MOTHERHOOD, STRESS, AND SEROTONIN RECEPTORS – INFLUENCE ON POSTPARTUM SOCIAL AND AFFECTIVE BEHAVIORS IN FEMALE LABORATORY RATS

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ABSTRACT

MOTHERHOOD, STRESS, AND SEROTONIN RECEPTORS – INFLUENCE ON POSTPARTUM SOCIAL AND AFFECTIVE BEHAVIORS IN FEMALE LABORATORY RATS

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Mammalian mothers show a unique suite of behavioral responses beginning around the time of parturition that are necessary for successful rearing of young. These include caring for offspring, high levels of aggression, and low anxiety. These behaviors emerge in response to the unique neurochemical milieu resulting from pregnancy and parturition. Studies in this dissertation test the hypothesis that changes in receptors for the neurotransmitter serotonin (5-HT) are part of this neurochemistry in female laboratory rats. Experiments in chapter one found that there are reproductive state-dependent changes in expression of central 5-HT receptors that may be responsible for peripartum behavioral responses. Specifically, females examined at parturition and early lactation showed less serotonin 2C receptor (5-HT2C) mRNA expression in the midbrain dorsal raphe (DR), more serotonin 2A receptor (5-HT2A) mRNA in the medial preoptic area (mPOA), and more serotonin 1A (5-HT1A) mRNA in the shell subregion of the nucleus accumbens (NAcSh) compared to nulliparous females. Receptor autoradiography confirmed that binding density of 5-HT2A was higher in the mPOA of recently parturient females and that binding density of 5-HT1A in the NAcSh was higher in lactating females at particular rostrocaudal levels. Such differences in 5-HT receptor expression were not found in maternally acting virgin females, suggesting that pregnancy and parturition are necessary for these changes in central 5-HT receptors to occur. Because pregnancy stress derails most behavioral adaptations of motherhood, follow up experiments then explored whether the

normative changes in 5-HT receptor expression across reproduction were prevented by daily application of mild-to-moderate stress beginning one week after mating. Stressed females showed lower maternal care and higher depression-like behaviors, which were correlated with 5-HT receptor mRNA in the mPOA, NAcSh and DR. Autoradiographic binding density of mPOA 5-HT2A receptors was not affected by pregnancy stress, although the stress reduced 5-HT1A binding in the NAcSh. Because the NAcSh is involved in motivation and reward processing, the last experiment directly tested whether 5-HT1A receptors in the NAcSh contribute to maternal caregiving and emotional behaviors. Long-term knock down of 5-HT1A in the NAcSh was established using an adeno-associated virus promoting shRNA against 5-HT1A mRNA. The 5-HT1A shRNA vector or a scrambled control vector was infused into the NAcSh during early pregnancy and mothers' later postpartum social and affective behaviors (i.e. caregiving, maternal motivation, aggression, anxiety- and depression-like behaviors) were observed. 5-HT1A knock down resulted in higher frequencies of self-grooming and sleeping away from the nest, delayed retrieval of displaced pups back to the nest, and increased anxiety-like behavior.

Overall, I found that female reproduction is associated with changes in serotonin receptor expression in numerous brain sites involved in postpartum behavior. Of particular interest, the normative change in 5-HT1A expression in the nucleus accumbens shell is altered in response to stress during pregnancy, and disrupting its expression reduces maternal motivation and increases postpartum anxiety-like behavior. Together, the results from this dissertation provide new insights into how the serotonergic system contributes to postpartum social and affective behaviors and offer a potential mechanism via the brain's reward system through which pharmacological treatments that affect the serotonin system (e.g., SSRIs) may work to alleviate postpartum affective disorders in women.

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CHAPTER 1: INTRODUCTION

Mammalian mothers show a unique suite of behavioral responses beginning around the time of parturition that are critical for successful reproduction. These include high maternal caregiving of their young, maternal aggression against intruders to the nest, and a dampening of anxiety-related behaviors that prevent over-reactivity to threats (Bridges, 2015; Fleming & Luebke, 1981; Lonstein et al., 2014). Under most conditions, these behavioral changes associated with motherhood initially depend on the hormonal fluctuations associated with pregnancy. In rats and a number of other mammals, these include a gradual rise in circulating estradiol across pregnancy that peaks at parturition, high levels of progesterone that peak at the end of pregnancy followed by a steep drop during parturition, and sharp bursts of oxytocin and prolactin during parturition (Bridges, 2015; Lonstein et al., 2014). Interfering with this hormonal profile severely disrupts females' ability to display caregiving behaviors (Bridges et al., 1978), indicating that this hormone combination is necessary for maternal caregiving to occur.

After the typical onset of caregiving, peripheral estradiol and progesterone appear to instead facilitate the waning of caregiving behavior as the postpartum period progresses, as postpartum removal of the ovaries increases licking and upright-crouched (i.e., kyphotic) nursing at the end of lactation when they should be declining before weaning (Grieb et al., 2017). In contrast, postpartum oxytocin (OT) continues to facilitate the display of postpartum caregiving behaviors such as retrieving pups to the nest (Okabe et al., 2017), whereas blocking OT signaling in various brain regions significantly disrupts this and other maternal behaviors (Bosch et al., 2005; Bosch et al., 2012; Grieb and Lonstein, under review; Pedersen et al., 1994; Van Leengoed et al., 1987). While ovarian and peptide hormones along with interactions with offspring also modulate a host of neurotransmitters - including dopamine, norepinephrine, and GABA - to

maintain mothers' postpartum social and emotional behaviors (Lonstein, 2007; Pederson et al., 1994; Numan and Stolzenberg, 2009; Rosenblatt et al., 1988), little research has examined any role for serotonin (5-HT), a neurotransmitter that is well known to influence social and affective behaviors in non-parous animals (reviewed in Graeff et al., 1996).

Overview of Midbrain Raphe Nuclei and Serotonin 1A, 2A, and 2C Receptors

In the mammalian brain, serotonin is synthesized from the amino acid, tryptophan (TPH), in cell bodies located in the midbrain and hindbrain raphe nuclei. Five nuclei residing in the hindbrain comprise the descending serotonergic pathway that mainly goes to the spinal cord, and these include the nucleus raphe obscurus (NRO; B2), nucleus raphe pallidus (NRPa; B1 and B4), nucleus raphe magnus (NRM; B3), neurons in the ventrolateral medulla (B1 and B3), and the area postrema. Four raphe nuclei, mainly residing in the midbrain, send ascending serotonergic projections to the forebrain, and these include the caudal linear nucleus (CLN; B8), median raphe nucleus (MRN, B8 and B5), a group of neurons just dorsal to the medial lemniscus, and the dorsal raphe nucleus (DR; B7 and B6). The DR is the largest collection of forebrain-projecting 5-HT neurons, with the DR of the rat brain containing 11,500 neurons that synthesize serotonin (Jacobs and Azmitia, 1992).

The behavioral effects of central serotonin depend on the receptor subtype activated, and at least three (5-HT1A, 2A, and 2C) of the 15 different 5-HT receptors known to date have been implicated in social and affective behaviors in rats and in humans. 5-HT1A receptors are inhibitory, leading to activation of Gi protein and, therefore, a reduction in neuronal activity when activated (Raymond et al., 1999). 5-HT1A receptors are highly expressed in limbic regions including the hippocampus, amygdala, and cingulate cortex (Chalmers and Watson, 1991). These

receptors are also highly abundant in the DR, where they are found on terminals and somata of 5-HT neurons and regulate the release of serotonin in the forebrain (Hamon et al., 1991). The 5-HT2 family of receptors is excitatory, and activation of these receptors leads to Gq protein activation (Chang et al., 2000). 5-HT2A and 2C receptors are not as highly expressed in the DR, but can be found with particularly high density in the CA3 region of the hippocampus, frontal cortex, the amygdala, and the striatum (Pompeiano et al., 1993). 5-HT2A receptors are also found in the hypothalamus in greater amounts than are 5-HT2C receptors (Pompeiano et al., 1993). Based on the high abundance of these three receptors in cortical and limbic regions, it is unsurprising that they contribute to the display of various social and emotional behaviors in non-parous mammals (to be reviewed below).

Role of Serotonin in Social Behaviors

Serotonin has been widely implicated in numerous social behaviors in humans and non-human animals (Graef et al., 1996). Importantly, serotonergic neurons in the DR project to forebrain regions that are key regulators of prosocial and affective behaviors, including the medial prefrontal cortex (mPFC), the nucleus accumbens (NAc), the medial preoptic area (mPOA), the bed nucleus of the stria terminalis (BST), and the amygdala (Berk & Finklestein, 1981; Halberstadt & Balaban, 2006; Van Bockstaele et al., 1993). For instance, the mPFC regulates numerous reproductive behaviors in female rats, evidenced by the fact that excitotoxic lesions of it disrupt pup retrieval, pup licking, and proceptive sexual behaviors (Afonso et al., 2007). The mPOA is also critical for maternal caregiving, with lesions disrupting pup retrieval and nest building (Jacobson et al., 1980) and also reducing bar pressing in rats to gain access to pups (Lee et al., 2000). Regions of the limbic system, such as the dorsal BST and central and

basolateral amygdala, are highly involved in reproduction and also play a role in social investigation in juvenile male mice (Maaswinkle et al., 1996) and adult male and female rats (Dumais et al., 2016), social affiliation in male prairie voles (Lei et al., 2017), and social vigilance in female California mice (Duque-Wilckens et al., 2018). I will first discuss the specific role of serotonin on these behaviors in non-human mammals, and then turn to what is known about serotonin's role in these processes in humans.

Laboratory Rodents

Given that most if not all brain sites regulating social behaviors receive serotonergic innervation, it is unsurprising that manipulating this neurotransmitter affects a large suite of social behaviors, including sexual behavior, social play behavior, and aggression, and this has (not surprisingly) been most studied in males. With regards to sexual behavior, serotonin is generally considered to inhibit copulation. For example, serotonin release in the lateral hypothalamus reduces sexual motivation and increases sexual satiety during the post-ejaculatory interval in male laboratory rats (Lorrain et al., 1999). Selective serotonin reuptake inhibitors (SSRIs), which increase central serotoninergic signaling after long-term use, also reduce sexual motivation in male rats (Matusczyk et al., 1998). Infusing serotonin directly into the mPOA, a brain site that regulates sexual behavior, also impairs male copulation (Verma et al., 1989). Conversely, reducing serotonergic activity by inhibiting synthesis of serotonin facilitates male sexual behavior by increasing ejaculatory activity (Kondo & Yamanouchi, 1997). Although these studies provide evidence for an inhibitory role of serotonin on copulatory behaviors themselves, serotonin release during sexual behavior may promote the rewarding properties of reproduction. In support, depleting serotonin reduces the rewarding properties of sexual experience in males,

as demonstrated by a reduction in conditioned place preference to a context paired with a sexual stimulus (Straiko et al., 2007). Additionally, serotonin in the nucleus accumbens, an important region for reward processing, is increased during male sexual activity (Ahlenius et al., 1987). Different serotonin receptor subtypes may mediate these seemingly contrasting effects of serotonin on the components of sexual activity. For example, activating inhibitory 5-HT1A receptors stimulates sexual behavior and decrease ejaculation latency in male rats, while also increasing mounting behavior in female rats that were administered testosterone (Haensel et al., 1991). In contrast, activating 5-HT1A receptors inhibits sexual behavior in female rats by reducing proceptive and receptive behaviors (Mendelson & Gorzalka, 1986). Serotonin acting on excitatory 5-HT2 receptors instead facilitates sexual behavior in female rats, since blocking these receptors inhibits sexual receptivity and lordosis (Mendelson and Gorzalka, 1985). In males, administering a non-specific 5-HT2 agonist, DOI, inhibits copulation by increasing ejaculation latency (Foreman et al., 1989; Klint & Larsson, 1995). Thus, serotonergic influence on reproductive behavior may be opposite in males and females, with the inhibitory 1A receptor promoting copulation males but the excitatory 2 receptors promoting it in females.

Serotonin regulates non-sexual social behaviors as well, with 5-HT1B receptor antagonism in the NAc abolishing conditioned place preference for a socially paired context in mice (Dolen et al., 2013). Juvenile play (another highly motivating social behavior) is greatly reduced in rats with a genetic knockdown of the serotonin transporter (Homberg et al., 2007), and is reduced following acute SSRI treatment (Paksepp et al., 1987; Knutson et al., 1996). Central lesions of serotoninergic fibers using 5,7-DHT increases play in dominant rats but decreases play in non-dominant rats (Knutson & Paksepp, 1997). Mice lacking the serotonin transporter (SERT) gene also show disruptions in social behavior. Specifically, SERT knockout

mice are unable to distinguish between a novel and familiar mouse in a social recognition task, and basal firing rate of DR serotonin neurons is also significantly reduced (Veenstra-VanderWeele et al., 2012). Taken together, these studies provide support for the regulation of a variety of social behaviors by serotonin.

Arguably the largest research effort has been given to studying the role of serotonin in male aggression, with the overall consensus indicating an inverse relationship between the two (Duke et al., 2013). This "serotonin deficiency" hypothesis of aggression is supported by negative correlations between trait-like impulsive aggression and/or violence with cerebrospinal fluid (CSF) concentrations of the serotonin metabolite 5-HIAA in humans and non-human primates (for reviews see Berman et al., 1997; Krakowski et al., 2003). Studies of non-human primates have shown that serotonin influences social behaviors along two axes – dominant/submissive and agnostic/affiliative. Experimental manipulations that lower serotonergic function are most often found to increase dominance, while those that increase serotonergic function lower dominance and increase affiliative behavior (Chamberlain et al., 1987; Raleigh et al., 1991). Additionally, dominant monkeys have higher blood platelet serotonin, a possible peripheral indication of neuronal 5-HT function, and platelet serotonin levels fall as dominance is lost (Raleigh & McGuire, 1991).

Many studies in rodents have also demonstrated that reducing serotonin using neurotoxins for 5-HT neurons, or heightening brain serotonin by increasing tryptophan hydroxylase, the precursor to 5-HT, respectively increases or decreases aggressive behaviors in males (see Miczek et al., 2002 for review). Additionally, systemically administering 5-HT1A and 1B (inhibitory receptors) agonists or 5-HT2A and 2C (excitatory receptors) antagonists reduces aggression in male rodents (Blanchard et al., 1988; Lindgren & Kantak, 1987; Sijbesma et al.,

1991; for additional review see Olivier, 2004). While the majority of studies support this conclusion, particularly those that incorporate brain-wide manipulations of serotonin, many studies fail to tease apart the differential pre- and postsynaptic effects of 5-HT receptor pharmacological manipulations. For example, systemically activating 5-HT1A receptors reduces male aggression, but peripherally injecting S-15535 (an agonist to somatodendritic 5-HT1A receptors but a competitive antagonist at 5-HT1A heteroreceptors), reduces serotonin release and reduces male aggression (de Boer and Koolhaas, 2005). Studies in hamsters also support an inhibitory role for serotonin on aggression (Ferris et al., 1999), although recent findings discovered a sex difference in the serotonergic control of aggression, with systemic injection of fluoxetine increasing the duration of aggression in females while significantly reducing aggression in males (Terranova et al., 2018). Additionally, activating 5-HT1A receptors in the anterior hypothalamus almost completely abolishes aggression in males, but significantly increases it in females (Terranova et al., 2018). Of note, no studies have explored a sex difference in serotonergic modulation of aggression in other laboratory rodents, even though sex differences in aspects of the serotonergic system have been known to exist since the 1980s (Carlsson & Carlsson, 1988; as discussed in more detail below)

In humans

Serotonin also influences social behaviors including social affiliation, sexual behavior, and aggression in humans, and seems to do so in a similar fashion as in non-human mammals. For example, volunteers treated with the SSRI, paroxetine, for one week showed increased social cooperation during a partner puzzle task compared to placebo administered volunteers (Knutson et al., 1998). Although social cooperation did not significantly differ between the SSRI and

placebo groups after 4 weeks of administration, plasma levels of paroxetine were positively correlated with social cooperation during this time point. Acute administration of tryptophan facilitates the recognition of happy facial expressions and reduces processing of disgusted faces (Murphy et al., 2006). Interestingly, these effects were only present in women, while tryptophan administration had no effect on facial recognition in men. Women given tryptophan supplementation also showed reduced startle reactivity to neutral, pleasant, and unpleasant pictures. Conversely, reducing serotonin levels by tryptophan depletion attenuates the attractiveness of positive faces, while also attenuating the negative intensity of threatening faces (Beacher et al., 2011). This suggests that reducing serotonin impairs the ability to perceive and interpret social stimuli. Acutely administering an SSRI also reduces steady-state visually evoked potentials, which is a measure of cortical processing, in the frontal and occipital cortices following visualization of unpleasant images (Kemp et al., 2004). Because acute SSRI administration causes short-term attenuations in serotonin release due to activation of raphe 5-HT1A autoreceptors (Czachura et al., 2000), these results suggest that reductions in central serotonin impair the cortical activation that accompanies the processing of social stimuli. Together, these results suggest that serotonin regulates the processing of socially salient stimuli across various contexts.

Regarding sexual behavior, serotonin appears to have the same inhibitory effect in humans as it does in rodents. For instance, there have been many reports of low sexual motivation and arousal in men and women taking SSRIs (Aldrich et al., 1996; Montejo-Gonzalez et al., 1997). Several case studies have also reported erectile dysfunction, genital anesthesia, and ejaculatory anhedonia following chronic (4 months to 2 years) treatment with a variety of SSRIs (Reisman et al., 2017). Additional evidence for an inhibitory effect of serotonin on sexual

behavior in humans is that daily treatment with SSRIs such as sertraline and fluoxetine delays ejaculation in men who experience premature ejaculation (Waldinger & Olivier, 2004). SSRI influence on male ejaculation may be regulated by serotonergic mechanisms that differ from those that regulate depression, given that the antidepressant effects of SSRIs only appear after 4 to 6 weeks of chronic administration while the effects of SSRIs on male ejaculation can be seen at shorter treatment time points (Waldinger & Olivier, 2004). Overall, the inhibitory effects of serotonin on human sexual behavior appear to be similar to those found in laboratory rodents.

As stated in the previous section, serotonin has historically been negatively correlated with aggression based on many studies showing that highly aggressive human subjects have low CSF levels of 5-HIAA, the metabolite of serotonin (Brown et al., 1979). Low serotonin is also associated with violent, impulsive, and suicidal behavior, as well as personality measures of hostility and aggression (Cleare & Bond, 1997; Cremniter et al., 1989). In addition, 5-HT1A receptor binding potential in the dorsal raphe nucleus is significantly negatively correlated with lifetime aggression score, and this finding was true for both men and women (Parsey et al., 2002). Since inhibitory 5-HT1A receptors are presynaptic in the DR, this suggests that higher inhibitory control over serotonin release has anti-aggressive effects, in contrast to the majority of studies indicating an inverse relationship between serotonin and aggression. It is important to note that in this study, though, all individuals were healthy and did not meet criteria for any psychiatric disorders and were not on any medications. Therefore, individual differences in serotonin release via 5-HT1A receptor expression may influence individual variation in aggressive tendencies differently than in individuals with psychopathological forms of aggression.

Serotonin and Steroid Hormones

Although the review above indicates an important role for serotonin in social behaviors mostly studied in male mammals, serotonin may differentially contribute to these behaviors during female reproduction. The serotonergic system is heavily influenced by ovarian hormones, which are critical for the onset of maternal caregiving. As described earlier in this chapter, steroid hormones follow a particular pattern of release during the peripartum period in rats and numerous other non-primate mammals, involving a gradual rise in estradiol during the middle and end of pregnancy and a decline following parturition. Progesterone, on the other hand, stays high throughout almost all of pregnancy and rapidly declines right around parturition (Bridges, 2015). Many midbrain serotonergic neurons express estrogen and progesterone receptors (Alves et al., 1998; Nomura et al., 2005; VanderHorst et al., 2005), and these hormones are known to influence the serotonergic system of non-parous mammals; therefore, the hormonal changes associated with pregnancy, parturition, and lactation very likely influence the maternal serotonergic system. Indeed, estradiol injections increase TPH2 mRNA in the DR of ovariectomized virgin rats (Lu et al., 1999), and increase DR serotonin synthesis (Hiroi et al., 2011). Estradiol also increases the expression of 5-HT2A receptors in the NAc, the anterior frontal cortex, anterior cingulate cortex, and olfactory cortex of virgin female rats (Sumner and Fink, 1995), while it decreases 5-HT1A receptor expression in the amygdala and hippocampus (Osterlund et al., 2000). Chronic progesterone increases serotonergic activity within the brain of female macaques and guinea pigs by increasing TPH2 protein and decreasing 5-HT1A autoreceptor mRNA within the DR (Lu et al., 1999; Pecins-Thompson & Bethea, 1999; Lu & Bethea, 2002; Hiroi & Neumaier, 2009). Thus, elevations in estradiol and progesterone during

late pregnancy may produce behaviorally relevant increases in mother's central serotonergic activity.

Administration of either estrogen or progesterone also reduces serotonin transporter mRNA (Pecins-Thompson & Bethea, 1998), and reduces the serotonin degradation enzyme monoamine oxidase A in the brain of rhesus macaques and rodents (Gundlah et al., 2002; Smith et al., 2004), suggesting that once serotonin is released when these hormones are elevated, it remains in the synapse for a longer amount of time. Spontaneous firing rate of serotonin cells in the DR is also increased during the peripartum period, and this is due to the progesterone metabolite allopregnanolone (Robichaud & Debonnel, 2005). Interestingly, co-administration of estradiol and progesterone for 14 days significantly increases serotonin release within the hypothalamus, while administering either hormone alone does not have this effect (Lu et al., 1999). Therefore, the changes in estradiol and progesterone throughout pregnancy and parturition may together alter serotonergic activity in peripartum females. Either E alone or E+P also reduce expression of genes related to cell death in the DR, suggesting a neuroprotective effect of reproductive hormones on the serotonergic system, potentially during the peripartum period (Bethea et al., 2009). In fact, our lab recently found that cell death in the DR is relatively low when cells are born during late pregnancy, when both E and P are relatively high, compared to those that are born during the first week of lactation when E and P are much lower (Holschbach & Lonstein, 2018). Thus, ovarian and other hormones released across pregnancy, parturition, and lactation may profoundly influence serotonin release, serotonin receptor expression, and DR function in ways that help initiate and/or maintain the display of postpartum caregiving and affective behaviors.

Serotonin and Postpartum Behaviors

Postpartum females show a unique collection of behavioral responses, including maternal care towards offspring, high maternal aggression, and low emotionality, and serotonin release in the larger maternal behavior neural network - including the NAc, mPOA, and BST (Lonstein et al., 2014; Numan and Insel 2003) - may play a role. During the first week of lactation, when mothering and aggression are high and anxiety is low, there is higher serotonin turnover in the mPOA and both the dorsal and ventral subregions of the BST compared to non-parous female rates (Lonstein et al., 2003; Smith et al., 2013). This pattern suggests that serotonin release in these regions may contribute to postpartum behaviors. Indeed, mice with life-long knockdown of tryptophan hydroxylase 2 (TPH2, rate-limiting enzyme for serotonin synthesis) show little to no postpartum maternal behavior or nest building, and offspring survival is greatly reduced (Angoa-Perez et al, 2014). Serotonin coming specifically from the DR appears to be important for lactation, because both pre- and postpartum lesions of the serotonergic cells in the DR reduce pup survival and decrease prolactin secretion in rats (Barofsky et al., 1983a; Barofsky et al., 1983b). Although serotonin is clearly necessary for caregiving, postpartum emotional behaviors, such as maternal aggression and anxiety-like behaviors, were not tested in these studies. A more recent study from our lab selectively lesioned the serotonin cells in the medial DR two days after parturition and found that lesions altered the temporal patterning of kyphosis (i.e., arched-back/upright crouched nursing), reduced pup licking, and prominently reduced maternal aggression. The same study found that DR serotonin lesions during pregnancy reduced maternal aggression to an even greater extent (Holschbach et al., 2018). This effect may be mediated by a reduction in serotonin fiber innervation of the hypothalamus, as the lesions reduced fiber length

in the anterior hypothalamus (Holschbach et al. 2018), where serotonin is known to influence aggression in both males and females (Terronova et al., 2018).

Although it is clear that an intact midbrain serotonin system is necessary for normal mothering, there has yet to be an in-depth characterization across female reproduction of serotonin receptor expression in the DR or in its major projections sites. Such an exploration could help clarify what receptors are involved in the onset and maintenance of maternal caregiving and affective behaviors. Broadly, studies using peripheral drug injections have shown that central 5-HT1A, 2A, and 2C receptors contribute to postpartum caregiving behaviors in female rats. 5-HT2A and 2C receptors generally have opposing effects on mothering, with systemic blockade of 5-HT2A receptors during early lactation disrupting retrieval (Chen et al., 2014) while systemic activation of 5-HT2C similarly disrupts the behavior (Zhao et al., 2009). 5-HT2C activation also reduces arched back nursing (kyphosis). Of note, pup retrieval and kyphosis are not affected when a 5-HT2C receptor agonist is injected directly into the mPFC, NAc, or mPOA (Wu et al., 2016), suggesting that other brain sites are responsible for the behavioral effects of 5-HT2C activation, or that a collection of sites act together for 5-HT2C receptors' effects. Supporting this possibility, systemic activation of 5-HT2C receptors during the first week of lactation decreases basal Fos expression in the DR (Wu et al., 2016), so 5-HT2C receptor activation might influence overall neuronal activity and, therefore, serotonin release from the DR. Because 5-HT2C receptors in the DR directly affect central serotonin release (Queree et al., 2009), it is possible that activating 5-HT2C receptors in the DR disrupts postpartum caregiving by affecting serotonin output to the forebrain. This is plausible because 5-HT2C receptors in the DR are found almost exclusively on GABAergic neurons (Serrats et al.,

2004), and injecting a 5-HT2C agonist into the DR decreases 5-HT neuronal firing and increases Fos expression selectively in DR GABA neurons (Boothman et al., 2006).

With regards to maternal aggression, serotonin appears to be facilitatory, which is in contrast to the majority of work in male aggression suggesting an inverse relationship between serotonin and aggression. Reducing overall serotonin output with serotonin-specific lesions to the DR significantly reduces maternal aggression towards a male intruder male (Holschbach et al., 2018). Pharmacological manipulations have implicated 5-HT1A, 2A, and 2C receptors in maternal aggression, with intracerebroventricular (i.c.v.) injection of 8-OH-DPAT, a 5-HT1A receptor antagonist, decreasing maternal aggression in rats. Furthermore, activating postsynaptic 5-HT1A receptors in the amygdala, dorsal periaqueductal gray, or median raphe also reduces maternal aggression (de Almeida & Lucion, 1994; de Almeida & Lucion, 1997). In contrast, 5-HT2A and 2C receptors can either increase or reduce maternal aggression depending on the site of injection- infusing a mixed 5-HT2A/2C agonist into the dorsal PAG reduces aggression in postpartum females, yet the same drug appears to increase maternal aggression when injected into the central amygdala (de Amleida et al., 2005; de Almeida et al., 2006). Thus, it is likely that these site-specific effects of 5-HT2A/2C agonists may depend on whether 2A or 2C receptors are predominantly expressed in particular brain regions. Reproductive-state changes in 5-HT receptor expression in particular brain regions may also explain the site-specific effects of these pharmacological manipulations; however, this has yet to be determined.

Serotonin and Affective Behaviors

In non-human mammals

While 5-HT and its receptors clearly influence postpartum caregiving and maternal aggression, they might do so indirectly by regulating postpartum affective behaviors. Serotonin depletion studies indicate that reducing central serotonin increases anxiety-like behaviors in laboratory rodents (Mosienko et al., 2012). Furthermore, genetic manipulations that knockdown expression of serotonin-related genes, such as those for the serotonin transporter (Holmes et al., 2003a; Holmes et al., 2003b) or 5-HT1A receptor (Ramboz et al., 1998; Toth, 2003; Zhuang et al., 1999) significantly alter serotonin release and affect anxiety-like behavior. In fact, 5-HT receptors have been common pharmaceutical targets for treating anxiety and depression, and downregulating 5-HT1A receptors is proposed to underlie the alterations in 5-HT output seen in individuals with major depression (reviewed in Drevets, 2007). In rats, activating 5-HT1A receptors in the DR reduces stress-induced anxiety-like behaviors (Kennett et al., 1987) and rats with a genetic "resistance" to depression-like symptoms ("Flinders resilient rats") show higher 5-HT1A receptor density in many brain regions compared to controls (Nishi et al., 2008). 5-HT1A receptor binding in the cortex, CA1, and DR is also reduced by early life stress, a paradigm that induces later depression-like behaviors in rodents, and these alterations are reversed if offspring are given fluoxetine in adulthood (Leventopoulos et al., 2009; Sillaber et al., 2008; Czeh et al., 2006).

The 1A receptor is not the only one relevant for affective behaviors, as 5-HT2C antagonists have potent, and immediate, antidepressant effects in mice by reducing immobility in the forced swim test, with a faster onset (five days) than that of SSRIs (several weeks) (Opal et al., 2014). 5-HT2C antagonists also increase brain derived neurotrophic factor (BDNF) in the

medial prefrontal cortex within a few days, which is also associated with chronic SSRI antidepressant action (Opal et al., 2014). This same study found that 5-HT2C blockade specifically in the ventral tegmental area (VTA) was sufficient to induce the same antidepressant effects and increased BDNF in the medial prefrontal cortex. In addition, combining selective serotonin reuptake inhibitor (SSRI) treatment with a 5-HT2C antagonist reduces the amount of time that SSRIs require for symptom relief in human patients suffering from anxiety or depression (Cremers et al., 2007).

While forebrain 5-HT2C receptors have been implicated in the potent antidepressant effects of 5-HT2C antagonists, these receptors specifically in the DR also modulate affective behaviors. For instance, 5-HT2C receptor blockade reduces anxiety under some conditions by increasing serotonergic activity in the DR (Craige et al., 2015). Postsynaptic 5-HT2C receptors are highly localized on GABA neurons in the DR, and activating them increases GABA release. Importantly, injecting GABA directly into the DR reduces forebrain serotonin release and increases anxiety-like behaviors in mice, thus DR 5-HT2C receptors likely regulate anxiety-like behaviors by influencing GABA release in the DR (Xiao et al., 2017). Together, these results not surprisingly suggest that 5-HT receptors play an important role in maintaining 5-HT output, and alterations in 5-HT activity within the DR can dysregulate affective behaviors.

In humans

One of the first examples of serotonin's involvement in human affective state was the lowering of mood after acute tryptophan depletion (ATD) (Young et al., 1985). Since then, approximately half of all published studies of ATD in healthy volunteers show a similar subclinical reduction in mood, characterized by self-reported boredom and reduced interest in

rewards (reviewed in Young & Leyton, 2002). Women report lowered mood following ATD more often than men (Murphey et al., 2006), which is consistent with the higher rate of mood disorders found in women compared to men (Bebbington, 1998; Kessler et al., 1993; Wolk and Weissman, 1995). Others have found sex differences in serotonin synthesis in humans (M>F) (Nishizawa et al., 1997; Sakai et al., 2006), central 5-HT1A receptor binding potentials (M<F) (Jovanovic et al., 2008; Parsey et al., 2002) and serotonin transporter binding potentials (M<F) (Jovanovic et al., 2008). Because 5-HT1A receptors are associated with depression in humans and depression-like behavior in rodents (Kennett et al., 1987; Lesch et al., 1991; Nishi et al., 2009), higher 5-HT1A receptor binding potential in women may help to explain sex differences in mood and affective disorders.

Indeed, alterations in 5-HT receptor expression and functionality in specific brain sites have been found in patients with anxiety disorders and depression. For example, 5-HT1A receptor binding potential is significantly lower in the insula and anterior cingulate cortex of individuals with social anxiety disorder (Lanzenberger et al., 2007), major depressive disorder (Sullivan et al., 2005), and panic disorder (Neumeister et al., 2004). Furthermore, in healthy volunteers, anxiety scores are negatively correlated with 5-HT1A binding potential in the anterior cingulate cortex, occipital cortex, and dorsolateral prefrontal cortex (Tauscher et al., 2001). Fewer studies have examined 5-HT2A or 2C binding in individuals with affective disorders, although individuals with bipolar disorder had less mRNA expression of 5-HT2A receptors in the dorsolateral prefrontal cortex compared to control individuals (Lopez-Figueroa et al., 2004). Disruptions in genes related to the serotonergic system have also been associated with affective behavior. For instance, polymorphism in the gene that encodes 5-HT2A receptors in humans has been linked with SSRI efficacy (Cusin et al., 2002) and panic disorder (Inada et

al., 2003), while polymorphisms of the serotonin transporter gene have been implicated in risk for developing major depression (Risch et al., 2009; Kendler et al., 2005; Owens and Nemeroff, 1994). In sum, these findings provide support for a complex role of specific serotonin and its receptors in human affective disorders.

Serotonin, Stress, and Postpartum Affective Disorders

While alterations in serotonergic signaling are often linked to abnormal social and affective responding, there is still a dearth of research studying the contribution of serotonin to dysregulations in these behaviors specifically during the postpartum period. Such disruptions in emotional postpartum emotional behavior can be seen in individuals with postpartum affective disorders such as postpartum anxiety and depression. Additionally, stressful life events during pregnancy are prominent risk factors for developing postpartum psychiatric disorders including anxiety and depression (Faisal-Cury, 2004; Robertson et al., 2004). In laboratory rodents, pregnancy stress disrupts caregiving behaviors and maternal motivation, increasing the amount of time postpartum dams spend off the nest and reducing the amount of time spent arch-back nursing (kyphosis) (Leuner et al., 2014; Smith et al., 2004). These studies also found more depressive-like behaviors and increased HPA axis reactivity in stressed dams, the latter of which is normally blunted during the postpartum period (Slattery & Neumann, 2008; Tizabi & Aguilera, 1992). Interestingly, treatment with SSRIs or other serotonergic drugs is common, and often effective, for women experiencing anxiety or depression during pregnancy (reviewed in De Crescenzo et al., 2014; Logsdon et al., 2003; Suri et al., 2001; Wisner et al., 1999), and studies in laboratory rodents also show a reversal of some physiological effects of chronic pregnancy stress when an SSRI is administered after parturition (Gemmel et al., 2016; Pawluski et al., 2012;

Salari et al., 2016). Pharmacological treatment options may also be further improved by examining the normative peripartum changes in the serotonergic system, or whether stress during pregnancy affects this system, particularly because serotonergic drugs are so often prescribed as treatment for peripartum affective disorders.

While it is unknown how pregnancy stress affects or alters the activity of the peripartum serotonergic system, both acute and chronic stress does affect serotonin in non-parous rodents, and it does so in a sex-specific way. Acute stressors can activate DR neurons via the abundant CRH receptors in the DR (Chalmers et al., 1995), which is important for appropriate behavioral responses that help animals cope with the particular stressor (Johnson et al., 2004). On the other hand, chronic stress can lead to anxiety-like and depressive-like symptoms, and can permanently alter the activity and physiology of the stress response and serotonergic systems. For instance, chronic stress increases 5-HT1A receptor expression in the CA1 region of the hippocampus of male rats but has no effect on expression of 5-HT1A receptors there in females, while 5-HT2C receptor expression in the hippocampus is significantly increased in both males and females after chronic stress (Pitychoutis et al., 2012). Additionally, social isolation stress increased 5-HT1A receptor binding in the hippocampus of male rats but has no effect on 5-HT1A binding in this region in female rats (Schiller et al., 2006). Of note, this study and others have found that unstressed control rats show sex differences in baseline serotonin release (Mitsushima et al., 2006) and the expression of some 5-HT receptors in several brain regions (Schiller et al., 2006; Zhang et al., 1999), suggesting that the serotonergic system might differentially regulate anxiety and depression-like behaviors (and proneness) even in unstressed males and females.

Overview of Dissertation and Experiments

Given the literature review above, the overall hypothesis of my dissertation studies is that 5-HT receptors are sensitive to female reproductive state and are critical modulators of female social and affective behaviors displayed during the postpartum period. Therefore, the purpose of the following experiments was to first determine the normative changes in expression of three major serotonin receptors (5-HT1A, 2A, and 2C) in the laboratory rat forebrain and midbrain across female reproduction and in response to maternal experience alone (Chapter 2), and whether the normal expression of these receptors is altered by chronic pregnancy stress (Chapter 3). Based on my significant finding of a 250% increase in 5-HT1A expression in the NAc of recently parturient females compared to diestrous virgins, in Chapter 4 I directly examined the contribution of NAc 5-HT1A receptors to the display of postpartum caregiving, aggression, anxiety-like, and depression-like behaviors by using viral-vector mediated knockdown. This work was predicted to reveal a contribution of 5-HT receptors to the normative expression of, and stress-induced maladaptions in, maternal caregiving and affective behaviors. The results are expected to not only be valuable for understanding basic mechanisms involved in serotoninergic regulation of animal behavior, but also have important implications for understanding the etiology and treatment of human postpartum affective disorders.

CHAPTER 2: EXPRESSION OF FOREBRAIN AND MIDBRAIN SEROTONIN 1A, 2A, AND 2C RECEPTORS ACROSS FEMALE REPRODUCTION

In most female rats and other mammals, the postpartum period is characterized by high maternal caregiving, high maternal protection of young, and low anxiety (Lonstein et al., 2014). These behavioral responses are initiated and maintained through lactation by changes in many steroid hormones, neuropeptides, and neurotransmitters that begin to fluctuate well before the offspring are born (Bridges, 2015).

The neurotransmitter serotonin (5-HT) plays a vital role in many social and affective behaviors including sexual behavior, parenting, play, aggression, anxiety, and depression (see Kiser et al., 2012). Reproductive state influences the central serotonergic system, such that the system is more excitable during the peripartum period (for reviews see Pawluski et al., 2019; Lonstein, 2019), with more serotonin metabolism in forebrain regions that are involved in the display of maternal caregiving behaviors. More specifically, research indicates that 5-HT turnover is significantly higher in the medial preoptic area and bed nucleus of the stria terminalis in dams sacrificed during the first week of lactation compared to non-parous females (Lonstein & Hull, 2003; Smith et al. 2013). This increase in serotonin metabolism in the forebrain is likely due to increased activity within the dorsal raphe, the midbrain site providing the majority of the forebrain's serotonin (Vertes et al., 1991). In support, spontaneous firing rate of DR serotonin cells begins to increase during pregnancy, peaks just before parturition, and remains relatively high during early lactation (Klink et al., 2002). Administering an analogue of the progesterone metabolite allopregnanolone increases spontaneous firing rates of serotonin cells in the DR of female rats, indicating that allopregnanolone may be responsible for increased DR excitability (Robichaud & Debonnel, 2005). Estradiol and progesterone may also contribute to upregulation

of the central serotonin system, as administering estradiol to ovariectomized female rats increases serotonin synthesis and increases TPH2, the rate-limiting enzyme for serotonin production (Charoenphandhu et al., 2011; Donner & Handa et al., 2009). Progesterone given subcutaneously or daily for 14 days also increases serotonergic activity within the brain of female macaques and guinea pigs by increasing TPH2 protein and decreasing 5-HT1A autoreceptor mRNA within the DR (Hiroi and Neumaier, 2009; Lu et al., 1999; Lu & Bethea, 2002; Pecins-Thompson & Bethea, 1999).

Although there appears to be more serotonin output to, and metabolism in, some forebrain regions during the early postpartum period, it is unknown whether the ability of these brain regions to *respond* to serotonin is also affected by reproductive status. There are at least 15 receptors that bind serotonin and have different patterns of expression and functions in the brain (see Nichols, 2008 for review). Of the many serotonin receptors, three in particular - 5-HT1A, 2A, and 2C - have been shown to affect postpartum behaviors including maternal caregiving and maternal aggression. For example, activating 5-HT1A receptors either centrally or specifically in the amygdala, dorsal periaqueductal gray, or median raphe decrease maternal aggression (de Almeida & Lucion, 1994; de Almeida & Lucion, 1997; Ferreira et al., 2000). 5-HT2A and 2C receptors appear to have a more complex role in regulating maternal aggression, as infusing a mixed 2A/2C agonist into the dorsal PAG reduces aggressive behaviors in postpartum females, while infusing the same drug into the amygdala increases aggressive behaviors (de Almeida et al., 2005; de Almeida et al., 2006). However, these studies did not tease apart the differential effects of 5-HT2A and 5-HT2C receptors. In addition, the effects of 5-HT2A and 2C activation on maternal aggression may depend on the amount of expression or the sensitivity of these receptors during the postpartum period. Together, the extant literature indicates a complex role

for 5-HT receptors in maternal behaviors, and highlights the need for additional research to determine whether changes in expression or density of these receptors instead underlies their involvement in postpartum behaviors.

With regards to maternal caregiving behaviors, studies using atypical antipsychotic drugs that affect 5-HT2A and 2C receptors have found alterations in maternal responsiveness. For example, acutely administering the antipsychotic drug clozapine, a 5-HT2A antagonist, to postpartum female rats increases the latency to approach pups (Li et al., 2004). Acutely *activating* 5-HT2A receptors with the agonist TCB-2 also disrupts caregiving by disrupting retrieval of pups to the nest, while simultaneous activation of both 5-HT2A and 2C receptors almost completely abolishes pup retrieval (Gao et al., 2018). The acute effects of 5-HT2A receptor activation on caregiving may be mediated by the ventral bed nucleus of the stria terminalis, the central amygdala, or the dorsal raphe, as Fos immunoreactivity was significantly increased in these regions following acute 2A activation. However, postpartum dams with chronic TCB-2 administration did not show prolonged disruptions in pup retrieval, nor did they show the same Fos activation in the aforementioned brain sites. While there are a number of studies showing that 5-HT2A and 2C receptors are involved in maternal caregiving, there is currently only one study that examined the role central 5-HT1A receptors in caregiving behaviors. This study did find that central 5-HT1A blockade disrupted pup retrieval (Ferreira et al., 2000).

Expression of 5-HT1A and 2A, and 2C receptors is sensitive to steroid hormones such as estradiol and progesterone. For example, 5-HT2A receptor expression in the NAc and some cortical regions is increased in females receiving estradiol injections (Sumner & Fink, 1995), and estradiol increases immunolabeling of 5-HT2C receptors in the hippocampus (Berumen et al.,

2011). 5-HT1A receptor expression, on the other hand, is reduced in the hippocampus and limbic sites following estradiol administration in female rats (Osterlund et al., 2000) and is significantly reduced in DR serotonin neurons following estradiol administration in non-human primates (Pecins-Thompson & Bethea, 1999). Together, these findings suggest that expression of some 5-HT receptors could fluctuate across pregnancy, parturition, and the postpartum period in response to changing levels of steroid hormones.

There have been no previous studies characterizing the mRNA expression patterns of serotonin receptors in the brain across female reproductive states, so the primary goal of the experiments in this chapter is to determine the expression pattern of three serotonin receptors (1A, 2A, 2C) across female reproduction in selected midbrain and forebrain sites involved in social and affective behaviors in laboratory rats. In Experiment 2a, the following sites were analyzed for these receptor mRNAs: the medial prefrontal cortex (mPFC), nucleus accumbens shell (NAcSh), medial preoptic area (mPOA), anterior hypothalamus (AH), dorsal and ventral subregions of the anterior bed nucleus of the stria terminalis (dBST and vBST), ventral tegmental area (VTA), median raphe (MR), and dorsal raphe (DR).

Because females that undergo pregnancy, parturition and lactation not only experience hormonal changes but also maternal experience, Experiment 2b in this chapter analyzed whether 7 days of maternal experience in the absence of pregnancy and parturition was sufficient to produce changes in central 5-HT receptor mRNA similar to the expression pattern of postpartum females. To accomplish this, I used the maternal sensitization model (Rosenblatt, 1969). This model involves exposing nulliparous females to young pups until they are maternally responsive. Virgin maternal behaviors include retrieving pups when they are placed into the cage, licking pups, and hovering over them. Although these maternally sensitized females do not show the full

repertoire of maternal behavior, these females show lasting changes in the brain in response to pup stimuli, including increased dopamine release (Afonso et al., 2008) and increased Fos immunoreactivity in the mPOA compared to non-sensitized virgins (Numan & Numan, 1994).

Because mRNA and protein levels in the brain do not always correlate with one another (reviewed in Greenbaum et al., 2003), Experiment 2c of this chapter used receptor autoradiography to analyze the brain sites in Experiment 2a that showed significant differences among groups in 5-HT receptor mRNA to determine whether receptor binding follows the same patterns. Reasons for a possible mismatch between mRNA and protein expression include the complexity of post-transcriptional mechanisms involved in converting mRNA to protein that makes it difficult to predict protein concentrations from mRNA, as well as differences in the half-lives of different proteins once they are transcribed (Greenbaum et al., 2003). Additionally, binding analysis will provide insight into how much mRNA has been translated into functional protein, and may provide a more causative association between differences in 5-HT receptor expression and their potential contribution to reproductive behaviors. I hypothesize that there will be reproductive state-dependent changes in 5-HT receptor expression in at least some of the brain sites analyzed. Given that estradiol and progesterone administration affect mRNA expression of these receptors, I predict that 5-HT1A and 2A receptor mRNA and binding will be higher in some brain sites at parturition and early lactation compared to other times of reproduction. Additionally, I predict that the DR and MR will have less 5-HT2C receptor mRNA and binding in recently parturient and lactating females compared to virgins, as this receptor is expressed on GABA neurons in the DR, which disinhibit serotonin release there and elsewhere in the brain.

Experiment 2a – Effects of Female Reproductive State on Serotonin 1A, 2A, and 2C mRNA Expression

Methods

Subjects

In Experiment 2a, subjects were female Long-Evans rats (*n* =7-10/group) descended from rats purchased from Harlan Laboratories (Indianapolis, IN), born and raised in our colony at Michigan State University. Females were housed with 1 or 2 same-sex littermates in clear polypropelyne cages (48 cm x 28 cm x 16 cm) containing wood chip bedding, food (Tekland rat chow, Indianapolis, IN) and water ad libitum. The room was maintained on a 12 hr light/dark cycle (lights on at 0700 hr) with temperature kept at 22 ± 1 °C. After reaching 65 days old, females in the mated groups were placed in a cage with an experienced male breeder from the colony (Harlan Laboratories, Indianapolis, IN) on the morning of proestrus (determined by daily vaginal smearing and cytology) and housed with the male until the next day. Vaginal cytology was used to confirm the presence of sperm, and therefore successful insemination. Subjects were then again housed 2-3 to a cage until sacrifice, or were singly housed 5-7 days before parturition (for the parturient and postpartum groups). Subjects were euthanized with CO_2 and rapidly decapitated in the afternoon when in diestrous (DV), on pregnancy day 10 (P10), no later than three hours after birth of the first pup (Part), or on postpartum day 7 (PP7) (*n* = 8-10/group).

Real Time RT-PCR

Brains were stored at – 80 °C until sectioning, and then sliced coronally at 300 micrometers using a cryostat. The following nine brain sites of interest were punched bilaterally using a 1-mm micro-puncher (Harris Micropunch, Hatfield, PA) - medial prefrontal cortex

(mPFC; atlas plates ~7-10), nucleus accumbens shell (NAcSh; plates ~14-21), medial preoptic area (mPOA; plates ~18-20), anterior hypothalamus (AH; plates ~23-27), dorsal and ventral subregions of the anterior bed nucleus of the stria terminalis (dBST and vBST; plates ~17-20), ventral tegmental area (VTA; plates ~43-45), median raphe (MR; plates ~49-53), and dorsal raphe (DR; plates ~49-53) (Paxinos & Watson, 2006). Tissue was homogenized in RLT Plus buffer (74134, Qiagen, Valencia, CA) containing β-mercaptoethanol by pulsed sonication for 30s at 25% amplitude (Fisher Scientific, Pittsburgh, PA). Next, mRNA was extracted using the RNeasy Plus Mini Kit (74134, Qiagen, Valencia, CA) per the manufacturer's instructions. Extracted mRNAs were quantified using a Genequant 100 spectrometer (General Electric, Marlborough, MA) by measuring the 260 nm absorbance values. The ratio of 260 nm to 280 nm absorbance values was also used to check for mRNA contamination. 100 ng of mRNA was then converted to cDNA using a high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA) per the manufacturer's instructions. After conversion to cDNA, samples were stored at – 20 °C until real time RT-PCR analysis.

Three serotonin receptors were analyzed: the inhibitory 5-HT1A receptor (Forward primer – GGC GCT TTC TAT ATC CCG CT; Reverse primer– GGT GCC GAC GAA GTT CCT AA), the excitatory 5-HT2A receptor (Forward – CTT CCA ACG GTC CAT CCA CA; Reverse – CAG GAA GAA CAC GAT GCC CA) and the excitatory 5-HT2C receptor (Forward – CTG ATG CAC CTA ATC GGC CT; Reverse – TCC GGG AAT TGA AAC AAG CG). Hypoxanthine-guanine phosphoribosyltransferase (HPRT), (Forward – GAA ATG TCT GTT GCT GCG TCC; Reverse – GCC TAC AGG CTC ATA GTG CAA) was used as the control gene for all analyses based on its relatively constant expression in all cells independent of experimental conditions (Tan et al., 2012). All receptors were run in separate wells containing 5

ng cDNA, SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) and 200 nM (5-HT2A, 5-HT2C, and HPRT) or 400 nM (5-HT1A) of both the forward and reverse primers. Each sample was run in triplicate for every subject, with all groups equally represented on each plate. An ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) was used for quantification with the following settings: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A dissociation curve was run for each sample to ensure that only a single product was transcribed for each well. The ΔΔCT method was used to calculate the fold change between groups (Schmittgen and Livak, 2008).

Data Analysis

PCR data were analyzed using Analyses of Covariance controlling for PCR plate using group as the main factor and PCR plate as a categorical covariate. Additionally, mRNA expression in reproducing groups of females was expressed as percent fold change in comparison to the diestrous virgin group, which was set equal to 1.0.

Results

Several brain sites showed statistically significant reproductive state-related changes in 5-HT receptor mRNA expression (Figure 1). 5-HT1A receptor mRNA expression was 250% higher in recently parturient females compared to diestrous virgins in the NAc shell ($F = 3.22$, $p = 0.040$). It was also found that 5-HT2A receptor mRNA expression in the mPOA was 50% higher in recently parturient females compared to diestrus virgins, and expression of this receptor remained high in the early postpartum females ($F = 9.686$, $p < 0.001$). Groups did not differ in 5-HT2A mRNA in any other brain site analyzed here. For the 5-HT2C receptor, its mRNA

expression in the DR was significantly less in recently parturient females compared to DVs ($F = 7.47$, $p = 0.008$) and it further dropped during the early postpartum period. There were no significant differences among groups in any of the three 5-HT receptors analyzed in the PFC, dorsal or ventral BST, AH, or MR (Table 1).

	5-HT1A						
	DV	**P10**	**Part**	**PP7**	**F**	**p**	**Partial Eta^2**
mPFC	1.00 ± 0.20	1.04 ± 0.21	0.95 ± 0.17	1.07 ± 0.17	0.21	0.89	0.02
NAcSh	1.00 ± 0.66	1.18 ± 0.66	2.39 ± 0.57	1.53 ± 0.57	3.22	0.04*	0.30
mPOA	1.00 ± 0.17	1.07 ± 0.16	1.09 ± 0.15	0.85 ± 0.17	0.92	0.45	0.10
dBST	1.00 ± 0.10	0.69 ± 0.10	0.70 ± 0.11	0.64 ± 0.09	1.67	0.19	0.11
vBST	1.00 ± 0.20	1.52 ± 0.23	1.04 ± 0.22	1.02 ± 0.22	0.89	0.46	0.08
AH	1.00 ± 0.11	1.00 ± 0.11	0.97 ± 0.12	1.02 ± 0.11	0.12	0.95	0.02
VTA	1.00 ± 0.10	0.88 ± 0.10	0.94 ± 0.10	0.97 ± 0.10	0.16	0.92	0.02
MR	1.00 ± 0.27	1.14 ± 0/25	1.11 ± 0.29	1.43 ± 0.27	0.28	0.84	0.04
DR	1.00 ± 0.10	0.95 ± 0.10	0.70 ± 0.10	0.73 ± 0.10	1.39	0.27	0.13
	5-HT2A						
	DV	**P10**	**Part**	**PP7**	**F**	**p**	**Partial Eta^2**
mPFC	1.00 ± 0.16	0.82 ± 0.17	0.91 ± 0.14	0.92 ± 0.14	0.40	0.75	0.04
NAcSh	1.00 ±0.26	0.65 ± 0.26	0.85 ± 0.22	0.86 ± 0.22	2.15	0.12	0.22
mPOA	1.00 ± 0.09	1.22 ± 0.08	1.44 ± 0.08	1.29 ± 0.09	5.39	0.01*	0.38
dBST	1.00 ± 0.11	1.10 ± 0.11	0.83 ± 0.12	1.10 ± 0.11	0.21	0.89	0.01
vBST	1.00 ± 0.23	1.50 ± 0.26	1.31 ± 0.25	1.34 ± 0.25	0.81	0.50	0.08
AH	1.00 ± 0.14	0.98 ± 0.14	1.05 ± 0.16	1.10 ± 0.14	0.21	0.89	0.03
VTA	1.00 ± 0.12	0.73 ± 0.11	0.86 ± 0.11	0.80 ± 0.11	1.38	0.27	0.13
MR	1.00 ± 0.19	1.04 ± 0.18	0.97 ± 0.21	1.02 ± 0.19	0.54	0.66	0.08
DR	1.00 ± 0.15	1.00 ± 0.15	1.17 ± 0.16	0.92 ± 0.14	0.25	0.86	0.03
	5-HT2C						
	DV	**P10**	**Part**	**PP7**	**F**	**p**	**Partial Eta^2**
mPFC	1.00 ± 0.11	0.83 ± 0.11	0.75 ± 0.11	0.81 ± 0.11	1.40	0.26	0.13
NAcSh	1.00 ± 0.12	0.70 ± 0.12	0.77 ± 0.11	0.79 ± 0.12	3.10	0.05*	0.28
mPOA	1.00 ± 0.12	0.96 ± 0.10	0.81 ± 0.10	1.03 ± 0.11	0.35	0.79	0.04
dBST	1.00 ± 0.17	1.64 ± 0.17	1.42 ± 0.18	1.60 ± 0.16	1.74	0.17	0.11
vBST	1.00 ± 0.29	1.17 ± 0.32	0.83 ± 0.31	0.88 ± 0.31	0.18	0.91	0.02
AH	1.00 ± 0.11	1.20 ± 0.11	1.10 ± 0.12	1.38 ± 0.11	1.26	0.31	0.14
VTA	1.00 ± 0.13	0.98 ± 0.13	0.98 ± 0.13	1.07 ± 0.13	0.16	0.92	0.02
MR	1.00 ± 0.22	0.92 ± 0.21	1.13 ± 0.24	1.06 ± 0.22	0.34	0.80	0.05
DR	1.00 ± 0.13	0.96 ± 0.13	0.52 ± 0.13	0.37 ± 0.12	3.07	0.04*	0.25

Table 1: 5-HT receptor mRNA in various brain sites across reproductive stages. 5-HT1A, 5-HT2A, and 5-HT2C mRNA levels (ΔΔCT ± SEM) in the medial preoptic area (mPFC), nucleus accumbens shell (NAcSh), medial preoptic area (mPOA), dorsal bed nucleus of the stria terminalis (dBST), ventral BST (vBST), anterior hypothalamus (AH), ventral tegmental area (VTA), median raphe nucleus (MR), and dorsal raphe nucleus (DR) of virgin females in diestrous (DV), pregnancy day 10 (P10), soon after parturition (Part), and postpartum day 7 (PP7). ΔΔCT values are normalized to DV, which is set to 1.00. *indicate differences in mRNA expression between groups, $p < 0.05$.

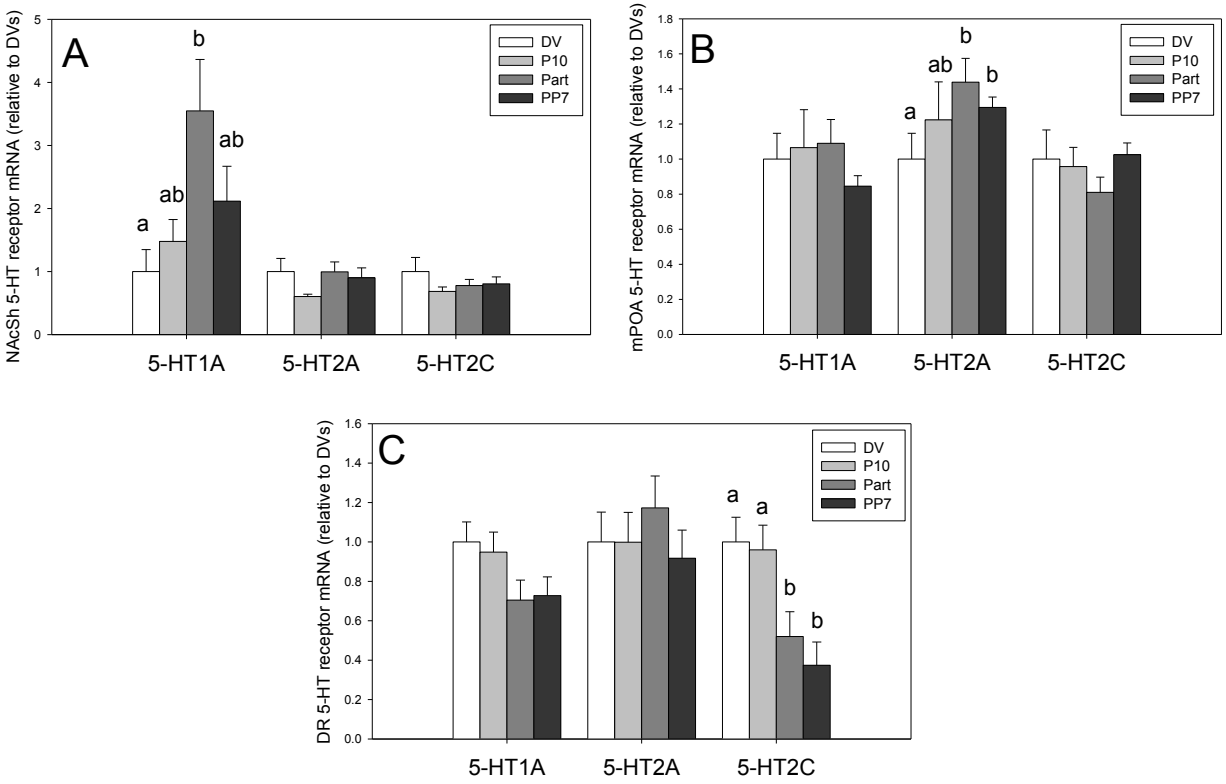

Figure 1: 5-HT receptor mRNA in brain sites across female reproductive states. 5-HT1A, 5-HT2A, and 5-HT2C mRNA in diestrous virgins (DV), pregnancy day 10 (P10), soon after parturition (Part), or on postpartum day 7 (PP7) in the NAcSh (A), mPOA (B), and DR (C). Bars represent ΔΔCT of each group normalized to DV. Letters above the bars indicate significant differences between groups, $p < 0.05$.

Ligand	[Ligand] nM	Pre-incubation protocol	Buffer	Incubation Temp	Incubation Time	Wash Time	Exposure	Blank	References
[H3] MDL100907	0.4	30 min at room temp	50 mM Tris-HCl (pH 7.4)	Room temp	60 min	2x10 min	35 days	10µM spiperone	Johnson et al., 1996; Lopez-Gimenez et al., 1997
[H3] mesulergine	4.8	15 min at room temp	170 mM Tris-HCl (pH 7.7)	Room temp	120 min	2x10 min	30 days	10µM mianserin	Pazos et al., 1985; Hoyer et al., 1986
[H3]8-OH-DPAT	2.0	15 min at room temp	50 mM Tris-HCl (pH 7.4)	Room temp	60 min	2x10 min	35 days	10µM 5-HT	

Table 2: Incubation conditions for the radioligands used in Experiment 2c.

Experiment 2b – Effects of Maternal Sensitization on Serotonin 1A, 2A, and 2C Receptor mRNA Expression

In order to tease apart the differential contribution of reproductive hormones versus experience with offspring for the effects of reproduction on 5-HT receptor mRNAs found in Experiment 2a, brains from a group of maternally sensitized nulliparous female rats and a control group of non-sensitized nulliparous rats were analyzed for 5-HT1A mRNA in the NAcSh, 5-HT2A mRNA in the mPOA, and 5-HT2C mRNA in the DR.

Methods

Subjects

Subjects were female Long Evans rats (n = 8-10/group) descended from rats purchased from Harlan Laboratories (Indianapolis, IN), born and raised in our colony at Michigan State University. All housing conditions were similar to those used in Experiment 2a.

Ovariectomy

Virgin females from both the experimental and control groups were weighed and anesthetized by an intraperitoneal injection of 90 mg/kg ketamine (Henry Schein Animal Health, Dublin, OH) followed by an intraperitoneal injection of 8 mg/kg xylazine (Lloyd laboratories, Shenandoah, IA). Incisions were made through the skin and underlying muscle in the dorsolateral flanks and the ovaries were externalized. The distal portion of the uterine horn was bilaterally tied with silk suture and the ovaries were removed. Incisions in the muscle layer were sutured closed and incisions in the skin closed with surgical staples. After surgery, subjects were placed on a heating pad within their home cage until they recovered from anesthesia. Subjects

received postoperative care including twice-daily subcutaneous injections of buprenorphine (0.015 mg/kg) for one day after surgery and then left singly housed either until sacrifice (non-sensitized group) or until maternal sensitization began (sensitized group).

Maternal Sensitization

Beginning a week after ovariectomy, and using methods similar to previous studies from our lab (Lonstein et al., 1999; Smith et al., 2013), the sensitized subjects were exposed in their home cage to three freshly fed 1-7 day old male and female pups obtained from surrogate dams in our colony. Each morning the cages were viewed by an observer for 15 min, and females' pup-directed behaviors (sniffing or retrieving each of the three pups, licking the pups, huddling over the pups) were scored as present or not present. The criteria for subjects to reach "full maternal behavior" was retrieving all three pups to a single location, licking them, and huddling over them on two consecutive days of testing (Bridges, 1984). Each morning the foster pups from the previous day were removed from the subjects' cages, and it was recorded if the pups were warm, to help confirm subjects' maternal or non-maternal state. Those pups were then placed with surrogate lactating rats for at least two days before being used again. To better be able to compare the sensitized females in this experiment to the day 7 postpartum females studied in Experiment 2a, sensitized females showing full maternal behavior after 2 consecutive days were then given an additional 5 days of experience with pups to reach 7 full days of caregiving. This was done by continuing to place three freshly fed pups in the sensitized females' cages day and observing the subjects for 15 min daily to verify the continuance of their full maternal behavior.

Sacrifice and Tissue Processing

Brains were stored at –80 °C until sectioning, and then sliced coronally at 500 μm using a cryostat. The following three brain sites where significant reproductive-state changes were present were punched bilaterally using a 1-mm micro-puncher (Harris Micropunch, Hatfield, PA) - nucleus accumbens shell (NAcSh), medial preoptic area (mPOA), and dorsal raphe (DR). Tissue was homogenized in RLT Plus buffer (74134, Qiagen, Valencia, CA) containing β-mercaptoethanol by pulsed sonication for 30s at 25% amplitude (Fisher Scientific, Pittsburgh, PA). Next, mRNA was extracted using the RNeasy Plus Mini Kit (74134, Qiagen, Valencia, CA) per the manufacturer's instructions. Extracted mRNAs were quantified using a Genequant 100 spectrometer (General Electric, Marlborough, MA) by measuring the 260 nm absorbance values. The ratio of 260 nm to 280 nm absorbance values was also used to check for mRNA contamination. 100 ng of mRNA was then converted to cDNA using a high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA) per the manufacturer's instructions. After conversion to cDNA, samples were stored at – 20 °C until real time RT-PCR analysis.

Real-Time PCR

The same three serotonin receptors from the first experiment were analyzed: the inhibitory 5-HT1A receptor (Forward primer – GGC GCT TTC TAT ATC CCG CT; Reverse primer– GGT GCC GAC GAA GTT CCT AA), the excitatory 5-HT2A receptor (Forward – CTT CCA ACG GTC CAT CCA CA; Reverse – CAG GAA GAA CAC GAT GCC CA) and the excitatory 5-HT2C receptor (Forward – CTG ATG CAC CTA ATC GGC CT; Reverse – TCC GGG AAT TGA AAC AAG CG). HPRT (Forward – GAA ATG TCT GTT GCT GCG TCC; Reverse – GCC TAC AGG CTC ATA GTG CAA) was used as the control gene for all analyses.

All receptors were run in separate wells containing 5 ng cDNA, SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) and 200 nM (5-HT2A, 5-HT2C, and HPRT) or 400 nM (5-HT1A) of both the forward and reverse primers. Each sample was run in triplicate for every subject, with all groups equally represented on each plate. An ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) was used for quantification with the following settings: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A dissociation curve was run for each sample to ensure that only a single product was transcribed for each well. The ΔΔCT method was used to calculate the fold change between the two groups (Schmittgen and Livak, 2008).

Data Analysis

PCR data were analyzed using Analyses of Covariance controlling for PCR plate using group as the main factor and PCR plate as a covariate. Additionally, mRNA expression in the sensitized virgin group was expressed as percent fold change in comparison to the non-sensitized virgin group, which was set equal to 1.0.

Results

Maternally sensitized virgin females did not differ from non-sensitized virgin females in expression of 5-HT1A, 2A, or 2C mRNA in the NAc, mPOA, or DR (Figure 2). This suggests that the hormones or reproduction, rather than simply maternal experience, alters expression of these serotonin receptors.

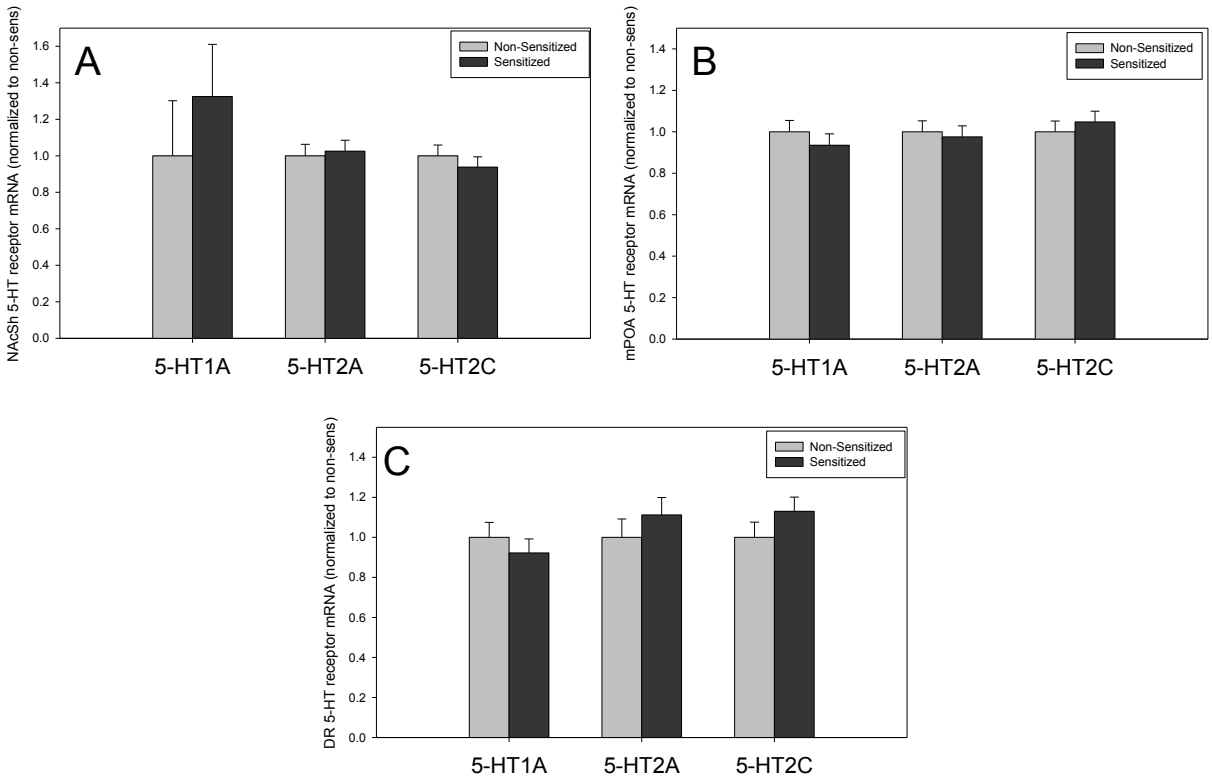

Figure 2. 5-HT receptor mRNA in brain sites in maternally sensitized and non-sensitized females. 5-HT1A, 5-HT2A, and 5-HT2C mRNA in ovariectomized females with 7 days of maternal experience (sensitized) and ovariectomized females without maternal experience (non-sensitized) in the NAcSh (A), mPOA (B), and DR (C). Bars represent ΔΔCT of the sensitized group normalized to the non-sensitized group.

Experiment 2c – Effects of Female Reproductive State on Serotonin 1A and 2A Receptor Autoradiograph Binding Density

Because brain mRNA and protein levels do not always correlate with one another, receptor autoradiography (autoRAD) was used to determine receptor binding in the brain regions where 5-HT receptor mRNAs differed across reproduction in Experiment 2a above. Specifically, I examined 5-HT1A receptor binding in the NAcSh, 5-HT2A receptor binding in the mPOA, and 5-HT2C receptor binding in the DR in diestrous virgin, recently parturient, and postpartum day 7 female rats.

Methods

Subjects

Long Evans rats (n = 7/group) were euthanized with CO_2 and rapidly decapitated on a day of diestrous, within three hours after birth of the first pup, or on postpartum day 7 (PP7). Brains were extracted and frozen. Brains were sliced coronally with a cryostat into 10 series of 15 µm-thick sections that were mounted onto glass slides and frozen at -80º until processing.

Receptor Autoradiography

One series of slides was removed from the -80˚C freezer and allowed to thaw at room temperature for 1 hr. The slide-mounted sections were fixed in 1% paraformaldehyde for 2 min and then pre-incubated in Tris-HCl buffer (see Table 3 for details on incubation and washing conditions for each radioligand) at room temperature for 30 min. Sections were then incubated in a solution containing Tris-HCl buffer and either 0.4 nM [3H]MDL100.907 (Metis Laboratories,

Valley Stream, NY) or 2 nM [3H]8-OH-DPAT (Perkins-Elmer, Waltham, MA) for 1-2 hrs. A control set of slides was pre-incubated in a blocking agent (see Table 2) in order to determine the background signal and nonspecific binding of each radioligand. After incubation, sections were washed twice at 4˚C in Tris-HCl buffer for 10 min each. The sections were then dipped for a few sec into 4˚C distilled water. Slides were covered and left overnight to dry at room temperature. The following day, slides were moved to autoradiography cassettes (Fisher Scientific, Pittsburgh, PA) each containing a set of tritiated standards (ARI 0133, American Radiolabeled Chemicals) and exposed to a tritium-sensitive phosphorscreen (BAS-IP TR 2040E, GE Healthcare) at room temperature for 30 days. The screens were scanned using an Amersham Typhoon IP Biomolecular Imager (GE Healthcare, product # 29187194) at 10 μm resolution and 4000 PMT.

Data Analysis

Autoradiographic slides were run in two sets, and were analyzed using Analyses of Covariance (controlling for set) to compare binding among the three groups. Additionally, repeated measures Analyses of Variance were used to determine within-subject differences in autoradiographic binding across rostrocaudal level of each region. Using ImageJ, a ROI containing each region of interest was drawn on digitized images of the phoshorscreens, resulting in 4 boxes through the NAc (2.28-1.2mm from bregma) and 3 boxes through the mPOA (-0.15 - -0.45mm from bregma). 5-HT2C in the DR could not be analyzed due to non-specific binding of the 5-HT2C ligand. Control slides containing blocking peptides were also processed and scanned to subtract out background binding for each radioligand. Optical density of the receptor binding of interest in each brain region was compared between the three groups of females. For all

analyses, a significant main effect was determined by $p < 0.05$, and in such cases, LSD post-hoc tests were conducted to determine differences between pairs of groups.

Results

Total 5-HT1A receptor binding density (adding all four levels of the NAc shell) did not significantly differ by reproductive state ($F_{(2, 20)} = 1.55$, $p = 0.24$). However, analysis of binding density across rostrocaudal extent revealed that 5-HT1A binding in the most rostral section of the NAc shell (~2.28 mm from bregma) was significantly higher in PP7 dams compared to recently parturient dams (repeated-measures ANOVA $F_{(2,16)} = 3.22$, $p = 0.067$; LSD post-hoc $p = 0.027$). In the most medial section analyzed (~1.56 mm from bregma), 5-HT1A binding tended to be higher at parturition compared to DVs (ANOVA $F_{(2,16)} = 3.03$, $p = 0.077$; LSD post-hoc $p = 0.042$).

5-HT2A binding density in the mPOA was significantly higher at parturition compared to virgins and PP7 dams ($F_{(2, 21)} = 17.4$, $p < 0.0001$) and was significantly higher at every rostrocaudal level analyzed (LSD post-hoc $p < 0.005$).

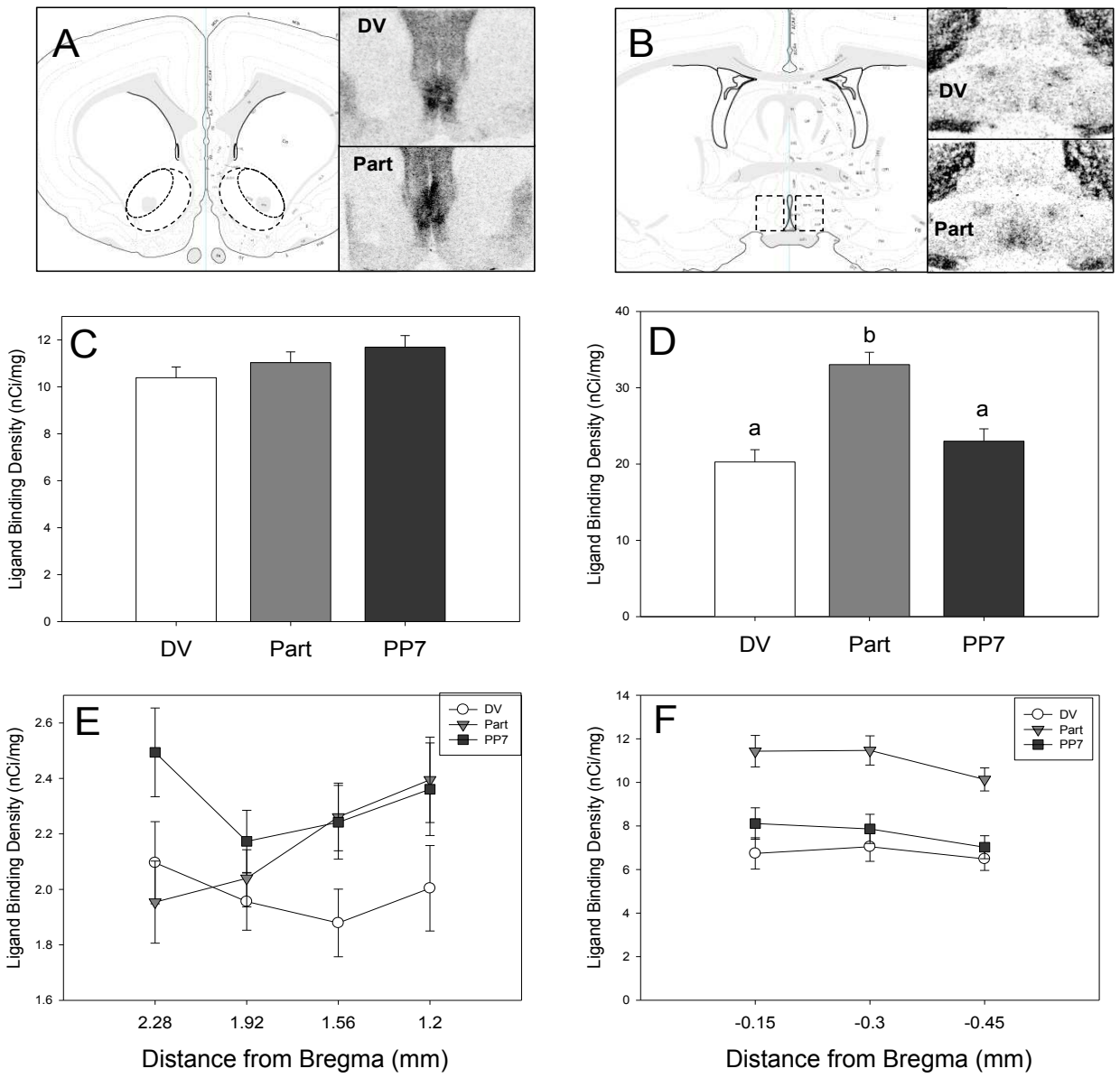

Figure 3. 5-HT receptor binding in the NAc and mPOA across female reproductive states. Representative radiograms of 5-HT1A binding in the NAc (A) and of 5-HT2A binding in the mPOA (B) of a diestrous virgin (DV) and a recently parturient dam (Part). Total 5-HT1A binding density (Mean ± SEM) in the NAcSh of DV, Part, and postpartum day 7 (PP7) collapsed across rostrocaudal section (C). Total 5-HT2A binding density (Mean ± SEM) in the mPOA of DV, Part, and PP7 females collapsed across rostrocaudal section (D). Total 5-HT1A binding (Mean ± SEM) across rostrocaudal level of the NAcSh of female rats as DV, Part, and PP7 (E). Total 5-HT2A binding (Mean ± SEM) across rostrocaudal level of the mPOA of female rats as DV, Part, and PP7 (F). Letters above the lines indicate significant differences between groups.

Discussion

Consistent with previous research from our laboratory and others, the findings from these experiments indicate that maternal state is characterized by changes in the central serotonin system, both at the level of the serotonergic DR and in its forebrain projection sites. Specifically, I found that when compared to diestrus virgins, 5-HT1A mRNA expression in the NAcSh was significantly higher in recently parturient dams, that 5-HT2A mRNA in the mPOA was higher at both parturition and early lactation, and that 5-HT2C mRNA was significantly lower in the DR in recently parturient dams (and even lower during early lactation). The autoradiographic binding results supported some of these findings, such that 5-HT2A binding in the mPOA was significantly higher in recently parturient dams compared to diestrous virgins. However, despite the fact that Experiment 2a found that 5-HT2A mRNA remained high during early lactation, this experiment found that binding density of this receptor in early lactating dams was comparable to that of virgins.

With regards to 5-HT1A binding in the NAc shell, it was not significantly higher in reproductive rats compared to virgins, although some sections analyzed did show higher levels in 5-HT1A binding at parturition or early lactation. Together, these results suggest that that alterations in the ability of forebrain sites in the larger maternal behavior network to respond to serotonin around the time of parturition or soon thereafter may contribute to the unique behavioral responses seen during this period of reproduction.

Reproductive State Influences 5-HT Receptors in the DR

The serotonergic system arising from the DR is generally upregulated during the peripartum period, with an increase in firing rate of serotonin cells and increased serotonin

release in some forebrain sites that regulate maternal caregiving (Klink et al., 2002; Smith et al., 2013). Serotonin release is partly modulated by the various 5-HT receptors within the DR that respond to serotonin coming from local DR serotonergic neurons, as well as respond to serotonin inputs from other raphe nuclei projecting to the DR (Sprouse & Aghajanian, 1986; Liu et al., 2000). Inhibitory 5-HT1A receptors are commonly expressed on the soma and dendrites of serotonergic neurons as a way of reducing serotonin release when it becomes too high (Hopwood & Stamford, 2001); on the other hand, excitatory 5-HT2A and 2C receptors can be found on many non-serotonergic neurons within the DR (Serrats et al., 2004; Liu et al., 2000). Although reproductive state did not appear to influence the expression of 5-HT1A or 2A receptor mRNA in the DR, we found significantly less 5-HT2C receptor expression at parturition and a further reduction at postpartum day 7 compared to diestrous virgins. 5-HT2C receptors are most commonly expressed on GABAergic neurons within the DR, and activating DR 5-HT2C-expressing neurons using electrophysiology causes a marked reduction in serotonin release and an increase in Fos immunoreactivity in GABA neurons within the DR (Boothman et al., 2006). Therefore, reduced 2C expression at parturition suggests a reduction in the ability for serotonin to influence intra-DR GABA release, which would presumably lead to reduced GABAergic signaling within the DR, disinhibition of 5-HT neurons, and greater 5-HT release. Although 5-HT2C binding in the DR could not be analyzed in this experiment, lower 5-HT2C binding density in the DR during lactation would further suggest disinhibition of the DR at this time.

Reproductive State Influences 5-HT Receptors in the mPOA

The DR is responsible for ~80% of the forebrain's serotonin, projecting to sites including the prefrontal cortex, nucleus accumbens, and many hypothalamic regions (Vertes et al., 1991).

5-HT turnover in the mPOA and the adjacent BST is elevated during early lactation (Lonstein et al., 2003; Smith et al., 2011). Both sites regulate maternal caregiving and other postpartum behaviors (Jacobson et al., 1980; Perrin et al., 2007), suggesting that serotonin release in these brain areas may contribute to caregiving behaviors. The current experiment found that the mPOA had reproductive state-related changes in 5-HT receptor expression and binding density. Specifically, 5-HT2A receptor mRNA was higher in recently parturient and early-postpartum females compared to diestrous virgin females, while 5-HT2A binding density was significantly higher just in recently parturient mothers. These findings are consistent with a microarray study reporting higher 5-HT2A gene expression in the mPOA of postpartum rats sacrificed approximately 2 days after parturition when compared to virgin females (Akbari et al., 2013), and they collectively suggest an increase in responsiveness of the mPOA to serotonin during the peripartum period and early expression of caregiving behaviors.

5-HT2A signaling in the mPOA is known to be involved in female reproduction. Injecting the 5-HT2A receptor antagonist perinperone into the mPOA inhibits female sexual receptivity in laboratory rats (Mendelson & Gorzalka, 1985), and it is possible that the sharp increase in 5-HT2A expression and binding density during parturition facilitates sexual receptivity during postpartum estrus (Connor & Davis, 1980a, 1980b; Dewsbury, 1990). This possibility should be considered in light of the fact that 5-HT2A receptor binding density is not significantly higher during proestrus, the day of the rodent estrus cycle when females are sexually receptive, compared to other phases of the estrus cycle (Sumner & Fink, 1997), though.

With regards to maternal behavior, centrally administering the 5-HT2A and dopamine receptor antagonist, clozapine, increases the latency of postpartum dams to investigate pups (Li et al., 2004). Concurrent administration of quinpirole to block the dopamine effects of clozapine

does not alleviate the retrieval deficits, indicating that it is the 5-HT2A inhibition that disrupts postpartum caregiving. 5-HT2A receptor involvement in clozapine-induced pup retrieval deficits was further proven when co-administration of DOI (a 5-HT2A agonist) attenuated pup retrieval. Interestingly, DOI on its own also disrupts pup retrieval and increases Fos expression in the mPOA; however, activating 5-HT2A specifically in the mPOA during lactation does not affect maternal caregiving (Gao et al., 2018). Because activity of the mPOA is generally pro-maternal, and 5-HT2A activation elevates postsynaptic potentials, activating 5-HT2A receptors in this region during a time when postpartum females already show robust pup-retrieval behaviors may have produced a ceiling effect in studies when the agonist was injected. Since only the 5-HT2A agonist was injected into the mPOA in that study, it is unknown whether inhibiting these receptors would instead disrupt maternal caregiving similar to the systemic injection of the 5-HT2A antagonist. Additionally, the 5-HT2A-modulating drugs in these studies were injected on postpartum days 4, 6, and 8, which is after these receptors are the most densely expressed based on the findings here in the current study that 5-HT2A binding was elevated only at parturition and soon thereafter.

Female ovarian hormones, including progesterone and estrogens are likely candidates for influencing the serotonergic system during the peripartum period to promote maternal caregiving. This is especially supported by the finding that 5-HT2A mRNA is only increased in females that gave birth to pups but not in those that only had maternal experience with pups (i.e., maternally sensitized). In the female rat, estradiol rises throughout pregnancy, and peaks right around parturition in preparation of the postpartum estrus (Bridges, 1984). Furthermore, administering estradiol and progesterone to nulliparous ovariectomized female rats induces maternal caregiving (Rosenblatt 1984). Ovarian hormones directly influence the serotonergic

system by acting on estrogen and progesterone receptors in the DR (Alves et al., 1998). ERβ-expressing serotonin neurons in the DR also supply ~50% of the serotonergic fibers that project to the mPOA (Lu et al., 2001), suggesting that mPOA-projecting DR 5-HT neurons are sensitive to estrogens. Indeed, acute systemic estradiol administration increases TPH2 mRNA in the DR of ovariectomized virgin rats (Lu et al., 1999), and increases DR serotonin synthesis (Hiroi et al., 2011).

Estradiol's effect on serotonin receptor expression depends on the brain region and the specific receptor subtype. Expression of 5-HT2A receptors is particularly influenced by estradiol. Ovariectomized female rats given estradiol have higher 5-HT2A receptor binding density in the frontal cortex, cingulate cortex, nucleus accumbens, caudate-putamen, and ventromedial hypothalamus compared to ovariectomized females given vehicle (Sumner et al., 1999). Interestingly, the mPOA was also analyzed in this study but no such increase in 5-HT2A receptor binding density was found following estradiol administration (Sumner et al., 1999). This suggests that while high levels of estradiol are capable of increasing the expression of 5-HT2A receptors in some areas of the forebrain, it does not do so in the mPOA. Therefore, high estradiol during the peripartum period likely does not explain the significant increase in 5-HT2A mRNA and binding density at parturition found in my study.

Instead, other hormones and neuropeptides elevated during the peripartum period could be responsible. These include oxytocin and prolactin, both of which are released at very high levels around the time of parturition. Blockade of either oxytocin or prolactin release during parturition disrupts the onset of maternal behavior (Van Leengoed et al., 1987; Zarrow et al., 1971), while their release promotes the onset of caregiving in nulliparous females (Bridges et al., 1985; Bridges, 1990; Pedersen et al., 1982). Suckling increases systemic prolactin release, and

also increases serotonin synthesis in the DR and metabolism in the medial preoptic area (Johnston et al., 1984). Thus, prolactin release during the peripartum period may increase mPOA responsiveness to serotonin. While no studies have shown a direct link between prolactin secretion and 5-HT2A expression anywhere in the brain, the suckling-induced increase in serotonin in the mPOA combined with the peripartum increase in mPOA serotonin binding sites may promote caregiving. Further support for this suckling-induced serotonergic mechanism in the mPOA is my finding in Experiment 2b that maternally sensitized females, which do not lactate and are not suckled by their foster pups (Lonstein et al. 1999), did not show higher 5-HT2A receptor mRNA compared to control females.

Reproductive State Influences 5-HT Receptors in the NAcSh

I also found that parturient females had ~250% more 5-HT1A receptor mRNA in the NAcSh when compared to that of diestrous virgin females. However, when collapsing all four NAc sections analyzed, 5-HT1A receptor binding density was not significantly higher at parturition or during early lactation. Nonetheless, the rostral-most section of the NAc had significantly more 5-HT1A binding in postpartum day 7 dams compared to recently parturient dams, and recently parturient dams showed significantly more 5-HT1A binding in the mid-caudal sections of the NAcSh compared to diestrous virgins. Similar to the mPOA, the NAc receives dense innervation from DR serotonergic neurons (Van Bockstaele et al., 1993), and the NAc expresses at least six subtypes of serotonin receptors (Sumner & Fink, 1995; Filip & Cunningham, 2002; Di Matteo et al., 2008).

No previous studies have investigated the effects of ovarian hormones on 1A expression in the NAc, but neurons in the NAc do express estrogen receptors (Le Saux et al., 2006). 5-

HT2A in the NAc is significantly increased in females following estradiol administration. This is interesting given that recent parturition, a time characterized by high estradiol circulation, did not significantly affect mRNA for 5-HT2A in the NAc in the current experiment. 5-HT1A receptor mRNA and binding in several forebrain sites is affected by chronic estradiol administration (Osterlund et al., 2000), although no studies have examined the effects of estrogens on 5-HT1A in the NAc. This peripartum increase in NAcSh 5-HT1A receptor mRNA is likely not the result of maternal experience alone, given that mRNA for this receptor subtype did not differ in maternally sensitized ovariectomized females compared to non-sensitized ovariectomized females. Thus, increased 5-HT1A mRNA (and autoradiographic binding in the rostral and mid-caudal NAc) may be related to hormones or peptides other than estradiol that are increased around the time of parturition.

The NAc is necessary for appetitive aspects of maternal behavior, with lesions of the NAc abolishing pup retrieval, an indicator of maternal motivation (Li & Fleming, 2003a). The NAc is also involved in the consolidation of maternal memory of a caregiving experience, because lesioning the NAc either before or after initial exposure to pups reduces maternal behaviors and pup retrieval when exposed to pups 10 days later (Li & Fleming, 1999). Spikes in dopamine (DA) release in the NAc are essential for maternal motivation and appetitive maternal behaviors. For example, DA is released in the NAc when dams are performing behaviors directed at pups, such as approaching, sniffing, and retrieving (Robinson et al., 2011), and is higher in postpartum dams that exhibit the most licking and grooming of pups (Champagne et al., 2004). 5-HT1A receptors in the NAc may influence these behaviors by affecting DA release, since injecting serotonin directly into the NAc increases extracellular DA there, and non-specific blockade of 5-HT1 family receptors attenuates this increase in DA (Parsons & Justice, 1993).

The cell types in the NAc that express 5-HT1A receptors are unknown, though 5-HT1A receptors in many brain sites are found on calbindin- and parvalbumin-containing GABAergic interneurons (Aznar et al., 2003) or GABAergic pyramidal cells (Sprouse & Aghajanian, 1988). Because the 5-HT1A receptor is an inhibitory G-protein coupled receptor, higher expression of these receptors in the NAc at parturition may result in less activation of GABAergic interneurons and, therefore, disinhibition of the NAc. At the same time, 5-HT1A receptors in the NAc could be expressed on GABAergic projections to brain sites that stimulate the NAc, releasing these sites from GABAergic inhibition and creating a feed-forward mechanism for NAc stimulation. This disinhibition of the NAc at parturition may increase pup approach and motivation to care for offspring. In support of this theory, increasing inhibition of the NAc with the GABAergic agonist, muscimol, delays pup retrieval in lactating rats (Numan et al., 2005), suggesting that disinhibition of NAc is necessary during the peripartum period to promote normal maternal motivation.

Conclusion

These experiments in Chapter 2 demonstrate for the first time that a particular pattern of expression of 5-HT1A, 2A, and 2C receptors exists among pregnancy, parturition, and lactation in female rats. This occurs in brain regions involved in maternal caregiving and affective behaviors, and tends to occur most around parturition when the brain is being prepared for motherhood. These changes are not solely due to maternal experience with offspring, and are more likely the result of the dramatic fluctuations in hormones during the peripartum period. These results further suggest that serotonin acting on forebrain and midbrain 5-HT receptors may contribute to the onset of postpartum behaviors.

CHAPTER 3: EFFECTS OF PREGNANCY STRESS ON POSTPARTUM SOCIAL AND AFFECTIVE BEHAVIORS AND BRAIN SEROTONIN RECEPTOR BINDING DENSITY

Introduction

The postpartum period is characterized by changes in behavior and the brain, including the serotonergic system (Lonstein, 2019). Studies in this dissertation have already shown that serotonin receptor expression is altered by reproductive status, and overall the serotonin system appears to be upregulated and more excitable during parturition and early lactation (Klink et al., 2002; Robichaud & Debonnel, 2006). Serotonin has been highly implicated in anxiety and depression in humans, with alterations in both serotonin release and 5-HT receptor affinity and activity have been linked to human affective disorders (Akimova et al., 2009; Drevets et al., 2007; Naughton et al., 2000). In rodents, chronic stress leads to increased anxiety-like and depression-like behaviors and alters serotonin (Grippo et al., 2005; Pitychoutis et al., 2012), although little research has examined the effects of chronic stress during pregnancy on the central serotonergic system in postpartum humans or laboratory rodents.

In humans, increased stress during pregnancy is a risk factor for developing postpartum anxiety or depression, which can have deleterious effects on developmental outcomes of infants. Maternal stress and anxiety during pregnancy negatively predicts infant behavioral reactivity (Davis et al., 2004) and problem behavior in children (Gutteling et al., 2005). These problem behaviors even persist into later childhood (Gutteling et al., 2005), suggesting long-term impact of stress during pregnancy. Rodent models have also shown effects of gestational stress on offspring behavior in adulthood. For example, gestational stress is associated with adult anxiety-like behavior (Brunton & Russell, 2010; Van den Hove et al., 2005; Vallee et al., 1997), greater

HPA axis response to acute stress (Takahashi & Kalin, 1991; Weinstock et al., 1992; Brunton & Russell, 2010), and abnormal social and reproductive behaviors (Lee et al., 2007; Holsen et al., 1995; Frye & Orecki, 2002a; Frye & Orecki, 2002b). Thus, it is important to understand the biological underpinnings of postpartum affective disorders for the wellbeing of both mothers and infants.

In laboratory rodents, chronic stress during pregnancy is known to negatively affect postpartum caregiving and increase postpartum depression-like behaviors. For example, both acute and chronic predator stress (exposure to a cat) during pregnancy reduces the number of pups later retrieved to the nest and the time sniffing pups (Patin et al., 2002). Repeated restraint or swim stress during pregnancy each increase time spent away from the nest and reduce arched-back nursing (kyphosis) (Smith, 2004; Haim et al., 2014; Leuner et al., 2014). Gestational stress also reduces licking behavior in naturally high-licking mothers to levels found in naturally low-licking dams (Champagne & Meaney, 2006). Many of these studies also found alterations in postpartum affective behaviors, including increased floating in the forced swim test (Smith, 2004; Haim et al., 2014; Leuner et al., 2014), a measure of behavioral despair (Porsolt et al., 1977), increased anxiety-like behavior in the open-field test, and altered HPA axis function (Champagne & Meaney, 2006; Smith et al., 2004). Chronically injecting pregnant rats with corticosterone, which is released when animals experience a stressor, also disrupts caregiving by increasing the amount of time spent off the nest and reducing the amount of time nursing, and continuing this corticosterone administration into the postpartum period exacerbates these effects (Brummelte & Galea, 2010). Similar to chronic pregnancy stress, chronic corticosterone administration during pregnancy also increases floating in the forced-swim test (Brummelte & Galea, 2010). Given these effects on behavior, it is unsurprising that stress during pregnancy

alters the postpartum brain, including the limbic system and reward pathways. For example, stress during pregnancy reduces structural plasticity, characterized by diminished dendritic length and spine density, within the basolateral amygdala (BLA), medial prefrontal cortex (mPFC), and nucleus accumbens shell (Haim et al., 2014; Haim et al., 2016; Leuner et al., 2014). Because the BLA, mPFC, and NAc regulate various aspects of maternal motivation and pup approach behaviors (Afonso et al., 2007; Lee & Fleming, 1999; Numan, 2005; Numan, 2010), changes in this region following gestational stress may underlie at least some of the disruptions in maternal caregiving.

Little research has focused on the effects of stress during pregnancy on the central serotonergic system, even though chronic stress affects this system and causes a depression-like phenotype in non-parous animals. For example, chronic unpredictable stress reduces sucrose consumption and decreases spontaneous firing activity of DR serotonin neurons by ~35% in male laboratory rats, suggesting that overall serotonin release throughout the brain may be reduced (Bambico et al., 2009). At the level of DR projection sites, chronic stress increases 5-HT1A receptor expression in the CA1 region of the hippocampus of male rodents, but this effect is not seen in females (Pitychoutis et al., 2012). 5-HT2C receptor expression in the hippocampus is significantly increased in both males and females following chronic stress. This provides evidence that 5-HT1A and 2C receptor expression, both of which showed reproductive state-dependent changes in expression in Chapter 2, are sensitive to stress. Alterations in the serotonergic system are also commonly found among humans with anxiety and/or depression, with reduced serotonin release and diminished receptor sensitivity being correlated with depression-like symptoms (Mann et al., 1996). 5-HT1A receptor binding is also reduced in individuals with major depressive disorder (Drevets et al., 1999). Polymorphisms in the

serotonin transporter (5-HTT) and 5-HT2C receptor genes have also been correlated with depression in humans, including (Owens & Nemeroff, 1994; Lyddon et al., 2013).

While it is becoming evident that derailments in serotonergic activity might contribute to postpartum affective disorders, it has yet to be determined whether changes in this system that deviate from the normal peripartum pattern underlie the behavioral effects of peripartum affective disorders. Chapter 2 of this dissertation revealed that the expression of 5-HT receptors normatively changes across female reproduction in 3 of the 8 brain sites examined. 5-HT2A receptor expression in the medial preoptic area is significantly higher in parturient and PP7 dams compared to diestrous virgins, 5-HT1A receptor expression in the nucleus accumbens is 250% higher in recently parturient dams compared to virgins, and 5-HT2C receptor expression in the DR is significantly lower at parturition with a further reduction in dams during the first week of lactation. The purpose of this chapter is to determine whether stress during pregnancy prevents or even reverses the normative postpartum expression of forebrain and midbrain 5-HT receptor mRNA found in Chapter 2. This experiment will use a novel, repeated variable stress paradigm to examine postpartum social and affective behaviors and analyze whether pregnancy stress also alters the normative postpartum expression of central 5-HT receptors, which might suggest a role for these receptors in regulating the stress-induced disruption of postpartum behaviors.

Experiment 3a – Effects of Repeated Variable Stress During Pregnancy on Postpartum Caregiving and Affective Behaviors

Methods

Subjects

Subjects were 20 female Long-Evans rats descended from rats purchased from Harlan Laboratories (Indianapolis, IN), born and raised in our colony at Michigan State University. Females were housed with 1 or 2 same-sex littermates in clear polypropylyne cages (48 cm x 28 cm x 16 cm) containing wood chip bedding, food (Tekland rat chow, Indianapolis, IN) and water ad libitum. The room was maintained on a 12 hr light/dark cycle (lights on at 0700 hr) with temperature kept at 22 +/- 1 °C. After reaching 65 days old, females in the mated groups were placed in a cage with a male breeder (Harlan Laboratories, Indianapolis, IN) on the morning of proestrus (determined by vaginal smearing and cytology) and housed with the male until the next day. Vaginal smearing and cytology were used to confirm the presence of sperm, and therefore successful copulation. Subjects were again housed 2-3 to a cage until pregnancy day 7 (P7), when rats were singly housed and randomly assigned to one of two groups – pregnancy stressed or pregnancy non-stressed (n = 10/group).

Repeated Variable Stress (RVS)

Repeated variable stress (RVS) began on P7 for the stressed group of subjects. This paradigm was originally developed by Koenig et al. (2005) and has been adapted here from work in female rats by Galea and colleagues (2010). This variable stress method is known to not cause litter loss during pregnancy or thereafter. Two mild-moderate stressors were applied each day from pregnancy days 7-20 and consisted of: (1) restraint in a ventilated cylindrical Plexiglas™

restrainer for 1 hr; animals were gently placed inside the restrainer, secured, and remained there for 1 hr until being returned to their home cage (2) exposure to a cold environment; subjects in their home cages were placed inside a cold room (4° C) for 4 hr, and were then removed and placed back in the animal facility, (3) overnight food deprivation; the standard rat chow and water were removed from the lid of the subjects' home cage overnight and were replaced the next morning, (4) Cage tilt; the home cage of each subject was tilted at 45° for 4 hr, (5) wet bedding; subjects in their home cage with the existing bedding dampened by 250 mL of distilled water, they were placed into a clean home cage with fresh, dry bedding after 4 hr, (6) White noise; subjects were placed in our behavior testing room across from our laboratory and exposed to 80 dB of white noise for 1 hr, (7) Tail pinch; a plastic clothespin was placed at the base of the tail for 5 min, (8) Continuous light overnight; subjects were placed in a room in the animal facilities with lights on during the normal dark phase of the subjects. This lasted overnight, and the next day subjects were placed back on our main colony with regular light cycles, (9) Strobe light; subjects were moved to our behavior testing room and exposed to a strobing light at 1 flash/sec in an otherwise dark room, during the light cycle for 1 hr.

An example of a typical schedule of stress applications is presented in Table 3 where each subject underwent 2 of the 9 possible stressors each day until P20. After RVS ended, subjects were left alone for the 1-3 days until parturition (designated as PP0). In the afternoon of PP0 or the morning of PP1 (at least 2 hours before the first behavior observation), litters were culled to 8 pups (4 males and 4 females). Because pup behavior itself may be altered due to gestational stress (Patin et al., 2004), and pup behavior can affect the maternal care given by the dam (Del Cerro et al., 2010), each subject (regardless of group) received an equal number of pups given birth to by stressed and unstressed dams.

Maternal Caregiving Behavior and Retrieval Testing

Beginning the day after parturition, which is designated as postpartum day 1 (PPD1), subjects were observed daily for maternal behavior for 30 min every morning between 0900-1000 and again in the afternoon between 1400-1500 on PPD1-8 (see Figure 4). During these observations, experimenters stood quietly in the colony room and nests were first scored on a scale of 0-3 (0 = no nest, 1 = poor nest, 2 = partial nest, 3 = complete nest with high walls; Lonstein & Fleming, 2002) and maternal behaviors were then recorded by hand every 30 sec. Recorded behaviors included both maternal (licking, mouthing, retrieving, and sniffing pups, nest-building, nursing) and non-maternal (self-grooming, eating/drinking, resting alone, exploring) behaviors. To ensure the stress paradigm did not tremendously disturb the growth of infants, pups were cross-fostered daily to surrogate dams from the colony and litter weights were recorded each afternoon. In addition, dam weights were taken on postpartum days 2, 4, and 6 to assess general health. To assess maternal motivation, retrieval tests immediately followed the afternoon observations on PPD3 and PPD5. For retrieval tests, litters were removed from the home cage for 15 min and placed in an incubator set to nest temperature (34 ºC). After 15 min, the eight pups were scattered in the cage on the opposite side of the nest and the time for the dams to retrieve each pup and begin hovering over the nest was recorded. If a dam did not retrieve her pups to the nest site after 5 min, the experimenter grouped the pups and placed them back in the nest. After all pups were gathered back to the nest site (either by the subject or by the experimenter) maternal behaviors were observed every 30 s for an additional 10 min.

Anxiety-like behaviors

On the afternoon of PPD4, subjects were transported in their home cage (with their litter present) to a behavior testing room illuminated by one 100W light bulb. The elevated plus-maze was elevated 50 cm from the floor and made of black plastic with four arms emerging from a 10 X 10 cm center square. Arms were 10 cm wide by 50 cm long, two of which had 40-cm high walls, 2 of which had no walls. Approximate illumination on the open arms was 28 lux, and approximate illumination on the closed arms was 2 lux (Miller et al., 2011). Each subject was removed from their home cage and placed in the central square of an elevated plus maze facing an open arm. The home cage with the litter was brought to the adjacent room. A low-light-sensitive video camera suspended above the maze relayed images to a Magnavox DVD recorder to monitor activity in the maze from the adjacent room. An experimenter recorded subjects' behavior with computerized data acquisition software. Recorded behaviors included time spent in the open and closed arms, and the frequency of entries into each arm (Pellow et al, 1985). After testing, dams were gently removed from the maze and returned to their home cage containing their pups, and were carried back to the colony room. On the afternoon of PPD6, subjects were tested in a light-dark box. The light-dark box was made of white and black opaque Plexiglas chambers (20 X 30 X 30 cm light chamber, 30 X 30 X 30 cm dark chamber) connected by a 10 X 10 cm door in the middle of the wall. The light chamber of the box was illuminated to ~624 lux (Miller et al., 2010). Subjects with their litters were carried to the same behavior testing room described above. Subjects were placed in the light chamber of the box, and behavior was video recorded and scored for 10 min. Behaviors scored included the latency to enter the dark chamber, frequency of stretches into the light chamber, and time spent in the light chamber (Miller et al., 2010). After testing, dams were gently removed from the light-dark box and

returned to their home cages with their litters. Following both tests, the dam's anxiety-like behaviors were scored by an experimenter blind to experimental group using data acquisition software (Solomon Coder).

Maternal Aggression

On the afternoon of PPD7 subjects were tested for aggressive behavior toward an unfamiliar male intruder to the home cage. Males 45-55 days old from our colony were used as intruders. Males were placed into the subjects' home cage with the litters present. The tests were video recorded and intruders were removed after 10 min. Intruder males were sacrificed immediately following testing. The dams' behaviors were scored using data acquisition software (Solomon Coder), and included latency to attack the intruder, frequency of attacks, duration of each attack, and other aggressive behaviors directed at the intruder (sniffing, aggressive grooming, kicking, boxing) (Lonstein and Stern, 1997; Bosch et al., 2005).

Saccharin Preference Test

On the morning of PPD8, subjects were habituated to the saccharin solution by being given 24-hr access to two bottles, one containing water and another containing 0.1% saccharin. Bottle placement was counterbalanced across groups, and each bottle was weighed and the position swapped after 12 hours, then removed and weighed again after an additional 12 hours. On PPD9 immediately following the 24-hr exposure period, food and water was removed from the subjects' home cage for 4 hours. After the 4-hr food and water deprivation, pups were removed from the nest and placed in an incubator set at nest temperature (34°C), and dams' food was replaced and they were again given access to a bottle filled with water and a bottle of 0.1%

saccharin solution. After 1 hour, bottles were removed and weighed, and pups were placed back into the home cage.

Forced Swim Test (FST)

The FST (adapted from Leuner et al., 2014) was used to assess depressive-like behaviors in the dams. On the afternoon of PPD8, subjects were moved from the animal facilities to the behavior testing room described above for a pre-swim test. Rats were placed into a Plexiglas™ cylinder filled with water high enough so that the tail could not touch the bottom of the container. The test was digitally recorded from a suspended camera for 15 min, after which the subjects were removed from the apparatus, towel dried, and returned to their home cage with their pups. On the afternoon of PPD9, at least one hour after saccharin preference testing, rats were again removed from the animal facilities and placed into the same Plexiglas™ cylinder filled with water. The test was digitally recorded for 10 min, after which the subjects were removed from the apparatus, towel dried, and returned to their home cage. The total time spent swimming and immobile (floating in the water only making movements necessary to maintain the head above water) were measured using behavior coding software (Solomon Coder). The percentage of time spent floating during the 10-min test was used as the measure of depression-like behavior.

Sacrifice and Blood Collection

Postpartum dams in both the pregnancy stressed and non-stressed groups were sacrificed immediately following the FST on PPD9. Subjects were euthanized with CO_2, rapidly

decapitated, and trunk blood was collected and centrifuged for 15 min at 10,000 rpm. Blood plasma was stored at -20°C.

Data Analyses

Undisturbed maternal behaviors were analyzed using Repeated Measures ANOVAs to compare the frequency of maternal behaviors both between groups (stressed, non-stressed) and across postpartum days. T-tests were also used to compare summed frequencies of maternal behaviors on all postpartum days between the groups. Data for anxiety tests and aggression were analyzed using two-tailed t-tests to compare stressed and non-stressed conditions. In a few cases where groups had significantly unequal variances, the nonparametric Mann Whitney-U test was used to compare groups. For all analyses, a significant main effect was determined by $p < 0.05$. Significant main effects in Repeated Measures ANOVAs were followed up with post-hoc tests comparing groups at the different time points.

Results

Maternal Caregiving Behavior

Repeated variable stress during pregnancy significantly reduced litter size when measured at parturition ($t = 2.64$, $p = 0.015$), consistent with at least one other study implementing a similar chronic stress paradigm during pregnancy (Gotz et al., 2008). While litter size at parturition was affected by RVS, litter weight gain across the postpartum period ($t = 0.025$, $p = 0.88$), as well as dam weight gain across lactation ($t = 0.051$, $p = 0.83$), did not differ between stressed and unstressed dams.

As previously reported by several others (Haim et al., 2014; Smith et al., 2014), RVS during pregnancy significantly altered maternal caregiving. T-tests comparing summed frequencies of behaviors across the 8-day observation period revealed a significant reduction in frequency of time in the nest (all nursing postures + hovering over pups in the nest; $t = 2.51$, $p = 0.023$) in stressed dams compared to controls, although nursing frequency on its own was unaffected by RVS (see Table 5). Additionally, stressed dams had a higher frequency of non-pup directed behaviors (self-grooming, feeding, sleeping away from pups, exploring the cage) compared to non-stressed controls ($t = -2.45$, $p = 0.026$). While not statistically significant, stressed dams also tended to lick their pups less frequently ($t = -1.79$, $p = 0.092$), but explore the cage ($t = 2.02$, $p = 0.059$) and sleep away from the nest ($t = 1.90$, $p = 0.077$) more frequently.

Pup Retrieval

RVS during pregnancy did not significantly affect the latency for dams to retrieve the first pup to the nest (averaged across both tests) ($t = -1.509$, $p = 0.151$). However, during the 10-minute undisturbed maternal observations immediately following the retrieval tests, stressed dams had a significantly lower frequency of licking pups compared to controls ($t = -2.47$, $p = 0.025$) and a significantly higher frequency of exploring the cage ($t = 2.64$, $p = 0.018$).

Anxiety-like behavior

Stressed and non-stressed dams did not significantly differ in the percentage of time they spent in the open arms of the EPM (two-paw: $t = 1.01$, $p = 0.33$; four-paw: $t = 0.84$, $p = 0.42$). Stressed dams showed more general locomotor activity in the EPM compared to non-stressed dams, as indicated by a higher number of closed-arm entries (two-paw: $t = 2.30$, $p = 0.037$; four-

paw: t = 2.24, p = 0.042) and more total arm entries into either types of arms (t = 2.10, p = 0.055). In the light-dark box test, groups did not significantly differ in the amount of time spent in the light chamber of the box (t = 0.777, p = 0.45) or their latency to enter the dark chamber (t = 0.972, p = 0.35).

Maternal aggression

Stressed dams showed less maternal aggression in the form of a lower total duration of attacking compared to controls (t = -2.18 p = 0.0.44) and tended to spend more time sniffing the intruder male (t = 1.88, p = 0.078). Means and standard errors for all other recorded behaviors, which did not significantly differ between groups, can be found in Table 6.

Depression-like behavior in the saccharin preference and forced swim tests

Dams that received RVS during pregnancy showed a significantly higher percentage of time spent floating in the forced swim test compared to non-stressed controls (t = 4.82, p = 0.0003), although the latency to begin floating was unaffected by stress (t = -0.484, p = 0.636). Stressed dams also showed a significantly lower preference for 0.1% saccharin over water compared to controls during the 1-hr test (t = -2.55, p = 0.022).

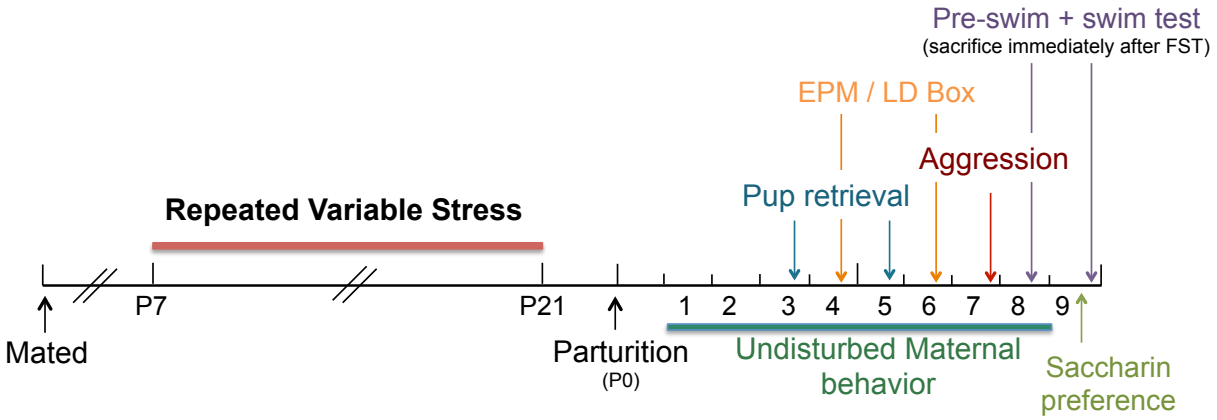

Figure 4: Schematic representation of experimental timeline used to determine the effects of repeated variable stress during pregnancy on postpartum caregiving and affective behaviors. Pregnant females either went through a repeated variable stress paradigm from pregnancy days 7-20, or were left unhandled until parturition. Following birth, undisturbed maternal behavior observations were conducted twice daily. On PPDs 3 and 5, dams' retrieval performance was assessed. On PPDs 4 and 6, anxiety-like behavior was assessed in the elevated plus maze and light/dark box, respectively. On PPD 7, maternal aggression towards an unfamiliar male rat was assessed. Finally, on PPDs 8 and 9, dam's depression-like behaviors were observed using the saccharin preference test and the forced swim test.

Pregnancy Day	AM (~9:00am)	PM (~2:00pm)
7	Cage tilt (4 hr)	Light (overnight)
8	Restraint (1 hr)	Cold exposure (4 hr)
9	Strobe light (1 hr)	Food deprivation (overnight)
10	Restraint (1 hr)	Strobe light (1 hr)
11	Wet bedding (4 hr)	Light (overnight)
12	White noise (1 hr)	Restraint (1 hr)
13	Tail pinch (5 min)	Food deprivation (overnight)
14	Strobe light (1 hr)	Wet bedding (4 hr)
15	Cold exposure (4 hr)	Tail pinch (5 min)
16	White noise (1 hr)	Light (overnight)
17	Cage tilt (4 hr)	Strobe light (1 hr)
18	Wet bedding (4 hr)	Food deprivation (overnight)
19	Tail pinch (5 min)	Wet bedding (4 hr)
20	Cold exposure (4 hr)	Cage tilt (4 hr)

Table 3: Sample schedule of RVS stressors. Pregnant females will receive pseudo-random schedule of two mild-moderate stressors each day from pregnancy day 7 to pregnancy day 20.

	Non-stressed	Stressed	Group (t; *p*)	PPD (F; *p*)	Group*PPD (F; *p*)
Pup Total	10.58 ± 0.67	8.08 ± 0.67	2.64; 0.015*	NA	NA
# Males	5.08 ± 0.48	4.00 ± 0.49	1.56; 0.130	NA	NA
# Females	5.58 ± 0.5	4.08 ± 0.59	1.93; 0.067	NA	NA
% Change in Pup Weight	13.87 ± 0.95	14.09 ± 1.04	0.025; 0.878	3.45; 0.006*	0.36; 0.749
Dam Weights	305.15 ± 11.16	309.53 ± 15.79	0.051; 0.830	3.21; 0.071	0.33; 0.720

Table 4: Effects of RVS during pregnancy on dam and litter health across lactation. Means ± SEMs of pup numbers at parturition, percent change in pup weight, and dam weight by the end of lactation. *indicates $p < 0.05$

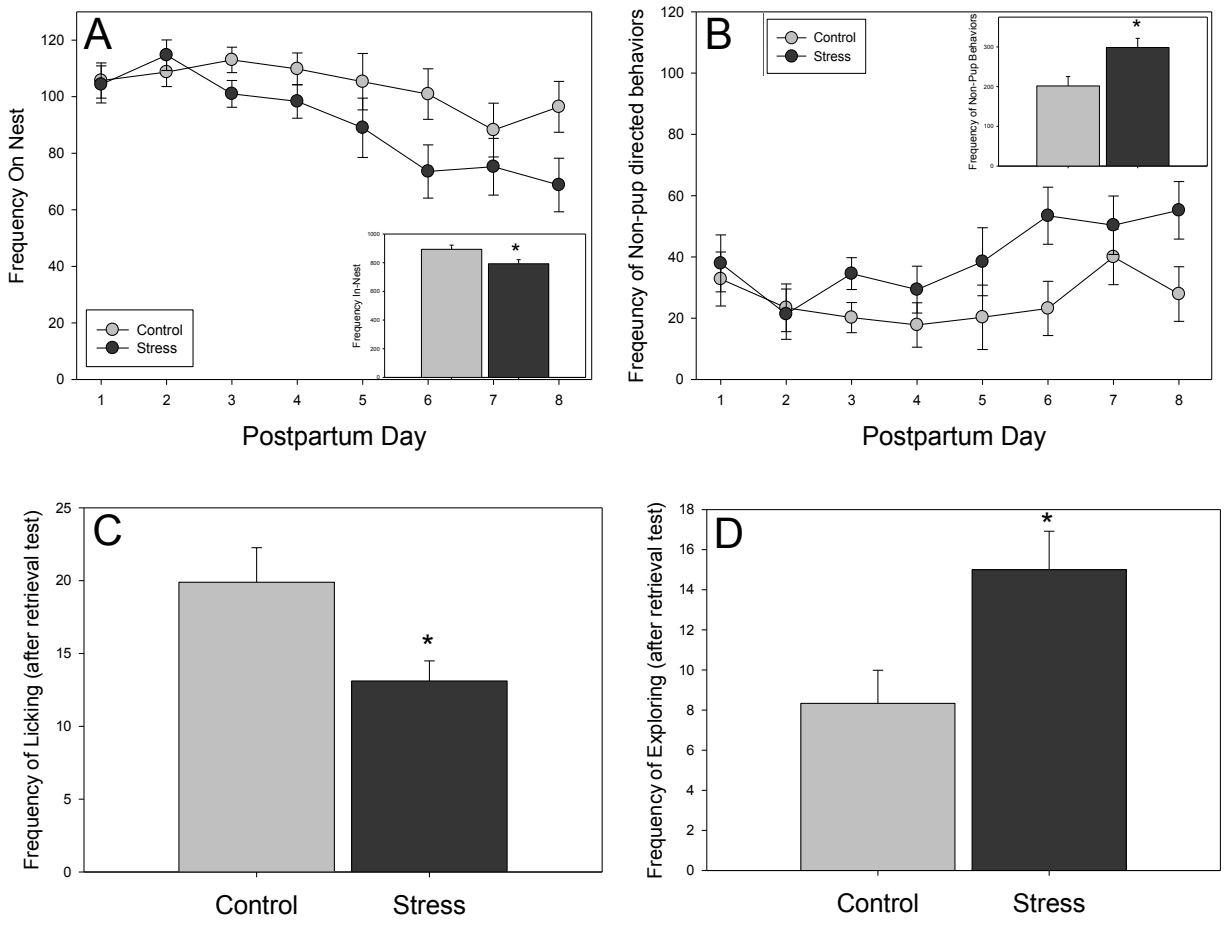

Figure 5: Effects of repeated-variable stress on maternal caregiving behaviors. Frequency of behaviors in the nest (A) and non-pup directed behaviors (B) in non-stressed control dams and pregnancy stressed dams (Means ± SEMs). Frequency of licking pups (C) and exploring the cage (D) during the 10 min observation immediately following retrieval tests in non-stressed control dams and pregnancy stressed dams (Means ± SEMs). *indicates significant group difference, $p < 0.05$

	Non-stressed	Stressed	Group (t; *p*)	Time (F; *p*)	Group*Time (F; *p*)
Crouch	550.1 ± 20.4	486.6 ± 44.8	1.34; 0.20	1.96; 0.13	0.81; 0.52
Hover	175.6 ± 11.4	170.7 ± 21.8	0.21; 0.84	1.8; 0.24	0.38; 0.72
On Side Nursing	26.4 ± 6.7	13.8 ± 5.1	1.48; 0.16	2.4; 0.023*	0.32; 0.95
Blanket Nursing	75.9 ± 13.3	53.9 ± 9.1	1.33; 0.20	0.97; 0.42	0.39; 0.75
Licking	97.9 ± 6.1	76.1 ± 10.9	1.79; 0.09	0.08; 0.19	0.49; 0.077
Nesting	18.6 ± 3.8	26.1 ± 7.8	-0.89; 0.38	5.88; 0.001*	1.3; 0.25
Feeding	30.0 ± 9.9	55.9 ± 12.5	-1.63; 0.12	1.31; 0.25	1.37; 0.22
Grooming	55.0 ± 8.0	76.8 ± 9.7	-1.75; 0.10	1.08; 0.38	0.34; 0.93
Inactive	48.8 ± 11.7	82.4 ± 13.4	-1.90; 0.075	5.96; 0.001^	0.45; 0.87
Exploring	46.9 ± 6.4	69.2 ± 9.2	-2.02; 0.06	3.19; 0.025^	0.53; 0.68
Tail Chasing	6.3 ± 2.5	10.1 ± 3.1	-0.98; 0.34	3.63; 0.001*	0.48; 0.85
In Nest behaviors	828 ± 25.5	724.9 ± 32.8	2.51; 0.023*	4.99; 0.002*	1.198; 0.32
Non –pup Directed behavior	205.6 ± 31.2	320.6 ± 35.3	-2.45; 0.026*	1.71; 0.11	0.53; 0.81

Table 5: Frequency (Mean ± SEM) of maternal behaviors displayed by non-stressed and stressed dams during two 30 min observations each day on postpartum days (PPD) 1-8. *indicates significant decrease in behaviors across PPD, ^indicates significant increase in behaviors across PPD.

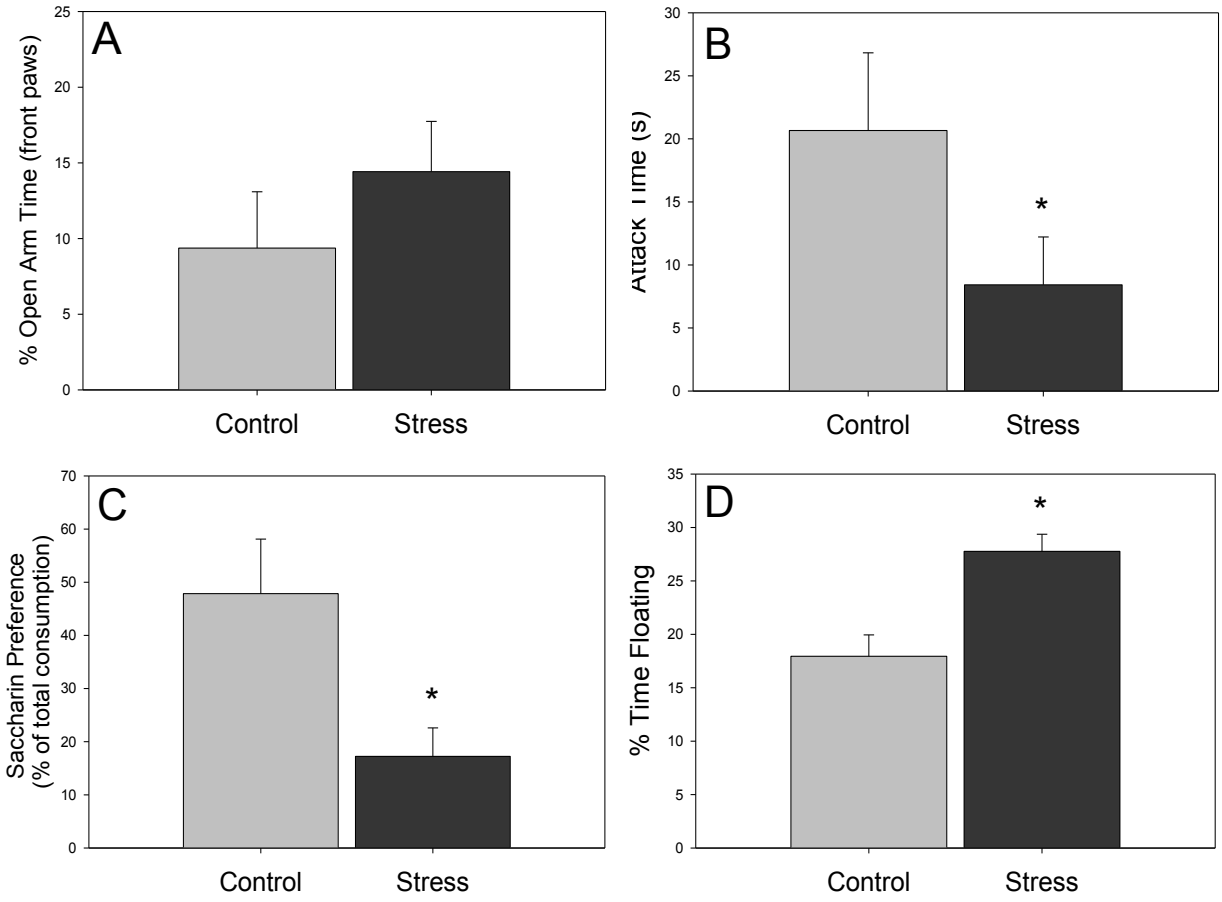

Figure 6: Effects of RVS during pregnancy on anxiety-like behavior, maternal aggression, and depression-like behavior. Percentage of time spent in the open arm of the Elevated Plus Maze (A), total duration of time spent attacking male intruder (B), percentage of preference for 0.1% saccharin (C), and percentage of time floating (immobile) in the forced swim test (D). *indicates significant difference between control and stressed dams, $p < 0.05$

EPM	Control	Stress	t	p
# Open Arm Crosses - Front Paws	5.1 ± 1.5	9.7 ± 1.8	-1.89	0.079
# Open Arm Crosses - Four Paws	2.3 ± 0.9	3.9 ± 1.5	-0.87	0.400
# Closed Arm Crosses - Front Paws	9.4 ± 1.5	14.0 ± 1.3	-2.30	0.037*
# Closed Arm Crosses - Four Paws	7.3 ± 1.4	11.8 ± 1.4	-2.24	0.041*
% Time Open Arm - Front Paws	9.4 ± 3.7	14.4 ± 3.3	-1.01	0.330
%Time Open Arm - Four Paws	4.5 ± 2.4	7.8 ± 2.9	-0.84	0.420
Light/Dark Box				
Frequency of Light entries	1.1 ± 0.6	1.3 ± 0.5	-0.30	0.770
Frequency of Stretches into Light	17.1 ± 4.0	14.7 ± 2.8	0.50	0.630
Latency to enter Dark	4.4 ± 0.7	5.9 ± 1.4	-0.97	0.350
%Time in Light	1.4 ± 0.7	2.4 ± 1.1	-0.78	0.450
Maternal Aggression				
Frequency of Attacks	11.2 ± 3.4	4.2 ± 1.4	1.91	0.074
Frequency of Bites	3.9 ± 1.2	4.6 ± 1.5	-0.35	0.730
Frequency of Lateral Threats	.002 ± 1.1	0.8 ± 0.4	1.07	0.300
Attack Latency	224.3 ± 66.3	284.2 ± 74.1	-0.60	0.560
Total attack duration	21.9 ± 7.3	5.5 ± 1.9	2.18	0.044*
Total lateral threat duration	2.9 ± 1.5	0.7 ± 0.4	1.47	0.160
Total duration sniffing intruder	54.7 ± 7.0	69.9 ± 8.8	-1.35	0.190
Depression-Like Behaviors				
% Floating	18.0 ± 2.0	27.8 ± 1.6	-4.82	<0.0005*
% Preference for Saccharin	47.9 ± 10.2	17.2 ± 5.4	2.55	0.022*

Table 6: Anxiety-like behaviors, maternal aggression behaviors, and depression-like behaviors (Means ± SEMs) in control and stressed dams. *indicates statistical significance between groups.

Chapter 3b – Effects of Pregnancy Stress on Postpartum Serotonin 1A and 2A Receptor Binding

Methods

Subjects

Using methods identical to those in Experiment 3a above, another cohort of 20 female Long Evans rats from our colony (n = 10/group) were randomly assigned to one of two groups – pregnancy stressed, pregnancy non-stressed. Rats from both pregnancy groups were mated with a breeder and group housed 2-3 per group until pregnancy day 7 (P7).

Repeated Variable Stress (RVS)

RVS was conducted identical to the methods found in Chapter 3a. No behavior testing was conducted in this cohort in order to determine the effects of RVS, unconfounded by frequent handling and behavior testing, on the 5-HT receptor binding.

Tissue Processing and Receptor Autoradiography

On PPD7, all rats and their pups were euthanized via CO_2 and rapidly decapitated. Trunk blood was collected and subjects' brains were removed and kept at – 80 °C. Brains (n = 6/group) were sliced coronally in 15 µm-thick sections and mounted onto glass slides until processing.

Glass slides containing 15-µm sectioned rat brain tissue were removed from the -80˚ C freezer and allowed to thaw at room temperature for 1 hr. The slide-mounted sections were then fixed in 1% paraformaldehyde for 2 minutes and then pre-incubated in 50 mM Tris-HCl buffer (pH 7.4) at 4˚C for 30 min. Sections were then incubated in a solution containing 50 mM Tris-HCl buffer and either 0.4 nM [3H]MDL100.907, (Metis Laboratories, Valley Stream, NY) or 2 nM [3H]8-OH-DPAT (Perkins-Elmer, Waltham, MA) for 1-2 hours (depending on radioligand).

After incubation, sections were washed twice at 4˚ C in 50 mM Tris-HCl buffer for 10 minutes each. The sections were then dipped for a few seconds into 4˚C distilled water. Slides were left overnight to dry at room temperature. The following day, slides were moved to autoradiography cassettes (Fisher Scientific, Pittsburgh, PA) each containing a set of tritiated standards (ARI 0133, American Radiolabeled Chemicals) and exposed to a tritium-sensitive storage phosphorscreen for 35 days. Storage phosphor screens were then scanned at 10 μm and 4000 PMT using a Typhoon phosphorimager (GE Lifesciences).

Data Analyses

Autoradiographic data from Experiment 3b were analyzed using t-tests to compare main effects between the two groups. Additionally, repeated measures Analyses of Variance were used to determine differences in autoradiographic binding across rostrocaudal level of each region. Using ImageJ, a box containing each region of interest was drawn on digitized images of the phoshorscreens, resulting in four ovals through the NAc (2.28-1.2mm from bregma) and three boxes through the mPOA (-0.15 - -0.45mm from bregma). Control slides containing blocking peptides were also processed and scanned to subtract out background binding for each radioligand. Optical density of the receptor of interest in each brain region was compared between stressed and non-stressed dams. For all analyses, a significant main effect was determined by $p < 0.05$, and in such cases, post-hoc tests were conducted between groups (repeated measures ANOVAs).

Results

Repeated variable stress during pregnancy did not alter the total autoradiographic binding of 5-HT2A receptors across all three levels of the medial preoptic area (main effect of group: $F_{(1,1)} = 0.006$, $p = 0.938$). Repeated measures ANOVA analysis of binding density of 5-HT2A receptors across rostrocaudal extent was also not significantly different at any level between stressed and non-stressed subjects ($F_{(1, 9)} = 0.017$, $p = 0.900$), although 5-HT2A binding was significantly lower in the most caudal section of the mPOA compared to the most rostral ($F_{(1, 9)} = 5.32$, $p = 0.022$). There was also no interaction between group and level ($F_{(1, 9)} = 1.22$; $p = 0.319$). 5-HT1A receptor binding density collapsed across all four levels of the NAc did not significantly differ between stressed and unstressed dams (main effect of group: $F_{(1, 11)} = 508$, $p = 0.50$). However, there was an almost significant interaction between group rostrocaudal levels of the NAc ($F_{(3,27)} = 2.96$, $p = 0.063$). Post-Hoc ANCOVAs revealed that 5-HT1A binding in the most rostral section of the NAc shell (2.28mm from bregma) and the most caudal section (1.2 mm from bregma) tended to be less in stressed dams compared to unstressed dams (rostral: $F_{(1,11)} = 3.09$, $p = 0.117$; caudal: $F_{(1,11)} = 3.01$, $p = 0.121$).

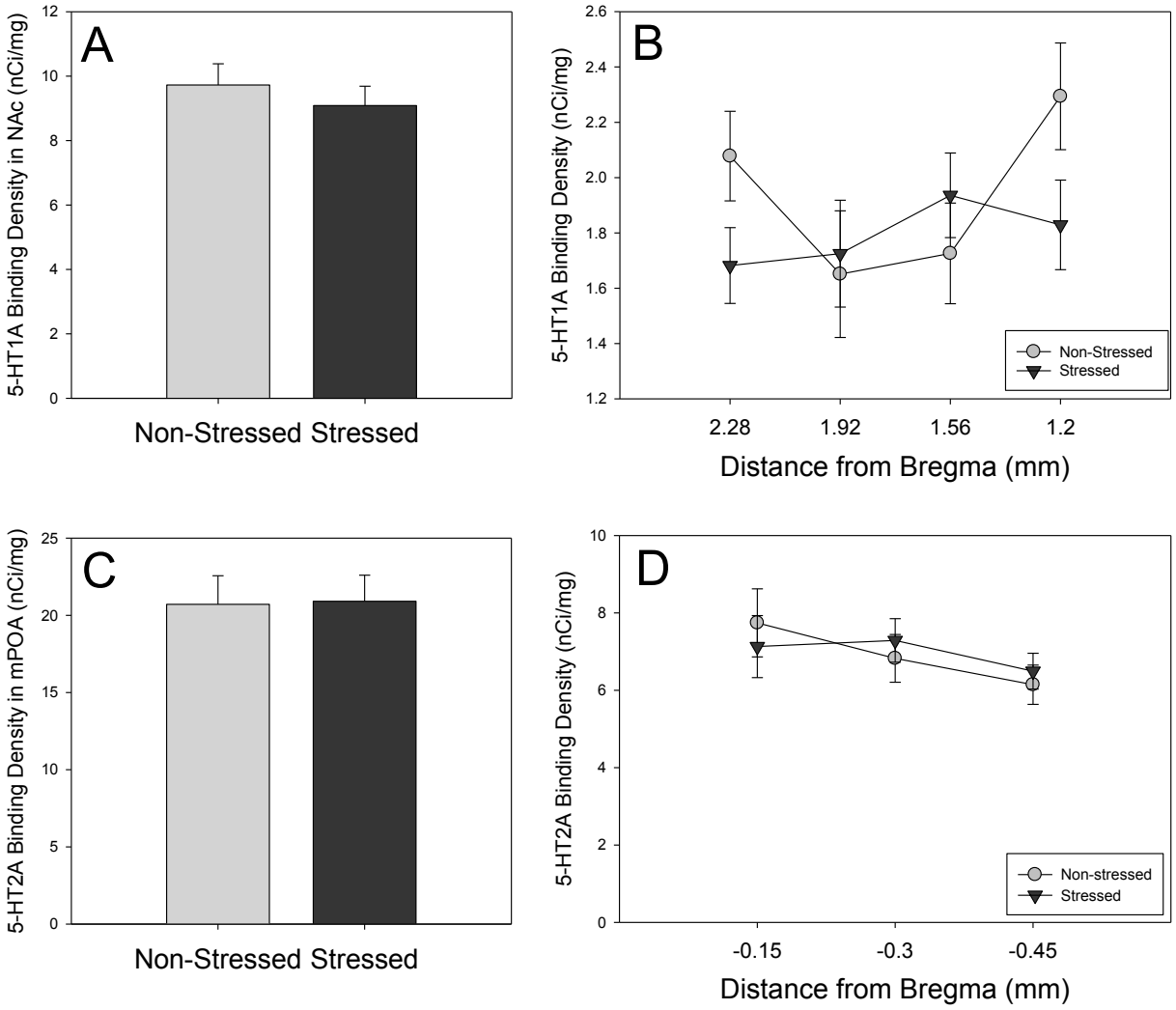

Figure 7: Nucleus accumbens 5-HT1A and medial preoptic area 5-HT2A receptor binding density in non-stressed and stressed dams. RVS during pregnancy did not significantly alter binding density of 5-HT1A in the NAc (A-B) or 5-HT2A in the mPOA (C-D).

Discussion

Repeated variable stress during pregnancy significantly altered postpartum caregiving and affective behaviors, and these behavioral disruptions coincided with some small changes in 5-HT receptor binding. RVS also resulted in significantly smaller litters at birth, which has been reported by some (Smith et al., 2004; Leuner et al., 2014), but not all (Haim et al., 2014) studies utilizing chronic stress paradigms. Our RVS paradigm did not significantly affect litter weight gain across the first week of lactation, and this suggests that RVS does not significantly impact lactation. Additionally, this study did not find a significant reduction in dam weight across the first week of lactation. This is similar to other findings after chronic restraint stress during pregnancy (Leuner et al., 2014),

RVS significantly reduced the total frequency of on-nest behaviors (all nursing postures and hovering), and significantly increased the frequency of non-pup directed behaviors such as feeding, self-grooming, and sleeping away from the nest. These results appear to be quite reliable, as others have reported similar findings following either chronic pregnancy restraint or swim stress (Haim et al., 2014; Smith et al., 2004; Leuner et al., 2014), as well as after pre- and postpartum corticosterone administration (Brummelte & Galea, 2010). I found that mild/moderate RVS did not affect the frequency of kyphosis or the total amount of time spent in any nursing posture, although chronic peripartum administration of high corticosterone and chronic gestational swim stress do (Brummelte & Galea, 2010; Leuner et al., 2014). These contrasting findings may be explained by the severity of the swim stress compared to the repeated variable stress paradigm used here in this experiment.

RVS during pregnancy did not affect the latency to retrieve pups to the nest, although stressed dams took longer to retrieve their pups on the second test compared to the first, which

differs from the faster retrieval by the non-stressed dams on the second test. Female rats will typically perform better at pup retrieval after subsequent tests; therefore, this effect may reflect slight deficits in maternal memory. Brief removal of pups from the nest increases maternal caregiving behaviors once pups are returned (reviewed in Lehmann & Feldon, 2000); however, stressed dams spent less time licking their pups once they were retrieved to the nest, and explored the cage more compared to non-stressed dams. Because this was not found during the twice-daily undisturbed observations, this result suggests impairment in maternal motivation only under conditions that would normally elicit more caregiving responses. To our knowledge, we are the first to report this effect of stress during pregnancy on maternal caregiving after brief separation and disruptions in brain reward circuitry may be involved.

Repeated variable stress during pregnancy did not significantly affect anxiety-like behavior in the elevated plus maze or light-dark box. Chronic corticosterone administration also does not alter anxiety-like behavior in postpartum females tested in an open field (Brummelte & Galea, 2010). Of note, pregnancy stressed dams did show more locomotor activity in the EPM, indicated by the total number of entries into both types of arms of the maze. Increased locomotor activity has been reported in the open field test following hormone withdrawal, a model of depression-like phenotype in postpartum rats (Galea et al., 2001), but not following chronic gestational corticosterone administration (Brummelte & Galea, 2010).

Although pregnancy stress did not affect anxiety-like behavior in either the EPM or light-dark box, stress did increase depression-like behavior. Stressed dams spent significantly more time floating in the forced-swim test compared to unstressed dams. This has also been reported after chronic corticosterone administration during pregnancy and lactation (Brummelte et al., 2006; Brummelte & Galea, 2010) or after chronic restraint stress or swim stress during

pregnancy (Smith et al., 2004; Haim et al., 2014; Leuner et al., 2014). Females that received 23 days of estradiol injections (to mimic pregnancy) also float more in the forced swim test than females that continued to receive estradiol injections after the 23-day "pregnancy" period, which is interesting given that estradiol and progesterone activate the serotonergic system (Galea et al., 2001; Lu et al., 1999; Pecins-Thompson et al., 1999). These findings collectively suggest that endocrine conditions that promote serotonergic signaling may protect against depression-like symptoms, or that withdrawal or reduction in serotonergic activity (under hormone-deprived conditions) contribute to depression-like behavior. I also found that anhedonia, or reduced motivation for and enjoyment of rewarding stimuli, was higher in pregnancy-stress dams compared to controls, as indicated by a reduction in preference for 0.1% saccharin solution. These findings, together with the increase in non-pup directed behaviors suggest that pregnancy stress disrupts the circuitry regulating a variety of highly motivated responses.

Because RVS during pregnancy altered a number of behaviors associated with motivation, this may indicate alterations in the nucleus accumbens, a brain region well studied for its role in reward processing. NAc activation, particularly in the shell subregion, promotes "liking" of sweet solutions, as well as increases food intake (for review see Pecina & Berridge, 2005); therefore, the NAc is a likely candidate where stress may reduce the rewarding properties of saccharin, pups, and other natural rewards. In support of this theory, Leuner and colleagues found that chronic pregnancy stress reduced structural plasticity within the NAc shell, but not the core (Leuner et al., 2014) and these functional changes in the NAc shell could underlie some of the behavioral changes. Typically, the DA system in the NAc has been implicated in regulating reward and motivation, and serotonin affects DA release in the NAc (Parsons & Justice, 1993). Serotonin-dopamine interactions in the NAc have been implicated in depression-like behavior in

non-parous rats. In support, Flinders Sensitive Line rats that have been selectively bred to show high depression-like behavior (i.e., increased floating, or passive coping), have reduced concentration of DA and its metabolites in the NAc compared to control rats, and also do not show the typical increase in DA release in the NAc after local infusion of serotonin (Zangen et al., 2001). This suggests that the NAc of control rats responds to serotonin release, but this response is inhibited in rats that show depression-like behavior. Additionally, systemic administration of the selective serotonin reuptake inhibitor, paroxetine, reduces depression-like symptoms and restores the serotonin-induced increase in DA response in the NAc of FSL rats. Taken together, these findings suggest reduced ability of the NAc to respond to serotonin may underlie the display of depression-like symptoms such as passive coping.

The current experiment examined 5-HT1A receptor expression in the NAc shell of postpartum females that were stressed or unstressed, and found no effects. This was unexpected given the reductions in motivated behaviors found in this experiment, and NAc involvement in motivated behaviors. Additionally, Chapter 2 of this dissertation found a more NAc 5-HT1A mRNA in recently parturient females compared to virgins, although total binding density did not significantly differ between rats in different reproductive states. The mesocorticolimbic dopamine system, with cell bodies arising in the midbrain ventral tegmental area (VTA) and releasing DA into the nucleus accumbens (NAc), is extensively involved in regulating motivated behaviors, including maternal caregiving. For example, DA is released during appetitive, or goal-oriented, behaviors such as pup approach and pup retrieval (Champagne et al., 2004; Robinson et al., 2011), and blocking DA receptors in the NAc severely disrupts these same aspects of maternal behavior and also reduces pup licking (Keer et al., 1999; Numan et al., 2005). Serotonin infusion into the NAc increases DA release in the NAc, implicating a role for serotonin in this

reward pathway. While total 5-HT1A receptor binding in the NAc was not significantly altered in stressed dams compared to unstressed dams, the rostrocaudal pattern of binding density was slightly altered in the NAcSh in pregnancy stressed postpartum females, such that binding density in the rostral NAcSh was reduced in stressed dams. The functions of the NAcSh can be separated by rostrocaudal gradients, with the rostral portion more heavily influencing feeding behavior (Reynolds & Berridge, 2001). A reduction in 5-HT1A binding sites specifically in the rostral NAcSh may explain the significant effects on saccharin preference in stressed dams compared to unstressed dams.

Although Chapter 2 of this dissertation showed that postpartum dams still had more 5-HT1A mRNA expression in the NAcSh at this time compared to virgins, it is likely that the more dramatic elevation at parturition aids in initiating maternal behaviors, such as pup-approach and pup licking, at parturition. RVS may have more significantly altered the parturition-induced increase in 5-HT1A expression, leading to disruptions in maternal behaviors lasting throughout early lactation. Future studies may examine the effects of RVS on 5-HT1A expression at parturition instead of early lactation. In addition, DA signaling in the NAc may have instead been disrupted in pregnancy stressed dams, leading to reductions in appetitive maternal behaviors. Slight alterations in postpartum 5-HT1A binding density in the NAc following RVS could also disrupt DA release here, since serotonin-induced increase in DA in the NAc is mediated by 5-HT1 receptors (Parsons & Justice, 1993).

Another likely neural candidate for mediating the stress-induced disruptions in maternal behavior is the medial preoptic area of the hypothalamus (mPOA). A previous chapter in this dissertation showed that 5-HT2A receptor mRNA and binding is significantly higher in the mPOA of parturient rats when compared to diestrous virgins, and others have shown higher

serotonin turnover in the mPOA during early lactation (Lonstein et al., 2003) compared to before mating. The serotonergic system is sensitive to stress, and a single stressful event is capable of reducing 5-HT2A receptor mRNA and protein in the hippocampus of male rats (Dwivedi et al., 2005). I hypothesized that repeated variable stress during pregnancy would significantly reduce the normative peripartum increase in 5-HT2A receptors in the mPOA and implicate an involvement for these receptors in stress-induced disruption in maternal caregiving behavior. Receptor autoradiography revealed that pregnancy stressed females did not differ from non-stressed females in postpartum binding of 5-HT2A receptors in the mPOA, though. While this finding may indicate that repeated variable stress during pregnancy does not significantly alter postpartum 5-HT receptor expression in the mPOA, 5-HT2A binding here is at its very highest right after parturition rather than on PPD7 when my subjects in these experiments were sacrificed. This suggests that 2A receptors in the mPOA may be more important for the onset of normal caregiving behavior instead of the maintenance of maternal behaviors. The mPOA as a whole is just as necessary for the onset of maternal behavior as it is for the maintenance of maternal behavior. Electrolytic lesions (Jacobson et al., 1980) or knife cuts to destroy dorsolateral connections of the mPOA/vBST (Numan & Callahan, 1980) during pregnancy prevent the onset of maternal behaviors at parturition. Additionally, the mPOA is primed by reproductive hormones such as estradiol and oxytocin during pregnancy as these neuroendocrine regulators ramp up leading to parturition (for review see Bridges et al., 2015), and the actions of both estradiol and oxytocin in the mPOA at parturition are also required for the initiation of caregiving behavior at parturition (Numan et al., 1997; Fahrbach et al., 1985; Pedersen et al., 1994). While the neuroendocrine influences on the mPOA have been well studied, the contribution of neurotransmitter systems in the mPOA for the onset of caregiving has not been

identified, although their role in ongoing maternal behaviors during lactation is substantial (Hansen et al., 1990; Numan et al., 2007; Smith & Lonstein, 2013). Future studies should seek to further understand the contribution of the serotonergic system in the mPOA for the onset of maternal behavior, either by utilizing a serotonin-specific neurotoxin such as 5,7-DHT to lesion 5-HT projections to the mPOA during pregnancy or by injecting a viral vector construct to knockdown 5-HT2A receptor expression during late pregnancy, followed by observation of behavior at parturition. Additionally, while alterations in serotonin receptor 1A and 2A binding were minimal following RVS during pregnancy, alterations in affinity of serotonin to 5-HT receptors in the NAc and mPOA may still be altered and should be studied in future experiments.

In humans, depression and anxiety are often correlated with altered output of DR serotonin to the forebrain (Stockmeier et al., 1998). Since RVS did not alter 5-HT receptor binding in the forebrain DR projection sites studied here, but did alter many behaviors that are influenced by serotonin, it is possible that overall serotonin release from the DR was instead affected by RVS, leading to alterations in postpartum caregiving and affective behavior. Experiment 2a in this dissertation found lower 5-HT2C receptor mRNA in recently parturient dams compared to virgins, with a further reduction in PP7 lactating dams. 5-HT2C receptors in the DR are localized on inhibitory GABA neurons, at least in male rats (Boothman et al., 2006). Thus, reduced ability of 5-HT2C receptors to activate GABA neurons during the peripartum period may indicate an increase in DR serotonergic activity during this time. Indeed, serotonin turnover is higher in the BST and mPOA of lactating dams compared to virgins, giving some support for a postpartum increase in serotonin release. Lesions to DR serotonin neurons either during pregnancy or two days after birth did not significantly alter postpartum anxiety-like behavior, but they did significantly reduce maternal aggression (Holschbach et al., 2018).

Depression-like behavior was not analyzed in the aforementioned experiment; therefore, it is unknown whether peripartum serotonin lesions increase postpartum depression-like symptoms. 5-HT receptor binding could indicate whether serotoninergic signaling from the DR is disrupted after RVS. In the behavior-tested cohort of dams in this study, 5-HT2C receptor mRNA was significantly negatively correlated with saccharin preference, such that dams that had reduced preference for saccharin (i.e., higher depression-like symptoms) had higher 5-HT2C mRNA in the DR. These findings could suggest that altered postpartum 5-HT2C receptor expression is responsible for the stress-induced increase in postpartum depression-like symptoms. 5-HT2C receptor binding could not be analyzed in this experiment due to the inability to successfully run autoradiography for my 2C ligand. However, future research should examine the effects of pregnancy RVS on DR 5-HT2C receptor expression.

In sum, this experiment showed that a novel repeated variable stress paradigm significantly alters postpartum caregiving behavior and increases depression-like behaviors in lactating female rats. Although we found minimal alterations in 5-HT receptor binding in the mPOA or NAc at the end of the first week postpartum that could explain these behavioral effects, these behavioral disruptions could instead be due to changes in 5-HT receptor affinity or disruptions in overall serotonin output.

CHAPTER 4: EFFECTS OF 5-HT1A RECEPTOR KNOCKDOWN IN THE NUCLEUS ACCUMBENS SHELL ON POSTPARTUM SOCIAL AND AFFECTIVE BEHAVIORS

Previous chapters in this dissertation have shown alterations in serotonin receptor mRNA and binding in various regions of the maternal brain responsible for the onset and maintenance of caregiving behaviors. One such region is the nucleus accumbens, which is a key brain site for processing the salience of rewards (reviewed in Volkow & Morales, 2015). The nucleus accumbens receives dopaminergic input from the ventral tegmental area, and this circuit regulates response to both natural rewards such as food and sex, and the rewarding properties of exogenous compounds such as drugs of abuse (Volkow & Morales, 2015). The nucleus accumbens is also critical for the display of maternal behavior during the postpartum period, as mothers find their pups highly rewarding. For example, mother rats will bar-press at high frequencies for access to their pups, and will form a preference for a space where they previously had access to their pups (conditioned place-preference) (Fleming et al., 1994). Lesioning or inactivating the NAc also reduces licking (Keer and Stern, 1999) and disrupts appetitive maternal behaviors such as retrieving (Li & Fleming, 2003; Numan et al., 2005), and lesions have no effect on consummatory behaviors such as nursing. Dopamine release in the NAc during the postpartum period promotes maternal motivation to care for pups. While there are no significant changes in dopamine turnover in the NAc across pregnancy and lactation (Lonstein et al., 2003; Winokur et al., 2019), there are phasic changes in dopamine release while postpartum dams are retrieving or licking pups (Afonso et al., 2009; Champagne et al., 2003; Robinson et al., 2011).

While the changes DA release in the NAc are transient and occur just before or within seconds of starting an appetitive behavior towards pups, changes in accumbens DA receptor expressions are more long lasting. We recently found that DA receptor 1 mRNA in the NAc shell

is significantly increased at postpartum day 7 compared to virgin female rats, and remains higher than virgins at least until postpartum day 19 (Grieb et al., 2019). In contrast, D2 mRNA in the NAc is significantly lower in recently parturient and late lactating dams compared to virgins (Grieb et al., 2019). D2 receptors in the NAc are responsible for opposing aversive responses (Hikida et al., 2010; Kravitz et al., 2012), thus their activation in response to DA release in anticipation of or during active maternal behaviors likely suppresses aversion to pups, while increased D1 receptor expression in the NAc during lactation promotes the rewarding properties of pups. DA receptors in the NAc are involved in the display of maternal caregiving behaviors, because infusing DA D1 or D2 receptor antagonists directly into the shell region of the NAc also disrupts maternal behavior (Keer & Stern, 1999; Li et al., 2003; Numan et al., 2005). Haloperidol, a D2 antagonist, also disrupts pup retrieval and pup licking and increases Fos expression in the NAc, and pretreatment with quinpirole (a D2 agonist) reinstates maternal motivation and reduces Fos expression in the NAc shell (Zhao & Li, 2010). Because D2 receptors are inhibitory, these findings suggest that, during motherhood, DA acting at D2 receptors inhibits neuronal activation in the NAc shell to promote maternal motivation. While these studies provide evidence for a role of the DA system in the NAc in the regulation of maternal caregiving, it is important to note that none of these studies have examined the role of NAc DA or DA receptors in regulating postpartum affective behavior such as anxiety-like or depression-like behavior, even though this system is involved in affective behavior in non-parous rodents (for review, see Nestler et al., 2006).

While the role of dopamine in maternal motivation has been fairly well characterized, other neurotransmitter systems within the NAc have been overlooked with regards to postpartum caregiving. The NAc receives heavy serotonergic input from the dorsal and median raphe nuclei

(Van Bockstaele et al., 1993), and serotonin infused into the NAc increases local dopamine release (Parsons & Justice, 1993). Several classes of 5-HT receptors are expressed on neurons in the NAc, including 5-HT2A, 5-HT2C, 5-HT1B and 5-HT1A (Sumner & Fink, 1995; Filip & Cunningham, 2002; Di Matteo et al., 2008), but the 1 family of receptors is especially important for this serotonin-induced increase in DA release because it can be attenuated with simultaneous infusion of a non-specific 5-HT1 receptor antagonist infused into the NAc (Parsons & Justice, 2003). Although the role of 5-HT1A receptors in the NAc has not been well-studied regarding social behavior in laboratory rodents, systemic manipulation of 5-HT1A activity does affect social investigation and aggressive behavior in males. For example, blocking 5-HT1A receptors produces anti-aggressive effects in male mice (Sanchez, 1990), while activating 1A receptors with a low dose agonist significantly increases social investigation such as anogential sniffing and approach behavior (Bell & Hobson, 1993; Dunn et al., 1989), and 5-HT1A activation also facilitates copulation in male rats (Fernández-Guasti; 1992). 5-HT1A receptors have also been extensively implicated in maternal aggression (De Almeida & Lucion, 1997). Few studies have examined the involvement of 1A receptors on other postpartum behaviors, such as maternal caregiving or anxiety-like and depression-like behaviors, although one study did find disruptions in pup retrieval following systemic injections of buspirone, a 5-HT1A agonist (Ferreira et al., 2000). Still, the dearth of studies examining the role of 5-HT1A in caregiving is surprising, given that maternal 5-HT1A receptor deficiency has drastic effects on pup development and later emotional reactivity (van Velzen & Toth, 2010; Gleason et al., 2010). Based on cross-fostering done in this study, increased offspring stress responsiveness depends on both pre- and postnatal maternal 5-HT1A receptor genotype, suggesting that maternal care may be altered as a result of maternal 5-HT1A deficiency.

The goal of the current study is to test the hypothesis that high 5-HT1A receptor expression in the nucleus accumbens regulates postpartum social and affective behaviors. I found in Chapter 2a that 5-HT1A expression is higher by 250% in the NAc of recently parturient rats compared to diestrus virgins, and, this may be required for the expression of high maternal care, low anxiety, and high maternal aggression. To test my hypothesis, an adeno-associated viral vector construct knocking down 5-HT1A receptors (or a control construct) was injected into the NAcSh during pregnancy. After parturition, I examined maternal caregiving behavior, maternal motivation to retrieve pups, postpartum anxiety-like and depression-like behavior, and maternal aggression.

Methods

Subjects

Subjects were 20 female Long-Evans rats purchased from Envigo Laboratories (Indianapolis, IN). Females were housed with 1 or 2 other females in clear polypropylene cages (48 cm x 28 cm x 16 cm) containing wood chip bedding, food (Tekland rat chow, Indianapolis, IN) and water *ad libitum*. The room was maintained on a 12 hour light/dark cycle (lights on at 0600 hr) with temperature kept at 22 +/- 1 °C. After reaching 65 days old, females were placed in a cage with a male breeder (Envigo) on the morning of proestrus (determined by vaginal smearing and cytology) and housed with the male until the next day. Vaginal smearing and cytology was used to confirm the presence of sperm, and therefore successful copulation. Subjects were again housed 2-3 to a cage until pregnancy day 7 (P7), when rats underwent stereotaxic surgery.

Surgery and Viral Vector Injection

On pregnancy day 7, female rats were weighed and anesthetized with ketamine (90 mg/kg IP; Butler, Dublin, OH) and xylazine (8 mg/kg IP; Butler, Dublin, OH) and placed in a Kopf stereotaxic apparatus. The scalp was retracted, and two holes were drilled into the skull above both sides of the nucleus accumbens shell (A/P = 1.6 mm, M/L = +/- 1.10 mm from bregma). 0.75 μL of htr1a-shRNA (n = 16) or the scrambled control vector solution (*n* = 11) (Vector Biolabs, Malvern, PA) was slowly injected (~0.75 μL / 2 mins) into the NAc through a Hamilton syringe at 7.3 mm ventral from the skull. This viral construct contains shRNA for the rat *htr1a* gene, which instructs the infected cells to stop making 5-HT1A mRNA. The needle remained in the NAc for 7 min on each side, and was then slowly retracted. The scalp was closed with surgical staples. Subjects received postoperative care including twice-daily subcutaneous injections of buprenorphine (0.015 mg/kg) for one day after surgery and then left undisturbed until parturition. On the day of parturition (PPD 0), litters were culled to 8 pups per subject (4 males and 4 females).

Maternal Behavior and Retrieval Testing

Beginning the day after parturition, which was designated as postpartum day 1 (PPD1), subjects were observed daily for maternal behavior for 30 min daily at 0900 and 1400 on PPD1-12 (see Figure 8). During these observations, nests were scored on a scale of 0-3 (0 = no nest, 1 = poor nest, 2 = partial nest, 3 = complete nest with high walls; Lonstein and Fleming, 2002) and maternal behaviors were recorded every 30 sec. As described in the Chapter 3, recorded behaviors included both maternal (licking, mouthing, retrieving, and sniffing pups, nest-building, nursing) and non-maternal (self-grooming, eating/drinking, resting alone, exploring) behaviors.

To ensure the stress paradigm did not tremendously disturb the growth of infants, litter weights were recorded each afternoon. In addition, dam weights were taken each afternoon to assess general health. To assess maternal motivation, retrieval tests immediately followed the afternoon observations on PPD3 and PPD5. Litters were removed from the cage for 15 min and placed in an incubator set to nest temperature (34 ºC). After 15 min, pups were scattered in the cage on the opposite side of the nest and the time for the dams to retrieve each pup and hover over the nest was recorded. If a dam did not retrieve her pups to the nest site after 5 min, the experimenter grouped the pups and placed them back in the nest. After all pups were gathered back to the nest site (either by the subject or by the experimenter) undisturbed maternal behaviors were observed every 30 s for 10 min.

Anxiety-like behaviors

On the afternoon of PPD4, subjects were transported in their home cage (with their litter present) to a behavior testing room illuminated by one 100W light bulb. The elevated plus-maze was elevated 50 cm from the floor and made of black plastic with four arms emerging from a 10 X 10 cm center square. Arms were 10 cm wide by 50 cm long, two of which had 40-cm high walls, 2 of which had no walls. Approximate illumination on the open arms was 28 lux, and approximate illumination on the closed arms was 2 lux (Miller et al., 2011). Each subject was removed from their home cage and placed in the central square of an elevated plus maze facing an open arm. The home cage and the litter remained in the adjacent room. A low-light-sensitive video camera suspended above the maze relayed images to a Magnavox DVD recorder to monitor activity in the maze from the adjacent room. An experimenter recorded subjects' behavior with computerized data acquisition software. Recorded behaviors included time spent

in the open and closed arms, and the frequency of entries into each arm (Pellow et al, 1985). After testing, dams were gently removed from the maze and returned to their home cage containing their pups, and were carried back to the colony. On the afternoon of PPD6, subjects were tested in a light-dark box. Subjects with their litters were carried to the same behavior testing room described above. The light chamber of the box was illuminated to ~624 lux (Miller et al., 2010). Subjects were placed in the light chamber of the box, and behavior was video recorded and scored for 10 min. Behaviors scored included the latency to enter the dark chamber, frequency of stretches into the light chamber, and time spent in the light chamber (Miller et al., 2010). After testing, dams were gently removed from the light-dark box and returned to their home cages with their litters.

Maternal Aggression

On the afternoon of PPD7 subjects were tested for aggressive behavior toward an unfamiliar male intruder to the home cage. Males 50-60 days old from our colony were used as intruders. Males were placed into the subjects' home cage with the litters present. The tests were video recorded and intruders were removed after 10 min. Intruder males were sacrificed immediately following testing. The dams' behaviors were scored using data acquisition software. An attack was scored if the dam initiated a tumble and pin sequence (Lonstein and Gammie, 2002). Behaviors scored included latency to attack the intruder, frequency of attacks, and duration of each attack, and other aggressive behaviors directed at the intruder (sniffing, boxing, kicking, biting, upright posturing, lateral threat) (Lonstein and Stern, 1997; Bosch et al., 2005).

Saccharin Preference Test

At 0800 hrs on the morning of PPD8, subjects were given access to 2 bottles, one containing water and another containing 0.1% saccharin for 24 hours. Bottle placement was counterbalanced across group, and each bottle was weighed and the position swapped after 8 hours, then removed and weighed again after an additional 12 hours. At 0800hr on PPD9 immediately after the 24 hour exposure period ended, pups were removed from the subjects' home cage and placed in an incubator set at nest temperature (34°C) and were again given access to a bottle filled with water and a bottle of 0.1% saccharin solution (Fernandez et al., 2014; Green et al., 2009). After 1 hour, bottles were removed and weighed, and pups were placed back into the home cage for at least an hour before the next maternal behavior observation.

Forced Swim Test (FST)

The FST (adapted from Leuner et al., 2014) was used to assess depressive-like behaviors in dams. On the afternoon of PPD8, subjects were moved from the animal facilities to the behavior testing room described above for a pre-swim test. Rats were placed into a Plexiglass™ cylinder filled with water ~25°C high enough so that the tail could not touch the bottom of the container. The test was digitally recorded from a suspended camera for 15 min, after which the subjects were removed from the apparatus, towel dried, and placed in a clean cage (without their pups) with heating pads until fully dry. Once dry, subjects were carried back to the animal facilities and their pups were placed back into their home cage. On the afternoon of PPD9, rats were again removed from the animal facilities and placed into the same Plexiglass™ cylinder filled with water. The test was digitally recorded for 10 min, after which the subjects were removed from the apparatus, towel dried, and returned to their home cage (without their pups)

with a heating pad until fully dry. Once dry, subjects were carried back to the animal facilities and their pups were placed back into their home cage. The percentage of time spent immobile (floating in the water only making movements necessary to maintain the head above water) was measured using a laptop and behavior coding software.

Sacrifice and Tissue Collection

Postpartum dams in both the 5-HT1A-shRNA-injected and scramble-injected groups were sacrificed immediately following the afternoon maternal behavior observation on PPD12. Subjects were IP injected with sodium pentobarbitol (200 mg/kg) and transcardially perfused through the heart with 4% paraformaldehyde in 0.1M phosphate buffered saline (PSB). Brains were extracted and post-fixed overnight, then transferred to 30% sucrose in 0.1M PBS until sectioning. Brains were sectioned at 40 μm using a freezing microtome into four series. One full series was processed to determine virus injection sites (see immediately below).

Immunohistochemistry for Green-Fluorescent Protein

To verify injection localization, one series of tissue from each animal containing the NAc (~2.52mm – 1.2mm from bregma) was rinsed in 0.1M PBS 6 times for 8 min each. Next, sections were blocked in 3% normal donkey serum (NDS) in PBS+TritonX for 1 hr, then incubated in rabbit anti-GFP (1:1000) in 3% NDS in PBST overnight at 4 °C. The next day, sections were rinsed again 6 times for 8 min each, then incubated in Donkey-anti-rabbit AlexaFluor 488 secondary antibody (A21206, Fisher Scientific, Pittsburgh, PA; 1:500) for 2 hr at room temperature, followed by slide mounting and coverslipping with FluoromountG (ThermoFisher). The entirety of the nucleus accumbens (core and shell) was scanned for GFP-ir

cells under 10X magnification using a Nikon Eclipse E600 light microscope. Any subjects that had GFP-ir cells outside the nucleus accumbens shell (n = 6) were removed from further data analyses.

In vivo determination of 5-HT1A receptor knockdown

Level of 5-HT1A receptor knockdown after NAcSh infusion of the shRNA was determined *in vivo* in a separate cohort of female Long-Evans rats (Envigo Laboratories), with the goal of reaching at least 50% knockdown (Khvorova et al., 2003; Reynolds et al., 2004). Surgery and infusions were identical to that described above. Within 3 hrs of parturition, these 5-HT1A-knockdown- and Scramble-injected dams (n = 6/group) were weighed, rendered unconscious with CO_2 and rapidly decapitated. Brains were removed from the skull and stored at -80 °C until sectioning. Brains were cut coronally into 300-µm-thick sections using a cryostat (Leica CM1950, Nussloch, Germany) to obtain 3 sections that included the NAc (2.26-1.2 mm from bregma). The tissue was punched bilaterally from the sections using first a 0.5 mm micropuncher to dissect the NAc core subregion, and then a 1-mm-diameter micropuncher was used to dissect the shell (Harris Micropunch, Hatfield, PA). To determine 5-HT1A mRNA levels, the tissue was homogenized in RLT buffer (74134, Qiagen, Valencia, CA) containing β-mercaptoethanol by pulsed sonication for 30 sec at 25% amplitude (Fisher Scientific, Pittsburgh, PA). mRNAs were then extracted using the RNeasy Plus Mini Kit (74134, Qiagen, Valencia, CA) per the manufacturer's instructions. The extracted mRNAs were quantified using a Gene Quant 100 spectrophotometer (General Electric, Marlborough, MA) by measuring the 260 nm absorbance values. 100 ng of mRNAs were then converted to cDNA using a high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA) per the manufacturer's

instructions. After conversion to cDNA, samples were stored at -20 °C until being analyzed with real time RT-PCR.

5-HT1A mRNA was run in triplicate and included cDNA, primers, and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a 25-µL reaction. All primers were from Integrated DNA Technologies (Coralville, Iowa). A QuantStudio 5 Real Time PCR System (Applied Biosystems, Foster City, CA) was used for quantification, with the following settings: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. A dissociation curve was run for each sample to ensure that only a single product was transcribed. To analyze changes in 5-HT1A mRNA, two transcripts were run: 5-HT1A (400 nM primers: Forward primer – GGC GCT TTC TAT ATC CCG CT; Reverse – GGT GCC GAC GAA GTT CCT AA) and our control gene, HPRT-1 (200 nM primers: Forward- GAA ATG TCT GTT GCT GCG TCC; Reverse – GCC TAC AGG CTC ATA GTG CAA). PCR products of each primer set were sequenced at the RTSF Genomics Core at Michigan State University to confirm specificity. During quantification, a no-template control was run alongside the samples to ensure that no primer-dimer amplification had occurred. In addition, mRNA samples that were not run through the reverse transcription kit were run simultaneously to ensure no gDNA contamination. Amplification efficiencies were calculated for each primer set, and each was within the accepted range (1.90- 2.10) to use the ΔΔCT method to calculate fold change between groups, with 5-HT1A normalized to HPRT-1 (Schmittgen & Livak, 2008).

Data Analyses

Undisturbed maternal behaviors were analyzed using Repeated Measures ANOVAs to compare the frequency of maternal behaviors both between groups (5-HT1A-shRNA and scramble) and across postpartum days. T-tests were also used to compare summed frequencies of

maternal behaviors from all postpartum days between groups. Data from the anxiety and aggression tests were analyzed using two-tailed t-tests to compare viral knockdown and scramble conditions. In a few cases where groups had significantly unequal variances, nonparametric Mann Whitney-U tests were used to compare groups. For all analyses, a significant main effects and interactions were determined by $p < 0.05$.

Results

NAcSh infusions and degree of knockdown

5-HT1A shRNA resulted in a ~60% knock down of 5-HT1A mRNA in the NAcSh ($t = 2.474$; $p = 0.051$). In the behavior tested groups, 10/16 subjects were included in the KD group; four subjects were removed due to significant viral infection in the NAc core in addition to the shell, one subject was removed due to significant viral infection in the lateral septum, and one was removed due to lack of viral infection at all. GFP expression was typically found in the dorsomedial NAcSh. The most rostral infection was found at ~2.52mm from bregma, and the most caudal expression was found at ~1.20mm from bregma. Representative photomicrograph can be found in Figure 9.

Maternal and Litter Health

Number of pups in each litter at parturition was not significantly affected by prepartum 5-HT1A viral vector knockdown ($t = -0.580$, $p = 569$). Pup weights across the 12-day testing period also did not significantly differ between knockdown and scramble-injected dams ($F_{(1,1)} = 1.464$, $p = 0.240$). As expected, pup weight significantly increased across days ($F_{(1,10)} = 1322.49$, $p < 0.0001$), but there was no significant interaction between pup weight gain across days and

group ($F_{(1,10)} = 1.045$, $p = 0.408$). Dam weights also did not significantly differ across days between knockdown- and scramble-injected dams ($F_{(1,1)} = 1.251$, $p = 0.279$), though there was the expected increase in dam weight gain across the 12-day testing period ($F_{(1,10)} = 47.91$, $p < 0.0001$). There was no significant interaction between dam weight gain across days and group ($F_{(1,10)} = 1.177$, $p = 0.310$).

Maternal Behavior

There were significant effects of 5-HT1A knockdown in the NAc shell on undisturbed maternal behaviors, such that1A-KD mothers generally spent more time performing non-pup-oriented behaviors and less time with their pups. The 1A-KD mothers had a significantly higher frequency of self-grooming ($t_{(18)} = 3.011$, $p = 0.007$), and tended to spend less time in the nest with pups ($t_{(19)} = -1.599$, $p = 0.126$). Additionally, 1A-KD mothers had a higher frequency of all non pup-directed behaviors summed together (self-grooming, sleeping away from pups, feeding, exploring the cage, and nesting) ($F_{(1,1)} = 7.588$, $p = 0.013$) averaging across postpartum day. Means and standard errors for all behaviors scored during undisturbed observations can be found in Table 8. As others have shown previously, many behaviors changed in frequency across testing day as the pups aged. Means, SEMs, and statistics for these can also be found in Table 8.

KD mothers also showed disruptions in maternal motivation during retrieval testing. 1A-KD dams took significantly longer to retrieve all 8 pups pups back to the nest ($F_{(1,1)} = 5.125$, $p = 0.036$) and there was a significant interaction between group and which pup was retrieved, such that 1A-KD dams started to significantly diverge from scramble-injected dams by retrieval of the 5^{th} pup, and continued to take significantly longer to retrieve each pup thereafter. The 1A-KD dams also took more time to hover over all 8 pups in the nest after completing the 8^{th} retrieval

($F_{(1,8)} = 5.589$, $p = 0.005$). 1A-KD and scramble-injected mothers did not differ in the frequency of maternal caregiving behaviors during the 10 min observation period immediately following the retrieval test, but1A-KD dams did have a significantly higher frequency of nesting in the 10 min following the retrieval tests compared to controls ($t_{(18)} = 2.253$, $p = 0.037$).

Anxiety-Like Behavior

5-HT1A knockdown in the NAcSh significantly increased anxiety-like behavior in the light/dark box, with. 1A-KD dams showing a significantly longer latency to enter the light chamber ($t_{(15)} = 2.486$, $p = 0.029$), entered the light chamber less frequently, ($t_{(15)} = -4.379$, $p = 0.001$), and had a significantly lower percentage of time spent in the light chamber ($t_{(15)} = -3.892$, $p = 0.001$). Knockdown did not affect behavior in the EPM. Means and standard errors for all variables for the EPM can be found in Table 9.

Maternal Aggression

5-HT1A knockdown in the NAcSh tended to increase the number of lateral threats during maternal aggression testing ($U = 1.859$, $p = 0.062$) and did not significantly affect any other aggressive behaviors (Table 9)

Depression-like Behavior

5-HT1A KD dams did not significantly differ from scramble-injected dams in depression-like behaviors. KD subjects and scramble subjects had similar preference for 0.1% saccharin over water in the two-bottle choice test, both during the initial 24-hr exposure period ($t = -1.21$, $p = 0.244$) and the 1-hr test ($t = 0.109$, $p = 0.924$). KD subjects and scramble subjects also spent a similar percentage of time floating in the forced swim test ($t_{(19)} = -0.603$, $p = 0.554$).

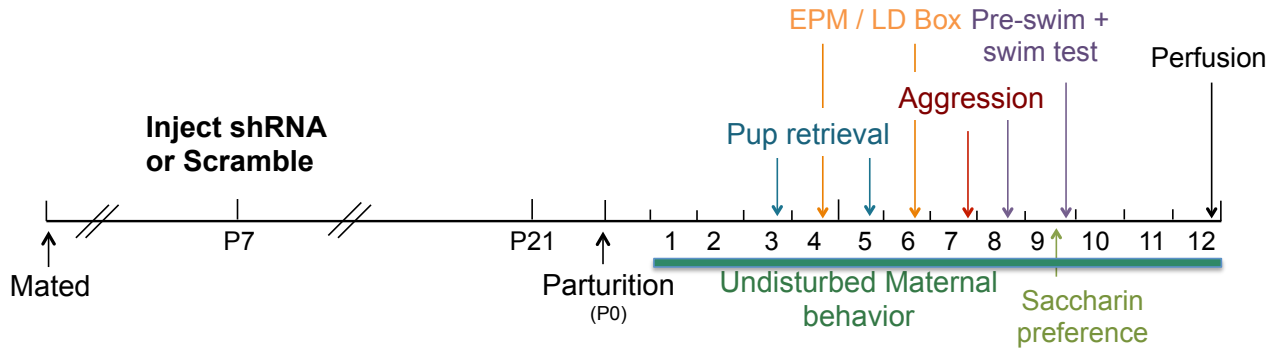

Figure 8: Schematic representation of experimental timeline used to determine the effects of 5-HT1A knockdown in the nucleus accumbens shell on postpartum caregiving and affective behaviors. Pregnant females were injected with the 5-HT1A shRNA or scramble vector on pregnancy day 7. Following birth, undisturbed maternal behavior observations were conducted twice daily. On PPDs 3 and 5, dams' retrieval performance was assessed. On PPDs 4 and 6, anxiety-like behavior was assessed in the elevated plus maze and light/dark box, respectively. On PPD 7, maternal aggression towards an unfamiliar male rat was assessed. Finally, on PPDs 8 and 9, dam's depression-like behaviors were observed using the saccharin preference test and the forced swim test.

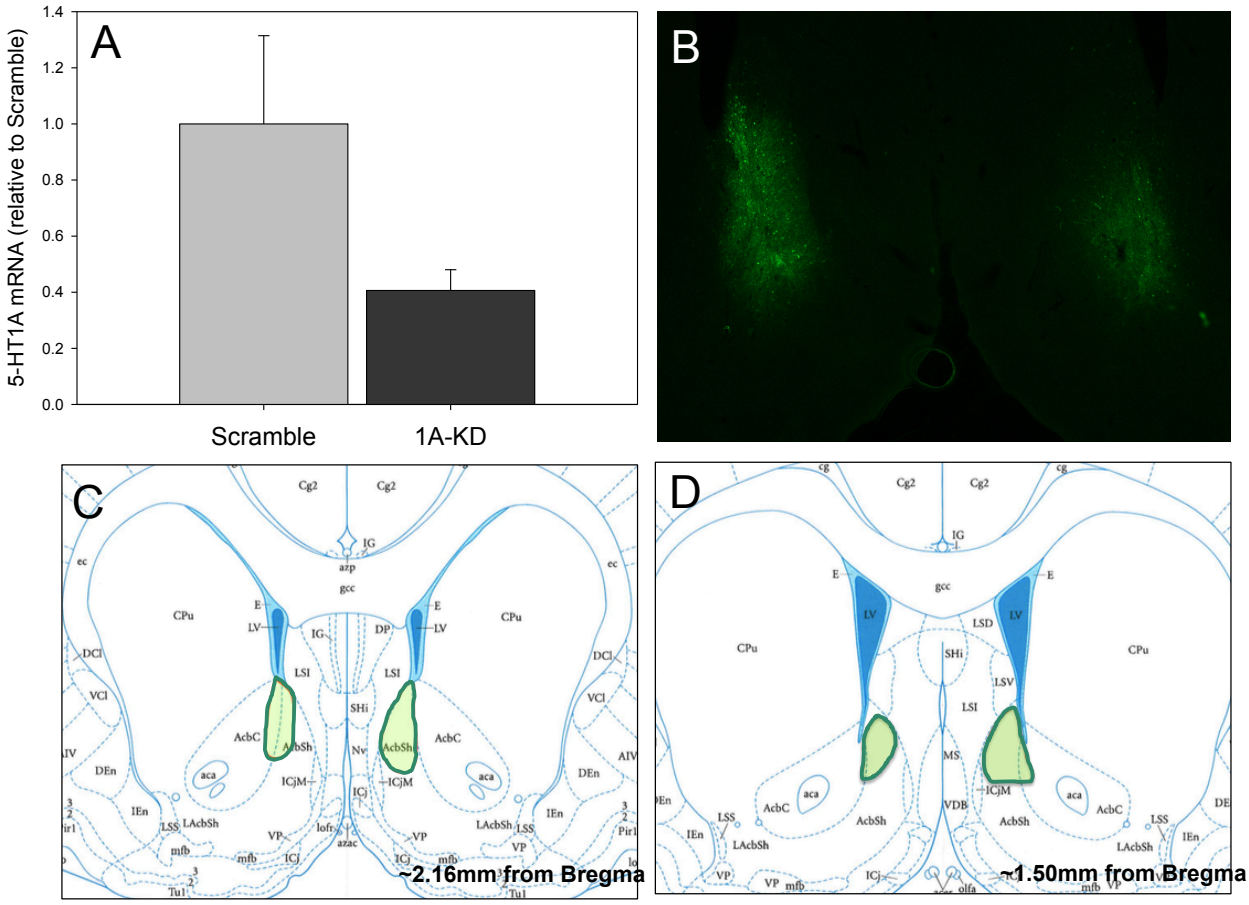

Figure 9: 5-HT1A mRNA and GFP immunoreactivity in the nucleus accumbens shell. 5-HT1A shRNA resulted in a ~60% reduction in 5-HT1A mRNA in the NAcSh of recently parturient dams compared to scramble injected recently parturient dams (A). Representative photomicrograph of GFP immunoreactivity in the NAcSh of a postpartum dam (B), schematics of average GFP spread in the rostral (C) and medial (D) NAcSh.

	Scramble	KD	Group (t, *p*)	Time (F, *p*)	Group*Time (F, *p*)
Pup Number	11.1 ± 1.1	11.4 ± 0.9	-0.16; 0.88	NA	NA
# Males at birth	5.6 ± 1.0	6.1 ± 0.7	-0.44; 0.67	NA	NA
# Females at birth	5.6 ± 0.7	5.2 ± 0.7	0.27; 0.79	NA	NA
Pup weight across lactation	125.6 ± 3.8	130.4 ± 3.4	0.84; 0.37	1085; <0.00001*	0.64; 0.79
Dam weight across lactation	286.9 ± 4.7	297.37 ± 4.2	0.66; 0.43	41.2; <0.00001*	1.56; 0.11

Table 7: Effects of 5-HT1A knockdown during pregnancy on dam and litter health across lactation. Means ± SEMs of pup numbers at parturition, percent change in pup weight, and dam weight by the end of lactation. *indicates $p < 0.05$

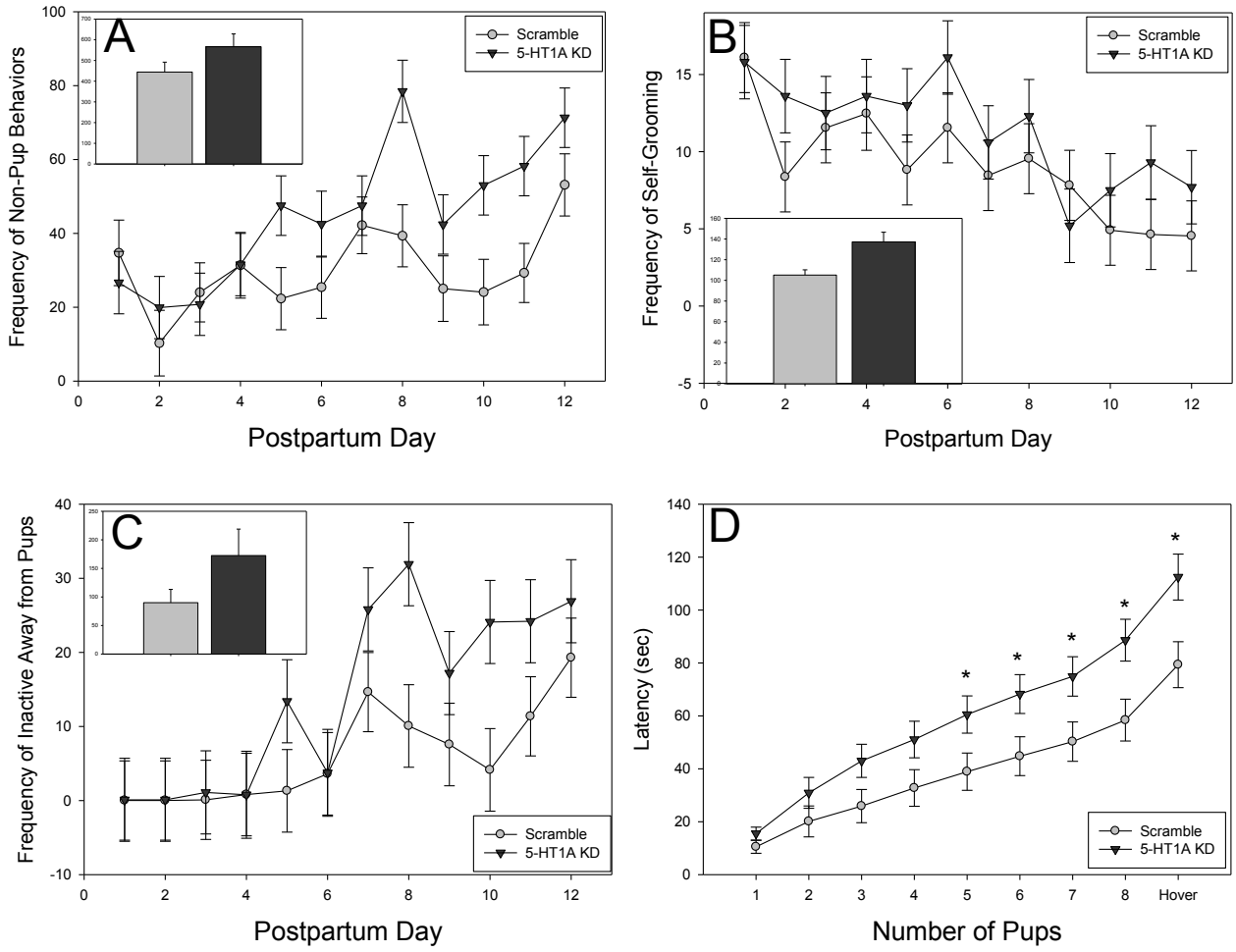

Figure 10: Effects of 5-HT1A knockdown in the nucleus accumbens shell on maternal caregiving and maternal motivation. Frequency (Mean ± SEMs) of dams' non-pup directed behaviors (A), self-grooming (B), sleeping away from the nest (C), and latency to retrieve displaced pups back to the nest (D). *indicates statistically significant difference between groups, $p < 0.05$

	Scramble	1A-KD	Group (t; *p*)	Group (F, *p*)	Time (F,*p*)	Group* Time (F, *p*)
Crouch	858.9 ± 40.1	799.3 ± 55.9	0.88; 0.39	0.77; 0.39	14.1; <0.0001*	0.98; 0.47
Hover	353.2 ± 21.1	344.6 ± 22.5	0.28; 0.78	0.44; 0.52	3.66; <0.0001*	0.97; 0.47
On-Side Nursing	353.5 ± 47.1	356.4 ± 40.0	-0.05; 0.96	0.001; 0.97	8.36; <0.0001*	0.85; 0.59
Blanket Nursing	327.5 ± 40.9	270.9 ± 31.7	1.08; 0.30	1.34; 0.26	0.83; 0.61	1.14; 0.33
Licking	145.7 ± 10.6	156.5 ± 12.0	-0.68; 0.51	0.03; 0.86	1.57; 0.11	1.32; 0.21
Nesting	26.1 ± 5.8	20.1 ± 3.1	0.89; 0.38	0.008; 0.93	1.99; 0.03*	3.02; <0.001*
Feeding	128.6 ± 27.3	146.8 ± 26.2	-0.48; 0.64	0.98; 0.33	5.33; <0.0001*	0.96; 0.49
Grooming	105.1 ± 5.1	137.2 ± 9.4	-3.01; 0.007*	6.84; 0.017*	3.87; <0.0001*	0.48; 0.91
Inactive (sleeping away from pups)	90.0 ± 23.2	172.5 ± 46.3	-1.64; 0.12	4.3; 0.05*	6.48; <0.0001*	1.22; 0.28
Exploring	69.4 ± 5.8	85.8 ± 9.9	-1.43; 0.17	0.73; 0.40	0.73; 0.40	0.77; 0.67
Tail Chasing	10.5 ± 4.5	2.2 ± 0.7	1.64; 0.12	1.16; 0.29	1.76; 0.06	0.97; 0.47
In-Nest Behaviors	1893.0 ± 38.9	1771.2 ± 67.5	1.60; 0.13	2.57; 0.13	10.26; <0.0001*	0.95; 0.49
Non-pup behaviors	444.1 ± 47.8	566.3 ± 62.0	-1.58; 0.13	7.59; 0.013*	6.02; <0.0001*	1.71; 0.07
All Nursing Postures	1539.8 ± 53.4	1426.6 ± 65.3	1.35; 0.19	1.88; 0.19	3.12; <0.001*	1.57; 0.11

Table 8: Frequency (Mean ± SEM) of maternal behaviors displayed by 5-HT1A KD dams and scramble injected dams during two 30 min observations each day on postpartum days (PPD) 1-12. *indicates statistically significant difference, $p < 0.05$

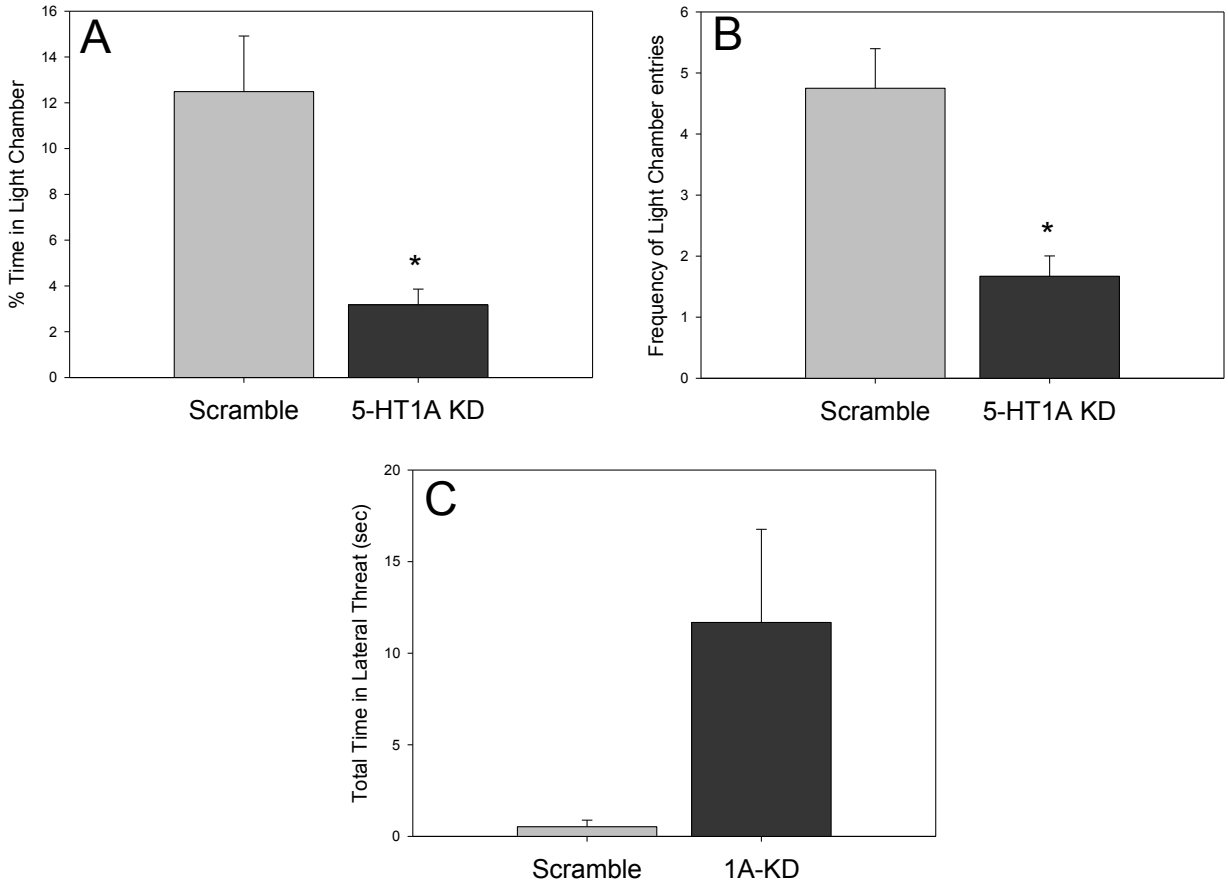

Figure 11: Effects of 5-HT1A knockdown on anxiety-like behavior and maternal aggression. 5-HT1A KD dams were more anxious in the light/dark box compared to scramble injected dams (A-B), and tended to spend more time displaying lateral threats toward an unfamiliar male (C). *indicates statistical significance between groups, $p < 0.05$

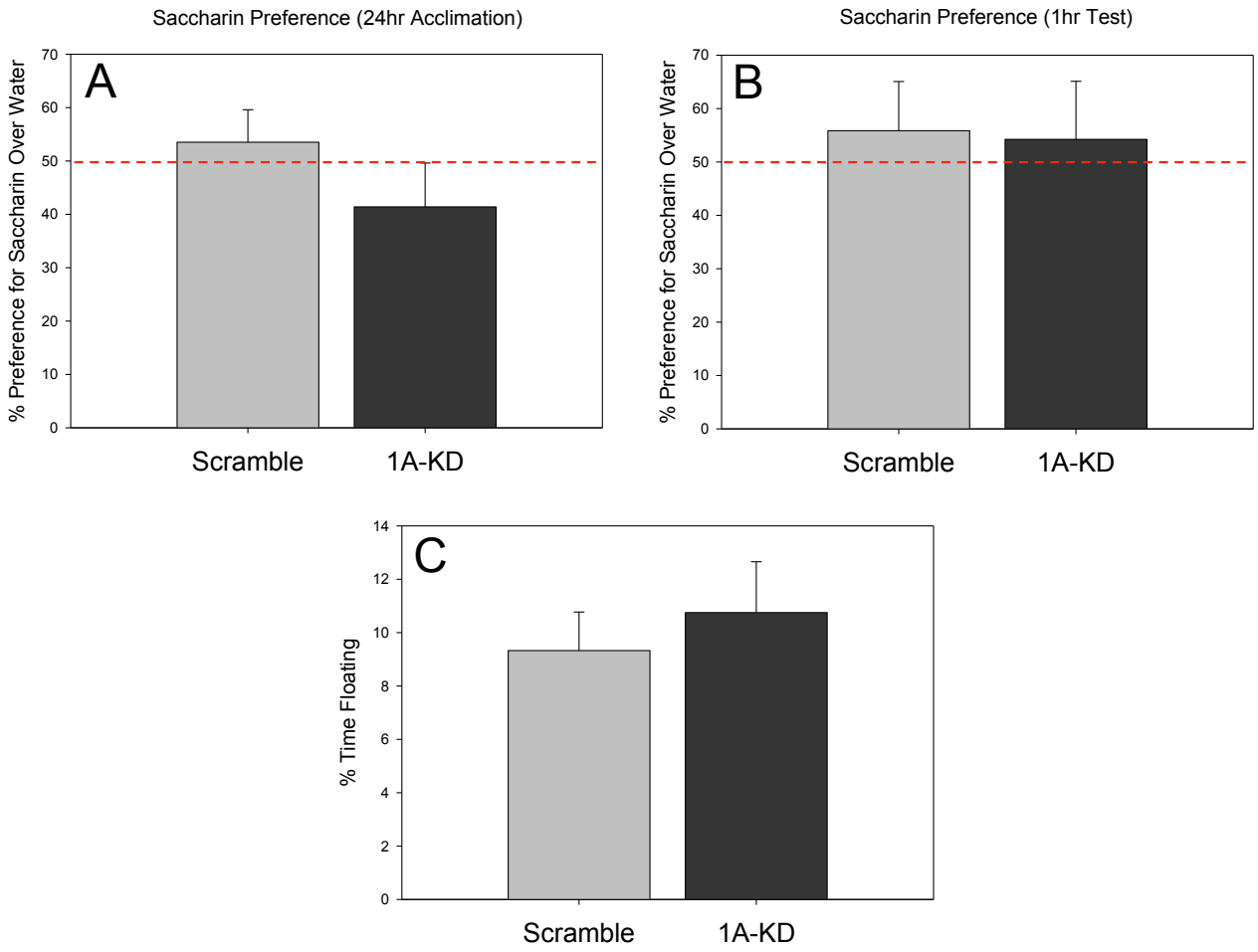

Figure 12: Effects of 5-HT1A knockdown on postpartum depression-like behaviors. Percentage preference for saccharin solution after 24-hr acclimation period (A) and during the 1-hr test (B). Percentage of time dams spent floating (immobile) in the forced swim test (C).

EPM	Scramble	1A-KD	t	p
# Open Arm Crosses - Front Paws	13.25 ± 0.98	14.8 ± 1.8	-0.704	0.492
# Open Arm Crosses - Four Paws	4.625 ± 0.5	5.3 ± 1	-0.564	0.581
# Closed Arm Crosses - Front Paws	17.375 ± 1.96	17.1 ± 1.17	0.126	0.901
# Closed Arm Crosses - Four Paws	13.125 ± 1.44	13.56 ± 0.56	-0.291	0.775
% Time Open Arm - Front Paws	20.43 ± 2	21.92 ± 3.54	-0.341	0.737
%Time Open Arm - Four Paws	8.89 ± 1.17	9 ± 1.57	-0.056	0.956
Light/Dark Box				
# Light entries	4.75 ± 0.65	1.67 ± 0.33	4.379	0.001*
# Stretches into Light	31 ± 2.99	30.67 ± 2.46	0.087	0.932
# Rears in Light	12.38 ± 3.36	2.33 ± 0.85	3.061	0.008*
Latency to enter Dark	6.68 ± 0.97	4.38 ± 0.84	1.794	0.092
%Time in Light	12.49 ± 2.43	3.17 ± 0.69	3.892	0.001*
Depression-like behavior				
% Time Floating (immobile)	9.32 ± 1.45	10.75 ± 1.90	-0.603	0.554
% Preference 1st 12hr	50.2 ± 8.21	54.06 ± 9.46	-0.309	0.761
% Preference 2nd 12hr	54.91 ± 6.45	35.53 ± 9.54	1.734	0.100
% Preference 24hr	53.51 ± 6.07	41.39 ± 8.26	1.202	0.246
% Preference 1hr Test	55.85 ± 9.23	54.22 ± 10.89	0.114	0.910
Total Consumption - 24hr Acclimation	68.65 ± 3.74	73.06 ± 4.26	-0.781	0.446
Total Consumption - 1hr Test	7.28 ± 2.3	11.17 ± 2.98	-1.051	0.306

Table 9: Anxiety-like behaviors and depression-like behaviors (Means ± SEMs) in 5-HT1A knockdown and control injected dams. *indicates statistical significance between groups. [a]indicates Mann-Witney U test.

Maternal Aggression	Scramble	1A-KD	t	p
Frequency of Attacks	1.78 ± 0.57	3.7 ± 1.74	-1.000	0.330
Frequency of Lateral Threats	0.25 ± 0.16	2.5 ± 1.04	1.86a	0.101
Frequency of Kicks	0.38 ± 0.18	3.7 ± 1.61	1.441a	0.203
Frequency of Pins	0.78 ± 0.32	3.1 ± 1.5	0.913a	0.4
Frequency of Bites	1.89 ± 0.51	1.3 ± 0.5	0.825	0.421
Frequency of Sniffing intruder	45.89 ± 2.89	44.1 ± 4.17	0.345	0.735
Latency to Attack	352.22 ± 66.91	323.4 ± 59.07	0.324	0.750
Latency to Lateral Threat	455.16 ± 73.24	395.9 ± 62.58	0.619	0.544
Latency to Bite	330.78 ± 62.58	419.6 ± 56.78	-0.995	0.334
Latency to Pin	439.87 ± 64.48	389.56 ± 65.99	0.543	0.594
Total Attack Duration	7.47 ± 3.19	8.2 ± 4.57	-0.132	0.897
Total Lateral Threat Duration	0.53 ± 0.36	11.68 ± 5.08	1.853a	0.101
Total Sniff Duration	118.02 ± 4.48	111.94 ± 15.61	0.816a	0.447

Table 10: Maternal aggression related behaviors (Means ± SEMs) in 5-HT1A knockdown and control injected dams. *indicates statistical significance between groups. a indicates Mann-Witney U test.

Discussion

The present study provides evidence for a role of 5-HT1A receptors in the nucleus accumbens shell (NAcSh) in the expression of maternal caregiving and other postpartum behaviors. Specifically, disrupted expression of 5-HT1A receptors in the NAcSh significantly increased non pup-directed behaviors such as self-grooming and sleeping away from pups, and significantly delayed the latency to retrieve pups to the nest. These effects may be due to serotonin's influence on dopamine release in this brain site. Dopamine in the NAcSh has been repeatedly implicated in the display of maternal motivation and appetitive maternal behaviors such as pup retrieval and pup licking (Numan & Stolzenberg, 2005). Selective lesions to VTA-NAc DA neurons severely impair pup retrieval (Hansen, 1992), antagonizing either D1 or D2 receptors in the NAc shell severely disrupts pup retrieval (Numan et al., 2005; Keer and Stern, 1999; Silva et al 2003), and behaviors such as approaching and retrieving pups significantly increase phasic DA release in the NAc (Hansen et al., 1993; Robinson et al., 2011). Serotonin infusion into the NAc causes increased DA release in the NAc (Parsons & Justice, 1993), suggesting that serotonin receptors in the NAc may directly affect DA output from the VTA.

Medium spiny neurons (MSNs) in the NAc are generally separated into two groups that modulate reward and motivation via different pathways – excitatory D1-expressing MSNs comprise the direct pathway and are involved in promoting the rewarding properties of a stimulus, while inhibitory D2-expressing MSNs make up the indirect pathway that regulates aversive responses to a stimulus (reviewed in Volkow et al., 2015). Thus, DA regulates reward by activating the pathway that reinforces a reward and inhibiting the pathway that mediates aversion. 5-HT1A receptors are not highly expressed in the NAcSh, and there are no studies characterizing the cell types that express them, so it is unknown which pathway 5-HT1A

receptors influence to promote maternal motivation. However, because 5-HT1A receptors are inhibitory, they probably co-localize with inhibitory D2 receptors to help suppress aversion to pups.

5-HT1A knockdown in the NAcSh also significantly increased maternal self grooming, and several studies in male laboratory rodents have reported dopamine receptor effects in the NAcSh on oral movements such as self-grooming and spout licking (Cools et al., 1995; Prinssen et al., 1994). The same is found after NAc injection of the non-specific 5-HT2 antagonists methysergine or cyproheptadine (Gaffori & Van Ree, 1985). Increased self-grooming has been suggested to reflect anxiety-like behavior because it is often increased following stressors or administration of stress-related hormones (Soubrie, 1971; Gispen et al., 1981). This suggests that 1A-KD mothers may be more emotionally reactive and/or more anxious, which is supported by my finding that 5-HT1A knockdown in the NAc shell also significantly increased anxiety-like behavior in the light-dark box. This was not found in the elevated plus maze, though this discrepancy may be due to the larger anxiogenic experience associated with the light dark box paradigm: prior elevated plus maze exposure reduces anxiety when animals are tested again in the EPM, but prior exposure to an EPM or even the light/dark box itself either does not change or sometimes increases later anxiety in the light/dark box (at least in male rats: Barry et al., 1987; Rodgers & Shepherd, 1993). The NAc is an important mediator of approach and reward as well as avoidance and fear. It receives glutamatergic input from the amygdala (Jackson et al., 2001; Mogenson et al., 1980; Stuber et al., 2011) and the prefrontal cortex (Jackson et al., 2001; Pennartz et al., 1994), and Fos expression in the caudal NAcSh is increased following stress (Duncan et al., 1996; Kreibich et al., 2009). Glutamate release in the caudal portion of the medial NAcSh (~ 1.2-0.4mm from bregma) is a particularly important mediator of fearful behavior, as

blocking AMPA receptors in the caudal NAcSh increases vocalizations, escape behavior, and defensive treading, while blocking glutamate receptors in the rostral NAcSh (~2.4-1.6mm from bregma) increases feeding (Richard & Berridge, 2011). Glutamate receptor blockade in the medial portion of the NAcSh (~1.8-1.2mm from bregma) affects both feeding and fear, and 5-HT1A shRNA injections in the current experiment were localized to this medial portion of the NAcSh. 5-HT1A knock down resulted in increased fear and anxiety-like behavior while not affecting feeding behavior, suggesting that loss of 5-HT1A in the medial NAcSh shifts affective valence toward fear/avoidance.

The increased anxiety-like behavior and reduced motivation to care for offspring in 5-HT1A KD dams suggest alterations in the pathways that regulate approach and avoidance. The now classic approach-avoidance model of caregiving proposes that the brain undergoes neurochemical changes during pregnancy and parturition that inhibit fear/anxiety/avoidance pathways and excite approach pathways to promote maternal caregiving of the possibly noxious pups (Numan, 2007). Serotonin receptors in the NAcSh are well situated to modulate the balance of these two opposing behavioral states, particularly within the context of maternal behavior. The NAcSh receives dense input from the dorsal raphe nucleus (Van Bockstaele et al., 1993) and expresses many different serotonin receptor subtypes (Di Matteo, 2008). Serotonin infused into the NAc increases DA release in the NAc to modulate the behavioral output. Thus, the balance of 5-HT receptors here may determine the neurotransmitter systems that lead to appropriate behavioral responses. 5-HT1A receptors, in particular, have been widely known to affect anxiety-like behavior and impulsivity. 5-HT1A receptor knockout mice exhibit more anxiety-related responses (Heisler et al., 1998; Ramboz et al., 1998; Zhuang et al., 1999), are more reactive in fear conditioning paradigms (Klemenhagen et al., 2006), and show increased freezing

after shock (Klemenhagen et al., 2006). In addition, genetic over-expression of central 5-HT1A receptors in developing mice reduces anxiety-like behavior and increases serotonin content in the NAc (Bert et al., 2005). Heterozygote 5-HT1A knockouts also have a high anxiety-like phenotype, suggesting that receptor downregulation may be a sufficient risk factor for the development of psychiatric disorders. 5-HT1A receptor downregulation may be particularly significant for the development of peripartum affective disorders such as postpartum anxiety, which may be typically avoided due to my finding that parturition is accompanied by a 250% increase in 5-HT1A receptor expression in the NAc.

In contrast to its effects on anxiety-like behavior, and the role of 5-HT1A receptors in depression-like behaviors in both humans and rodents (reviewed in Celada et al., 2004), my results indicate that 5-HT1A receptors specifically in the NAcSh do not influence depression-like behaviors in postpartum female rats. NAc 5-HT1 receptors mediate the serotonin-induced increases in DA in the NAc (Parsons & Justice, 1993), so it is surprising that 5-HT1A knockdown did not significantly alter depression-like symptoms in this experiment, particularly given that other DA-dependent behaviors were affected. Perhaps this might be explained by potential differences in the regulation of depression-like symptoms in non-parous rodents (which are most studies) compared to postpartum rodents. There is also a dearth of studies examining 5-HT1A expression in the NAc in non-parous males or females with depression-like symptoms; therefore the contribution of 5-HT1A specifically in the NAc to depression-like behaviors is still undetermined. A previous chapter in this dissertation did find that postpartum female rats exhibiting depression-like symptoms resulting from pregnancy stress only tended to show concurrent reductions in 5-HT1A receptor binding in the most rostral and caudal sections of the NAc shell. Thus, the appearance of postpartum depression-like symptoms in those animals may

not have resulted from significant loss of 5-HT1A binding sites in the NAc, although binding affinity has not been tested.

Consistent with the above findings from the forced swim test, 5-HT1A knockdown dams also did not differ in their preference for 0.1% saccharin solution compared to scrambled-injected dams either during the 24-acclimation period or the 1-hr 2-bottle choice test. However, knockdown dams tended to prefer saccharin less (below 50% preference) during the dark phase of the acclimation period, while scrambled-control dams preferred saccharin to a higher degree (above 50% preference). As mentioned in other chapters above, the NAc is well known for its role in reward processing and implicated in anhedonia (i.e., the lack of enjoyment in rewarding stimuli). Rats that have been bred for high depression-like behavior in the forced swim test show reduced concentration of dopamine and its metabolites in the NAc compared to control rats (Zangen & Nakash, 2001). Furthermore, treatment with either desipramine or nefazodone, both of which show affinity for the serotonin transporter and 5-HT1A receptors, reduces immobility in the forced swim test and restores extracellular levels of DA in the NAc (Dremencov et al., 2004).

Other studies in rodents have commonly used chronic stress paradigms to induce depression-like responses, and findings from these studies reveal altered morphology of medium spiny neurons (MSNs) in the NAc (Bessa et al., 2013), and altered expression of genes related to nervous system development and function in the NAc (Hodes et al., 2015). The postpartum period in females is characterized by changes in neuronal plasticity in order to allow for experience-dependent maintenance of maternal care (Fleming et al., 1999). Thus, stress or other environmental perturbations that might alter circuit plasticity increase the risk of undoing the behavioral adaptations that allow for optimal maternal care and low emotionality. A study in

postpartum females with pregnancy stress-induced anhedonia and concurrent reductions in maternal care found reductions in dendritic length and spine density of MSNs in the NAc shell, but not core (Haim et al., 2014). Neuronal morphology of NAc MSNs is positively regulated by dopamine (Meredith et al., 1995), and 5-HT1A knockdown altered maternal behaviors that are mediated by NAc DA. Thus, 5-HT1A knockdown in the NAc may have produced alterations in DA contributing to the behavioral deficits in KD dams. Alternatively, 5-HT1A receptors have also been shown to positively mediate the neurotrophic effects of serotonin in the hippocampus (Yan et al., 1997; Rojas et al., 2014). Therefore, loss of 5-HT1A receptors in the NAc may also lead to altered morphology of MSNs and a reduction in efficacy of the neurons in sending signals to other brain regions.

5-HT1A receptors are involved in the display of maternal aggression (De Almeida & Lucion, 1997; De Almeida & Lucion, 1994). In postpartum females, serotonin facilitates maternal aggression, since lesioning dorsal raphe serotonin neurons either during pregnancy or after parturition significantly reduces the duration of attacking (Holschbach et al., 2018) as does activating 5-HT1A receptors in the dorsal or median raphe, thereby reducing serotonin release (De Almeida & Lucion, 1997; da Veiga et al., 2010). Furthermore, 5-HT1A receptor agonism in the medial septum increased lateral threats at low doses. I found that 5-HT1A knockdown specifically in the NAcSh did not significantly alter the latency to begin attacking a male intruder, nor the total time that dams spent attacking, so the effects of 5HT1A receptor on attack must be site-specific. 1A-KD dams did, however, exhibit more lateral attacks (arched-back position laterally facing the male). Lateral attacks are considered a defensive behavior, while offensive behaviors exhibited by lactating females are jump/bite attacks and tumble/pin attacks. Exposure to a cat reduces the frequency of lateral attacks while offensive displays were not

altered in lactating dams that are later exposed to an unfamiliar male intruder (Lucion and de Almeida, 1996). This suggests that under conditions that increase stress (e.g., exposure to a cat), defensive behaviors decline. 5-HT1A knockdown dams had significantly higher anxiety-like behavior compared to scramble injected dams, and this may have contributed to the reduction in defensive behaviors when faced with an unfamiliar male intruder.

In sum, this experiment is the first to provide evidence for 5-HT1A receptors in the nucleus accumbens shell in regulating postpartum caregiving and affective behaviors. Specifically, reduced 5-HT1A expression in the NAcSh of mother rats significantly increases non pup-directed behaviors, delays retrieval of displaced pups back to the nest, and significantly increases postpartum anxiety-like behavior in some paradigms. These results provide new insights into how the serotonergic system contributes to postpartum social and affective behaviors, and describe a potential mechanism through which pharmacological treatments that affect the serotonin system (e.g., SSRIs) may work to alleviate postpartum affective disorders, particularly anxiety, and rescue maternal caregiving in highly anxious mothers.

CHAPTER 5: GENERAL DISCUSSION

Overall summary of findings

Chapter 1 of this dissertation discussed the critical role of serotonin and several of its receptor subtypes in a variety of social and affective behaviors, including postpartum behaviors. It also identified a lack of knowledge regarding normative 5-HT receptor expression across female reproductive states. Given that the scientific literature shows that function of the serotonergic DR is upregulated in lactating females, that serotonin receptor manipulation affects maternal caregiving, and that serotonin turnover is increased in some forebrain regions in the maternal behavior network (Lonstein, 2019; Pawluski et al., 2019), I hypothesized that forebrain 5-HT receptor expression also changes across female reproduction and may be necessary for expression of social and affective behaviors during the postpartum period.

Chapter 2 of this dissertation took an exploratory approach to determine whether 5-HT1A, 2A, and 2C mRNA expression in selected forebrain and midbrain sites was affected by female reproductive status, in order to gain insight into where changes in these receptors may be important for the onset and/or maintenance of maternal behaviors. I found higher 5-HT1A mRNA in the NAcSh of recently parturient females compared to diestrous virgins, higher 5-HT2A mRNA and receptor binding density in the mPOA in recently parturient dams compared to virgins, and less 5-HT2C mRNA in the DR of recently parturient and early lactating dams compared to virgins. These reproductive state-dependent changes in central serotonin receptor mRNA were not found in maternally sensitized virgin females compared to non-sensitized virgin females. These results suggest that pregnancy and/or parturition, rather than maternal experience alone, are required for the changes in 5-HT1A, 2A, and 2C mRNA expression found in mother rats.

Chapter 2 revealed several brain targets for examining the effects of stress during pregnancy on changes in serotonin receptor expression in Chapter 3, which used a novel repeated variable stress paradigm during pregnancy to examine its effects on postpartum caregiving, anxiety- and depression-like behaviors, as well as 5-HT1A autoradiographic binding in the NAc and 5-HT2A binding in the mPOA. Analysis of 5-HT2C binding in the DR was not possible due to an inability to successfully run autoradiography for this ligand (although I will continue to attempt this in the coming months). Repeated variable stress during pregnancy significantly reduced time that dams' spent in the nest with pups, increased non-maternal behaviors such as eating and drinking, and produced robust depression-like behavior. Experiments from that chapter also indicated that, although maternal behavior was impaired following pregnancy stress, 5-HT2A binding density in the mPOA was not altered. However, it is still unclear whether sensitivity or other aspects of receptor functioning not examined in this dissertation were affected by stress or contribute to stress-induced disruptions in caregiving behavior. I also found that stress during pregnancy tended to reduce 5-HT1A binding density in the most rostral and caudal NAc shell, which along with the over 2-fold increase in 5-HT1A mRNA in the NAcSh at parturition led me to choose this region as the target for viral vector manipulation in the final experiment of this dissertation.

In the final experiment of this dissertation (Chapter 4), I injected a viral construct expressing shRNA targeting the 5-HT1A mRNA (which knocks down 5-HT1A expression) bilaterally into the NAcSh during early pregnancy. After parturition, I observed a suite of maternal and affective behaviors across the early postpartum period. 5-HT1A knockdown in the NAcSh increased the time that dams' spent not interacting with pups, including sleeping outside the nest and self-grooming, significantly delayed retrieval of displaced pups back to the nest, and

significantly increased postpartum anxiety-like behavior. These changes in behavior implicate 5-HT1A receptors in the NAcSh as an important regulator of postpartum behavior and perhaps as a novel target for the treatment of postpartum disruptions in affective responses, particularly postpartum anxiety-like behavior.

Future directions and relevant considerations

Hormonal contributions to 5-HT receptor expression

As discussed at the end of Chapter 2, hormonal fluctuations during the peripartum period likely contribute to the reproductive state-dependent changes in 5-HT receptor expression in the NAc, mPOA, and DR. This is further evidenced by the lack of 5-HT receptor expression differences between ovariectomized virgin females with maternal experience with pups and virgins with no maternal experience. Estradiol is a likely candidate for altering 5-HT receptor expression during the peripartum period, as it is known to do so in non-parous females (Sumner & Fink, 1995; Sumner et al., 1999). However, previous research has found no effect of estradiol administration alone on expression of 5-HT2A mRNA in the mPOA of nulliparous female rats, although Experiment 2a of this dissertation is not the first to report increased 5-HT2A mRNA during lactation in rats (Akbari et al., 2013). 5-HT1A binding, on the other hand, is reduced in the amygdala, hippocampus, perirhinal cortex, and motor cortex of nulliparous females following estradiol administration (Le Saux & Di Paolo, 2005; Osterlund et al., 2000). While this contrasts with results from Experiment 2a showing an increase in NAc 5-HT1A receptors, no studies have examined the effects of estradiol on 5-HT1A expression in the NAc of nulliparous females. Additionally, 5-HT1A is expressed highly on pyramidal neurons in the cortex and hippocampus, while 5-HT1A receptors in the NAc are likely expressed on medium spiny neurons, which

account for 90-95% of all neurons in the NAc (Smith & Bolam, 1990). Thus, estradiol may have differential effects on 5-HT receptors depending on the cell types in which they are expressed. Alternatively, there are many hormones and peptides that are changing across pregnancy, and the net sum of these may alter 5-HT receptors differently than estradiol alone. To further elucidate the contribution of ovarian hormones in altering expression of 5-HT receptors, additional studies should be performed on pregnancy terminated (removal of uterus and fetuses) females. This paradigm results in rapid decline of progesterone and rise in estradiol secretion typical of parturition, and leads to immediate onset of maternal behavior when pups are presented 48 hr after surgery (Rosenblatt & Siegel, 1975). Results from this type of study could indicate that although estradiol alone may not be capable of altering receptor expression, the full hormonal profile associated with parturition or the act of giving birth itself is responsible for alterations in 5-HT receptor mRNA.

Of the other hormones and peptides fluctuating during the peripartum period that may contribute to 5-HT receptor expression and function during this time, glucocorticoids may be worth attention. Glucocorticoid release is high at the end of pregnancy and during parturition (Atkinson & Waddell, 1995), and corticosterone application to brain slices reduces electrophysiological activity of presynaptic 5-HT1A receptors in the DR (Laaris et al., 1995). Adrenalectomy, on the other hand, increases 5-HT1A binding in the CA2-CA4 and the dentate gyrus of the hippocampus in male rats, and binding is reduced following corticosterone supplementation (Kuroda et al., 1994). Taken together, these findings suggest that high levels of glucocorticoids may alter responsiveness, expression, or binding of 5-HT receptors in the brain. While 5-HT1A receptor expression is higher in the NAc at parturition, and glucocorticoids seem to downregulate expression and function of 5-HT1A receptors, at least in studies in the

hippocampus of male rats, this may reflect sex differences in the effects of glucocorticoids on 5-HT receptors, differences in pre- vs. postsynaptic receptors, or differences in the cell types that postsynaptic receptors are expressed on. Future research could examine the effects of adrenalectomy during gestation on 5-HT receptor mRNA and binding at parturition and during lactation to determine the influence of glucocorticoids on peripartum 5-HT receptor expression.

With regards to clinical implications of these findings, a further understanding of the hormonal contribution to peripartum 5-HT receptor expression may prove useful in treatment for postpartum depression, which has long been thought to result from withdrawal of ovarian hormones following parturition (although there is not yet strong evidence to support this) (Arpels, 1996; Hendrick et al., 1998; Sichel et al., 1995). Brain regions involved in maternal caregiving and emotional behavior, such as the mPOA, NAc, and DR, express receptors for estrogen (Liposits et al., 1990; Lu et al., 2001) and progesterone (Alvea et al., 1998; Numan et al., 1999). Thus, estrogens (E) and progesterone (P) released during the peripartum period can act directly on these brain sites to modulate postpartum social and affective behavior. Laboratory rodent models have shown that hormone withdrawal, either by discontinuing chronic E injections, or via ovariectomy, produces depression-like responses (Galea et al., 2001; Stoffel & Craft, 2004). Another hormone withdrawal paradigm used a chronic hormone regimen of both E and P to better model the human hormonal profile. This study found that withdrawal of both hormones resulted in increased learned helplessness and increased anxiety-like behavior, while reducing behavioral despair in the forced-swim test (Suda et al., 2008). While these laboratory rodent models provide some evidence that ovarian hormone withdrawal contributes to anxiety- and depression-like responses, they do not explain why only some women develop postpartum anxiety or depression, even though all women experience the drop in estrogen and progesterone

following delivery. Additionally, there is no consistent evidence to support that women with postpartum depression experience a more rapid or drastic reduction in ovarian hormones (Chatzicharalampous et al., 2011; Heidrich et al., 1994; O'Hara et al., 1991). In fact, the strongest predictors of postpartum depression are psychosocial, and include past history of psychopathology, psychological disturbance during pregnancy, or low social support (Beck, 2001; Lancaster et al., 2010; O'Hara & Swain, 1996). Thus, ovarian hormones interacting with other neural systems might underlie the effects of estrogen and progesterone withdrawal on depression-like symptoms in rodent models and some women with PPD but are probably not extremely strong determinants on their own.

As described previously, function and expression of a variety of 5-HT receptors is sensitive to ovarian hormones, and altered receptor signaling has been implicated in the development of depression in humans and laboratory rodent models (Brown & Linnoila, 1990; Grippo et al., 2005; Lopez-Figueroa et al., 2004; Stockmeier et al., 1998). Interestingly, 5-HT1A receptor activation may contribute to estradiol-induced reduction in depression-like symptoms in the forced swim test. Concurrent administration of the 5-HT1A antagonist, WAY 100635, with estradiol blocks the antidepressant-like action of estradiol (Estrada-Camarena et al., 2005). Therefore, alterations in serotonin receptor expression may instead contribute to the anti-depressant effects of estradiol administration during the postpartum period.

Characterization of 5-HT1A receptor-expressing neurons in the NAcSh

It would be informative if future studies characterize the cell types that 5-HT1A receptors are expressed in the nucleus accumbens. Receptor binding and *in situ* hybridization studies indicate relatively low abundance of these receptors in the NAc, which has led to a dearth of

more descriptive information regarding the cell types these receptors are localized on and to where these neurons from the NAc project. The NAc is comprised of mainly GABAergic medium spiny neurons (MSNs) (Smith & Bolam, 1990), so 5-HT1A receptors are presumably located on GABA neurons there. As discussed previously there, are two pathways from the NAc that modulate reward processing. The direct pathway is comprised of D1-expressing MSNs that promote approach to rewarding stimuli, while the indirect pathway is comprised of D2-expressing MSNs that inhibit aversive responses (Hikida et al., 2010; Kravitz et al., 2012). Therefore, to better understand the role of 5-HT1A receptors in the NAc on postpartum and other behaviors, it would be useful to know whether either of these populations of neurons expresses 5-HT1A receptors. Alternatively, 5-HT1A receptors in the NAc may be expressed on GABAergic interneurons instead of projection neurons, therefore influencing both pathways simultaneously. A subpopulation of cholinergic interneurons accounts for approximately 1% of neurons in the NAc (Tepper & Bolam, 2004), and these neurons communicate with MSNs via muscarinic and nicotinic receptors (Rahman & McBride, 2002). These cholinergic neurons contain the serotonin receptor interacting protein p11, which regulates the localization of certain 5-HT receptors such as 5-HT1B, 5-HT4, and 5-HT1A (Hensler et al., 1996; Svenningsson et al., 2006; Warner-Schmidt et al., 2009). Therefore, 5-HT1A receptors may also be expressed in this population of NAc cholinergic neurons. As a complement to cell phenotyping studies, anterograde tracing could also be utilized to determine where 5-HT1A receptor-expressing neurons in the NAcSh are projecting to throughout the brain. Creating and injecting a viral construct that expresses a fluorescent protein only in neurons that contain the promoter region of the htr1a gene may work to visualize projection sites of 5-HT1A-containing NAc neurons. Similarly, a 5-HT1AiCre transgenic mouse line (Sahly et al., 2007) could be used to express a

fluorescent tracer specifically in NAc neurons that express 5-HT1A receptors. These approaches would be particularly useful in combination with D1/D2 co-localization, given that these two neural circuits do not appear to be as divergent as was once hypothesized (reviewed in Kupchik & Kalivas, 2017). These analyses would provide insight into a novel neural mechanism through which serotonin interacts with the mesocorticolimbic system to modulate motivated behaviors.

5-HT1A receptors in the approach/avoidance model of maternal behavior – implications for postpartum affective behaviors

Initiation of maternal caregiving requires a shift from avoidance of neonates to approach (Numan, 2005). The medial preoptic area is a key integrative site where hormones prime neurons to respond to a variety of pup stimuli and produce the appropriate behavioral output (i.e., maternal behavior). This model also proposes two pathways that work in opposition to one another – the avoidance pathway that is active in virgin females and drives aversive responses to pup stimuli, while the approach pathway is active in parturient or otherwise maternally-experienced females and drives caregiving responses to pup stimuli (Kinsley & Bridges, 1990). The NAc is a component of the approach pathway, and its activation by DA helps initiate and sustain maternal motivation to care for offspring (Numan and Stolzenberg, 2009). Interestingly, the drug addiction literature describes a similar role for the NAc in approach/avoidance with regard to motivation for a particular stimulus. DA acting on both pathways results in approach toward and interaction with a rewarding stimulus, while also suppressing avoidance of the stimulus. While drugs of abuse, such as cocaine, "hijack" the reward system by increasing DA release in the NAc (Gratton & Wise, 1994), serotonin is also involved in some of the effects of cocaine. For example, cocaine increases locomotor activity, and administering a 5-HT1A

antagonist can prevent the locomotor stimulant effects of cocaine treatment (Muller et al., 2007). Although central pretreatment with a 5-HT1A agonist decreases self-administration of low doses of cocaine (Peltier & Schenk, 1992), this result may be explained by a reduction in overall serotonin release due to activation of 5-HT1A autoreceptors in the DR. Injecting 5-HT1A agonist directly into the NAc does facilitate some reward-related behaviors, such as male copulation (Fernandez-Guasti et al., 1992), though examination of the effects of NAc 5-HT1A activation on other motivated behaviors is lacking.

Results from 5-HT1A knockdown in the NAc of postpartum females suggest alterations in the ability of the NAc to fully suppress avoidance responses, as exhibited by an increase in pup retrieval latencies, an increase in non pup-directed behaviors, and an increase in anxiety-like behavior. Thus, the NAc is also an important "switch box" for mediating the competing drives for approach/avoidance, and serotonin in the NAc may be important for balancing the two pathways and contributing to optimal behavioral output. This model may have clinical relevance for the display of postpartum anxiety- or depression-like behavior in humans. Postpartum depression is typically characterized by feelings of despair, sadness, anxiety, fears, and compulsive thoughts (Sichel, 2002) and also disrupts the ability of affected mothers to care for and bond with their infants (Behrendt et al., 2016; Dubber et al., 2014; Licata et al., 2016). These symptoms suggest alterations not only in affective responding but also in motivation to provide maternal care. Major depressive disorder is also characterized by loss of motivation, and individuals experiencing PPD often describe a loss of enjoyment in activities that they once found rewarding, and these symptoms are contributed to DA dysregulation (Szczypinski & Gola, 2018). Individuals with untreated MDD show reduced NAc response to rewards (Pizzagalli et al., 2009), and deep brain stimulation of the NAc reduces ratings of depression and anxiety

symptoms (Bewernick et al., 2010). Because altered serotonergic signaling has been shown to contribute to MDD (reviewed in Naughton et al., 2000), serotonin may act in the NAc to affect motivation-related depression symptoms. Serotonin infusion into the NAc increases DA release in the NAc, and infusing a non-specific 5-HT1 antagonist blocks the serotonin-induced increase in NAc DA (Parsons & Justice, 1993). Therefore, disruptions in serotonin-dopamine interactions, possibly in the NAc, may contribute to the display of this particular symptom pattern. Thus, the novel finding that 5-HT1A receptor knockdown in the NAc disrupts caregiving and increases emotionality in postpartum rats may provide a target for the development of more specific treatment options for women experiencing postpartum affective disorders.

In sum, in this dissertation I found that female reproduction is associated with changes in serotonin receptor expression in numerous brain sites involved in postpartum behavior. Of particular interest, the normative increase in 5-HT1A expression in the nucleus accumbens shell may be necessary for the low anxiety-like behavior and high maternal care characterized by the postpartum period. Together, the results from this dissertation provide new insights into how the serotonergic system contributes to postpartum social and affective behaviors and offer a potential mechanism via the brain's reward system through which pharmacological treatments that affect the serotonin system (e.g., SSRIs) may work to alleviate postpartum affective disorders in women.

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