NORADRENERGIC MODULATION OF THE VENTRAL BED NUCLEUS OF THE STRIA TERMINALIS: EFFECTS ON MATERNAL AND ANXIETY-RELATED BEHAVIORS

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ABSTRACT

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Postpartum rats spontaneously display pup-directed behaviors and exhibit lower anxiety-related behaviors compared to nulliparous rats. The ventral bed nucleus of the stria terminalis (BSTv) is a neural site critical for dams' maternal behaviors and, in male rats, for regulating anxiety-related behaviors. Neurotransmitters modulating the BSTv in females have not been previously identified. The BSTv contains one of the highest concentrations of norepinephrine (NE) in the brain and, in male rats, the release of (NE) in the BSTv is known to increase anxiety. It is also known that NE depresses neural excitability when applied to the BSTv. Given that: 1) maternal behavior is dependent on a functional BSTv, 2) NE inhibits the BSTv, 3) NE increases anxiety in male rats, and 4) dams exhibit pup-directed care and low anxiety, I hypothesized that dams maintain naturally low levels of NE release in their BSTv that account for their maternal responsiveness and low anxiety-related behaviors. To test this hypothesis, I examined the effects of increasing NE release in the BSTv on maternal behaviors, the effects of increasing or decreasing NE release in the BSTv on anxiety-related behaviors, and the concentrations of endogenous monoamines in female rats. Yohimbine or idazoxan (alpha-2 autoreceptor antagonists that increase NE release) significantly disrupted retrieving behaviors in postpartum rats. Idazoxan, but not yohimbine, also reduced the time dams spent nursing pups. Yohimbine also decreased exploration of the open arms in an elevated plus maze (EPM) when administered systemically or within the BSTv. Idazoxan, surprisingly, did not mimic the anxiogenic effects of yohimbine. Additionally, clonidine (an alpha-2 autoreceptor agonist that decreases NE release)

did not affect anxiety-related behaviors in an EPM when administered systemically or within the BSTv. Finally, the concentrations of NE, dopamine, and serotonin were measured in the BSTv, medial preoptic area (mPOA), and dorsal bed nucleus of the stria terminalis (BSTd). Numerous differences in monoamine levels were found. Some notable results include postpartum rats containing higher levels of NE and turnover of serotonin compared to postpartum rats that had their litter removed or diestrous virgins. Together, these results demonstrate that increasing NE release in the BSTv of postpartum rats disrupts maternal behavior and increases anxiety-related behaviors. Furthermore, differences in NE and serotonin may account for the behavioral differences between postpartum and virgin rats.

I dedicate this dissertation to Dr. Dwayne Hamson. You are the model of an ideal scientist, philanthropist, and human being. You taught me what it means to love science and to love life. For that, I am forever grateful. Thank you.

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Introduction

Parenting is a universally characteristic behavior of mammals necessary for the healthy physical, social, emotional and cognitive development of neonates. Rats are an example of a uniparental species that produce altricial offspring; newborn pups are unable move, regulate body temperature, urinate, or defecate without help from a parent. Numerous well-characterized pup-directed behaviors have been described in mother rats (Wiesner & Sheard, 1933). After parturition ends, dams construct a nest to shelter neonates. In the relatively rare event that the pups are scattered or if the dam moves the nest, the mother retrieves pups by grasping them with her incisors by the nape of the neck and moving each pup to the nest site. Once grouped inside the nest, dams lick and groom their pups to clean and facilitate the excretion of urine and feces. Dams eventually become quiescent and adopt an arched-back nursing posture over the pups, termed kyphosis (Stern, 1996). In addition to maternal behaviors, mother rats display changes in other behaviors that are necessary for successful rearing of offspring. Compared to non-postpartum rats, dams also exhibit heightened aggression towards intruders (Erskine et al., 1980; Ferreira & Hansen, 1986; Mayer et al., 1987) and reduced anxiety-related behaviors (Bitran et al., 1991; Ferreira et al. 1989; Fleming and Luebke 1981; Hard & Hansen, 1985). These behaviors may increase the likelihood that dams protect pups from potential predators and engage in risks that benefit the litter, such as exploring new territories that yield needed resources. Additionally, a state of low anxiety in postpartum rats may be necessary for the display of high aggression (Lonstein, 2007; although see Bosch et al., 2005).

Not all rats, however, spontaneously display these pup-directed behaviors. Male and adult virgin female rats do not immediately care for pups and, in fact, actively avoid or attack them (Fleming & Rosenblatt, 1974; Rosenblatt, 1967; Weisner & Sheard, 1933). This

immediate onset of maternal behavior observed in lactating rats depends on the hormonal events of gestation (for review, see Numan & Insel, 2003). Plasma levels of progesterone and estrogen shift dramatically throughout pregnancy and are critical for spontaneous maternal care. Early in gestation, estrogen levels remain low but then rise rapidly around day 18 of pregnancy until birth (day 22). A period of postpartum estrus follows in as little as 8 hours after parturition; if the female fails to copulate during this period, she enters a constant state of postpartum diestrus. Progesterone levels, conversely, are elevated throughout gestation, peak on day 16, and then quickly drop on day 20. Additionally, prolactin is secreted in daily surges during early gestation and then remains low between days 5 and day 20 before sharply spiking on the morning of parturition (Morishige et al., 1973). This pattern of increasing estrogen, drop of progesterone, and secretion of prolactin is necessary for the onset of maternal behavior.

Manipulating the levels of estrogen, progesterone, and prolactin provide evidence for the necessity of these hormones. Removing the uterus during pregnancy is one method of altering hormone secretion. A hysterectomy performed late in gestation (between day 16 and 19) stimulates the onset of maternal behavior when females are presented with healthy pups 48 hours after surgery (Rosenblatt & Siegel, 1975). This procedure induces a withdrawal in progesterone that stimulates a rise in estrogen, similar to what occurs at the end of gestation (Bridges et al., 1978; Rosenblatt & Siegel, 1975). Removing the ovaries along with the uterus, which prevents any surge in estrogen and prolactin, negates the facilitating effect of a hysterectomy (Rosenblatt & Siegel, 1975; Siegel & Rosenblatt, 1975). Providing injections of exogenous estrogen at the time of surgery, however, stimulates maternal behavior when the ovaries are removed (Siegel & Rosenblatt, 1975). Furthermore, simulating the hormonal events of gestation with exogenous estrogen, progesterone, and prolactin hastens maternal care in ovariectomized nulliparous rats

(Moltz et al., 1970; Zarrow et al., 1971; Bridges, 1984).

Although hormones are necessary for the immediate pup-directed behaviors observed in parturient rats, there can also be a non-hormonal basis for maternal behavior. Exposing nulliparous rats to pups through a period of cohabitation will elicit infant-directed care (Fleming and Rosenblatt, 1974; Leblond, 1938; Rosenblatt, 1967; Stern, 1983; Wiesner & Sheard, 1933). Termed *maternal sensitization*, males and virgin females will cease avoidance and eventually display parental behaviors when presented with freshly-nourished pups for a period of four to seven days. Although incapable of nursing, these rats will build nests, retrieve pups to a nest, lick pups, and hover over the litter. Additionally, once maternal behavior is established, regardless of reproductive state, removal of the ovaries (Rosenblatt et al., 1979), pituitary gland (Obias, 1957), or adrenal glands (Rees et al., 2004; Thoman & Levin, 1970) does not abolish maternal behavior. This evidence clearly suggests that hormones are required for the onset, but not maintenance, of pup-directed care in laboratory rats.

Instead, the maintenance of maternal behavior is largely reliant on sensory stimuli emitted from pups. Pups elicit attention from a mother through tactile, olfactory, visual (Beach & Jaynes, 1956), and auditory (Herrenkohl & Rosenberg, 1972) sensory modalities. The tactile feedback dams receive from pups, however, appears particularly important. Retrieval behavior is blocked by temporarily removing sensation in the snout of mother rats by injecting lidocaine into the mystacial pads or by cutting the trigeminal nerve (Kenyon et al., 1981, 1983; Stern & Kolunie, 1991). These effects are only temporary, as dams retrieve after the anesthetic wears off (Kenyon et al., 1981) or days after trigeminal dissection (Stern & Kolunie, 1991). In addition, tactile input on dams' ventral surface is necessary for eliciting kyphosis. Once pups are grouped to a nest site, mother rats engage in a period of hovering over the litter before transitioning into

quiescent nursing. Preventing pups from suckling inhibits the display of kyphosis. This can be accomplished by suturing the pups' mouths closed (Stern & Johnson, 1990) or by removing dams' teats through a surgical procedure called thelectomy (Stern et al., 1992). If pups cannot attach to the dams' nipples, the mothers spend more time hovering, licking pups, and engaging in non pup-directed behaviors such as grooming and exploration.

Brain regions regulating maternal behaviors

Neural sites modulating the unique behaviors of postpartum rats are complex and only partially understood. The expression of maternal behaviors such as retrieving pups to a nest, licking and grooming pups, and nursing pups, require an interplay of numerous neural systems and will only be briefly summarized here (refer to Figure 1). Numan (2007) suggests a model of how these brain regions interact to stimulate maternal behavior in postpartum rats or induce pup-withdrawal in nulliparous rats. In the non-maternal nulliparous rat, odors from pups are recognized by the olfactory bulbs, which, in turn, project to the medial amygala. Pup-related information is then sent from the medial amygdala to the anterior and ventral medial hypothalamic nuclei before finally synapsing in the periaqueductal gray (PAG). These neural sites likely inhibit the expression of maternal behavior as lesions of the olfactory bulbs (Fleming & Rosenblatt, 1974), medial amygdala (Fleming et al., 1980; Numan et al., 1993; Sheehan et al., 2001), and anterior/ventromedial hypothalamic nuclei (Bridges et al., 1999; Sheehan et al., 2001) all stimulate pup-directed behaviors in nulliparous rats. Connections from the anterior/ventromedial nuclei to the PAG may inhibit maternal behavior by increasing anxiety and initiating a withdrawal response in nulliparous rats from pups. In support, lesions of the PAG do not inhibit pup-directed approach behaviors (Lonstein & Stern, 1997) but do reduce anxiety-

related behaviors (Lonstein et al., 1998). Thus, non-maternal rats may have an anxiogenic response to pups that prevents them from initiating maternal behaviors.

In contrast, stimuli from pups produce different effects in other neural regions in maternally-behaving rats. Regions such as the medial preoptic area (mPOA) and adjacent ventral bed nucleus of the stria terminalis (BSTv) are essential for stimulating pup-directed care. First, these brain regions contain estrogen receptor (Pfaff & Keiner, 1973; Shugrue et al., 1997; Simerly et al., 1990) and prolactin (Bakowska & Morrell, 1997) that become more numerous during gestation, which may be important for the onset of maternal behavior. Second, as will be described in detail later, damage to the mPOA and/or BSTv inhibits the expression of most maternal behaviors. Third, the mPOA and BSTv project to brain regions such as the anterior hypothalamus and PAG (Numan & Numan, 1996), which may promote anxiety and inhibit maternal behavior. Importantly, Lonstein & De Vries (2000) have shown that neurons in the mPOA and BSTv expressing Fos after dams are reunited with pups are co-labeled with the rate-limited enzyme for GABA synthesis. The mPOA/BSTv may, thus, inhibit brain areas associated with anxiety.

In addition to inhibiting brain areas that may promote avoidance behavior from pups, other neural systems may promote approach behavior to pups. The mesolimbic dopamine system has particular importance, as the release of dopamine into the nucleus accumbens from the ventral tegmental area is necessary for infant-directed behaviors (Keer & Stern, 1999, Numan et al., 2005; Silva et al., 2003). Projections from the nucleus accumbens synapse in the ventral pallidum, where information is sent to motor cortices and brainstem structures that initiate maternally relevant movements (Mogenson & Yang, 1991). Neurons in the BSTv and mPOA may contribute to the continuation of maternal behavior by relaying sensory information from

pups to the ventral tegmental area (Numan & Smith, 1984). This system primarily regulates the retrieval component of maternal behavior, while other behaviors, such as nursing, have different neural substrates including the periaqueductal gray (for review, see Stern & Lonstein, 2001).

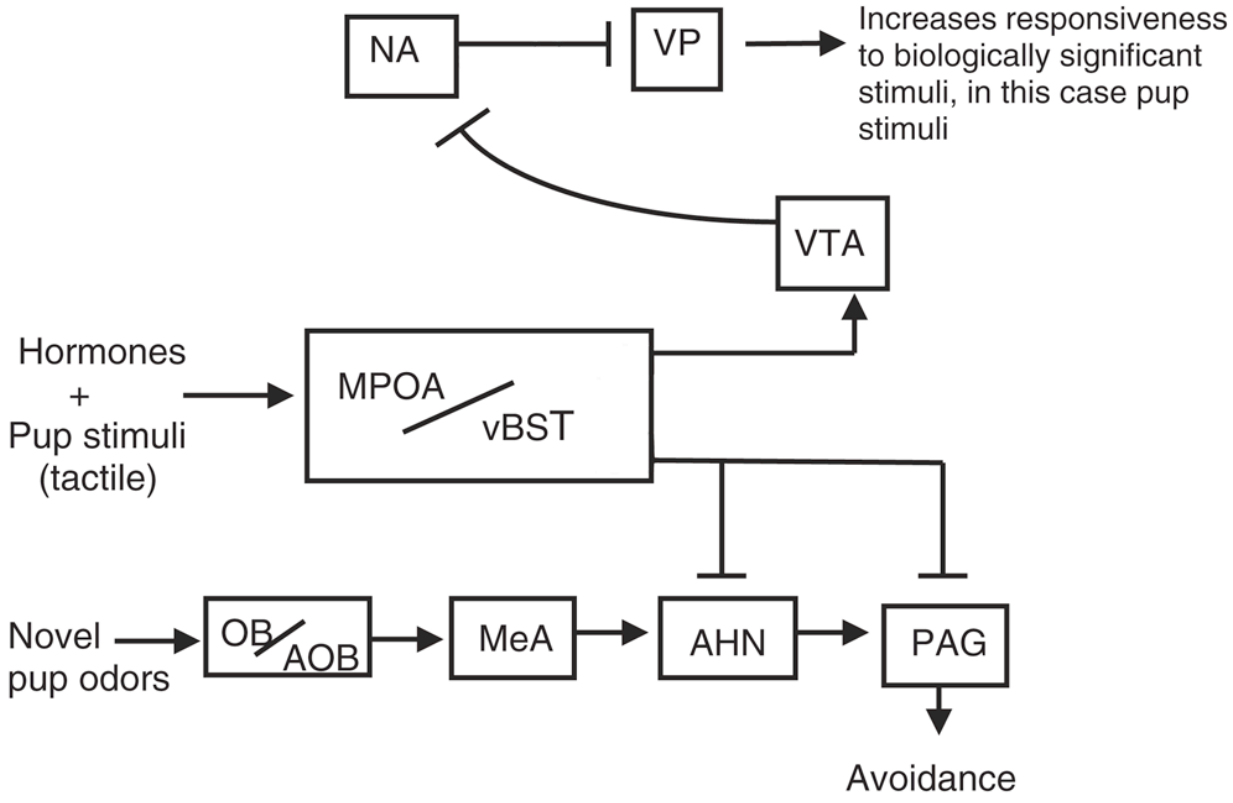

Figure 1 - Theoretical overview of neural connections relevant for the expression of maternal behavior. Arrows indicate excitatory connection and flat lines indicate inhibitory connections. Abbreviations are nucleus accumbens (NA), ventral pallidum (VP), ventral tegmental area (VTA), medial preoptic area (MPOA), ventral bed nucleus of the stria terminalis (vBST), main and accessory olfactory bulb (OB/AOB), medial amydala (MeA), anterior hypothalamic nucleus (AHN), and periaqueductal gray (PAG). Adopted from Numan (2007).

Effects of damaging the mPOA and BSTv have been extensively documented. Early studies in postpartum rats demonstrated that lesions to the mPOA produce severe deficits in maternal behavior (Jacobson et al., 1980; Numan, 1974; Numan et al., 1988), including poor nest-construction, disrupted retrieving, reduced licking, and reduced weight gained by the litter. Lesions also inhibit maternal behavior when made during gestation (Lee et al., 2000) and in maternally-behaving sensitized virgins (Gray and Brooks, 1984). Knife-cuts severing the lateral connections of the mPOA also mimic these deficits (Franz et al., 1986; Miceli et al., 1983; Numan, 1974; Numan et al., 1985; Terkel et al., 1979). It is unclear if lesions or knife-cuts disrupt nursing; although numerous studies report reduced nursing (Lee et al., 2000; Numan, 1974), others do not (Franz et al., 1986; Jacobson et al., 1980; Miceli et al., 1983). Size of the lesion may be important, as smaller areas of destruction (Jacobson et al., 1980) did not affect nursing while more widespread lesions did (Numan, 1974). Additionally, studies reporting severe deficits in nursing did not group pups to the nest after dams failed to retrieve. Typically, at least four pups are required for a female to initiate quiescent nursing (Stern et al., 1992). If pups remained individually scattered throughout the cage, dams may not have received adequate tactile sensation to begin kyphosis.

Many lesions targeting the mPOA were large and some included portions of the adjacent BSTv (Numan, 1974; Numan et al., 1988). Thus, damage to the BSTv may have contributed to the observed deficits in pup-directed behaviors. Site-specific destruction of the BSTv produces similar deficits in maternal behavior as mPOA lesions, although the deficits may not be as severe. Numan & Numan (1996) reported that half of the dams receiving BSTv lesions did not retrieve any pups postoperatively while the remaining dams began retrieving some pups five days after surgery. None of these rats displayed nursing behavior but whether the lesions

completely eliminated nursing is difficult to determine because the litters of non-retrieving dams were never grouped to the nest. In addition, knife cuts made laterally to the mPOA that successfully disrupt maternal behavior also remove connections to the BSTv. An examination of Fos in maternally behaving lactating rats or sensitized virgin rats indicates increased Fos-immunoreactivity (IR) in the BSTv, further suggesting that neural activity in this region is associated with maternal behavior (Numan & Numan, 1994).

Furthermore, implanting hormones directly into the BSTv and mPOA can shorten the length of time before non-maternal rats display pup-directed care. Estradiol implanted directly into the neighboring mPOA of pregnant and virgin rats stimulates maternal behavior more rapidly than does vehicle (Fahrbach & Pfaff, 1986; Numan et al., 1977), and it has been suggested that this effect may actually be partly due to the implanted estradiol spreading to the BSTv (Numan & Insel, 2003). Additionally, prolactin infused into the mPOA of ovariectomized, estrogen-treated virgins stimulates the onset of maternal behavior (Bridges et al., 1990). Although infusions were aimed at the mPOA, many terminated in the BSTv and had similar effects of promoting maternal behavior. These data provide further support for the involvement of the mPOA and BSTv in promoting pup-directed behaviors.

Changes in anxiety across reproductive state

Maternal care towards pups is not the only behavioral change to occur in postpartum rats. As described earlier, postpartum rats exhibit lowered anxiety-related behaviors compared to non-postpartum rats. This reduction in anxiety may contribute to the maintenance of maternal behavior. Indeed, virgin rats actively avoid pups before becoming tolerant of their presence (Fleming & Luebke, 1981). This avoidance may be a reaction to potentially anxiogenic stimuli

emitted from pups. In lactating rats, however, the change in anxiety is not solely related to cues from pups. Instead, dams exhibit a general reduction in anxiety-related behaviors. In an open field, parturient rats are faster to emerge from their cage into the field, explore more once in the field, and spend more time in the center of the apparatus compared to virgin females (Fleming and Luebke 1981; Toufexis et al. 1999), all of which are suggested to reflect decreased anxiety (Parmigiani et al. 1999). Lactating rats also show significantly less freezing behavior after exposure to an auditory stimulus (Hard & Hansen, 1985; Toufexis et al., 1999), greater consumption of water during a punished drinking test (Ferreira et al., 1989), faster entry into the open arm of a T-maze (Bridges et al., 1972), greater exploration in the open arms of an elevated plus maze (Bitran et al., 1991; Kellogg & Barrett, 1999; Lonstein, 2005), and decreased burying of an electrified probe (Picazo and Fernandez-Guasti 1993) when compared to virgins.

A recent set of experiments conducted in our laboratory (Lonstein, 2005) examined the sensory regulation mediating differences in anxiety-related behavior between virgin and lactating rats. Similar to Bitran et al. (1991), we demonstrated that lactating rats spent a greater percentage of time in the open arms of an elevated plus maze than did diestrous virgins, indicating dams' lower anxiety (Pellow et al., 1985). In addition, lactating females that were separated from their young for as little as four hours spent a lower percentage of time in the open arms compared to parturient rats that were not separated before the test, indicating that contact with pups prior to testing reduced dams' anxiety. Thelectomy (removal of the nipples) did not prevent this reduction in anxiety-related behaviors, suggesting that suckling is not required for dams' attenuated anxiety. Dams, however, must have physical contact with their litter because placing pups inside a wire mesh cage that prevents a female from receiving complete tactile stimulation does not maintain low anxiety. These results suggest that very recent tactile

stimulation from the young, but not suckling, is necessary to produce the anxiolytic state seen in lactating females.

Neural sites regulating anxiety-related behaviors

Relatively little is known about the brain regions that contribute to dams' reduced anxiety-related behaviors. Research from male rats provides insight into this question. Not surprisingly, males exposed to anxiogenic stimuli exhibit increased Fos-IR in widespread areas of the brain. These include the cortex, bed nucleus of the stria terminalis, paraventricular nucleus, amygdala, hypothalamus, septum, and periaqueductal gray (Dielenberg et al., 2001; Duncan et al., 1996; Emmert and Herman 1999; Salome et al., 2004; Silveira et al., 1993). Systemic injections of anxiogenic drugs in rats elicit Fos reactivity in many of these same brain sites, helping to confirm their involvement in anxiety-related behaviors (Singewald et al., 2003; Singewald & Sharp, 2000; Thompson & Rosen, 2006).

In addition to maternal behavior, the BSTv is also known to mediate anxiety-related behaviors in rats. Categorized as the "extended amygdala," the BST was originally proposed to modulate unconditioned anxiety-related processes while the amygdala modulates conditioned fear-related processes (Walker & Davis, 1997). This view has been modified to suggest that the BST is not solely involved in unconditioned anxiety but also long-term, as opposed to short-term, conditioned fear responses (Walker & Davis, 2008). Nevertheless, lesioning the BST or infusing anxiety-modulating drugs into the BST mediate anxiety and/or fear responses as measured by startle reflex. This is not surprising given the interconnected nature of the BST and amygdala (for review, see Walker & Davis, 2008). In addition, regions of the dorsal BST (BSTd) have long been implicated in "coping" mechanisms to stressors. Lesions to the BSTd or

entire BST, encompassing both the BSTd and BSTv, increase immobility in male and female rats exposed to a forced swim test (Pezuk et al., 2008; Schulz & Canbeyli, 2000). Thus, the BST likely relays potentially hazardous information from the environment to regions of the hypothalamus mediating the stress response (Choi et al., 2007; Choi et al., 2008). Furthermore, the ventral portion of the BST also projects to brain sites modulating anxiety-related behaviors, including the anterior and ventromedial hypothalamus and periaqueductal gray (Numan & Numan, 1996), which are also important for maternal behavior.

Evidence from our laboratory suggests that the BSTv is not only important for infant-directed behaviors, but also dams' anxiety-related behaviors. I previously found that low-anxiety dams have greater numbers of Fos-IR cells in the BSTv compared to dams rendered anxious by having their litter removed four hours before testing in an elevated plus maze (Smith & Lonstein, 2008). Importantly, the increase in Fos depended on both the presence of pups and exposure of dams to the elevated plus maze (EPM). Control dams that remained in contact with pups but were not exposed to the EPM did not have high Fos-IR in the BSTv (see Figure 2). These data suggest that activity in the BSTv may contribute to reduced anxiety, as measured by the EPM. Furthermore, these data underscore the importance of recent stimulation from pups for reduced anxiety-like behaviors, as four hours of separation from the litter increases mothers' anxiety to that of diestrous virgins (Lonstein, 2005; Smith & Lonstein, 2008). Interaction with young likely affects neuronal activity in the BSTv, which may modulate anxiety and other aspects of emotionality in lactating rats.

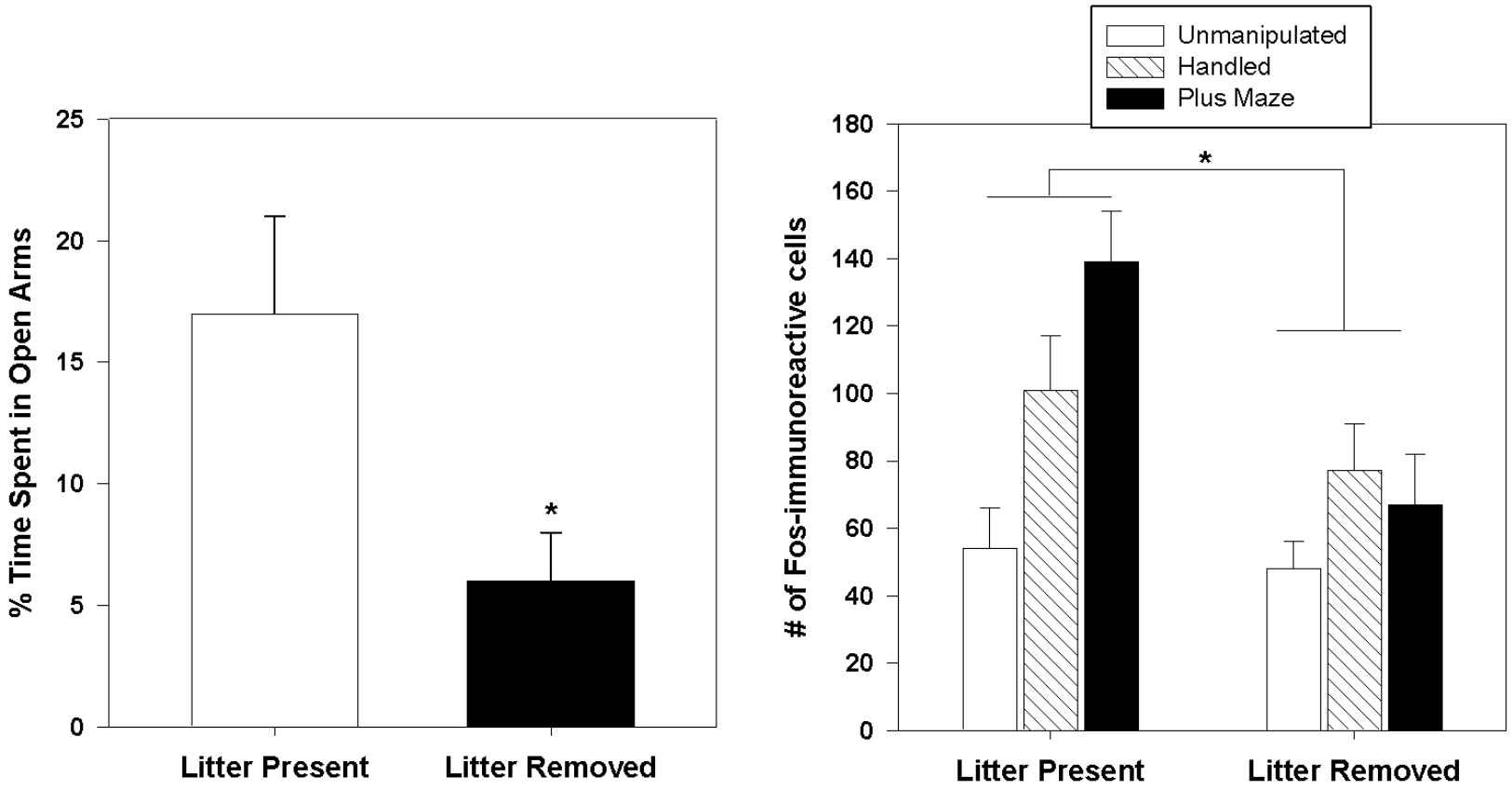

Figure 2 - (Left panel) Percentage of time (± SEM) dams spent in the EPM. (Right panel) Number of Fos-IR cells (± SEM) in the BSTv of dams exposed to no stimulation, handling, or an EPM. Dams in both panels were either left alone with their litter or had their litter removed four hours before testing. * indicates statistical significant determined at $p \leq 0.05$.

Noradrenergic Effects in the BSTv on Maternal and Anxiety-Related Behaviors

Despite data demonstrating the importance of the BSTv for maternal behavior and anxiety in dams, little is known about underlying neurotransmitter systems in this site that affect these behaviors. Cell bodies in the BSTv synthesize numerous neurochemicals, with the most ubiquitous being GABA. The far majority of neurons in the BSTv are GABAergic (Muganini & Oertel, 1985; Stefanova et al., 1997; Sun & Cassell, 1993), a phenotype potentially necessary for regulating maternal behavior. After lactating rats are exposed to pups, ~60% of Fos-IR neurons in the BSTv are colocalized with GAD, the rate-limiting enzyme in GABA synthesis (Lonstein & De Vries, 2000). Our laboratory also found that ~60% of Fos-IR cells in the BSTv are colocalized with GABA after dams are exposed to an elevated plus maze (Smith and Lonstein, unpublished data). Other neurons in the BSTv not identified as GABAergic may contain such neurochemicals as glutamate (Kocsis et al., 2003), CRH (Ju & Han, 1989; Swanson et al., 1983), galanin, neurotensin (Zahm et al., 2001), and substance P (Ju & Han, 1989; Shimada et al., 1989).

Not only are efferent projections of the BSTv of many phenotypes, but afferent projections modulating the BSTv are also numerous and extensive. One neurochemical system that may act in the BSTv to particularly influence maternal behavior and anxiety in mother rats, however, is the noradrenergic system. The BSTv contains one of the highest concentrations of noradrenergic terminals in the mammalian brain (Fendt et al., 2005; Kilts & Anderson, 1986; Woulfe et al., 1990), and in rats, electron microscopy confirms that these terminals form synapses with BSTv dendrites (Phelix et al., 1992). Sources of this noradrenergic input originate primarily from medullary A1 and A2 neurons, with only a small input from the locus coeruleus (Aston-Jones et al., 1999; Riche et al., 1990; Roder & Ciriello, 1994; Sawchenko & Swanson,

1982; Woulfe et al., 1990). Microdialysis demonstrates that norepinephrine (NE) is tonically released in the BSTv, and that this release is significantly decreased by autoreceptor agonists (Forray et al., 1997).

The release of NE into the rat BSTv is inhibitory and this NE-induced inhibition may affect maternal motivation. Direct application of NE to the BSTv reduces neuronal excitability in 70% of neurons while increasing excitability in only 2% of neurons (Casada & Dafny, 1993). NE may promote inhibition of the BSTv through numerous mechanisms. First, galanin, an inhibitory neuropeptide (Kask et al., 1995), is synthesized in the BSTv (Melander et al., 1986) and receives noradrenergic innervation (Kozicz, 2001). Galanin may serve an inhibitory role in the BSTv but its action is not necessarily correlated with the release of NE (Morilak et al., 2003), making it unclear if galanin is responsible for the NE-induced inhibition of the BSTv. Second, NE attenuates the release of glutamate into the BSTv (Forray et al., 1999). Thus, one effect of NE may be to blunt excitatory inputs to this brain region. Third, exposing BSTv neurons to NE increases the frequency of GABA inhibitory postsynaptic currents (IPSC), which inhibits excitatory neurons in the BSTv that project to the VTA (Dumont & Williams, 2004). Thus, the release of NE in this region may result in lowered dopaminergic activity in the VTA. As previously discussed, disrupting the release of dopamine into the nucleus accumbens, which receives projections from the VTA, inhibits maternal behavior (Keer & Stern, 1999, Numan et al., 2005; Silva et al., 2003).

Norepinephrine may modulate maternal behavior, although to date, studies examining NE in postpartum rats are equivocal. Entire hypothalamic NE concentrations decrease shortly after parturition while MHPG, a metabolite of NE, increases, suggesting that metabolism of NE increases after giving birth (Moltz et al., 1975). Interestingly, these same fluctuations are

observed in sensitized virgin female rats that behave maternally towards infants (Rosenberg et al., 1976). In addition, depleting NE throughout the brain of prepartum rats disrupts the parturitional onset of behaviors including nest-building and nursing (Rosenberg et al., 1977). The same preparation in postpartum rats, however, does not affect maternal behaviors. This indicates that NE is necessary for the onset of maternal behavior, but is not required thereafter. Evidence from mice that are unable to synthesize NE also demonstrate the importance of this neurotransmitter for the care of infants. Mice lacking the gene for *Dbh* do not retrieve, nurse, or clean pups, nor do they build adequate nests (Thomas & Palmiter, 1997). Administration of an NE precursor drug shortly before parturition, but not after birth, reinstates maternal behavior and is no longer required once infant care is established. One conclusion from these data in rats and mice is that increased noradrenergic metabolism facilitates the initiation of maternal behavior, and that depleting NE in the brain disrupts this process. In contrast, depleting NE after parturition has little or no effect, possibly because noradrenergic tone is already normally low in mothers.

Interpreting these studies, however, requires numerous caveats. First, NE levels were measured in the entire homogenized hypothalamus of rats and not in other brain regions. It is unknown if NE release in other brain sites mirrors that in the hypothalamus. Second, NE was depleted using 6-hydroxydopamine (6-OHDA), a toxin that also destroys dopaminergic neurons and nerve terminals. In Rosenberg et al. (1977), 6-OHDA was injected without an agent to protect dopaminergic neurons. Blocking the effects of dopamine throughout the hypothalamus and forebrain disrupts maternal behavior, particularly active behaviors such as retrieval and licking (Hansen, 1994; Keer & Stern, 1999; Miller & Lonstein, 2005; Numan et al., 2005). Third, severing the ascending noradrenergic pathways specifically innervating the hypothalamus

during gestation does not greatly affect maternal behaviors, with the exception of nest building (Bridges et al., 1982). This implies that noradrenergic innervation of the hypothalamus is not necessary for the onset or maintenance of maternal care. Fourth, it has been suggested that because *Dbh*$^{-/-}$ mice also overproduce dopamine, their impaired maternal behaviors may be attributed to an excess of dopamine instead of a lack of NE (Numan & Insel, 2003). Indeed, mice selectively bred for high aggressiveness, which show more maternal neglect than outbred controls, have significantly higher Fos expression in dopamine-rich brain areas (Gammie et al., 2008). These issues necessitate further research into the NE's role in maternal behavior

In addition to pup-directed care, NE release in the BSTv may also impact postpartum anxiety. The literature on male rodents strongly supports this general hypothesis. NE increases in the BSTv after male rats are exposed to numerous anxiogenic stimuli, including TMT, the fearful component of fox odor (Fendt et al., 2005). Infusion of clonidine, an alpha-2 autoreceptor agonist that decreases NE release from the presynaptic neuron (Fielding & Lal, 1981), prevents this increase (Fendt et al., 2005), as well as prevents the display of anxiety-related behaviors (Schweimer et al., 2005). Postsynaptic noradrenergic antagonists also reduce anxiety: rats infused with alpha-1 or beta-adrenergic receptor antagonists into the BSTv display attenuated anxiety-related behaviors after experiencing immobilization stress (Cecchi et al., 2002). Therefore, increasing noradrenergic activity in the BSTv promotes anxiety-related behaviors in male rats, while decreasing noradrenergic activity reduces their anxiety-related behaviors. Additionally, systemic administration of yohimbine (an alpha-2-autoreceptor antagonist that increases the release of NE) significantly increases the acoustic startle response in lactating rats, while clonidine (an alpha-2-autoreceptor agonist that decreases the release of NE) tends to reduce startling in both lactating and virgin rats (Toufexis et al., 1999). These data

suggest that decreased noradrenergic activity reduces anxiety in both male and postpartum female rats.

Changes in Central Noradrenergic Function During Lactation

How changes in noradrenergic activity in the BSTv of postpartum female rats are associated with changes in maternal and anxiety-related behaviors is a primary goal of this dissertation. There is precedence for reproductive state affecting the noradrenergic system in another physiological system, the hypothalamo-pituitary-adrenal (HPA) axis. Dams are in a state of tonic hypercorticalism that is necessary to satisfy the metabolic demands of nurturing infants (Lightman et al., 2001; Stern & Voogt, 1973; Walker et al., 1992). Despite high plasma levels of glucocorticoids throughout the postpartum period, dams show reduced HPA activity in response to numerous stressors (for review, see Slattery & Neumann, 2008). This is also behaviorally relevant because hormones produced by the HPA axis influence behaviors in maternal rats. Adrenalectomy, which eliminates the synthesis of corticosterone, mildly disrupts some postpartum behaviors; dams adrenalectomized late in pregnancy lick and groom pups less often and spend less time in the nest compared to control females (Rees et al., 2004). Time of adrenalectomy, however, is critical, because females adrenalectomized before gestation actually display more infant-directed behaviors than females receiving sham surgeries (Thoman & Levine, 1970). In contrast to facilitating affects of corticosterone (Rees et al., 2004), intracerebroventricular infusions of CRF in maternally-behaving sensitized virgin rats disrupts maternal behavior and increases the occurrence of infanticide (Pedersen et al., 1991). In addition, CRF increases anxiety in non-lactating rats (for review, see Lonstein, 2007) as well as startle in late postpartum lactating rats (Toufexis et al., 2004). HPA hyporesponsivness, though,

is not a likely factor contributing to dams' reduced anxiety because rats in late pregnancy, which also have HPA hypoactivity, exhibit elevated anxiety-related behaviors compared to lactating rats (Neumann, et al., 1998). Thus, the HPA axis mediates the maintenance of specific maternal behaviors and lactation but not anxiety-related behaviors in dams.

One mechanism reducing HPA responsiveness in dams is attenuated noradrenergic activity. Lactating rats have fewer alpha-2 noradrenergic receptors in the paraventricular nucleus (PVN) compared to virgins, and this may contribute to dams' blunted stress response (Toufexis et al., 1998). Indeed, 6-OHDA lesions of the PVN, which eliminates noradrenergic input, reduce the physiological stress response in virgin rats to levels observed in dams (Toufexis & Walker, 1996). This preparation in lactating rats offers no further reduction in the stress response. Thus, lactating rats have reduced noradrenergic tone in the PVN compared to diestrous virgins that contributes to reproductive state differences in stress responsiveness and behavioral changes associated with HPA suppression. A similar difference between lactating and virgin rats may also exist in the noradrenergic activity of the BSTv, and contribute to differences in maternal and anxiety-related behaviors.

Reproductive state is not the only influence on NE release throughout the brain. Recent tactile stimulation from infants may also contribute to the reduced noradrenergic tone in lactating rats. Transneuronal retrograde tracing from the mammary glands results in infected neurons in the A1 regions of the medulla, locus coeruleus, and the BST itself (Gerendai et al., 2001). Because the BSTv receives noradrenergic input from A1 and locus coeruleus neurons, the possibility exists that suckling or other types of somatosensory stimulation from pups could affect the activity of both NE-synthesizing and BSTv neurons. That is, sensory inputs from the mammary glands and ventrum may reduce NE release in the BSTv, which both promotes

maternal responses but prevents anxiety-related behavior. There is precedence for this because tactile sensation alters neuronal activity in NE-synthesizing neurons and the subsequent release of NE in brain regions (Foote et al., 1980; Kalen et al., 1988; Waterhouse et al., 1998). Attenuated NE release in the BSTv may explain why females that tactilely interact with their pups are less anxious than those separated from their litter (Lonstein, 2005). I hypothesize that this naturally occurs within the maternal BSTv in response to contact with infants.

In sum, research on the neurobiology of maternal and anxiety-related behaviors implicates both the BSTv and norepinephrine. I hypothesize that postpartum state and infant contact result in tonically low NE release in the BSTv of lactating rats, facilitating maternal behavior and reducing anxiety-related behaviors, which are both necessary for the survival of young.

Overview of dissertation chapters

Specific neurochemical systems that innervate the BSTv to regulate postpartum behaviors are largely unknown. Evidence shows that the BSTv receives dense noradrenergic inputs that may have effects on maternal and anxiety-related behaviors. To date, however, there are no experiments examining infusions of noradrenergic agonists or antagonists in the BSTv of female rats, and subsequent changes in maternal or anxiety-related behaviors. The following experiments will examine how manipulating NE release in the BSTv alters maternal and anxiety behaviors in female rats. In addition, HPLC will be used to elucidate differences between maternal and virgin rats in NE content and turnover within the BSTv. Chapter 1 determined the effects of infusing alpha-2 autoreceptor antagonists into the BSTv and mPOA on maternal behavior in postpartum rats. Chapter 2 elucidated the effects of infusing alpha-2 agonists and

antagonists into the BSTv on anxiety-related behavior of postpartum and virgin female rats tested in an elevated plus maze. Finally, in Chapter 3, tissue samples from the BSTv, BSTd, and mPOA of postpartum and virgin rats were analyzed for catecholamines. Together, these experiments will begin to elucidate the role of noradrenergic activity within the BSTv for mothers' maternal and anxiety behaviors and suggest that dams' naturally low anxiety may permit the expression of maternal behavior via a trade-off mediated norepinephrine release in the BSTv.

Chapter 1: Effects of increased release of norepinephrine in the BSTv and mPOA on maternal behavior

Introduction

Previous evidence has established the BSTv as a critical structure for the onset and maintenance of maternal behaviors. The BSTv is dense with estrogen receptors (ER) (Pfaff & Keiner, 1973; Simerly et al., 1990) that likely contribute to maternal responsiveness following the surge of estrogen during gestation. The BSTv also has dense projections that terminate in brain regions associated with motivation, such as the ventral tegmental area (Numan & Numan, 1996). Neural activity in the BSTv may, thus, enhance mothers' attraction to stimuli emitted by pups. Conversely, as detailed in the General Introduction, disrupting the BSTv inhibits the expression of maternal behavior as electrolytic or excitotoxic lesions of the area eliminate nest building and retrieving (Numan & Numan, 1996). These deficits are also mimicked by severing the lateral connections of the BSTv and adjoining mPOA (Numan et al., 1985). In addition, Fos expression in the BSTv is stimulated by pup presence in maternally behaving dams and sensitized virgin rats. (Numan & Numan, 1994).

Interestingly, part of this Fos expression overlaps where the dense plexus of noradrenergic fibers are found in the BSTv (Numan & Numan, 1994; Fendt et al., 2005). To date, no experiments have investigated the effects of manipulating NE release exclusively in the BSTv on maternal behavior. There is, however, indirect evidence that noradrenergic activity may be relevant to mother-infant interactions. Severing the lateral connections of the BSTv and mPOA with a knife coated in a retrograde tracer reveal cell-body labeling in the locus coeruleus

and nucleus of the solitary tract, brain regions that are dense with NE-producing neurons. These connections are important for maternal behavior and NE may have a regulatory role in the expression of pup-directed behaviors. In addition, transneuronal tract tracers injected into the mammary glands of female rats results in infected neurons in the A1 regions of the medulla, locus coeruleus, and the BST itself (Gerendai et al., 2001). This suggests that suckling and/or tactile sensation from pups may affect the release of NE in the BSTv.

NE has a stimulatory role for the onset of pup-directed behaviors around the time of parturition (Moltz et al., 1975; Rosenberg et al., 1976, 1977; Thomas & Palmer, 1997). NE mediates numerous processes in areas of the hypothalamus, particularly the paraventricular nucleus (PVN), that are relevant to maternal behavior (Daftary et al., 2000). These processes include the release of oxytocin (Bealer & Crowley, 1998, 1999; Crowley et al., 1987), prolactin (Andersson et al., 1981), and CRF (for review, see Russell et al., 2008). These hormones are not only important for peripheral functions such as milk letdown and the stress response, but also for mediating postpartum behaviors including infant care and anxiety (Insel & Harbaugh, 1989; Neumann et al., 1993; Neumann, 2003; Pedersen & Prange, 1979; Walker et al., 2001). To date, though, the role of NE in the BSTv on maternal behavior is unknown. The BSTv does not directly mediate the release of these hormones and, thus, likely affects postpartum behaviors through different neural pathways than those of the PVN. In addition, the BSTv is essential for the maintenance of maternal behavior (Numan & Numan, 1996) whereas the PVN is more important for the initiation of maternal behavior and its destruction has little effect once maternal behavior is established (Insel & Harbaugh, 1989). Therefore, the release of NE in the BSTv may be responsible for mediating components of postpartum maternal behaviors different from those mediated by NE in hypothalamic structures necessary for the behavior's onset.

In contrast to the onset of maternal behavior, the function of noradrenergic activity in the BSTv may be to inhibit postpartum maternal behavior. This is based on evidence demonstrating that application of NE to the BSTv reduces neuronal excitability (Casada & Dafny, 1993) and that NE in the BSTv is correlated with reduced cellular activity among excitatory neurons that project to the VTA (Dumont & Williams, 2004). NE release into the BSTv may result in the downstream inhibition of the VTA, which has been shown to inhibit maternal behavior (Numan & Smith, 1984). Further, depleting NE in the hypothalamus during pregnancy does not disrupt maternal behavior except for minor deficits in nest-building (Bridges, 1982). In sum, this evidence demonstrates or suggests that: 1) the BSTv receives noradrenergic input from the medulla and locus coeruleus, 2) sensory input from pups may alter the release of NE into the BSTv, 3) eliminating NE from the hypothalamus does not block the onset of maternal behavior, 4) NE has an inhibitory role in the BSTv, and 5) inhibition of the BSTv reduces activity of motivationally-relevant circuits that promote maternal behavior. Thus, I hypothesize that mother rats maintain low noradrenergic tone in the BSTv compared to non-mother rats, which is necessary for the normal expression of pup-directed behaviors.

Chapter 1 will examine the effects of pharmacologically increasing NE release in the BSTv on infant-directed behaviors in postpartum rats. In Experiment 1a, lactating rats will be infused with yohimbine, a noradrenergic autoreceptor antagonist that increases the release of NE, which is expected to disrupt their established maternal behavior. Experiment 1b will determine the effects of intra-BSTv infusion of idazoxan, a selective autoreceptor antagonist, on maternal behaviors. This will be done because yohimbine has mild affinity for serotonin and dopamine receptors, particularly the 5-HT1A and D2 receptor (Kleven et al., 2005; Newman-Tancredi et al., 1998; Winter & Rabin, 1992). Idazoxan is expected to mimic the effects of yohimbine,

which will provide evidence that the release of NE in the BSTv affects pup-directed behaviors. Finally, Experiment 1c will examine the effects of infusing idazoxan into the neighboring mPOA. The mPOA is critical for the expression of maternal behavior (Numan, 2006) and this experiment will determine if infusions of idazoxan into the mPOA inhibit pup-directed care. If intra-mPOA infusions of idazoxan do not reproduce the effects of intra-BSTv infusions, then it would be unlikely that noradrenergic antagonists applied to the BSTv have their effects by spreading to the mPOA.

Methods

Subjects

Long-Evans female rats, descended from male and female rats purchased from Harlan Laboratories (Indianapolis, IN), were born and raised in our colony. Food and water were continuously available. Colony room temperature was set at approximately 22^{o}C with a 12:12 light/dark cycle beginning at 0800 daily. Beginning at approximately 70 days of age, females designated to the postpartum group were monitored daily with a vaginal impedance meter until a day of proestrus. Females were then placed with a sexually experienced male overnight. Pregnant females were group housed (2-3 females per cage) until day of surgery (between days 15-18 of pregnancy) after which they were housed alone. The day of parturition was assigned as Day 0 postpartum. Within 24 hr after parturition, the litter was culled to include 4 males and 4 females.

Stereotaxic Sugery

Females were anesthetized using intraperitoneal injections of ketamine (90 mg/kg; Butler

Co.) and intramuscular injections of xylazine (4 mg/kg; Butler Co.). A solution of 3% lidocaine was injected subcutaneously along the midline of the skull after the scalp was shaved and cleaned with ethanol. All surgeries were performed using a Kopf stereotaxic instrument. Stainless steel 22-gauge bilateral guide cannulae (Plastics One, Roanoke, VA) were implanted into the BSTv of pregnant or virgin rats. Coordinates for the BSTv (A/P 0.0, M/L ± 1.2) and mPOA (A/P 0.0, M/L ± 0.75) were modified from Swanson's (1998) atlas of the rat brain. Cannulae were kept patent with a dummy stylet that extended 1 mm beyond the end of the cannulae. A dust cap was then screwed over the stylet to cover the entire apparatus. Three jeweler's screws (Plastics One) were placed into the skull to help anchor the cannula with the application of dental acrylic. Once the surgery ended, the analgesic buprenorphine (0.015 mg/kg; Reckitt Benckiser) was injected intraperitoneally and the animal was placed on a heating pad until recovery.

Drug Infusions

On day 1 postpartum, dams were habituated to the infusion and testing procedure. Pups were removed from the home cage, weighed, and placed in an incubator. Dams were transported to a testing room in their home cage and allowed to acclimate for 2 hours. The testing room was lit with a 100W bulb that provided moderate levels of light. A cardboard folder was placed on the cage over the site of the dams' nest to reduce the amount of light over this side. At the end of the 2-hour period, females were placed in a carrying cage and transported to the infusion room. Dams were handled for approximately 5 minutes, during which their dust cap and stylet were removed, cleaned with ethanol, and replaced. Subjects were returned to their home cages in the testing room and allowed 10 minutes before being reunited with their litter. Pups were scattered

away from the nest and the latency to retrieve all pups to the nest was recorded. Any dam that did not retrieve all pups to the nest within 4 minutes was removed from further testing. After returning the litter to the nest, mother rats were allowed 20 minutes to interact with pups in the testing room before being returned to the colony room.

On day 2 postpartum, rats were returned to the testing room in their home cage. Litters were removed, weighed, and placed in an incubator. Two hours later, dams were transferred to a clean carrying cage and moved to the infusion room. All drug infusions into the BSTv or mPOA were delivered through a 28-gauge injector connected to a 0.5 µl Hamilton syringe (Hamilton, Reno, NV) by clear polyethylene tubing. Drugs were continuously infused over a 1 min period. After the infusion, the injector remained in the cannulae for an additional minute, and then removed. Females were given intra-BSTv infusions of yohimbine (0, 1, or 3 µg/0.125 µl per hemisphere; Experiment 1a) or idazoxan (0, 5, or 10 µg/0.125 µl per hemisphere; Experiment 1b), or intra-mPOA infusions of idazoxan (0, 5, or 10 µg/0.125 µl per hemisphere; Experiment 1c). Doses were based on each drug's ability to affect behavior when infused into the brain (Cervo, et al., 1990; Dodge & Badura, 2002; Gulia et al., 2002; Guo et al., 1996). Subjects were returned to their home cage and were not disturbed for 10 minutes. During this time, pups were expressed of urine and feces and weighed.

Maternal Behavior Testing

Upon reunion with the mother, pups were scattered in the home cage away from the nest. Behaviors were recorded on a laptop using custom-made data acquisition software. The frequency and duration of pup-directed behaviors were recorded, including retrieving to the nest, sniffing, licking, hovering over the pups, and nursing. Care was taken to differentiate between

dams' hovering over pups and the arched-back nursing posture indicative of kyphosis. Behaviors not oriented towards pups were also measured, including general exploration of the cage, self-grooming, eating, drinking, nest building, and laying inactive away from pups. If the female did not retrieve all pups to the nest within 10 minutes, the experimenter briefly paused the observation and manually moved the remaining pups to the nest. All behaviors were observed for a total of 45 minutes. After testing, litters were weighed to assess the quantity of milk letdown. Dams and pups were then returned to the colony room.

Infusion Placement Analysis

1-7 days after testing, subjects received an overdose of 0.7 ml sodium pentobarbital and were perfused through the heart with 100 ml of 0.9% saline. The brains were removed and postfixed overnight in 10% formalin followed by submersion in 20% sucrose for 2 days. Brains were then sectioned into 40 μm slices on a freezing microtome and stained with Neutral Red. Site of infusion were analyzed with a Nikon E600 microscope at 400x magnification.

Infusions placed within the BSTv (Experiments 1a and 1b) and mPOA (Experiment 1c) were included in the final data analysis. For the Experiments 1a and 1b, an infusion was considered a "hit" if: 1) it terminated in the ventral portion of the BST and 2) it terminated between 0.0 and -0.51 mm from Bregma (Swanson, 1998). Although the BSTv extends caudally from -0.51 mm, noradrenergic fibers are densest in the rostral BSTv and norepinephrine content rapidly decreases in the posterior BSTv (Fuentealba et al., 2000; Phelix et al., 1992). Because the pharmacological agents used in these experiments affect noradrenergic receptors, only infusions placed in noradrenergic-rich regions of the BSTv were included. For the Experiment 1b, an infusion was considered a "hit" if it terminated within the mPOA between -0.11 and -0.88

mm from Bregma.

Data Analysis

Behavioral variables were analyzed with one-way ANOVAs. Data from the each experiment were separately analyzed. Statistical significance was indicated by $p \leq 0.05$. In the case of significant effects, pairwise comparisons were performed using Fisher's LSD post-hoc tests that were Bonferroni corrected with significance at $p \leq 0.016$. Variables analyzed included latencies to retrieve the first pup and all the pups, total number of pups retrieved, total activity (cumulative time spent retrieving, mouthing pups, nest-building, exploring the cage, eating, drinking, licking the pups, and self-grooming), latency to hover over the litter, time spent in kyphosis, total time with pups (hovering over plus kyphosis), and litter weight gain. In addition, the percentage of time spent with pups and the percentage of time spent nursing were also analyzed. The percentage of time dams remained in contact with pups only included time after mothers first hovered over the litter. Similarly, the percentage of time dams spent nursing only included time after mothers grouped all pups to the nest (or had pups grouped by the experimenter).

Results

Experiment 1a – Maternal behaviors after intra-BSTv infusion of yohimbine

19 out of 23 dams infused with yohimbine were included in these analyses. Four females had misplaced cannulae and were excluded from the results. These missed infusions were located in the dorsal BST and in the caudal BST (posterior to -0.51 from Bregma). Representations of infusions placed in the BSTv are represented in Figure 3.

Infusions of yohimbine into the BSTv of postpartum rats severely disrupted maternal behavior, particularly retrieving. Dams receiving yohimbine had significantly longer latencies to begin retrieving ($F(2,16) = 5.34$, $p = 0.017$; Table 1) and the number of dams retrieving all pups in 10 min. differed significantly by dose ($\chi^2(2,19) = 13.14$, $p = 0.0014$). All 6 vehicle-infused rats retrieved the entire litter within 10 minutes but only 2/7 and 0/6 dams receiving 1 and 3 μg, respectively, retrieved all pups to the nest. Given these results, it is not surprising that rats infused with yohimbine retrieved significantly fewer pups compared to rats infused with vehicle ($F(2,16) = 9.09$, $p = 0.0023$; Figure 4). Importantly, females infused with yohimbine made initial contact with pups at reunion as quickly as controls ($F(2,16) = 2.44$, $p = 0.12$) and showed the same duration of total activity as controls ($F(2,16) = 3.01$, $p = 0.073$; Figure 4). This suggests that deficits were specific to mother-infant interactions and not the result of an inability to locate pups or a general impairment in locomotion.

Not all aspects of maternal behavior were inhibited by yohimbine. After any unretrieved pups were manually returned to the nest by the experimenter, the percentage of time rats spent nursing did not differ ($F(2,16) = 0.57$, $p = 0.58$) and the raw amount of time dams spent nursing was similar (Table 1) . Although nursing did not differ, the quality of milk-letdown may have been compromised because the litters of yohimbine-infused dams did not gain as much weight as control dams (Table 1). The percentage of time spent with pups once dams initiated hovering also did not differ among groups ($F(2,16) = 0.17$, $p = 0.85$). In addition, both groups had similar durations of licking (Table 1)

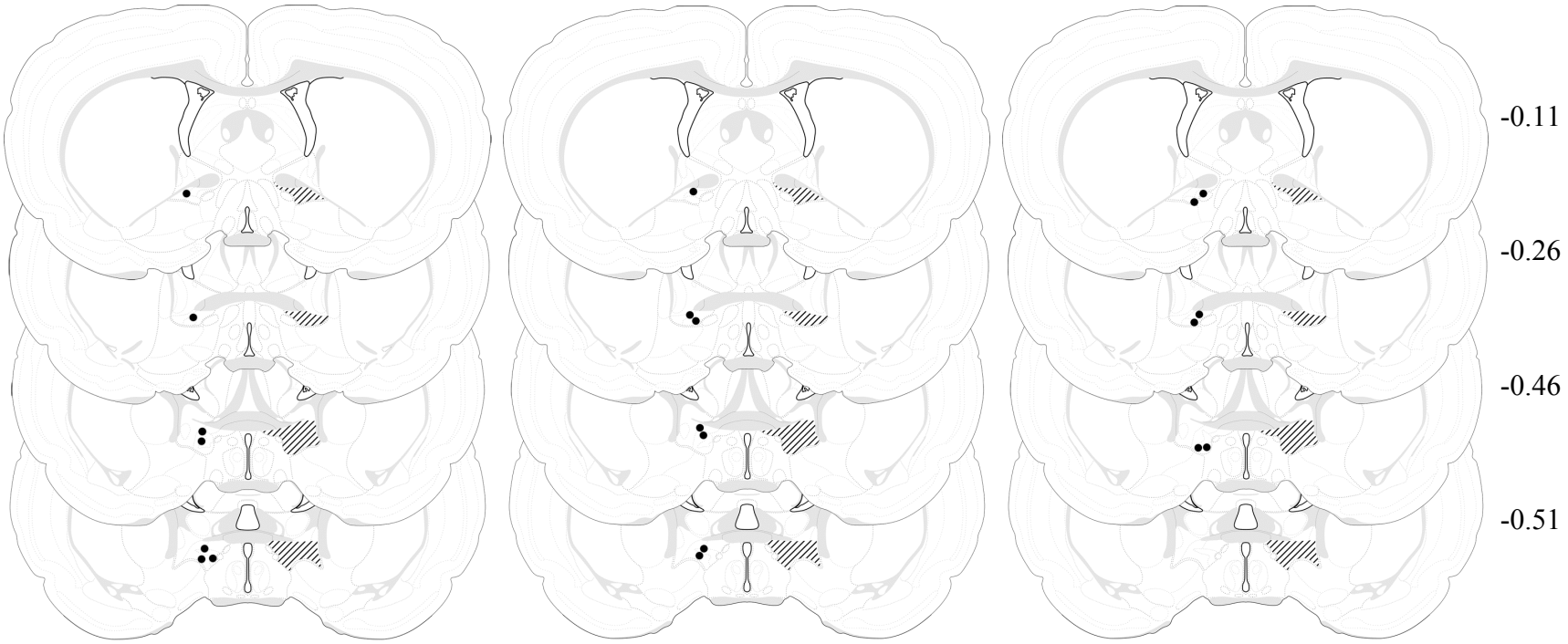

Figure 3 - Intra-BSTv infusion placements for dams receiving 0 (left), 1 (middle), and 3 (right) µg of yohimbine. Each unilateral dot indicates the bilateral infusion placement of one subject. The area of the BSTv is demarcated by diagonal lines. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

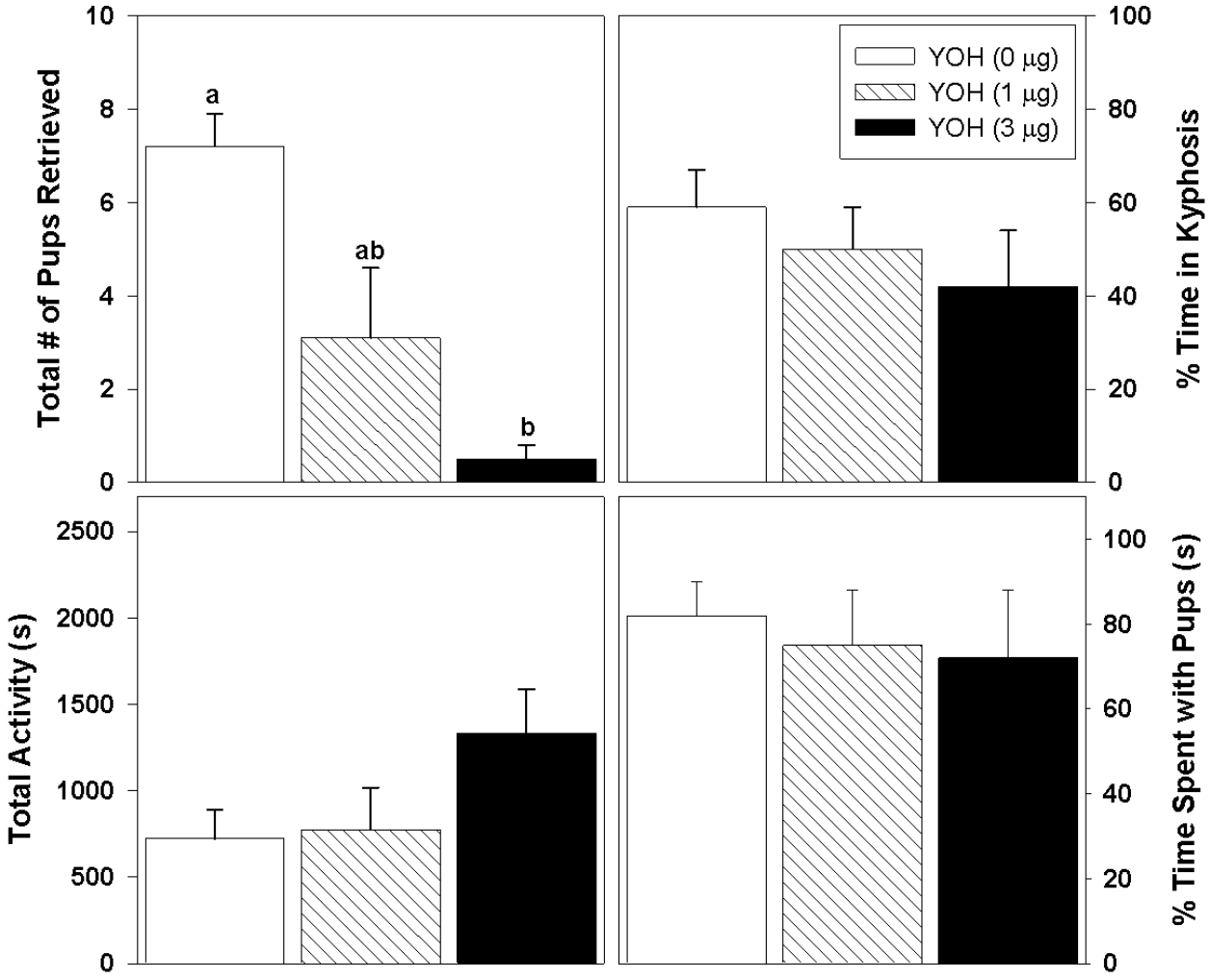

Figure 4. Effects of intra-BSTv infusion of yohimbine on total number of pups retrieved (upper left), percentage of time dams spent in kyphosis (upper right), dams' total activity (lower left), and percentage of time dams were in contact with pups (lower right). Statistical significance determined at $p \leq .05$. Groups with different letters (a,b,c) are statistically different from one another ($p \leq .016$).

	Yohimbine				
	0 µg	1 µg	3 µg	*F* (2,16)	*p*
Sample Size	6	7	6		
Latency (s)					
First contact with pups	1 ± 0	5 ± 2	9 ± 4	2.44	0.12
Retrieve first pup	8 ± 2^{a}	344 ± 121^{ab}	442 ± 105^{b}	5.34	0.017
Retrieve all pups	170 ± 88^{a}	469 ± 86^{b}	600 ± 0^{b}	8.67	0.0028
Hover from reunion	23 ± 8^{a}	35 ± 9^{a}	837 ± 356^{b}	5.62	0.014
Nurse from reunion	536 ± 138	706 ± 247	1187 ± 308	1.84	0.19
Duration (s)					
Hovering	649 ± 155	563 ± 156	189 ± 64	3.11	0.072
Licking	260 ± 145	123 ± 68	116 ± 87	0.61	0.56
Nursing	1,486 ± 190	1,207 ± 242	887 ± 265	1.53	0.25
Nest building	7 ± 5	4 ± 2	20 ± 10	1.83	0.19
Self-grooming	191 ± 39^{a}	364 ± 58^{ab}	453 ± 58^{b}	5.94	0.012
Exploring	227 ± 128	272 ± 62	714 ± 156	5.16	0.019
Total time with pups	2,140 ± 194	1,773 ± 317	1,121 ± 304	3.16	0.070
Litter weight gain (g)	3 ± 0^{a}	2 ± 0^{b}	1 ± 0^{bc}	8.55	0.0030

Table 1 - Maternal and non-maternal behaviors of postpartum rats after intra-BSTv infusion of yohimbine. Groups with different letters (a,b,c) are statistically different from one another ($p \leq .016$).

Experiment 1b - Maternal behaviors after intra-BSTv infusion of idazoxan

25 out of 34 dams infused with yohimbine were included in these analyses. Nine females had misplaced cannulae and were excluded from the results. These missed infusions were located in the nucleus accumbens, lateral preoptic area, dorsal BST, and in the caudal BST (posterior to -0.51 from Bregma). Representations of infusions placed in the BSTv are represented in Figure 5.

Intra-BSTv infusions of idazoxan also disrupted maternal behavior, although not quite to the same extent as yohimbine. Once the litter was returned to the home cage, dams in all groups had similar latencies to make contact with pups and begin retrieving (Table 2). Once retrieving began, however, not all females receiving idazoxan successfully retrieved the entire litter. The number of dams retrieving all pups in 10 min. differed significantly by dose ($\chi^2(2,25) = 6.91$, $p = 0.032$). All 9 dams receiving vehicle retrieved within 10 minutes, whereas 4/7 and 4/9 females retrieved after receiving low and high doses of idazoxan, respectively. The total number of pups retrieved was significantly different among groups ($F(2,22) = 3.99$, $p = 0.033$; Figure 6), with dams infused with 10 µg retrieving fewer pups than vehicle-infused rats. Mothers receiving the 5 µg of idazoxan did not differ from the other doses.

Idazoxan also decreased the percentage of time dams spent with pups ($F(2,22) = 3.59$, $p = 0.045$) but post-hoc analysis did not reveal specific differences among any two groups. In contrast to yohimbine, dams infused with idazoxan did not display normal nursing behaviors once pups were grouped to the nest. Compared to controls, the high dose of idazoxan significantly reduced the percentage of time mothers spent in kyphosis ($F(2,22) = 4.12$, $p = 0.030$) and, in concordance with yohimbine, idazoxan also decreased the weight gained by the litter (Table 2). The decrease in time in contact with pups is reflected by an increase in general

activity. Dams receiving the high dose of idazoxan had higher levels of total activity ($F(2,22) = 5.40$, $p = 0.012$) and spent more time grooming ($F(2,22) = 5.79$, $p = 0.0096$) than controls. Neither the duration of licking pups nor hovering over them were affected by idazoxan.

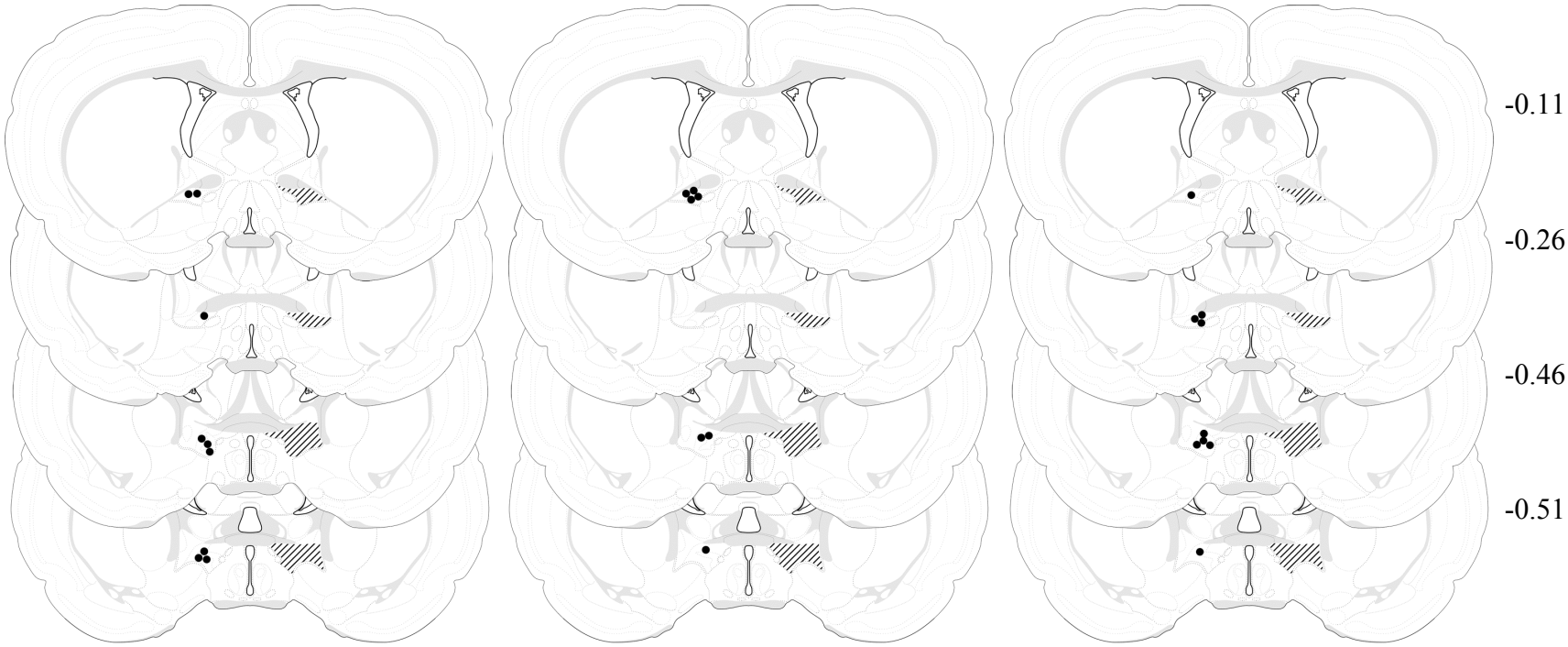

Figure 5 - Intra-BSTv infusion placements for dams receiving 0 (left), 5 (middle), and 10 (right) µg of idazoxan. Each unilateral dot indicates the bilateral infusion placement of one subject. The area of the BSTv is demarcated by diagonal lines. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

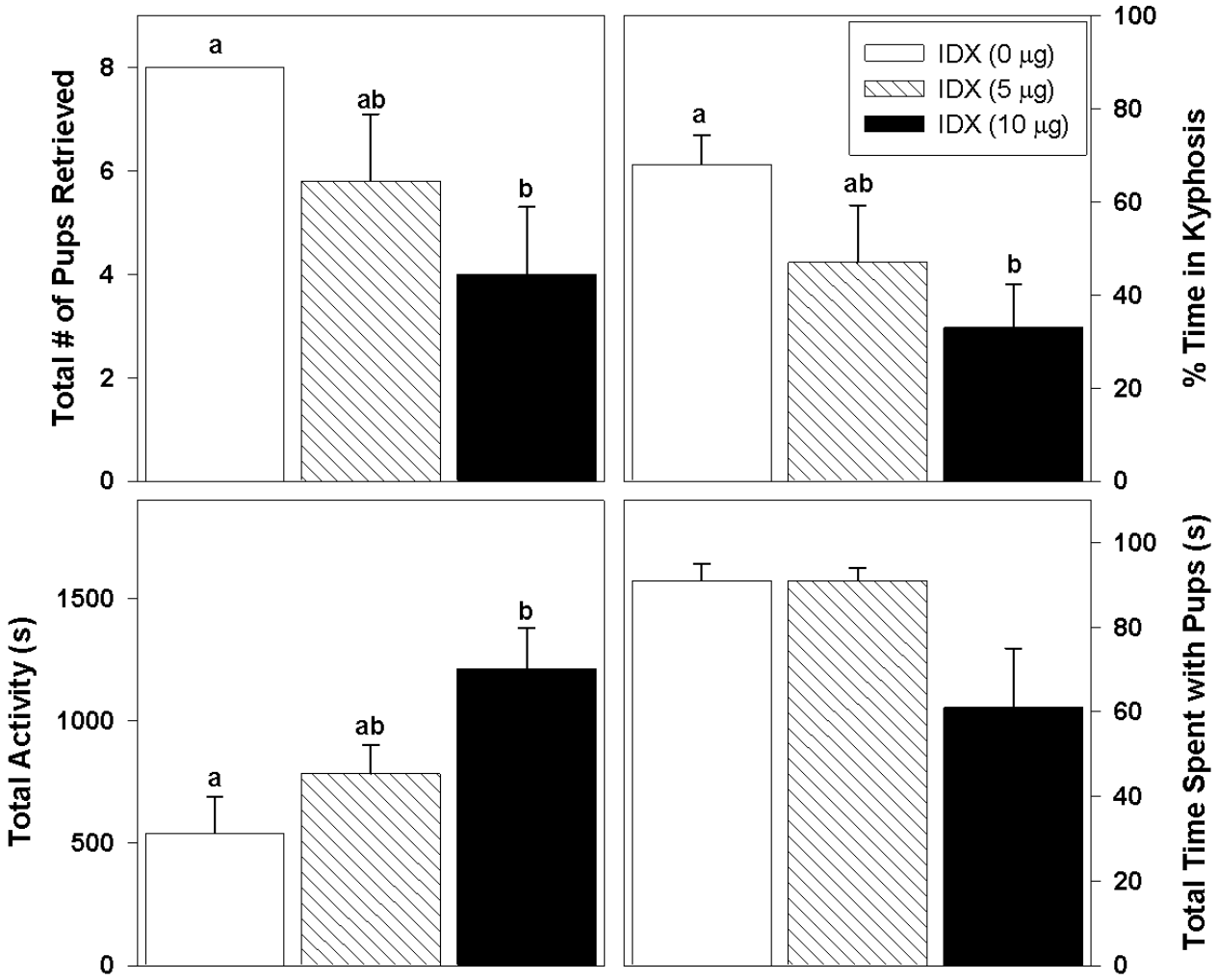

Figure 6 - Effects of intra-BSTv infusion of idazoxan on total number of pups retrieved (upper left), percentage of time dams spent in kyphosis (upper right), dams' total activity (lower left), and percentage of time dams were in contact with pups (lower right). Statistical significance determined at $p \leq .05$. Groups with different letters (a,b) are statistically different from one another ($p \leq .016$).

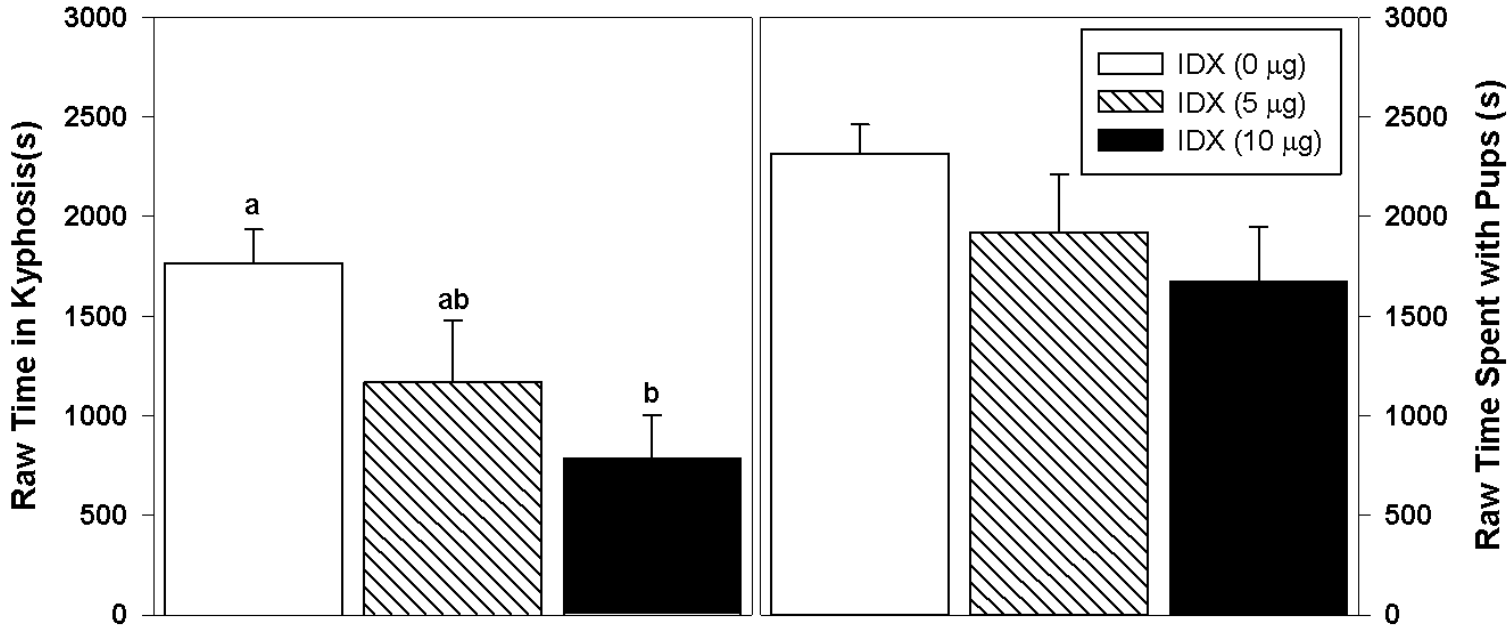

Figure 7 - Effects of intra-BSTv infusion of idazoxan on the raw amount of time in kyphosis (left) and raw amount of time spent with pups (right). Statistical significance determined at $p \leq .05$. Groups with different letters (a,b) are statistically different from one another ($p \leq .016$).

	Idazoxan				
	0 µg	5 µg	10 µg	F (2,22)	p
Sample Size	9	7	9		
Latency (s)					
First contact with pups	5 ± 2	5 ± 3	3 ± 1	0.26	0.77
Retrieve first pup	25 ± 17	119 ± 82	170 ± 83	1.34	0.28
Retrieve all pups	98 ± 39[a]	249 ± 95[ab]	406 ± 81[b]	4.86	0.018
Hover	73 ± 61	344 ± 267	521 ± 299	0.83	0.45
Nurse from reunion	419 ± 112[a]	863 ± 241[ab]	1,156 ± 245[b]	3.55	0.046
Duration (s)					
Hovering	546 ± 114	508 ± 195	554 ± 149	0.025	0.98
Licking	192 ± 70	177 ± 72	130 ± 49	0.27	0.76
Nursing	1767 ± 167[a]	1166 ±	787 ± 214[b]	5.15	0.015
Nest building	81 ± 49	28 ± 14	89 ± 74	0.33	0.73
Self-grooming	111 ± 37[a]	196 ± 42[ab]	432 ± 103[b]	5.79	0.0096
Exploring	137 ± 48	326 ± 94	467 ± 151	2.50	0.11
Total time with pups	2,317 ± 144	1,921 ± 293	1,671 ± 276	1.98	0.16
Litter weight gain (g)	2 ± 0[a]	1 ± 1[ab]	1 ± 0[b]	4.08	0.031

Table 2 - Maternal and non-maternal behaviors of postpartum rats after intra-BSTv infusion of idazoxan. Groups with different letters (a,b) are statistically different from one another ($p \leq .016$).

Experiment 1c – Maternal behaviors after intra-mPOA infusions of idazoxan

15 out of 21 dams infused with idazoxan into the mPOA were included in these analyses. Five females had misplaced cannulae and were excluded from the results. One dam's data was lost in a computer malfunction during behavioral observations. Missed infusions were located in the optic chiasm, nucleus accumbens, and lateral preoptic area. Representations of infusions placed in the BSTv are represented in Figure 8.

Infusions of idazoxan into the mPOA disrupted retrieving. Dams receiving the drug were slower to make contact and begin retrieving pups compared to controls (see Table 3). Four out of the 7 rats in the drug group initiated retrieving but 2 of those dams carried a total 1 and 4 pup(s) back to the nest, respectively. The number dams retrieving all pups in 10 min. differed significantly by dose ($\chi^2(2,19) = 5.66$, $p = 0.017$). All 8 controls retrieved all pups to their nest while only 2/7 drug-infused rats retrieved all. The total number of pups retrieved was also reduced in dams receiving idazoxan ($t(1,13) = 14.80$, $p = 0.002$; Figure 9). Drug-infused dams spent a lower percentage of time in contact with pups ($t(1,13) = 5.43$, $p = 0.037$; Figure 10) and spent less time nursing; the latency to begin nursing was longer (see Table 3) and the percentage of time in kyphosis was reduced ($t(1,13) = 18.90$, $p = 0.0008$; Figure 9). Litters of dams treated with idazoxan also gained less weight than control litters (Table 3)

In addition, non-pup directed behaviors were affected by idazoxan. Drug-infused females displayed higher levels of total activity ($t(1,13) = 16.96$, $p = 0.0012$: Figure 9), including self-grooming and exploration of the cage (Table 3).

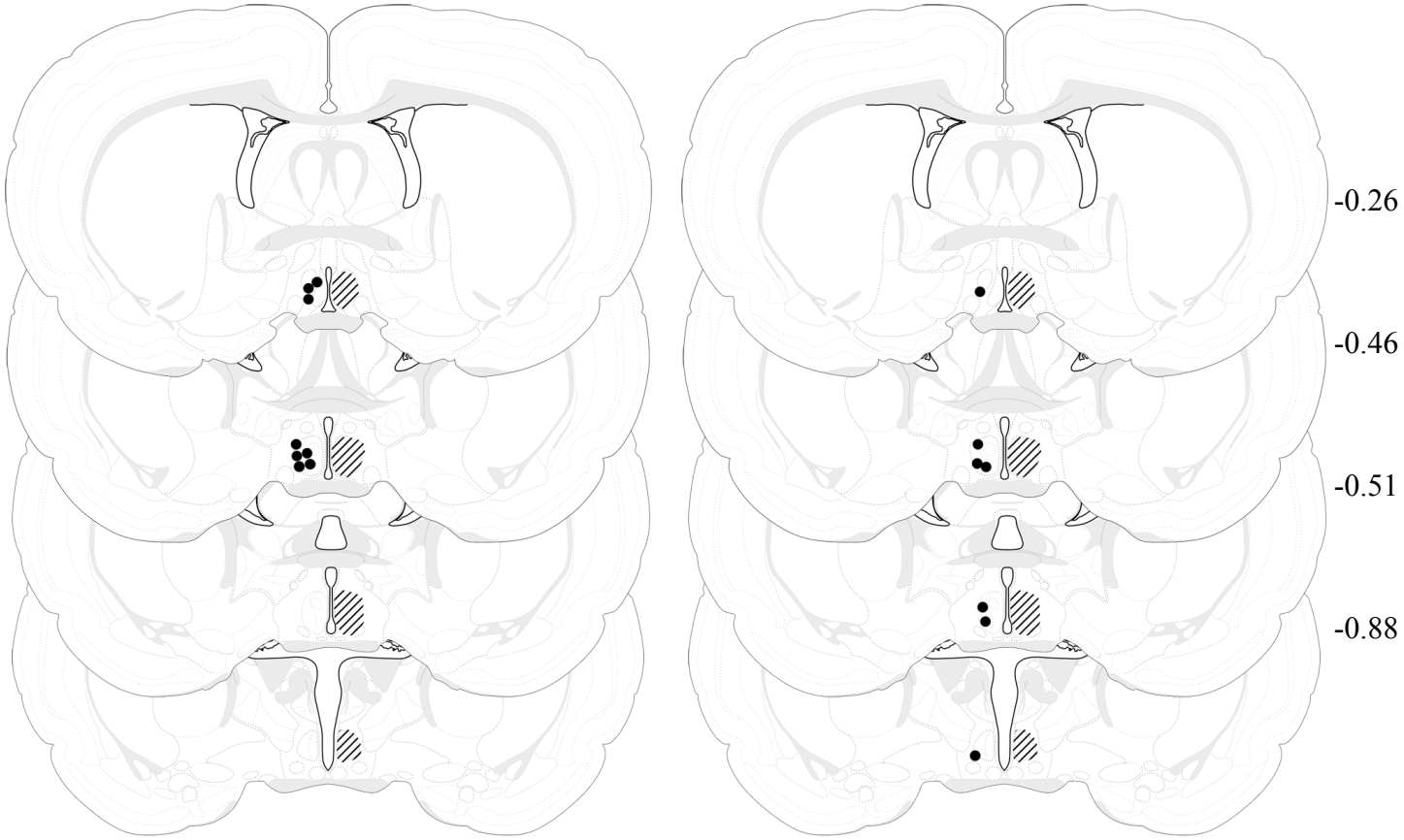

Figure 8 - Intra-mPOA infusion placements for dams receiving 0 (left) and 10 (right) µg of idazoxan. Each unilateral dot indicates the bilateral infusion placement of one subject. The area of the mPOA is demarcated by diagonal lines. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

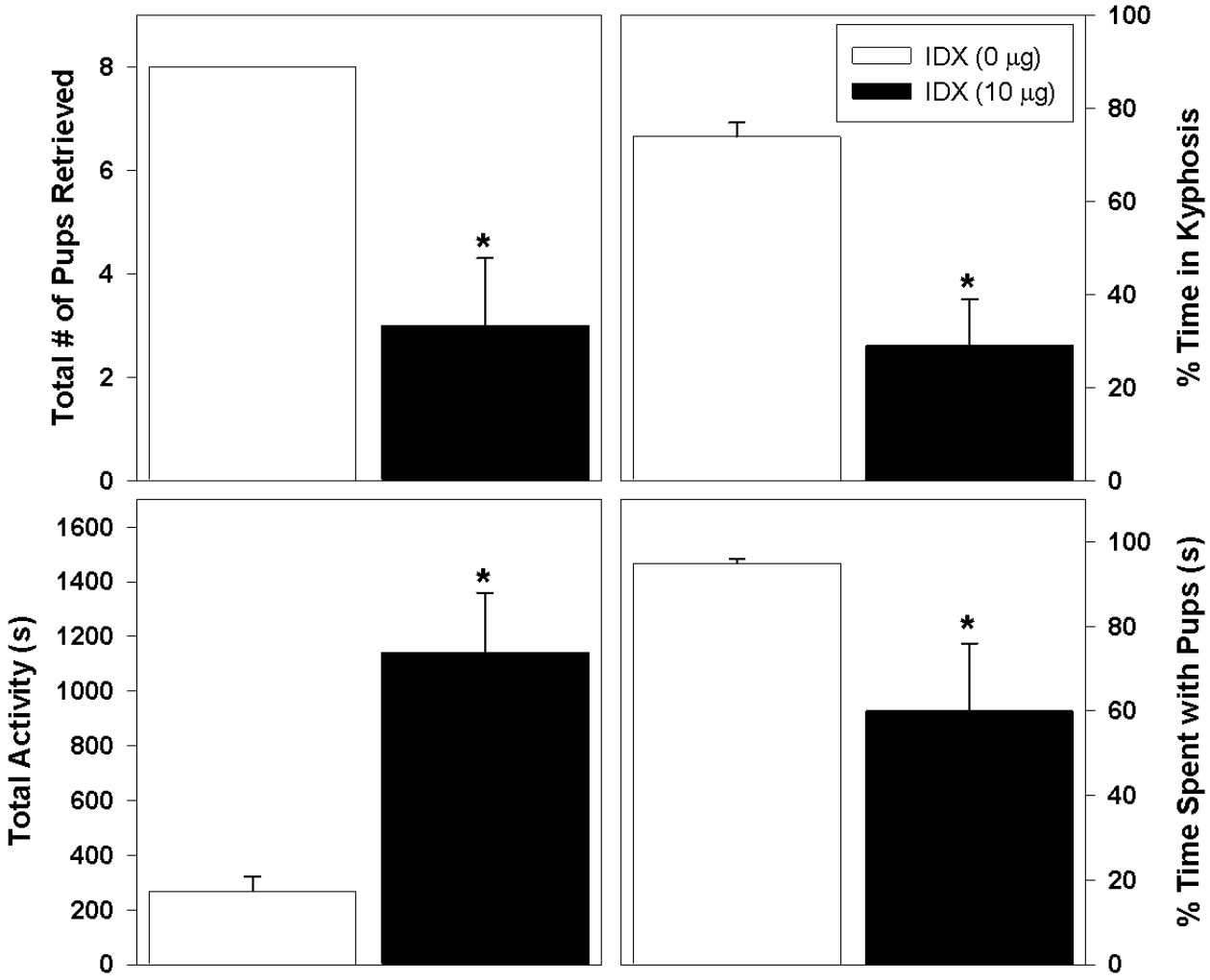

Figure 9 - Effects of intra-mPOA infusion of idazoxan on total number of pups retrieved (upper left), percentage of time dams spent in kyphosis (upper right), dams' total activity (lower left), and percentage of time dams were in contact with pups (lower right). Statistical significance determined at $p \leq .05$.

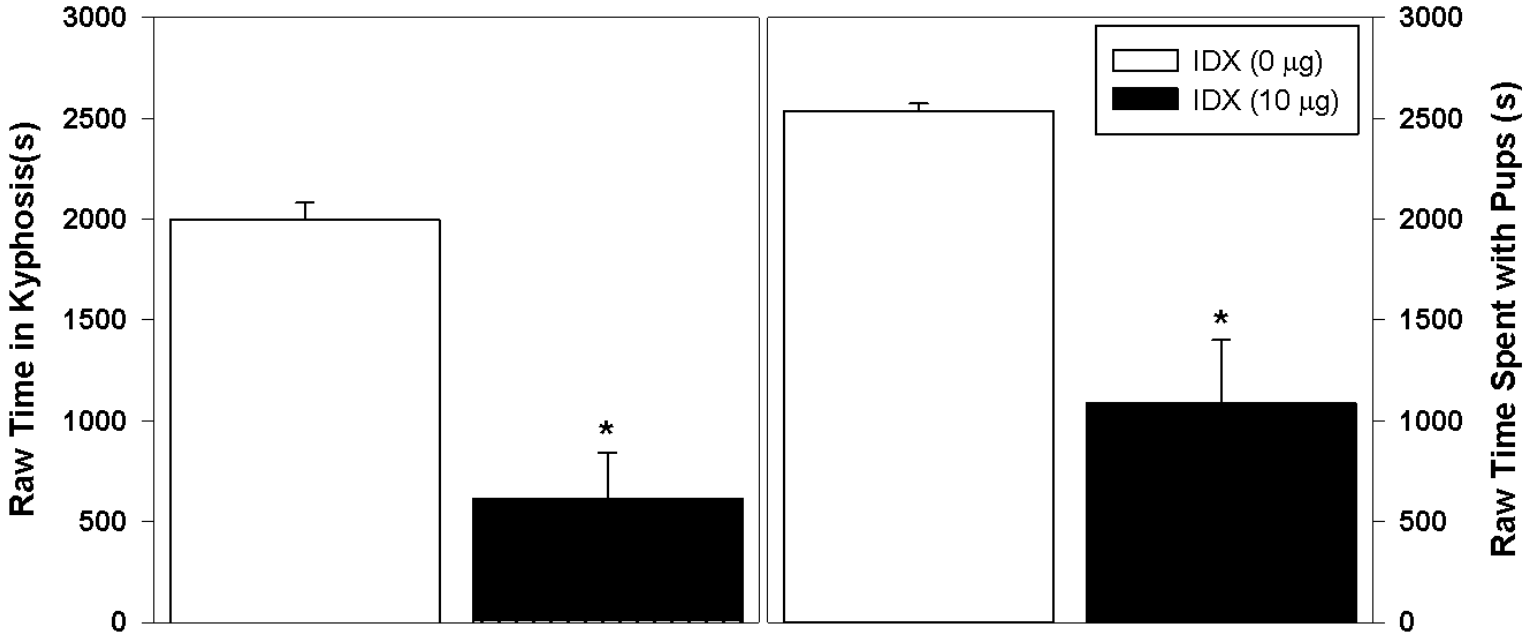

Figure 10 - Effects of intra-mPOA infusion of idazoxan on the raw amount of time in kyphosis (left) and raw amount of time spent with pups (right). * indicates statistical significance at $p \leq .05$.

	Idazoxan			
	0 µg	10 µg	*t* (1,13)	*p*
Sample Size	8	7		
Latency (s)				
First contact with pups	1 ± 0	11 ± 4	5.52	0.035
Retrieve first pup	3 ± 1	313 ± 104	10.22	0.007
Retrieve all pups	33 ± 3	543 ± 51	117.56	<0.0001
Hover	77 ± 42	858 ± 386	4.65	0.050
Nurse from grouping to nest	529 ± 57	1,458 ± 239	16.24	0.0014
Duration (s)				
Hovering	349 ± 42	155 ± 53	8.34	0.013
Licking	68 ± 26	48 ± 29	0.26	0.62
Nursing	1,996 ± 86	615 ± 228	35.78	<0.0001
Nest building	1 ± 1	5 ± 3	1.86	0.20
Self-grooming	85 ± 14	442 ± 73	26.54	0.0002
Exploring	88 ± 21	621 ± 136	17.33	0.0011
Total time with pups	2,537 ± 37	1,086 ± 314	24.27	0.0003
Litter weight gain (g)	3 ± 0	1 ± 0	22.11	0.0004

Table 3 - Maternal and non-maternal behaviors of postpartum rats after intra-mPOA infusion of idazoxan.

Discussion

These experiments demonstrate that increasing the release of NE in the BSTv or mPOA with alpha-2 autoreceptor antagonists disrupts numerous components of maternal behavior. In particular these data show that: 1) infusion of yohimbine into the BSTv inhibited dams' ability to initiate retrieving and reduced the total number of pups retrieved but had no effect on licking, nursing, or the total amount of time spent in contact with pups; 2) infusion of idazoxan into the BSTv reduced the total number of pups retrieved and the percentage of time spent nursing but did not affect licking, or time spent in contact with pups; 3) infusions of idazoxan into the mPOA disrupted dams' initiation and completion of retrieving, reduced nursing, and reduced the amount of time spent in contact with pups but did not affect licking. In addition, the litters of dams infused with yohimbine into the BSTv and mPOA or idazoxan infused into the mPOA did not gain as much weight as the litters of dams infused with vehicle. Although examining the BSTv was the primary objective of this chapter, the mPOA was included as a control site for infusions into the BSTv. Clearly, noradrenergic antagonists disrupted maternal behavior when infused into either the BSTv or mPOA. For simplicity, behavioral results pertaining to the BSTv and mPOA will be discussed together, however, I will propose that drug infusions into the BSTv did not produce their effects by diffusing into the mPOA (and vice versa). Instead, I will suggest that increasing the release of NE in either the BSTv or mPOA inhibits pup-directed behaviors.

Effects of increasing the release of NE in the BSTv and mPOA on maternal behavior

This is the first study to measure the effects of increasing NE in the BSTv and mPOA on ongoing postpartum maternal behavior. These results generally correlate with previous experiments that inhibit the BSTv and mPOA. Lesions of either the BSTv (Numan and Numan,

1996) or mPOA (Jacobson et al., 1980; Numan, 1974) disrupt retrieving as do knife cuts that sever the lateral connections of these neural sites (Franz et al., 1986; Miceli et al., 1983; Numan et al., 1985; Terkel et al., 1979). Temporary inactivation of the mPOA with the local anesthetic bupivacaine also inhibits maternal behavior during the early postpartum period (Pereira & Morrell, 2009). My data differ slightly in that disruptions in licking were not found. Interestingly, the more selective autoreceptor antagonist, idazoxan, effectively reduced the percentage time dams spent nursing pups. As discussed in the General Introduction, disagreement exists if lesions or knife cuts to the BSTv or mPOA inhibit nursing. Some reports have found that BSTv (Numan and Numan, 1996) and mPOA (Numan, 1974) lesions reduce nursing but others show little or no disruption (Jacobson et al., 1980). This disagreement may exist because previous studies did not examine nursing behaviors after pups were grouped to the nest. Indeed, ventral stimulation from at least four pups are generally required to induce kyphosis in mother rats (Stern et al., 1992). Dams receiving damage to the BSTv or mPOA in these studies, then, may not have nursed because of the lack of stimulation from pups. The current experiments address this issue by reporting the percentage of time nursing after pups are grouped to the nest, regardless of retreival performance, thus, suggesting a direct effect of idazoxan on nursing.

In addition to pup-oriented behaviors, non-maternal behaviors were also measured to determine if deficits in maternal care are due to general impairments in locomotor activity. There is precedence for this concern because the BSTv and mPOA have known projections to brain regions involved in general motor activity, such as the ventral pallidum (Grove, 1988; Numan & Numan, 1996). It is unlikley, however, that impaired locomotion from infusions of yohimbine or idazoxan prevented retreiving in my studies because there was no difference in

total activity (including self-grooming and exploration of the cage) in rats receiving intra-BSTv infusions of yohimbine compared to vehicle. Although differences in total activity were observed in mothers infused with idazoxan into the BSTv or mPOA, these females showed *increased* levels of activity, including exploration and self-grooming. Dams displayed 45% and 225% more activity than controls at the low and high dose, respectively. These dams' greater total activity is reflected by a decrease in the total amount of time spent with pups (mPOA group only) and percentage of time spent nursing. Thus, instead of interacting with pups, idazoxan-infused dams spent more time engaging in non pup-directed behaviors. In addtion, it is important to note that total activity during the first ten minutes of testing did not differ between dams infused with vehicle or idazoxan (data not shown). This suggests that levels of activity between groups were similar before control dams initiated quiescent nursing.

Neural consequences of increasing NE release in the BSTv and mPOA

Increasing noradrenergic tone in the BSTv and mPOA impairs maternal behavior but exactly how remains unknown. The two regions share similar efferents as revealed by anterograde tracing; these efferents are widespread but there is particularly dense labeling in the lateral septum, paraventricular hypothalamic nucleus, ventromedial hypothalamic nucleus, premamillary nucleus, periaqueductal gray (PAG), and ventral tegmental area (VTA) (Dong & Swanson, 2006; Numan & Numan, 1996; Simerly & Swanson, 1988). Additionally, retrograde tracers applied to some of these projection sites are co-labeled with Fos in the BSTv/mPOA of maternally-behaving dams (Numan & Numan, 1997). In other words, neurons that express Fos when dams interact with pups also project to these neural sites. Recall that NE inhibits neuronal excitability in the BSTv (Casada & Dafny, 1993) and that the release of NE into the BSTv

inhibits excitatory projections to one of these brain areas, the VTA (Dumont and Williams, 2004).

In addition, neurons in the mPOA may be inhibited by application of NE. Infusions of NE into the mPOA reduces the display of female sexual behavior (Caldwell & Clemens, 1986), but whether NE produces this effect by depressing neurons in the mPOA is unknown because stimulating A1 noradrenergic neurons can both excite or inhibit the mPOA (Kim et al., 1987, 1988). Future research is needed to clarify the role of NE on neuronal activity in the mPOA. Nevertheless, considering the similar connections of the BSTv/mPOA and the possibility that NE produces similar effects in these regions, increasing the release of NE in the BSTv/mPOA may affect the same downstream brain sites and inhibit maternal behavior. As discussed in the General Introduction, one of these brain sites may be the VTA. A portion of dopaminergic projections from the VTA terminate in the nucleus accumbens, which is suggested to be important for the expression of reward-seeking behavior (for review see Berridge, 2007). The nucleus accumbens, in turn, alters motor behaviors through its connections with the ventral pallidum (Hooks & Kalivas, 1995; Mogenson & Yang, 1991). Evidence suggests that pups are reinforcing stimuli to mother rats and that this relationship is at least partially regulated by the mesolimbic dopamine system. In an operant chamber, maternally-behaving rats press a bar for the presentation of a pup (Lee et al., 2000). Non-maternal rats, in contrast, quickly extinguish bar-pressing behavior when the reinforcing stimulus is changed from food to a pup. Furthermore, experiments using a conditioned place paradigm demonstrate that rats in the early postpartum period (day 8) spend more time in a chamber associated with pups than a chamber associated with cocaine (Mattson et al., 2001). Inactivation of the mPOA prevents maternally-behaving rats from bar-pressing for pups (Lee et al., 2000) and prevents place preference for

chambers with pup-associated cues (Pereira & Morrell, 2010). Although these studies did not investigate inhibition of the BSTv, inactivation of the mPOA prevents dams' pup-associated behaviors and suggests that the mPOA (and possibly the adjacent BSTv) projects to brain areas associated with processing reward salience.

Interestingly, Lee et al. (2000) found no effect of lesioning the nucleus accumbens on maternal behaviors or dams' bar-pressing for pups. This may appear counterintuitive because one may presume that the release of dopamine excites outputs of the nucleus accumbens to promote motivated motor behaviors. The opposite may be true, however, as dopamine inhibits GABA release from the nucleus accumbens to the ventral pallidum (Mogenson & Yang, 1991). In other words, dopamine from the VTA releases the ventral pallidum from inhibition that may allow rats to engage in motivated behaviors. Supporting data demonstrate that blocking dopamine binding in the nucleus accumbens disrupts maternal behavior (Keer & Stern, 1999; Numan et al., 2005; Silva et al., 2003), which possibly allows the accumbens to inhibit the ventral pallidum. The function of the BSTv and mPOA, then, would be to excite the VTA, which inhibits GABAergic outputs of the nucleus accumbens and stimulates rats to approach pups (an idea originally proposed by Numan [2007]).

In addition to potentially depressing approach behaviors toward pups, the release of NE into the BSTv or mPOA may promote withdrawal behaviors from pups. Recall from the General Introduction that the BSTv and mPOA have projections to brain regions that promote anxiety, such as the anterior/ventromedial hypothalamic nuclei, amygdala, mammillary region, and PAG (Numan & Numan, 1996), and that neurons expressing Fos during maternal behavior are colabeled with the rate-limiting enzyme for GABA (Lonstein & De Vries, 2000). It is possible that the BSTv and mPOA of mother rats normally release GABA onto these neural sites to

inhibit possible anxiogenic consequences. Thus, inhibiting the BSTv and mPOA with NE may allow uninhibited activity of these sites to increase anxiety. Dams infused with idazoxan and yohimbine may then regard pup stimuli as anxiogenic and not engage in retrieving (tested directly in Chapter 2). Although a reasonable possibility, an increase in pup-specific withdrawal is probably not the only factor contributing to the deficits in maternal behavior. First, dams infused with yohimbine into the BSTv did not retrieve pups but spent as much time nursing and in contact with pups compared to normal controls. Second, as will be discussed in the next section, many dams infused with idazoxan into the BSTv (but not mPOA) initiated retrieving but did not complete the task. This latter point suggests that dams were not averse to initially approaching pups but had deficits in maintaining interest in pups sufficient to retrieve all to the nest. In support, there were no differences in the latency to begin retreiving pups between rats receiving idazoxan or vehicle into the BSTv. These points do not exclude that an increase in anxiety disrupted retrieving and/or nursing but do not suggest that any anxiogenic consequences were the sole source of poor maternal care in drug-infused dams. It should be noted that dams receiving intra-mPOA infusions of idazoxan were slower to begin retrieving, which may have been the result of heightened anxiety in dams.

Differential effects of yohimbine and idazoxan on maternal behavior

Although infusions of yohimbine and idazoxan into the BSTv inhibited maternal behavior, they did not produce completely identical effects. Yohimbine prevented 71% and 100% of dams from retrieving all pups to the nest at the low and high dose, respectively. Idazoxan instead prevented only 43% and 56% dams from retrieving all pups at the low and high dose, respectively. In other words, at the highest dose, yohimbine abolished retrieving to the

nest in all dams while idazoxan was much less effective. Another difference between the drugs was that yohimbine completely prevented *any* retrieving in 67% dams while idazoxan completely prevented any retrieving in only 22% dams. Also, data regarding nursing behaviors supports the possibility that yohimbine and idazoxan have unique effects on maternal care. Once pups were returned to the nest, idazoxan, but not yohimbine, disrupted the percentage of time dams spent in kyphosis. Exactly why retrieving differed and particularly why idazoxan had the unique effect of decreasing the display of kyphosis are unknown. One possible mechanism for the observed differences between yohimbine and idazoxan are the drugs' selectivity for neurotransmitter receptors. In the current experiments, the more selective drug for the alpha-2 autoreceptor (idazoxan) disrupted retrieving to a lesser extent than the less selective drug (yohimbine). In addition, only the more selective drug disrupted the display of kyphosis.

Yohimbine's additional effects on serotonergic and dopaminergic systems may account for these discrepancies. As previously stated, yohimbine acts as a weak agonist on 5-HT1A receptors and as an antagonist on D2 receptors (Millan et al., 2000; Scatton et al., 1980). Although idazoxan has some affinity for the 5-HT1A receptor, it is approximately one-third of yohimbine's affinity (Winter & Rabin, 1992) and I am unaware of any study examining the affinity of idazoxan to the D2 receptor.

The 5-HT1A receptor is located both post- and presynaptically (Hayes & Greenshaw, 2011) but the majority of these receptors are presynaptic and function as autoreceptors; infusions of selective 5-HT1A agonists inhibit the release of serotonin (Hjorth & Sharp, 1991). Interestingly, although yohimbine is a 5-HT1A agonist, it increases serotoinin release when infused into the hippocampus (Broderick, 1997), frontal cortex (Cheng et al., 1993), and lateral ventricles (Brannan et al., 1991). These findings seem counterintuitive because the parsimonious

conclusion is that 5-HT1A agonists reduce serotonin release by stimulating the autoreceptor. Alpha-2 noradrenergic receptors are also found on axon terminals containing serotonin (Maura et al., 1982; Saito et al., 1996; Trendelenburg et al., 1994) and application of alpha-2 agonists reduce serotonin release (Tao & Hjorth, 1992). Further, the release of serotonin increases after infusions of alpha-2 antagonists (Tao & Hjorth, 1992), which includes yohimbine (Dawson et al., 1987). It is not surprising, then, that idazoxan also stimulates serotonin release (Tao & Hjorth, 1992) even though it has low affinity for the 5-HT1A receptor compared to yohimbine (Winter & Rabin, 1992). Unfortunately, the effects of yohimbine and idazoxan on serotonin concentrations in the BSTv have not been examined. The effects of increasing the release of serotonin by alpha-2 autoreceptor antagonists likely cause inhibition of the BSTv because 5-HT decreases the excitability of BSTv neurons, possibly through inhibiting glutamatergic activity (Guo & Rainnie, 2010; Rainnie, 1999). There may, thus, be a quantitative difference between yohimbine and idazoxan. If yohimbine stimulates alpha-2 and 5-HT1A autoreceptors to a greater extent than idazoxan, yohimbine could cause a greater depression of neural activity in the BSTv.

In addition to effects on the 5-HT1A receptor, yohimbine's but not idazoxan's affinity for the D2 receptor may explain yohimbine greater effects on some maternal behaviors. The BSTv does contain D2 receptors (Bouthenet et al., 1987, 1991; Wamsley et al., 1989). Peripheral injection of yohimbine increases dopamine release in the frontal cortex (Millan et al., 2000) and increases the dopamine turnover in the stratium (Scatton, 1980). These findings may not be surprising given that D2 receptors are located presynaptically and can act as autoreceptors (Mercuri et al., 1997; Usiello et al., 2000). Hence, yohimbine likely increases the release of dopamine by disinhibiting the inhibitory action of the autoreceptor. Importantly, Scatton (1980)

found that other alpha-2 antagonists (with little or no selectivity for the D2 receptor) did not have the same effect on dopamine turnover. This could suggest that yohimbine's effects may partly result from its binding to the D2 receptor. This is very unlikely because evidence from the nucleus accumbens (Keer & Stern, 1999; Numan et al., 2005) and medial preoptic area (Miller & Lonstein, 2005) demonstrates that decreasing the release of dopamine *inhibits* retrieving. Thus, if yohimbine is increasing the release of dopamine in the BSTv, one could expect it to facilitate pup-directed behaviors. It is important to note that too much DA can inhibit maternal behavior, as cocaine (Zimmerberg & Gray, 1992; Johns et al., 1998; Vernotica et al., 1999) or apomorphine (Stern & Protomastro, 2000) disrupts maternal behavior.

Interestingly, Miller & Lonstein (2005) reported that infusions of raclopride, a D2 antagonist, into the mPOA region did not affect retrieving in dams but actually *facilitated* nursing. Albeit small, a number of these infusions were located in or very close to the BSTv. The authors postulate that activation of D2 receptors may be important for initiating the switch between actively retrieving pups and beginning quiescent nursing. Remember that yohimbine, but not idazoxan, is also a D2 antagonist. Although yohimbine disrupted retrieving (presumably by binding to alpha-2 autoreceptors) it may have permitted nursing by antagonizing the D2 receptor. Idazoxan's lack of D2 affinity would then disrupt both retrieving and nursing, which is what was observed. This effect of idazoxan would also explain why infusions into the mPOA also reduced nursing. Additional research is needed to determine how the D2 receptor is potentially involved in the control of nursing behaviors.

The direction of idazoxan's effects when infused into the mPOA were similar those found in the BSTv. All 8 control dams retrieved their pups to the nest while 2/7 (29%) idazoxan-infused dams retrieved. Of the 5 dams that did not retrieve, 3 did not retrieve any pups and 2

retrieved some pups (1 and 4 pups, respectively) but did not finish. Differential effects of infusions of yohimbine and idazoxan on maternal behavior cannot be determined because yohimbine was not infused into the mPOA. Nevertheless, a similar analysis of idazoxan's effects that were described for the BSTv applies to the mPOA. The mPOA is moderately immunoreactive for DβH fibers (Simerly et al., 1986) and contains alpha-2 receptors (Bruning et al., 1987; Unnerstall et al., 1985), suggesting that idazoxan produced its behavioral effects by binding to the alpha-2 receptor and increasing NE release. It is also possible that idazoxan's albeit low affinity for 5-HT1A accounted for, or contributed to, the disruption in maternal behavior. Application of selective 5-HT1A agonists into the mPOA affects female reproductive behaviors, with infusions of the agonist 8-OH-DPAT reducing lordosis in female rats (Uphouse & Caldarola-Pastuszka, 1993).

Spread of drugs to other neural sites

The possibility exists that yohimbine and/or idazoxan effected maternal behavior by diffusing into neural sites outside the BSTv. Areas adjacent to the BSTv, including the mPOA, nucleus accumbens, ventral pallidum, and lateral hypothalamus are implicated in the control of maternal behavior (for review, see Numan & Insel, 2003). Altering neural activity in these regions with yohimbine and idazoxan may have disrupted maternal behavior. Future experiments could examine the rate and distance of diffusion of 0.125 ul by visualizing tritiated yohimbine and idazoxan with autoradiography. It is also important to note that targeting the BSTv may not necessarily be as important as targeting the noradrenergic content of this region. The DβH-immunoreactive fiber plexus in the BSTv extends somewhat into adjacent brain regions, including the lateral hypothalamus, and ventral pallidum, and dorsal mPOA (as shown

in a photomicrograph in Fendt et al., 2005). More importantly, the tract of DβH fibers in the nucleus accumbens and ventral pallidum are described as being contiguous with those of the BSTv. Neural sites defined by brain atlases are certainly useful but, in this case, neurons that respond to NE to affect maternal behaviors may not be confined to one particular brain region.

However, I will argue it is unlikely that the drugs: 1) spread to these neural sites or 2) had significant effects if they did diffuse out of the BSTv. First, the infusion volume was relatively small. Dams in these experiments received 0.125 µl infusions while many studies use volumes of 0.5 µl or greater to alter neural activity in the BST (Alsene et al., 2011; Liu & Liang, 2009; Schweimer et al., 2005). Additionally, 0.5 µl of a dye infused into the BST before sacrifice is estimated to spread approximately 1 mm from the tip of the injector (Liu & Liang, 2009) and 1 µl of tritated lidocaine spreads ~1.7 mm (Martin, 1991). My volume was one-quarter of 0.5 µl (one-eighth of 1 µl) and effects on maternal behavior were observed within minutes after infusion; although one cannot assume that one-quarter of volume translates to one-quarter of diffusion, my infusions would be expected to have spread notably less than 1 mm from the injection site. The BSTv, at its largest area, has an approximate medial-lateral distance of 1 mm and dorsal-ventral distance of 1 mm. Intra-BSTv infusions terminating at or posterior to -0.46 from Bregma would be at least 1 mm from the nucleus accumbens, ventral pallidum, and lateral hypothalamus. Infusions around -0.11 from Bregma are significantly closer to the ventral pallidum and nucleus accumbens but they account for a relatively small portion (~20%) of all infusions. It is, thus, reasonable to suggest that yohimbine and idazoxan did not diffuse far beyond the BSTv if at all.

In addition to the small infusion volume, many brain sites surrounding the immediate area of the BSTv lack dense noradrenergic innervation. That is not to say surrounding areas lack

any noradrenergic content. The ventral pallidum (Chang, 1989; Berridge et al., 1997), nucleus accumbens (Berridge et al., 1997), lateral hypothalamus (Woulfe et al., 1990), and mPOA (Simerly et al., 1986) all contain fibers immunoreactive for DβH. Norepinephrine content in regions such as the nucleus accumbens and hypothalamus is relatively low compared to that found in the BSTv (Kilts & Anderson, 1986). Similarly, densities of alpha-2 receptors in the BST (Unnerstall et al., 1985) are approximately 2-3 times higher than that found in the ventral pallidum, nucleus accumbens, or lateral hypothalamus (Bruning et al., 1987). These densities for the BST may be underestimated because previous reports combined the dorsal BST with their analysis of the ventral BST, the former containing relatively little noradrenergic innvervation (Phelix et al., 1992). An analysis of my "missed" infusions emphasizes how specific infusions into the BSTv or mPOA had to be to inhibit maternal behavior. 2/3 females receiving yohimbine (low and high dose combined) near the BSTv, 3/4 females receiving idazoxan (high dose) near the BSTv, and 2/3 females receiving idazoxan near the mPOA retrieved all pups to the nest within 10 min. In addition, all except one of these infusions were placed just outside the anatomical border of the BSTv or mPOA.

It is even more reasonable to suggest that idazoxan and yohimbine infused into the BSTv spread to the mPOA to disrupt maternal behavior (and vice versa for infusions of idazoxan into the mPOA). This is also probably unlikely because, in addition to the small volumes of drugs used, maternal behavior was disrupted when infusions were centered in the most rostrodorsal BSTv and ventrocaudal mPOA. Infusions would have to spread over 1 mm to affect the alternative brain site. Instead, infusions of idazoxan into the mPOA most likely produced effects by increasing the release of NE within each region. This conclusion is based on the presence of DβH fibers and alpha-2 receptors and the similar connectivity of the BSTv and mPOA to

maternally-relevant neural sites.

Effects of reproductive state on noradrenergic activity

If increasing the release of NE in the mPOA and BSTv disrupts maternal behavior, then what is the evidence demonstrating that a natural difference in noradrenergic activity exists between postpartum and nulliparous rats? Although there are no studies examining these differences in the BSTv and mPOA (but will be address in Chapter 3), a downregulation of the noradrenergic system in dams has been reported in other neural sites. As discussed in the General Introduction, postpartum rats have fewer alpha-2 receptors in the PVN compared to virgin rats (Toufexis, et al., 1998). Reducing noradrenergic innervation of the PVN with 6-OHDA blunts the physiological stress response in virgin rats to levels observed in dams (Toufexis & Walker, 1996). It is parsimonius to consider that this change in alpha-2 receptors occurs more widespread throughout the brain, including the BSTv and mPOA.

Additional evidence suggests that interactions with pups has potential to affect activity of NE-producing neurons and their sites of projection. A transneuronal retrograde virus injected into the mammary glands of postpartum rats infects neurons in the noradrenergic A1 region of the medulla and locus coeruleus (Gerendai et al., 2001). Furthermore, infected neurons are found in the BST but only when animals are sacrified at a later time (implying that the virus may have traveled through A1 and locus coeruleus neurons before terminating in the BST). Thus, ventral stimulation and/or suckling from pups may change the synthesis, release, and/or metabolism of NE in nursing mothers. In support, suckling increases Fos-IR in the A1, locus coeruleus, and BST (Li et al., 1999). It is unlikely, however, that suckling is necessary to change noradrenergic activity because thelectomy does not disrupt maternal behavior (Moltz et al.,

1967) and rats without developed teats can be induced to display pup-directed behaviors (Rosenblatt, 1967). More general tactile stimulation from pups on the mother's ventrum may be adequate to cause these potential changes in the noradernegic system. It would be interesting to determine if lactating rats and maternally-sensitized non-lactating rats have similar noradrenergic content in their brains.

Conclusions

Increasing the release of NE in the BSTv and mPOA using the autoreceptor antagonists yohimbine and idazoxan significantly disrupts retrieving in postpartum rats. In addition, idazoxan, but not yohimbine, significantly reduced the percentage of time dams spent nursing as well as total time spent in contact with pups. High NE in these brain regions may disrupt dams' pup-approach behaviors by inhibiting excitatory projections that terminate in the VTA. NE may also change neural activity within those sites to increase anxiety and stimulate withdrawal from pups. Although yohimbine and idazoxan probably produced their effects by increasing the release of NE, additional research is needed to determine other neurochemicals affected by infusions of these drugs (e.g., serotonin). Furthermore, examining the extent of diffusion of these drugs is required to rule out the involvement of brain regions besides the BSTv and mPOA. The present experiments are the first to suggest that noradrenergic innveration of the BSTv and mPOA modulates ongoing maternal behaviors and greatly expands what is known about what neurotransmitters released in these sites are involved in maternal care.

Chapter 2: Effects of systemic and intra-BSTv infusions of a noradrenergic alpha-2 agonist or antagonists on female rats' anxiety-related behaviors

Introduction

Dams exhibit fewer anxiety-related behaviors compared to diestrous virgin rats in paradigms including the elevated plus-maze, open field, light-dark box, punished drinking test, and acoustic startle test (for review see Lonstein, 2007). Mothers' reduced anxiety can be eliminated if litters are removed for as little as two to four hours before testing (Lonstein, 2005; Neumann, 2003; Smith & Lonstein, 2008). Visualization of *c-fos* reveals that dams allowed access to pups show very high Fos-IR in the BSTv after exposure to an elevated plus maze compared to dams separated from their litter four hours before testing (Smith & Lonstein, 2008). This suggests that dams allowed physical contact with pups have higher neural activity in the BSTv in response to an anxiogenic event, and that this contributes to or is at least consistent with their low anxiety. Neurotransmitter systems that affect neural activity in the BSTv to regulate anxiety-related behaviors are likely numerous but one neurotransmitter with a notably large input to this site is norepinephrine (Brownstein & Palkovits, 1984; Kilts & Anderson, 1986). This dense innervation of noradrenergic fibers suggests that the release of NE into the BSTv may contribute to dams' reduced anxiety-related behaviors.

In support, evidence from male rats demonstrates that increased NE release in the BSTv is associated with heightened anxiety (Cecchi et al., 2002; Fendt et al., 2005). Likewise, reducing the amount of NE released with noradrenergic antagonists prevents anxiety. Low NE in the BSTv may also account for higher Fos expression in the BSTv of low-anxiety dams compared to high-anxiety dams (Smith & Lonstein, 2008); administration of NE directly to the

BSTv reduces excitability in 70% of neurons while increasing excitability in only 2% (Casada & Dafny, 1993), suggesting that low noradrenergic tone would permit neural activity. The majority of neurons in the BSTv are GABAergic (Muganini & Oertel, 1985; Stefanova et al., 1997) and increased activity of these projections throughout the brain may serve to inhibit regions that would possibly promote anxiety. Thus, naturally low noradrenergic tone in the BSTv of lactating rats, and subsequent high activity of GABAergic neurons may be partially responsible for dams' attenuated anxiety-related behaviors compared to non-lactating rats.

If so, I hypothesize that pharmacologically increasing NE release within dams' BSTv will prevent the effects of postpartum state or recent contact with pups on anxiety. Conversely, I hypothesize that decreasing NE release within the BSTv will further decrease anxiety in lactating female rats. Thus, the present experiments will examine if pharmacological manipulation of noradrenergic activity can increase or decrease anxiety-related behaviors in postpartum and diestrous virgin rats. Experiments 2a and 2b will determine if this can be achieved through systemic injections of noradrenergic autoreceptor agonists and antagonists. Clonidine is an alpha-2 agonist that stimulates presynaptic autoreceptors and reduces NE release (Lal & Fielding, 1981) and is predicted to increase the percentage of time rats spend in the open arms of an elevated plus maze (EPM). In male rats, systemic injections of clonidine have already been demonstrated to reduce anxiety-related behaviors (Johnston & File, 1989; Soderpalm & Engel, 1988).

Conversely, yohimbine is an alpha-2 antagonist that blocks these autoreceptors and increases NE release (Bremner et al., 1996) and is expected to decrease the percentage of time rats spend in the open arms. To help avoid a ceiling effect, postpartum rats receiving clonidine will be rendered more anxious by having their litter removed four hours prior to testing. To help

avoid a floor effect, postpartum rats receiving yohimbine will be allowed contact with pups until the time of testing. The inclusion of diestrous virgin rats will determine if reproductive state influences sensitivity to clonidine's and yohimbine's effects on plus-maze behavior. If virgin rats have increased noradrenergic tone compared to dams, diestrous virgins may be more sensitive than dams to lower doses of yohimbine and clonidine. The prediction that increased neurochemical tone translates to increased pharmacological sensitivity has been demonstrated in several neurotransmitter systems (Overstreet et al., 1996; Toufexis et al., 1999; Toufexis et al., 1998; although see Belzung, 2001).

I will further test the hypothesis that NE release in the BSTv affects anxiety by conducting Experiments 2c and 2d to elucidate how infusion of these noradrenergic drugs directly into the BSTv modulates anxiety. Dams and diestrous virgins implanted with bilateral cannulae aimed at the BSTv will receive microinfusions of clonidine or yohimbine before testing in the EPM. If the BSTv is affected by systemic injections of these drugs, then I hypothesize that the BSTv is the relevant site of action, and that direct intra-BSTv infusions will mirror these changes in anxiety. Experiment 2e will determine the effects of intra-BSTv infusion of idazoxan, a selective alpha-2 autoreceptor antagonist, on plus-maze behavior. This will be done because yohimbine has mild affinity for serotonin and dopamine receptors, particularly the 5-HT1A and D2 receptor (Kleven et al., 2005; Millan et al., 1998; Newman-Tancredi et al., 1998; Winter & Rabin, 1992). Idazoxan is expected to mimic the effects of yohimbine, which will provide evidence that the BSTv modulates anxiety-related behaviors through noradrenergic mechanisms. To further test the specificity of yohimbine, Experiment 2f will determine if the anxiogenic effects of intra-BSTv infusions of yohimbine can be blocked by pretreatment with intra-BSTv infusions of clonidine.

Methods

Subjects

Long-Evans female rats, descended from male and female rats purchased from Harlan Laboratories (Indianapolis, IN), were born and raised in our colony. Food and water were continuously available. Colony room temperature was set at approximately 22^{o}C with a 12:12 light/dark cycle beginning at 0800 daily. Beginning at approximately 70 days of age, females designated to the postpartum group were monitored daily with a vaginal impedance meter until a day of proestrus. Females were then placed with a sexually experienced male overnight. Pregnant females were group housed (2-3 females per cage) until one week before giving birth (Experiment 2a and 2b) or day of surgery (between days 15-18 of pregnancy; Experiment 2c-2f), after which they were housed alone. The day of parturition was assigned as Day 0 postpartum. Within 24 hr after parturition, the litter was culled to include 4 males and 4 females. A second group of rats, designated as virgins, were singly housed and received daily vaginal smears approximately one week before testing in the elevated plus maze. Virgins were tested one day after estrus; a subsequent vaginal smear post-testing confirmed females' diestrous state and the small number of females found to not be in diestrous were removed from analysis.

Experiment 2a and 2b – Effects of peripheral injection of clonidine and yohimbine

On day 7 postpartum, dams receiving clonidine were separated from their litters between 0800 hr and 1000 hr with the intention of increasing anxiety (Lonstein, 2005; Smith and Lonstein, 2008). To control for the nest perturbation induced by removing pups for the group receiving clonidine, dams receiving yohimbine had their litter mildly perturbed by the experimenter, which involved lifting the pups out of the cage and immediately replacing in the

nest. Four hours after separation from the litter or nest perturbation, females were injected intraperitoneally with clonidine (Sigma, USA; 0, 5, or 10 μg/kg dissolved in saline) or yohimbine (Sigma, USA; 0, 1, or 5 mg/kg dissolved in 2:1 propylene glycol:saline). These doses of clonidine and yohimbine were based on their ability to influence anxiety-related behaviors when systemically injected into male rats (Davis et al., 1979; Johnston & File, 1989; Soderpalm & Engel, 1988; Toufexis et al., 1999). Diestrous virgin rats were injected with clonidine or yohimbine without any prior manipulation on testing day.

Following injection, all animals remained in their home cage for 30 min before behavioral testing. Females were then carried in their home cage to a testing room ~ 6 m away. Anxiety-related behaviors were assessed with an elevated plus maze. The maze was made of black plastic and had four arms emerging from the center of the maze, measuring 50 cm in length by 10 cm in width. Two arms were completely open while the second set had 40 cm high walls outlining the arms. The entire maze was elevated 50 cm from the floor. A low-light sensitive video camera (Panasonic) recorded the image from a mirror that was suspended above the maze. The camera was connected to a videocassette recorder (Panasonic) that recorded and stored the movements of the rat. An experimenter simultaneously scored the movements of the rat during the behavioral test. The tape player, TV, and experimenter were all in an adjacent room. Scoring the animal's movements took place with a computerized data-acquisition system. The experimenter recorded the latency, frequency and duration of time spent in the open arms (OA), closed arms (CA), and center square. An entry was defined as a female placing her head and both front paws into a different arm of the maze. If a female fell off the maze, the test was briefly paused while she was replaced on that arm, and the test then resumed.

Measures recorded from the EPM include the percentage of time spent in each of the

open and closed arms, the total time spent in both arms, the percentage of entries made into each type of arm, the number of entries made into each type of arm, and the total number of entries into all arms. The results presented in the text will detail the percentage of open arm duration and entries, the number of closed arm entries, and the total number of arm entries, which are suggested to be most associated with anxiety and locomotor activity in rodents (Pellow et al., 1985). All other behaviors not described in the text will be summarized in tables.

Experiment 2c, 2d, 2e, and 2f – Intra-BSTv infusions of clonidine, yohimbine, or idazoxan.

Stereotaxic surgery occurred in pregnant rats between days 15 and 18 of gestation (for rats designated to the postpartum group) or in female virgin rats. Females were implanted with bilateral cannulae aimed at the BSTv and allowed to recover as described in Chapter 1. Between 1200 and 1500 hr on day 6 postpartum for dams, or on a day of estrus for virgin rats, rats were placed into a clean carrying cage and moved to the infusion room for habituation to the handling and infusion procedures. Females were handled for approximately 5 minutes, during which their dust cap and stylet was removed and cleaned with ethanol. Stylets and caps were replaced and the females were returned to her cage in the colony room. Infusion and behavioral testing occurred the next day. Dams receiving clonidine had their litter removed between 0800 and 1000 hr, to increase dams' anxiety. Females receiving yohimbine instead had their litter mildly perturbed by the experimenter, which involved lifting the pups out of the cage and immediately replacing them. Diestrous virgin rats had the experimenter's hand place in the cage to simulate the perturbation that lactating rats experience.

Four hours after separation from the litter or nest/cage perturbation, females were placed in a clean carrying cage and moved to the infusion room where clonidine (2c), yohimbine (2d),

idazoxan (2e), or vehicle was infused. Clonidine was infused at doses of 0, 100, or 1000 ng/125 nl per hemisphere, yohimbine at doses of 0, 1, or 3 µg/125 nl per hemisphere, and idazoxan (Sigma, USA) at doses of 0, 5, or 10 µg/125 nl per hemisphere. These doses of clonidine, yohimbine, and idazoxan were based on their ability to influence anxiety and other behaviors when infused site-specifically into the rat brain (Clark, 1991; Dodge & Badura, 2002; Fendt et al., 2005; Gulia et al., 2002; Guo et al., 1996; Schweimer et al., 2005). All drug infusions were performed as described in Chapter 1. Immediately after infusion, animals were placed in an elevated plus maze and their behavior videotaped for 10 minutes. Rats were perfused and brain tissue was prepared for analysis as described in Chapter 1.

For Experiment 2f, rats received intra-BSTv infusions of saline or 100 ng/0.125 µl of clonidine immediately followed by a separate intra-BSTv infusion of saline or 1 µg of yohimbine. The four treatment groups were: saline followed by saline, clonidine followed by saline, saline followed by yohimbine, and clonidine followed by yohimbine.

Data Analysis

For the elevated plus-maze data, any arm entry less than 1 s in duration was considered a keystroke error and removed from the analysis. Any rat that fell from the maze more than three times was also removed from analysis. Behavioral data from the elevated plus-maze were analyzed with two-way ANOVAs with reproductive state and dose of drug as factors. Data from the clonidine, yohimbine, and idazoxan experiments were separately analyzed. Statistical significance was indicated by $p \leq 0.05$. In the case of significant main effects of drug, pairwise comparisons were performed using Fisher's LSD post-hoc tests that were Bonferroni corrected with significance at $p \leq 0.016$. In the case of significant interactions, one-way ANOVAs were

used to detect differences in drug dose within each reproductive state. These were also Bonferroni corrected with significance at $p \leq 0.016$.

Results

Experiment 2a – Behaviors in the elevated plus maze after systemic injections of clonidine

There was a main effect of reproductive state on behavior in the elevated plus maze. Postpartum rats spent a higher percentage of time in the open arms ($F(1,36) = 30.04$, $p < 0.0001$; Figure 11) and had a higher percentage of open arm entries compared to virgins ($F(1,36) = 12.91$, $p = 0.001$). The total number of arms entered was also higher in dams than virgins ($F(1,36) = 58.02$, $p < 0.0001$), as was the number of entries made into the closed arms ($F(1,36) = 30.17$, $p < 0.0001$).

Contrary to what was expected, there was no main effect of clonidine on the percentage of time spent in the open arms ($F(1,36) = 0.91$, $p = 0.41$; Figure 11), nor was there an interaction between reproductive state and dose of clonidine on this measure ($F(1,36 = 0.71$, $p = 0.50$). Clonidine did not affect the percentage of entries into the open arms ($F(2,36) = 0.11$, $p = 0.90$) or total number of entries into all arms ($F(2,36) = 1.70$, $p = 0.20$). The number of entries into the closed arms was significantly lower in rats receiving clonidine ($F(2,36) = 4.60$, $p = 0.017$), and collapsed across reproductive state, rats injected with 10 µg/kg of clonidine had significantly lower closed arm entries compared to the rats injected with 5 µg/kg of clonidine. A significant interaction between reproductive state and dose of clonidine was also found on the number of entries into the closed arms ($F(2,36) = 4.68$, $p = 0.016$). One-way ANOVAs within reproductive state revealed that virgins injected with 5 µg/kg of clonidine entered the closed arms of the maze more frequently than virgins injected with either 0 or 10 µg/kg ($F(2,18) = 8.03$, $p = 0.0032$).

Clonidine did not affect closed arm entries made by dams ($F(2,18) = 0.37$, $p = 0.70$), suggesting that the main effect of clonidine on closed arm entries was due to effects in virgin rats only. Additional measures of behavior in the elevated plus maze are shown in Table 4.

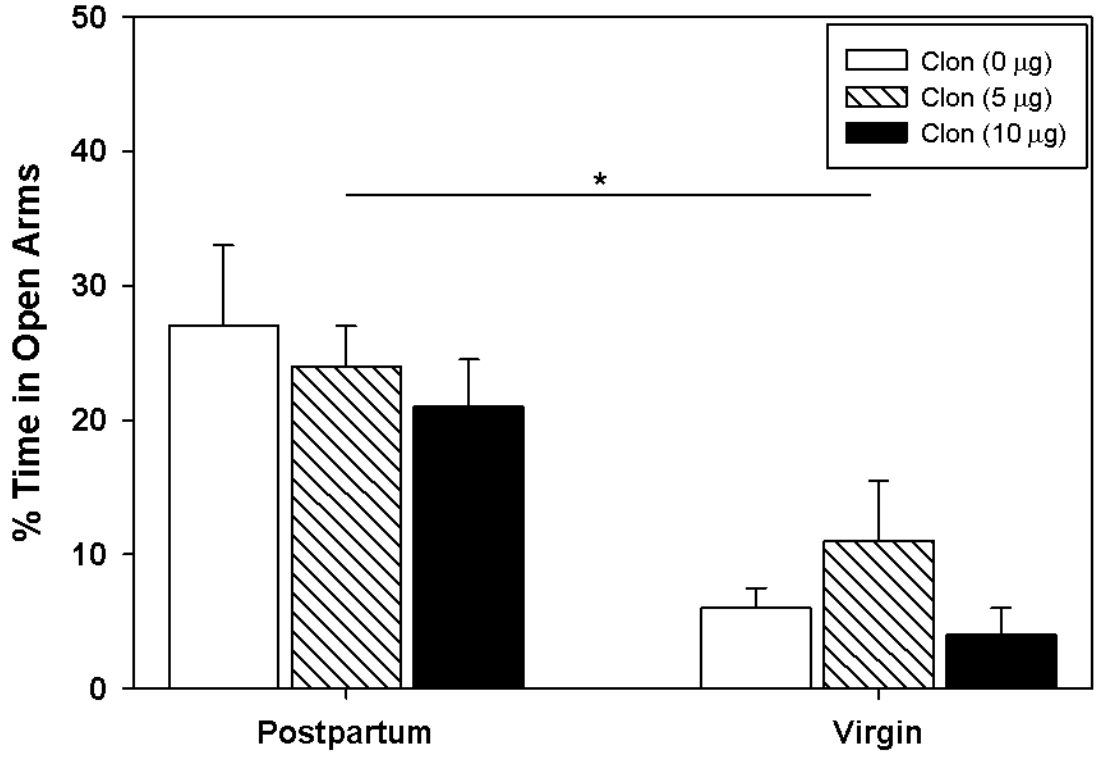

Figure 11 - Percentage of time (mean ± SEM) postpartum and virgin rats spent in the open arms of an elevated plus maze after systemic injection of clonidine. * indicates significant main effect of reproductive state, $p \leq 0.05$

	Virgin VEH	Virgin CLON (5 µg)	Virgin CLON (10 µg)	PP VEH	PP CLON (5 µg)	PP CLON (10 µg)	Significant Effects
Sample Size	7	7	7	7	7	7	
Time in open arms (s)	33 ± 9	59 ± 23	22 ± 10	138 ± 31	120 ± 15	109 ± 17	State
Time in closed arms (s)	516 ± 9	473 ± 25	538 ± 16	369 ± 34	390 ± 19	409 ± 20	State
Total time in arms (s)	548 ± 10	533 ± 7	561 ± 7	507 ± 8	511 ± 11	519 ± 7	State
Open arm entries	3 ± 1	6 ± 1	4 ± 1	14 ± 2	10 ± 1	13 ± 2	State
Percent open arm entries	49 ± 3	41 ± 4	35 ± 8	45 ± 3	42 ± 3	44 ± 5	-
Closed arm entries	9 ± 2^{a}	13 ± 1^{b}	7 ± 1^{a}	16 ± 1	15 ± 1	14 ± 2	State, Dose, State x Dose
Percent closed arm entries	70 ± 6	69 ± 4	71 ± 5	55 ± 5	59 ± 4	53 ± 4	State
Total arm entries	12 ± 2^{a}	20 ± 2^{b}	10 ± 2^{a}	29 ± 3	25 ± 1	27 ± 3	State, State x Dose

Table 4 - Behavior of diestrous virgin and postpartum rats (PP) in the elevated plus maze after systemic injections of clonidine or vehicle. Different letters (a,b) indicate statistical differences ($p \leq 0.016$) within each reproductive state. Significant main effects and interactions are indicated at $p \leq 0.05$.

Experiment 2b - Behaviors in the elevated plus maze after systemic injections of yohimbine

Consistent with Experiment 2a, there were main effects of reproductive state on behavior in the elevated plus maze. Postpartum rats spent a higher percentage of time in the open arms compared to virgin rats ($F(1,41) = 6.22$, $p = 0.017$; Figure 12). The percentage of entries made into the open arms did not differ between dams and virgins ($F(1,41) = 0.40$, $p = 0.53$) but dams made a higher number of closed ($F(1,41) = 12.07$, $p = 0.0012$) and total ($F(1,41) = 16.17$, $p = 0.0002$) arm entries compared to virgins.

Yohimbine significantly decreased the percentage of time rats spent in the open arms of the elevated plus maze ($F(2,41) = 16.65$, $p < 0.0001$; Figure 12) but did not affect the number entries made into the open arms ($F(2,41) = 1.51$, $p = 0.23$). Collapsed across reproductive state, rats receiving injections of 1 mg/kg of yohimbine spent significantly less time in the open arms compared to rats injected with 0 mg/kg. In addition, females receiving injections of 5 mg/kg of yohimbine spent less time in the open arms compared to those injected with either 0 mg/kg or 1 mg/kg. There was also a significant interaction between reproductive state and dose of yohimbine on time spent in the open arms ($F(2,41) = 3.52$, $p = 0.039$). One-way ANOVAs performed within reproductive state revealed that virgins injected with the low dose of yohimbine spent a lower percentage of time in the open arms compared to virgins receiving saline ($F(2,19) = 16.14$, $p < 0.0001$). Postpartum rats, however, did not spend less time in the open arms after receiving the low dose of yohimbine. This effect at the low dose in virgins but not dams may account for the significant main effect of reproductive state on the percentage of time rat spent in the open arms.

In addition, yohimbine significantly reduced closed arm entries ($F(2,41) = 14.89$, $p < 0.0001$) and total arm entries ($F(2,41) = 27.01$, $p < 0.0001$). Collapsed across reproductive state,

rats receiving 5 mg/kg of yohimbine had significantly decreased closed and total arm entries compared to the 0 or 1 mg/kg dose. There was no difference in closed or total arm entries, however, between rats receiving 0 or 1 mg/kg of yohimbine. In addition, there was no interaction between dose and reproductive state on closed arm entries ($F(2,41) = 1.67$, $p = 0.20$) but there was a significant interaction on total arm entries ($F(2,41) = 8.03$, $p = 0.0032$). ANOVAs performed within each reproductive state revealed that both doses of yohimbine reduced closed arm entries in virgins ($F(2,19) = 22.07$, $p < 0.0001$) but that only the high dose of yohimbine reduced closed arm entries in dams ($F(2,22) = 12.25$, $p = 0.0003$). Additional behavioral measures are shown in Table 5.

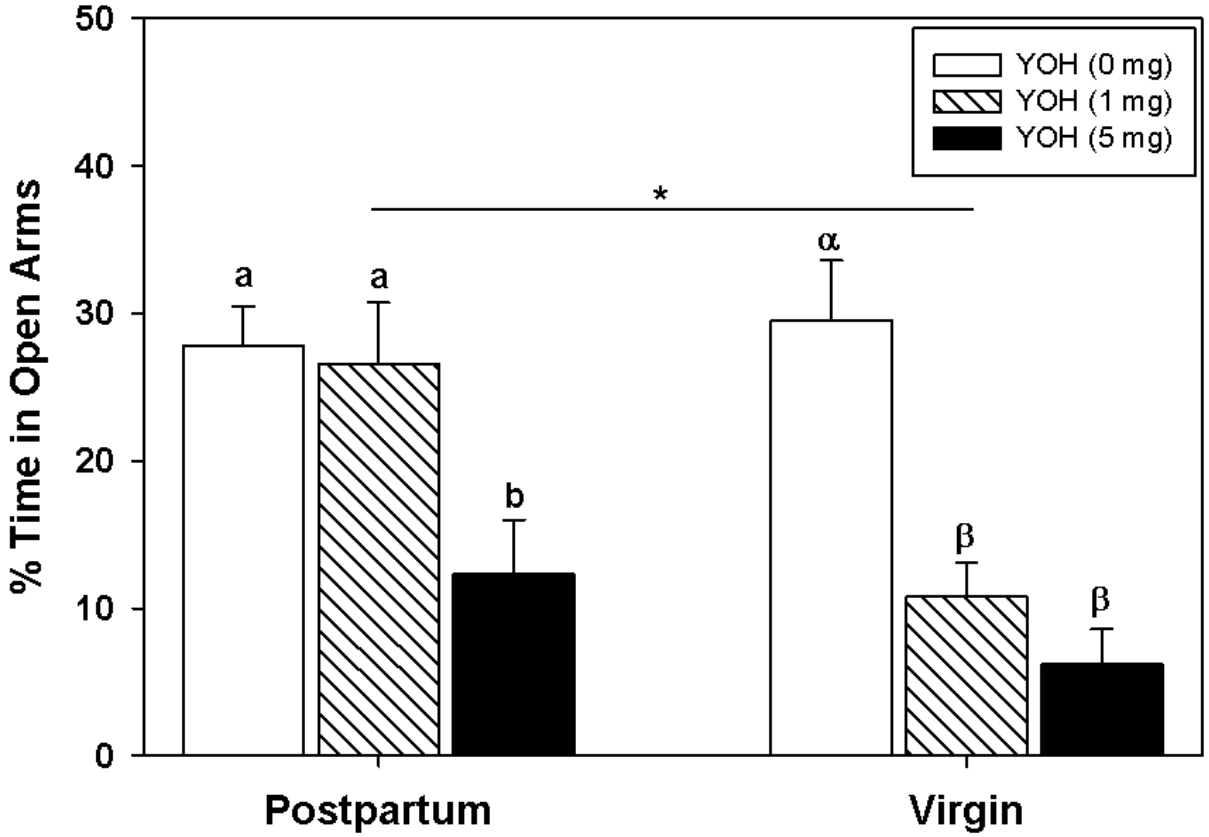

Figure 12 - Percentage of time (mean ± SEM) postpartum and virgin rats spent in the open arms of the elevated plus maze after systemic injection of yohimbine. * indicates significant effect of reproductive state, $p \leq 0.05$. Groups with different letters (a,b; postpartum) and greek letters (α,β; virgin) are statistically different from one another ($p \leq 0.016$) within each reproductive state.

	Virgin VEH	Virgin YOH (1 mg)	Virgin YOH (5 mg)	PP VEH	PP YOH (1 mg)	PP YOH (5 mg)	Significant Effects
Sample Size	7	8	7	9	8	8	
Time in open arms (s)	152 ± 23^{a}	57 ± 12^{b}	36 ± 14^{b}	$141 \pm 13^{\alpha}$	$139 \pm 21^{\alpha}$	$68 \pm 20^{\beta}$	State, Dose, State x Dose
Time in closed arms (s)	360 ± 22	484 ± 24	540 ± 15	369 ± 18	389 ± 26	489 ± 26	State, Dose
Total time in arms (s)	511 ± 14	541 ± 18	576 ± 5	510 ± 6	527 ± 7	557 ± 10	State
Open arm entries	13 ± 1^{a}	6 ± 1^{b}	3 ± 1^{b}	$14 \pm 1^{\alpha}$	$13 \pm 2^{\alpha}$	$5 \pm 1^{\beta}$	State, Dose, State x Dose
Percent open arm entries	49 ± 3	41 ± 4	35 ± 8	45 ± 3	42 ± 3	54 ± 4	-
Closed arm entries	14 ± 1	9 ± 2	4 ± 1	17 ± 1	18 ± 3	8 ± 3	State, Dose
Percent closed arm entries	51 ± 3	59 ± 4	65 ± 8	55 ± 3	58 ± 3	55 ± 4	-
Total arm entries	27 ± 2^{a}	15 ± 3^{b}	7 ± 2^{b}	$31 \pm 2^{\alpha}$	$31 \pm 4^{\alpha}$	$13 \pm 3^{\beta}$	State, Dose, State x Dose

Table 5 - Behavior of diestrous virgin and postpartum rats (PP) in the elevated plus maze after systemic injections of yohimbine or vehicle. Different letters (a,b) and greek letters (α,β) indicate statistical differences ($p \leq 0.016$) within each reproductive state. Significant main effects and interactions are indicated at $p \leq 0.05$.

Experiment 2c - Behaviors in the elevated plus maze after intra-BSTv infusions of clonidine

A total of 39 virgin female and 82 postpartum rats were infused with clonidine into the BSTv and tested in the elevated plus maze. 17 virgins were removed from analysis: 14 rats had misplaced infusions located outside of the BSTv, 3 rats were in estrus during testing. 46 postpartum rats were removed from analysis because of misplaced infusions. A large majority of "missed" infusions were located in the very caudal BST while some infusions terminated in the dorsal BST and ventral pallidum. Representations of "hit" infusion sites in the rats receiving clonidine are shown in Figures 13-15.

There was no main effect of reproductive state on the percentage of time spent in the open arms ($F(1,52) = 0.50$, $p = 0.49$; Figure 16). Dams, however, had a higher percentage of entries into the open arms ($F(1,52) = 7.78$, $p = 0.074$) compared to diestrous virgins. There was no difference between dams and virgins on the number of open ($F(1,52) = 1.39$, $p = 0.25$), closed ($F(1,52) = 0.75$, $p = 0.39$), or total ($F(1,52) = 0.023$, $p = 0.88$) arm entries.

In addition, there was no effect clonidine on the percentage of time rats spent in the open arms ($F(2,52) = 0.73$, $p = 0.49$; Figure 16) or on the percentage of open arm entries ($F(2,52) = 0.29$, $p = 0.75$). Clonidine did significantly reduce the number of open ($F(2,52) = 3.90$, $p = 0.026$), closed ($F(2,52) = 3.98$, $p = 0.025$), and total ($F(2,52) = 4.51$, $p = 0.016$) arm entries. For these open, closed, and total arm entries, rats receiving the 1000 ng dose entered the arm(s) significantly fewer times than rats receiving saline. Rats infused with 100 ng of clonidine did not differ from rats infused with 0 or 1000 ng. Additional behavioral measures are shown in Table 6.

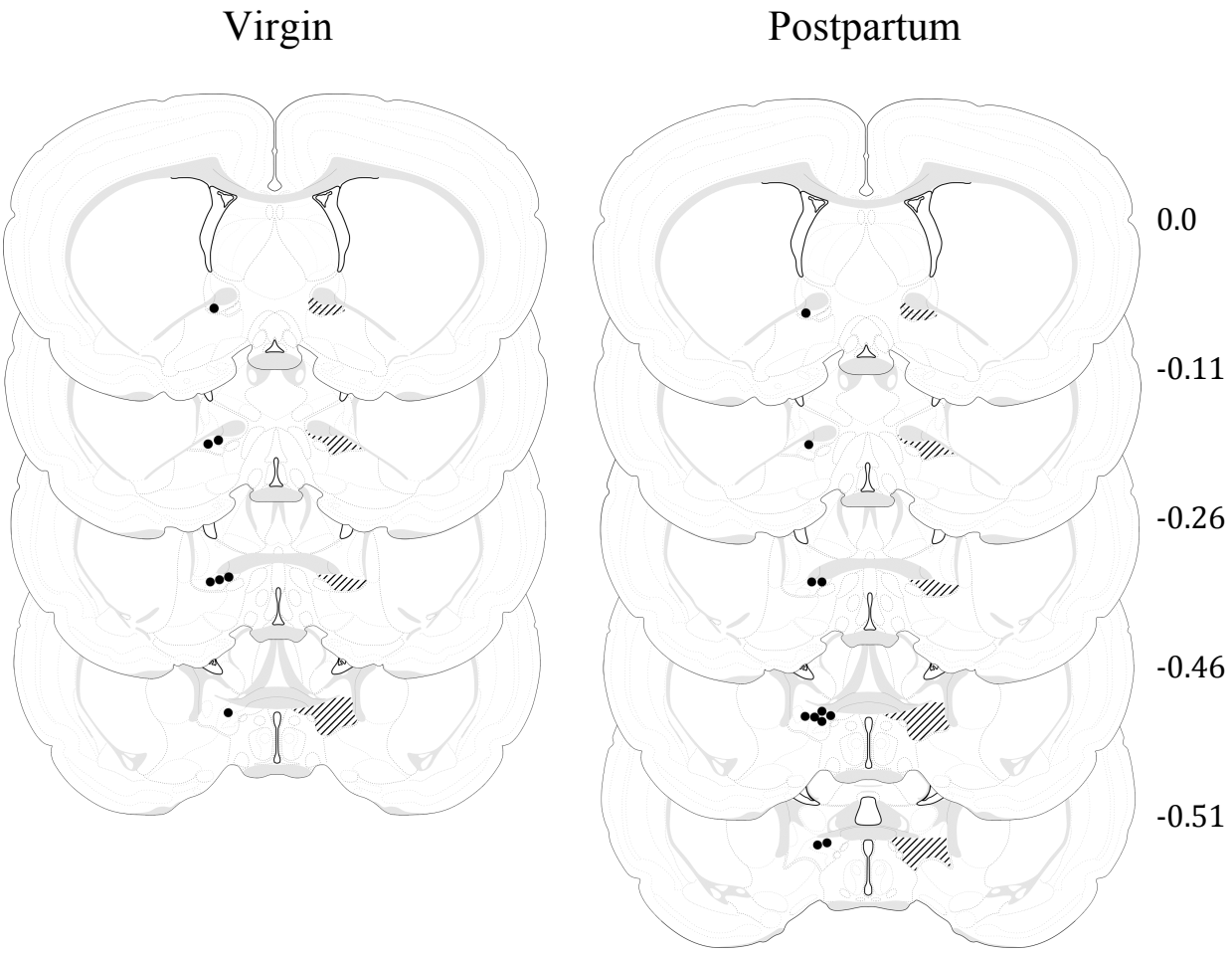

Figure 13 - Intra-BSTv infusion placements for virgin (left) and postpartum (right) rats receiving 0 ng of clonidine. Each unilateral dot indicates the bilateral infusion placement of one subject. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

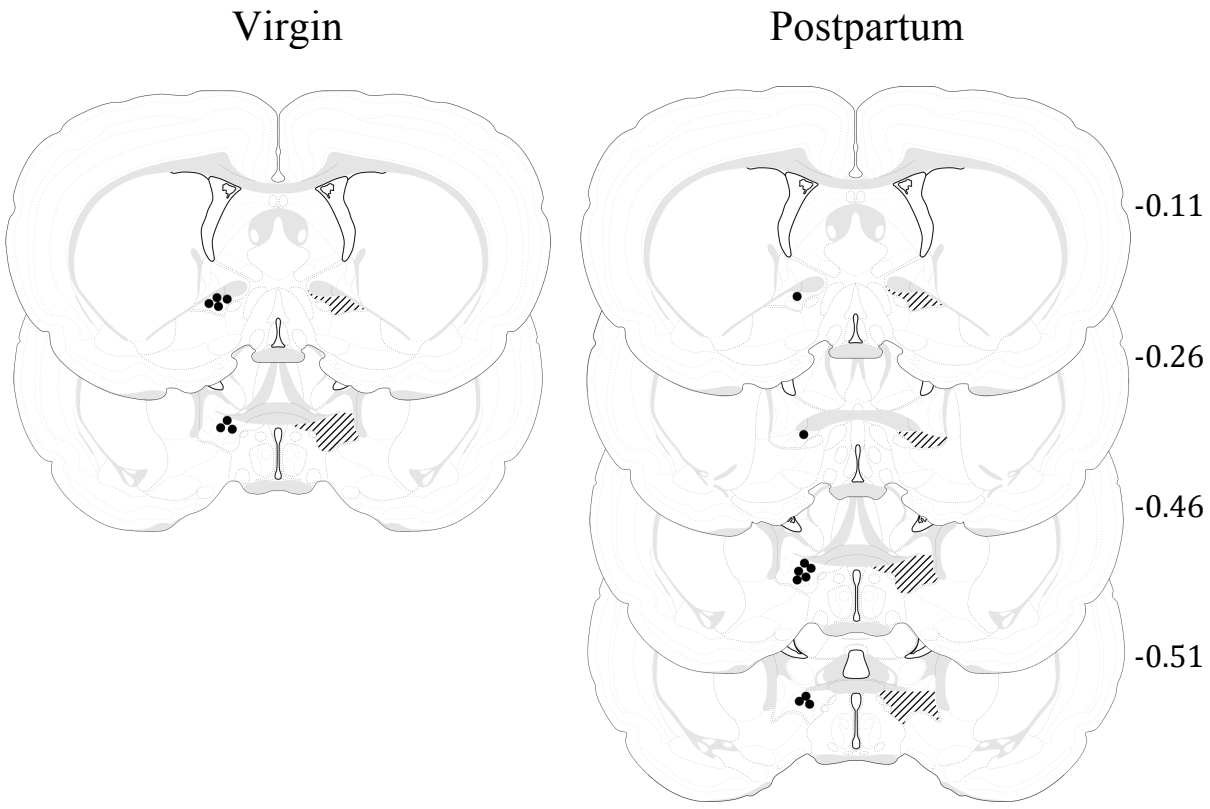

Figure 14 - Intra-BSTv infusion placements for virgin (left) and postpartum (right) rats receiving 100 ng of clonidine. Each unilateral dot indicates the bilateral infusion placement of one subject. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

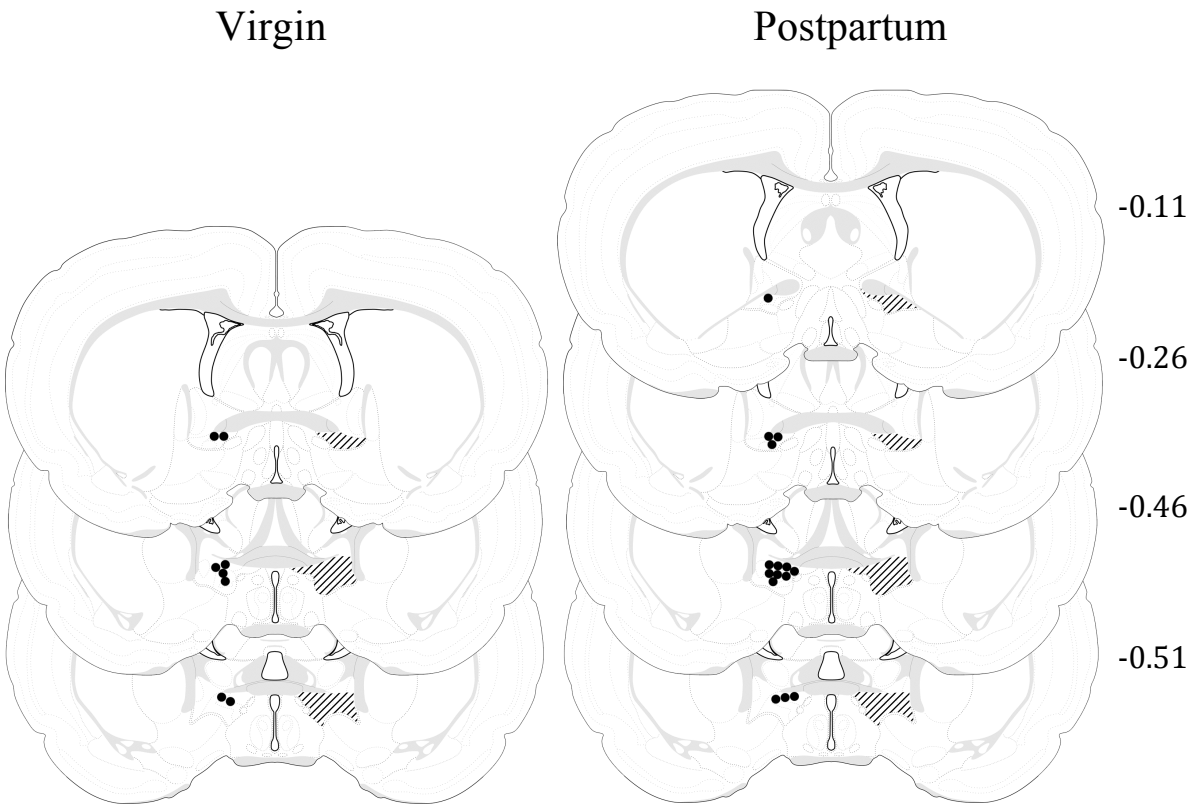

Figure 15 - Intra-BSTv infusion placements for virgin (left) and postpartum (right) rats receiving 1000 ng of clonidine. Each unilateral dot indicates the bilateral infusion placement of one subject. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

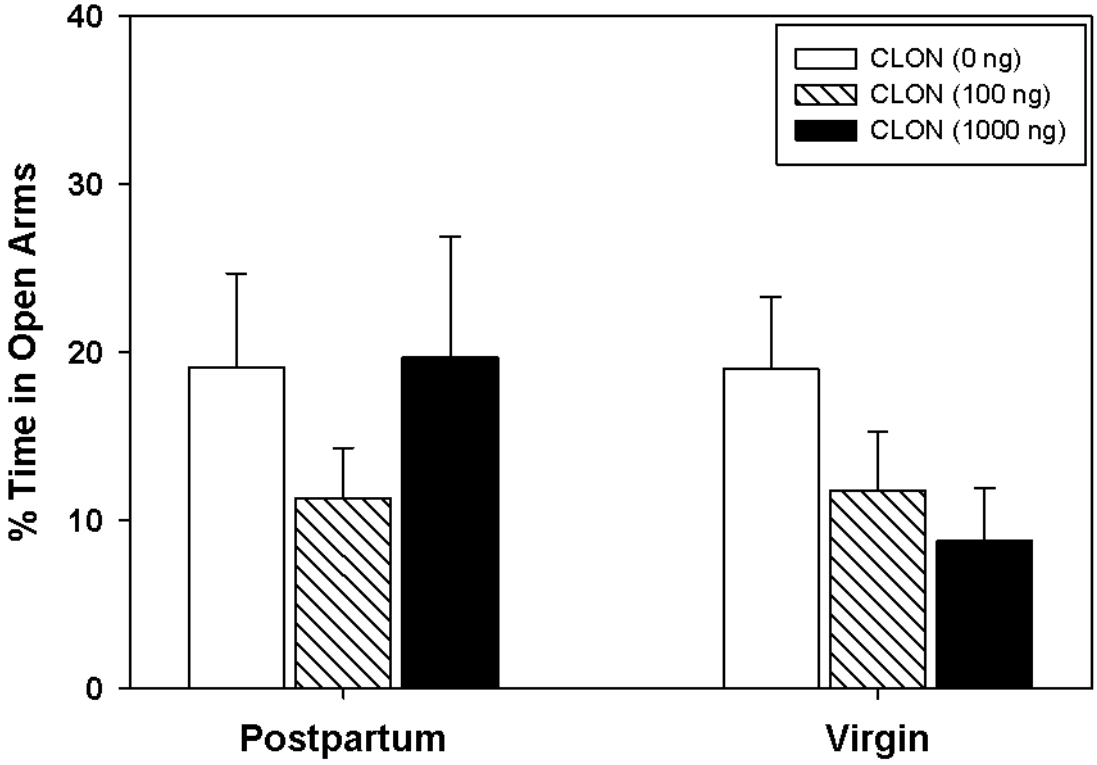

Figure 16 - Percentage of time (mean ± SEM) postpartum and virgin rats spent in the open arms of an elevated plus maze after intra-BSTv infusions of clonidine.

	Virgin VEH	Virgin CLON (100 ng)	Virgin CLON (1000 ng)	PP VEH	PP CLON (100 ng)	PP CLON (1000 ng)	Significant Effects
Sample Size	7	7	8	11	10	15	
Time in open arms (s)	98 ± 19	72 ± 28	52 ± 18	96 ± 28	58 ± 15	69 ± 16	-
Time in closed arms (s)	440 ± 35	498 ± 21	546 ± 45	434 ± 39	471 ± 28	451 ± 47	-
Total time in arms (s)	539 ± 21	571 ± 34	597 ± 41	530 ± 21	529 ± 21	520 ± 34	-
Open arm entries	10 ± 3	8 ± 2	5 ± 1	11 ± 2	9 ± 2	8 ± 1	Dose
Percent open arm entries	44 ± 2	39 ± 4	37 ± 4	47 ± 3	47 ± 2	51 ± 4	State
Closed arm entries	13 ± 3	12 ± 2	8 ± 1	12 ± 2	10 ± 2	7 ± 1	Dose
Percent closed arm entries	56 ± 2	61 ± 4	63 ± 4	53 ± 3	53 ± 2	49 ± 4	State
Total arm entries	23 ± 5	20 ± 3	13 ± 2	23 ± 3	18 ± 3	15 ± 2	Dose

Table 6 - Behavior of diestrous virgin and postpartum rats (PP) in the elevated plus maze after intra-BSTv injections of clonidine or vehicle. Significant main effects and interactions are indicated at $p \leq .05$.

Experiment 2d - Behaviors in the elevated plus maze after intra-BSTv infusions of yohimbine

A total of 41 virgin female and 45 postpartum rats were infused with yohimbine into the BSTv and tested in the elevated plus maze. Seventeen virgins were removed from analysis: twelve rats had misplaced infusions located outside of the BSTv, two rats had excessive damage around the infusion site during perfusion that included damage to the BSTv, mPOA, lateral preoptic area, and ventral pallidum, two rats were found to be in estrus during testing, and one rat fell off the maze multiple times. Twenty-one postpartum rats were removed from analysis because of misplaced infusions. The majority of misplaced infusions were located in the dorsal BST and caudal BSTv. Representations of "hit" infusion sites in rats receiving yohimbine are shown in Figures 17-19.

There were significant main effects of reproductive state. Dams spent a significantly greater percentage of time in the open arms ($F(1,42) = 7.64$, $p = 0.0084$; Figure 20) and had a higher percentage of entries into the open arms ($F(1,42) = 5.03$, $p = 0.030$) compared to virgins. The number of closed ($F(1,42) = 6.17$, $p = 0.017$) and total ($F(1,42) = 23.14$, $p < 0.0001$) arm entries was also higher in postpartum rats compared to virgins.

There were also main effects of yohimbine. The percentage of time spent in the open arms was significantly lower in rats receiving yohimbine ($F(2,42) = 4.68$, $p = 0.015$; Figure 20). Collapsed across reproductive state, rats receiving infusions of 1 μg of yohimbine spent significantly less time in the open arms compared to rats infused with 0 μg. Infusions of 3 μg of yohimbine tended to reduce open arm exploration but were not statistically significant. There was also a significant interaction between reproductive state and dose of yohimbine on time spent in the open arms ($F(2,42) = 4.14$, $p = 0.023$; Figure 20. One-way ANOVAs within reproductive state showed that dams ($F(2,21) = 6.64$, $p = 0.0058$), but not virgins ($F(2,21) =$

1.22, p = 0.32), spent less time in the open arms after receiving either dose of yohimbine. Therefore, this effect in dams, but not virgins, may account for the interaction and main effect of yohimbine infusion.

The percentage of open arm entries did not differ between doses of yohimbine (F(2,42) = 0.34, p = 0.71), nor was there an interaction between reproductive state or dose of yohimbine on this measure (F(2,42) = 0.55, p = 0.58). Yohimbine significantly reduced the number of closed (F(2,42) = 9.76, p = 0.0003) and total (F(2,42) = 16.77, $p < 0.001$) arm entries compared to vehicle. Collapsed across reproductive state, both doses of yohimbine significantly reduced closed and total arm entries compared to vehicle. The two doses of yohimbine did not differ between each other. Furthermore, there was no interaction found between reproductive state and dose of yohimbine for the number of closed (F(2,42) = 0.65, p = 0.52) or total (F(2,42) = 1.82, p = 0.17) arm entries. Additional behavioral measures are shown in Table 7.

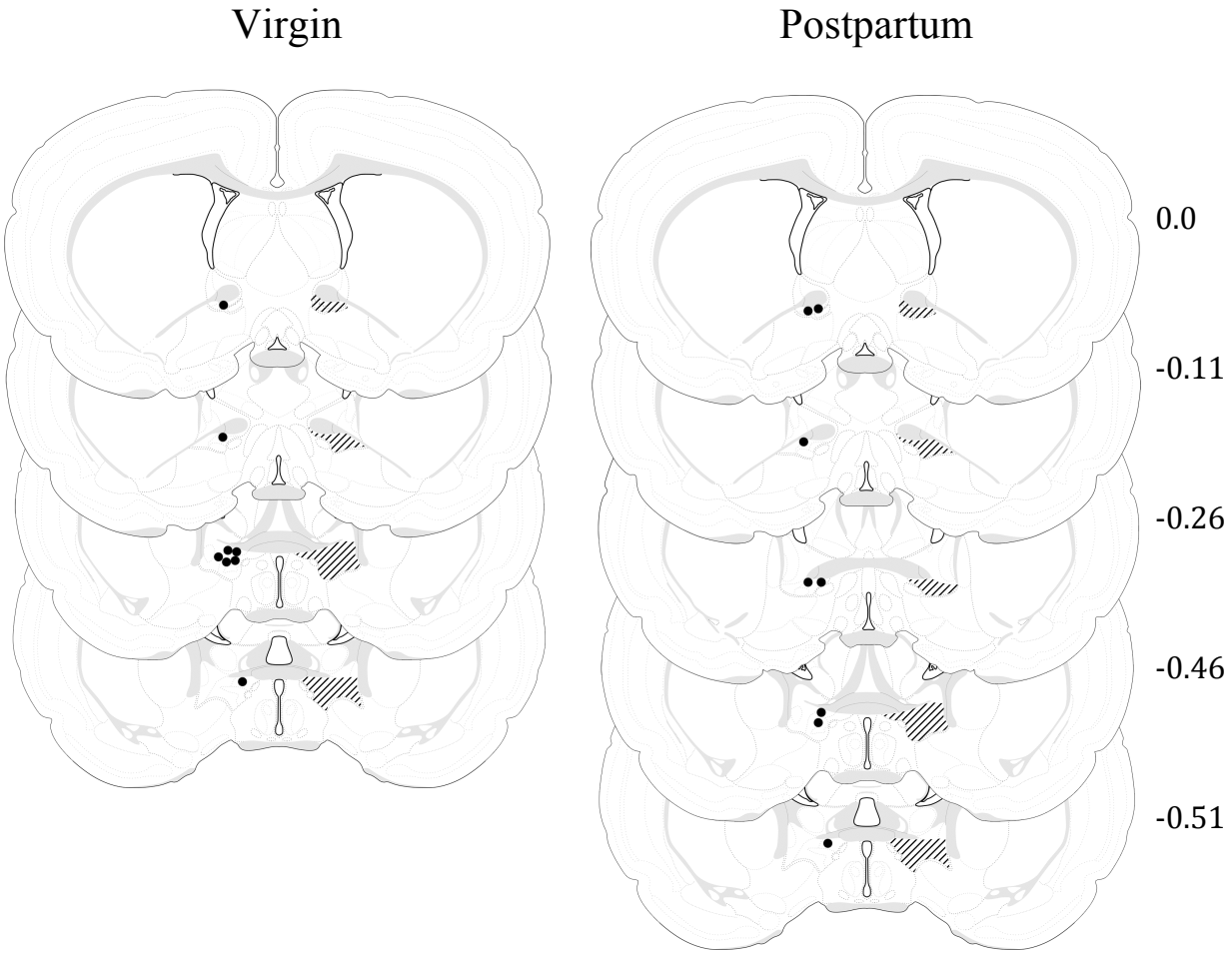

Figure 17 - Intra-BSTv infusion placements for virgin (left) and postpartum (right) rats receiving 0 μg of yohimbine. Each unilateral dot indicates the bilateral infusion placement of one subject. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

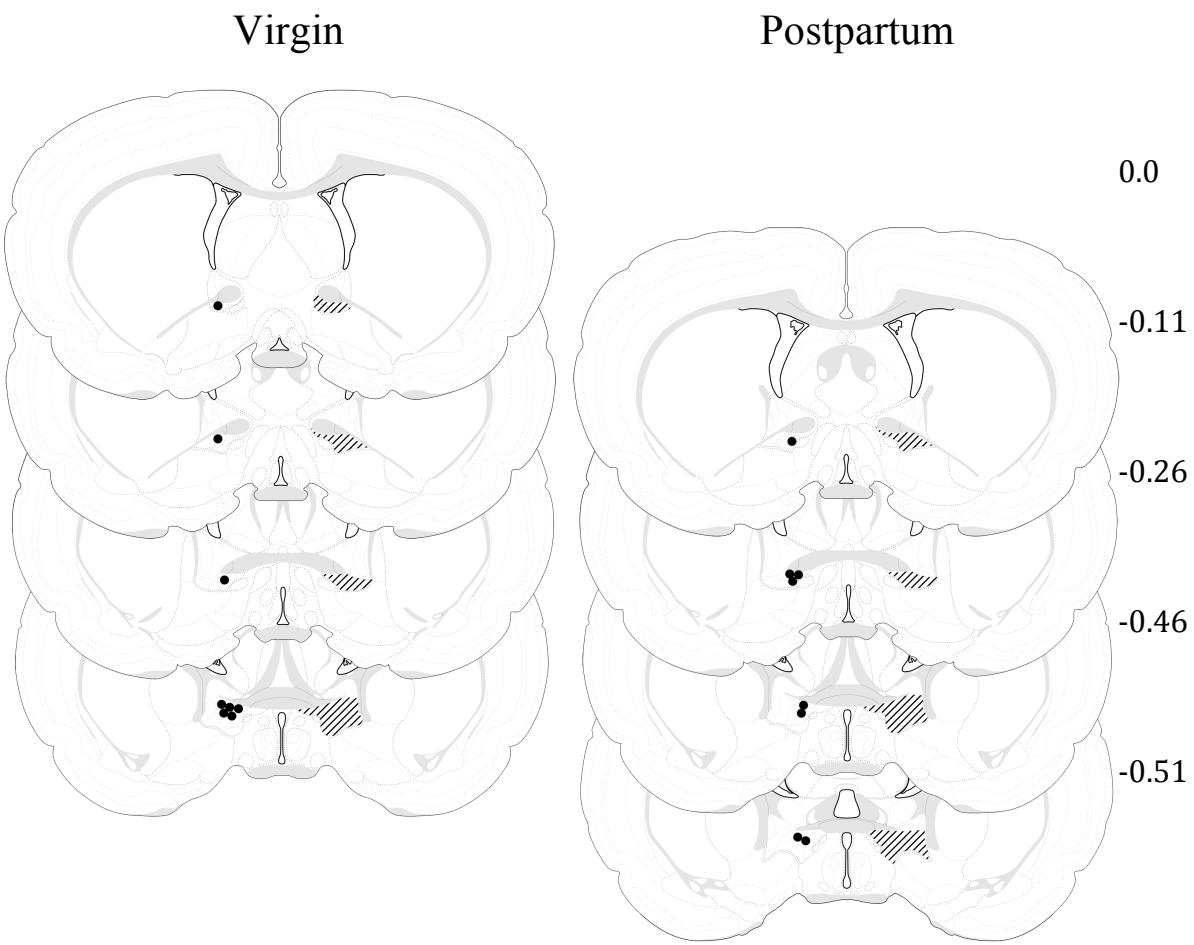

Figure 18 - Intra-BSTv infusion placements for virgin (left) and postpartum (right) rats receiving 1 μg of yohimbine. Each unilateral dot indicates the bilateral infusion placement of one subject. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

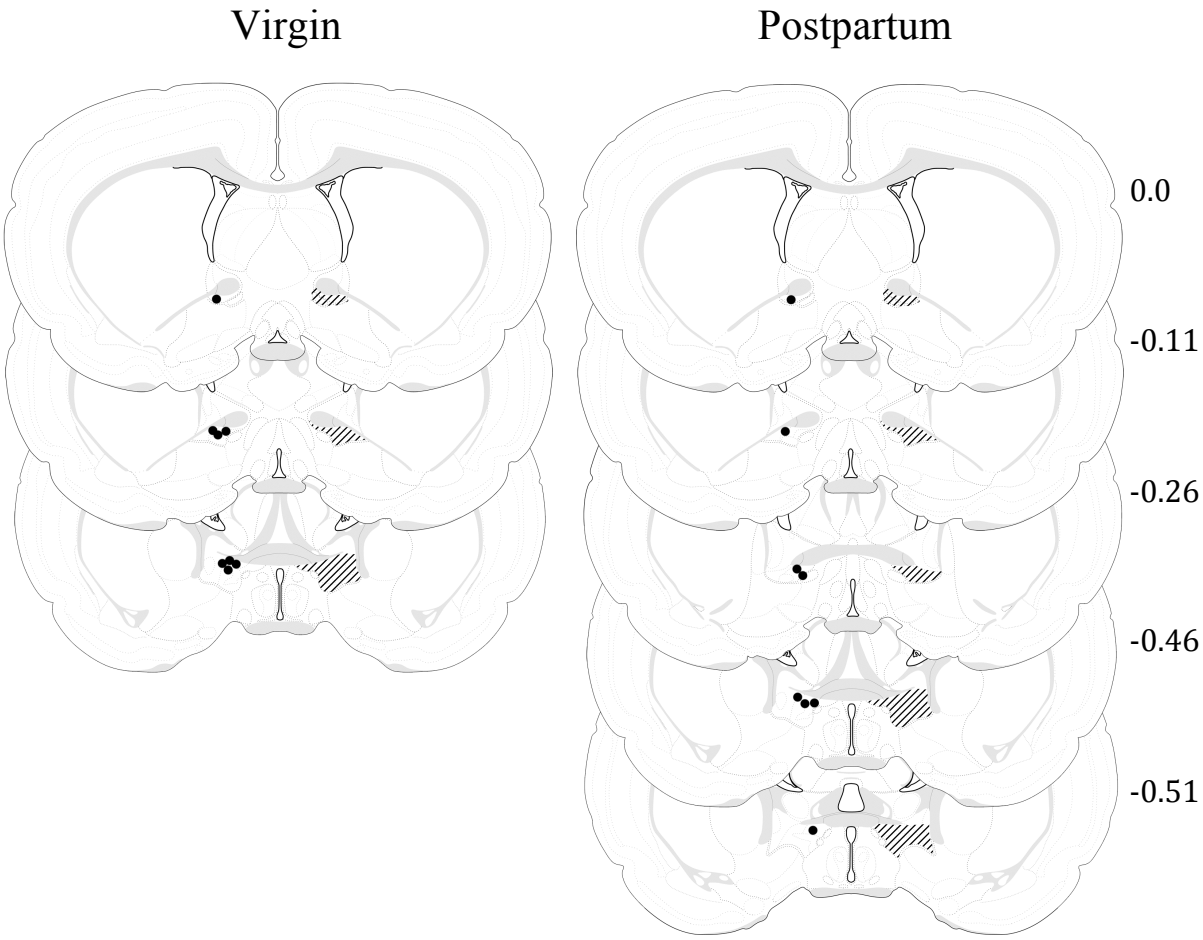

Figure 19 - Intra-BSTv infusion placements for virgin (left) and postpartum (right) rats receiving 3 μg of yohimbine. Each unilateral dot indicates the bilateral infusion placement of one subject. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

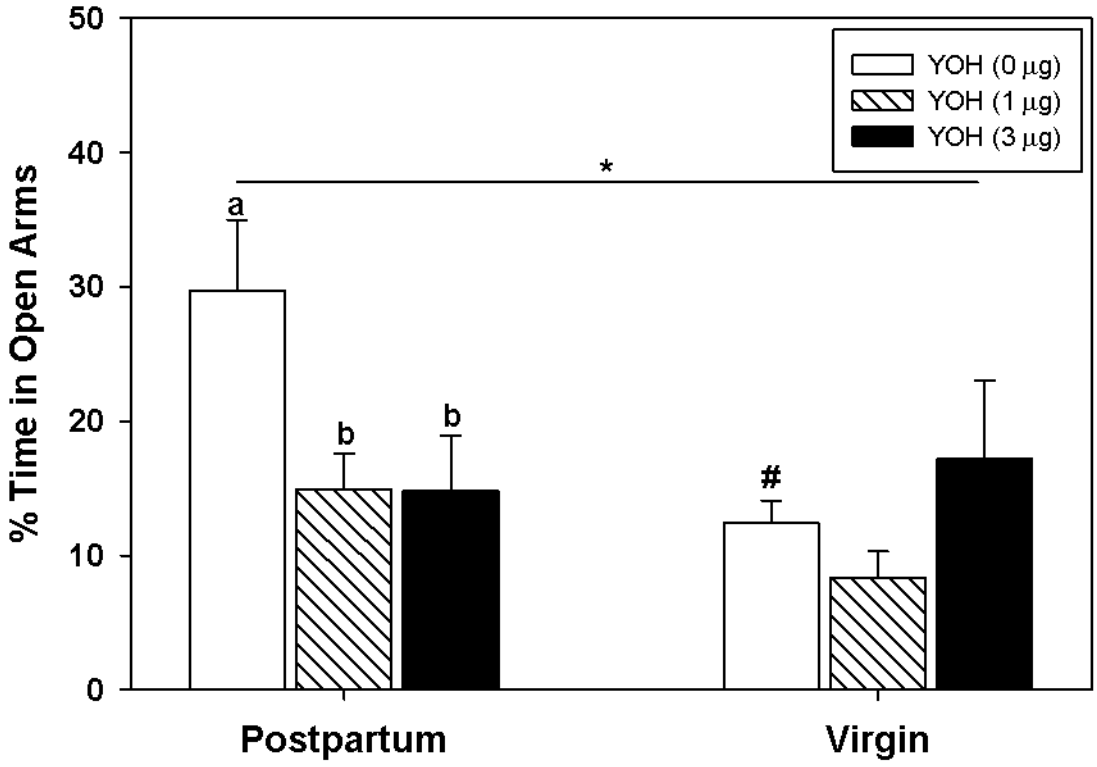

Figure 20 - Percentage of time (mean ± SEM) postpartum and virgin rats spent in the open arms of the elevated plus maze after intra-BSTv infusion of yohimbine. * indicates statistical significance of reproductive state at $p \leq .05$. # indicates site of significant interaction ($p \leq .05$). Postpartum groups with different letters (a,b) are statistically different from one another ($p \leq .016$).

	Virgin VEH	Virgin YOH (1 μg)	Virgin YOH (3 μg)	PP VEH	PP YOH (1 μg)	PP YOH (3 μg)	Significant Effects
Sample Size	8	8	8	8	8	8	
Time in open arms (s)	63 ± 9	42 ± 9	66 ± 23	$160 \pm 22^{\alpha}$	$76 \pm 17^{\beta}$	$75 \pm 20^{\beta}$	State, Dose, State x Dose
Time in closed arms (s)	448 ± 22	491 ± 27	373 ± 50	$333 \pm 30^{\alpha}$	$455 \pm 26^{\beta}$	$437 \pm 31^{\alpha\beta}$	Dose, State x Dose
Total time in arms (s)	511 ± 15	533 ± 18	439 ± 44	494 ± 15	531 ± 18	512 ± 22	-
Open arm entries	8 ± 1	5 ± 1	6 ± 1	$17 \pm 1^{\alpha}$	$10 \pm 1^{\beta}$	$9 \pm 1^{\beta}$	State, Dose, State x Dose
Percent open arm entries	42 ± 3	43 ± 7	47 ± 5	55 ± 3	49 ± 3	52 ± 4	State
Closed arm entries	12 ± 1	6 ± 1	7 ± 1	14 ± 1	10 ± 2	8 ± 1	State, Dose
Percent closed arm entries	51 ± 3	59 ± 4	65 ± 8	55 ± 3	58 ± 3	55 ± 4	-
Total arm entries	20 ± 2	12 ± 2	13 ± 2	31 ± 2	20 ± 3	17 ± 2	State, Dose

Table 7 - Behavior of diestrous virgin and postpartum rats in the elevated plus maze after intra-BSTv injections of yohimbine or vehicle. Different letters (a,b) and greek letters (α,β) indicate statistical differences ($p \leq .016$) within each reproductive state. Significant main effects and interactions are indicated at $p \leq .05$.

Experiment 2e - Behaviors in the elevated plus maze after intra-BSTv infusions of idazoxan

57 virgin female and 47 postpartum rats were infused with idazoxan into the BSTv and tested in the elevated plus maze. Fifteen virgins were removed from analysis: thirteen rats had misplaced infusions located outside of the BSTv, one rat was found to be in estrus during testing, and one rat fell off the maze more than three times. Five postpartum rats were removed from analysis because of misplaced infusions. The majority of misplaced infusions were located in the dorsal BST, caudal BSTv, and ventral pallidum. Representations of "hit" infusion sites in rats receiving idazoxan are shown in Figures 21-23.

There were no main effects or interactions related to reproductive state on behavior in the elevated plus maze. Dams tended to spend a higher percentage of time in the open arms than virgins ($F(1,58) = 3.77$, $p = 0.057$; Figure 24). There was no effect of reproductive state on the percentage of open arm entries ($F(1,58) = 1.93$, $p = 0.17$), closed ($F(1,58) = 0.84$, $p = 0.36$), or total ($F(1,58) = 2.01$, $p = 0.16$) arm entries.

In contrast to yohimbine, infusion of idazoxan did not affect the percentage of time rats spent in the open arms ($F(2,58) = 0.13$, $p = .88$; Figure 24), nor was there an interaction between reproductive state and dose of idazoxan on this measure ($F(2,58) = 0.98$, $p = .38$). The percentage of open arm entries was not affected by idazoxan ($F(2,58) = 0.18$, $p = .84$) and there was no significant interaction between dose and reproductive state ($F(2,58) = 1.02$, $p = 0.37$) on this measure. Idazoxan significantly reduced the number of closed ($F(2,58) = 7.99$, $p = .0009$) and total ($F(2,58) = 4.88$, $p = 0.011$) arm entries. Collapsed across reproductive state, only the highest dose of idazoxan reduced closed and total arm entries compared to the low dose and vehicle. Finally, there was no interaction between dose and reproductive state on either closed ($F(2,58) = 0.29$, $p = 0.75$) or total ($F(2,58) = 0.012$, $p = 0.99$) arm entries. Additional behavioral

measure are shown in Table 8.

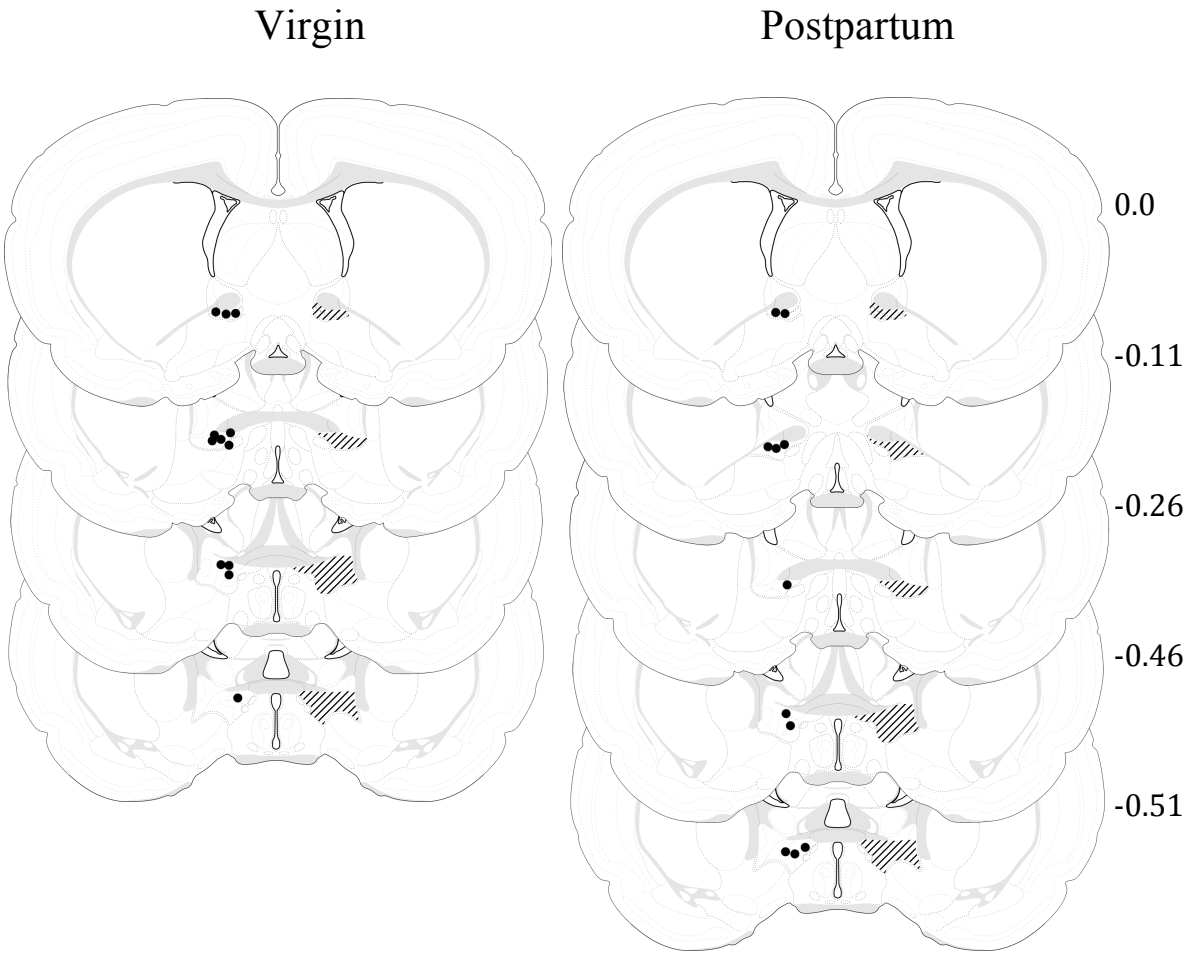

Figure 21 - Intra-BSTv infusion placements for virgin (left) and postpartum (right) rats receiving 0 μg of idazoxan. Each unilateral dot indicates the bilateral infusion placement of one subject. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

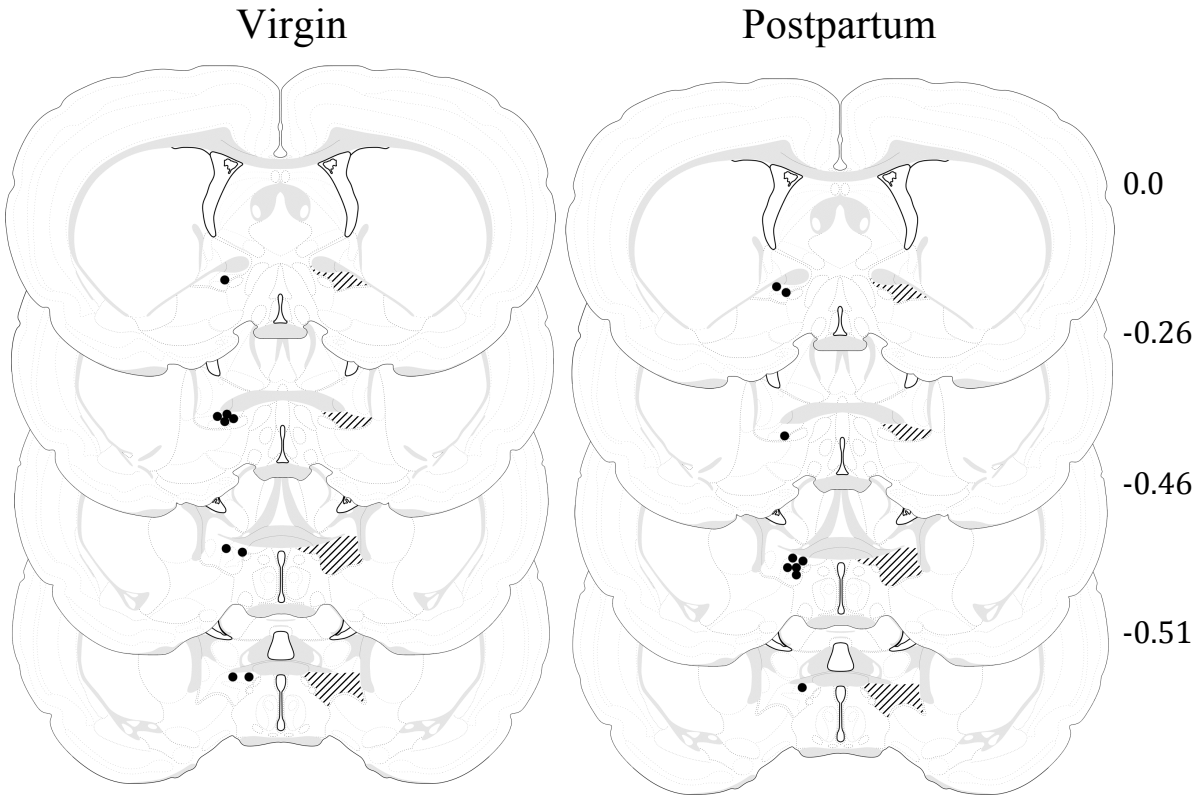

Figure 22 - Intra-BSTv infusion placements for virgin (left) and postpartum (right) rats receiving 5 μg of idazoxan. Each unilateral dot indicates the bilateral infusion placement of one subject. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

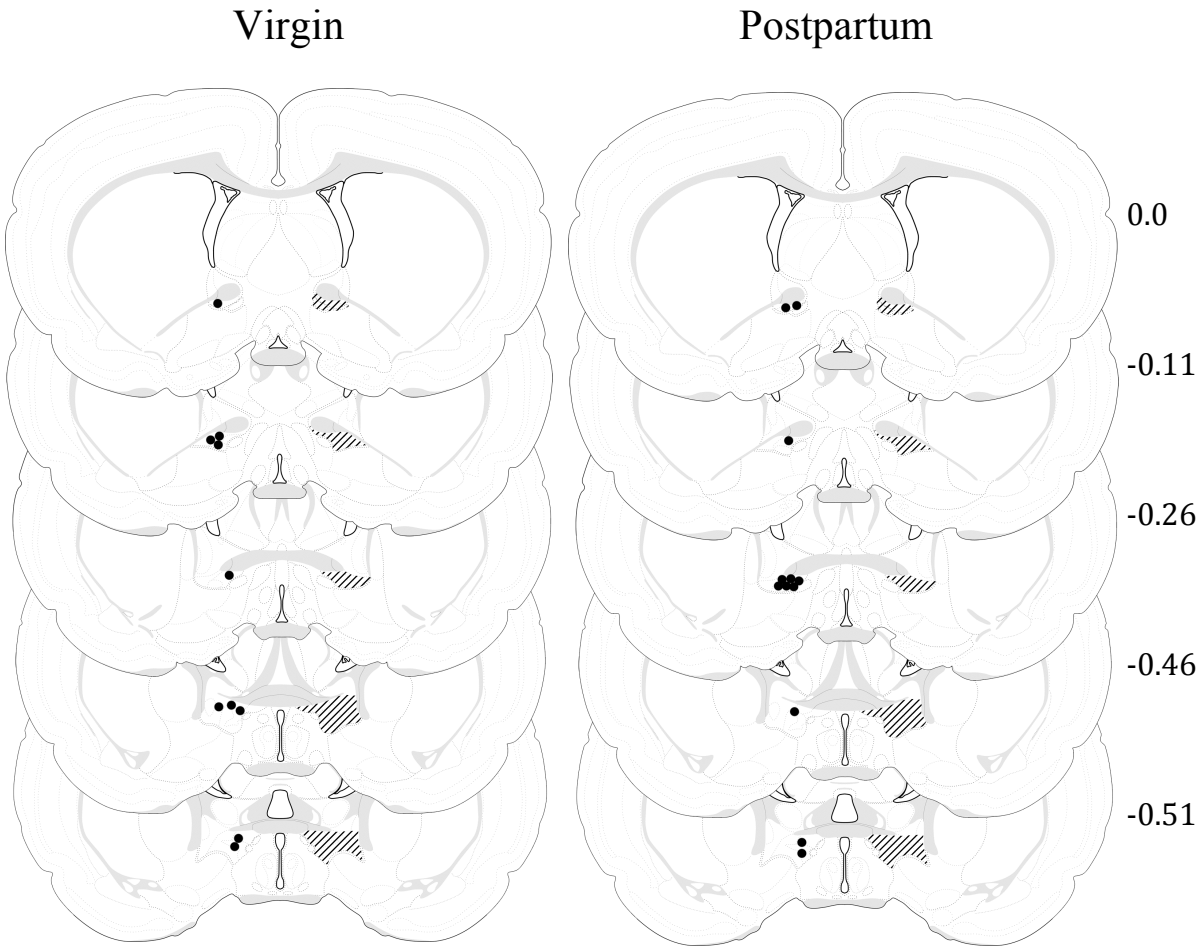

Figure 23 - Intra-BSTv infusion placements for virgin (left) and postpartum (right) rats receiving 10 μg of idazoxan. Each unilateral dot indicates the bilateral infusion placement of one subject. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

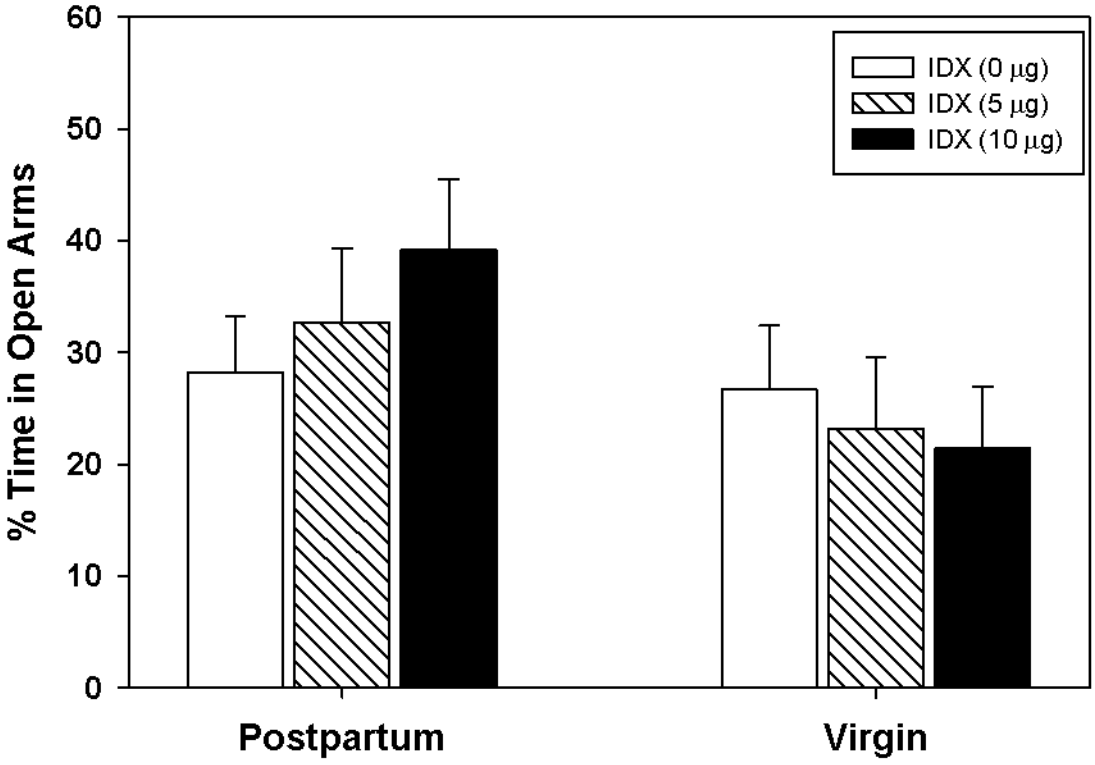

Figure 24 - Percentage of time (mean ± SEM) postpartum and virgin rats spent in the open arms of the elevated plus maze after intra-BSTv infusion of idazoxan.

	Virgin VEH	Virgin IDX (5 μg)	Virgin IDX (10 μg)	PP VEH	PP IDX (5 μg)	PP IDX (10 μg)	Significant Effects
Sample Size	13	9	10	11	9	12	
Time in open arms (s)	126 ± 28	110 ± 36	100 ± 26	136 ± 21	163 ± 34	172 ± 28	-
Time in closed arms (s)	351 ± 32	358 ± 41	396 ± 43	360 ± 33	332 ± 33	284 ± 41	-
Total time in arms (s)	477 ± 17	468 ± 32	496 ± 28	496 ± 12	495 ± 9	455 ± 35	State
Open arm entries	12 ± 2	7 ± 1	8 ± 1	13 ± 2	10 ± 1	12 ± 3	-
Percent open arm entries	45 ± 4	43 ± 3	42 ± 5	45 ± 3	47 ± 3	52 ± 4	-
Closed arm entries	13 ± 1	10 ± 1	9 ± 1	15 ± 2	11 ± 1	9 ± 2	Dose
Percent closed arm entries	55 ± 4	57 ± 3	58 ± 5	55 ± 3	53 ± 3	48 ± 4	-
Total arm entries	25 ± 3	17 ± 2	16 ± 3	28 ± 3	21 ± 2	20 ± 4	Dose

Table 8 - Behavior of diestrous virgin and postpartum rats (PP) in the elevated plus maze after intra-BSTv injections of idazoxan or vehicle. Significant main effects and interactions are indicated at $p \leq .05$.

Experiment 2f – Behaviors in the elevated plus maze after sequential intra-BSTv infusions of clondine and yohimbine

43 virgin female and 52 postpartum rats were infused with saline or clonidine followed by saline or yohimbine into the BSTv and tested in the elevated plus maze. 14 virgins were removed from analysis: 12 rats had misplaced infusions located outside of the BSTv, 1 rat was in estrus during testing, and 1 rat fell off the maze more than three times. 20 postpartum rats were removed from analysis because of misplaced infusions. Most "missed" infusions terminated in the dorsal BST, caudal BST, and ventral pallidum. Representations of "hit" infusion sites are shown in Figures 26-28.

There was no main effect of reproductive state on the percentage of time spent in the open arms ($F(1,53) = 0.68$, $p = 0.042$; Figre 29), the percentage of entries made into the open arms ($F(1,53) = 0.001$, $p = .057$), or the number of entries made into the open arms ($F(1,53) = 2.13$, $p = 0.15$). Dams, however, had fewer closed ($F(1,53) = 4.94$, $p = 0.031$) and total ($F(1,53) = 4.31$, $p = 0.043$) arm entries compared to virgins.

Intra-BSTv of saline, clondine, and/or yohimbine did not effect the percentage of time spent in the open arms ($F(3,53) = 0.68$, $p = 0.42$; Figure 29) but there was an interaction between drug infusion and reproductive state on this measure ($F(3,53) = 3.44$, $p = 0.023$). One-way ANOVAs within reproductive state revealed that virgins, but not dams, receiving saline followed by yohimbine spent significantly more time in the open arms than virgins receiving the other drug combinations ($F(3,25) = 4.12$, $p = 0.017$). There were also significant main effects of drug infusion on the percentage of entries into the open arms ($F(3,53) = 4.51$, $p = 0.0068$). Collapsed across reproductive state, females infused with clonidine followed by yohimbine had a higher percentage of entries into the open arms compared to females receiving two infusions of saline.

Drug infusions also affected open ($F(3,53) = 5.68$, $p = 0.0019$), closed ($F(3,53) = 17.82$, $p < 0.0001$), and total ($F(3,53) = 12.95$, $p < 0.0001$) arm entries. For simplicity, post-hoc analysis of these arm entries are not described here but are shown in Table 9. Additional behavioral measures are shown in Table 10.

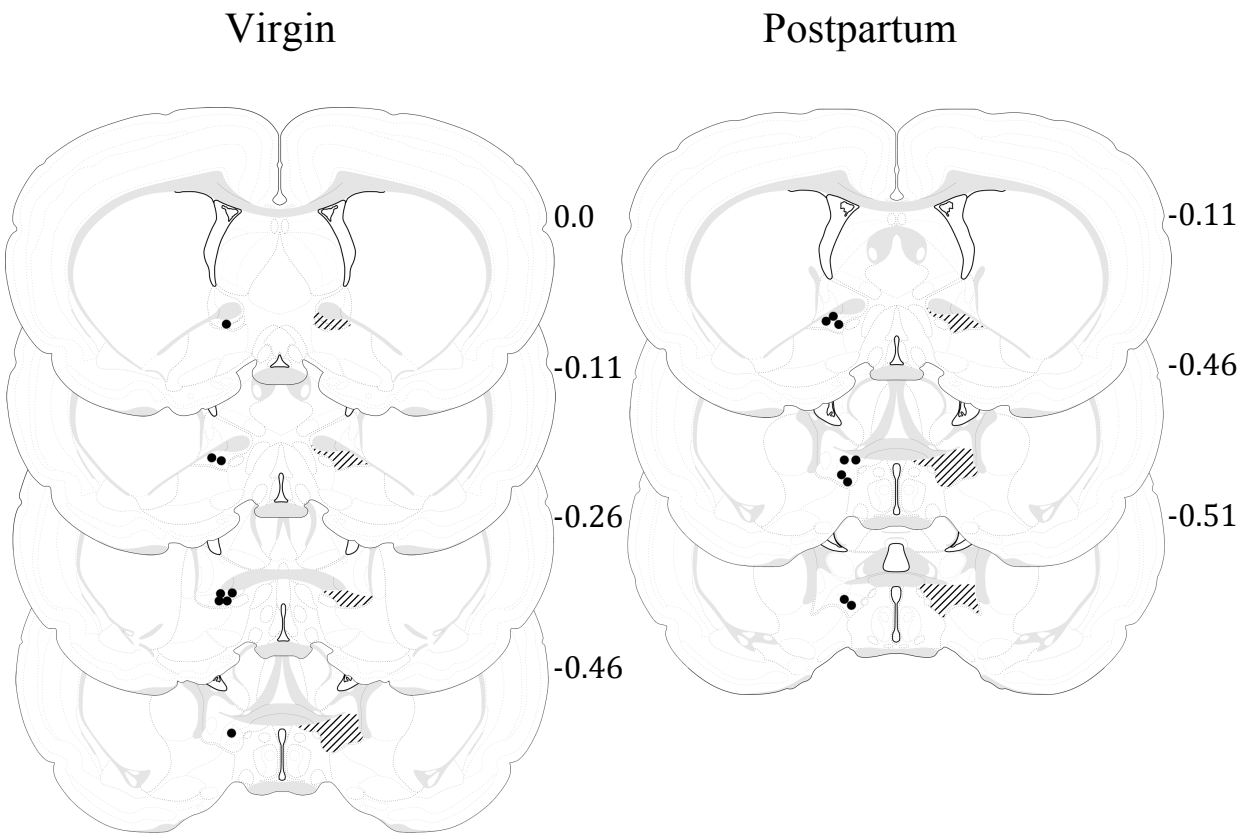

Figure 25 - Intra-BSTv infusion placements for virgin (left) and postpartum (right) rats receiving saline followed by saline. Each unilateral dot indicates the bilateral infusion placement of one subject. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

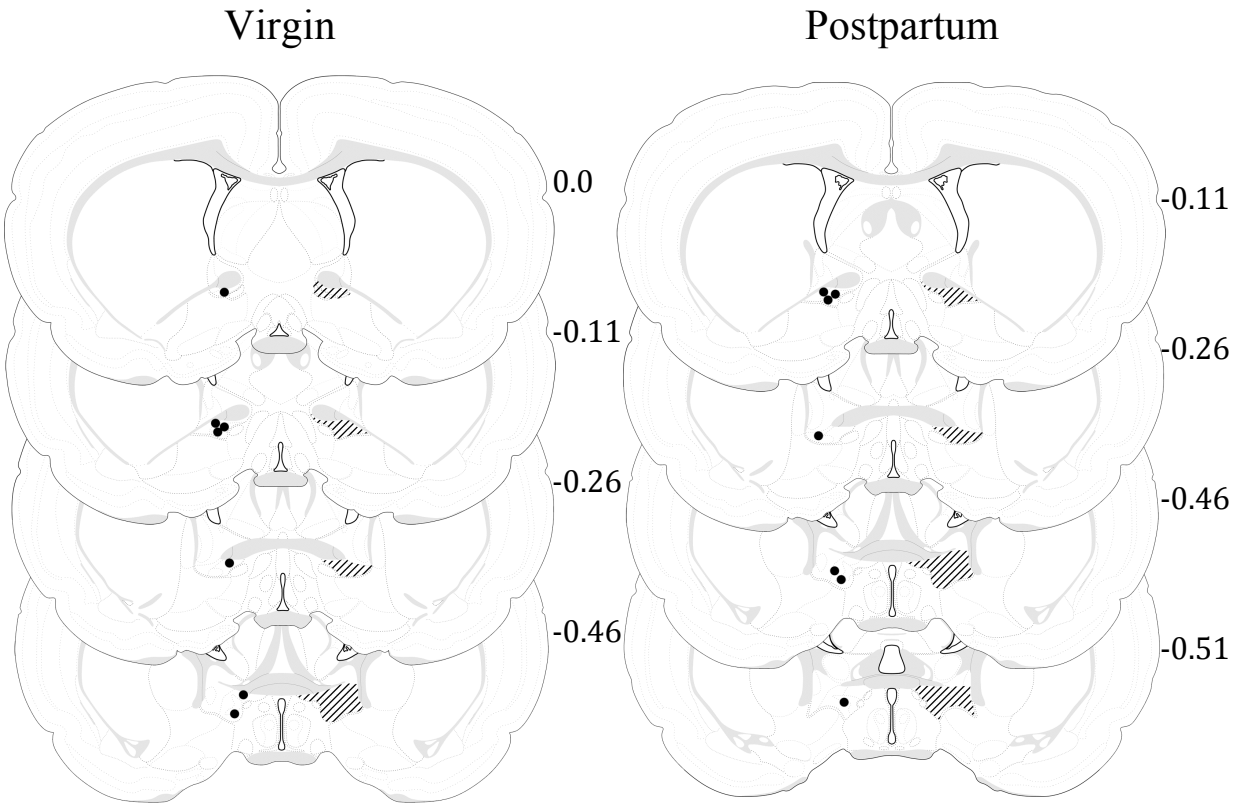

Figure 26 - Intra-BSTv infusion placements for virgin (left) and postpartum (right) rats receiving clonidine followed by saline. Each unilateral dot indicates the bilateral infusion placement of one subject. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

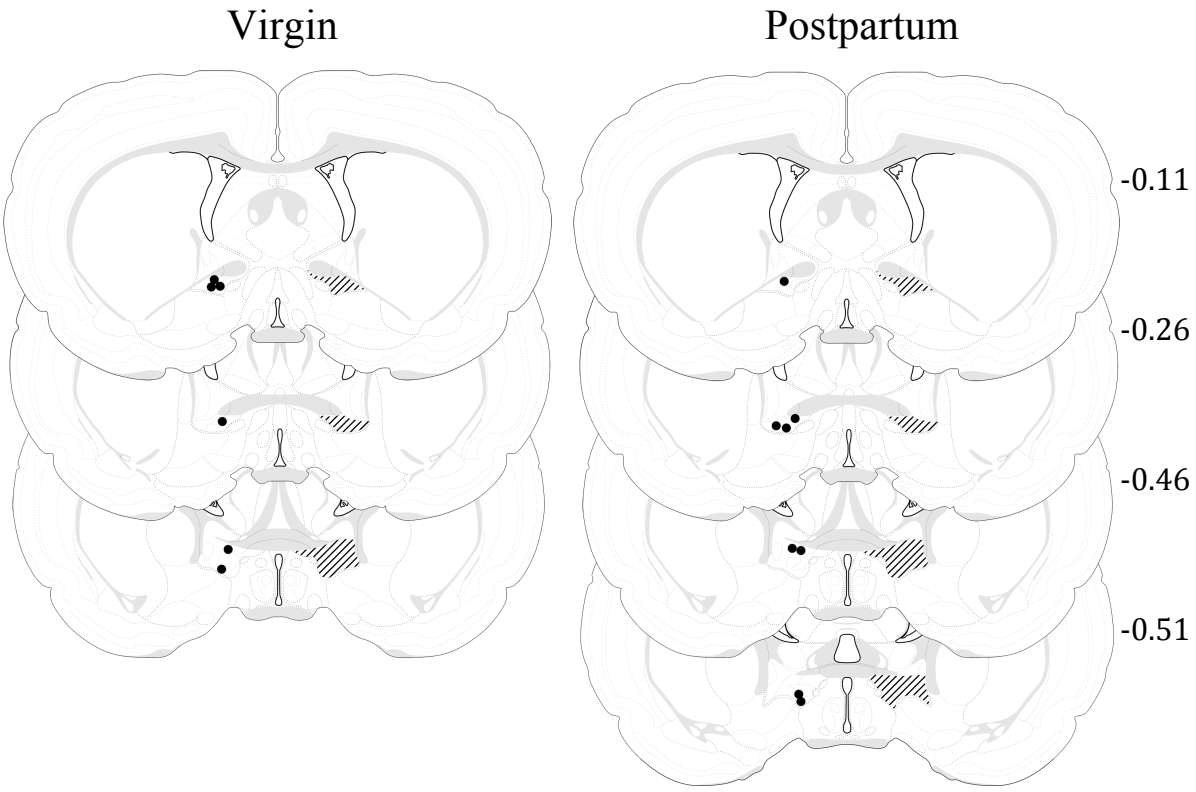

Figure 27 - Intra-BSTv infusion placements for virgin (left) and postpartum (right) rats receiving saline followed by yohimbine. Each unilateral dot indicates the bilateral infusion placement of one subject. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

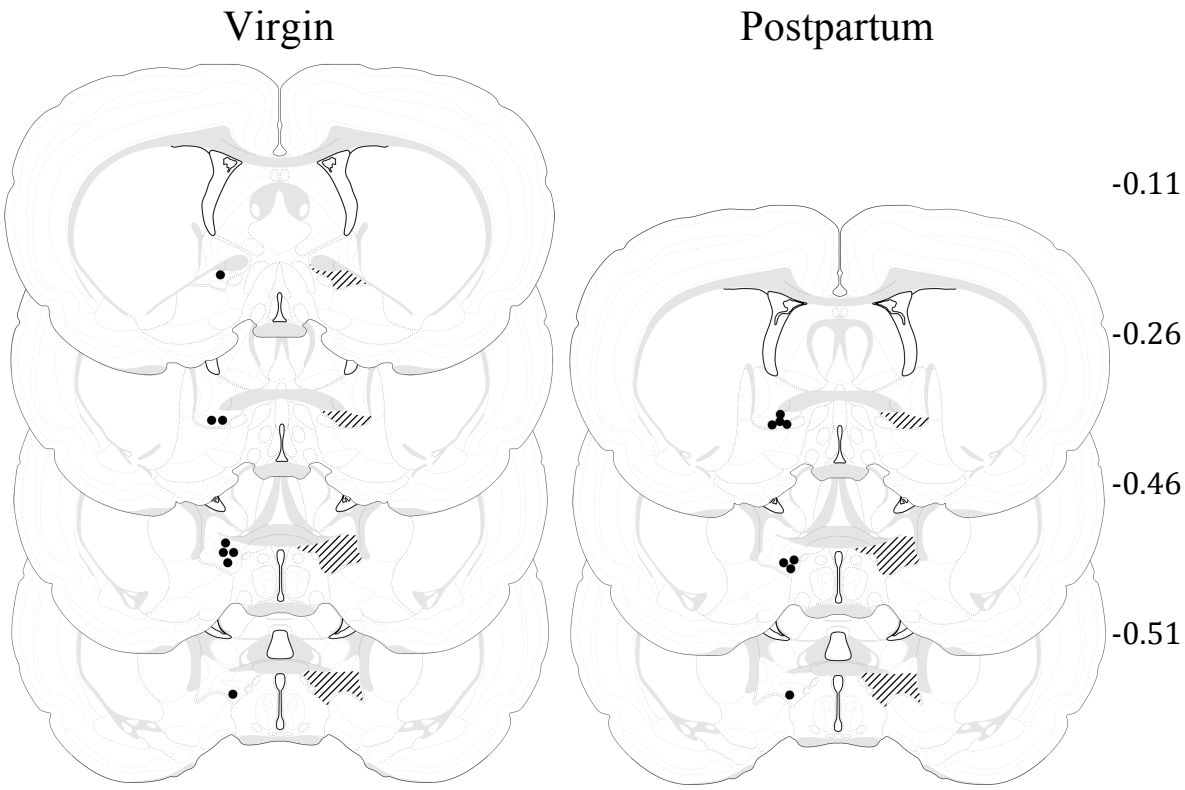

Figure 28 - Intra-BSTv infusion placements for virgin (left) and postpartum (right) rats receiving clonidine followed by yohimbine. Each unilateral dot indicates the bilateral infusion placement of one subject. Numbers on right indicate distance (mm) from Bregma according to Swanson (1998).

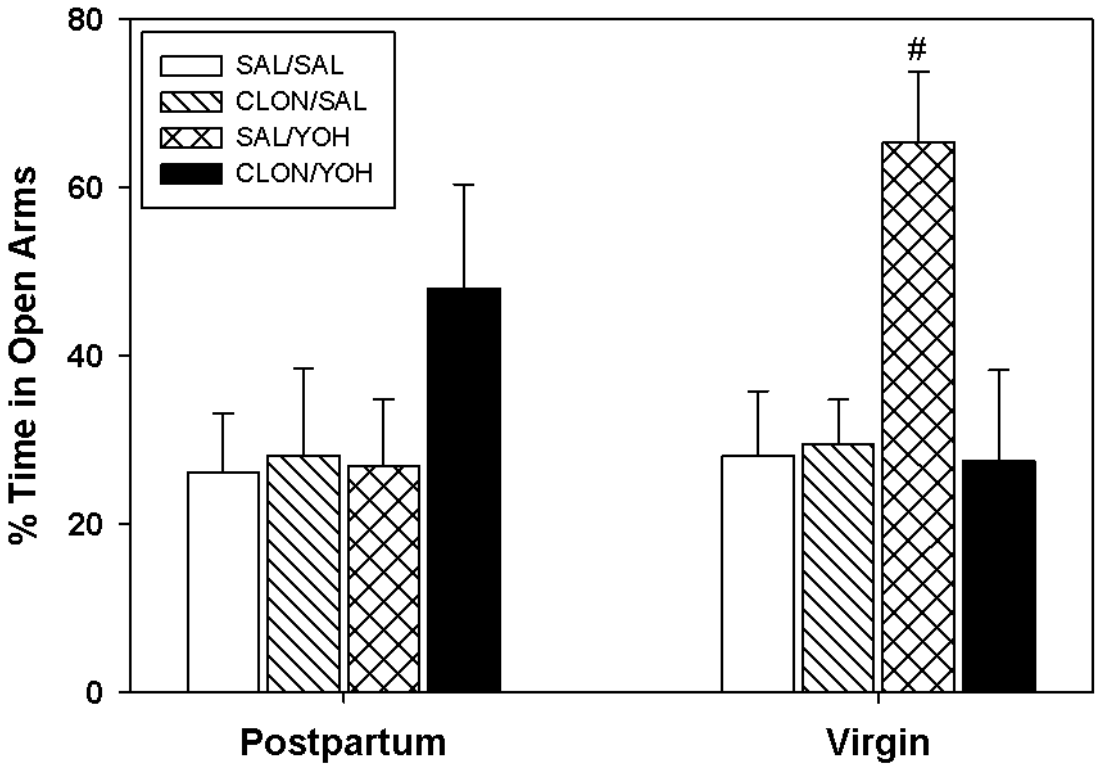

Figure 29 - Percentage of time (mean ± SEM) postpartum and virgin rats spent in the open arms of the elevated plus maze after intra-BSTv double-infusion of saline, clonidine, or yohimbine. # indicates site of significant interaction ($p \leq .05$).

	Saline/Saline	Clondine/Saline	Saline/Yohimbine	Clonidine/Yohimbine
Open arm entries	13 ± 1^{a}	11 ± 1^{a}	11 ± 1^{a}	7 ± 1^{b}
Closed arm entries	13 ± 1^{a}	9 ± 1^{b}	8 ± 1^{b}	5 ± 1^{c}
Total arm entries	26 ± 2^{a}	21 ± 2^{ab}	20 ± 2^{b}	12 ± 1^{c}

Table 9 - Number of arm entries rats made in the elevated plus maze after intra-BSTv double infusions of saline, clonidine, and/or yohimbine. Different letters (a,b,c) indicate statistical differences ($p \leq .0125$) collapsed across reproductive state.

	Virgin SAL/ SAL	Virgin CLON/ SAL	Virgin SAL/ YOH	Virgin CLON/ YOH	PP SAL/ SAL	PP CLON/ SAL	PP SAL/ YOH	PP CLON/ YOH	Significant Effects
Sample Size	8	7	6	8	9	7	8	8	
Time in open arms (s)	130 ± 37	145 ± 29	290 ± 37	144 ± 59	109 ± 23	129 ± 47	120 ± 28	212 ± 58	-
Time in closed arms (s)	332 ± 40^{ab}	342 ± 27^{ab}	162 ± 45^{b}	386 ± 62^{a}	370 ± 47	367 ± 65	377 ± 54	219 ± 53	State x Drug
Total time in arms (s)	462 ± 18	488 ± 17	452 ± 29	530 ± 14	480 ± 36	496 ± 29	497 ± 30	431 ± 30	-
Open arm entries	13 ± 1	12 ± 1	15 ± 1	7 ± 1	12 ± 2	11 ± 2	9 ± 2	7 ± 1	Drug
Percent open arm entries	45 ± 4	55 ± 4	61 ± 3	57 ± 3	49 ± 3	54 ± 2	52 ± 4	62 ± 4	Drug
Closed arm entries	16 ± 2	10 ± 1	10 ± 2	5 ± 1	11 ± 1	8 ± 1	7 ± 1	4 ± 1	State, Drug
Percent closed arm entries	54 ± 4	44 ± 4	38 ± 3	42 ± 3	50 ± 3	45 ± 2	47 ± 4	37 ± 4	State
Total arm entries	29 ± 2	22 ± 2	25 ± 3	12 ± 1	24 ± 3	20 ± 3	17 ± 3	11 ± 1	State, Drug

Table 10 - Behavior of diestrous virgin and postpartum (PP) rats in the elevated plus maze after intra-BSTv double infusions of saline, clonidine, and/or yohimbine. Different letters (a,b) indicate statistical differences ($p \leq .0125$) within each reproductive state. Significant main effects and interactions are indicated at $p \leq .05$.

Discussion

Systemic injections of clonidine or yohimbine and anxiety-related behaviors

Injections of clonidine in Experiment 2a were expected to decrease anxiety-related behaviors in females. This was not found, as clonidine had no effect on open-arm exploration in the EPM. These results are surprising given that, in male rats, systemic injections of clonidine reduces anxiety-related behaviors in the EPM (Soderpalm & Engel, 1988), elevated X maze (EXM; Handley & Mithani, 1984), and conditioned startle paradigm (Davis et al., 1979), and clonidine also blocks the anxiogenic properties of yohimbine (Chopin et al., 1986; Johnston & File, 1989; Pellow et al., 1987).

There are numerous possibilities for the discrepancies between previous research and results in this current experiment. On the issue of clonidine's lack of anxiolytic effects, not all reports using clonidine have found reductions in anxiety. Acute injections of clonidine are unable to increase drinking from an electrified water source (Fontana & Commissaris, 1992); only after 8 weeks of posttest injections of clonidine do rats exhibit increased responses in this paradigm. These authors suggest that repeated clonidine injections alter the function of postsynaptic alpha-2 receptors to affect behavior. Furthermore, clonidine does not reduce the ascoutic startle reflex in virgin or postpartum rats compared to injections of saline (Toufexis et al., 1999). These data suggest that clonidine may not be the most effective noradrenergic drug for suppressing anxiety-related behaviors. One possible consideration for the current experiment could be the very narrow range of effective doses of clonidine. Although doses of less than 5 µg/kg have not been examined in EPM or EXM performance, doses of more than 80 µg/kg (Soderpalm & Engel, 1988) in the EPM or 75 µg/kg in the EXM (Handley & Mithani, 1984) actually *increase* anxiety-related behaviors in male rats. Although doses used in Experiment 2a

were in or near Soderplam & Engel's (1988) effective anxiolytic range, they may have been too low for use in female rats. In support, females are less sensitive to some effects of clonidine than males; high doses of clonidine (250 μg/kg) sedate males to a greater extent than females when rats are given a nociceptive stimulus (Kiefel & Bodnar, 1992). Future experiments should examine effects of a higher range of doses on females' behaviors in the EPM.

In addition to the ineffectiveness of clonidine, dams separated from their litters 4 hours before testing did not exhibit reduced open-arm exploration when compared to virgins. There may be reasons for this absence of an effect. Previous studies showing a similar anxiety profile between dams separated from their litter 4 hours before testing and diestrous virgins used females that were otherwise unmanipulated (Lonstein, 2005; Smith and Lonstein, 2008, although see Figueira et al., 2008). Females in those experiments were moved directly from their home cages to the EPM. Subjects in this experiment, however, received i.p. injections immediately before testing. We have shown that injecting a dam with saline tends to decrease anxiety-related behaviors in the EPM when compared to dams that did not receive an injection (Cavanaugh & Lonstein, unpublished data). To clarify, separated dams expected to exhibit low open-arm exploration after i.p. injections, instead, spent unusually higher percentages of time in the open arms. The relatively gentle handling and restraint occurring during drug infusion may stimulate behavior that would increase time spent in the open-arms of the EPM in dams separated from their litter. Indeed, as will be discussed later, results from Experiment 2f show that intracranial infusions of saline immediately preceding infusions of an anxiogenic drug block that drug's anxiety-promoting effects in the EPM. Additional research is required to determine how the effects of receiving an injection can alter anxiety-related behaviors.

In contrast to clonidine, Experiment 2b demonstrated that peripheral injections of

yohimbine significantly decreased open-arm exploration in the EPM. Postpartum rats receiving the highest dose of yohimbine (5 mg/kg), but not the low dose (1 mg/kg), spent a lower percentage of time in the open arms compared to dams receiving vehicle. In virgin rats, both doses of yohimbine significantly reduced the percentage of time spent in the open arms. These results agree with previous reports showing that yohimbine increases anxiety-related behaviors in the EPM in male rats (Johnston & File, 1989; Pellow et al., 1985) and suggests that increasing NE release increases the display of anxiety-related behaviors. Furthermore, that the low dose of yohimbine increased anxiety-related behaviors in virgin but not postpartum rats indicates different sensitivities toward the drug. Perhaps dams' downregulated alpha-2 receptor densities, as is observed in the PVN (Toufexis et al., 1998), occurs elsewhere in the nervous system to account for these dose-dependent effects?

There was also a significant main effect of reproductive state on open-arm exploration in Experiment 2b. As expected, diestrous virgins spent a lower percentage of time in the open arms compared to postpartum rats. This effect, however, was likely caused by decreased open-arm exploration in virgins at the lowest dose, which was not observed in dams. No difference was found between virgins and dams injected with vehicle. This is surprising because, unlike animals injected with clonidine, these dams were not separated from their litter 4 hours before testing. Numerous studies show that dams exhibit reduced anxiety-related behaviors compared to virgins (Bitran et al., 1991; Ferreira, et al., 1989; Fleming & Luebke, 1981; Hard & Hansen, 1985), which includes experiments from this chapter (Experiment 2d). It is difficult to determine why diestrous virgins injected with vehicle spent more time in the open arms. As previously discussed, simply injecting females with vehicle can increase exploration of the open arms. It may be possible that injecting virgins with vehicle decreased their anxiety to that of postpartum

rats while yohimbine increased anxiety over-and-above the effect of receiving an injection. Furthermore, a ceiling effect in dams may have prevented injections of vehicle from producing additional increases in open-arm exploration. That is, dams were already at their lowest state of anxiety and effects of an injection could not increase time spent in the open arms. This is not a completely satisfactory answer, though, as virgins in Experiment 2a *did not* exhibit increased open-arm exploration after injections of vehicle. A few published reports do show no differences in open-arm exploration in postpartum rats compared to virgins (Neumann et al., 2000; Silva et al., 1997). It could be that, by chance, differences in measures of anxiety do not emerge between dams and virgins in every experiment. Furthermore, manipulations such as injections and/or brief restraint stress diminish the likelihood of detecting such a difference. It should be noted that studies reporting no differences in anxiety did not control for stage of the estrous cycle, which also may account for their results (Marcondes et al., 2001).

Intra-BSTv infusion of clonidine, yohimbine, or idazoxan and anxiety-related behaviors

Infusions of clonidine directly into the BSTv failed to alter anxiety-related behaviors in dams or virgins, which is dissimilar to studies in male rats showing reduced anxiety after infusions of clonidine into the BSTv (Fendt et al., 2005; Schweimer et al., 2005). These reports, however, examined intra-BSTv infusions of clonidine to study behavioral responses such as startle (Schweimer, et al., 2005) and freezing (Fendt, et al., 2005). It is possible that clonidine is not effective for studying long-term coping mechanisms needed for exploration in the elevated plus maze. This requires further investigation because 1) peripheral injections of clonidine increase open-arm exploration in males (Soderpalm & Engel, 1988) and 2) clonidine readily crosses the blood brain barrier (Khan et al., 1999), suggesting that this drug affects the brain to

decrease long-lasting tests of anxiety.

Also, similar to Experiment 2b, Experiment 2d demonstrated that increasing the release of NE in the BSTv with yohimbine altered behaviors in the EPM. Both low (1 μg) and high (3 μg) doses of yohimbine significantly reduced the percentage of time dams spent in the open arms. Additionally, a reproductive state difference was found in that postpartum rats spent a higher percentage of time in the open arms compared to diestrous virgins. Furthermore, examination of the interaction between dose of yohimbine and reproductive state showed that dams displayed fewer anxiety-related behaviors after infusions of vehicle compared to virgins. Results from Experiment 2d is consistent with previous reports that yohimbine increases anxiety-related behaviors (Johnston & File, 1989; Pellow et al., 1985). Intra-BSTv infusions of yohimbine did not, however, reduce open-arm exploration in virgin rats (as seen in Expeirment 2b). One possible reason is that a floor effect existed in virgins; their anxiety may have been high enough that yohimbine was unable to further reduce open-arm exploration, although their open-arm exploration still could have been lower. Additionally, neither peripheral nor intra-BSTv administration of yohimbine affected the percentage of entries made into the open arms in either reproductive state. A higher percentage of open arm entries may indicate lower anxiety (Pellow et al., 1985), but numerous studies in female rats report differences in the percentage of time spent in the open arms without finding a difference in the percentage of open arm entries (Mann, 2006; Marcondes et al., 2001; Miller et al., 2010; Smith & Lonstein, 2008).

Intra-BSTv infusions of the more selective alpha-2 antagonist, idazoxan, in Experiment 2e did not mimic the anxiogenic effects induced by yohimbine. If anything, dams receiving the higher dose of idazoxan had increased exploration of the open arms. This is surprising given idazoxan's ability to affect other behaviors when infused within the brain. Microinfusions of

idazoxan into discrete brain regions increase pain sensitivity (Ortiz et al., 2008), suppresses feeding (Alexander et al., 1993), and is known to alter NE release in the hippocampus and hypothalamus (Fulford & Marsden, 1997). Although idazoxan is a more selective alpha-2 antagonist than yohimbine, its ability to alter anxiety-related behaviors in rats has not been established and I am unaware of any study that used intra-cranial infusions to idazoxan to examine anxiety in laboratory rats. Peripheral injections of idazoxan do block the anxiolytic effects of clonidine (Soderpalm & Engel, 1988) and alcohol (Taksande et al., 2010) in the EPM. Idazoxan injections alone, however, do not increase anxiety-related behaviors compared to vehicle in the EPM (Moser, 1989; Soderpalm & Engel, 1988). Idazoxan's higher selectivity for the alpha-2 receptor is useful for evaluating yohimbine's mechanism of action, and idazoxan does alter behaviors in female rats (see Experiments 1b/c), but why is it is unable to affect behaviors in the EPM is unclear.

The ineffectiveness of idazoxan on anxiety prompted Experiment 2f. Here, clonidine (an alpha-2 agonist) was infused into the BSTv immediately before yohimbine (an alpha-2 antagonist). If pretreatment with clonidine blocked the anxiogenic effects of yohimbine, then it is reasonable to assume that yohimbine exerts its affects by binding to the alpha-2 receptor. Unfortunately, the results from Experiment 2f did not provide a decisive indication of yohimbine's mechanism of action. There were no main effects of reproductive state or drug on the percentage of time females spent in the open arms. A significant interaction revealed that diestrous virgins receiving saline followed by yohimbine spent a higher percentage of time in the open arms compared to other virgin groups. This finding, however, is very difficult to interpret because yohimbine would have been expected to *decrease* open-arm exploration. Additionally, pretreatment of saline *or* clonidine was able to prevent the anxiogenic effects of yohimbine.

These results suggest that two consecutive infusions of any drug (or vehicle) reduces females' anxiety-related behaviors in the EPM. This is also consistent with my findings in Experiment 2a suggesting that peripheral injections of saline reduced anxiety in dams separated from their litter 4 hours before testing. It may be possible that additional habituation to the handling and injection procedures could avoid any such effects, but repeated handling itself reduces anxiety (Meerlo et al., 1999; Vallee et al., 1997) and would confound the measure of interest (*e.g.,* anxiety).

Specificity of yohimbine on anxiety-related behaviors

Although yohimbine clearly reduced open-arm exploration, it also reduced general locomotion in the EPM, as indicated by a lower number of closed arm and total arm (open + closed) entries (Pellow et al., 1985). Yohimbine also decreases mobility in other paradigms, such as the holeboard test (Chopin et al., 1986). One could argue that yohimbine had no effect on anxiety *per se*, but rather prevented rats from physically moving into the open-arms. This, however, is extremely unlikely for numerous reasons. First, Experiment 1a in Chapter 1 examining maternal behavior showed that yohimbine had no effect on general activity compared to vehicle-infused dams and, at the highest dose, tended to increase activity. Second, independent of its effects on anxiety, yohimbine has anti-sedative effects. Indeed, clonidine at high doses decreases locomotor activity in rats and this can be reversed by administration of yohimbine (Drew et al., 1979). Third, drugs that are very well known to *reduce* anxiety-related behaviors, such as diazepam, can also reduce total arm entries in the EPM (Pellow et al., 1985). This indicates that motor behavior does not necessarily correlate with indicators of anxiety-related behaviors in the EPM. Together, these points suggest that yohimbine's anxiogenic

effects were not the consequence of impaired locomotion.

Diffusion of yohimbine to other brain regions

Peripheral injections of yohimbine increased anxiety-related behaviors in the EPM but the site of action is unknown. Site-specific infusions of yohimbine into the BSTv showed that this brain region is at least partly responsible for the anxiogenic effects of peripheral injections. It is possible, however, that yohimbine diffused out of the BSTv and produced its effects by altering neural activity in other brain sites. This issue has been largely addressed in the Chapter 1, so will only be briefly discussed here. First, infusion volumes of 0.125 µL used in this experiment are relatively small. Many studies commonly use volumes of at least 0.5 µL and the diffusion of this volume is estimated to spread ~1 mm (Liu & Liang, 2009). Given that the dimensions of the BSTv are approximately 1 mm in length, it is unlikely that infusions one-quarter of the volume used in Liu & Liang (2009) spread far beyond the boundaries of the BSTv. Furthermore, a two-way ANOVA (reproductive state x dose of yohimbine) of infusions placed outside of the BSTv reveals that the 3 µg dose of yohimbine actually *increased* the percentage of time dams and virgins spent in the open arms ($F(2,26) = 6.68$, $p = 0.0046$). Most of these missed infusions were placed in the dorsal BST or very caudal BST. Although the low sample sizes (n = 4 for rats receiving 3 µg of yohimbine) make interpreting the meaning of this effect difficult, clearly infusions of yohimbine located closely outside the BSTv do not account for the decreased percentage of time spent in the open arms.

Yohimbine's effects on serotonergic and dopaminergic systems

As discussed in Chapter 1 in regard to its effects on maternal behavior, yohimbine has

mild affinity for the 5-HT1A receptor (Winter & Rabin, 1992) and application of yohimbine increases serotonin release in numerous brain regions (Brannan et al., 1991; Broderick, 1997; Cheng et al., 1993). Additionally, increasing the release of serotonin within the BSTv causes decreased excitability of this area (Rannie, 1999). It is, thus, possible that yohimbine via its effects on 5HT1A receptors increased serotoinin release to inhibit the BSTv and increase anxiety-related behaviors. This explanation may be too simplistic because alpha-2 receptors can exist on axon terminals containing serotonin (Maura et al., 1982; Saito et al., 1996; Trendelenburg et al., 1994) and administration of selective alpha-2 antagonists, such as idazoxan, can also increase the release of serotonin (Tao & Hjorth, 1992). Nevertheless, a quantitative difference between the two drugs may account for the efficacy of yohimbine but not idazoxan; yohimbine may be able to depress neural activity in the BSTv to a greater extent than idazoxan by recruiting a larger amount of both NE and serotonin release, which would presumably result in increased anxiety. This possibility is only speculative and requires additional investigation.

It may also be the case that yohimbine's anxiogenic properties may be the result of its effects on the D2 receptor. Yohimbine is a weak antagonist of the D2 receptor (Scatton et al., 1980) whereas idazoxan has no known binding affinity. Unfortunately, there are conflicting reports of the D2 receptor's relationship to anxiety. Antagonism of the D2 receptor in male rats increases anxiety-like behaviors in the black-white box (Timothy et al., 1999) and in the open field test (Siemiatkowski et al., 2000). Systemic injection LY-171555, a specific D2 agonist, however, does not block the anxiogenic effects of yohimbine as measured by the EPM (Pellow et al., 1987). Additionally, injections of some D2 antagonists *increase* exploration of the open-arms in an EPM in mice (Rodgers et al., 1994) and increase punished responding in a conflict test in rats (Pich & Samanin, 1986). Given the disagreement regarding the D2 receptor and

anxiety, and that yohimbine has relatively low affinity for the D2 receptor, yohimbine is probably not producing anxiogenic effects solely through the D2 receptor. Nevertheless, additional research would help determine if and/or how D2 antagonists, particularly in the BSTv, affect anxiety-related behaviors in postpartum female rats.

Effects of NE in the BSTv on anxiety-related behaviors and the larger neural circuitry

Exactly where the BSTv projects to alter anxiety-related behaviors, and the phenotype of these projections remains unclear. The BSTv has projections to numerous brain regions regulating anxiety, which as previously discussed, include (but are not limited to) the amygdala, paraventricular nucleus, anterior/ventromedial hypothalamic nuclei, mammillary region, and periaqueductal gray (PAG) (Dong & Swanson, 2006a,b; Numan & Numan, 1996) and activity in many of these brain regions is associated with heightened states of anxiety. One role of the BSTv may be to inhibit these brain regions associated with stimulating anxiety. Indeed, as previously introduced, the majority of neurons in the BSTv are GABAergic (Muganini & Oertel, 1985; Stefanova et al., 1997; Sun & Cassell, 1993) and approximately 60% of neurons immunreactive for Fos in maternally-behaving dams are also labeled with GAD, the rate-limiting enzyme in GABA synthesis (Lonstein & De Vries, 2000). I also found that ~60% of Fos-immunoreactive neurons in the BSTv are colocalized with GABA in dams exposed to the EPM (Smith & Lonstein, unpublished data). It is currently unknown where efferent GABAergic neurons from the BSTv terminate, however, they can have both short and long projections (Fisher et al., 1988; Roland & Sawchencko, 1993; Sun & Cassell, 1993). Therefore, GABA-containing neurons in the BSTv may project as distal as the PAG.

In particular, the PAG is well known to modulate anxiety in rats and is considered to be a

"final common pathway" for all defensive behaviors (for review, see Vianna & Brandao, 2003). In male rats, electrical stimulation of the PAG induces freezing whereas lesions of the PAG in postpartum rats (and probably male rats) increases exploration of the open arms in an EPM (Lonstein et al., 1998). Infusion of a GABA-A receptor antagonist into the PAG, which prevents GABA-mediated inhibition of this site, decreases open-arm exploration (Miller et al., 2010). Inhibitory projections directly from the BSTv to the PAG may modify anxiety but the BSTv can inhibit the PAG indirectly. The anterior (Risold et al., 1994) and ventromedial (Canteras et al., 1994) hypothalamic nuclei, central amygdala (Rizvi et al., 1991), and paraventricular nucleus (Geerling et al., 2010) all project to the PAG and neural activity in these regions are associated with fear and/or anxiety-related behaviors (for review, see Vianna & Brandao, 2003).

Although ~60% of Fos-IR neurons in the BSTv are colabeled with GAD (or GABA) during maternal behavior or testing in the EPM, it is unclear what phenotype comprises the other ~40% of neurons. Excitatory projections to the VTA (Dumont & Williams, 2004), for example, suggest that a population of neurons in the BSTv contain glutamate (Georges & Aston-Jones, 2002). Numerous excitatory and inhibitory neuropeptides are also synthesized by the BSTv, including CRF (Moga et al., 1994), substance P, enkephalin, and galanin (Gray & Magnuson, 1987). Regardless of the exact neurotransmitter system(s) that yohimbine affects, it is parsimonious to still suggest that yohimbine depresses neural activity in the BSTv and that this resulted in a decrease in anxiety-related behavior in postpartum and virgin female rats. Recall that increasing the release of NE (Casada & Dafny, 1993) or 5-HT (Rainnie, 1999) in the BSTv inhibits neuronal excitability. These data complement previous experiments showing that high-anxiety dams have reduced Fos-immunoreactivity in the BSTv compared to low-anxiety dams after exposure to an EPM (Smith & Lonstein, 2008).

Conclusions

This is the first examination of the effects of noradrenergic agonism and antagonism on postpartum and virgin female rats' behavior in the EPM. Increasing the release of NE with yohimbine either systemically or within the BSTv significantly increased anxiety-related behaviors while decreasing the release of NE with clonidine had no effect on anxiety-related behaviors. Furthermore, BSTv infusions of idazoxan, a more selective alpha-2 autoreceptor antagonist, did not mimic yohimbine's ability to decrease open-arm exploration. This latter finding suggests that yohimbine's affinity for 5-HT1A and D2 receptors may contribute to its anxiogenic properties, and future research could help clarify this question. Although it is unlikely that yohimbine exerted its effects by spreading outside of the BSTv, additional research is needed to determine the extent of this possible diffusion. Nevertheless, these experiments provide support for noradrenergic activity in the BSTv mediating female rats' anxiety-related behaviors.

The primary hypothesis guiding this dissertation is that interactions with pups reduce the release of NE in the BSTv, which affects excitability of BSTv projections and postpartum behaviors regulated by the BSTv. As previously described in Chapter 1, tactile input from dams' ventral trunk synapse in NE-producing neurons of the medulla and BST itself (Gerendai et al., 2001). Somatosensation from pups may reduce NE release in the BSTv to disinhibit GABAergic neurons. GABA, then, released from the BSTv could inhibit brain regions that promote anxiety-related behaviors. Indeed, suckling stimuli increases Fos-IR in the medulla and BSTv (Li et al., 1999). Further investigation is required to: 1) identify projection sites of GABAergic neurons from the BSTv and 2) determine if sensory stimulation from pups can alter the activity of these neurons.

Chapter 3 –Monoamine content in the BSTv, BSTd, and mPOA of postpartum and virgin female rats

Introduction

Chapters 1 and 2 have identified NE as a possible neurotransmitter affecting infant-directed and anxiety-related behaviors in female rats. Chapter 1 demonstrated that increasing the release of NE in either the BSTv or mPOA inhibits components of maternal behavior. Chapter 2, although the interpretation is unclear, showed that increasing the release of NE systemically and within in the BSTv promotes anxiety. This latter finding, however, requires further study because only yohimbine (the drug with less selectivity for NE) could alter anxiety-related behaviors. These chapters demonstrate that exogenously manipulating the release of NE, and possibly 5-HT, can affect social and emotional behaviors. It is unknown, however, if these changes reflect the natural endogenous neurochemical milieu in postpartum and virgin rats. In other words, do mother rats who care for infants exhibit reduced anxiety because they have naturally lower levels of NE in their BSTv compared to nulliparous virgins?

There are few studies examining the monoamine content of the BSTv and mPOA across reproductive state in female rats. Lonstein et al. (2003) measured concentrations of dopamine (DA) and serotonin (5-HT) in the mPOA of virgin, pregnant, and postpartum rats. Although they did not analyze NE, DA was lower on the day of parturition compared to postpartum day 7. The ratio of DOPAC, a metabolite of DA, to DA was not different among groups, suggesting that DA metabolism in the mPOA does not change across reproductive state. Additionally, no differences were reported in concentrations of 5-HT in the mPOA. Moltz et al., (1975) examined changes in NE content in the hypothalamus during pregnancy and after parturition; shortly after

birth, NE decreases while MHPG, a metabolite of NE, increases. This study, however, homogenized the entire hypothalamus and did not analyze concentrations of NE in discrete brain regions. There are numerous other reports of changes in monoamines across reproductive state in brain regions including the, cortex, hypothalamus, striatum, hippocampus, thalamus, and cerebellum (Desan et al., 1988; Luine, 1993; Macbeth et al., 2008 Smolen et al., 1987). Little is known how altering concentrations of monoamines in these brain sites would affect maternal behaviors and these reports did not include analyses of the BSTv.

A comprehensive analysis comparing levels of NE, 5-HT, DA, and their metabolites between diestrous virgin and postpartum rats would clarify if differences in these monoamines could contribute to the observed behavioral differences in their maternal behavior and anxiety-related behaviors. I predict that diestrous virgins have naturally higher concentrations of NE and/or turnover of NE compared to postpartum rats, and that this partially underscores the differences in behavior between the two reproductive states. Furthermore, this experiment may elucidate which specific neurotransmitter system may have been underlying the effects of drug infusions in Chapter 2. If I find that only 5-HT differed in the BSTv and mPOA of virgin and postpartum rats, for example, then those results would suggest that yohimbine and idazoxan's behavioral effects were mediated through the serotonergic system. All conclusions would have be made with caution, as such results would be correlational and do not imply causation.

Chapter 3 aimed to quantify the concentrations of the following monoamines and their metabolites using high performance liquid chromatography with circular dichroism (HPLC-CD): norepinephrine and 3-methoxy-4-hydroxyphenylglycol (MHPG), serotonin and 5-hydroxyindoleacetic acid (5-HIAA), dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) in the BSTv, dorsal BST (BSTd), and mPOA of diestrous virgins and postpartum rats.

Furthermore, postpartum rats were divided into two groups: those that remained in contact with their litter until sacrifice and those that had their litter removed 6 hours before sacrifice. These manipulations may determine how concentrations of monoamines: 1) differ across reproductive state (excluding pregnancy) and 2) are affected by recent physical contact with pups. This second point may provide insight into the mechanism for why anxiety-related behaviors increase in dams that had their litters removed 4 hours before testing in an elevated plus maze or light-dark box (Lonstein, 2005; Smith & Lonstein, 2008; Miller, Piasecki, Lonstein, in preparation). The BSTd was included to act as a control site; in male rats, it projects to some of the same sites as the BSTv (Dong et al., 2001; Dong & Swanson, 2004) but contains very little noradrenergic innveration compared to the ventral subdivision (Phelix, et al., 1992). It is, thus, important to determine if concentrations in NE are indeed different between the dorsal and ventral portions of the BST in female rats.

Methods

Subjects

Rats were housed under the same conditions described in Chapter 1 and 2. Virgin females designated to be in postpartum groups were mated and their litters were culled as described in previous chapters. Rats were assigned to one of three groups: diestrous virgins (virgins; n = 9), postpartum rats that remained in contact with their litter before sacrifice (PP-pups; n = 9), and postpartum rats that had their litter removed 6 hours before sacrifice (PP-nopups; n=8). Females assigned to the virgin group received daily vaginal smears to determine the stage of the estrus cycle; they were sacrificed one day after estrus and a postmortem smear confirmed their diestrous state. Dams were sacrificed on either postpartum day 7 or 8. All rats

were singly housed one week before sacrifice.

Sacrifice and tissue collection

Immediately before sacrifice, rats were placed in a 1-gallon plastic bag that was prefilled with CO_2. Bags were then filled with additional CO_2 and sealed. When rats stopped moving, they were brought to a nearby room (~20 ft away) and decapitated using a guillotine. Brains were quickly removed and frozen in cold 2-methylbutane before being wrapped in foil and stored at -80ºC. Only one rat was decapitated at a time and new bags were used for each rat. The entire procedure from the time rats were placed in the bag to the time their brains were frozen required approximately 2.5 min.

Brains were then placed in a cryostat and 1 mm-thick sections were obtained beginning at approximately -0.15 mm from Bregma. The BSTv, BSTd, and mPOA were all punched from the same brain slice. Bilateral punches of each brain region (Figure 30) were made with an 18-gauge stainless steel tube and weighed. Once samples were collected from each brain, they were placed in centrifuge tubes filled with 200 nl of cold 0.1M perchloric acid and homogenized. Tubes were centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and stored at --80ºC until processing with HPLC.

Detection of monomamines

Monoamine analysis was conducted at a core facility on the Michigan State University campus using procedures described by King (2008). HPLC-CD was performed using a commercial system (ESA Biosciences, Inc, Chelmsford, MA) consisting of a solvent delivery module (model 584), an autosampler (model 542) cooled to 4^{o}C and a Coulochem III detector,

which was equipped with a 5021A conditioning cell (electrode I) and a 5011A high sensitivity analytical cell (electrode II and III). Both cells use flow-through porous graphite electrodes. The high surface area of the detection electrodes results in an almost 100% reaction of the electroactive compound. Hydrodynamic voltammograms were obtained to determine the optimum potential for detection. The highest signal-to-noise results were obtained when electrode I was set at +200 mV, electrode II at +100 mV and electrode III at -280 mV. Chromatograms were obtained by monitoring the reduction current for working electrode III. The monoamines and metabolites were separated on an HR-80 (C18, 3 μm particle size, 80 mm length x 4.6 mm I.D.) reversed-phase column (ESA Biosciences, Inc.). The mobile phase was a commercial Cat-A-Phase II (ESA Biosciences, Inc.) that consisted of a proprietary mixture of acetonitrile, methanol, phosphate buffer and an ion pairing agent (ca. pH 3.2). The optimum flow rate for the separation was 1.1 mL/min. The separation column was maintained at 35^{o}C. The limit of detection was 0.1 ng/ml for NE and DA and 0.5 ng/ml for 5-HT and 5-HIAA. Unfortunately, DOPAC and MHPG were not detectable using this HPLC-CD system and were not included in the data analyses below.

Data Analysis

Concentrations of monoamines were analyzed with a 3 x 3 ANOVA with brain site (BSTv, mPOA, and BSTd) and reproductive state (postpartum with pups, postpartum without pups, virgin) as factors. Statistical significance was indicated by $p \leq 0.05$. In the case of significant main effects, pairwise comparisons were performed using Fisher's LSD post-hoc tests that were Bonferroni corrected with significance at $p \leq 0.016$. Significant interactions were analyzed with one-way ANOVAs to detect differences in specific concentrations of monoamines

within each reproductive state and brain site. These were also Bonferroni corrected with significance at $p \leq 0.016$.

Results

Subjects

The dialysate from one subject in the PP-pups group was not analyzed for 5-HT or 5-HIAA, which left the PP-pups group with a sample size of $n = 8$ for these chemicals. Additionally, the BSTd from one virgin rat was improperly weighed after the tissue punch and was excluded from analysis, resulting in sample sizes for the virgin group of $n = 9$ for the BSTv, $n = 9$ for the mPOA, and $n = 8$ for the BSTd.

Norepinephrine

There was a significant main effect of reproductive state ($F(2,68) = 3.19$, $p = 0.048$) on tissue concentrations of NE. Post-hoc analysis revealed that PP-pups tended to have more NE than PP-nopups ($p = 0.030$) or virgin ($p = 0.071$) rats but these differences were not statistically significant at the Bonferroni-corrected levels. There were also main effects of brain site ($F(2,68) = 205.24$, $p < 0.0001$) on levels of NE (Table 11, Figure 31). Brain sites were all significantly different from each other; the BSTv contained the highest concentrations of NE compared to the mPOA, which was greater than the BSTd. There was no significant interaction between reproductive state and brain site ($F(4,68) = 1.78$, $p = 0.14$).

Dopamine

Concentrations of DA were significantly affected by reproductive state ($F(2,68) = 6.94$, $p = 0.0018$). PP-pups had more DA than PP-nopups and tended to have more DA than virgins ($p = 0.048$), but this latter comparison was not statistically significant. There was also a main effect of brain site ($F(2,68) = 189.60$, $p < 0.001$) on DA content (Table 11, Figure 32). The BSTd contained more DA than the BSTv and mPOA, which were similar to each other. There was also a significant interaction between reproductive state and brain site ($F(4,68) = 4.42$, $p = 0.0031$). A one-way ANOVA revealed that DA content in the BSTd of PP-nopups was significantly less than PP-pups and tended to be lower than virgins ($F(2,22) = 5.27$, $p = 0.014$). A significant difference in the concentration of DA was also found among groups in the mPOA ($F(2,22) = 3.99$, $p = 0.033$), with PP-pups tending to have more DA than PP-nopups or virgin rats, althoughpairwise group differences were not significant using the Bonferonni-corrected tests.

Serotonin, 5-HIAA, and Serotonin Turnover

There was no main effect of reproductive state on concentrations of 5-HT ($F(2,65) = 1.28$, $p = 0.29$) but there was a significant main effect of brain site ($F(2,65) = 4.57$, $p = 0.014$). The content of 5-HT was higher in the BSTv than mPOA but no difference was found between BSTv and BSTd or between the mPOA and BSTd. There was no significant interaction between reproductive state and brain site in 5-HT levels ($F(4,65) = 1.08$, $p = 0.38$) . A similar pattern was found for 5-HIAA. There was a non-significant trend towards a main effect of reproductive state ($F(2,65) = 3.08$, $p = 0.053$). A significant main effect of brain site, however, was found ($F(2,68) = 3.19$, $p = 0.048$); the BSTv and BSTd contained higher concentrations of 5-HIAA compared to the mPOA, but the BSTv and BSTd did not differ from each other (Table 11, Figure 33, 34).

Serotonin turnover (5-HIAA/5-HT) differed among groups (Table 11, Figure 35). There was a main effect of reproductive state ($F(2,65) = 16.14$, $p < 0.0001$) such that PP-pups had a significantly greater turnover compared to PP-nopups or virgins. Turnover in PP-nopups, however, did not differ from virgins. In addition, there was a main effect of brain site on 5HT turnover ($F(2,65) = 9.09$, $p = 0.0003$). Both the BSTv and BSTd had greater 5HT turnover compared to the mPOA, with the BSTv and BSTd not differing from each other. There was no significant interaction between reproductive state and brain site ($F(2,68) = 0.40$, $p = 0.81$)

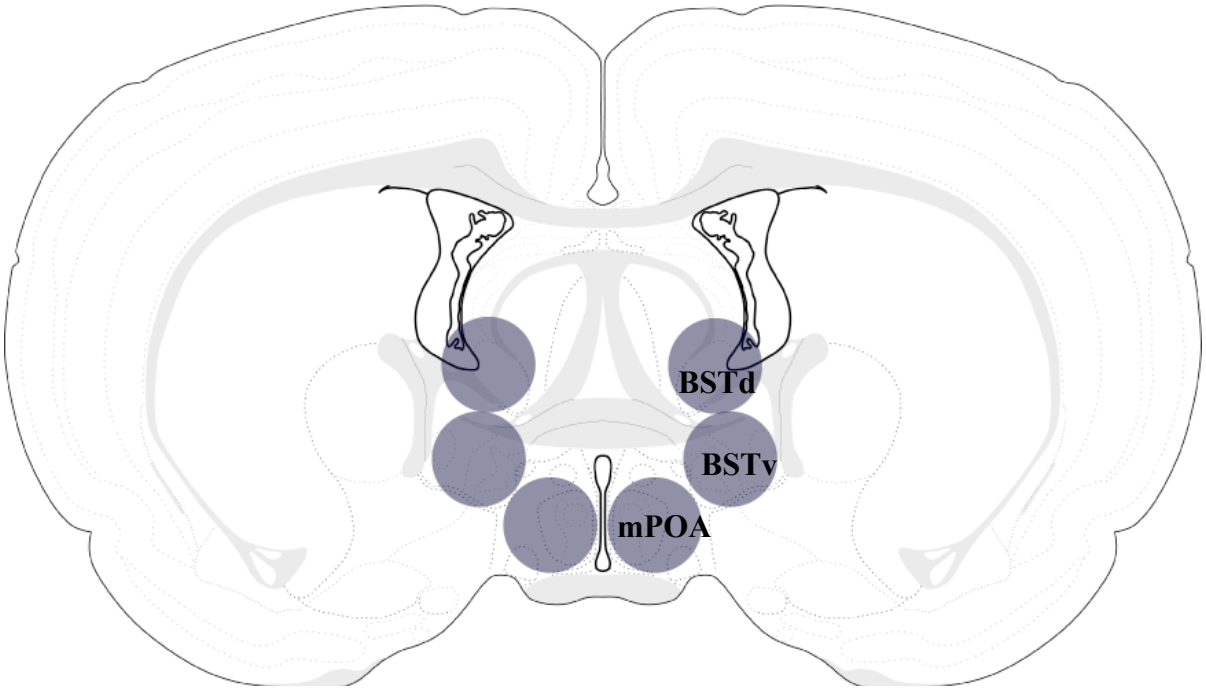

Figure 30 - Location of bilateral tissue punches including the BSTv, BSTd, and mPOA using an 18-gauge tube (modified from Swanson [1998]).

Monoamine	Brain Site	PP – pups	PP – no pups	Virgin	Significant Effects
NE	BSTv[a]	11.86 ± 0.75	9.57 ± 1.09	10.88 ± 0.75	State, Site
	mPOA[b]	4.70 ± 0.58	3.20 ± 0.35	2.48 ± 0.39	
	BSTd[c]	1.33 ± 0.17	1.64 ± 0.49	1.23 ± 0.20	
DA	BSTv[a]	1.10 ± 0.075	0.83 ± 0.046	1.19 ± 0.26	State, Site, State x Site
	mPOA[a]	0.72 ± 0.14	0.37 ± 0.042	0.36 ± 0.069	
	BSTd[b]	9.27 ± 0.85	5.45 ± 0.78	7.71 ± 0.88	
5-HT	BSTv[a]	0.67 ± 0.045	1.02 ± 0.13	0.93 ± 0.060	Site
	mPOA[b]	0.65 ± 0.12	0.64 ± 0.11	0.60 ± 0.13	
	BSTd[ab]	0.76 ± 0.087	0.83 ± 0.090	0.72 ± 0.13	
5-HIAA	BSTv[a]	0.75 ± 0.075	0.83 ± 0.12	0.75 ± 0.11	Site
	mPOA[b]	0.50 ± 0.077	0.36 ± 0.074	0.28 ± 0.11	
	BSTd[a]	0.76 ± 0.089	0.67 ± 0.072	0.48 ± 0.10	
5-HT Turnover	BSTv[a]	1.11 ± 0.061	0.79 ± 0.058	0.79 ± 0.11	State, Site
	mPOA[b]	0.88 ± 0.12	0.49 ± 0.082	0.41 ± 0.10	
	BSTd[a]	1.01 ± 0.066	0.81 ± 0.062	0.66 ± 0.10	

Table 11 - Concentrations of monoamines (ng/mg) as a function of brain site (BSTv, mPOA, and BSTd) and group of rats (PP-pups, PP-nopups, and Virgins). Main effects are statistically significant at $p \leq 0.05$. Brain sites that do not share letters (a,b,c) are significantly different from each other at $p \leq 0.016$.

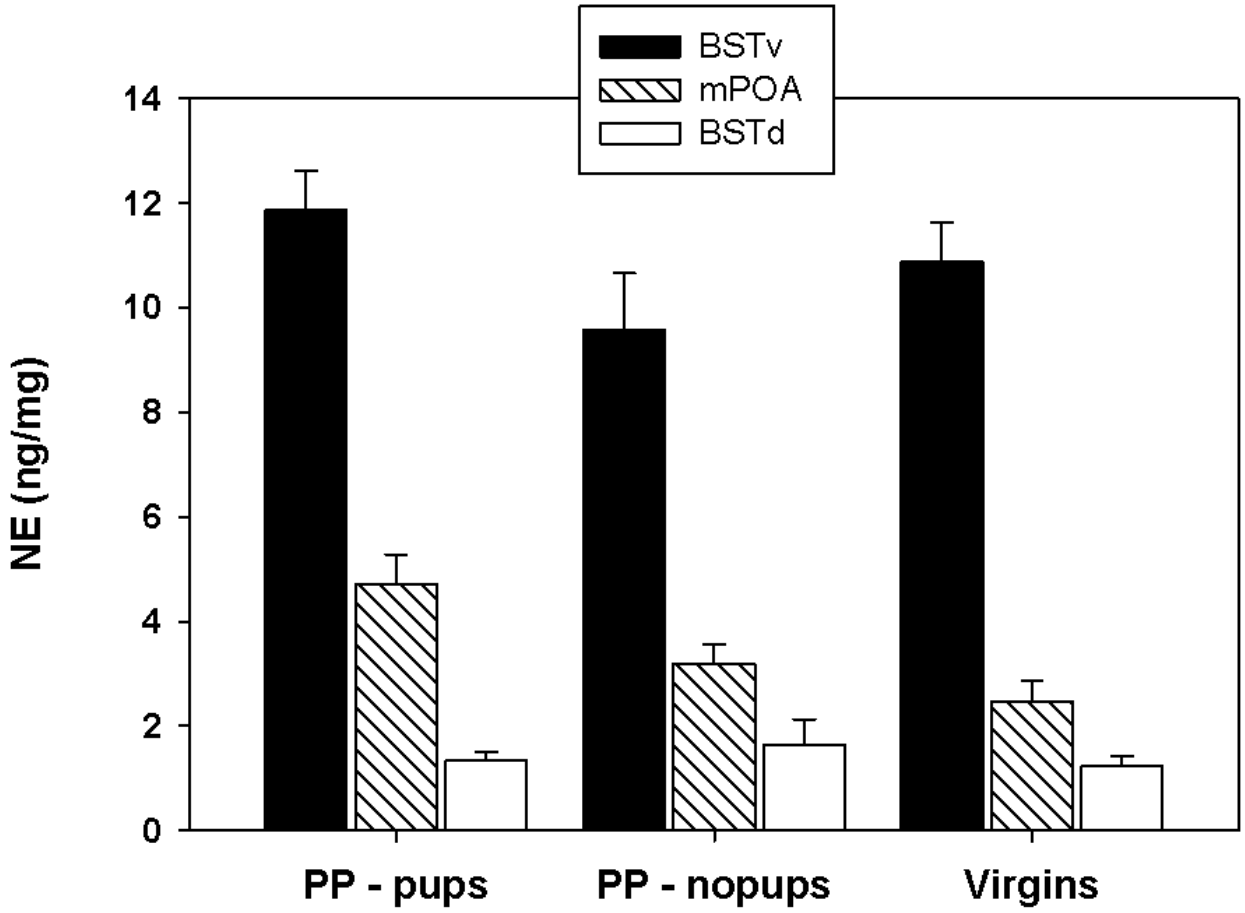

Figure 31 - Concentrations of NE (ng/mg) in the BSTv, mPOA, and BSTd of postpartum rats with pups (PP-pups), without pups (PP-nopups) and in diestrous virgins (Virgins). Significant main effects of brain site are shown in Table 11.

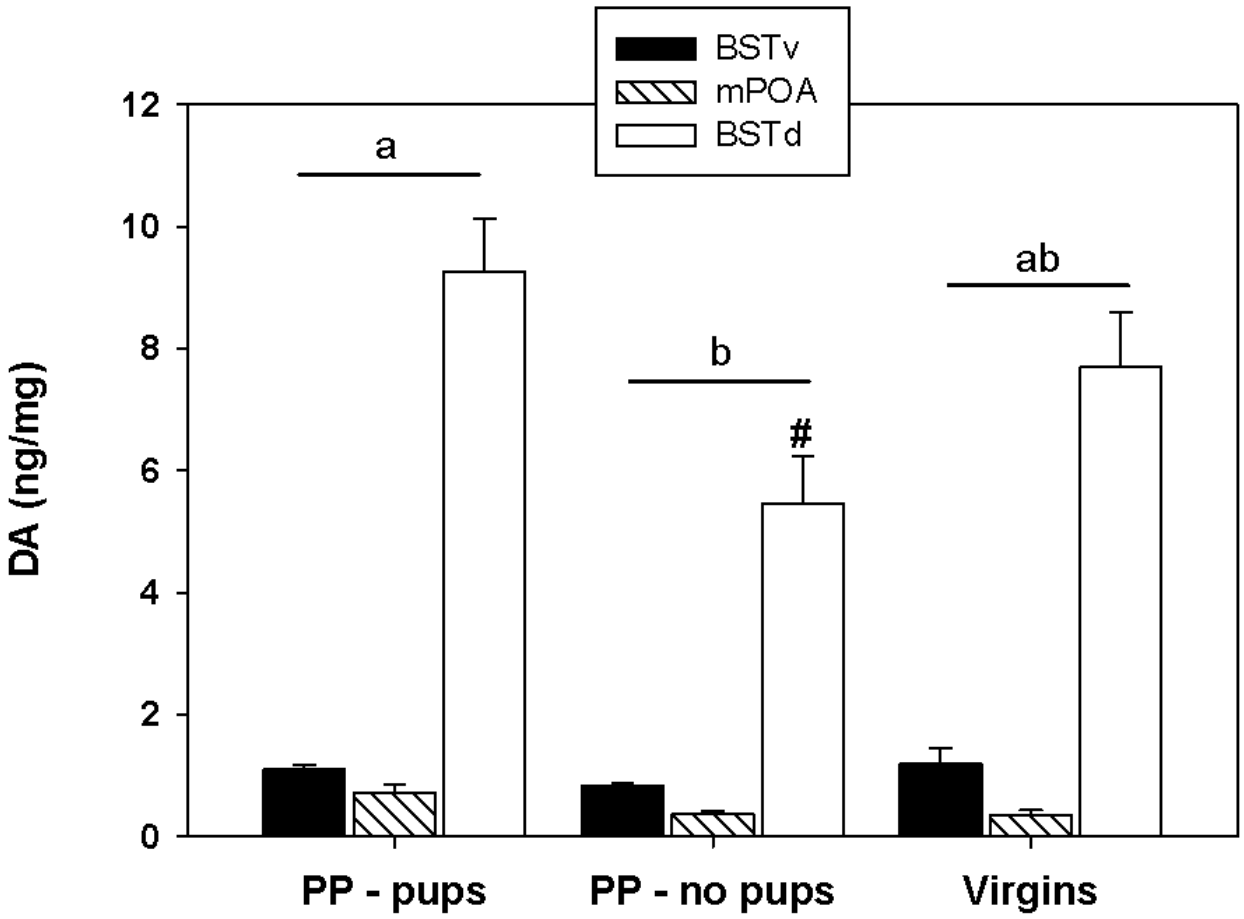

Figure 32 - Concentrations of DA (ng/mg) in the BSTv, mPOA, and BSTd of postpartum rats with pups (PP-pups), without pups (PP-nopups) and in diestrous virgins (Virgins). Significant main effects of brain site are shown in Table 11. Rat groups that do not share similar letters (a,b) are significantly different from each other. Main effects and interactions of reproductive state and brain sites are indicated at $p \leq 0.05$. Likely site of significant interactions is indicated by # (BSTd x reproductive state) and is statistically significant at $p \leq 0.016$.

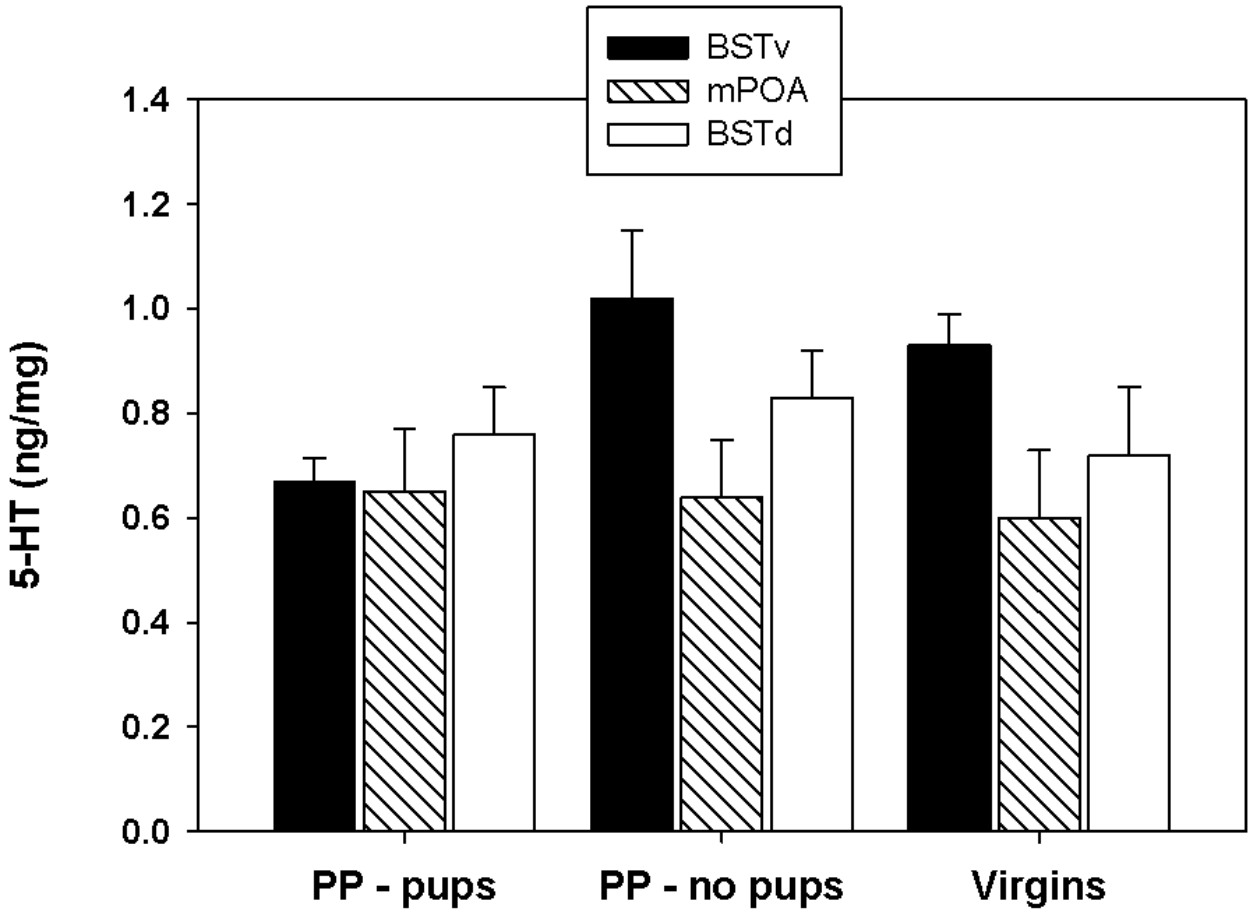

Figure 33 - Concentrations of 5-HT (ng/mg) in the BSTv, mPOA, and BSTd of postpartum rats with pups (PP-pups), without pups (PP-nopups) and in diestrous virgins (Virgins). Significant main effects of brain site are shown in Table 11.

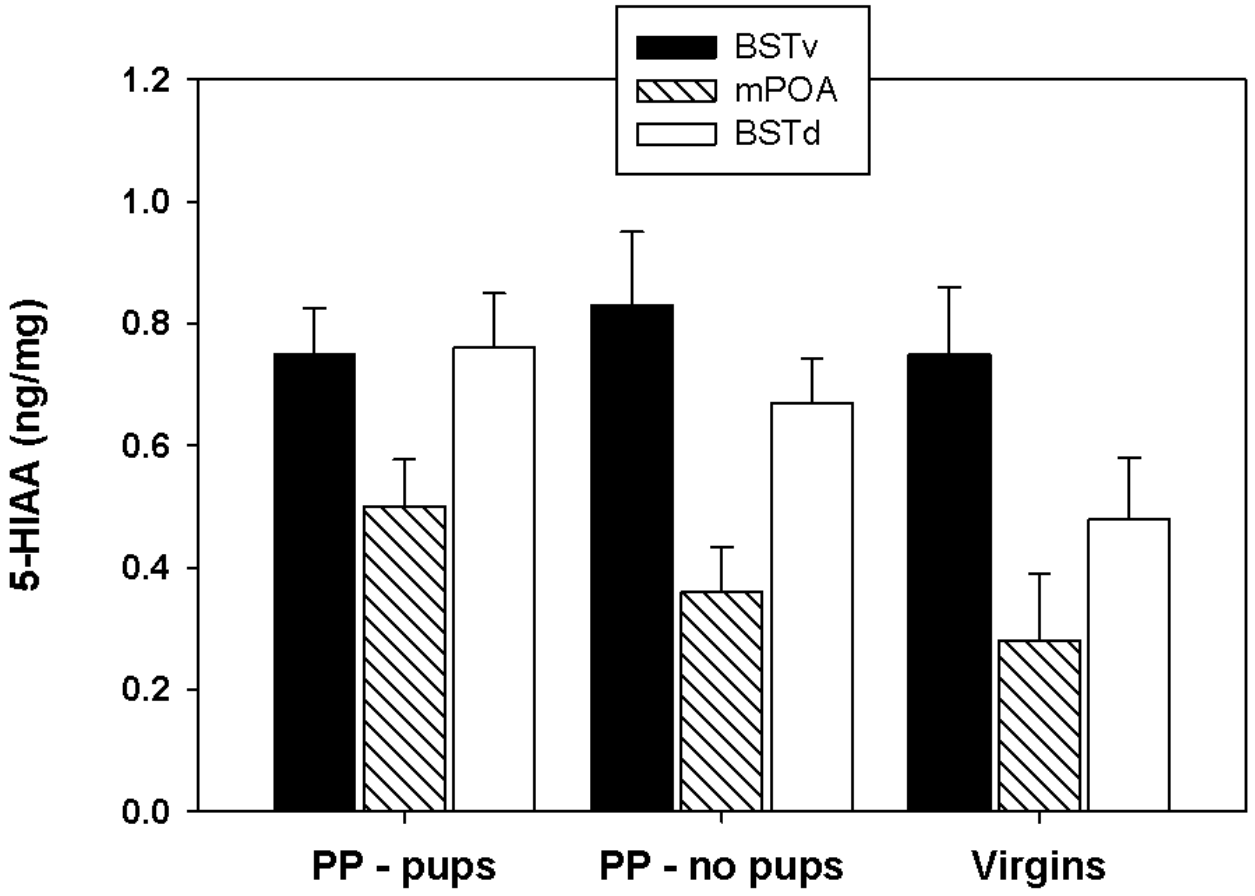

Figure 34 - Concentrations of 5-HIAA (ng/mg) in the BSTv, mPOA, and BSTd of postpartum rats with pups (PP-pups), without pups (PP-nopups) and in diestrous virgins (Virgins). Significant main effects of brain site are shown in Table 11.

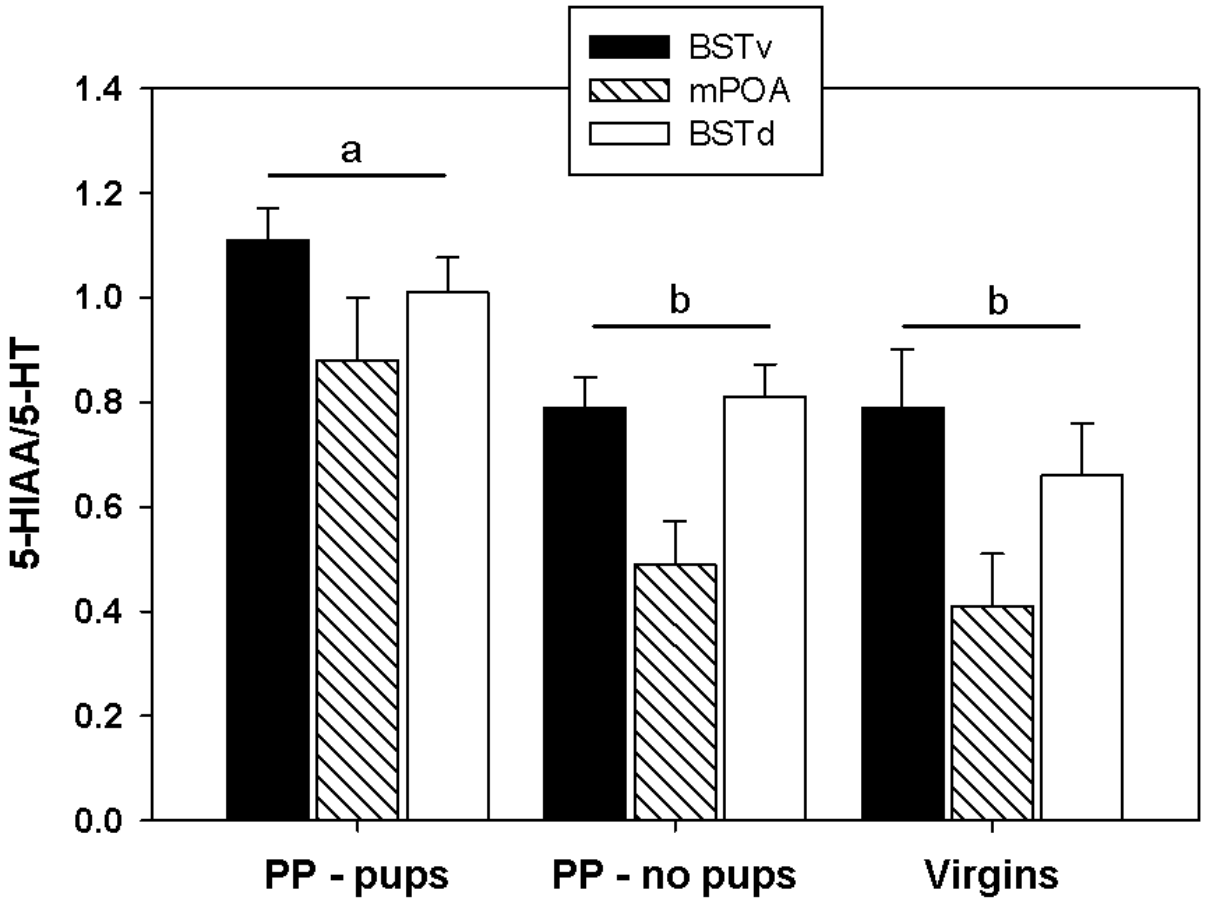

Figure 35 - Concentrations of 5-HT turnover in the BSTv, mPOA, and BSTd of postpartum rats with pups (PP-pups), without pups (PP-nopups) and in diestrous virgins (Virgins). Significant main effects of brain site are shown in Table 11.

Discussion

Results from Chapter 3 revealed numerous differences in monoamine concentrations among brain sites and female rats: (1) The BSTv contained the highest concentration of NE followed by the mPOA and lastly BSTd, 2) There was a significant main effect of reproductive state in NE levels but post-hoc analysis showed that the PP-pups group only tended to have more NE than PP-nopups and virgins, 3) The BSTv had more 5-HT than the mPOA but did not differ from the BSTd, 4) Both BSTv and BSTd, however, contained more 5-HIAA than the mPOA, 5) there was no significant difference among groups in levels of 5-HT or 5-HIAA, 6) 5-HT turnover in the BSTv and BSTd was greater than that in the mPOA, 7) The PP-pups group had greater 5-HT turnover than PP-nopups or virgins, 8) the BSTd contained more DA than the BSTv or mPOA, 9) females in the PP-pups group had more DA than PP-nopups, and lastly, 10) there was significantly less DA specifically in the BSTd of PP-nopups compared to PP-pups (and no difference compared to virgins). Unfortunately, MHPG and DOPAC were not detected using HPLC and so NE and DA turnover could not be determined.

This is the first study to compare monoamine concentrations in the BSTv, mPOA, and BSTd among virgin female rats and postpartum rats with and without pups before sacrifice. In general, these results agree with previous reports measuring monoamine content in these regions. Lonstein et al. (2003) did not find differences in 5-HT, 5-HIAA, or DA within the mPOA between dams with pups and diestrous virgins, which my results support. These authors also reported a significantly higher amount of 5-HT turnover in the mPOA of postpartum rats compared to virgins. The current results also show a similar difference in the mean amount of 5-HT turnover in the mPOA, although a comparison in my case was not justified because of a non-significant interaction between reproductive state and brain site ($p = 0.81$). High concentrations

of NE in the BSTv also agree with evidence showing high NE levels in this region in male rats (Funtealba et al., 2000). Additionally, that the BSTv contained more NE than the BSTd or mPOA supports evidence demonstrating high densities of DβH-immunoreactivity in the BSTv compared to other brain sites (Fendt et al., 2005; Kilts & Anderson, 1986; Woulfe et al., 1990).

Differing monoamine concentrations among female rats

One difference in monoamine concentrations among groups of rats was higher 5-HT turnover of females with pups (PP-pups) compared to those without pups (PP-nopups) or virgins. Because this depended on the presence of the litter, and not just reproductive state, it is likely that 5-HT turnover was affected by sensory stimulation that dams received from pups. The exact type of sensation remains unknown. Future experiments could examine 5-HT turnover when dams are allowed only distal or distal and proximal contact with their litter. Placing pups in a wire mesh cage, for example, would allow dams to smell, see, and hear infants but would prevent most tactile stimulation. In addition to sensory stimulation, the PP-pups were also allowed to nurse, which may have contributed to increased 5-HT turnover. Previous evidence gives precedence for 5-HT's involvement in lactation, particularly in prolactin secretion. Systemically inhibiting the synthesis of 5-HT prevents the suckling-induced rise in plasma prolactin (Kordon et al., 1973) and serotonergic activity in the hypothalmus, such as the mPOA, affect plasma prolactin levels. Indeed, 30 min of suckling increases 5-HT turnover in the mPOA (Johnston et al., 1984) and electrically stimulating the mPOA reduces the suckling-induced rise in prolactin secretion (Wiersma & Kastelijn, 1990). This suggests that 5-HT turnover reduces neural activity in the mPOA to permit continued prolactin secretion.

In addition to 5-HT turnover, concentrations of DA differed among groups of females.

PP-pups had more DA than PP-nopups but neither of these groups differed from virgins. This suggests that differences in DA concentrations depends on litter presence; it is possible that simply removing pups caused a change in DA content once females are maternal. Future experiments examining DA concentrations in maternally-sensitized virgins that had pups removed could address this issue. Nevertheless, similar to the discussion of 5-HT turnover, sensory stimulation from pups and/or the hormones of lactation may underlie the differences in DA content. It is particularly important to note that a significant interaction revealed that PP-pups had significantly more DA only in the BSTd compared to PP-nopups. Because of this interaction and the apparant lack of differences between groups in DA in the mPOA and BSTv, only the BSTd will be discussed further.

Relatively little is known how interactions with pups affects neural activity or DA concentrations in the BSTd. The BSTd contains fibers immunoreactive for tyrosine hydroxylase (TH) (Phelix et al., 1992), and these fibers originate from the VTA, substantia nigra, and retrorubral field (Hasue et al., 2002; Meloni et al., 2006). Stimuli from pups impacts this region as suckling elicits Fos-IR in the BST (Li et al., 1999). This may not be specifc to pup-related interactions because Fos-IR in the BSTd increases in response to pups or palpable food (Numan & Numan, 1995). Interestingly, Numan & Numan (1995) found that olfactory bulbectomy signficantly reduced Fos-IR in the BSTd but not BSTv or mPOA. The authors postulate that Fos-IR in intact dams results from olfactory information, regardless of whether it is associated with sensory cues from infants. Stimulation from pups may alter DA concentrations in the BSTd to affect another neurochemical, corticotropin-releasing factor (CRF). The BSTd is dense with neurons immunoreactive for CRF (Merchenthaler, et al., 1982; Swanson et al., 1983) and lactating rats exhibit blunted HPA activity (Neumann et al., 1998; Stern et al., 1973; Walker et

al., 1992) in response to stressors, which may be important for the demands of lactation and possibly fear or anxiety-related behaviors (for review, see Numan & Insel, 2003). Reduced corticosterone release in response to a stressor is also reflected by differences in levels of CRF mRNA within discrete brain regions. Injections of hypertonic saline, for example, increases CRF mRNA in the PVN of virgins but not lactating rats (Lightman & Young, 1989). Interestingly, removing dams' litters for 48 hrs increases CRF mRNA (Lightman & Young, 1989) levels to that of virgins, suggesting that suckling and/or sensory stimulation from pups maintains dams' blunted response. Although not examined in female rats, stress can increase CRF mRNA in the BSTd (Carrasco et al., 2008; Kim et al., 2006) and CRF activity in the BSTd is affected by DA. Intra-ventricular infusions of a D1 receptor antagonist blocks the increase in startle after rats are systemically injected with CRF (Meloni et al., 2006) and application of DA directly to the BSTd increases neuronal excitability (Kash et al., 2008). In this latter study, CRF antagonists blocked the DA-dependent rise in excitability. DA in the BSTd may increase activity of CRF-containing neurons, which may affect CRF synthesis and/or release.

It is unclear how this relates to my finding that DA tissue content within the BSTd was lower in the PP-nopups groups. I propose the following hypothetical model to explain the potential effects of DA on CRF mRNA expression in the BSTd. Evidence from previous research is in normal type while my own hypothetical elements are in italics. 1) Removing pups for 48 hours increases CRF mRNA in the brain (Lightman & Young, 1989), 2) in males, DA increases the excitability of CRF-containing neurons in the BSTd (Kash et al., 2008), 3) *DA receptor activity in the BSTd increases CRF mRNA levels,* 4) *in this chapter*, *reduced DA levels in the BSTd are the result of increased DA release from tissues stores and increased DA turnover*, 5) *increased DA turnover in the BSTd of PP-nopups females is a an early precursor to*

increased CRF mRNA in this brain region. I propose the increased metabolism of DA results in a series of events that returns postpartum CRF mRNA levels to that of virgins. Clearly, this model is particularly sensitive to point (4) being true. Reduced DA levels in PP-nopups *may* reflect increased DA turnover but it could represent other processes. DA synthesis or storage, for example, may decrease in PP-nopups, which could have no association to turnover. Unfortunately, the HPLC system used to analyze my samples could not detect DOPAC, which prevents any firm conclusions regarding DA metabolism. Additional research is required to determine if DA turnover is affected by removal of pups and if DA affects CRF mRNA in the BSTd.

Relationship between monoamine levels on rats' maternal and anxiety-related behaviors

Norepinephrine

Chapter 1 demonstrated that increasing the release of NE in the BSTv and mPOA inhibits dams' maternal care. Furthermore, Chapter 2 showed that increasing NE systemically or within the BSTv potentiates anxiety-related behaviors in the EPM. Results from Chapter 2, however, are complicated because only yohimbine (but not idazoxan) affected behavior in the EPM. Yohimbine has mild affinities for 5-HT1A and D2 receptors, suggesting its effects may be mediated through non-noradrenergic mechanisms. In addition, systemic injections of yohimbine may have increased anxiety-related behaviors by acting on brain regions other than the BSTv.

Naturally, examining the levels of NE is of most interest because yohimbine, idazoxan, and clonidine have the highest affinity for noradrenergic receptors. Despite that NE turnover could not be determined, analyizing NE levels alone may elucidate how this monoamine contributes to differences in behavior. Significant main effects of reproductive state and brain

site were found on the concentrations of NE. Post-hoc tests did not show a signficant difference among groups of rats, but PP-pups did tend to have more NE than PP-nopups or virgins. The meaning of this trend is difficult to determine, particularly without knowing MHPG levels. Reduced tissue levels of NE in PP-nopups and virgins could indicate lower NE content or higher NE release that would be reflected by increased NE turnover. An increase in NE turnover among PP-pups and virgins would then suggest that noraderenrgic activity is anxiogenic, consistent with the literature on male rats. Regardless, the trend of reduced NE in PP-nopups and virgins lend support that *some* difference in noradrenergic activity in these brain regions may contribute to behavioral differences between these groups. Also, NE content was nearly ten times higher in the BSTv than BSTd, supporting the hypothesis that yohimbine in part exerted its effects through noradrenergic mechanisms and in the ventral, but not dorsal, BST.

Dopamine

The largest difference between groups in DA was observed in the BSTd, with DA significantly lower in the BSTd of PP-nopups compared to PP-pups. The relationship between this difference and maternal and/or anxiety-related behaviors is unclear. Dopaminergic activity in the BSTd is not a likely contributer to maternal behaviors. Lesions of the BSTd do not disrupt maternal behavior (Numan & Numan, 1996) and Fos-IR is non-selectively induced there by pups as well as other stimuli (Numan & Numan, 1995). This is not to suggest that the BSTd has no role in pup-directed care. The BSTd receives olfactory information (Canteras et al., 1992,1995; Krettek & Price, 1978) and dopamine from the VTA (Hasue et al., 2002; Meloni et al., 2006), the latter being a brain region known to affect maternal behaviors (Hansen et al., 1991; Numan et al., 2009). Thus, although the BSTd is not *critical* for maternal care, it may contribute to these

behaviors, possibly by affecting responsiveness to olfactory cues from pups. In contrast, dopaminergic activity may inhibit maternal behavior.

In addition to maternal care, DA activity in the BSTd may affect anxiety-related behaviors. The BST is considered apart of the "extended amygdala" and is known to affect fear and anxiety, particularly startle and freezing behaviors (for review, see Walker & Davis, 2008). It is not clear, though, if dopamine contributes to the BSTd's effects on fear and anxiety. As previously described, DA enhances excitablity in the BSTd and this is blocked by CRF-receptor antagonism (Kash et al., 2008). CRF antagonists also reduce freezing and startle responses in rats (Walker & Davis, 2008) and male rats bred for high anxiety have increased CRF mRNA in the BSTd compared to lower-anxiety rats (Carrasco et al., 2008). Together, this evidence suggests increased DA transmission in the BSTd could promote anxiety-related behaviors by enhancing the effects of CRF. Data in this chapter supports this, assuming the observed reduction in DA tissue content in PP-nopups indicates increased release and metabolism (reflected by greater DA turnover). Challenging this hypothesis, however, is the lack of difference observed between PP-pups and virgins. Because virgins also exhibit increased anxiety-related behaviors compared to PP-pups, DA in their BSTd should be significantly higher than that found in PP-pups. It is, thus, unclear if the drop in DA levels within the BSTd of PP-nopups could contribute to differences in anxiety-related behaviors.

Serotonin

Groups did not differ in 5-HT or 5-HIAA. 5-HT turnover, however, was higher in PP-pups compared to PP-nopups or virgins. This finding is somewhat unexpected because 5-HT release, at least in the BSTv, decreases neuronal excitability (Rainnie, 1999). If 5-HT turnover is

comparable to the release of 5-HT, then these results would suggest PP-pups have more inhibition in these brain regions compared to PP-nopups and virgins. This conflicts with what is known about these ares; activity in the BSTv and mPOA is particularly important for maternal behaviors (Numan & Insel, 2003) while Fos-IR is elevated in the BSTv of low-anxiety dams (Smith & Lonstein, 2008). Yohimbine also increases the release of 5-HT (Brannan et al., 1991; Broderick, 1997; Cheng et al., 1993), which may contribute to the drug's effects on maternal and anxiety behaviors. These data present numerous considerations. First, PP-pups may have higher 5-HT turnover and this accounts for their pup-directed behaviors (compared to virgins) and low anxiety (compared to PP-nopups and virgins). Second, yohimbine may not exert its effects through serotonergic mechanisms. Third, high 5-HT turnover may be necessary for behaviors or bodily processes in PP-pups unrelated to maternal or anxiety-related behaviors, such as lactation.

I propose an alternative explanation for the change in 5-HT turnover. In the current experiment, dams were left with their litter, unmanipulated, before sacrifice. Dams did not have their pups removed from the cage nor were they required to engage in retrieving at any point before their brains were collected. It may be that 5-HT turnover was higher in PP-pups so as to *inhibit* behaviors such as retrieving. Casual observation indicated that most PP-pups were nursing immediately prior to sacrifice. It has been suggested that although the BSTv/mPOA are required for active maternal behaviors, these regions may require inhibition so mothers can initiate quiescent nursing (Lonstein & Stern, 1997; Miller & Lonstein, 2005; Numan & Insel, 2003). Increased serotonergic activity could facilitate this inhibition and stimulate quiesence. This hypothesis would also correlate with the findings that suckling increases 5-HT turnover in the mPOA (Johnston et al., 1984) while stimulating the mPOA reduces prolactin secretion (Wiersma & Kastelijn, 1990). Still, the drop in 5-HT turnover was also observed in virgins but

this effect may require that females underwent pregnancy and parturition and that pups are initially present.

Finally, it should be noted that monoamines may not be the only factor for behavioral differences across reproductive state. Differences in monoamine receptors may affect monoamines' affinity in brain areas including the BSTv and mPOA. Indeed, as discussed in the General Introduction, alpha-2 receptors are less dense in the PVN and supraoptic nucleus of lactating rats compared to virgins (Toufexis et al., 1998). Future research should consider examining how receptor densities change in the BSTv and mPOA of female rats across reproductive states. In particular, determing densities of alpha-2, 5-HT1A, and D2 receptors could aid in elucidating yohimbine's mechanism of action.

Conclusions

Numerous differences in monoamine levels were observed in the BSTv, mPOA, and BSTd among PP-pups, PP-nopups, and virgins. Notable findings include reduced tissue levels of DA in the BSTd of PP-nopups compared to PP-pups and increased 5-HT turnover in PP-pups compared to the other groups. Any function of low DA in females from the PP-nopups group is difficult to determine but may have effects related to how CRF influences anxiety-related behaviors. Furthermore, increased 5-HT turnover, particularly in the BSTv and mPOA, may inhibit active maternal behaviors (such as retrieving) and stimulate queiscent nursing. Additional research is needed to examine these possibilities. It is being discussed with a possible collaborator that the remaining supernatant collected from my tissue samples will be processed once again using different HPLC methods that can detect levels of MHPG and DOPAC. Without measuring concentrations of these metabolites, turnover rates of NE and DA remain

unknown. Nevertheless, data from this chapter suggest some monoaminergic mechanisms underlying behavioral differences among postpartum rats, postpartum rats separated from their litters, and diestrous virgins.

General Discussion

The BSTv has long been known to regulate maternal behaviors in females and anxiety-related behaviors in males. Although numerous neurotransmitter systems innervate this region, little is known what types are important for postpartum mothering and emotional states. Numan et al. (1990) indicated a possible role for NE by coating a "knife" with a retrograde tracer and then severing the lateral connections of the BSTv/mPOA, which disrupted maternal behavior. Of many brain sites, presence of tracer was particularly high in the locus coeruleus and A2 region of the medulla. The authors suggested that noradrenergic inputs to the BSTv and mPOA might alter maternal behaviors (also, see Numan & Insel, 2003). In this dissertation I confirmed that noradrenergic activity in the BSTv affects maternal behavior and, in agreement with studies in male rats, anxiety-related behaviors. Still, some results in my dissertation are not completely consistent with previous research in laboratory rats. I will address some of these issues here and discuss the broader implications of noradrenergic activity as it relates to emotional states in rats. Furthermore, as will be discussed, my results may also have implications for understanding postpartum mood disorders in women.

Based on evidence that NE inhibits neural activity in the BSTv (Casada & Dafny, 1993), I proposed that an uninhibited BSTv is required for pup-directed behaviors and low states of anxiety. This may appear to conflict with what is known about neural systems regulating maternal motivation and anxiety. Sites of projection from the BSTv promoting approach behavior towards pups, such as the mesolimbic dopamine system, require excitatory input, as inhibiting the VTA, nucleus accumbens, or ventral pallidum disrupts maternal behavior (Hansen et al., 1991; Numan et al., 2005a,b). In contrast, inhibiting brain regions associated with anxiety, including the PAG (Lonstein et al., 1998), reduces anxiety-related behaviors. It may seem

paradoxical that BSTv projections *both* stimulate maternal behavior and simultaneously reduce anxiety when more than half of neurons in the BSTv of maternally-behaving dams expressing Fos are also co-labeled with GAD (Lonstein & De Vries, 2000).

As suggested by others (Lonstein & De Vries, 2000; Numan & Insel, 2003), there are numerous explanations. First, the BSTv may contain at least two populations of neurons, one inhibitory and one excitatory. Although ~60% of neurons in dams are labeled with both Fos and GAD, ~40% are not. These remaining neurons may contain glutamate or other excitatory neurochemicals and the phenotype of these neurons are surely specific to their projections. GABAergic neurons may project primarily to areas that promote anxiety (such as the PAG) while excitatory neurons project to areas promoting pup-directed behaviors (such as the VTA). Indeed, glutamate antagonists infused into the VTA block neural activity induced by BSTv stimulation, suggesting that the BSTv has glutamatergic projections to the VTA (Georges & Aston-Jones, 2002). Second, GABAergic neurons in the BSTv may also be responsible for inhibiting active maternal behaviors when dams are being suckled, permitting them to engage in quiescent nursing. Lonstein & De Vries (2000) allowed dams to nurse before sacrifice; the high proportion of double-labeled cells may reflect inhibition of behaviors such as retrieving, licking/grooming pups, and nest-building. It would be interesting to examine the proportion of cells co-labeled for Fos and GAD in dams that were permitted to retrieve but not nurse. Regardless of GABA's function in the BSTv, these explanations generally support that noradrenergic inhibition of the BSTv disrupts maternal behavior and increases anxiety.

Another issue to consider is how NE levels naturally fluctuate in maternal and non-maternal rats. Evidence from male rats suggests NE is tonically released in the BSTv (Forray et al., 1997), which inhibits neural excitability (Casada & Dafny, 1993). Depressing BSTv activity

with tonically high NE release could explain why virgins are anxious and do not spontaneously exhibit maternal behaviors. Furthermore, reduced noradrenergic tone in the BSTv and mPOA, which is known to exist in the PVN (Toufexis et al., 1998), may account for dams' pup-directed care. Unfortunately, studies examining NE concentrations across reproductive states are scant. In fact, most evidence suggests the opposite: 1) hypothalamic NE metabolism increases at parturition (Moltz et al., 1975), 2) suckling increases NE levels in the bloodstream (Clapp et al., 1985), and 3) in Chapter 3, virgins and PP-nopups tended to have *lower* NE tissue content than PP-pups. Evidence from points (1) and (2), however, specifically examined parturition and nursing, which may not be relevant to the control of active maternal behaviors (*e.g.* retrieving, licking, and nest-building) during the postpartum period (see Chapter 1 Discussion). Furthermore, Chapter 3 measured relatively steady-state NE content in unmanipulated rats; changes in NE may only be elicited in response to specific stimuli such as reunion with pups or exposure to an EPM. Future research could, thus, measure monoamines when are females are given a maternal behavior test or placed in an EPM. Additionally, caution should be noted when interpreting studies measuring plasma NE levels (such as in Clapp et al., 1985). NE does not readily cross the blood brain barrier (MacKenzie et al., 1976) but does generally correlate well with CSF NE levels (Mautes et al., 2001; Ratge et al., 1985).

Such data could also address another question regarding anxiety: do interactions with pups produce a constant state of low anxiety *or* do they permit the display of low anxiety-related behaviors only in the face of an anxiogenic stimulus? Similarly, do PP-pups have constantly low levels of monoamines (that remain low during anxiety testing) or do PP-pups have a blunted monoamine response during anxiety testing? Or, is it a combination of both? This is difficult to conclude, particularly without knowing MHPG and DOPAC concentrations or how monoamines

are released on a minute-to-minute basis. Additionally, as discussed in Chapter 3, changes in monoamine levels may only reflect the presence or absence of pups and may have no relevance to behavior. The same question is applicable to the anxiogenic effects of yohimbine. Does yohimbine induce a state of constant "anxiety" or instead enhance anxiety-related behaviors only in response to normally anxiogenic stimuli? Data from humans may provide insight into this question. Patients with anxiety and/or phobic disorders have higher baseline levels of central and peripheral NE (Post et al., 1978; Sevy et al., 1989). Furthermore, yohimbine increases subjective feelings of anxiety when administered to patients with panic disorders (Gurguis et al., 1997) and healthy controls (Charney et al., 1983; Mattila et al., 1988) in the absence of anxiogenic stimuli. Thus, at least in humans, high noradrenergic activity is associated with unprovoked states of high anxiety. If this applies to rats, then pup stimulation may maintain a constantly low level of NE release to maintain low states of anxiety. Conversely, pup stimulation could maintain a higher threshold for an anxiogenic stimulus to elicit a response.

If, however, removing dams from their litters increases endogenous NE in a manner that alter anxiety behavior, then why do dams behave maternally upon reunion with the litter? It might be expected that this rise in NE levels would *inhibit* maternal care. It may be possible that upon reunion, distal pup stimuli immediately reduce noradrenergic tone in the BSTv. The sound, sight, or smell of pups could attenuate NE levels enough to initiate retrieving by dams. Second, intra-BSTv infusions of yohimbine and idazoxan may more chronically maintain unnaturally high levels of NE, which cannot be rapidly reduced after exposure to pups. Indeed, the half-life of yohimbine in brain tissue is approximately 8 hours (Hubbard et al., 1988), indicating a slow rate of clearance. Neural sites regulating maternal behavior are widespread and highly interconnected (Numan & Insel, 2003). It is entirely possible that brain regions responsive to

distal pup stimuli rapidly reduce noradrenergic activity in the BSTv to permit pup-seeking and retrieval behaviors.

Neural activity in the BSTv likely regulates anxiety-related behaviors similarly in males and females. Indeed, much of the research providing rationale for Chapter 2 is on male rats. The BSTv of male and female rats has similar afferent and efferent projections (Dong & Swanson, 2006a,b; Numan & Numan, 1996), very high noradrenergic content (see results from Chapter 3; Fuentealba, 2000; Kilts & Anderson, 1986), but most data regarding serotonin (Guo & Rainnie, 2010; Rainnie, 1999) and dopamine (Bouthenet et al., 1987, 1991; Wamsley et al., 1989) in the BSTv originates from studies in males. Given this, it is not surprising that systemic injections of yohimbine reduced open-arm exploration in postpartum and virgin female rats (Chapter 2) and male rats (Johnston & File, 1989; Pellow et al., 1985). There are no studies in males examining intra-BSTv infusions of yohimbine on behavior in the EPM but it would likely decrease open-arm exploration. Some data from Chapter 2 are not consistent with the anxiety literature in male rats. Intra-BSTv infusions of clonidine block fear-potentiated and light-enhanced startle in males (Schweimer et al., 2005), the latter being an indicator of anxiety. In contrast, clonidine did not reduce anxiety-related behaviors in postpartum or diestrous virgin rats. As discussed in Chapter 2, doses of clonidine may have been too low to affect females' behavior in the EPM but additional research is needed to address this issue. Regardless, high noradrenergic tone in the BSTv appears anxiogenic in both sexes. In males, an anxiogenic stimulus increases NE release (Fendt et al., 2005) and reducing NE (Schweimer et al., 2005) or blocking postsynaptic adrenergic receptors (Cecchi et al., 2002) increases open-arm exploration. This suggests a conserved neurochemical and anatomical organization in the BSTv between males and females.

Despite many similarities between male and female neurochemistry and anatomy, there

are behavioral differences. In rats, females display fewer anxiety-related behaviors in the EPM than do males (Johnston & File, 1991; Nasello et al., 1998), although some studies report no differences (Chadda & Devaud, 2005; Stock et al., 1999). These lack of differences, however, may be explained because females were either gonadectomized or in diestrus; only females in proestrus display more open-arm exploration compared to males (Marcondes et al., 2001). Additionally, sex differences are found in animal models of depression but results can vary depending on the paradigm and prior manipulations made to the animals (Dalla et a., 2009). Behavioral differences also exist in humans, such that major depression and anxiety disorders typically occur more often in women than men (for review, see Bekker & van Mens-Verhulst, 2007; Parker & Brotchie, 2010). If the BSTv significantly contributes to these emotional states, and is organized similarly between the sexes, how can behavioral differences exist? The most obvious answer lies in gonadal hormones, such as testosterone and estrogen. The BSTv contains fibers immunoreactive for aromastase (Jakab et al., 1993), is dense with estrogen receptors (Pfaff & Keiner, 1973; Simerly et al., 1990), and females have higher densities of GABAergic neurons than males (Stefanova et al., 1997, 1998). It is possible that high estrogen levels in proestrous virgins, for example, increases the activity of these GABA-containing neurons to inhibit brain regions that promote anxiety. In addition, there are reasons for differential activity in the BSTv in postpartum rats compared to virgin female or male rats. First, the hormonal events of gestation may change the BSTv in ways not yet identified. Adrenergic receptor densities may decrease in this region, as occurs in the PVN (Toufexis et al., 1998). Second, the unique experience of interacting with pups may affect noradrenergic activity in postpartum rats. If this latter point is true, then this change could occur in maternally-behaving virgin females and males.

Regardless of the exact mechanism, it is possible that pregnancy or the sensory input from infants affect the BSTv to regulate emotional states. This may not be limited to laboratory rats and a broader implication of this research is determining the biological basis of depression and anxiety in postpartum women. The postpartum period is generally associated with positive emotional states that include low incidences of depression and anxiety (for review, see Lonstein, 2007; Nonacs & Cohen, 1998). However, many women experience depression and/or anxiety disorders that have adverse consequences on both the mother and infant. In fact, a less extreme "postpartum blues," which can include depressive and anxiety symptoms, is reported in upwards of 75% of postpartum women (Nonacs & Cohen, 1998). Interactions with infants may be important in determining one's postpartum mental health. Women who breastfeed their child generally report better mood and perceive less stress than women who bottle-feed (Groer, 2005; Mezzacappa & Katkin, 2002). Whether changes in NE precipitate these moods, however, is unclear. Women who breastfeed exhibit blunted plasma NE levels after exposure to a stressor compared to postpartum women who bottle-feed (Altemus et al., 1995). Heinrichs et al. (2001), however, found that either breastfeeding or simply holding an infant decreases NE levels after exposure to stressors. That NE decreases in women who are in physical contact with their child, and that their mood is generally better, is consistent with the finding that: 1) recent contact with pups decreases anxiety-related behaviors in rats, even in the absence of suckling (Lonstein, 2005; Smith & Lonstein, 2008) and 2) increasing NE release disrupts maternal behaviors and increases anxiety (Chapters 1 and 2).

Could reduced noradrenergic activity in postpartum women account for their positive mood? fMRI does reveal increased activity in the BST when non-postpartum humans are threatened with predictable (Somerville et al., 2010) and unpredictable (Alvarez et al., 2011)

shocks. BST activity also increases when patients with diagnosed arachnophobia are presented images of spiders (Straube et al., 2007). Given the high noradrenergic innervation of the human BST (Farley & Hornykiewicz, 1977), and that many commonly prescribed anxiolytic drugs depress noradrenergic activity (Boehnlein & Kinzie, 2007, Noyes, 1985, Raskind et al., 2003), it would not be surprising if increased NE release in the BST potentiates anxiety in humans. It should be noted that elevating brain NE with selective norepinephrine reuptake inhibitors can also be anxiolytic (for review, see Baldwin et al., 2010), suggesting that abnormally high *or* low levels of NE have negative emotional consequences. Postpartum state and recent interaction with the infant may optimize mother's NE activity in the BST and elsewhere to prevent anxiety-inducing overactivity. In addition, high NE concentrations in the BST of women may negatively affect parental care. I am unaware of studies examining BST activity in postpartum women but evidence shows that women with postpartum blues have elevated levels of plasma MHPG (Doornbos et al., 2008). Future studies could examine BSTv activity in postpartum women who have recent physical contact with their infants compared to those separated from their infant for a relatively long period of time. Furthermore, these studies could also examine how stressors differentially affect BSTv activity in these participants.

Based on evidence that: 1) in rats, sensations from the mammary glands synapse in the BST as well as noradrenergic neurons in the medulla and locus coeruleus (Gerendai et al., 2001), and acute suckling increases Fos-IR in these areas (Li et al., 1999), 2) breastfeeding women have lower plasma NE than non-breastfeeding women in response to a stressor (Altemus et al., 1995), and 3) increasing the release of NE in the BST disrupts maternal behavior and increases anxiety (Chapter 1 and 2), I suggest that tactile stimulation from infants (breastfeeding or holding the infant) decreases the release of NE in the BSTv in mothers and contributes to their positive and

stable mood. Clearly, much more research is needed to better clarify this question but my dissertation does provide a framework of how NE can affect maternal and anxiety-related pathways (Figure 36) and future directions for research.

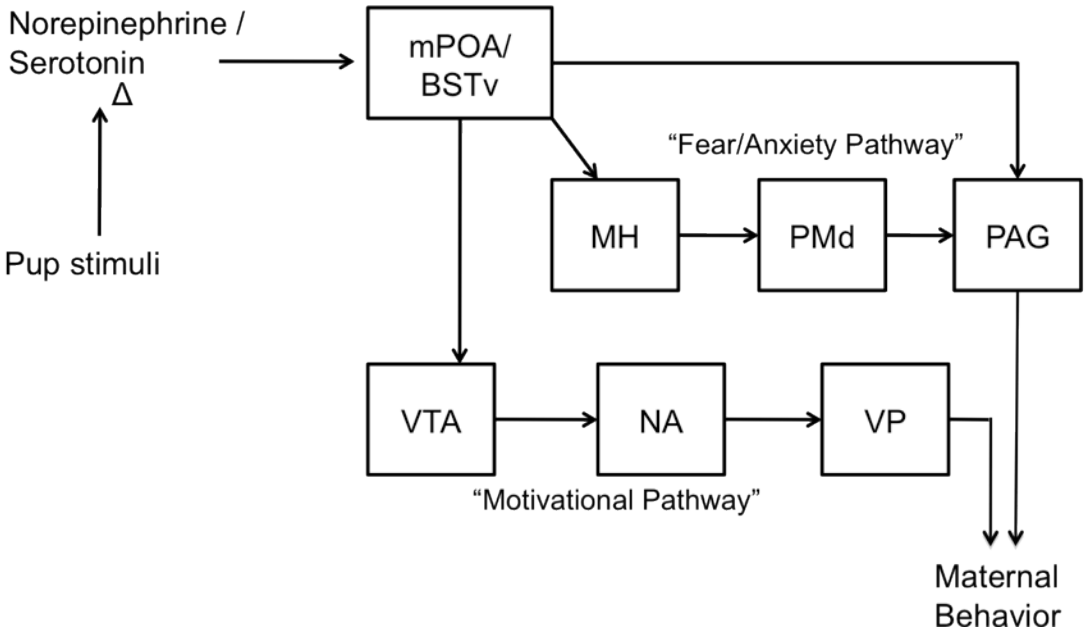

Figure 36 – Hypothetical diagram of neural networks regulating maternal and anxiety-related behaviors. The mPOA/BSTv inhibit brain sites promoting fear and/or anxiety while exciting sites involved in motivation. In a non-maternal rat, NE and 5-HT inhibit the mPOA/BSTv that results in low maternal responsiveness and higher levels of anxiety. In the maternal rat, pup stimuli alter NE and 5-HT activity to disinhibit the mPOA/BSTv. This results in high maternal responsiveness and low anxiety. Figure modified from Numan (2007).

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