22.14$, $p = <0.001$, $\eta^2_p = 0.55$; Fig 15D) less often than controls. Conversely, OTR-knockdown dams displayed almost twice as much non-pup directed behaviors compared to scrambled control dams ($F_{(1, 18)} = 18.87$, $p = <0.001$, $\eta^2_p = 0.51$; Fig 16A). For non-pup directed behaviors, OTR-knockdown dams ate/drank over two-times more often ($F_{(1,18)} = 19.22$, $p = <0.001$, $\eta^2_p = 0.52$; Fig 16B), as well as self-groomed ($F_{(1, 18)} = 7.80$, $p = 0.01$, $\eta^2_p =$

0.30; Fig 16C), and explored their homecage ($F_{(1, 18)}$ = 15.00, p = 0.001, η^2_p = 0.46; Fig 16D) more often than controls.

There was an interaction between group and postpartum day for nursing in any posture ($F_{(6, 108)}$ = 3.91, p = 0.01, η^2_p = 0.18; Fig 15C) and specifically kyphotic nursing ($F_{(6, 108)}$ = 3.54, p = 0.01, η^2_p = 0.17; Fig 15D), such that frequencies of both behaviors were similar on PPDs 2 and 3, but thereafter OTR-knockdown dams displayed lower frequencies than scrambled controls. There was also an interaction between group and postpartum day on the frequency of non-pup directed behavior ($F_{(6, 108)}$ = 4.22, p = <0.01, η^2_p = 0.19; Fig 16A), self-grooming ($F_{(6, 108)}$ = 4.04, p = <0.01, η^2_p = 0.18; Fig 16C) and exploring ($F_{(6, 108)}$ = 2.77, p = 0.04, η^2_p = 0.13; Fig 16D), such that the frequencies of these behaviors were similar on PPDs 2 and 3, but thereafter OTR-knockdown dams displayed higher frequencies than scrambled controls. There were expected main effects of postpartum day for several behaviors (see Table 8).

Pup retrieval

OTR-knockdown and control dams had similar latencies to retrieve their first pup ($t_{19)}$ = 0.23, p = 0.83, d = 0.10; Fig 17A) and to group all their pups ($t_{(19)}$ = 0.45, p = 0.66, d = 0.20; Fig 17B). There were also no differences between groups on maternal behaviors observed in the 10 mins after retrievals (Table 9).

Anxiety-like behaviors

OTR-knockdown dams spent 58% more time in the open arms of the elevated plus-maze (EPM) compared to controls ($t_{(20)}$ = 2.20, p = 0.04, d = 0.95; Fig 18A). There

was no effect of OTR knockdown on the number of closed arm entries in the elevated plus maze ($t_{(20)}$ =1.25, p = 0.23, d = 0.55; Table 10), nor the latency to enter the dark chamber ($t_{19)}$ = 0.49, p = 0.64, d = 0.23; Table 10), or time spent in the light chamber of the L/D box (although OTR-knockdown dams spent almost twice as long as controls in the light chamber) ($t_{(19)}$ = 1.90, p = 0.07, d = 0.84; Fig 18B). The effects of OTR knockdown on other behaviors scored in the EPM and L/D box are shown in Tables 10 and 11.

Maternal aggression

OTR knockdown greatly reduced maternal aggressive behaviors toward a male intruder. OTR knockdown lead to a shorter latency to attack ($t_{(16)}$ = 3.89, p < 0.01, d = 1.84; Fig 19A), an almost four-fold increase in the frequency of attacks ($t_{(16)}$ = 3.96, p <0.01, d = 1.95; Fig 19C), and three-fold longer duration per attack ($t_{(16)}$ = 2.65, p = 0.02, d = 1.31; Fig 19D) compared to controls. OTR knockdown lead to a six-fold increase in the total duration of attacks ($t_{(16)}$ = 3.52, p < 0.01, d = 1.58; Fig 19B). OTR knockdown also increased frontal attacks and lateral attacks (see Table 12).

Depressive-like behaviors

OTR-knockdown dams spent more time floating in the forced swim test compared to controls ($t_{(16)}$ = 2.70, p = 0.02, d = 1.28; Fig 20A). There was no effect of OTR knockdown on saccharin preference ($t_{(16)}$ = 1.03, p = 0.32, d = 0.48; Fig 20B).

Serotonin-immunoreactive fibers in the forebrain

OTR-knockdown dams had one-third the length of serotonin-ir fibers in the S1 compared to controls ($t_{(10)}$ = 4.64, p < 0.01, d = 2.68; Fig. 21B), but more than twice the length of serotonin-ir fibers in both the lOFC ($t_{(10)}$ = 3.28, p = 0.01, d = 1.89; Fig 22B) and mPOA ($t_{(10)}$ = 2.41, p = 0.04, d = 1.39; Fig 23A). There was no effect of OTR knockdown on serotonin-ir fibers in the M1 ($t_{(10)}$ = 0.50, p = 0.63, d = 0.29; Fig 23B). Collapsed across the two groups, serotonin-ir fiber length in the S1 was positively correlated with the frequency that dams were on the nest (r_{10} = 0.68, p = 0.02, Fig 21C) and their attack latency (r_{10} = 0.62, p = 0.04, Fig 21E), while serotonin-ir fiber length in the S1 was negatively correlated with the frequency of non-pup directed behaviors (r_{10} = -0.74, p < 0.01, Fig 21D) and the number of attacks (r_{10} = -0.73, p = 0.01, Fig 21F). Other significant relationships between serotonin-ir fiber length in the S1 and caregiving behaviors are reported in Table 13. Serotonin-ir fiber length in the lOFC was positively correlated with the percentage of time spent in the open arms of the elevated plus maze (r_{10} = 0.59, p = 0.04, Fig 22D) and the number of attacks (r_{10} = 0.66, p = 0.03, Fig 22C). There were no relationships between serotonin-ir fiber length in the mPOA and M1 on any postpartum behaviors.

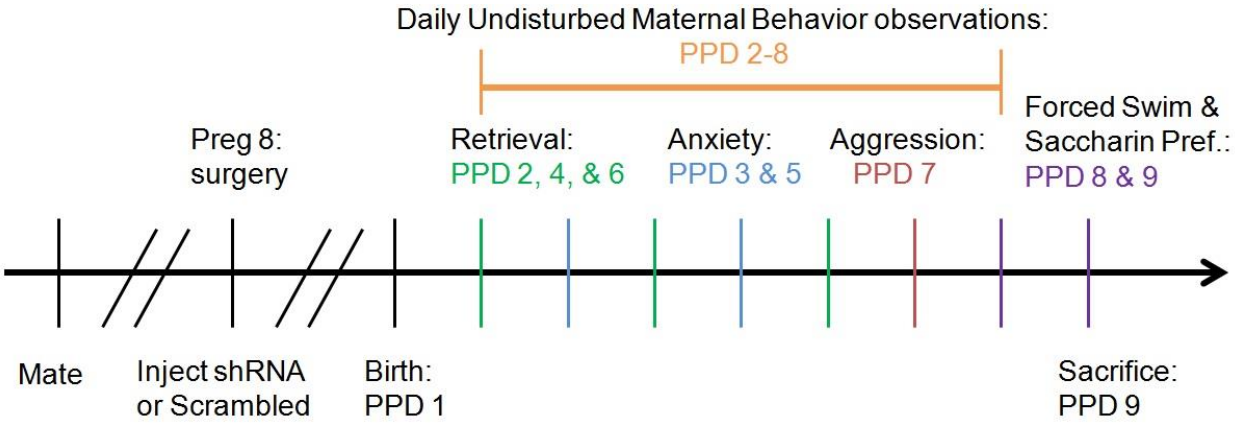

Figure 10: Schematic representation of the experimental timeline used to determine the effects of OTR knockdown in the dorsomedial tegmentum on postpartum socioemotional behaviors. Pregnant females were injected with either the OTR shRNA or scrambled vector on pregnancy day 8. Following birth (postpartum day 1; PPD 1), dams' undisturbed maternal behaviors were observed from PPDs 2 – 8 three times daily. On PPDs 2, 4, and 6, dams pup retrieval performance was assessed. On PPDs 3 and 5, dams' anxiety-like behavior was assessed in an elevated plus maze and light-dark box, respectively. On PPD 7, dams' aggressive behaviors towards an unfamiliar male rat were assessed using the resident-intruder paradigm. Finally, on PPDs 8 and 9, dams' depressive-like behaviors were observed using the saccharin preference test and the forced swim test.

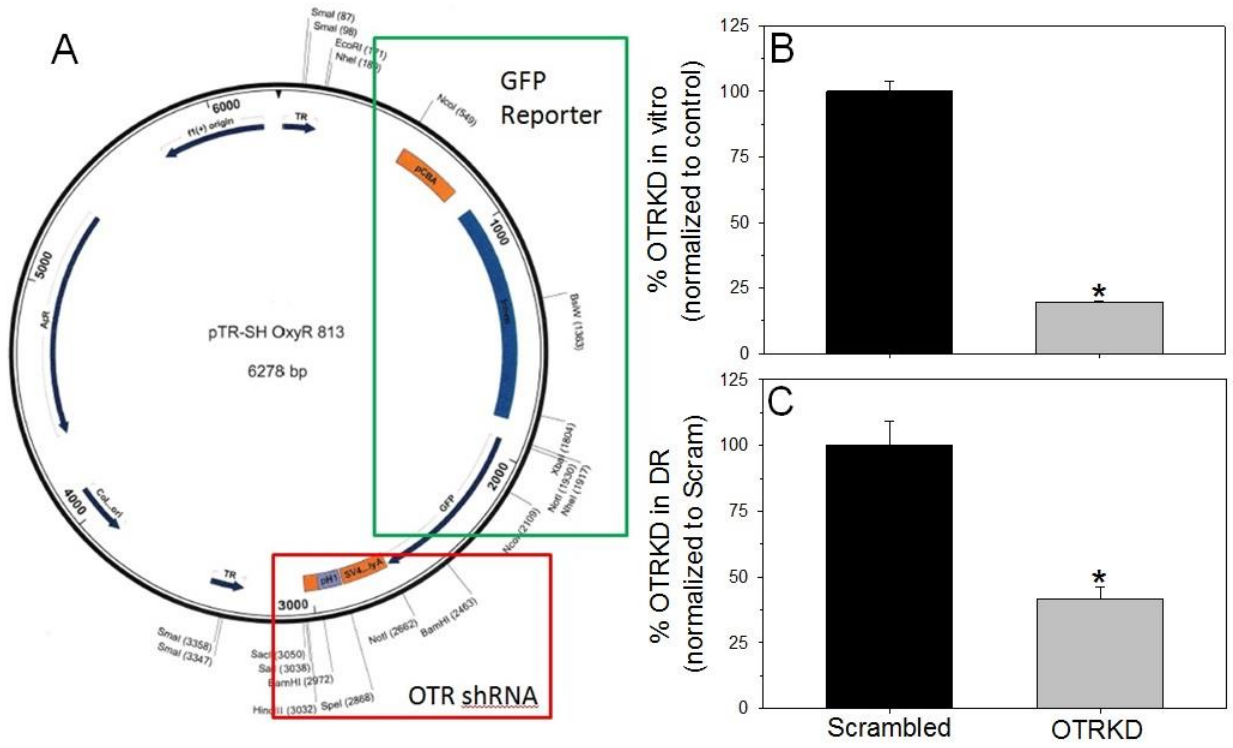

Figure 11: Viral construct successfully knocked down OTR expression *in vitro* and *in vivo*. (A) Schematic representation of the OTR shRNA vector used in Chapter 3. Percent OTR knockdown normalized to controls (Mean ± SEM) (B) *in vitro* and (C) *in vivo*. * indicates statistically significant differences between groups, $p < 0.05$.

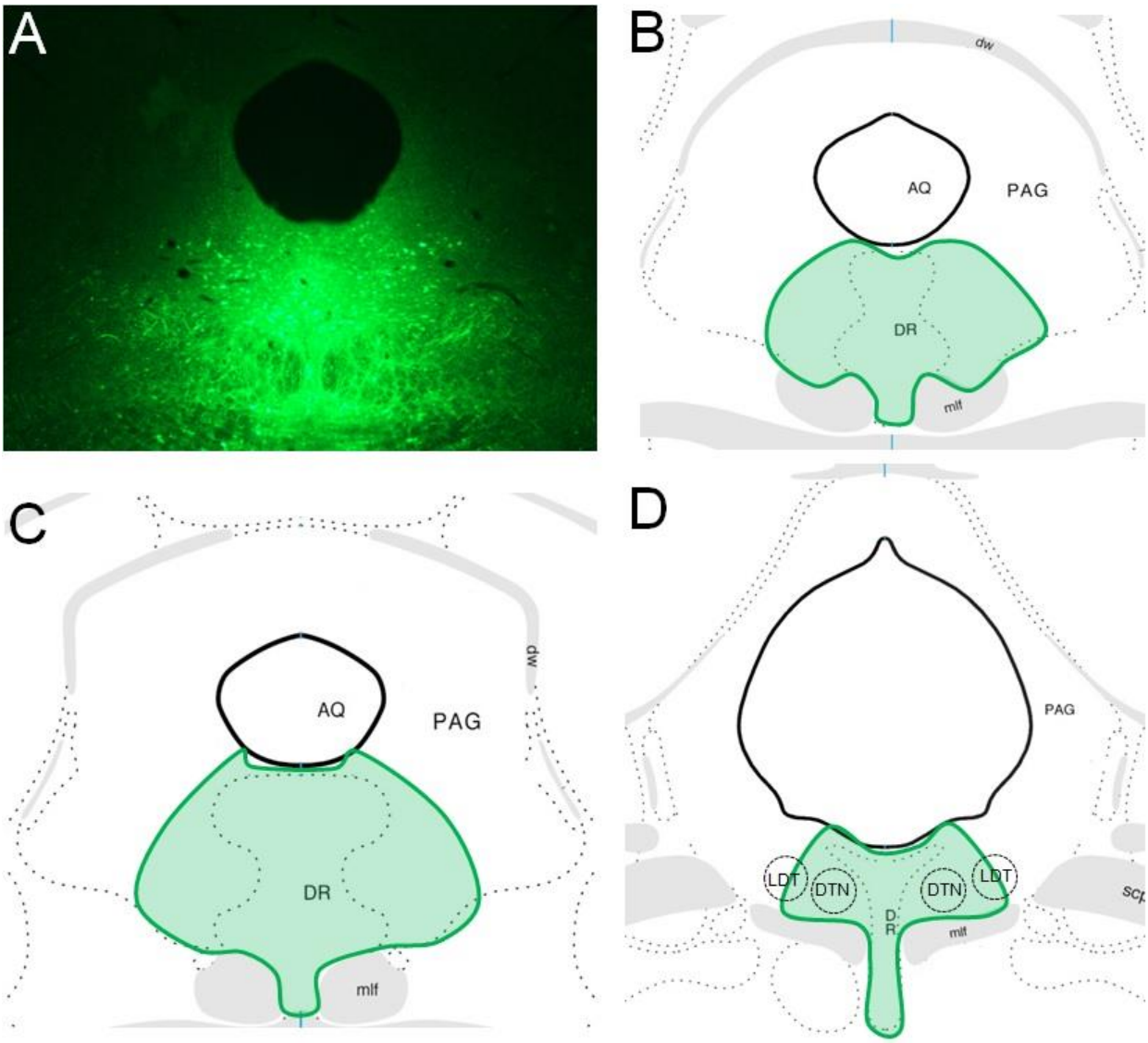

Figure 12: GFP immunoreactivity in the dorsomedial tegmentum of postpartum rats. A) Representative photomicrograph of GFP immunoreactivity in the dorsomedial tegmentum. Schematics of average GFP immunoreactive spread in the (B) rostral dorsal raphe, (C) medial dorsal raphe, and (D) caudal dorsal raphe. Modified images from (Swanson, 2004). DTN – dorsal tegmental nucleus. LDT – laterodorsal tegmental nucleus. AQ – cerebral aqueduct. DR – dorsal raphe.

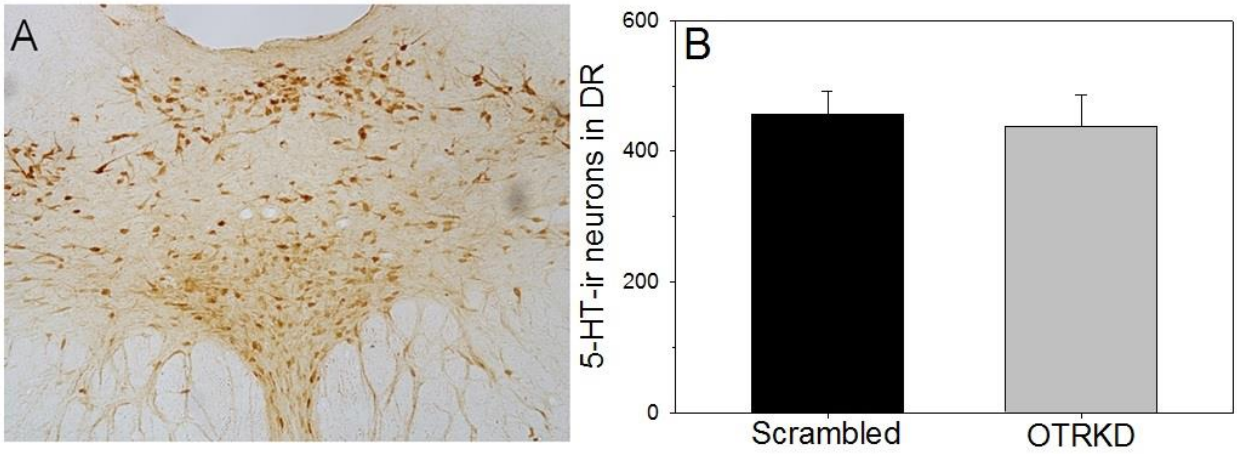

Figure 13: Number of serotonin-immunoreactive cells in the dorsal raphe of OTR shRNA-injected dams (OTRKD) and scrambled vector-injected dams. A) Representative photomicrograph of the serotonin immunoreactivity in the medial dorsal raphe. B) Number of serotonin-immunoreactive neurons in the dorsal raphe of OTR shRNA-injected (OTRKD) and scrambled vector-injected dams.

# of 5-HT-ir Cells	Scrambled (M ±SEM)	OTRKD (M ±SEM)	Group ($t_{(10)}$; p; d)
Total DR	457 ± 35	438 ± 49	0.46; 0.65; 0.27
Rostral DR	139 ± 13	148 ± 14	0.32; 0.76’ 0.20
Medial DR	231 ± 21	236 ± 29	0.14; 0.89; 0.08
Caudal DR	87 ± 9	54 ± 22	1.38; 0.21; 0.81

Table 7: Number of serotonin-immunoreactive neurons (Mean ± SEM) in the dorsal raphe of OTR shRNA-injected dams (OTRKD) and scrambled vector-injected dams.

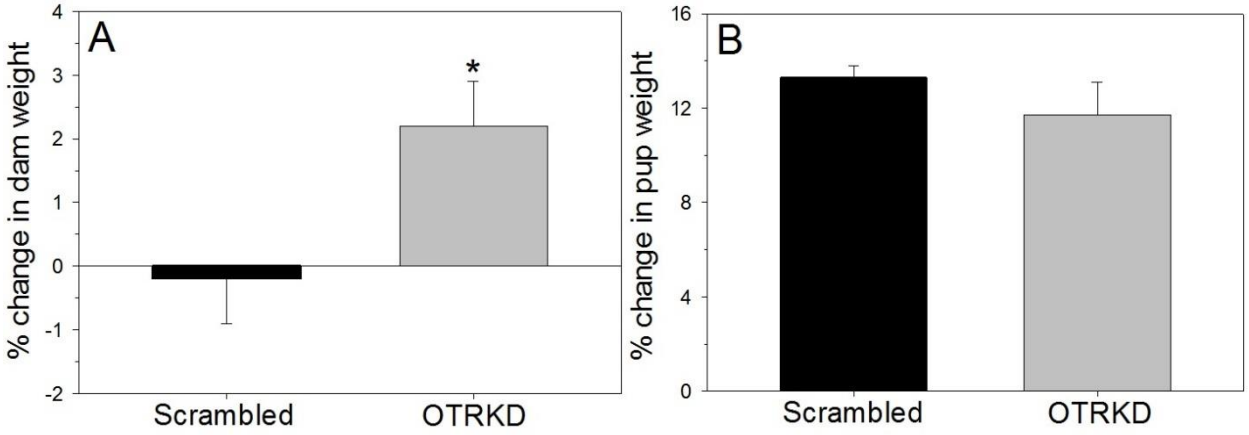

Figure 14: Effects of OTR knockdown in the dorsomedial tegmentum on maternal and litter health. Average percent change in (A) dam weights and (B) litter weights (Mean ± SEM) for OTR shRNA-injected (OTRKD) and scrambled vector-injected dams. * indicates statistically significant group difference, $p < 0.05$.

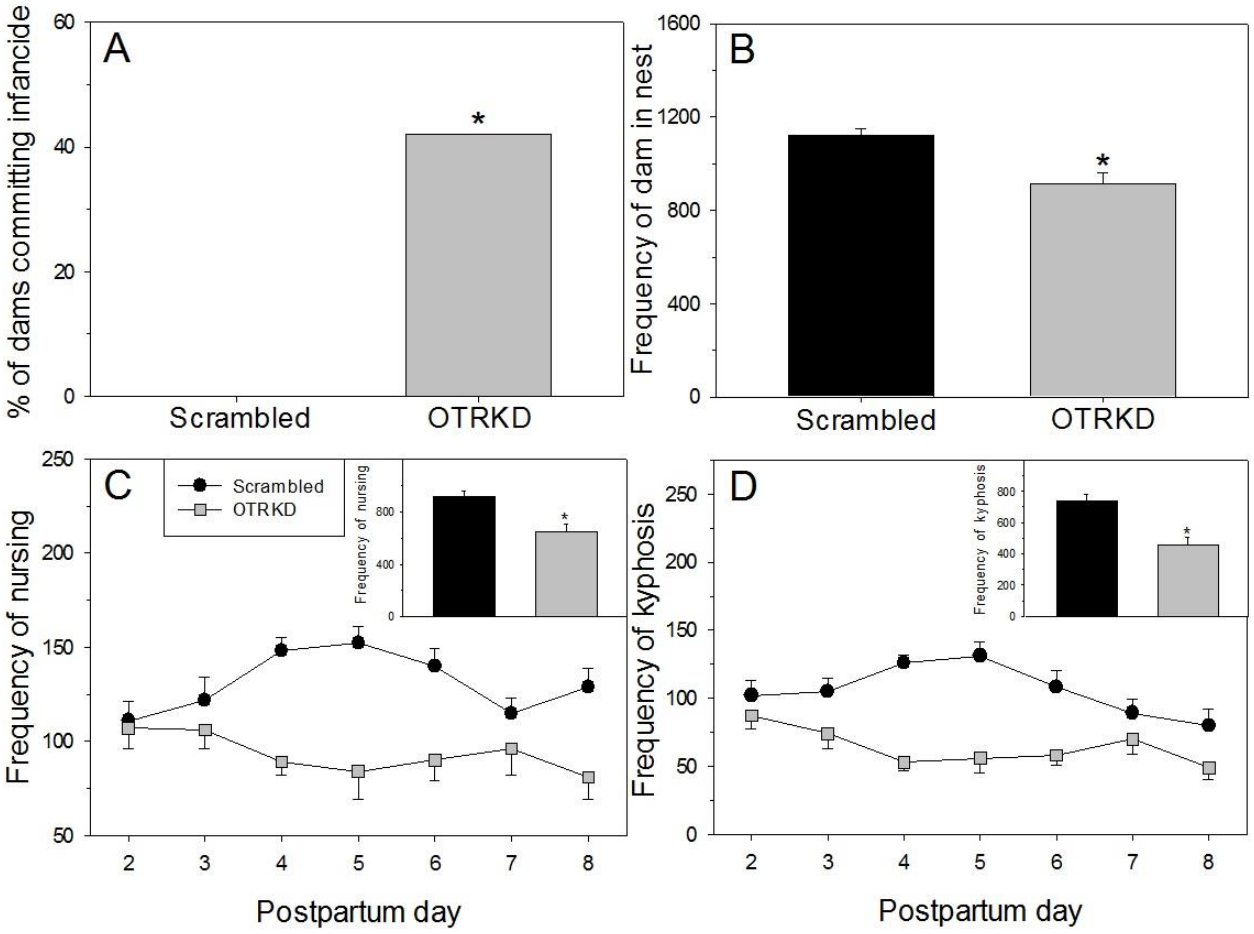

Figure 15: Effects of OTR knockdown in the dorsomedial tegmentum on maternal caregiving. A) Percentage of dams committing infanticide (Mean ± SEM) in OTR shRNA-injected (OTRKD) and scrambled vector-injected dams. Frequency of (B) dam in the nest, (C) nursing, and (D) kyphosis (Mean ± SEM) in these dams. * indicates statistically significant group difference, $p < 0.05$.

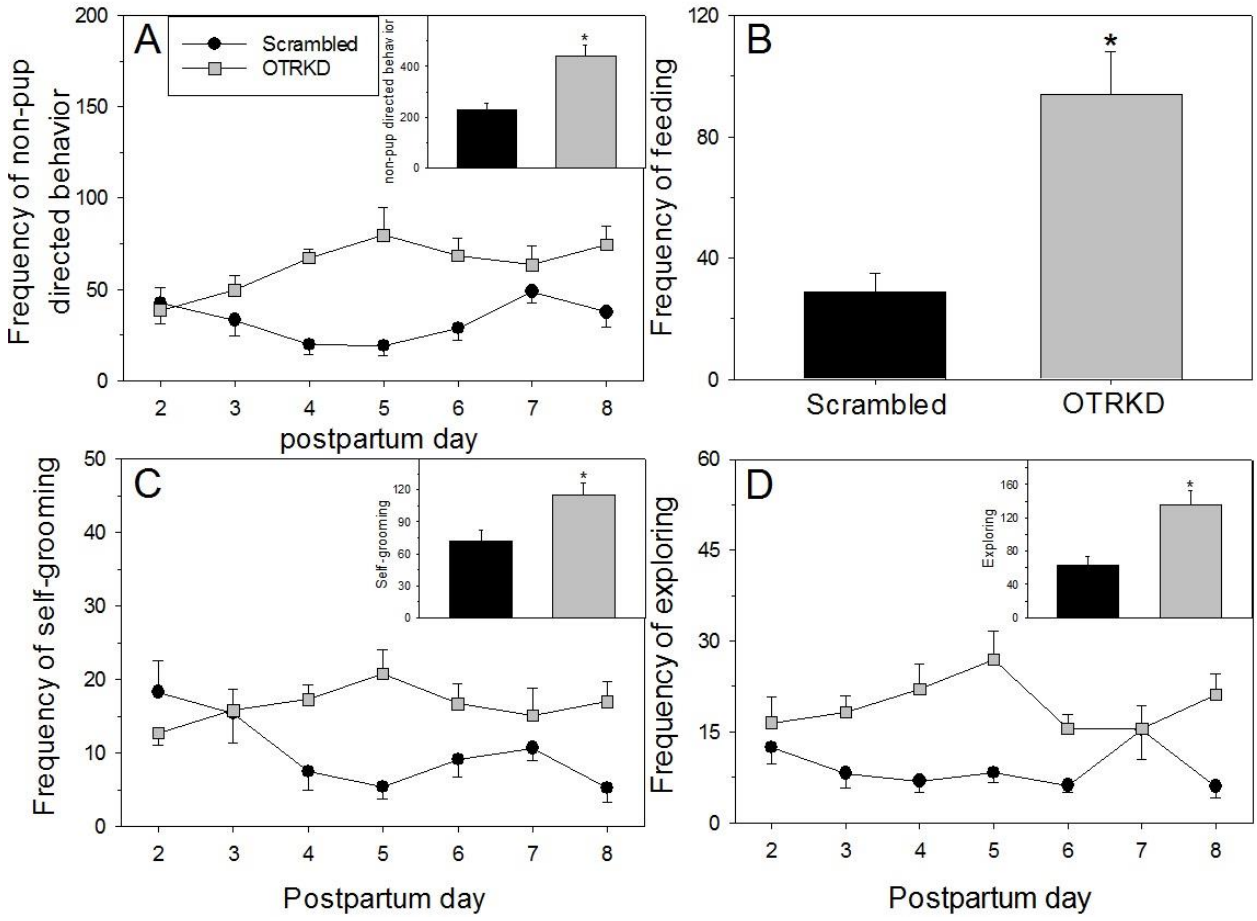

Figure 16: Effects of OTR knockdown in the dorsomedial tegmentum on non-pup direct behavior. Frequency of (A) non-pup directed behavior, (B) feeding, (C) self-grooming, and (D) exploring (Mean ± SEM) by OTR shRNA-injected (OTRKD) and scrambled vector-injected dams. * indicates statistically significant group difference, $p < 0.05$.

	Scrambled (M ± SEM)	OTRKD (M ± SEM)	Group ($F_{(1, 18)}$; p; η^2_p)	Time ($F_{(6)}$; p; η^2_p)	Interaction ($F_{6,108)}$; p; η^2_p)
Dam in nest	1124 ± 24	916 ± 44	17.56; <0.01; 0.49	4.86; <0.01*; 0.21	2.57; >0.05; 0.13
Nursing	918 ± 39	652 ± 56	15.16; <0.01; 0.46	0.82; 0.52; 0.04	3.91; 0.01; 0.18
Kyphosis	741 ± 37	455 ± 48	22.14; <0.01; 0.55	3.40; 0.01*; 0.16	3.54; 0.01; 0.17
Supine nursing	79 ± 20	76 ± 30	0.01; 0.92; <0.01	5.81; <0.01^;0.24	0.90; 0.45; 0.05
Prone nursing	97 ± 17	122 ± 12	1.33; 0.26; 0.07	1.72; 0.15; 0.09	1.36; 0.25; 0.07
Hovering over the litter	206 ± 31	264 ± 31	1.66; 0.21; 0.09	5.12; <0.01*; 0.16	1.01; 0.41; 0.05
Erect postures (hovering over + kyphosis)	947 ± 29	718 ± 40	21.19; <0.01; 0.54	8.68; <0.01*; 0.33	2.22 ; 0.07; 0.11
Passive postures (supine + prone nursing	177 ± 23	198 ± 35	0.25 ; 0.62; 0.01	3.42; 0.01^; 0.16	1.44; 0.23; 0.07
Licking pups	105 ± 19	140 ± 25	1.32; 0.27; 0.07	3.79; 0.01^; 0.17	0.81; 0.52; 0.04
Retrieval	0.9 ± 0.8	1.9 ± 1.1	0.52 ; 0.48; 0.03	N/A	N/A
Non-pup directed behavior	230 ± 26	442 ± 41	18.87; <0.01; 0.51	1.44; 0.22; 0.07	4.22; <0.01; 0.19
Nesting	13 ± 5	31 ± 8	3.29; 0.09; 0.16	1.66; 0.18; 0.09	1.54; 0.21; 0.08
Nest quality	38 ± 3	27 ± 5	4.67; 0.04; 0.21	4.55; <0.01*; 0.20	2.00; 0.10; 0.10
Self-grooming	72 ± 10	115 ± 12	7.80; 0.01; 0.30	0.84; 0.51; 0.05	4.04; <0.01; 0.18
Eating/drinking	29 ± 6	94 ± 14	19.22; <0.01; 0.52	2.02; 0.10; 0.10	2.27; 0.07; 0.11
Exploring	63 ± 10	136 ± 16	15.00; <0.01; 0.46	1.10; 0.36; 0.06	2.77; 0.04; 0.13
Sleeping away from pups	53 ± 12	56 ± 17	0.35; 0.56; 0.02	3.47; 0.01^; 0.16	0.56; 0.69; 0.03

Table 8: Frequency (Mean ± SEM) of maternal behaviors displayed by scrambled and oxytocin receptor knockdown dams during three 30-min observations each day on postpartum days 2 – 8. ^ indicates increasing frequency of behavior across postpartum day. *indicates decreasing frequency of behavior across postpartum day.

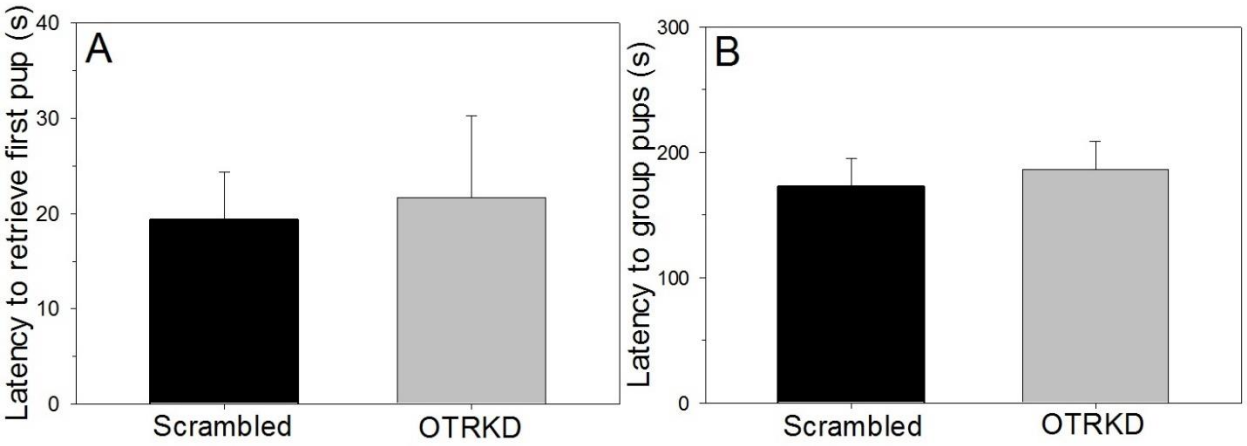

Figure 17: Effects of OTR knockdown in the dorsomedial tegmentum on retrieval behaviors. Latency to (A) retrieve the first pup and (B) group all the pups in the nest (Mean ± SEM) of OTR shRNA-injected (OTRKD) and scrambled vector-injected dams.

	Scrambled (Mean ± SEM)	OTRKD (Mean ± SEM)	$t_{(19)}$; p; d
Latency to retrieve first pup (s)	19 ± 5	27 ± 9	0.23; 0.83; 0.10
Latency to group all pups (s)	173 ± 23	186 ± 23	0.45; 0.66; 0.20
Kyphosis	0.6 ± 0.4	0.5 ± 0.3	0.31; 0.76; 0.14
Hovering over litter	16 ± 1	15 ± 1	1.14; 0.27; 0.53
Licking pups	9. ± 1	8 ± 1	1.52; 0.15; 0.67
Retrieval	0.2 ± 0.1	0.2 ± 0.2	0.39; 0.71; 0.16
Non-pup directed behavior	10 ± 1	11 ± 2	0.09; 0.93; 0.55
Nesting	1 ± 1	2 ± 0.4	0.77; 0.45; 0.34
Self-grooming	3 ± 1	3 ± 0.4	0.94; 0.36; 0.40
Eating/drinking	0.4 ± 0.2	0 ± 0	1.39; 0.20; 0.63
Exploring	7 ± 1	8. ± 1	1.26; 0.22; 0.57

Table 9: Average frequency (Mean ± SEM) of maternal behaviors displayed by OTR shRNA-injected dams (OTRKD) and scrambled vector-injected dams following retrieval testing. OTR knockdown in the dorsomedial tegmentum did not affect maternal behaviors during retrieval tests.

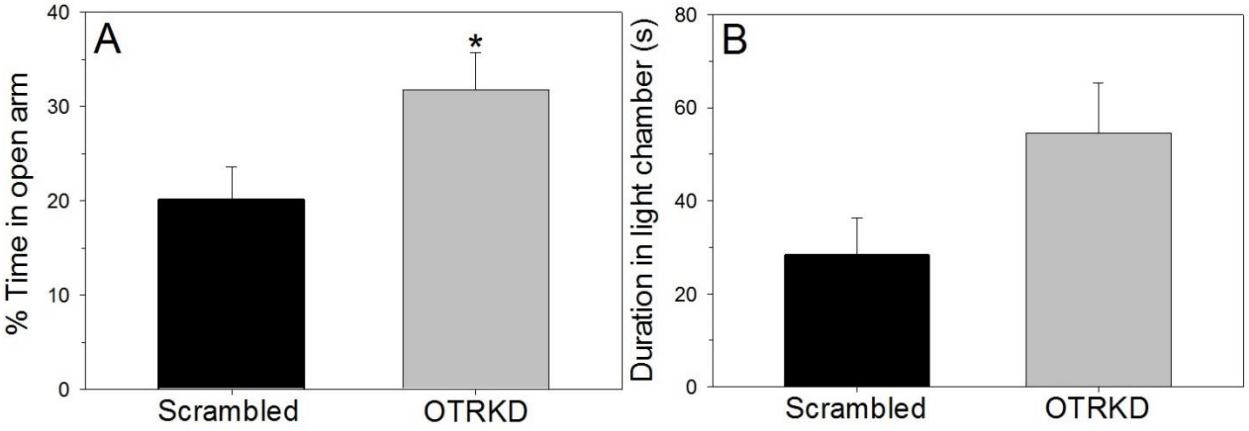

Figure 18: Effects of OTR knockdown on anxiety-like behaviors. A) Percentage of time (Mean ± SEM) spent in the open arms of an elevated plus maze by OTR shRNA-injected (OTRKD) and scrambled vector-injected dams. B) Duration of time spent in the light chamber (Mean ± SEM) of the light-dark box OTRKD and scrambled vector-injected dams. * indicates statistically significant group difference, $p < 0.05$.

	Scrambled (Mean ± SEM)	OTRKD (Mean ± SEM)	$t_{(20)}$; p; d
% entries into open arms	40 ± 2	50 ± 2	3.11; <0.01; 1.34
% time in open arms	20 ± 4	32 ± 4	2.20; 0.04; 0.95
Total arm entries	25 ± 3	37 ± 3	2.63; 0.02;1.15
Closed-arm entries	15 ± 2	19 ± 2	1.25; 0.23; 0.55

Table 10: Anxiety-like behaviors (Mean ± SEM) of OTR shRNA-injected dams (OTRKD) and scrambled vector-injected dams in an elevated plus maze. OTR knockdown in the dorsomedial tegmentum decreased anxiety-like behavior in the elevated plus maze.

	Scrambled (Mean ± SEM)	OTRKD (Mean ± SEM)	$t_{(19)}$; p; d
Latency (s) to enter the dark chamber	6 ± 1	7 ± 2	0.49; 0.64; 0.23
Duration (s) in light chamber	28 ± 8	55 ± 11	1.90; 0.07; 0.84
Frequency of transitions between chambers	2 ± 1	4 ± 1	2.05; >0.05; 0.91
Frequency of stretches in the light chamber	10 ± 2	15 ± 2	2.13; <0.05; 0.92
Frequency of rears in the light chamber	3 ± 1	4 ± 1	0.18; 0.86; 0.07

Table 11: Anxiety-like behavior s (Mean ± SEM) of OTR shRNA-injected dams (OTRKD) and scrambled vector-injected dams in a light-dark box. OTR knockdown in the dorsomedial tegmentum did not affect postpartum anxiety-like behaviors in the light-dark box.

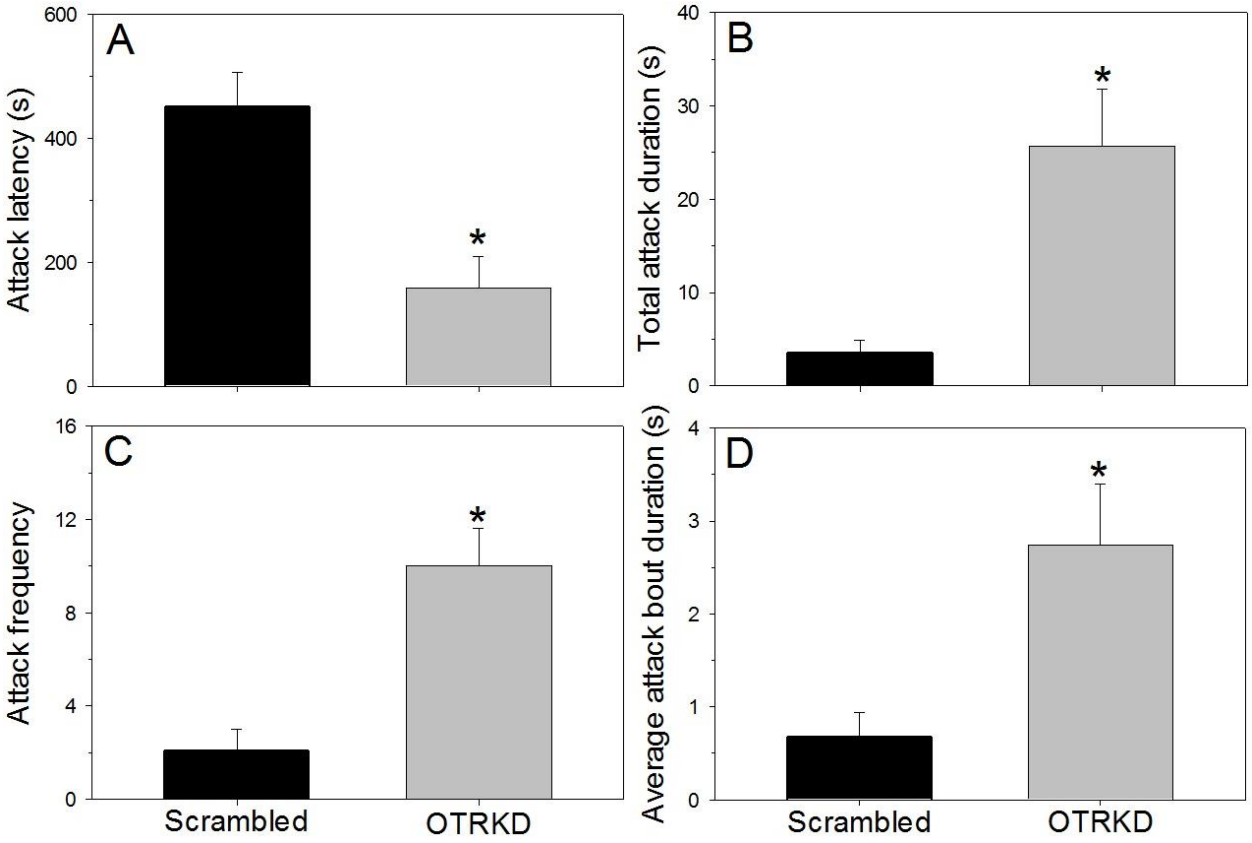

Figure 19: Effects of OTR knockdown in the dorsomedial tegmentum on postpartum aggression. A) Latency to attack the male intruder (Mean ± SEM) of OTR shRNA-injected (OTRKD) and scrambled vector-injected dams. Duration (Mean ± SEM) of (B) total attacks and (D) average attack duration of OTRKD and scrambled vector-injected dams. C) Frequency of attacks (Mean ± SEM) of OTRKD and scrambled vector-injected dams. * indicates statistically significant group difference, $p < 0.05$.

	Scrambled (Mean ± SEM)	OTRKD (Mean ± SEM)	$t_{(16)}$; p; d
Attack latency (s)	451 ± 56	159 ± 51	3.89; <0.01; 1.84
Attack frequency	2.1 ± 0.9	10.0 ± 1.6	3.96; <0.01; 1.95
Average attack duration (s)	0.7 ± 0.3	2.7 ± 0.7	2.65; 0.02; 1.31
Total attack duration (s)	3.5 ± 1.4	25.7 ± 6.1	3.52; <0.01; 1.58
Frequency of frontal attacks	2.3 ± 0.8	6.9 ± 1.7	2.28; 0.04; 1.12
Frontal attack duration	3.0 ± 1.4	19.6 ± 6.4	2.28; 0.04; 1.15
Frequency of lateral attacks	0.5 ± 0.5	3.1 ± 0.8	2.52; 0.02; 1.21
Lateral attack duration	0.5 ± 0.5	6.1 ± 1.9	2.52; 0.02; 1.27

Table 12: Aggressive behaviors (Mean ± SEM) of OTR shRNA-injected dams (OTRKD) and scrambled vector-injected dams. OTR knockdown in the dorsomedial tegmentum increased many postpartum aggressive behaviors.

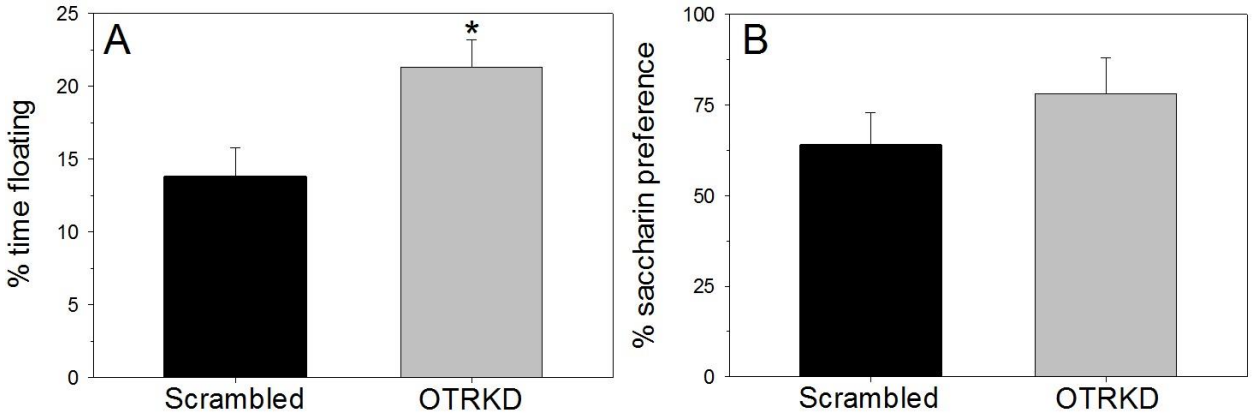

Figure 20: Effects of OTR knockdown in the dorsomedial tegmentum on depressive-like behaviors. A) Percentage of time spent floating (Mean ± SEM) in the forced swim test of OTR shRNA-injected (OTRKD) and scrambled vector-injected dams. B) Percent saccharin preference (Mean ± SEM) of OTRKD and scrambled vector-injected dams. * indicates statistically significant group difference, $p < 0.05$.

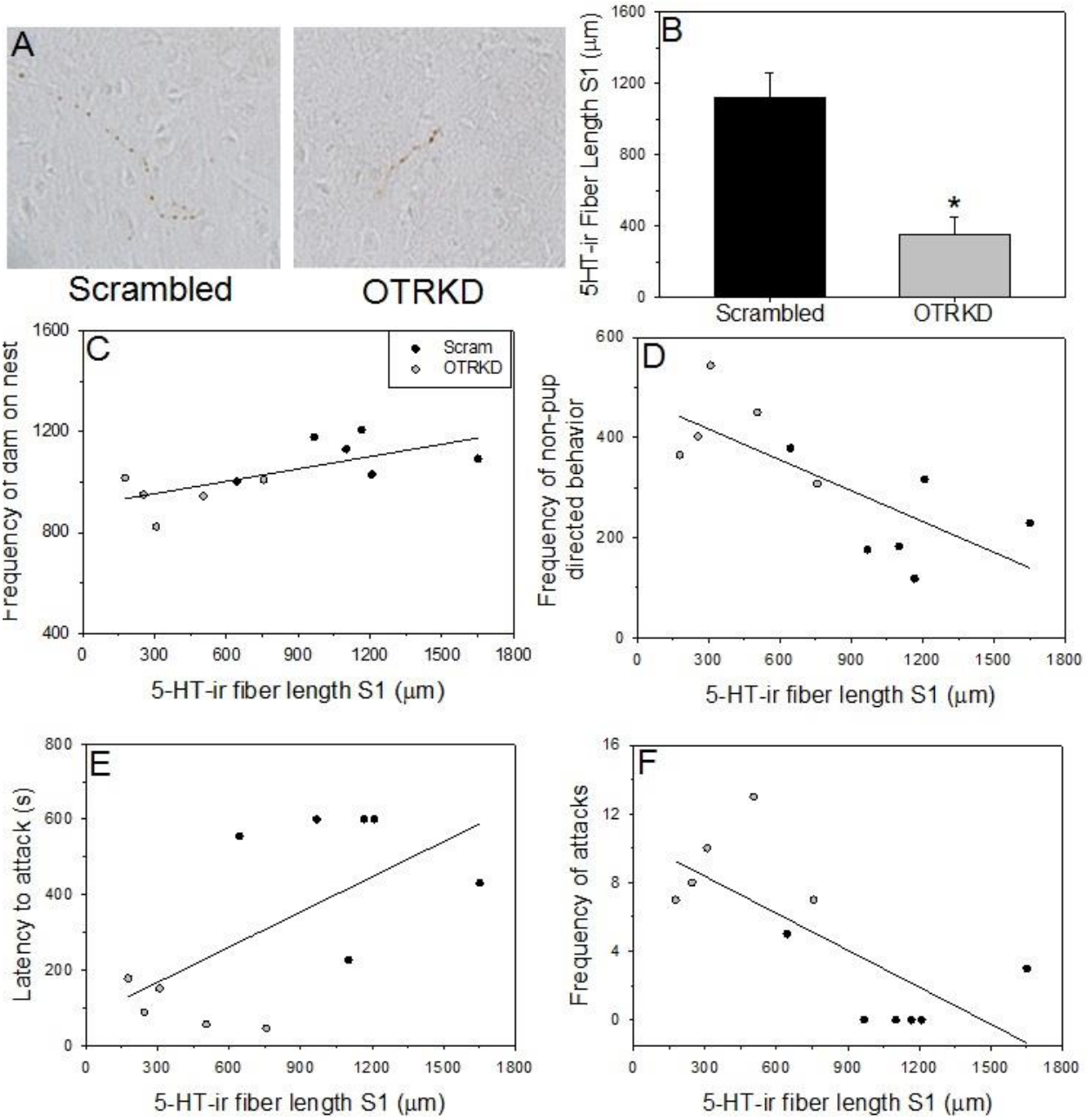

Figure 21: Effects of OTR knockdown in the dorsomedial tegmentum on serotonin immunoreactive fiber length in the primary somatosensory cortex. A) Representative photomicrographs from the primary somatosensory cortex of OTR shRNA-injected (OTRKD) and scrambled vector-injected dams B) Serotonin immunoreactive fiber length in the barrel field and trunk regions of the somatosensory cortex of OTRKD and scrambled vector-injected dams. Relationship between serotonin immunoreactive fiber length and frequency of (C) dam on nest, (D) non-pup directed behavior, and (E) number of attacks. F) Relationship between serotonin immunoreactive fibers and the latency to attack. * indicates statistically significant group difference, $p < 0.05$.

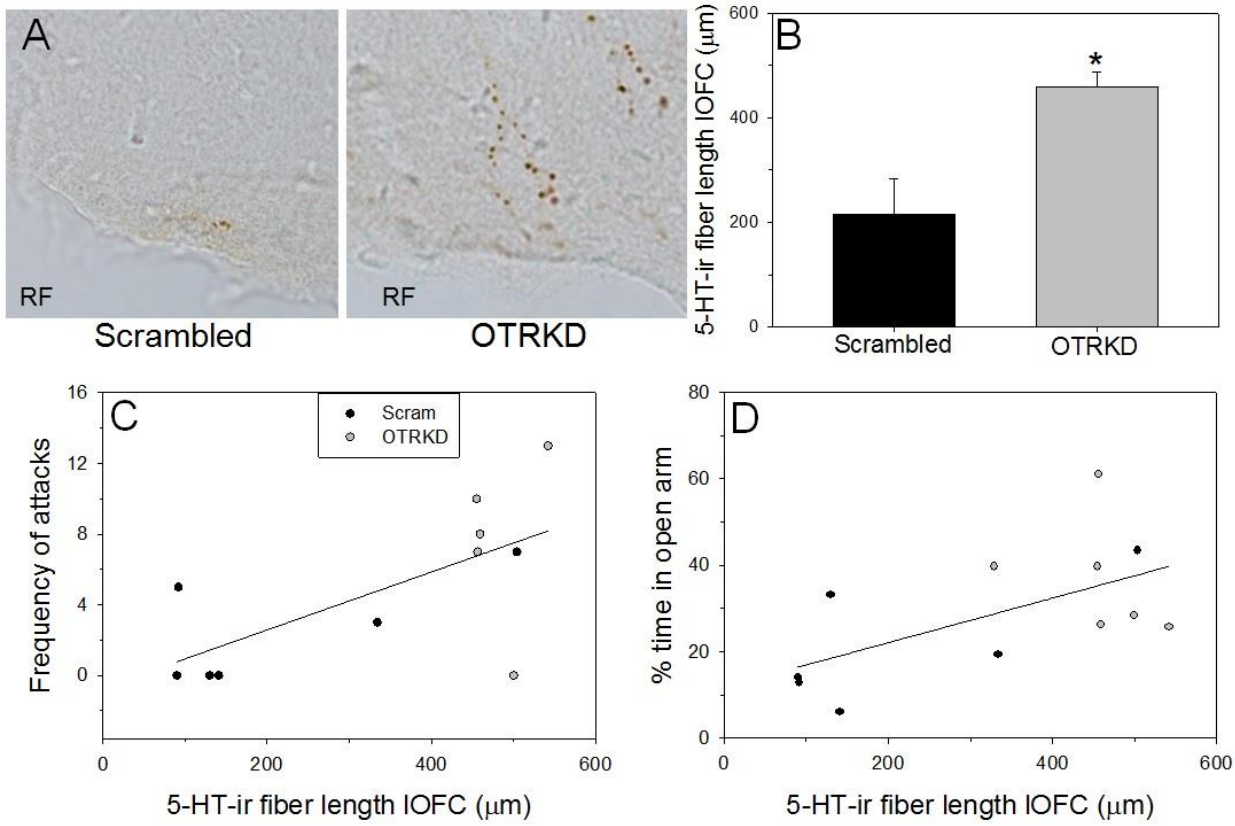

Figure 22: Effects of OTR knockdown in the dorsomedial tegmentum on serotonin immunoreactive fiber length in the lateral orbitofrontal cortex. A) Representative photomicrographs from the lateral orbitofrontal cortex of OTR shRNA-injected (OTRKD) and scrambled vector-injected dams. B) Serotonin immunoreactive fiber length in the lateral orbitofrontal cortex of OTRKD and scrambled vector-injected dams. Relationship between serotonin immunoreactive fiber length and (C) frequency of attacks and (D) the percentage of time spent in the open arms of the elevated plus maze. * indicates statistically significant group difference, $p < 0.05$. RF – Rhinal fissure.

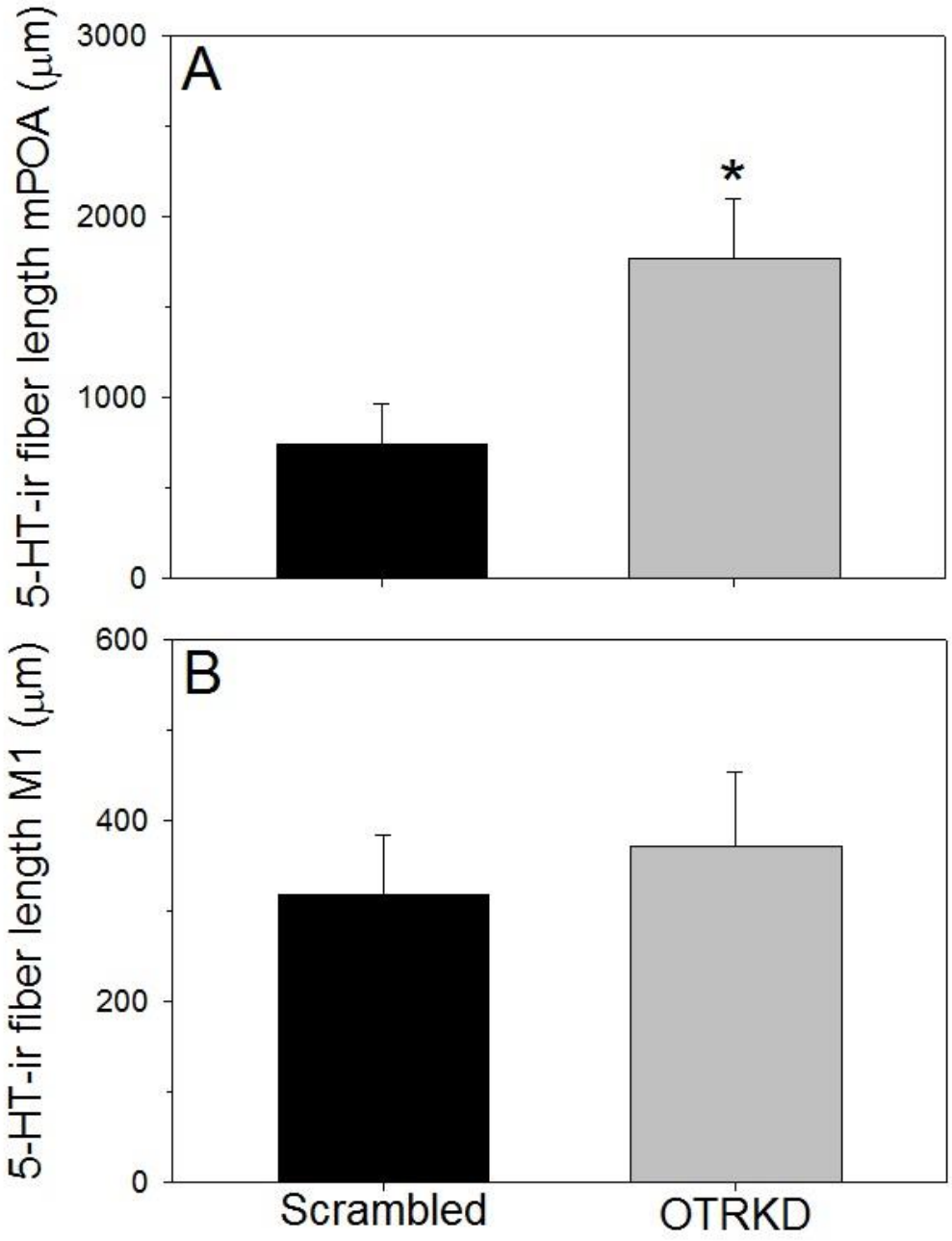

Figure 23: Effects of OTR knockdown in the dorsomedial tegmentum on serotonin immunoreactive fiber length in the medial preoptic area (top) and motor cortex (bottom). * indicates statistically significant group difference, $p < 0.05$.

5-HT-ir fibers	Behavior	r_{10}; p
S1	Hovering over the litter	-0.76; <0.01
	Kyphosis	0.75; <0.01
	Nursing	0.76; <0.01
	Dam in nest	0.68; 0.02
	Non-pup directed behavior	-0.74; <0.01
	Eating/drinking	-0.82; <0.01
	Self-grooming	-0.77; <0.01
	Licking pups	-0.68; 0.02
	Nesting	-0.72; 0.01
	Exploring	-0.77; <0.01
	Latency to attack	0.62; 0.04
	Number of attacks	-0.73; 0.01
lOFC	Number of attacks	0.66; 0.03
	% time in open arms	0.59; 0.04

Table 13: Correlations between length of serotonin-ir fibers in the S1 and lOFC and postpartum behaviors.

Discussion

Given the individual roles of oxytocin and the dorsomedial tegmentum in postpartum behaviors (Barofsky et al., 1983; Holschbach et al., 2018; Lonstein et al., 1998; Lonstein and Stern, 1997a), I hypothesized that OTR knockdown in this region would affect multiple aspects of dams' behavior. Specifically, I predicted that OTR knockdown in the dorsomedial tegmentum would decrease maternal caregiving and postpartum aggression, while increasing postpartum anxiety-like and depressive-like behaviors.

In partial support of these hypotheses, 5/12 of the OTR-knockdown dams committed infanticide compared to none of the controls. Infanticide is extremely rare in postpartum rats (Peters et al., 1991; Peters and Kristal, 1983) and more common in virgin rats (Jakubowski and Terkel, 1985; Peters et al., 1991; Peters and Kristal, 1983). In virgin rats, infanticide is eventually prevented by continual somatosensory contact with the pups, and receipt of just their distal cues is ineffective (Jakubowski and Terkel, 1985). This negative effect of somatosensory input from the pups on infanticide might be regulated by oxytocin, as oxytocin is released into the dorsomedial tegmentum in response to gentle brush-like stroking (Lund et al., 2002) and oxytocin suppresses infanticide in mice (Macbeth et al., 2010; McCarthy, 1990; Ragnauth et al., 2005; Rich et al., 2014) and dogs (Kockaya et al., 2018). This suggests that oxytocin released into the dorsomedial tegmentum and acting on OTRs, perhaps in response to somatosensory stimulation from the pups, prevents infanticide. Given this result, it would be of interest to see if infusion of oxytocin into the dorsomedial tegmentum of virgin female rats can decrease pup killing. Because the cases of infanticide occurred on PPDs 2 and 3, after

dams already had at least one full day of maternal experience, OTRs in the dorsomedial tegmentum might be imperative for experience-dependent somatosensory adaptations. During the postpartum period, the cortical area of S1 devoted to the ventrum expands, which depends on somatosensory stimulation from the pups (Rosselet et al., 2006; Xerri et al., 1994). Perhaps stimulation of OTRs in the dorsomedial tegmentum mediates this long-term change in the S1, while the hormones of parturition are primarily responsible for inhibiting infanticide within the first few days postpartum. There is already evidence that the dorsomedial tegmentum is critical in experience-dependent behavioral changes in mothers, as lesioning the serotonergic neurons of the DR after one day of pup experience prevents some of the experience-dependent changes in nursing behavior that occur across the postpartum period (Holschbach et al., 2018).

In addition to increased infanticide, OTR-knockdown dams also spent less time in the nest compared to controls, which was largely driven by a 39% decrease in the frequency of kyphotic nursing (no group differences in other nursing postures were found). The lower frequency of kyphotic nursing is likely caused by decreased oxytocin signaling in the PAGvl. Lesions of the PAGvl greatly reduce kyphotic nursing without affecting numerous other maternal behaviors (Lonstein et al., 1998; Lonstein and Stern, 1997a). Given that oxytocin increases cellular firing in the PAGvl (S. Ogawa et al., 1992), knocking down OTR expression in the PAGvl would be expected to decrease cellular firing of the PAGvl and kyphotic nursing.

Instead of spending time in the nest and nursing, OTR-knockdown dams unsurprisingly showed more non-pup directed behaviors; these include eating/drinking, self-grooming, and exploring the homecage. Particularly notable was the over three-fold

higher frequency of ingestive behaviors in OTR-knockdown dams compared to controls. This may be due to knockdown of OTRs expressed particularly on DR serotonergic neurons. Both serotonin and oxytocin are potent anorexigenics (Arletti et al., 1989; Sabatier et al., 2013; Voigt and Fink, 2015), and manipulations that increase central oxytocin (Arletti et al., 1989; Olson et al., 1991a) and serotonin signaling (Blundell and Leshem, 1975; Latham and Blundell, 1979) decrease ingestive behaviors, while those that decrease central oxytocin (Arletti et al., 1989; Leibowitz et al., 1981; Olson et al., 1991b) and serotonin signaling (Geyer et al., 1976; MacKenzie et al., 1979) increase ingestive behaviors. Therefore, knocking down OTRs on serotonergic neurons (which would reduce their activity) would be expected to enhance dams' ingestive behaviors. These data would be the first to suggest that oxytocin acting on serotonergic neurons in the DR is potently anorexigenic. Release of this anorexigenic signal and increased ingestion is likely the reason why OTR-knockdown dams gained a small amount of weight over testing while controls did not.

There was no effect of OTR knockdown on pup retrieval behaviors. This was not expected given the large population of TH-ir neurons expressing OTRs in the rostral DR (Chapter Two) and works showing that pup retrieval is driven by dopamine signaling (Keer and Stern, 1999; Li et al., 2004; Numan et al., 2005; Silva et al., 2001; Stern and Taylor, 1991; Zhao and Li, 2009). However, only slightly more than half of the dopamine neurons (~53%) in the DR were found here to express OTRs (Table 3). Perhaps this subpopulation, or any population, of dopaminergic neurons in the DR is not relevant to retrieval. The dopamine neurons the DR might be more relevant for increased wakefulness seen around the time of parturition (Branchey and Branchey, 1970; Kimura

et al., 1996), because DR dopamine neurons are active during wake states and lesioning them increases time spent sleeping (Cho et al., 2017; Lu et al., 2006). Future studies selectively disrupting these OTR/dopaminergic neurons in the DR, along with timing intervals between nursing bouts, would further elucidate their role in caregiving.

There was no relationship between serotonin-ir fiber length in the mPOA and caregiving behaviors. This is not unexpected, as the mPOA is critical for the active components of caregiving behavior (Lee et al., 1999; Numan, 1974; Numan et al., 1988; Numan et al., 1977; Numan and Stolzenberg, 2009; Pereira and Morrell, 2009) and there was no affect of OTR knockdown on pup licking and pup retrieval. However, disrupted kyphotic nursing after OTR knockdown was associated with changes in the serotonergic fiber innervation of the barrel field and trunk regions of the S1. Somatosensation is absolutely essential for nursing, as decreasing ventrum stimulation by placing a full spandex jacket on the dams completely prevents the onset of maternal behavior in inexperienced dams, and greatly decreases nursing and eventually leads to litter abandonment in experienced mothers (Morgan et al., 1992; Stern and Johnson, 1990). Serotonin released into the S1 increases cortical inhibition (Bassant et al., 1990; Nienborg and Jacob, 2018; Waterhouse et al., 1986b), which decreases S1 responses to tactile stimulation in laboratory rodents (Dugué et al., 2014; Waterhouse et al., 1986b). There was less than half the serotonergic-ir fiber length in the S1 of OTR-knockdown dams compared to controls, suggesting that the S1 of OTR knockdown dams might have been overly activate in responses to pup stimulation. This result might be surprising, given that Fos expression in the barrel and trunk regions of the S1 is greatly increased by pup tactile stimulation (Fleming et al., 1994b; Lonstein et al., 1997) and that pup stimulation is

essential for S1 plasticity (Rosselet et al., 2006; Xerri et al., 1994). However, unpleasant/nociceptive somatosensory stimulation activates the S1 in humans and rats as well (Bushnell et al., 1999; Jin et al., 2018; Trotter et al., 2016). Therefore, serotonin-mediated inhibition of the S1 might decrease unpleasant/nociceptive tactile stimulation from the pups and enhance caregiving behaviors.

In addition to affecting caregiving behaviors, OTR knockdown greatly increased maternal aggression, with >3-fold increase in the number of attacks and a >6-fold increase in the time spent attacking. The higher maternal aggression in the OTR-knockdown dams was likely due to lower oxytocin signaling in both the PAGvl and DR. Lesions of the PAGvl double the frequency of attacks (Lonstein et al., 1998; Lonstein and Stern, 1997a), and given that oxytocin increases cell firing in the PAGvl (S. Ogawa et al., 1992), knocking down OTR expression there would be expected to decrease activity, like the lesions, and increase postpartum aggression. The PAGvl might regulate maternal aggression through its reciprocal connections with the DR (Beitz et al., 1986; Fu et al., 2010; Ogawa et al., 2014). GABAergic neurons in the PAGvl synapse onto serotonergic DR neurons (Bagdy et al., 2000; Baraban and Aghajanian, 1980; Belin et al., 1979; Boothman and Sharp, 2005), and decreasing activation of these GABAergic neurons by knocking down OTRs could disinhibit DR serotonergic cells. This would be consistent with other work showing a positive role of serotonergic signaling from the DR in postpartum aggression (De Almeida and Lucion, 1994; Ferreira et al., 2000; Holschbach et al., 2018; Johns et al., 2005).

Higher maternal aggression in OTR-knockdown dams may be due to changes in serotonergic output from the DR to the S1. Serotonin-ir fiber length in the trunk and barrel

field regions of the S1 was found to be negatively associated with maternal aggression. Similar to caregiving, maternal aggression is maintained by recent somatosensory input from the pups (Erskine et al., 1978; Erskine et al., 1980a; Flannelly et al., 1986; Flannelly and Kemble, 1987; Stern and Kolunie, 1993; Svare and Gandelman, 1973). Given that serotonin enhances cortical inhibition (Bassant et al., 1990; Nienborg and Jacob, 2018; Waterhouse et al., 1986b), S1 output would be higher at low serotonin levels (Dugué et al., 2014; Trotter et al., 2016; Waterhouse et al., 1986b). This suggests that over-activation of the S1 might facilitate pup-driven maternal aggression.

In contrast to S1, serotonin-ir fiber length in the lOFC was positively associated with maternal aggression. The lOFC provides affective information about somatosensory cues (Francis et al., 1999; McGlone et al., 2012; Rule et al., 2002; Trotter et al., 2016) and lesions to the OFC are associated with higher aggression in humans (Coccaro et al., 2007; Damasio et al., 1994; Grafman et al., 1996) and rhesus macaques (Izquierdo et al., 2005; Pribram and Bagshaw, 1953). Consistent with the idea of serotonin-mediated inhibition in the cortex, global serotonin depletion increased BOLD signal in the lOFC of humans (Trotter et al., 2016), and modeling of the effects of serotonin into the lOFC suggest increased OFC activation under low serotonin states (Maia and Cano-Colino, 2015). Therefore, in this study, higher levels of serotonin would be predicted to increase inhibition of the lOFC and increase postpartum aggression. Interestingly, aggression might be affected by the lOFC's inhibition of S1 responses to aversive stimuli. Lesions of the OFC decrease habituation of the S1 to a noxious somatosensory stimulus (Rule et al., 2002), and noxious somatosensory stimulation is associated with increased activation of the OFC and decreased activation of the S1 (Rolls et al., 2003; Shirato et al., 2018).

Therefore, increased serotonin-mediated inhibition of the lOFC might disinhibit dams' S1 responses to the male intruder and increase their maternal aggression.

OTR knockdown in the dorsomedial tegmentum also lowered dams' anxiety-like behavior in an elevated plus maze. This was surprising given that oxytocin is a potent anxiolytic in rodents (Amico et al., 2004; Bale et al., 2001; Francis et al., 2000; Jurek and Neumann, 2018; Mantella et al., 2003; McCarthy et al., 1996; Sabihi et al., 2014b; Waldherr and Neumann, 2007; Windle et al., 1997; Windle et al., 2006), including postpartum female rats (Figueira et al., 2008; Neumann et al., 1999; Sabihi et al., 2014a). However, anxiety is increased by serotonin (Andrade and Graeff, 2001; Briley et al., 1990; File et al., 1979; Geller and Blum, 1970), so knocking down OTRs on serotonergic neurons would in fact be predicted to be anxiolytic. Serotonin-specific OTR knockdown would also be predicted to underlie reduced anxiety because OTR antagonism in the GABAergic PAGvl of postpartum rats increases anxiety-like behavior in an elevated plus maze (Figueira et al., 2008). Indeed, GABAergic neurons in the PAGvl synapse onto serotonergic neurons of the DR (Bagdy et al., 2000; Baraban and Aghajanian, 1980; Belin et al., 1979; Boothman and Sharp, 2005) and decreasing activation of these GABAergic neurons by knocking down OTR could disinhibit DR serotonergic cells and increase anxiety-like behaviors. Importantly, serotonin can be either anxiogenic (Anderson et al., 2002; Campbell and Merchant, 2003) or anxiolytic (Maisonnette et al., 2000; Zangrossi and Graeff, 1994), suggesting that anxiety-like behavior might be under the control of different subpopulations of serotonergic neurons from the DR (Lowry and Hale, 2010). Given that ~55% of serotonergic neurons in the female rat DR expressed OTRs (Table 4), the possibility arises that OTRs are expressed preferentially on anxiogenic

serotonergic neurons, while the ~35% of GABAergic neurons in the PAGvl expressing OTRs (Table 6) preferentially project onto anxiolytic serotonergic neurons. Cell-type specific knockdown of OTRs would help clarify this possibility.

Cortical contributions to the anxiolytic effects of OTR knockdown in the dorsomedial tegmentum may include the lOFC. The lOFC is a part of circuitry mediating negative affective states associated with anxiety (Etkin and Wager, 2007; Fox et al., 2010; Kalin et al., 2007; Kringelbach and Rolls, 2004; Milad and Rauch, 2007) and lesioning the lOFC cortex in rhesus macaques decreases behavioral inhibition and defensive responses to a snake-like stimulus, indicating decreased anxiety (Fox et al., 2010; Izquierdo et al., 2005; Kalin et al., 2007; Pribram and Bagshaw, 1953). Given that serotonin decreases lOFC activity (Maia and Cano-Colino, 2015; Trotter et al., 2016), it is not surprising that serotonin-ir fiber length in the lOFC was positively associated with time spent in the open arms of the elevated plus maze. The relationship between the OFC and anxiety-like behavior in rats, though, is inconsistent. This may be because lesions targeted the entire OFC (Lacroix et al., 2000; Orsini et al., 2015; Rudebeck et al., 2007), and medial OFC and lOFC have opposite roles on anxiety-like behavior (i.e., the medial OFC is associated with decreasing anxiety, while the lOFC is associated with increasing anxiety) (Fox et al., 2010; Kalin et al., 2007; Milad and Rauch, 2007). The higher capacity for serotonin release in the lOFC of OTR-knockdown dams would be expected to increase inhibition of the lOFC and decrease anxiety. Interestingly, this might be mediated by the lOFC's role in inhibiting S1 responses to somatosensory stimuli. As stated above, somatosensory cues from the pups are critical for dams' reduced anxiety-like behavior postpartum (Lonstein, 2005; Miller et al., 2011; Neumann, 2003) and the OFC can gate

S1 responses to somatosensory stimuli (including stimuli from the pups) (Rolls et al., 2003; Rule et al., 2002; Shirato et al., 2018). Therefore, increased serotonin-mediated inhibition of the lOFC might disinhibit dams' S1 responses to pup cues and decrease postpartum anxiety-like behavior.

OTR knockdown in the dorsomedial tegmentum also increased depressive-like behaviors as seen as more time spent floating. Oxytocin typically has antidepressant effects, though (Arletti et al., 1995; Arletti and Bertolini, 1987; Nowakowska et al., 2002; Ring et al., 2010; however, see Slattery and Neumann, 2010). SSRIs decrease immobility in the forced swim test (Detke et al., 1995; Page et al., 1999), and depleting serotonin with PCPA blocks the beneficial effects of SSRIs on immobility (Page et al., 1999). Similarly, cerebral spinal fluid levels of serotonin and its metabolite, 5-HIAA, are lower in depressed patients (Åsberg et al., 1977; Asberg et al., 1976; Hou et al., 2006), and artificially decreasing serotonin with a low tryptophan diet induces a depressive episode in two thirds of depression patients in remission (Delgado et al., 1990). Therefore, OTRs increase serotonin release and thereby decrease depressive-like behaviors in postpartum animals. Importantly, OTR knockdown did not affect saccharin preference, suggesting that anhedonia and behavioral despair are differentially regulated by OTRs in the dorsomedial tegmentum.

Overall, OTR signaling in the dorsomedial tegmentum is important in the regulation of numerous postpartum behaviors. OTR knockdown led to more infanticide, less nursing, more non-pup directed behaviors, greater maternal aggression, decreased anxiety-like behavior, and increased depressive-like behavior. Critically, these behavioral effects are likely not due to changes in the closely related vasopressin system, as OTR knockdown

in the dorsomedial tegmentum did not affect V1a mRNA expression, although this does not preclude cross-talk between the systems (Song and Albers, 2017). These changes in behavior might be related to altered serotonin output to forebrain sites, such as the S1 and lOFC. Given that both these sites are crucial for somatosensory processing and in changing affective states in response to somatosensory information, OTR signaling in the dorsomedial tegmentum might normally bias the serotonergic output of the dorsomedial tegmentum to maximize maternal caregiving behaviors and mood state, as well as limit defense of the nest and postpartum anxiolysis.

CHAPTER 4: GENERAL DISCUSSION

Overall summary

Chapter One discussed the critical roles of oxytocin, serotonin, the DR, and the PAGvl in the display of maternal behaviors. Given that the serotonergic DR is upregulated in postpartum females, that serotonin receptor manipulations interfere with caregiving, and that serotonin cells express OTRs, oxytocin might be a key neurotransmitter regulating the DR during reproduction. The PAGvl also expresses OTRs and regulates the DR, so it might also be important in DR regulation of maternal behavior. Therefore, I hypothesized that oxytocin signaling in the dorsomedial tegmentum would be vital for the onset and display of a suite of maternal behaviors in laboratory rats.

Chapter Two of this dissertation investigated how the oxytocin system in the midbrain dorsomedial tegmentum changes across reproduction, with a focus on the expression of OTRs on serotonergic, GABAergic, and dopaminergic neurons of the DR and PAGvl. There was higher OTR autoradiographic binding in the rostral DR, along with a greater percentage of serotonergic and dopaminergic neurons expressing OTRs in the face of a lower percentage of GABAergic neurons expressing OTRs in recently-parturient dams. Furthermore, oxytocin-ir fiber length in the DR and PAGvl were upregulated in PPD 7 females. Therefore, in Chapter Three the behavioral consequences of this upregulation were examined. Specifically, a viral construct expressing shRNA targeting the OTR mRNA (which knocks down OTR expression) was injected into the dorsomedial tegmentum. A suite of maternal behaviors were then examined across the early postpartum period. OTR knockdown in the dorsomedial tegmentum led to more infanticide, less nursing, more non-pup directed behavior, more maternal aggression,

decreased anxiety-like behavior, and increased depressive-like behavior. Finally, these changes in behavior might be related to altered serotonin output to forebrain sites, such as the S1 and lOFC.

Future directions

Future studies should target specific cellular phenotypes of the dorsomedial tegmentum. Given that lesions of the PAGvl decreases kyphotic nursing, increases maternal aggression, and decreases postpartum anxiety (Lonstein et al., 1998; Lonstein and Stern, 1997a), knocking down OTRs specifically on midbrain GABAergic neurons, which are largely located in the PAGvl, would be predicted to decrease kyphotic nursing, increase maternal aggression, and decrease postpartum anxiety-like behaviors. Alternatively, knocking down OTRs, specifically on serotonergic neurons, would be predicted to decrease postpartum anxiety-like behaviors and increase depressive-like behaviors. This would contrast with mice, where knocking out OTR expression specifically on serotonergic neurons does not affect maternal behavior (Pagani et al., 2015). However, as stated in Chapter One, oxytocin is not a potent stimulator of maternal behavior in most strains of mice, anyway (Macbeth et al., 2010; Pedersen et al., 2006; Rich et al., 2014). Additionally, their mice were of the C57BL/6J strain, which are spontaneously maternal and might have precluded any effects of OTR knockout (Jakubowski and Terkel, 1982; Mann et al., 1983).

As a complement to cell phenotype-specific manipulations, future studies should also specifically target serotonergic innervation of the S1 and lOFC. A simple tool to use would be 5,7–dihydroxytryptophan (5,7-DHT), a neurotoxin that can be used to selectively

lesion serotonergic projections. An injection of 5,7-DHT into the lOFC would be predicted to increase infanticide and postpartum aggression, while decreasing postpartum anxiety-like behaviors. Alternatively, another experiment could inject 5,7-DHT into the S1, and see if, as predicted, there is an increase in infanticide, non-pup directed behaviors, and postpartum aggression, with a decrease in kyphotic nursing.

Relevant considerations

Source of dorsomedial tegmentum oxytocin

While parvocellular oxytocin neurons of the PVN project to the dorsomedial tegmentum (Buijs, 1978; Sofroniew, 1983a), oxytocin can also be released from the soma and dendrites of magnocellular neurons (Ludwig and Leng, 2006; Ludwig et al., 2002; Pow and Morris, 1989; Tobin et al., 2011). Somato-dendritic oxytocin release is important for self-regulation of oxytocin neurons (Kombian et al., 1997; Lambert et al., 1993; Oliet et al., 2007; Rossoni et al., 2008), including in the suckling-induced milk-ejection reflex in postpartum animals (Lambert et al., 1993; Moos et al., 1984; Moos et al., 1989; Rossoni et al., 2008), which results in 2.5 – 3-fold higher concentrations of oxytocin in the PVN and SON (Neumann et al., 1993).

In addition to the milk-ejection reflex, dendritic release of oxytocin can also coordinate many neural systems through “bulk flow” signaling via the interstitial fluid and cerebral spinal fluid (Ludwig and Stern, 2015; Son et al., 2013). This might be especially true in the postpartum period, where the readily-releasable somatodendritic vesicles containing oxytocin are doubled in the PVN (de Kock et al., 2003). Dendritic fibers from oxytocin neurons from the PVN and SON surround and penetrate the ependymal walls of

the ventricular system (Buijs, 1978; Dubois-Dauphin et al., 1989), which likely allows oxytocin release directly into ventricular circulation (Veening et al., 2010). Furthermore, during lactation, glial-like processes extending from local astrocytes that would otherwise preclude passage of oxytocin between neurons retract, allowing nearly 80% of oxytocin neurons to be juxtaposed to other oxytocin neurons (Bonfanti et al., 1993; Hatton and Tweedle, 1982; Montagnese et al., 1987; Theodosis and Poulain, 1984; Theodosis et al., 1981; Theodosis, 2002; Theodosis and MacVicar, 1996; Theodosis and Poulain, 1989). These morphologic changes in the PVN and SON are critical for the oxytocin-mediated positive feedback necessary for cell-to-cell coordination of burst firing that results in the bolus release of oxytocin, such as what occurs during parturition or milk ejection (Coles and Poulain, 1991; Leng and Shibuki, 1987). These glial retractions also allow oxytocin to more freely diffuse throughout the interstitial space and into the cerebral spinal fluid for bulk flow and long-range oxytocin signaling. Given the 2.5 – 3-fold increase in local oxytocin release in the PVN and SON at parturition and during suckling (Neumann et al., 1993), the retracted glial coverage of oxytocin neurons in those sites (Theodosis, 2002; Theodosis and MacVicar, 1996), and the dendritic fibers that penetrate the ependymal walls (Buijs, 1978; Dubois-Dauphin et al., 1989), the postpartum period is likely a unique physiological state in which long-range bulk flow of oxytocin is in a particularly strong position to influence females' behavior.

Interpretation of changes in oxytocin and serotonin immunoreactive fibers

Oxytocin-ir fiber length was used as an indicator of capacity for local oxytocin release. Lactating rodents have higher oxytocin-ir fiber density than do virgins females in

many sites of the forebrain (Caldwell et al., 1987; Jirikowski et al., 1989; Knobloch et al., 2012), and this is positively associated with the levels of oxytocin released in those sites (Neumann and Landgraf, 1989; Neumann et al., 1993). Similarly, serotonin-ir fiber length was used as a measure of the capacity for serotonin release. Manipulations that decrease central serotonin levels (such as PCPA and neurotoxin administration) decrease serotonin-ir fiber length and serotonin turnover (Edwards et al., 1986; Hritcu et al., 2007; Tohyama et al., 1988; Wallace et al., 1982), while manipulations that increase central serotonin levels increase serotonin-ir fiber length and serotonin release (Bel and Artigas, 1995; Celada and Artigas, 1993; Nielsen et al., 2006). This occurs without changing serotonin transporter-ir fiber levels (Dewar et al., 1992; Graham et al., 1987; Nielsen et al., 2006), suggesting that serotonin-ir fiber length is not necessarily a measure of serotonin fiber length, but rather is a measure of capacity for serotonin release. However, while oxytocin- and serotonin-ir fiber length or density can provide clues to the capacity for oxytocin and serotonin release, it might also relate to previous neurotransmitter release. For example, oxytocin-ir fibers might be higher in the DR and PAGvl of PPD 7 dams because there was a recent decrease in oxytocin release and a concomitant increase in oxytocin storage. Conversely, instead of indicating reduced capacity for release, lowered serotonin-ir fibers in the S1 might reflect recent serotonin release. Given this caveat, it will be critical for future work to analyze release and turnover of oxytocin and serotonin, perhaps using microdialysis, to fully understand how the release of these neurochemicals change across female reproductive state and potentially influence postpartum behavior.

Dissociation of oxytocin receptor's effect on postpartum behavior

OTR knockdown in the dorsomedial tegmentum greatly affected numerous postpartum behaviors. It increased infanticide, decreased maternal caregiving, increased postpartum aggression, decreased anxiety-like behaviors, and increased depressive-like behaviors. However, none of the behavioral effects presented here can easily be attributed to just one site within the dorsomedial tegmentum. Indeed, there are reciprocal connections between the DR and PAGvl (Beitz et al., 1986; Fu et al., 2010; Ogawa et al., 2014), which might suggest a complex role of dorsomedial tegmentum OTR signaling in balancing activity between sites during female reproduction (Proposed model in Fig 24). Furthermore, the shRNA vector also hit the dorsal portion of the median raphe, the dorsal tegmental nucleus (DTN) and the laterodorsal tegmental nucleus (LDT) (Fig 12). OTR knockdown in the median raphe is not predicted to have any effects, as there are no OTRs in the median raphe in the rat (Yoshimura et al., 1993; Chapter Two observation). OTR knockdown in the DTN probably did not affect postpartum behavior, given that the DTN is a key node in the navigational circuitry (Clark et al., 2013; Sharp et al., 2001) and there were no effects of OTR knockdown on dams' navigation of the homecage during pup retrievals. Finally, OTR knockdown in the LDT also probably did not affect postpartum behavior because the LDT is critical for dopamine related reward behavior (Lammel et al., 2014; Lammel et al., 2012), but there were no effects of OTR knockdown on pup retrieval, which is driven by dopamine signaling (Keer and Stern, 1999; Li et al., 2004; Numan et al., 2005; Silva et al., 2001; Stern and Taylor, 1991; Zhao and Li, 2009). To dissociate the role of OTR signaling in the dorsomedial tegmentum, future studies would

benefit from targeting specific areas and cellular phenotypes of the dorsomedial tegmentum.

Maternal behavior, somatosensation, and serotonin

Maternal behavior is influenced by pup cues that impinge upon all maternal sensory modalities (Fleming and Rosenblatt, 1974a, 1974b; Herrenkohl and Rosenberg, 1972; Smotherman et al., 1974; Stern and Johnson, 1989, 1990). However, somatosensation is the only modality that is absolutely essential for postpartum behaviors. Decreasing perioral stimulation that dams receive from pups by muzzling or injecting lidocaine into the mystacial pads decreases licking behavior, hovering over the litter, and pup retrieval (Stern and Johnson, 1989; Stern and Kolunie, 1989), while decreasing ventrum stimulation received from the pups greatly decreases kyphotic nursing (Stern and Johnson, 1990). Most notably, blocking the ability to perceive both perioral and ventral stimulation completely prevents the onset of maternal caregiving in rats (Morgan et al., 1992). Recent somatosensory stimulation from the pups is also critical for high postpartum aggression (Erskine et al., 1978; Erskine et al., 1980a; Flannelly et al., 1986; Flannelly and Kemble, 1987; Stern and Kolunie, 1993; Svare and Gandelman, 1973) and low postpartum anxiety-like behaviors (Lonstein, 2005; Miller et al., 2011; Neumann, 2003).

Somatosensation is modified by the serotonergic fibers that innervate the S1 (Beaudet and Descarries, 1976; Dori et al., 1996; Fuxe, 1965; Kirifides et al., 2001; Waterhouse et al., 1986a). In the S1, serotonin fibers are densely packed in the superficial layers and less so in its deeper layers (Beaudet and Descarries, 1976; Lamour et al.,

1983). Serotonin inhibits the S1 by activating excitatory serotonin 2A receptors on inhibitory interneurons and activating inhibitory serotonin 1A receptors on pyramidal neurons (Bassant et al., 1990; Foehring et al., 2002; Nienborg and Jacob, 2018; Waterhouse et al., 1986b). Serotonin-mediated inhibition of the S1 seems relevant for behavior as iontophoretic serotonin application decreases neuronal firing rate of the S1 in response to tactile stimulation in female rats (Waterhouse et al., 1986b), and optogenetically activating serotonergic neurons of the DR decreased responsivity of the S1 to tactile stimulation in mice (Dugué et al., 2014). Conversely, in humans, depletion of the serotonin precursor tryptophan, which would acutely decrease serotonin availability, increases female participants' S1 BOLD signal in response to gentle tactile stimulation (Trotter et al., 2016). Overall, these studies suggest that serotonin released into the S1 increases cortical inhibition. Without this, the S1 might be overly activated in OTR-knockdown mothers in response to pup stimulation. Fos expression in the barrel and trunk regions of the S1 is greatly increased by pup tactile stimulation (Fleming et al., 1994b; Lonstein et al., 1997) and pup stimulation is essential for S1 plasticity (Rosselet et al., 2006; Xerri et al., 1994). However, unpleasant/nociceptive somatosensory stimulation also activates the S1 in humans and rats, as well (Bushnell et al., 1999; Jin et al., 2018; Trotter et al., 2016). Therefore, serotonin-mediated inhibition of the S1 might decrease unpleasant/nociceptive tactile stimulation from the pups and enhance caregiving behaviors.

Serotonin might also be important for providing affective information regarding social touch through the lOFC. The lOFC is activated in response to both nociceptive and pleasant touch (Francis et al., 1999; McGlone et al., 2012; Rule et al., 2002; Trotter et al.,

2016). The OFC can gate S1 responses to somatosensory stimuli from the pups (Rolls et al., 2003; Rule et al., 2002; Shirato et al., 2018), and serotonin from the DR projects to the lOFC, where it increases cortical inhibition (Linley et al., 2013; Maia and Cano-Colino, 2015; Trotter et al., 2016). With a higher capacity for serotonin release into the lOFC of OTR-knockdown dams, the lOFC would be predicted to be more inhibited, leading to less inhibition of the S1. Therefore, increased serotonin-mediated inhibition of the lOFC might disinhibit dams' S1 responses to pup cues and increase maternal aggression and decrease postpartum anxiety-like behavior.

In the skin of rodents and humans, there are c-fibers that respond to innocuous somatosensory stimulation and code pleasant touch (i.e., C tactile fibers) (Ackerley et al., 2014; Djouhri, 2016; Fang et al., 2005; Leem et al., 1993; Löken et al., 2009; McGlone et al., 2014; Wessberg and Norrsell, 1993). These fibers are likely part of dorsomedial tegmental circuitry mediating postpartum behaviors. Nociceptor signaling c-fibers and C tactile fibers innervate the same columns of the spinal cord (Li et al., 2011; Light et al., 1979; Perl, 1984), which then project to the PAGvl (Clement et al., 1996; Keay et al., 1994; Parry et al., 2008). Consistent with an overlapping pathway, suckling stimulation from pups induces Fos expression in the PAGvl but not most other brain sites (Lonstein and Stern, 1997a, 1997b). Given that GABAergic neurons in the PAGvl synapse onto serotonergic neurons of the DR (Bagdy et al., 2000; Baraban and Aghajanian, 1980; Belin et al., 1979; Boothman and Sharp, 2005), C tactile fiber-mediated signaling in the PAGvl might directly influence DR activity and postpartum behaviors in response to gentle/pleasant pup stimulation. In support, serotonin depletion enhanced activation of

the S1 and lOFC in response to C tactile fiber-specific stimulation in female human subjects (Trotter et al., 2016).

Proposed model for midbrain dorsomedial tegmentum oxytocin effects on postpartum behavior

Oxytocin in reproductive females biases neuronal systems to respond positively to pup cues (Marlin et al., 2015; Valtcheva and Froemke, 2018; Yu et al., 1996). Therefore, OTR signaling in the dorsomedial tegmentum likely also biases circuitry to enhance postpartum behavior (hypothetical model shown in Fig 24). In this model that is partly based on my findings and the existent literature but otherwise is highly hypothetical, OTRs expressed on the GABAergic neurons in the PAGvl are hypothesized to preferentially inhibit lOFC-projecting serotonergic neurons. Therefore, OTR knockdown in the dorsomedial tegmentum would disinhibit these serotonergic neurons and increase serotonin output to the lOFC. This knockdown would be predicted to increase maternal aggression and decrease postpartum anxiety-like behaviors. Alternatively, the serotonergic neurons projecting to the S1 are hypothesized to be OTR expressing. Therefore, OTR knockdown in the dorsomedial tegmentum would lead to decreased activation of these serotonergic neurons and decreased serotonin output to the S1. This knockdown would be predicted to increase infanticide, decrease maternal caregiving behaviors, increase non-pup directed behaviors, and increase maternal aggression. Given that serotonin inhibits cortical activation, this hypothetical model would suggest that dorsomedial tegmental OTR knockdown would increase inhibition of the lOFC and decrease inhibition of the S1. Finally, the lOFC can inhibit somatosensory processing in

the S1, so increased inhibition of the lOFC would be expected to disinhibit the S1. Disinhibition of the S1 would lead to hyper activation of the S1 in OTR-knockdown dams. This proposed model of OTRs exciting S1-projecting serotonin neurons versus inhibiting of lOFC-projecting serotonin neurons might explain why the DR does not have increased Fos expression during lactation (Lonstein and Stern, 1997a; Smith and Lonstein, 2008), even though serotonergic neurons in the DR are reported to fire ~63% more at this time (Klink et al., 2002).

Overall, given that there is long-term plasticity in the S1 in postpartum rats that requires somatosensory input from the pups (Rosselet et al., 2006; Xerri et al., 1994), over activation of the S1 might lead to enhanced expansion of the face and trunk regions of the S1 and render OTR-knockdown dams hyper responsive to pup tactile cues across the postpartum period. Consistent with this idea, OTR-knockdown was associated with increased infanticide after 1 - 2 days of maternal experience and there were interactions between caregiving behaviors and postpartum day, such that the frequencies of caregiving behaviors were similar between OTR-knockdown dams and controls on PPDs 2, but thereafter diverged. These results suggest that OTR-mediated serotonin release in the S1 and lOFC might be critical to long-term neural plastic changes underlying maternal behaviors. Importantly, this model is likely translatable to human mothers because there is a negative linear relationship between the total duration of breastfeeding during the first six months of the postpartum period and the incidence of child abuse in women (Strathearn et al., 2009). Therefore, ventrum stimulation concurrent with oxytocin release might be critical for behaviorally relevant long-term changes in the S1 for both rats and humans. Using this proposed model in future studies might provide essential clues about

the role of OTR signaling in the dorsomedial tegmentum on postpartum plasticity in the S1.

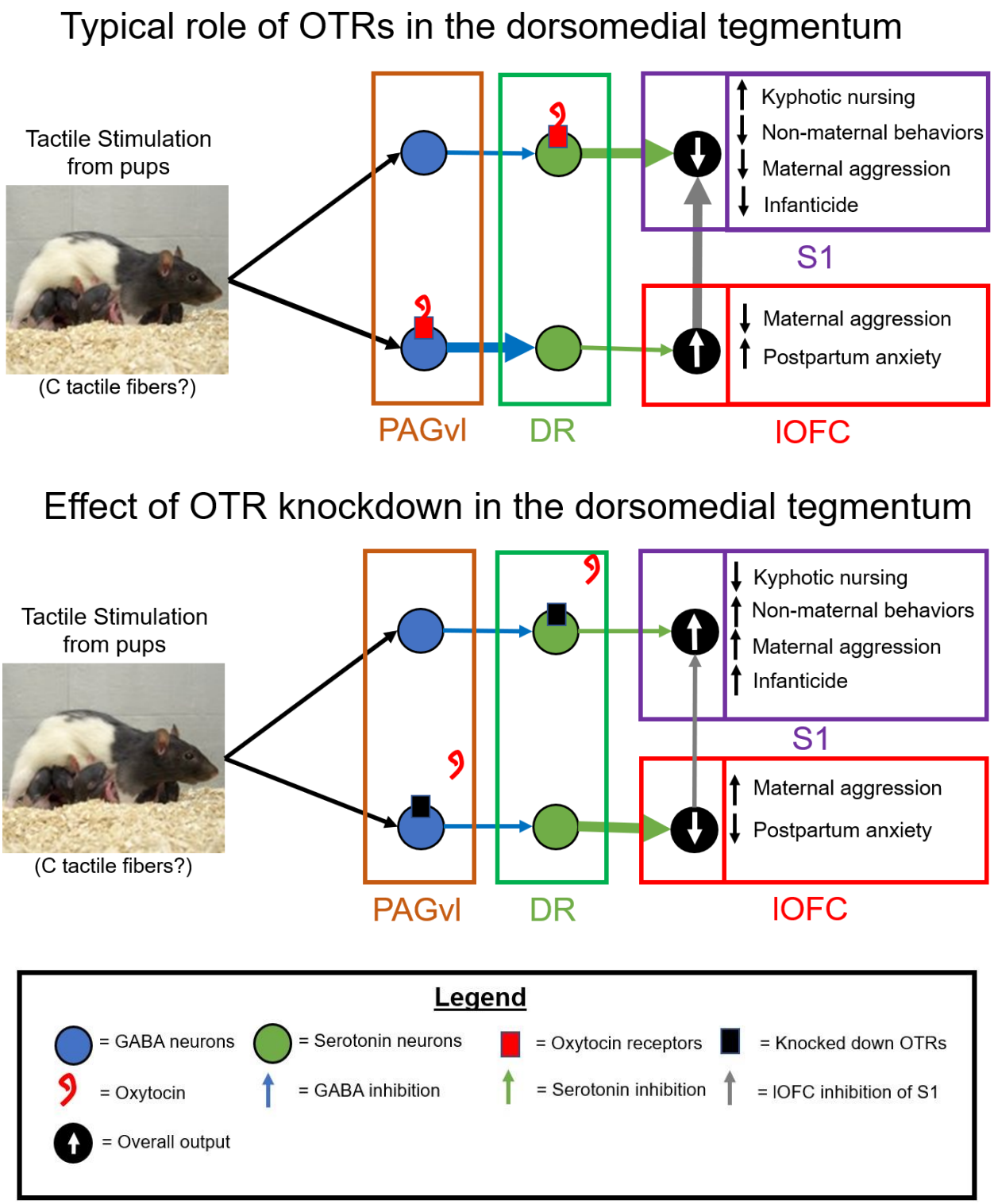

Figure 24: Schematic of the proposed role of OTRs in the dorsomedial tegmentum. A) Schematic of the proposed role of OTRs in the dorsomedial tegmentum under normal conditions. B) Schematic of the proposed effects of OTR knockdown on the circuitry of the dorsomedial tegmentum.

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