PERIPARTUM PLASTICITY IN THE SEROTONERGIC DORSAL RAPHE: IMPLICATIONS FOR POSTPARTUM SOCIOEMOTIONAL BEHAVIOR AND PHYSIOLOGY

By

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ABSTRACT

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Postpartum rats are highly maternal and show high aggression and low anxiety compared to nulliparous rats. To promote these dramatic changes in behavior, new mothers experience equally dramatic endocrine changes that elicit widespread neural plasticity. This neural plasticity includes cell birth and death in several regions of the peripartum forebrain, but such plasticity has never been reported in the dorsal raphe (DR), a midbrain site that provides most of the forebrain's serotonin. Because 1) postpartum lesions of the dorsal raphe reduce offspring development and survival, 2) serotonin affects postpartum social behaviors including caregiving and aggression, and 3) serotonin modulates anxiety in nulliparous males and females, I hypothesized that motherhood alters DR plasticity and serotonin synthesis/metabolism to support postpartum changes in socioemotional behaviors. To test this hypothesis, I examined effects of reproductive state and maternal experience on DR cell proliferation, newborn cell survival, cell death, and many aspects of the serotonin synthesis/metabolism pathway, then tested postpartum social and emotional behavior after lesioning the serotonergic DR. I discovered that although an equal number of cells are born in the DR of virgin, pregnant, and postpartum rats, fewer cells survived into the late postpartum period compared to cells surviving into the early postpartum period. These late postpartum females also had the highest levels of cell death within the DR. Next, I determined that interacting with the litter reduced cell survival and increased cell death in the DR of late postpartum rats. These effects were not due to high maternal corticosterone

because adrenalectomized and sham-operated postpartum rats had equivalent DR cell survival. DR newborn cell survival and cell death were related to changes in serotonin synthesis and metabolism because late postpartum rats also had lower levels of serotonin's precursor (5-HTP) and metabolite (5-HIAA) than early postpartum rats. To begin to test the functional significance of these changes in neuroplasticity and neurochemical function, I performed serotonin-specific DR lesions using a saporin-conjugated toxin targeting the serotonin transporter. Lesioning the DR altered numerous postpartum behaviors. During undisturbed observations, lesioned animals actively nursed pups (in kyphosis) more and licked pups less. Lesioning the DR did not greatly affect anxiety-like behavior, but did reduce maternal aggression. These data demonstrate that the DR is a site of significant peripartum plasticity, and that, along with this plasticity, there are concurrent changes in local serotonin synthesis and metabolism. These neurochemical changes may guide postpartum behavioral adaptations because lesioning the DR of new mothers had numerous effects on postpartum social behaviors. Taken together, these data suggest that the DR is an integral part of the maternal neural network that guides the initiation, modulation, and regression of postpartum behaviors.

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KEY TO ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HTP	5-hydroxytrytophan
ADX	adrenalectomy
ADX+Cort	adrenalectomy with corticosterone replacement
aq	cerebral aqueduct
BDNF	brain derived neurotrophic factor
BrdU	5-bromo 2-deoxy uridine
BSTd	dorsal bed nucleus of the stria terminalis
BSTv	ventral bed nucleus of the stria terminalis
DG	dentate gyrus
DR	dorsal raphe nucleus
DRd	dorsal subregion of the dorsal raphe nucleus
DRi	interfascicular subregion of the dorsal raphe nucleus
DRlw	lateral wings of the dorsal raphe nucleus
DRv	ventral subregion of the dorsal raphe nucleus
GABA	λ–amino butyric acid
HPLC	high performance liquid chromatography
hr	hours
IP	inraperitoneal
ir	immunoreactive
kg	kilogram

M	mean
µg	micrograms
mg	milligrams
ml	milliliter
mPOA	medial preoptic area
MS/DBv	medial septum-vertical limb of the diagonal band nucleus
MR	median raphe nucleus
NAc	nucleus accumbens core
NAsh	nucleus accumbens shell
NeuN	neuronal nuclei antigen
nm	nanometers
PD	pregnancy day
PFA	paraformaldehyde
PMT	photomultiplier
PPD	postpartum day
ROI	region of interest
s	seconds
SEM	standard error of the mean
SERT	serotonin transporter
SI	primary somatosensory cortex
SVZ	subventricular zone
TPH2	tryptophan hydroxylase 2
TUNEL	Terminal dUTP-Nick End Labeling

CHAPTER 1: INTRODUCTION

New mothers undergo dramatic changes in behavior and physiology to support the survival of their offspring. Some of the most dramatic behavioral changes include tremendously motivated caregiving, maternal aggression toward intruders that could threaten the nest site or young, and low anxiety to help the mothers focus on the needs of the offspring. These maternal behaviors have been best characterized in postpartum Norway rats (*Rattus norvegicus*) and studied for over 80 years (Wiesner & Sheard, 1933). This rich history of scientific exploration of parental behaviors has focused on females because, unlike male and nulliparous female rats that actively avoid or attack pups (Fleming & Rosenblatt, 1974; Rosenblatt, 1967; Weisner & Sheard, 1933), postpartum females are highly motivated to care for young (Lonstein et al., 2015; Hansen, 1994; Pereira et al., 2005; Pereira & Ferreira, 2015). These highly motivated postpartum rats build nests and retrieve displaced pups to this shelter by orally grasping the nape of their neck. After gathering pups to the nest, dams lick, groom, and nurse their pups. Dams nurse their pups in a variety of postures, including an arched back posture termed kyphosis or crouching (Stern, 1996), by lying prone on top of the pups, and by lying supine alongside the pups in a cat-like position with her ventrum exposed (Figure 1).

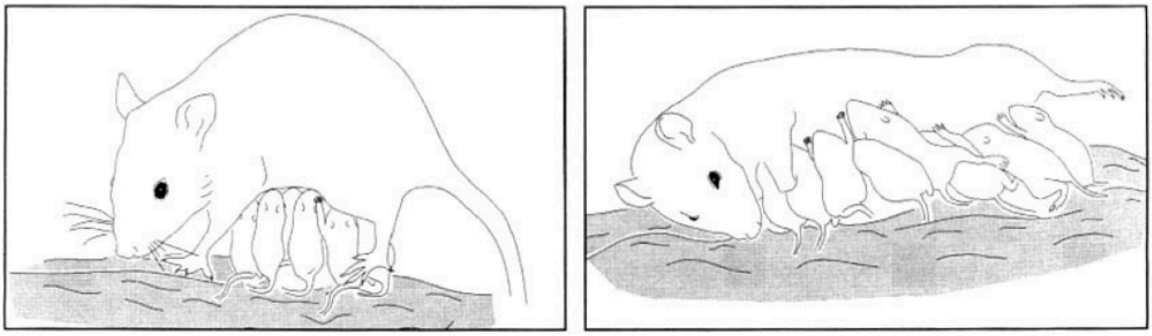

Figure 1: **Depictions of two distinct nursing positions: crouching (kyphosis; left panel) and supine nursing (right panel).** Adapted from Behavior of the Laboratory Rat: A Handbook with Tests.

Unlike nonpostpartum females, postpartum rats also attack intruders (Erskine et al., 1980; Ferreira & Hansen, 1986; Mayer et al., 1987). Maternal aggression is highest during the first week postpartum and very low or completely absent by the end of lactation (Flannelly & Flannelly, 1987; Caughey et al., 2011). Maternal aggression is similar to territorial aggression shown by males during resident-intruder tests; maternal aggression is often characterized by a quick, intense attack toward the head and neck of the intruder, but behaviors also include lateral threat postures, kicking, boxing, lunging, pinning, and biting (Lonstein & Gammie, 2002; Olivier & Mos 1992). In addition to high caregiving and aggression, early postpartum rats also show very low anxiety-related behaviors compared to diestrus virgins (Bitran et al., 1991; Ferreira et al., 1989; Fleming & Luebke, 1981; Hard & Hansen, 1985) whether anxiety is measured in an open field (Fleming & Luebke, 1981; Toufexis et al., 1999), a T-maze (Bridges et al., 1972), or an elevated plus maze (Bitran et al., 1991; Kellogg & Barrett, 1999; Lonstein, 2005). Dams also show decreased burying of an electrified probe (Picazo & Fernandez-Guasti, 1993) and less noise-induced startle (Hard & Hansen, 1985; Toufexis et al., 1999). All of these postpartum behaviors are initiated during pregnancy by circulating hormones that allow for successful gestation and delivery and also establish and fine-tune maternal neural networks.

The endocrine events of pregnancy that facilitate the onset of postpartum caregiving (Numan & Insel, 2003) include the rapid rise of estrogen on pregnancy day (PD) 18, the decline in high gestational progesterone beginning on PD 20, and the daily prolactin surges from PD 5 to PD 20 followed by its rapid rise on the day of parturition (Morishige et al., 1973). Recreating these hormonal events earlier during pregnancy (PD 16-19) by removing the uterus (Bridges et al., 1978) makes previously pregnant females quickly maternal toward foster pups (Rosenblatt & Siegel, 1975). In contrast, hysterectomy does not facilitate maternal behavior in the absence of

endogenous or exogenous estrogen (Rosenblatt & Siegel, 1975; Siegel & Rosenblatt, 1975). Moreover, recreating these hormonal events by providing exogenous estrogen, progesterone, and prolactin reduces the latency for nulliparous rats to care for pups (Moltz et al., 1970; Zarrow et al., 1971; Bridges, 1984), although nulliparous rats will eventually care for pups even without hormonal intervention after prolonged cohabitation (which is termed *maternal sensitization*) (Fleming & Rosenblatt, 1974; Leblond, 1938; Rosenblatt, 1967; Stern, 1983; Wiesner & Sheard, 1933).

After maternal behaviors are established - whether through pregnancy, exogenous hormone exposure, or prolonged exposure to young - hormones are no longer necessary (Rosenblatt et al., 1979; Obias, 1957; Rees et al., 2004; Thoman & Levin, 1970; Lonstein, 2005). Instead, the most important determinant of postpartum behavior after its initiation is sensory input provided by the young. Contact with young is essential for nearly every postpartum behavioral or physiological response. Whereas blinding, deafening, or preventing olfaction has very minor effects on caregiving or aggression (Stern, 1996; Lonstein et al., 2015), preventing tactile contact with pups dramatically impairs most aspects of postpartum behavior (Stern & Johnson, 1989; Stern & Johnson, 1990; Lonstein, 2005; Smith & Lonstein, 2008). Preventing perioral sensation interferes with pup retrieval and reduces pup licking, crouching, and pups' weight gain (Kenyon et al., 1981; Kenyon et al., 1983; Stern & Johnson, 1989; Stern & Kolunie, 1991). Tactile contact with the mother's ventrum is also critical for postpartum behavior; when pups are prevented from providing this input dams fail to crouch over them and instead continue licking the pups for extended periods of time (Stern & Johnson, 1990; Stern et al., 1992) and fail to show the normal decrease in postpartum anxiety (Lonstein, 2005). In fact, both low postpartum anxiety and high postpartum aggression require recent contact with young because

both wane within 4-8 hours after separation from pups (Erskine et al. 1978a; Ferreira and Hansen 1986; Mayer et al. 1987b; Stern and Koulnie 1993 Gandelman and Simon 1980; Lonstein & Gammie, 2002; Lonstein, 2005; Lonstein, 2007).

Once postpartum behaviors are initiated, they are not static, but rather change synchronously with the development of the young. That is, behavior of both mother and young change throughout lactation as the young develop and become more independent. When the mothers' postpartum stage is matched with litter age, the postpartum decline in maternal behavior begins during the second week after parturition (Grota & Ader, 1969; Moltz & Robbins, 1965). However, giving early postpartum dams foster pups that are older than her own hastens the decline in many but not all maternal behaviors. This result suggests that both elapsed postpartum time and pup cues contribute to the decline in mothering (Reisbick et al., 1975; Rosenblatt, 1969; Wiesner & Sheard, 1933). Maternal aggression is also affected by both postpartum stage of the mother and the developmental stage of the pups (Giovenardi et al., 2000), and I recently showed that the two factors also interact to control postpartum anxiety (Holschbach, Grieb, & Lonstein, *in prep*). Although it is often suggested that the postpartum reduction in anxiety permits maternal aggression (Lonstein, 2001), highly anxious postpartum rats are more aggressive than postpartum rats bred for low anxiety (Bosch et al., 2005), showing that anxiety and aggression can be controlled separately. It is well known that the socioemotional behaviors required for successful mothering change across the postpartum period to suit the developmental needs of the offspring, but the neurobiology underlying the decline in postpartum caregiving is very poorly understood. It is clear, though, that the medial preoptic area (mPOA) plays a role in the decline, as well as the initiation, of postpartum caregiving (Pereira & Morrell, 2009). The work in this dissertation suggests that the serotonergic midbrain

dorsal raphe (DR) also participates in the initiation, modulation, and termination of postpartum behaviors.

Motherhood and the dorsal raphe (DR)

The neurobiology underlying postpartum social and emotional behaviors includes a diverse network of brain regions and neurochemical systems (Neumann, 2003; Lonstein & Miller, 2008; Sisk et al., 2013; Lonstein et al., 2014; Pereira et al., 2015), and this likely includes the DR and its production of serotonin. The DR contains approximately half of the serotonergic neurons in the brain and while several raphe nuclei send descending projections to the hindbrain and spinal cord, the DR and median raphe (MR) mostly innervate the midbrain and forebrain, with eighty percent of forebrain serotonin being produced by the DR (Steinbusch, 1981; Hensler et al., 1994; Lowry et al., 2008). Evidence for a role of serotonergic DR neurons in maternal behaviors includes their higher basal firing rate in pregnant and postpartum rats compared to virgin females (Klink et al., 2002). Moreover, DR activity, as indicated by Fos expression, is higher in recently parturient and lactating rats than in virgin, pregnant, or non-lactating postpartum females without pups (Lin et al., 1998). Similarly, DR Fos expression increases in the paternal mouse DR after interaction with pups (de Jong et al., 2009), signifying DR involvement in parental behaviors beyond the physiological changes associated with pregnancy or lactation. Beyond these natural changes associated with reproduction and parental experience, acute postpartum treatment with the serotonin-2A/2C receptor antagonist, clozapine, disrupts retrieval of pups and reduces licking and nursing of pups and these effects on maternal behaviors are prevented by pretreatment with the selective serotonin-2A/2C receptor agonist, dimethoxy-4-iodoamphetamine (Zhao et al., 2009; Zhao et al., 2010).

In addition to caregiving, serotonin and the DR have been strongly linked to maternal aggression. Infusing serotonin receptor agonists into the lateral ventricles or various brain regions, including the periaqueductal grey and the ventral prefrontal cortex, reduces maternal aggression, whereas infusing autoreceptor agonists into the DR reduces serotonin output and increases maternal aggression (DeAlmeida & Lucion, 1994; Almeida et al., 2005; Almeida et al., 2006; Lonstein & Gammie, 2002; Veiga et al., 2010). In contrast, infusing serotonin receptor agonists into the central amygdala or the medial septum increases maternal aggression (Almeida & Lucion, 1994; Almeida et al., 2005). Although generally reducing neural serotonin increases aggression in male and postpartum rats (Olivier & Mos, 1992; Veiga et al., 2010), some results suggest that serotonin increases maternal aggression (Johns et al., 2005; Almeida et al., 2005). These discrepancies may lie in the brain region and receptor that is targeted or differences between acute and chronic treatments. In addition to brain region and receptor targeted, serotonin's role in aggression also apparently depends on context. For example, although serotonin is positively related to normal levels of aggression in rats, serotonin is negatively associated with pathological aggression / violence in rats and mice (de Boer et al., 2009; van der Vegt et al., 2003). In light of these and other data, Bower & colleagues proposed a comprehensive hypothesis about the role of serotonin in aggression, suggesting that serotonin promotes appropriate, adaptive aggression but maladaptive, trait-like aggression (e.g. violence) is inversely associated with serotonin (de Boer et al., 2015). Although acutely reducing DR serotonergic output by applying serotonin-1A receptor agonists increases maternal aggression (Veiga et al., 2010), permanent reductions of DR serotonin have yet to be explored in this model and may have distinct effects.

Despite a somewhat alarming lack of data concerning the role of serotonin in postpartum anxiety, there are myriad links between serotonin and anxiety in other models. For example, increasing serotonin synthesis by overexpressing its synthesizing enzyme, tryptophan hydroxylase 2 (TPH2) in ovariectomized female rats reduces anxiety, but only if estrogen had been chronically replaced (Hiroi et al., 2011). Similarly, the anxiolytic effects of SSRI treatment are prevented by ovariectomy and restored by estrogen replacement (Charoenphandhu et al., 2011). This may be because serotonin coordinates the anxiolytic effects of estrogen: estrogen increases the expression of the serotonin transporter (SERT) and serotonin-1B autoreceptor and blocking the serotonin-1B autoreceptor prevents the anxiolytic effects of estrogen (Charoenphandu et al., 2011; Hiroi et al., 2011; Donner & Handa, 2009). Serotonin autoreceptors in the DR modulate anxiety with agonists being anxiolytic and antagonists being anxiogenic in nulliparous male and female rats (for review, see McDevitt et al., 2011). In male rats, DR expression of the serotonin-1B autoreceptor is negatively correlated with anxiety (Hiroi & Neumaier, 2009; Kaiyala et al., 2003) further suggesting that activating DR serotonin autoreceptors (thus, reducing DR serotonin output) reduces anxiety. By contrast serotonin-1A receptor knockout mice show higher anxiety in a variety of behavioral tests because of reduced serotonin-1A heteroreceptors in the forebrain (Gross et al., 2002; Gross et al., 2000; Heisler et al., 1998; Parks et al., 1998). Moreover, we have shown that handling and/or exposure to an elevated plus maze increases Fos-immunoreactivity in the DR of postpartum rats and the number of Fos-immunoreactive serotonin-immunoreactive neurons in the lateral wings of the DR (DRlw) tends to predict the percent entries and percent time in the open arms of the elevated plus maze (Holschbach, Smith, & Lonstein, *unpublished data*). Thus, DR serotonin is a very strong candidate to regulate peripartum anxiety.

Lastly, DR serotonin is critical for postpartum physiology. Endocrine responses to suckling (which include surges in prolactin and oxytocin) are prevented by lesioning serotonergic innervation of the hypothalamus, lesioning the DR, reducing DR activity by infusing a glutamate receptor antagonist, or by applying serotonin antagonists either peripherally or within the hypothalamus (Crosignani et al., 1979; Moos and Richard, 1983; Barofsky, 1983; Bodnar et al., 2009). The increased energy burdens of pregnancy require increased food intake; increased serotonin could be problematic in this regard because serotonin reduces food intake in nonpregnant females, but, interestingly, this effect is prevented during pregnancy (Lingis et al., 2012). Serotonin also reduces HPA overactivity in obese male mice (Kurhe et al., 2015), although acute SSRI treatment increases corticosterone in male rats (Hestermann et al., 2014). Importantly, serotonin-1A receptor antagonists reduce HPA response to acute stress in male but not female rats (Goel et al., 2014), so extrapolation of data concerning the role of serotonin in the HPA response from male rodents is not always valid.

The DR is exquisitely sensitive to the hormones that initiate maternal behavior. The DR expresses receptors for glucocorticoids, estrogen, progesterone, and oxytocin (Alves et al., 1998; Spaethling et al., 2014; Heydendael & Jacobson, 2009; Vincent & Jacobson, 2014). The DR also expresses prolactin receptors during lactation, though prolactin receptor expression is very low or undetectable in nulliparae (Brown et al., 2011). Glucocorticoid receptor expression is especially high in the DR and corticosterone increases DR SERT expression (Zhang et al., 2002) and reduces autoinhibition of serotonergic DR neurons by serotonin in male rats (Laaris et al., 1995; Judge et al., 2004; Fairchild et al., 2003), so it may also facilitate the increased activity of the maternal DR. Although estrogen does not affect the number of serotonergic neurons in the DR of ovariectomized females (Kunimura et al., 2015), estradiol and estrogen receptor agonists

increase DR TPH2 expression in ovariectomized female rats (Charoenphandhu et al., 2011; Donner et al., 2009). Gonadal hormones, including estrogen, progesterone, and testosterone, also reduce serotonin-1A autoreceptor mRNA and serotonin-1A receptor binding in the DR (Pecins-Thompson & Bethea, 1999; Zhang et al., 1999; Lu & Bethea, 2002; Hiroi & Neumaier, 2009) and inhibit metabolism of serotonin by monoamine oxidase A (Bethea et al., 2002; Smith et al., 2004). Similarly, progesterone's metabolite, allopregnanalone, reduces the efficacy of inhibitory λ–amino butyric acid (GABA) transmission and thereby increases the firing rate of serotonergic DR neurons (Robichaud et al., 2005; Friedman et al., 1993). On the other hand, oxytocin has a neuromodulatory role in the DR, where it increases the firing rate of serotonergic DR neurons in response to histamines in mice (Spaethiling et al., 2014). Given the DR's sensitivity to pregnancy-related hormones, motherhood likely resculpts the DR to promote neurochemical changes that support postpartum behavior and physiology.

In sum, serotonin's role in caregiving, aggression, anxiety, and endocrine system function suggest that the serotonergic DR plays a strong regulatory role for new mothers. Given that potential role in postpartum behavior and physiology, one could predict that reproduction and motherhood affect plasticity in the serotonergic DR, possibly through the endocrine changes that support pregnancy and lactation.

Peripartum plasticity

Given the dramatic endocrine and behavioral changes associated with the peripartum period, it may not be surprising that it is associated with remarkable nervous system plasticity (Galea et al., 2006; Pawluski & Galea, 2007; Pawluski et al., 2009; Levy et al., 2011; Larsen & Grattan, 2012). One such source of plasticity is neurogenesis - the proliferation, differentiation,

maturation, survival, and integration of neuronal precursors and neurons. Neurogenesis has often been observed in the adult mammalian subventricular zone (SVZ) and subgranular zone of the hippocampal dentate gyrus (DG) (Vellema et al., 2010; Lois & Alvarez-Buylla, 1994; Dayer et al., 2003; Knoth et al., 2010; Taupin 2007). Fascinatingly, neurogenesis changes during motherhood. In laboratory rodents, SVZ cell proliferation increases briefly during pregnancy (PD 7 and PD 19 in mice; PD 21 in rats), and again postpartum (PPD 7 in mice), while DG cell proliferation and short-term cell survival decrease during the early postpartum period (PPD 2 and PPD 8) in rats (Shingo et al., 2003; Larsen & Grattan, 2012; Larsen & Grattan, 2010; Furuta & Bridges, 2003; Pawluski & Galea, 2007; Leuner et al., 2007; Brummelte & Galea, 2010). It may be surprising that these changes in neurogenesis in the SVZ and DG are in opposite directions, but the two neurogenic regions are responding to different peripartum hormones. The increased SVZ proliferation is caused by high prolactin (Shingo et al., 2003; Larsen & Grattan, 2010), while the reduced DG proliferation and short-term survival is due to high corticosterone (Leuner et al., 2007). While the functional significance of adult neurogenesis is often obscure or unknown (discussed in Bonfanti & Perretto, 2011), these new cells in mothers are known to mediate some postpartum behaviors. For example, preventing the increase in SVZ proliferation in mice with an antimitotic agent during pregnancy increases postpartum anxiety and impairs maternal behavior when tested under stressful conditions (Larsen & Grattan, 2010), and reducing DG cell proliferation with exogenous corticosterone in rats increases mothers' depressive-like behaviors and impairs maternal behavior (Brummelte & Galea, 2010). Furthermore, exposing a father rat to his own adult offspring increases immediate early gene expression in SVZ and DG cells that were born during his litter's gestation, and reducing both SVZ and DG neurogenesis by preventing the increased paternal prolactin impairs offspring recognition (Mak & Weiss, 2010).

Thus, cells added to the SVZ and DG during pregnancy and lactation are already known to contribute to parental socioemotional behaviors, but changes outside these well-studied forebrain proliferative zones remain mostly unexplored.

Investigating peripartum cell addition outside the classic neurogenic zones (i.e., in the brain areas more closely associated with the behaviors of interest in postpartum females) would provide valuable insight into the role of this type of neuroplasticity in female reproduction and behavior. Although many laboratories have now demonstrated adult neurogenesis in the hypothalamus (Xu et al., 2005; Kokoeva et al., 2007; Ahmed et al., 2008; Fowler et al., 2008; Mohr & Sisk, 2013), cortex (Bernier et al., 2002; Dayer et al., 2005; Czéh et al., 2006), and elsewhere (Shapiro et al., 2009; Lieberwirth et al., 2012; Bauer et al., 2005; Zhao et al., 2003; Zhao et al., 2009), almost all research studying peripartum neurogenesis has been restricted to the two classic neurogenic zones discussed above. The only study to look outside the classic neurogenic zones in reproductive females reported that maternal experience led to a very small, but statistically significant, increase in postpartum cell survival in the nucleus accumbens and bed nucleus of the stria terminalis, two areas necessary for maternal behavior (Akbari et al., 2007). There have been no reports of peripartum addition of cells anywhere in the midbrain, including the DR, which is the main source of serotonergic innervation of the forebrain and elsewhere and is the focus of the studies included in this dissertation.

Overview of dissertation chapters

Research on new brain cells outside of the SVZ and DG in mothers is sparse. While effects of maternal experience on postpartum cytogenesis have been tested in some forebrain regions, as discussed above, effects of reproductive state on cell proliferation and survival

outside of the SVZ and DG have never been reported. To my knowledge, cytogenesis within the DR of an adult animal has never been studied in any animal model, although previous reports do indicate dense labeling of a pro-differentiation marker in the DR of adult male monkeys (Vinet et al., 2002) and adult neurogenesis in other midbrain regions (e.g. the substantia nigra) of male mice (Zhao et al., 2003; Zhao et al., 2009). Previous reports also indicate that the adult brain can support newborn serotonergic neurons, as transplants of serotonergic fetal midbrain into adult brains survive and grow to innervate surrounding tissue (Zhou & Azmitia, 1990; Ueda et al., 1996; Daszuta et al., 1988). Moreover, electrophysiological measures and immunohistochemical detection of immediate early genes suggest that the DR is activated by maternal experience (Klink et al., 2002; Lin et al., 1998), but aside from one paper that found reduced serotonin-immunoreactivity in the lateral wings of the DR (DRlw) in postpartum mice compared to cycling nulliparae (Jury et al., 2015), neurochemical measures of serotonin synthesis, content, and metabolism within the DR have not been reported,.

The following experiments will examine how motherhood alters neuroplasticity and neurochemical processing in the DR and what role serotonergic DR neurons play in postpartum behavior. In Chapter 2, we determined how reproductive state, maternal experience, and adrenal hormones affected cell proliferation, newborn cell survival, and cell death in the DR and then determined how reproductive state affects serotonin content, TPH2 expression, and levels of serotonin's precursor, 5-HIAA, and metabolite, 5-HTP, in the DR. In Chapter 3, we began to determine how such structural and chemical plasticity might be involved in postpartum behavior by selectively lesioning serotonergic neurons in the DR and studying postpartum caregiving, maternal aggression, and postpartum anxiety. As discussed above, the DR produces most forebrain serotonin, a neurochemical that facilitates postpartum behavior and physiology, which

makes motherhood-associated neuroplasticity and neurochemical changes in the DR of great interest to the field of postpartum neurobiology and DR serotonin is also involved in a wide range of behaviors not studied in this proposal, making the implications of broad interest for future studies in many subfields of neuroscience (discussed in Chapter 4).

CHAPTER 2: Motherhood and infant contact regulate neuroplasticity in the serotonergic dorsal raphe of female laboratory rats

Abstract

The maternal brain undergoes widespread plasticity to establish and then maintain the remarkable postpartum changes in behavior and physiology required for successful rearing of young. This plasticity includes the birth and death of new cells in several forebrain regions, but midbrain sites that may show such plasticity have never been examined. Using bromodeoxyuridine (BrdU) to label mitotic cells and TUNEL to label dying cells, we found that the midbrain dorsal raphe nucleus (DR) exhibited significant neuroplasticity in response to motherhood. Most newborn cells were neurons (NeuN-ir) and newborn cell survival was significantly regulated by reproductive state - cells born during the first week postpartum were less likely to survive compared to cells born during late pregnancy. This reduction in newborn cell survival required maternal experience because removing the litter at parturition increased DR newborn cell survival and reduced cell death. Unlike cytogenesis in the hippocampus, DR newborn cell survival was unaffected by postpartum adrenalectomy. These effects of reproductive state and motherhood on DR plasticity were associated with changes in DR concentrations of serotonin's precursor, 5-HTP, and its metabolite, 5-HIAA. These results demonstrate for the first time that cytogenesis occurs in the DR of an adult mammal, that DR neuroplasticity is influenced by female reproductive state and motherhood, and that this neuroplasticity is associated with changes in the neurochemical pathway for serotonin synthesis and metabolism. Because serotonin is strongly linked with postpartum behavior and physiology, DR neuroplasticity may be vital for the ability of new mothers to care for their offspring.

Introduction

Maternal female mammals experience some of the most extraordinary behavioral and physiological modifications that can occur in adult organisms (Sisk et al., 2013; Lonstein et al., 2014). These modifications include sensitive caregiving of young, maternal aggression toward threats, reduced anxiety, lactation, and blunted endocrine responses to many stressors. To support such notable changes in behavior and physiology, mothers undergo equally remarkable neuroplasticity.

Of the many forms of neuroplasticity, cytogenesis and cell death are two primary mechanisms contributing to profound changes in adult brain structure, connectivity, and chemical functioning (Gould, 2007; Migaud et al., 2010; Bonfanti & Peretto, 2011). A handful of studies have shown that this is true for the maternal forebrain. For example, pregnant and postpartum rodents have a prolactin-mediated increase in cytogenesis in the subventricular zone (SVZ) compared to nulliparae (Shingo et al., 2003; Furuta & Bridges, 2005; Larsen & Grattan, 2010), and postpartum maternal experience increases newborn cell survival in the nucleus accumbens and bed nucleus of the stria terminalis (Akbari et al., 2007). On the other hand, postpartum rats also experience a corticosterone-mediated decrease in cytogenesis in the hippocampal dentate gyrus (DG) (Galea et al., 2014; Pawluski et al., 2009; Pawluski & Galea, 2007; Leuner et al., 2007) and pregnant mice have more cell death in the DG and prefrontal cortex compared to nulliparae (Erbil et al., 2014). These results indicate that neuroplasticity in the maternal forebrain is extensive and likely contributes to the many postpartum adaptations required to rear the young (Larsen & Grattan, 2010; Brummelete & Galea, 2010).

Peripartum neuroplasticity has never been reported in the midbrain, though, perhaps because studies of cytogenesis in the mammalian brain almost invariably focus on forebrain sites

apposing or close to the ventricular system where cytogenesis is traditionally thought to occur (Kokoeva et al., 2007). However, the lining of the midbrain cerebral aqueduct is a highly proliferative niche during early development, adulthood, and aging (Fischer et al., 2011; Zhao et al., 2003; Zhao et al., 2009). One midbrain site adjacent to the cerebral aqueduct that likely drives postpartum behavior and physiology is the dorsal raphe nucleus (DR). The DR contains 80% of forebrain-projecting serotonergic neurons (Steinbusch, 1981; Lowry et al., 2008). Lesioning the DR of postpartum rats increases pup mortality (Barofsky et al., 1983), and maternal behavior is impaired by knocking out *Pet-1* (transcription factor for serotonergic neuron differentiation) or *TPH2* (enzyme necessary for neuronal serotonin synthesis) (Lerch et al., 2008; Angoa et al., 2014). Moreover, maternal caregiving and aggression are decreased by pharmacologically manipulating serotonin signaling (Ferreira et al., 2000; Zhao & Li, 2009; Veiga et al., 2010; Chen et al., 2014). Postpartum physiological events, including the suckling-induced prolactin and oxytocin surges necessary for milk production and release, also rely on DR serotonergic efferents to the hypothalamus (Crosignani et al., 1979; Moos and Richard, 1983; Bodnar et al., 2009). Given serotonin's role in postpartum behavior and physiology, one might predict that reproduction and motherhood affect plasticity in the serotonergic DR.

We here tested the hypothesis that motherhood affects neuroplasticity in the DR of adult female rats. To determine this, mitotic cells were labeled with 5-bromo 2-deoxyuridine (BrdU) and dying cells were identified using Terminal dUTP-Nick End Labeling (TUNEL) across reproductive states, in postpartum rats with or without maternal experience, and in adrenalectomized mothers to examine if corticosterone affects postpartum DR cytogenesis as it does in the hippocampus. We also colocalized BrdU and Neuronal Nuclei antigen (NeuN), a common marker of neurons (Mullen et al., 1992) to phenotype 12-day old neurons. Lastly, to

evaluate whether changes in DR plasticity contemporaneously occur with changes in DR serotonin synthesis and metabolism, we measured numerous aspects of the serotonin neurochemical pathway across reproductive states. We found that reproduction and motherhood alter DR newborn cell survival, cell death, and serotonin synthesis and metabolism.

Materials & Methods

Subjects

Female Long-Evans rats, descended from rats purchased from Harlan Laboratories (Indianapolis, IN), were born and raised in our colony. Subjects were housed in clear polypropylene cages (48 cm x 28 cm x 16 cm) with one or two female littermates from weaning at 21 days of age until the experiment began. Animals were provided with food and water ad libitum and wood shavings for bedding and experienced a 12:12 light/dark cycle (0700 hr lights on). Pregnant females were singly housed approximately five days before the expected day of parturition, and litters were culled to contain four males and four females within 24 hours after birth or were permanently removed from the homecage within one hour after the completion of parturition. The day of parturition was considered PPD 0. All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee at Michigan State University.

BrdU injections

To measure cytogenesis in the DR and elsewhere in the brain, female rats were injected with BrdU, a thymidine analogue that is permanently incorporated into the DNA of dividing cells (Gratzner et al., 1976; Gratzner et al., 1978; Taupin, 2007; Cavanagh et al., 2011). Rats were injected once with BrdU dissolved in 0.9% sterile saline solution (IP 150 mg/ kg bodyweight (Taupin, 2007); 10 mg/mL; Sigma-Aldrich, St. Louis, MO) either on a day of diestrus, on pregnancy day (PD) 19, or on postpartum day (PPD) 7 or 8 (Figure 2). Subjects were sacrificed one day after BrdU injection to examine cell proliferation or 12 days later to

examine cell survival. This duration was chosen for several reasons. First, newborn cells migrate from the SVZ lining the cerebral aqueduct to the substantia nigra (SN) within 10 days (Zhao et al., 2003), and because the SN is much farther from the SVZ than the DR, newborn cells still residing within the DR 12 days after injection are unlikely to be simply migrating through the DR. Second, 12 days is sufficient for the emergence of NeuN expression (e.g. Kuhn et al., 1996), which we utilized to phenotype newborn neurons. Finally, 12-day survival was a good choice ecologically because it allowed multiple estrous cycles to occur, and in reproductive females allowed one measure of cell survival during the onset and maintenance of postpartum behaviors and another as these behaviors wane. Moreover, 12 days allows a measurement of postpartum cell survival without artifacts of parturition and before weaning. To assess the effects of maternal experience with the litter on DR cytogenesis, postpartum rats with or without pups since giving birth to them were injected twice with BrdU on PPD 7 and 8. Finally, to assess the effects of adrenal hormones on postpartum DR cytogenesis, intact and adrenalectomized female rats were injected with BrdU twice on PPD 7 and 8. All injections were given during the light photophase.

Adrenalectomy

Lactation is characterized by high circulating corticosterone and this hormone is known to reduce cytogenesis in the hippocampus of postpartum rats (Leuner et al., 2007). We examined effects of corticosterone and other adrenal hormones on cytogenesis in the maternal DR by comparing sham-operated animals (Sham) with two groups of dams that were adrenalectomized on PPD 1. Subjects were anesthetized with ketamine (90 mg/kg, ip) and xylazine (8 mg/kg, ip) and the adrenal glands were removed (or exposed but left intact for Sham dams) through bilateral

dorsal incisions made through the skin and muscle layers (Leuner et al., 2007; Rees et al., 2004). Litters were fostered to surrogate dams during surgery and given back to their mothers immediately following recovery from anesthesia. One group of adrenalectomized dams received a very low dose of corticosterone in their drinking water to maintain general neuronal health (ADX; 15 μg/mL in 0.5% saline, 0.4% ethanol; Leuner et al., 2007) and the other received full corticosterone replacement (ADX+Cort; 100 μg/mL; Rees et al., 2004). Because adrenalectomy can reduce milk production and output, and thereby litter weight gain and pup health (Leuner, 2007; Anderson et al., 2014), litters were weighed and cross-fostered daily among the three groups of dams involved in the experiment and an additional group of unmanipulated surrogate lactating dams from our colony.

BrdU Immunohistochemistry

Subjects were overdosed with sodium pentobarbital and perfused with 150 mL of 0.9% saline followed by 4% paraformaldehyde (PFA) either one day (to examine cell proliferation, n = 8 per group) or 12 days (to examine short-term cell survival, n = 10 per group) after BrdU injection at 1400 hr. Brains were postfixed overnight in 4% PFA, and stored in 20% sucrose until they were sectioned into 40-μm-thick sections on a freezing microtome, collected into four alternate series, and stored in a sucrose-based cryoprotectant at -20° C. BrdU IHC was performed on free-floating sections as described previously (Ahmed et al., 2008; Mohr & Sisk, 2014) using a monoclonal mouse anti-BrdU primary antiserum (0.10 μg/mL; Roche Diagnostics, Germany) and biotinylated horse-anti-mouse secondary antiserum (6 μg/mL; Vector Laboratories, Burlingame, CA).

BrdU-immunoreactive (BrdU-ir) cells in the DR were counted under 200 x magnification. The DR is a heterogeneous site with numerous subregions, so analyses were done within four distinct subregions of the DR (DRd, DRv, DRlw and DRi) as described previously (Kelly et al., 2011) by counting all BrdU-ir cells in five consecutive sections in a series through the DR for each animal using a Nikon E400 light microscope with assistance of an atlas of the laboratory rat brain (Swanson, 1998). We found that the effects of reproductive state and maternal experience were not subregion specific, so the data were then collapsed across subregions to reflect effects on the entire DR as reported below. We also quantified the number of BrdU-ir cells in the median raphe (MR), as well as in several forebrain sites previously examined in this literature. Results from the DR are presented and discussed in detail but BrdU-ir cells in the MR were in incredibly low abundance (every group's Abercrombie-corrected mean was <10 cells). Moreover, no significant differences among groups in cell proliferation or survival were found in any of the forebrain sites, and so these sites are not discussed but their data can be found in Tables 1 & 2.

Alternate sections from three of the females injected with BrdU on day 19 of pregnancy and three of the females injected with BrdU on PPD 7 to measure short-term cell survival were used to determine whether newborn cells in the DR had a neuronal phenotype. Dual-label immunofluorescence was performed on free-floating sections as described previously (Mohr & Sisk, 2014) using a cocktail of monoclonal rat anti-BrdU primary antiserum (1 μg/mL; Serotec) and monoclonal mouse anti-NeuN primary antiserum (clone A60, 1:1000), followed by a cocktail of biotin-SP-conjugated AffiniPure goat anti-rat secondary antiserum (1.3 μg/mL; Jackson ImmunoResearch, West Grove, PA) and Alexa Fluor 405 goat-anti-mouse secondary antiserum (4 μg/mL; Invitrogen, Grand Island, NY), and finally Cy-2 conjugated streptavidin

(1.8 μg/mL; Jackson ImmunoResearch, West Grove, PA). BrdU- and NeuN-ir nuclei were visualized using an Olympus FluoView 1000 Filter-based Laser Scanning Confocal Microscope configured on an Olympus IX81 automated inverted microscope platform using a blue diode laser (405 nm) and band pass emission filters for wavelength selection. High-resolution confocal fluorescence was collected through a single, variable pinhole aperture and recorded using three high-sensitivity photomultiplier (PMT) detectors. Z-stack images were collected at 2.65 μm intervals through five sections containing the DR per animal. Thirty BrdU-ir nuclei in the DR were randomly identified for each animal and colocalization with NeuN was determined via 3D reconstruction using a Z-stack orthogonal viewer.

TUNEL

Cell death was measured using an *In Situ* Cell Death Detection Kit (TMR Red; Roche Applied Sciences, Indianapolis, IN) to perform a TUNEL assay. TUNEL is a frequently used method that labels the fragmented DNA of dying cells (Duan et al., 2003). TUNEL+ nuclei were visualized on the same confocal microscope using a green Helium Neon gas laser (543 nm) to visualize TUNEL. Z-stack images were collected at 2.65 μm intervals through five sections containing the DR per animal and TUNEL+ nuclei were quantified using Image-J. To examine the phenotype of dying DR cells in virgin, PPD8 and PPD19 females, the tissue was double-labeled to identify dying neurons. Immunohistochemical labeling was processed using NeuN antiserum (monoclonal mouse anti-NeuN; clone A60, 1:1000), as described above. NeuN-immunoreactivity was detected using a blue diode laser (405 nm). Colocalization of TUNEL and NeuN was confirmed via 3D reconstruction using a Z-stack orthogonal viewer.

Serotonin IHC

Adult female rats were sacrificed as diestrus nulliparae, on PPD 8, or on PPD 19 (13-20 females per group). These groups were chosen because they corresponded to the days of sacrifice we had used to examine the effects of reproductive state on newborn cell survival and cell death in the DR. Females were overdosed with pentobarbital and perfused, and their brains were harvested and prepared for IHC as described above. IHC was performed on free-floating sections using a monoclonal rabbit anti-serotonin primary antiserum (1:10,000; Protos BiotechCorp, NY) and a biotinylated goat-anti-rabbit secondary antiserum (1:1000; Vector Laboratories, Burlingame, CA). Serotonin immunoreactivity in the DR was measured on five consecutive sections in a 1 in 4 series per animal using NIS-Elements to quantify the percent area of a standardized region of interest (ROI) covered by immunoreactivity similar to that described elsewhere (Smith et al., 2013).

TPH2 Western Blotting

Diestrus nulliparous, PPD 8, and PPD 19 rats (8 per group) were narcotized by a brief (< 90 sec) exposure to carbon dioxide at 1400 hr. Their brains were harvested, flash frozen in isopentane, and stored at -80 ^{o}C until being sectioned into 500-µm-thick sections on a cryostat. Sections were mounted onto microscope slides and stored at -80 ^{o}C until the DR was microdissected and homogenized in 50 µL RIPA buffer (Santa Cruz Biotechnology SC-24948, Santa Cruz, CA). Samples were denatured at 100 ^{o}C for five minutes, electrophoresed on 10 % Bis-Tris Plus precast gels (BG00100; Thermo Scientific; Rockford, IL) for 50 min at 165 V using a Powerpac Basic (Bio-Rad, Hecules, CA) and transferred to polyvinylidene difluoride membranes using an iBlot system (Invitrogen #iB4010, Grand Island, NY). Membranes were

stored in ultrapure water overnight at 4 °C, saturated in iBind Solution, then loaded into an iBind Western Blotting system that drew 2 mL each of the following solutions: 1) primary antiserum diluted 1:500 in iBind solution (rabbit anti-TPH2, Thermo Scientific PA1-778, Rockford, IL) 2) iBind solution, 3) secondary antiserum diluted 1:1000 in iBind Solution (goat anti-rabbit Cell Signaling #70745, St. Louis, MO). Finally, 6 mL of iBind solution was drawn through the membranes. Membranes were then temporarily placed in ultrapure water and ir bands were detected using a chemiluminescence kit (Bio-Rad #170-5060, Hercules, CA) and placed on films (Optimum blue Sensitive X-ray film, XR-0810-100; Life Scences Products; Frederick, CO) that were developed and fixed with a Kodak X-OMAT 100A Processor (Kodak Co., Rochester, NY). After developing, films were scanned (PSC 1410, Hewlett Packard) and images of TPH2-ir bands were analyzed using Image J (National Institutes of Health, Bethesda, MD, USA) to determine their integrated density. Membranes were rinsed 3 times in ultrapure water, then they were processed similarly for GAPDH using appropriate primary (1:500; mouse anti-GAPDH; Millipore #MAB374, Temecula, CA) and secondary (1:4000; rabbit anti-mouse, Sigma-Aldrich #A9044, St. Louis, MO) antisera. TPH2 integrated density was standardized to corresponding GAPDH integrated density for each subject, as described previously (Ragan & Lonstein, 2014).

High Performance Liquid Chromatography (HPLC)

Diestrus nulliparous, PPD 8, and PPD 19 rats (9 per group) were narcotized with carbon dioxide and brains were harvested, flash frozen in isopentane, and stored at -80 °C until they were sectioned into 500-µm-thick sections using a cryostat. Sections were mounted onto microscope slides that were stored at -80 °C until the DR was microdissected and homogenized in 200 nL cold 0.1 M perchloric acid. Samples were centrifuged at 10,000 rpm for 10 min, then

the supernatant was collected and stored at -80 ^{o}C until monoamine analysis was conducted using HPLC at a core facility at Michigan State University (Smith et al., 2013). The limits of detection for serotonin, its precursor (5-HTP), and its metabolite (5-HIAA) were all 0.5 ng / mL. Norepinephrine and dopamine in the DR were also measured but did not differ among the groups studied (data not shown).

Data analyses

To estimate the total number of BrdU-ir and TUNEL+ nuclei in the immunohistochemical analyses, an Abercrombie correction factor of 0.93 was applied based on our section thickness and the average measured DR nuclear diameter of 3.1 μm (Abercrombie, 1946). The number of BrdU-ir cells was not normally distributed among the groups of females compared across reproductive states or adrenal status, so these data were analyzed using Kruskall-Wallis tests followed by Mann Whitney U tests as the post-hoc analyses. TUNEL+ cells in virgin, PPD8, and PPD19 females were analyzed using one-way ANOVAs, followed by Tukey HSD tests for post-hoc analyses. Independent samples t-tests were used to analyze the number of BrdU-ir and TUNEL+ cells in mothers with or without their litters since parturition. Serotonin pathway measures from IHC, Western Blot, and HPLC were analyzed using one-way ANOVAs, followed by Tukey HSD tests for post-hoc analyses. $P < 0.05$ was considered statistically significant.

Results

Cytogenesis and cell death across reproductive state

As has been reported previously in adult male mice (Zhao et al., 2003; Zhao et al., 2009), the dorsal and lateral SVZ surrounding the cerebral aqueduct was densely populated by BrdU-ir cells in most of our female rats (Figure 3A, 3I). There were also BrdU-ir cells within the DR of all three groups of females (Figure 3B, 3E). While some newborn DR cells likely migrated from the nearby SVZ, pairs of BrdU-ir nuclei were frequently observed (Figure 3B), suggesting that these cells may have divided within the DR itself (Kokoeva et al., 2007).

Although reproductive state did not affect the number of proliferating (1-day old) BrdU-ir cells in the DR (χ^2 (2 N = 24) = 1.54, p = .46; Figure 2C), the number of BrdU-ir cells surviving until 12 days after injection was significantly affected by reproductive state (χ^2 (2 N = 32) = 8.66, p = .034; Figure 2D). PPD 19 females (i.e., those injected with BrdU early postpartum) had fewer BrdU-ir cells in the DR compared to PPD 8 females (i.e., those injected with BrdU during late pregnancy) (p = .005). Most of the 12-day old BrdU-ir cells were also NeuN-ir (Figure 3E-H). The percentage of BrdU-ir cells that were neurons (i.e., NeuN-ir) was similar between the late postpartum (range = 43-57%; mean = 51%) and early postpartum (range = 30-83%; mean = 62%) females. By contrast, none of the BrdU-ir nuclei lining the aqueduct were NeuN-ir (Figure 3I).

We used TUNEL to measure DR cell death occurring in the face of this decreased cell survival (Figure 4A-D). We found a non-significant effect of reproductive state on the number of dying cells ($F_{2,24}$ = 1.03, p = .37), despite 34% more TUNEL+ nuclei in the DR of the PPD 19 rats compared to PPD 8 rats (Figure 4E). The percentage of TUNEL+ cells that were neurons

(i.e., NeuN-ir) was stable at between 51 and 62 % across reproductive state ($F_{2,22} = 0.37$, $p = .70$, Figure 4D).

Cytogenesis and cell death in postpartum rats with or without pups

Removal of the litter at parturition significantly increased the number of 12-day old BrdU-ir cells in the DR of PPD 19 rats ($t_{(17)} = 2.44$, $p = .026$, Figure 5A). Furthermore, litter removal reduced the number of TUNEL+ cells in the postpartum DR ($t_{(17)} = 2.90$, $p = .01$, Figure 5B). Thus, the postpartum reduction in newborn cell survival in the DR requires experience with offspring, rather than simply being pregnant and giving birth.

Cytogenesis in postpartum rats with or without adrenal glands

We confirmed that adrenalectomy successfully reduced circulating corticosterone, with most ADX dams having non-detectible plasma levels of the hormone while all Sham and ADX+Cort dams had measureable corticosterone (χ^2 (2 N = 32) = 9.50, $p = .009$). Expectedly, ADX dams had significantly lower plasma corticosterone levels compared to the combined groups of control dams (0.56 ± 3.82 vs. 37.96 ± 14.13 ng/ml; $t_{(30)} = 2.56$, $p = .017$). Consistent with the literature (Tucker, 1994; Leuner et al., 2007), daily relative weight gains were lower in litters fed by ADX females than by control dams, and corticosterone replacement partially alleviated that deficit ($F_{(2,28)} = 10.49$, $p = .0005$, Figure 5C). However, adrenalectomy did not affect newborn cell survival ($F_{(2,25)} = 1.63$, $p = .22$, Figure 5D), suggesting that the effects of maternal experience on newborn cell survival in the DR were not mediated by the dams' circulating adrenal hormones.

Neurochemical measures across reproductive state

Neither the immunohistochemical ($F_{(2,38)} = 0.31$, $p = .74$, Figure 6A) nor the HPLC ($F_{(2,26)} = 1.74$, $p = .20$, Figure 6D) analyses of DR serotonin revealed significant differences across reproductive states. Western blotting for TPH2 also revealed no differences ($F_{(2,20)} = 1.10$, $p = .36$, Figure 6B). In contrast, the HPLC analysis showed that early postpartum rats had higher levels of the serotonin precursor, 5-HTP ($F_{(2,26)} = 6.79$, $p = .005$, Figure 6C), and serotonin metabolite, 5-HIAA ($F_{(2,26)} = 8.53$, $p = .002$, Figure 6D), in the DR compared to the diestrus nulliparae or late postpartum rats. Serotonin turnover (5-HIAA/serotonin) followed a similar pattern and tended to differ among the groups ($F_{(2,26)} = 3.27$, $p = .055$).

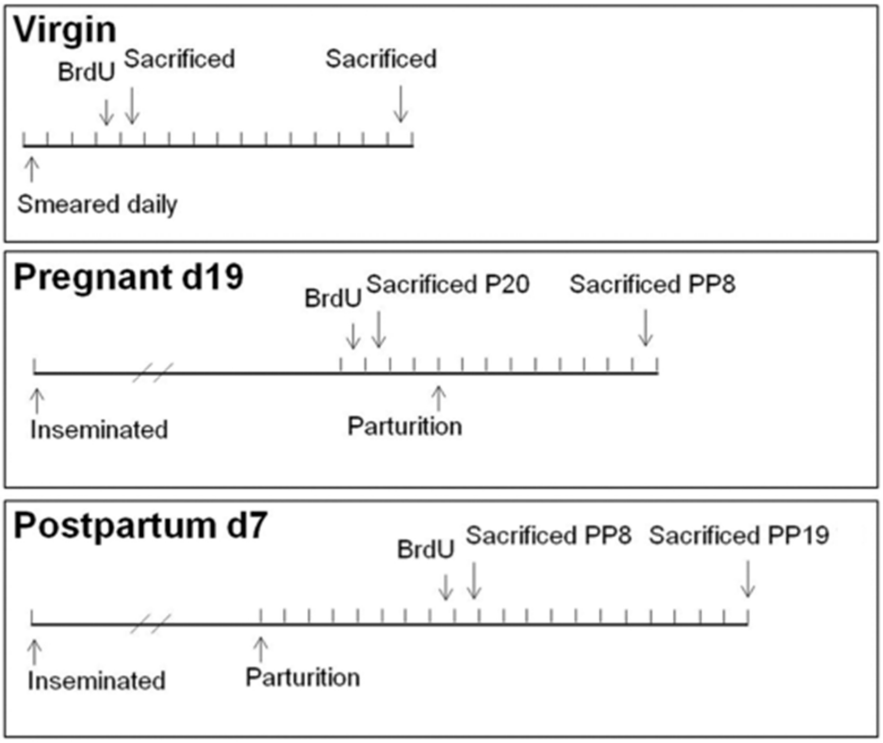

Figure 2: Schematic representation of the experimental time course to determine the effects of reproductive state on cell proliferation and survival. Adult female rats were injected with the mitotic marker BrdU as diestrus virgins, on day 19 of pregnancy, or on day 7 postpartum. Females were sacrificed one day later to measure cell proliferation, or 12 days later to measure cell survival.

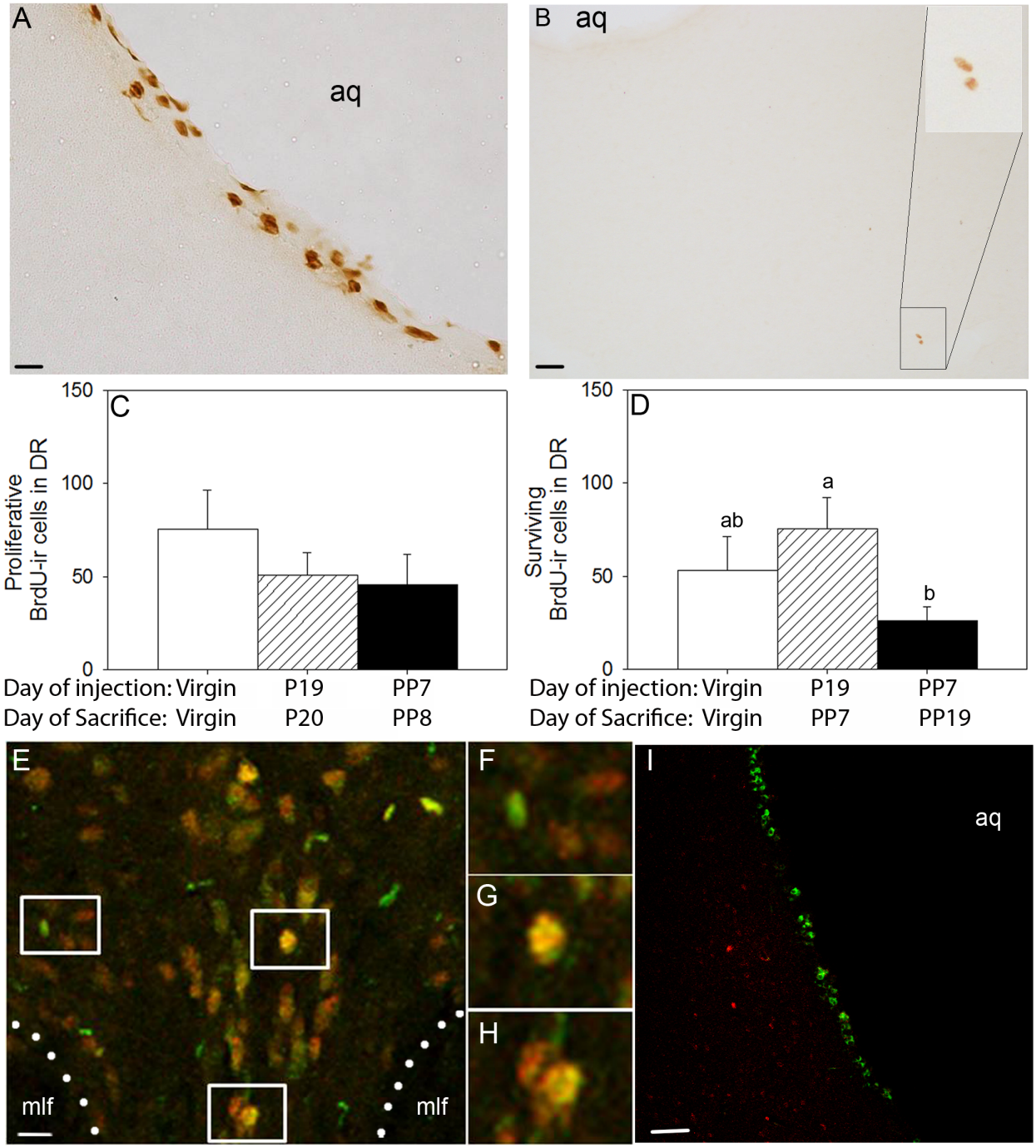

Figure 3: Reproductive state significantly affected newborn cell survival in the DR. A, Photomicrograph of BrdU-ir nuclei lining the lateral border of the midbrain cerebral aqueduct (aq) of a representative PPD 19 female rat sacrificed 12 days after injection. Scale bar, 50 μm. B, Photomicrograph of BrdU-ir nuclei within the DR of a representative PPD19 female rat sacrificed 12 days after injection. Scale bar, 20 μm. C, Reproductive state did not significantly affect cell proliferation in the DR when BrdU-ir nuclei were quantified one day after BrdU **Figure 3 cont'd:** injection into diestrus virgins, PD 19 females, or PPD 7 females. D, Reproductive state did significantly affect newborn cell survival in the DR when BrdU-ir nuclei

were quantified 12 days after BrdU injection. E, Photomicrograph showing BrdU (green) and NeuN (red) immunofluorescence and colocalization (yellow) in the DR. White frames indicate magnified inlays. Scale bar, 100 μm. F, Photomicrograph showing BrdU-ir nucleus that does not contain NeuN colocalization and three NeuN-ir nuclei that do not contain BrdU. G, Photomicrograph showing BrdU-ir/NeuN-ir nucleus. H, Photomicrograph showing BrdU-ir/NeuN-ir nucleus near a NeuN-ir nucleus that does not contain BrdU. I, Photomicrograph showing BrdU-ir nuclei densely lining the lateral border of the aq. No NeuN immunoreactivity is seen in this proliferative niche, where mature neurons do not reside. Scale bar, 50 μm. Bars show group means and SEMs. Unique letters above bars indicate statistically significant differences between groups.

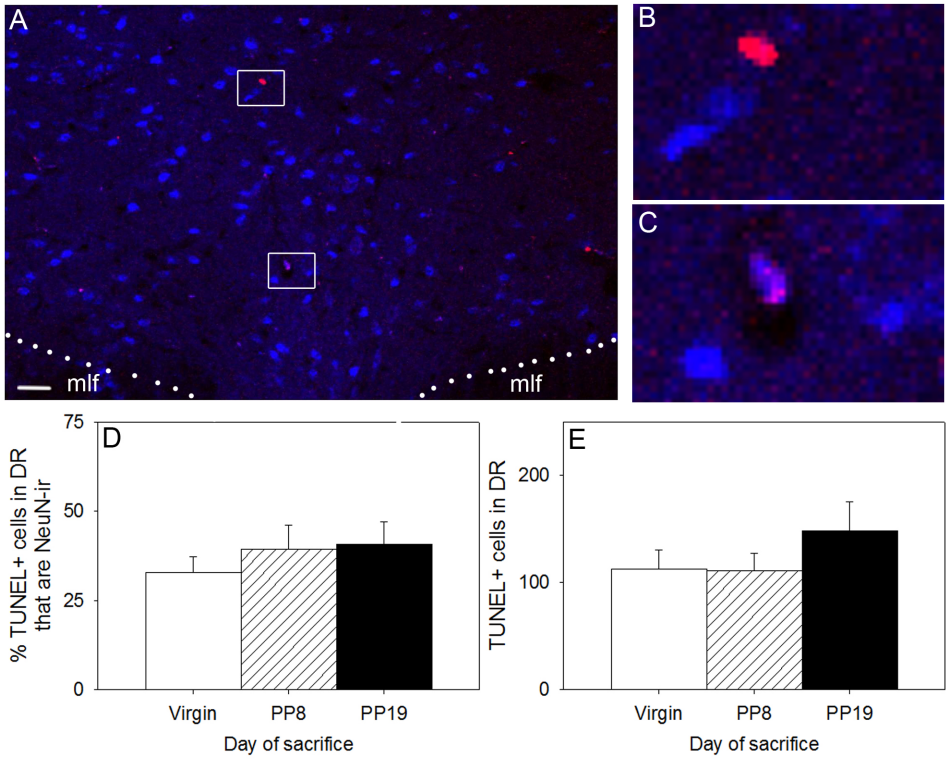

Figure 4: Reproductive state did not affect cell death in the DR. A, Photomicrograph showing TUNEL+ nuclei (red), NeuN immunofluorescence (blue), and NeuN/TUNEL colocalization (pink) in the DR. White frames indicate magnified inlays. Scale bar, 100 μm. B, Photomicrograph showing TUNEL+ nucleus that does not contain NeuN. C, Photomicrograph showing TUNEL+/NeuN-ir nucleus. D, Reproductive state did not affect the percent of TUNEL+ cells that were NeuN-ir (colocalized nuclei / total TUNEL+ nuclei). E, Reproductive state did not significantly affect the number (Mean and SEM) of TUNEL+ nuclei in the DR of adult female rats, despite a 34% increase in late postpartum females. Asterisks indicate significant differences between groups.

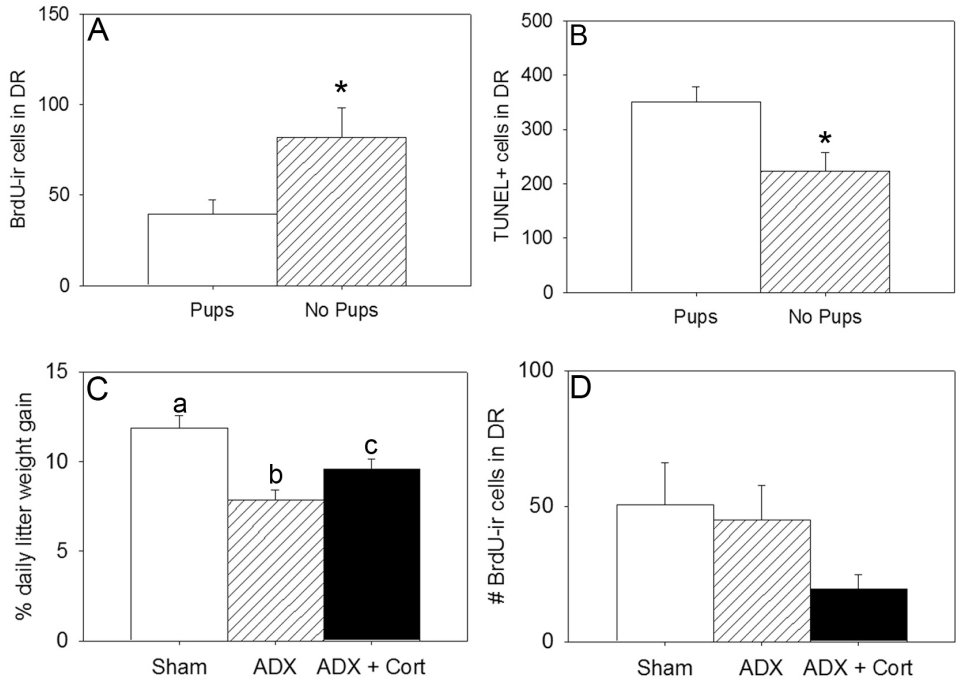

Figure 5: Maternal experience affected newborn cell survival and cell death in the DR, but adrenal hormones did not. A, Removing pups immediately after parturition significantly increased 12-day survival of maternal DR cells born on PPD 7. B, Removing the pups soon after parturition reduced later cell death in the maternal DR. C, Adrenalectomy and corticosterone replacement affected litter weight gain. Litters gained more weight (average % weight gain per day) when interacting with sham-operated females compared to litters fed by adrenalectomized females ($p = .0001$) or corticosterone-replaced females ($p = .014$); litters fed by corticosterone-replaced females also tended to gain more than litters fed by adrenalectomized females ($p = .059$). D, Adrenalectomy and corticosterone did not significantly affect on the number of BrdU-ir nuclei in the DR of postpartum females. Bars show group mean and SEMs. Asterisks indicate significant differences between groups.

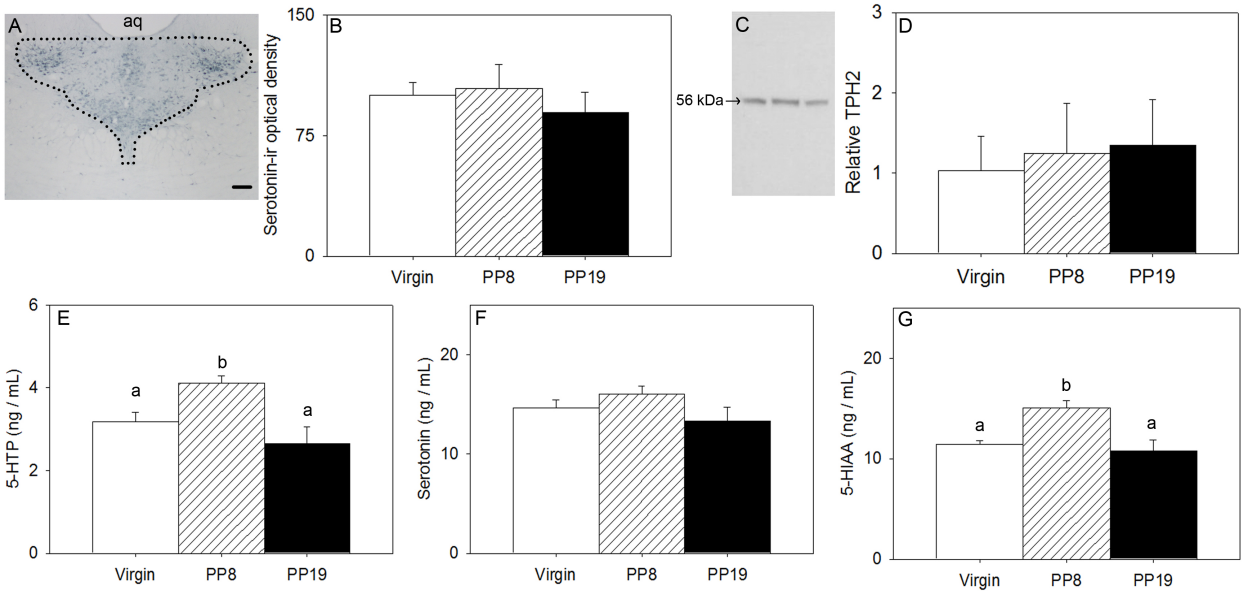

Figure 6: Reproductive state affected DR levels of serotonin's precursor and metabolite, but not serotonin or its synthesizing enzyme, TPH2. A, Photomicrograph showing serotonin immunoreactivity in the DR of a representative PPD 8 rat. Scale bar, 100 μm. B, Diestrus virgins, PPD 8, and PPD 19 female rats had similar percentage of total area within the DR covered by serotonin immunoreactivity. C, TPH2-ir bands from a Western Blot of microdissected DR obtained from representative diestrus virgin, PPD 8, and PPD 19 female rats. There was a single band at 56 kDa. D, Reproductive state did not affect DR TPH2 immunoreactivity. E, Reproductive state affected DR levels of serotonin's precursor, 5-HTP, quantified using HPLC. PPD 8 rats had more DR 5-HTP than diestrus nulliparae or PPD 19 rats. F, There was no effect of reproductive state on DR levels of serotonin quantified using HPLC. G, Reproductive state affected DR levels of serotonin's metabolite, 5-HIAA, quantified using HPLC. PPD 8 rats had more DR 5-HIAA than diestrus nulliparae or PPD 19 rats. Bars show group mean and SEMs. Unique letters above bars indicate significant differences between groups.

Brain site	Diestrus	BrdU	BrdU	*P*

	nulliparae	injected on PD 19	injected on PPD 7	
NAc: proliferation	36 ± 9	49 ± 9	54 ± 16	.366
survival	51 ± 12	39 ± 9	62 ± 13	.772
NAsh: proliferation	43 ± 11	41 ± 12	54 ± 16	.159
survival	45 ± 15	36 ± 10	48 ± 14	.924
BSTd: proliferation	51 ± 10	47 ± 8	50 ± 8	.851
survival	54 ± 9	48 ± 10	45 ± 13	.851
BSTv: proliferation	27 ± 5	34 ± 7	37 ± 6	.423
survival	41 ± 8	37 ± 8	35 ± 10	.726
mPOA: proliferation	70 ± 11	97 ± 18	74 ± 11	.250
survival	107 ± 18	84 ± 17	56 ± 15	.611
DG: proliferation	251 ± 87	264 ± 95	94 ± 20	.274
survival	86 ± 28	74 ± 27	78 ± 24	.951

Table 1: Reproductive state did not significantly affect cell proliferation or newborn cell survival in the forebrain regions examined. Adult female rats were injected with BrdU as virgins, on PD 19 or on PPD 7 and sacrificed one day later to measure cell proliferation (first line for each brain region) or 12 days later to measure newborn cell survival (second line for each brain region). BrdU-ir nuclei were counted in several forebrain regions involved in postpartum behavior and physiology, including the nucleus accumbens core (NAc) and shell (NAsh), the dorsal bed nucleus of the stria terminalis (BSTd), the ventral BST (BSTv), and the medial preoptic area (mPOA).

Brain site	Maternally experienced	Pups removed	*P*
NAc	149 ± 20	247 ± 45	.086
NAsh	159 ± 26	227 ± 40	.121
BSTd	76 ± 16	149 ± 32	.102
BSTv	33 ± 14	62 ± 18	.110
mPOA	83 ± 14	120 ± 22	.326
DG	103 ± 23	117 ± 22	.682

Table 2: Maternal experience did not significantly affect newborn cell survival in the forebrain regions examined. Postpartum females whose pups were removed immediately after parturition or remained with them for the duration of the experiment were injected with BrdU on PPD 7 and 8 and sacrificed 12 days later. BrdU-ir nuclei were counted in the nucleus accumbens core (NAc) and shell (NAsh), the dorsal bed nucleus of the stria terminalis (BSTd), the ventral BST (BSTv), and the medial preoptic area (mPOA).

Discussion

Successful rearing of newborns requires striking changes in behavior and physiology that are supported by plasticity in the maternal brain. Endocrinologically reducing cytogenesis in the SVZ or DG impairs maternal behavior and increases anxiety-like or depression-like behavior in laboratory rodents (Brummelte & Galea, 2010; Larsen & Grattan, 2010; Larsen & Grattan, 2012). In addition, separating dams from their offspring daily reduces cell proliferation in the maternal DG and SVZ, depletes serotonin in both sites and the DR, and increases depression-like behaviors (Sung et al., 2010). Despite intriguing links between serotonin and postpartum behavior and physiology, there have been no reports of motherhood-induced neuroplasticity within the DR. We hypothesized that motherhood modifies the DR via changes in cytogenesis and cell death, which could alter its neurochemical function in a manner that could support parenting.

We found that reproduction and parental experience sculpt the maternal DR by affecting both newborn cell survival and cell death, with concurrent changes in the serotonin synthetic and metabolic pathway. Specifically, fewer DR cells born during the early postpartum period survived 12 days (until late lactation, PPD 19) when compared to the number of cells born during late pregnancy that survived 12 days (until the early postpartum period, PPD 8). This reduced DR cell survival is unique among the eight brain sites analyzed suggesting a particularly specific modulation of brain plasticity. During late lactation when dams showed this reduced DR cell survival, there was a concomitant (but non-significant) 34% increase in DR cell death and decreased levels of the serotonin precursor, 5-HTP, and metabolite, 5-HIAA, compared to early postpartum dams. Despite significant differences in 5-HTP, the product of the rate-limiting

step in serotonin synthesis, serotonin itself was stable across reproductive state, probably because its metabolism into 5-HIAA changed across groups in the same direction. These concurrent changes in DR newborn cell survival and serotonin synthesis/metabolism suggest these events are related. Serotonin increases newborn cell survival in the DG and SVZ by stimulating 5-HT1A receptors (Gould, 1999; Soumier et al., 2010; Jahanshahi et al., 2011) and may act similarly in the DR. If so, early postpartum enhancement of local serotonin could support survival of newborn DR cells, whereas newborn cells fail to survive in the late postpartum DR when serotoninergic activity has waned. Of course, the reciprocal relationship is possible - changes in survival of newborn DR cells may affect serotonin. This could occur if newborn cells increase local neurotrophic factors, such as brain derived neurotrophic factor (BDNF). Transplanting mitotic cells into the adult brain increases local BDNF (Blurton-Jones et al., 2009) and administering BDNF elevates serotonin content and reuptake in DR neurons (Siuciak et al., 1998; Celada et al., 1996; Zhou et al., 2000; Goggi et al., 2002; Martinowich & Lu, 2008). In this scenario, a drop in newborn cell survival across lactation would reduce BDNF in the DR, thereby reducing serotoninergic activity.

Decreased DR serotonin synthesis/metabolism as lactation progresses would presumably limit serotonergic activity at DR terminal sites, including the medial preoptic area and bed nucleus of the stria terminalis (Lowry et al., 2008), which have high serotonin turnover in early postpartum dams and are essential for postpartum behavior (Lonstein et al., 2003; Smith et al., 2013; Numan and Insel, 2003; Pereira and Morrell, 2011). Postpartum behaviors and associated physiological processes are not static across the postpartum period, but change synchronously with offspring development (Deschamps et al., 2003; Lonstein et al., 2005; Caughey, 2011; Giovenardi, 2000). These temporal changes presumably rely on altered neurochemistry in many

brain areas (Pereira and Ferreira, 2015), and our results suggest this includes neuroplasticity-associated changes in DR serotoninergic activity.

One specific effect of neuroplasticity-associated changes in DR serotonergic activity could be related to serotonin's role in somatosensation (Arora & Chopra, 2013; Nagakura et al., 2009; Hole & Berge, 1981; Kulkarni & Robert, 1982). DR serotonin innervates the primary somatosensory cortex (SI) (Kirifides et al., 2001; Simpson et al., 2003; Sheikhkanloui-Milan et al., 2010), which expands and contracts across lactation to heighten and then reduce ventrum sensitivity to tactile input from young (Xerri et al., 1994; Rosselet et al., 2006). Contact with offspring is necessary to maintain most postpartum behavioral and physiological adaptations (Stern, 1996; Lonstein & Gammie, 2002; Numan et al., 2006; Lonstein, 2007), and removing offspring prevents this SI plasticity (Xerri et al., 1994). We similarly found that removing the offspring increased DR newborn cell survival and reduced cell death of late postpartum females. Thus, postpartum experience with young induces plasticity in the DR in ways that could support maternal behavior and physiology by directly influencing serotonergic output (Ferreira et al., 2000; Zhao & Li, 2010; Veiga et al., 2011; Bodnar et al., 2009) or indirectly by affecting plasticity elsewhere in the brain (Gould, 1999; Soumier et al., 2010; Jahanshahi et al., 2011). Reduced DR cell survival in maternally experienced postpartum females was also unique among the 8 brain regions analyzed here, although there was a general pattern among brain sites in that dams with pup contact had fewer BrdU-ir cells in each brain region that was only significant in the DR. This specificity is not due to reduced statistical power in other brain sites because all data are from the same population of animals. Similar to the reciprocal relationship between DR neuroplasticity and serotonin mentioned above, the temporal changes in mother-young interactions may guide newborn cell survival and cell death, and the affected cytogenesis and

cell death may then drive dams' late postpartum behavior to promote weaning of the litter. In support, learning enhances survival of DG cells born before but not during training (Gould et al., 1999), and these cells may then participate in learning and memory (Shors et al., 2002).

Numerous steroids and peptides differing across female reproductive states influence cell birth and death (Bridges & Grattan, 2003; Galea et al., 2006; Pawluski et al., 2009; Larsen & Grattan, 2012). Reducing corticosterone in dams by removing offspring (Walker et al., 1992) or adrenalectomy increases DG cell proliferation (Leuner, 2007). Exogenous corticosterone prevents the adrenalectomy effects (Leuner, 2007) and causes a further decrease in DG cell proliferation in intact dams (Brummelte, 2010). The DR is dense with glucocorticoid receptors (Heyendael & Jacobson, 2009) so we hypothesized that the high postpartum corticosterone was responsible for reducing DR newborn cell survival. However, ADX did not increase DR cytogenesis. In addition to glucocorticoids, the DR responds to many hormones of gestation and lactation (Shughrue et al., 1997; Mitra et al., 2003; Alves et al., 2000; Morimoto et al., 1996; Bethea et al., 2000). Prolactin is very high until PPD 15 in rats (Amenomori et al., 1970; Hansen et al., 1983), and is neurogenic (Pawluski et al., 2009; Levy et al., 2011; Larsen & Grattan, 2012), so would not be expected to reduce survival of cells born on PPD 7. Although it is possible that declining prolactin after PPD 15 promoted cell death by the time we counted cells on PPD 19, this seems unlikely because removing the young increased DR cell survival but rapidly reduces prolactin due to the lack of suckling (McNeilly, 2006). Ovarian hormones may instead be involved. The DR is sensitive to ovarian hormones, which increase DR TPH2 (Donner & Handa, 2009), reduce serotonin-1A autoreceptors (Pecins-Thompson & Bethea, 1999; Zhang et al., 1999; Lu & Bethea, 2002; Hiroi & Neumaier., 2009), and inhibit serotonin metabolism (Smith et al., 2004). Estradiol and progesterone are high during pregnancy or

parturition and temporarily decline thereafter (Smith & Neill, 1977; Hansen et al., 1983; Pawluski et al., 2009), so the postpartum absence of these neurogenic hormones could have contributed to the low newborn cell survival (Hughes et al., 2009; Barha et al., 2011). Removing the litter reversed this effect, possibly because the lack of suckling elevates ovarian hormones via the resumption of estrus cyclicity (McNeilly, 2006).

The frequent occurrence of pairs of BrdU-ir nuclei within the DR suggests these cells divided *in situ* because such similar migratory paths from the SVZ seem unlikely (Kokoeva et al., 2007). Cells born outside of the SVZ and hippocampus are less likely to become neurons compared to those within them (Lledo et al., 2006; Migaud et al., 2010) but ~55% of 12-day old BrdU-ir cells did express NeuN, indicating neuronal phenotype (Mullen et al., 1992). The remaining BrdU-ir cells could be neuroblasts too immature to express NeuN or cells that matured into glia. The latter could be relevant because astrocytes provide trophic factors that maintain high numbers of serotonergic DR neurons (Eriksen et al., 2002; Tajuddin et al., 2003). It will be valuable in future studies to determine whether gliogenesis also occurs in the maternal DR to better understand the diversity of its plasticity, as well as to determine whether these newborn cells are functionally integrated and activated by mother-young interactions (cf., Mohr and Sisk, 2013).

These experiments demonstrate the unique nature of the midbrain serotonergic DR during female reproduction. We show for the first time that cytogenesis occurs in the DR of an adult mammal, although previous reports do indicate dense labeling of a pro-differentiation marker in the DR of adult male monkeys (Vinet et al., 2002). Moreover we have shown that this plasticity is influenced by female reproductive state and motherhood, and that the plasticity is associated with changes in serotonin synthesis and metabolism. Reciprocal relationships between DR

plasticity and neurochemical changes - and between those phenomena and postpartum behavior - could be crucial for the onset, maintenance, and termination of the behavioral and physiological adaptations of motherhood. Similar plasticity may occur within the female DR during other reproductive milestones, including puberty and reproductive senescence.

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CHAPTER 3: Postpartum serotonin-specific lesions of the dorsal raphe nucleus impair maternal caregiving and aggression in female laboratory rats

Abstract

The survival and wellbeing of mothers and their young require high levels of maternal caregiving behaviors, aggression toward potential threats, and a low anxiety state that prevents mothers from overreacting to environmental disturbances. Maternal caregiving, aggression, and anxiety are all affected by pharmacological manipulation of central serotonin signaling but no experiments have examined these behaviors after permanently disrupting serotonin signaling from its primary source in the midbrain dorsal raphe (DR). In this experiment, serotonin-specific lesions of the DR were induced in postpartum day (PPD) 2 rats using a saporin-conjugated neurotoxin targeting the serotonin transporter. Undisturbed maternal behaviors and retrieval of scattered pups were observed before and numerous times after lesioning. Maternal aggression toward a male intruder, and anxiety-like behavior in an elevated plus maze and light dark box, were also tested. We found that serotonin-specific DR lesions altered numerous postpartum behaviors. During undisturbed observations, lesioned animals crouched over the litter more but showed less supine nursing compared to control dams. Lesioned dams also licked their pups less on some days and showed shorter attack bouts during maternal aggression compared to controls. Groups did not differ in anxiety-related behaviors. This experiment indicates new roles for DR serotonin in the social behaviors of postpartum females.

Introduction

Some of the most intense shifts in adult behavior and physiology occur during the peripartum period. These include the unique suite of socioemotional behaviors expressed by postpartum females, such as high maternal caregiving, high aggression, and low anxiety, which are all critical for the survival and wellbeing of both the mother and young. These behavioral characteristics are highly predictable and fully established at parturition by the hormonal fluctuations during pregnancy (Rosenblatt & Lehrman, 1963; Numan et al., 2006; Lonstein et al., 2014), but are maintained thereafter by sensory input from the young with very little contribution of postpartum gonadal, adrenal, or pituitary hormones (Stern, 1966; Hansen & Ferreira, 1986; Rees et al., 2004; Lonstein, 2007; de Sousa et al., 2010; Grieb et al., *in prep*). In addition to these critical behavioral changes, physiological changes prepare mothers to feed and nurture young during this very demanding period. For example, postpartum females have a reduced HPA axis stress response, and they instead show chronically elevated basal corticosterone to support the metabolic requirements of lactation (Stern & Voogt, 1973; Walker et al., 1992; Toufexis et al., 1998; Neumann et al., 2000). These behavioral and physiological changes make postpartum females exquisitely sensitive to infant cues and highly motivated to care for and protect young (Lonstein, 2007; Lonstein et al., 2014)

The neurobiology underlying postpartum behavior and physiology involves many neurochemical systems, including serotonin. Evidence for heightened serotonin metabolism during the early postpartum period has been found in the cerebrospinal fluid of laboratory rats (Spielman et al., 1985) and in forebrain regions that are important for postpartum adaptations. These sites include the medial preoptic area (Lonstein et al., 2003) and the bed nucleus of the stria terminalis (Smith et al., 2013), which are critical for postpartum caregiving and for

regulating anxiety (Numan et al., 2005; Pereira & Morrell, 2009; Smith et al., 2012; Smith et al., 2013; Kenny et al., 2014; Kuroda & Numan, 2014; Dobolyi et al., 2014; Brown & Brown, 2015; McHenry et al., 2015), and the hippocampus (Desan et al., 1988; Glaser et al., 1992) which regulates HPA axis activity (Stern et al., 1973; Tu et al., 2005; Windle et al., 2013) and suckling increases serotonin metabolism in the mPOA (Johnston et al., 1984). The forebrain's main source of serotonin is the dorsal raphe (DR; Lowry et al., 2008). During the postpartum period, serotonergic DR neurons are more excitable (Klink et al., 2002) and have more serotonin-synthesis products compared to cycling virgins (see Chapter 2). It follows that serotonin from the DR could play a critical role for new mothers.

Given that serotonin affects numerous aspects of postpartum behavior and physiology, there is a surprisingly small literature concerning the role of the DR or serotonin in postpartum socioemotional behavior, but the extant data are compelling. Postpartum caregiving in mice is impaired by knockout of *Pet-1*, the transcription factor necessary for the early-life differentiation of serotonergic neurons, or *TPH2*, the enzyme necessary for neuronal synthesis of serotonin (Lerch et al., 2007; Angoa-Perez et al., 2014). In addition to these permanent genetic manipulations, maternal caregiving in rats is affected by more acute manipulations, including administration of serotonin receptor agonists and antagonists (Li et al., 2004; Zhao & Li, 2009; Zhao & Li, 2010; Chen et al., 2014). For example, injecting a serotonin 2a/2c receptor antagonist impairs maternal retrieval and nest building and reduces licking of pups (Zhao & Li, 2010). Not only is maternal caregiving affected by serotonin, but maternal aggression is reduced by infusing serotonin receptor agonists into many of the brain sites tested or by reducing serotonergic output of the DR using autoreceptor agonists (Ieni et al., 1985; Almeida & Lucion, 1994; Olivier et al., 1995; Almeida & Lucion, 1997; Almeida et al., 2005; Almeida et al., 2006;

Lonstein & Gammie, 2002; Veiga et al., 2007; Veiga et al., 2010). Serotonin plays a strong regulatory role with regard to both social and emotional behaviors, including affective communication (see Wohr et al., 2015 for review). Additionally, serotonin autoreceptors in the DR modulate anxiety, with agonists being anxiolytic and antagonists being anxiogenic (McDevitt et al., 2011) and many treatments that increase serotonin signaling reduce anxiety (Donner & Handa, 2009; Charoenphandhu et al., 2011; Hiroi et al., 2011). While these studies demonstrate that serotonin generally plays an important role in postpartum caregiving and aggression, we know almost nothing about what part serotonin specifically origination from the DR plays in postpartum behavior.

Two groups have lesioned the DR of postpartum rats to study postpartum behavior and physiology, but both studies left many questions unanswered (Barofsky et al., 1983; Yurino et al., 2001). Barofsky and colleagues (1983) induced serotonin specific lesions by injecting 5,7-dihydroxytryptamine (5,7-DHT) into the DR. 5,7-DHT killed many, but certainly not all serotonergic neurons in the DR of these animals, resulting in a ~50% decrease in serotonin content in the forebrain and eliminating or reducing the suckling-induced prolactin surge needed for lactation. Although serotonin-specific lesioning of the DR did not seem to affect nursing (Barofsky et al., 1983) postpartum dams were only considered to be nursing if at least 3 pups remained attached to the teats after dams were lifted by the tail, so the behavioral repertoire involved in nursing was not observed in this experiment. Barofsky and colleagues (1983) also found that lesioning the DR did not affect pup retrievals, which were considered successful as long as eight scattered pups were gathered to the nest site within 45 minutes. It is completely unsurprising that the DR lesioned rats were able to successfully complete the task under these circumstances because retrieval of 8 scattered pups usually takes less than 5 minutes (Li &

Fleming, 2003; Miller & Lonstein, 2005; Zhao & Li, 2009). Despite these negative findings, lesioning the maternal DR reduced litter growth, and led to high rates of offspring mortality (Barofsky et al., 1983), suggesting that postpartum caregiving was somehow interrupted in a rather devastating way. Importantly, because 5,7-DHT is internalized and retrogradely transported (Zhou & Azmitia, 1983), these lesions would have killed serotonergic neurons innervating the DR, including from the median raphe (MR), which also provides serotonin to the forebrain (Kohler & Steinbusch, 1982; Kohler et al., 1982; Vertes et al., 1999). Thus, this group was unable to identify independent contributions of DR serotonin and MR serotonin to any of the behavioral or physiological effects.

In contrast, Yurino and colleagues (2001) induced radiofrequency lesions that were not serotonin-specific, but were locally restricted to the rostral half of the DR and did not directly affect the MR (Yurino et al., 2001). They reported that lesioning the DR had no effect on pup retrieval or the frequency of other postpartum caregiving behaviors, including licking of pups, when examined during 30-minute retrieval tests. These non-lactating, postpartum females did not cohabitate with pups, and instead only interacted with them during the retrieval tests, so there were no measures of offspring development or undisturbed maternal behavior possible in their study. Separation from pups causes a dramatic burst of maternal behavior in the first few hours after reunion (Champagne et al., 2003; Claessens et al., 2012; Reis et al., 2014; Stamatakis et al., 2015) so these brief observations by Yurino et al. (2001) do not resemble normally occurring maternal behavior, making it very difficult to interpret the results of this study because the high maternal motivation following separations would obscure effects that could be devastating to a full-time mother who must feed her young.

The goal of the current study was to fill this gap in the literature by much more thoroughly testing the role of DR serotonin in postpartum behavior. We infused a saporin-conjugated antiserum targeting the serotonin transporter (SERT) to selectively kill serotonergic neurons (Rattray et al., 1999; Gravitt & Marson, 2007; Shen et al., 2007; Nattie et al., 2004; Dias et al., 2007; Su et al., 2014). Once saporin enters the cells, it inactivates ribosomes, leading to cell death. Because SERT is not retrogradely transported under normal physiological conditions (Lau et al., 2008; DesCarries & Riad, 2012), Anti-SERT-Saporin only attacks serotonergic somata within the targeted region. Before and after lesioning the DR, we carefully observed undisturbed maternal behaviors. After lesioning the DR, we also examined retrieval of the scattered litter of pups, postpartum anxiety, and maternal aggression. We directly tested the hypothesis that serotonin originating from the DR plays a critical role in the social and emotional behaviors of postpartum female rats.

Methods

Subjects

Female Long-Evans rats descended from rats purchased from Harlan Laboratories (Indianapolis, IN) were born and raised in our colony, as described previously (Smith et al., 2012; Holschbach et al., *in prep*). Briefly, subjects were housed in clear polypropylene cages (48 cm x 28 cm x 16 cm) with one or two female littermates starting at weaning (21 days of age). All animals were provided with wood shavings for bedding and food and water *ad libitum* and experienced a 12:12 light/dark cycle (0600 hr lights on). Subjects were impregnated between 75-100 days old by being paired overnight with experienced male breeders in our colony during proestrus (determined by vaginal smear). After mating, subjects were housed with another

pregnant female until being singly housed approximately 6 days before parturition. Within 12 hours of parturition on postpartum day (PPD) 0, all litters were culled to contain 4 male and 4 female pups. All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee at Michigan State University.

Stereotaxic surgery

On PPD 2, all subjects were anesthetized with ketamine (90 mg/kg, ip) and xylazine (8 mg/kg, ip) and fitted into a stereotaxic frame. Lesions were induced by injecting anti-serotonin transporter saporin (Anti-SERT-Saporin; 1 μL, 0.1 M; Advanced Targeting Systems, San Diego, CA) into the DR, 8.2 mm posterior and 6.7 mm ventral of bregma. Control animals were injected with an inactive control conjugate provided by the vendor. This is the first time Anti-SERT-Saporin has been used in the DR, but it has previously been infused into the ventricular system (Shen et al., 2007) and descending raphe nuclei (Nattie et al., 2004; Dias et al., 2007; Su et al., 2014) to examine serotonin's role in urethrogenital reflexes, the baroreflex, and ventilatory responses. Litters were fostered to surrogate dams during surgery and given back to their own mothers following recovery from anesthesia. One subject attacked pups soon after their return, so thereafter the pups were placed in dams' cages two at a time and dams were only given more pups after accepting the previous two. In two cases the full litter was not returned until the following morning (one control and one lesioned dam), but the full litter was in place at least two hours before the next undisturbed observation began. Litters were weighed and cross-fostered among subjects and surrogate dams from our colony daily to ensure that the lesions could not

persistently disrupt the growth of the pups. Dams' body weight and core temperature were also measured every other day as indicators of subjects' general health.

Undisturbed maternal behavior observation

Subjects were observed in their home cage with their litter twice a day before surgery on PPD 1 and then after surgery from PPD 3-8. Nest quality was rated each day on a 4-point scale (Numan & Corodimas, 1985) and then the subjects' undisturbed behaviors were recorded every 30 sec for 30 min between 0800-0900 and 1300-1400 hr. Recorded behaviors included both maternal behaviors (licking, mouthing, retrieving, and sniffing pups, nest-building, nursing in one of three distinct postures - kyphosis, prone, supine) and nonmaternal behaviors (self-grooming, eating/drinking, resting away from pups, exploring) (Lonstein et al., 1998).

Pup retrieval

Immediately following the afternoon undisturbed observations on PPD 3, 5, and 7, litters were removed from their homecage and kept warm in an incubator set to nest temperature (34 °C) for 15 min, after which they were scattered in the subjects' homecage. The dams' latency to retrieve each pup to the nest site and to hover over the full litter was recorded. If the litter was not gathered after five min (which occurred for one control and one lesioned dam), the litter was placed into the nest site by the observer. Behavioral observations were conducted for ten min after the litter was gathered to the nest site.

EPM

After the final undisturbed observation on PPD 8 (1400-1500 hr), subjects were tested on an elevated plus maze as described previously (Holschbach et al., 2015; Smith et al., 2013; Miller et al., 2010). Briefly, subjects were carried in their home cages to a 10 x 10 ft room lit by a 100-watt light bulb that provided ~28 lx on the center square and open arms and ~2 lx at the end of the closed arms of a black Plexiglass plus maze similar to that of Pellow et al. (1985). Subjects were placed in the central square of the elevated plus maze, facing an open arm, and behaviors were video recorded and continuously scored using a custom-made analysis program (Miller & Lonstein, 2005; Smith et al., 2012). Entries and exits from arms occurred when the subject's head and two front paws crossed into or out of an arm. The percentage of time and percentage of entries into the open arms of the elevated plus maze were used to indicate anxiety-like behavior, and the number of entries into closed arms was used to indicate locomotion.

L/D box

The next day between 0930-1030 hr, subjects were tested in a light-dark box as reported previously (Miller et al., 2011). Briefly, subjects were placed into the light chamber of a light-dark box, which was illuminated at 624 lx compared to the 3 lx dark chamber. Behaviors in the light-dark box included the duration of time spent in the light chamber, the number of full-body transitions between chambers, frequency of rears in the light chamber (100 x # of rears / time spent in the light chamber), number of stretches from the dark chamber into the light chamber, latency to enter the dark chamber, and latency to reenter the light chamber. These behaviors are commonly used to measure anxiety in this apparatus (Costall et al.1989; Crawley, 1981; Bourin

& Hascöet, 2003; De Angelis, 1992; Hascöet & Bourin, 1998). Light-dark box behaviors were video recorded and continuously scored.

Maternal aggression

Between 1500 and 1600 hr on PPD 9, subjects were tested for maternal aggression toward a male intruder that was placed into the home cage with her litter present (Lonstein and Stern, 1998; Lonstein et al., 1998). Intruders were naïve, post-pubertal (51-54 days old) male rats from our colony. Aggressive behaviors (lateral threats, aggressive grooming, kicking, boxing, biting, and attacking [lunging at then pinning or biting] the intruder) and nonaggressive behaviors (sniffing) were video taped and continuously scored for ten min. Stimulus males were sacrificed immediately after testing, but no intruders were visibly injured.

Olfactory detection of and carrying food

Because the serotonin lesions had the potential to affect maternal behavior nonselectively by affecting dams' olfactory sensitivity or ability to use their mouths to carry and manipulate objects (see Numan & Corodimas, 1985), immediately after the intruder test, subjects were placed into a clean cage with fresh bedding. After at least 30 min of habituation, a single Fruit Loop™ was buried underneath the bedding. The subject's latency to find the fruit loop and pick it up to ingest it within a 3 min test was scored.

Perfusion, tissue collection, and immunohistochemistry

Approximately 5-10 min after the conclusion of the olfactory detection test, subjects were deeply anesthetized with sodium pentobarbital. Subjects were transcardially perfused with 200

mL 0.9 % saline and 150 mL 4 % paraformaldehyde in PBS. Brains were harvested and postfixed overnight in 4 % paraformaldehyde in PBS at 4 °C then incubated in 20 % sucrose in PBS for 2-4 days before they were sectioned into 40 μm thick sections using a freezing, sliding microtome. Sections were then stored in a sucrose based cryoprotectant at -20 °C until immunohistochemical processing. Serotonergic neurons were identified using immunohistochemistry as described previously (Leach et al., 2013; Holschbach & Lonstein, *in prep*) using a rabbit antiserum raised against serotonin (1:10,000; Protos Biotech Corporation, New York, NY) and a biotinylated goat-anti-rabbit secondary antiserum (3 μg/mL; Vector Laboratories, Burlingame, CA). Serotonin immunoreactivity was assessed by obtaining optical density measurements from five sections through the DR of each subject. Serotonin immunoreactivity was also measured in the MR to confirm local specificity of the lesion. The DR is a heterogeneous site with distinct subregions, so analyses were done within three distinct subregions of the DR on five rostrocaudal levels as described previously (Kelly et al., 2011; Holschbach & Lonstein, 2015). Images of the ventral subregion (DRv), the dorsal subregion (DRd) and the lateral wings (DRlw) of the DR and images of the MR were captured at 100 x magnification and the average light level was kept constant across all sections. Boxes of a standardized size for each site covering the area of interest were superimposed upon the images and the area covered by pixels above a standardized light threshold optimized to capture dark immunoreactivity was calculated using NIS-Elements as described previously (Smith et al., 2014). Because the subregions differ in area, weighted averages of the subregions of the DR corresponding to their sizes were used to calculate the total percent immunoreactivity in the whole DR.

Data analyses

Repeated measures ANOVAs using day and group as factors were used to analyze maternal behaviors during the undisturbed observations and retrieval tests, and to assess the dams' and litters' physiological measurements. Independent samples t-tests were used to compare sham and lesioned groups in their DR serotonin immunoreactivity, anxiety-related behaviors, and aggressive behaviors. Statistical significance was indicated by $p < .05$. One control subject was missing maternal behavior data from PPD 3 because she was aggressive toward pups after surgery, but she readily accepted the full litter by the morning of PPD 4 and was included in all analyses thereafter.

Results

Serotonin immunoreactivity in the DR and MR

Anti-SERT-saporin significantly reduced serotonin immunoreactivity in the DR without affecting it the MR. Overall, the DR area covered by serotonin immunoreactivity was 57% less in lesioned dams than control dams ($t_{(19)} = 3.73$; $p = .001$; Figure 7A-C). When intensely immunoreactive neurons in each subregion of the DR were counted, the lesions caused a 64% reduction in the number of cells in the DRv ($t_{(19)} = 4.85$; $p = .0001$; Figure 7D), a 58% reduction in the DRd ($t_{(19)} = 3.86$; $p = .001$), and a 48% reduction in the DRlw ($t_{(19)} = 3.20$ $p = .005$). However, the lesions spared the serotonergic neurons in the MR ($t_{(19)} = 0.50$; $p = .62$; Figure 7E).

Physiological measures

Lesions did not affect dams' or pups' general health because control and lesioned subjects had similar changes in body weight across the experiment (main effect of PPD: $F_{(1,19)} =$

.001; $p = .974$) and similar core body temperature (main effect of PPD: $F_{(1,19)} = 2.59$; $p = .124$), although by PPD 7 the lesioned group was 0.62 °C colder than control dams and core temperature on this day was significantly correlated with serotonin-immunoreactivity in the DR ($r = .466$, $p = .033$). Daily litter weight gains were also equivalent in litters being fed by control and lesioned dams (Table 3).

Undisturbed maternal and nonmaternal behaviors

The control and lesioned groups did not differ in their total nursing (main effect of group: $F_{(1,19)} = 0.79$; $p = .38$; Figure 8A), but did differ in the postures they nursed in. The lesioned group showed significantly more kyphosis (main effect of group: $F_{(1,19)} = 1.65$; $p = .05$; Figure 8B) and tended to show less supine nursing (main effect of group: $F_{(1,19)} = 3.39$; $p = .08$; Figure 8C) compared to controls. There was also a significant interaction between group and test day on kyphosis, such that control animals reduced the frequency of kyphosis over time but lesioned females failed to do so ($F_{5,86} = 4.54$; $p = .05$). There was also a significant interaction between group and test day on licking the pups, such that the lesioned group licked less by the end of the experiment than the control group did ($F_{(4,81)} = 2.59$; $p = .04$; Figure 8D). The lesions also reduced the frequency of self-grooming (main effect of group: $F_{(1,19)} = 5.69$; $p = .03$; Figure 8E). As would be expected, the frequency of most maternal behaviors changed over the course of the first week of lactation, with significant main effects of PPD found for crouching ($F_{5,86} = 2.69$; $p = .03$), supine nursing ($F_{2,44} = 6.12$; $p = .003$), licking the pups ($F_{4,81} = 3.25$; $p = .01$), and numerous other behaviors (see Table 3).

Latency to retrieve pups

Lesioned dams retrieved the first pup as quickly as control dams (9 ± 3 vs. 5 ± 1 sec; $F_{(1,17)} = 1.24$; $p = .20$). Similarly, lesioned dams gathered the entire litter into the nest site as quickly as control dams (90 ± 14 vs 78 ± 12 sec; $F_{(1,17)} = 0.31$; $p = .52$). Only two rats in the entire experiment, one control and one lesioned dam, failed to retrieve the entire litter within 5 min (Figure 9). Control and lesioned dams did not differ in the frequency of any behavior during the ten-minute observations following retrieval (data not shown).

Latency to find and eat food

Control and lesioned dams were equally likely to find and ingest the hidden Fruit Loop™ (75% and 89%, respectively, χ^2 (2 N = 17) = .562, $p = .453$) and lesioned dams discovered and ingested the food as quickly as control dams (112 ± 41 vs 81 ± 31 sec; $t_{(15)} = 0.62$; $p = .54$).

Anxiety-like behavior in the elevated plus maze and light-dark box

Control and lesioned dams were similar in the percentage of time they spent within, ($t_{(19)} = 0.70$; $p = .50$) and the percentage of entries made into ($t_{(19)} = .70$; $p = .50$), the open arms of the elevated plus maze. They were also similar in the number of entries into the closed arms ($t_{(19)} = .57$; $p = .57$). Similarly, control and lesioned dams spent an equal amount of time in the light chamber ($t_{(19)} = .29$; $p = .28$) of the light-dark box. Other behaviors were also similar between the two groups (Tables 4 and 5).

Maternal aggression

The average duration of attack bouts was significantly lower in lesioned dams than control dams ($t_{(19)} = 2.51$; $p = .03$) and the total duration of attacking tended to be similarly affected by the lesion ($t_{(19)} = 1.98$; $p = .07$). In fact, almost all aggressive behaviors were shown less to some degree by lesioned animals than control animals (Table 6).

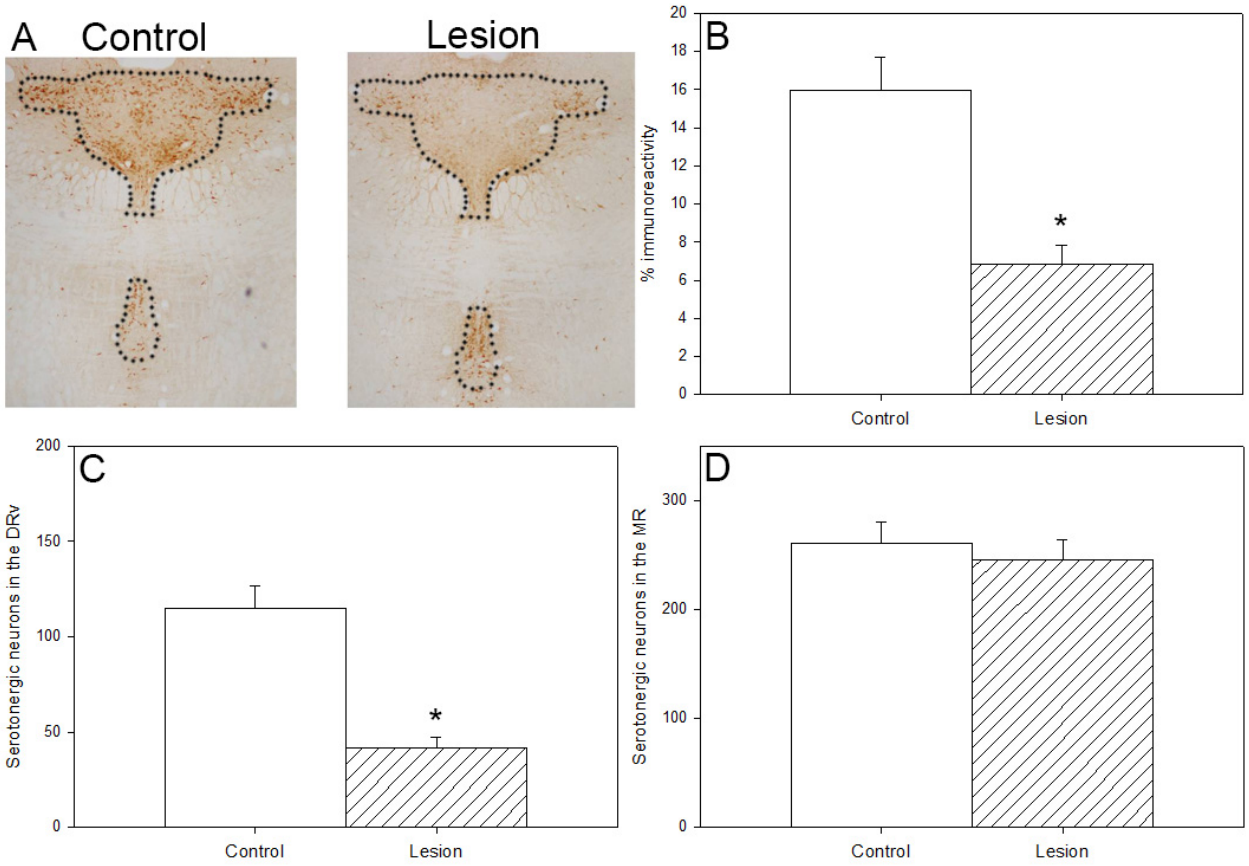

Figure 7: Quantification of the extent of DR lesions. A, Photomicrograph of serotonin immunoreactivity in the DR of a control (left) and a lesioned (right) postpartum rat. B, Percent area of the DR covered by dark serotonin immunoreactivity in control and lesioned dams. C, Number of darkly immunoreactive neurons in the DRv of control and lesioned dams. Other subregions of the DR followed a similar pattern. D, Number of darkly immunoreactive neurons in the MR of control and lesioned dams. Bars show group mean and error bars represent standard errors. Asterisks indicate significant differences.

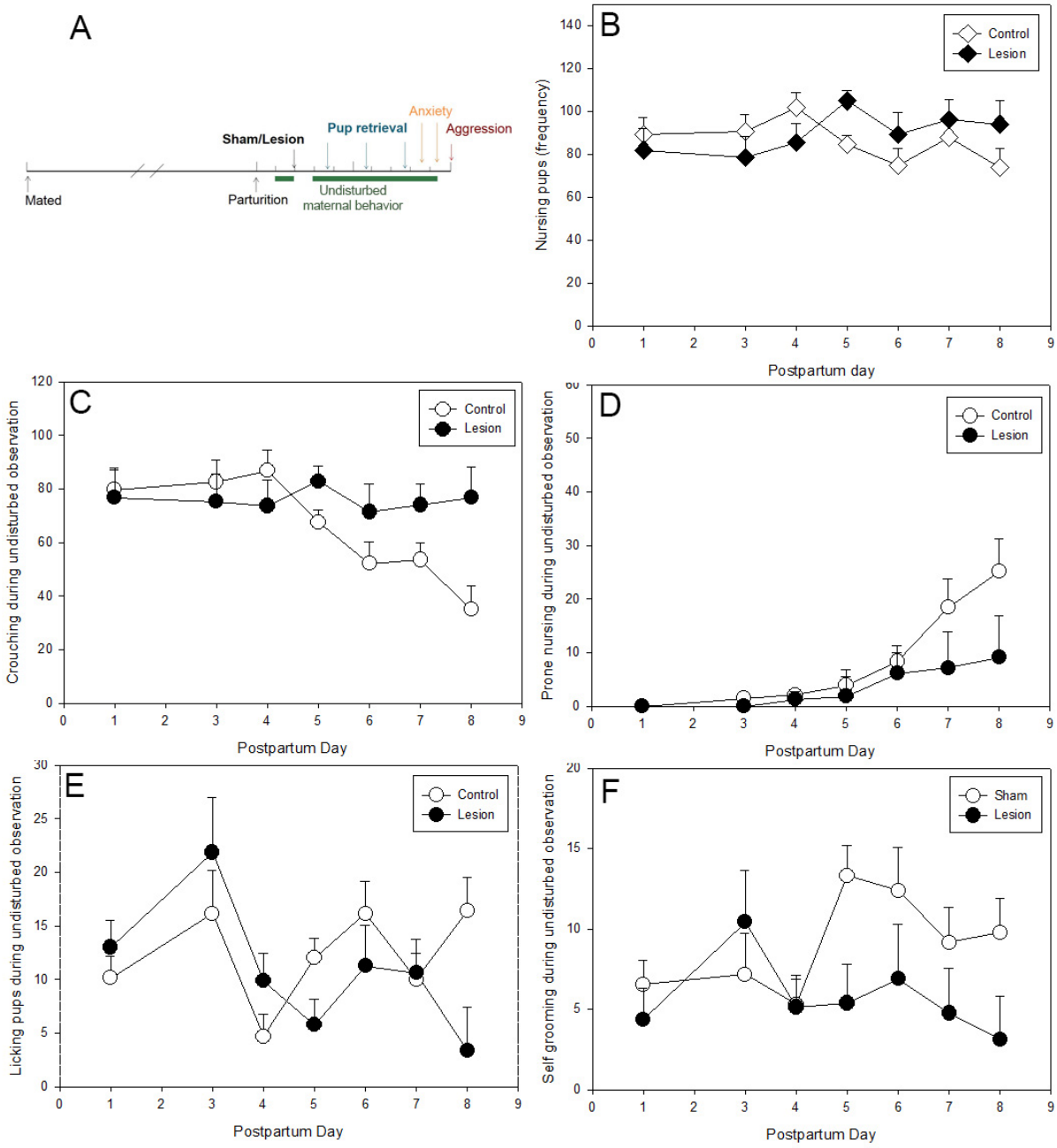

Figure 8: Experimental design and effects of serotonergic DR lesions on undisturbed postpartum behaviors. A, Schematic representation of the experimental time course to determine effects of serotonergic DR lesions on postpartum behavior. Subjects were observed in their homecage on PPD 1 then serotonin-specific lesions were performed on PPD2 and undisturbed behaviors continued on PPD 3-8. Pup retrievals were performed on PPD 3, 5, and 7. Anxiety was tested on PPD 8 in an elevated plus maze and on PPD 9 in a light dark box and then aggression was tested when an intruder male was placed into her homecage. Within one hour of the aggression test, latency to find and eat a fruit loop was measured then the animal was sacrificed and perfused. B, Frequency of total nursing in control and lesioned dams.

Figure 8 cont'd: C, Frequency of crouching in control and lesioned dams. D, Frequency of supine nursing in control and lesioned dams. E, Frequency of licking in control and lesioned dams. F, Frequency of self grooming in control and lesioned dams. Line plots show group mean and error bars show standard errors. Asterisks near the group key represent significant main effects of group. Asterisks above data points indicate days on which behaviors differed significantly between groups based on post hoc tests after significant interactions. Differences approaching significance are indicated by +.

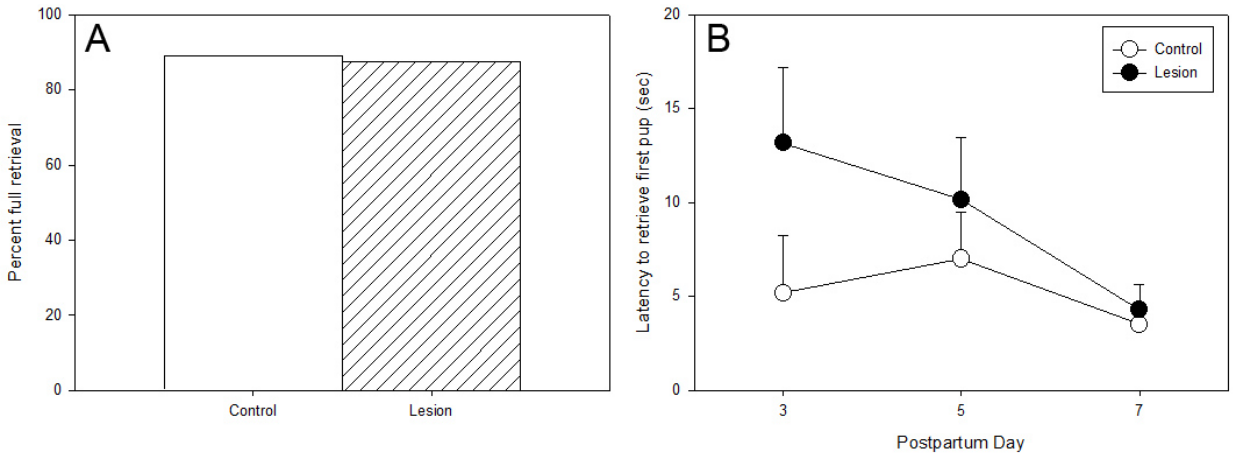

Figure 9: Lesioning serotonergic neurons in the DR had no effect on pup retrieval. A, Percent of control and lesioned dams completing retrievals of all 8 pups within five minutes. B, Latencies to retrieve first pup on PPD 3, 5, & 7 by control and lesioned dams. Bars show group mean and error bars represent standard errors.

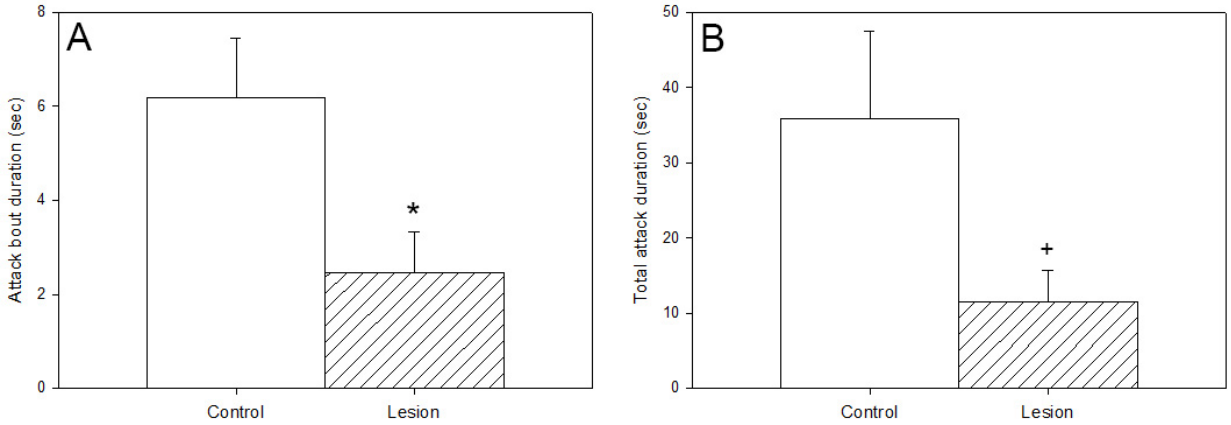

Figure 10: Lesioning serotonergic neurons in the DR reduced duration of attacks during maternal aggression. A, Attack bout durations for control and lesioned dams. B, Total attack duration for control and lesioned dams. Bars show group mean and error bars represent standard errors. Asterisks indicate significant differences. Differences approaching significance are indicated by +.

	Sham (n = 13)	**Lesion (n = 8)**	**Significant main effects**
Frequency			
Exploring	20 ± 2	26 ± 4	None
Eating/drinking	44 ± 9	35 ± 8	None
Resting alone	9 ± 5	9 ± 4	None
Nest-building	12 ± 3	19 ± 6	PPD: $p = .022$
Prone nursing	94 ± 13	75 ± 10	PPD: $p = .019$
Total nursing	641 ± 24	648 ± 15	None
On nest	810 ± 12	797 ± 20	PPD: $p = .002$
Relative litter weight gain	13.4 ± 0.9	13.4 ± 1.2	Postnatal day: $p = .005$
Nest quality (0-3)	2.43 ± .08	2.38 ± .05	PPD: $p = .0001$

Table 3: Frequency of maternal and nonmaternal behaviors (M ± SEM) of sham-operated and lesioned postpartum rats. No significant group differences were detected in these behaviors, although the frequency of some behaviors changed across the first 8 days of the postpartum period.

	Sham	Lesion	t (19)	*p*
Percentage				
Entries into open arms	42 ± 2	39 ± 3	0.70	0.495
Time in open arms	29 ± 4	25 ± 5	0.67	0.495
Frequency				
Total arm entries	27 ± 2	25 ± 3	0.65	0.526
Closed-arm entries	16 ± 1	14 ± 2	0.58	0.571

Table 4: Anxiety-like behaviors (M ± SEM) of control and lesioned postpartum rats in an elevated plus maze. Serotonin-specific DR lesions did not affect postpartum anxiety-like behaviors in the elevated plus maze.

	Sham	Lesion	t (19)	*p*
Frequency				
Transitions between light and dark chambers	4 ± 1	5 ± 1	0.75	0.465
Stretches into light chamber	18 ± 2	22 ± 3	1.01	0.323
Rears in light chamber	9 ± 2	8 ± 3	0.26	0.801
Latency (s)				
Enter dark chamber	7 ± 1	5 ± 1	1.11	0.281
Enter light chamber	322 ± 62	226 ± 67	1.01	0.324
Duration (s)				
In light chamber	55 ± 14	49 ± 14	0.29	0.773

Table 5: Anxiety-like behaviors (M ± SEM) of control and lesioned postpartum rats in a light dark box. Serotonin-specific DR lesions did not affect postpartum anxiety-like behaviors in the light dark box.

	Sham	Lesion	t(19)	*p*
Latency (s)				
First attack	209 ± 54	249 ± 81	0.44	0.667
Duration (s)				
Aggressive grooming	1 ± 1	0 ± 0	0.89	0.387
Lateral threat	19 ± 9	14 ± 6	0.32	0.756
Sniffing	60 ± 5	67 ± 16	0.54	0.599
Frequency				
Attacks	5 ± 1	3 ± 1	0.75	0.463
Bites	1 ± 1	1 ± 1	0.99	0.335
Pins	5 ± 2	3 ± 1	0.95	0.354
Kicks	5 ± 2	5 ± 2	0.18	0.862
Percent showing behavior				
Bury/barricade male	69	50	$\chi^2 = 0.78$	0.378

Table 6: Aggressive and nonaggressive behaviors (M ± SEM) of sham-operated and lesioned postpartum rats. Serotonin-specific DR lesions reduced attack bout duration but did not significantly affect other behaviors in the intruder test.

Discussion

This experiment is the first in-depth analysis of postpartum socioemotional behaviors after serotonin-specific lesions of the DR. Postpartum lesions killing serotonergic neurons in the DR and/or MR increased mortality of pups in previous studies (Barofsky et al., 1983; Rowland et al., 1973), but the behavioral impact of these lesions on the mothers was largely unknown. We hypothesized that serotonin from the DR is an important determinant of postpartum socioemotional behaviors because of the myriad links between serotonin and postpartum caregiving and aggression, and between serotonin and anxiety discussed above. Thus, we predicted that lesioning the serotonergic DR would impair maternal caregiving and aggression and increase postpartum anxiety. We found that the lesioned rats nursed their pups differently than controls by using more active postures, licked their pups less by the end of the experiment, and were less aggressive toward a male intruder. Despite these effects on social behavior, the lesion did not affect anxiety-related behaviors. These results suggest that serotonin from the DR plays a critical role in postpartum social behaviors, while emotional behaviors may be more strongly influenced by other neurotransmitter systems (Neumann, 2003; Lonstein et al., 2014) or by serotonin from another source, such as the MR (Andrade et al., 2013).

In contrast to other studies that performed DR lesions in postpartum females (Barofsky et al., 1983; Yurino et al., 2001), utilization of Anti-SERT-Saporin allowed a serotonin-specific lesion that was locally restricted to somata within the DR. Indeed, infusing Anti-SERT-Saporin into the DR of postpartum rats reduced the number of serotonergic neurons in the DR whereas the MR remained fully intact. In past experiments, serotonin specific lesions using 5,7-DHT infusions affected both the DR and the MR because this toxin is taken up by somata and

terminals and retrogradely transported (Barofsky et al., 1983), whereas radiofrequency lesions were restricted to the DR but presumably killed both serotonergic and non-serotonergic neurons (Yurino et al., 2001). The neurochemical and regional specificity of the lesions used in this study allow more conclusive findings about the role of DR serotonin in postpartum behavior and physiology. The late emergence of many of the behavioral effects, which often arose a few days after lesioning the DR, suggests that Anti-SERT-Saporin likely took a couple of days to kill the serotonergic neurons, as has been shown previously (Nattie et al., 2004).

Not only did we use more selective methodology to induce the DR lesions, but also conducted a much more detailed investigation of behavioral effects thereafter. This yielded some similar results, as well as novel findings, compared to the previous lesion studies. Some converging data include that in all studies, control and lesioned dams successfully retrieved their scattered young whether they were given 30-45 min (Barofsky et al., 1983; Yurino et al., 2001) or five min (present study) to do so. We also showed that control and lesioned dams spent an equal amount of time nursing, similar to previous studies (Barofsky, 1983), but because we did detailed behavioral analyses we found that reducing DR serotonin increases frequency of kyphosis and tends to decrease the frequency of the supine nursing position (i.e., dam laying on her side). Intriguingly, we recently found in a separate experiment that expression of serotonin's synthesizing enzyme, TPH2, within the DR was positively correlated with the frequency of supine nursing in unlesioned postpartum dams (Holschbach et al., in prep), further implicating DR serotonin in the regulation of nursing postures. It may do so in some manner related to serotonin's role in increasing behavioral and neural recovery after spinal cord injury (Ghosh & Pearse, 2015). Moreover, lesioning the serotonergic input to the motor cortex increases the amount of neural activity required to initiate changes in movement in adult rats whereas

stimulating the DR had the opposite effect (Scullion et al., 2013). Lesioning the DR may have similarly disrupted serotonergic neuromodulation in the motor cortex to result in sustained bouts of the immobile kyphosis posture for longer than normal. Alternatively, lesioning the DR could have affected dams' nursing posture indirectly through altered somatosensation. Serotonin affects the perception of touch (Arora 2014; Koyama 1994; Hole 1977), in part, through innervation, organization, and reorganization of the somatosensory cortex (Kirifides et al., 2001; Simpson et al., 2003; Sheikhkanloui-Milan et al., 2010; Ganzer et al., 2013), which expands during the early postpartum period to heighten sensitivity to infant contact (Xerri et al., 1994; Rosselet et al., 2006; Rosselet et al., 2008). A lack of serotonin to the SI might then be predicted to prevent this expansion and retraction, thereby making lesioned rats less sensitive to the tactile input from their offspring and prevent their postural adaptations once offspring are able to suckle from dams laying in a passive supine posture.

In addition to its effects on nursing, Anti-SERT-Saporin lesions also reduced maternal licking during the undisturbed observations. Licking of pups and other maternal caregiving behaviors are also impaired by genetic and pharmacological manipulations that reduce serotonergic capacity or signaling (Lerch et al., 2008; Li et al., 2004; Li et al., 2005; Zhao & Li, 2010; Chen et al., 2014). These effects of DR serotonin loss on licking may be specific to maternal licking of pups because increasing synaptic serotonin with SSRIs reduces operant licking for sucrose, water, or quinine (Mathes et al., 2013) and paw-licking in response to injury (Korneyev & Seredenin, 1993) perhaps by impairing rhythmic tongue movements in addition to reducing the number of licks (Das & Fowler, 1995; Das & Fowler, 1996). Despite effects of serotonin on maternal licking during undisturbed observations, DR-control and lesioned dams licked their litters with similar frequency immediately after the retrieval test in this experiment

and in a study using radiofrequency lesions (Yurino et al., 2001). This suggests that serotonin is less critical for maternal behavior following separations from young, when caregiving is especially high because maternal motivation is elevated (Hansen, 1994; Champagne et al., 2003; Pereira & Ferreira, 2006; Claessens et al., 2012; Reis et al., 2014; Stamatakis et al., 2015). This is the case for the role of midbrain dopamine systems in maternal behaviors, such that depleting striatal dopamine prevents maternal retrieval of pups even if pups were coated in sugar or made more vocal, but a 3-6 hour separation preceding testing completely restored retrieval behavior in the dopamine- depleted dams (Hansen, 1994). Not only does serotonin affect licking/grooming of pups but also self grooming. In male mice, increasing brain serotonin either through genetic mutations to SERT or knockdown or knockout of MAO-A, which metabolizes serotonin into 5-HIAA, increases self grooming (Kyzar et al., 2012; Kyzar et al., 2015). Consistent with these data, we found that serotonergic DR lesions reduced self-grooming in postpartum lactating rats.

Previous research has indicated that serotonergic terminals in the hypothalamus are critical for suckling-induced prolactin and oxytocin surges (Crosignani et al., 1979; Moos and Richard, 1983; Bodnar et al., 2009) and that serotonin lesions appear to impair development of the young due to a deficit in lactation (Barofsky et al., 1983; Rowland et al., 1978). In contrast, we found that lesioning the serotonergic DR had no effect on the growth of neonates. Several differences between previous studies and ours may account for this difference. First, our experiment only killed serotonergic neurons within the DR whereas previous lesions infusing 5,7-DHT into raphe nuclei (Barofsky et al., 1983) or the third ventricle (Rowland et al., 1978) could not achieve this level of specificity. Although the MR has less than one third the number of cells compared to the DR (Vertes & Crane, 1997), both cell groups provide serotonergic innervation of the hypothalamus that causes the endocrine responses to suckling (Rowland et al.,

1978). Because the DR is one of the main targets of the MR (Vertes et al., 1999), and 5,7-DHT is often used to lesion serotonergic neurons from their terminals (e.g. Rowland et al., 1978), 5,7-DHT lesions of the DR would have killed serotonergic neurons in both regions and this could account for their more dramatic physiological effects on lactation. Moreover, our lesions were least effective in the DRlw, which provides a great deal of hypothalamic and adrenal serotonin (described in Lowry, 2008) and this could further explain the sustained lactation in our lesioned rats. One previous experiment did have DR-specific lesions that affected both serotonergic and nonserotonergic neurons, but neither the lesioned nor the sham-operated control group were lactating in that study (Yurino et al., 2001) so no comparisons can be made to our work. Second, given the high occurrence of pup mortality reported in these studies (Barofsky et al., 1983; Rowland et al., 1978), it is also possible that continuous feeding from a lesioned dam exacerbated any effects of the lesion on newborns' weight gain. If so, our daily cross fostering may have prevented any cumulative effect. Moreover, any preventative effect of cross fostering may have been even further strengthened by the behavioral changes in our lesioned dams. Serotonin-specific lesioning of the DR increased the frequency of kyphosis, which is also elicited at a higher frequency by hungry pups (Stern & Keer, 2002), and may represent a behavioral adaptation made by the lesioned dams to overcome any reduction in lactation.

There are various lines of evidence that implicate serotonin in maternal aggression. In general, increased release or heteroreceptor binding of serotonin is associated with reduced maternal aggression, but the specific effects depend upon the receptor and region targeted. Systemic increases in serotonin or its efficacy including genetic manipulations, serotonin reuptake inhibition, and peripheral treatments with serotonin-1A or -2C receptor agonists reduce maternal aggression (Olivier, 1992; Hahn-Holbrook, 2011). Similarly, central injections of the

serotonin-2A/2C receptor agonist into the lateral ventricles or the midbrain periaqueductal grey, and infusions of serotonin-1B receptor agonists into the prefrontal cortex, reduce maternal aggression (Almeida et al., 2005; Almeida & Lucion, 2005; Veiga et al., 2010). Consistent with these findings, infusions of serotonin-1A receptor agonists into the DR - where they activate inhibitory autoreceptors to reduce serotonin release - increase maternal aggression (Veiga et al., 2010). Serotonin seems to play a similar role in aggressive behavior of males. For example, SSRI treatments reduce aggression in stressed rats (Ossowska et al, 2002) or aggressive dogs (Dodman et al., 1996). In contrast, we found that serotonin-specific lesions of the DR reduced the duration of maternal attacks. This result does seem at odds with the preponderance of data concerning serotonin and either maternal aggression or aggression more generally. In fact, a meta-analysis studying the role of serotonin in aggression found that most experiments show that increasing serotonin reduces aggression (64% vs 13% showing increased aggression) and this effect did not depend on species or sex (Carrillo et al., 2009). Importantly, this meta-analysis did not take into account the hormonal status of subjects. This is important because serotonin depletion increases territorial aggression in gonadally intact males, but not castrated males (Svensson et al., 2015; Studer et al., 2015), so perhaps the state of postpartum diestrus characterized by low estrogens may partially explain our possibly unexpected result. Some exceptions include that CSF levels of serotonin and its metabolite are positively correlated with territorial aggression and in male rats (Van der Vegt et al., 2003), injecting of serotonin-2A/2C receptor agonists into the central amygdala increases maternal aggression (Almeida et al., 2005), SSRI treatments also increase maternal aggression after SSRI treatment (Johns et al., 2005).

There is an extensive literature indicating a role for the DR in anxiety-like behaviors, but the relationship is complex and the literature is often contradictory. Some studies have shown

that serotonin-specific lesions of the DR have no effect on anxiety-like behavior of male rats in an open field (Lieben et al., 2006) or an elevated plus maze (Rex et al., 2003). Still others have reported that a similar lesion both reduces the latency for male rats to explore the open arms of an elevated plus maze, suggesting an anxiolytic effect, and reduces the latency to escape from the open arms, suggesting an panicogenic effect (Sena et al., 2003). Moreover, anxiety after depleting forebrain serotonin seems to depend on the extent of the lesion, with anxiolytic effects of modest lesions (Hall et al., 1999) and anxiogenic effects of extensive lesions affecting targets of both the DR and the MR (Briley et al., 1990). Others find that serotonin depletion increases anxiety in nulliparous females, but not males, and thereby eliminates the sex difference in anxiety-like behavior on the elevated plus maze (Naslund et al., 2013).

In addition to DR lesions, serotonin autoreceptors in the DR modulate anxiety, with agonists being anxiolytic and antagonists being anxiogenic (McDevitt & Neumaier, 2011; Higgins et al., 1992). Viral and genetic manipulations suggest that autoreceptors in the rostral DR play a greater role than the caudal DR (Clark et al., 2002; Clark et al., 2004; McDevitt et al., 2011) and that the serotonin-1B autoreceptors play a greater role than the serotonin-1A autoreceptors (Richardson & Jones et al., 2011; Clark et al., 2002; Clark et al., 2004; McDevitt et al., 2011). Although these studies suggest that decreased serotonergic output from the DR due to autoreceptor activation can be anxiolytic, it is important to consider that there are many reports contradicting this view. For example, TPH2 overexpression reduces anxiety whereas knockdown of TPH2 reduces the efficacy of anxiolytic treatments (Hiroi et al., 2011; Charoenphandhu et al., 2011; Donner et al., 2009).

The present study was the first analysis of postpartum anxiety after a lesion to the DR, and our negative result suggests that serotonin from outside of the DR, potentially from the MR

or even the periphery, is sufficient for the normal reduction in postpartum anxiety. In fact, we have shown that while the level of TPH2 in the DR of postpartum rats is positively correlated with supine nursing, TPH2 in the MR is positively correlated with time spent in the open arms of an elevated plus maze (Holschbach et al., in prep). There is a great deal of overlap in the serotonergic projections from the DR and MR to forebrain sites, but the MR provides almost all of the serotonin in the hippocampus (Kohler & Steinbusch, 1982), and serotonin in the hippocampus is anxiolytic (Graeff et al., 1996). Serotonergic projections from the MR to the hippocampus confer resilience to internal physiological signals that could be anxiogenic (Graeff et al., 1991) and treatments that increase serotonin in the MR reduce anxiety (Yoshida et al., 2009). The MR also provides most of the serotonergic efferents to the lateral septum and the medial septum-vertical limb of the diagonal band nucleus (MS/DBv; Kohler et al., 1982; Vertes et al., 1999), and septal injections of presynaptic serotonin 1A agonists are anxiogenic (Micheau & Marrewijk, 1999), suggesting that activation of serotonergic innervation from the MR to the septum is anxiolytic. Although blood concentrations of serotonin in healthy postpartum women are negatively correlated with their anxiety, and postpartum women have higher circulating serotonin than nonpostpartum women only when the mothers have lower anxiety (Sekiyama et al., 2013), this circulating serotonin is almost certainly from a peripheral source because most serotonin in the body is synthesized in the gastrointestinal tract (Gherson & Tack, 2007; Mawe et al., 2006; Erspamer, 1957) and neural contributions to circulating serotonin is only quantifiable levels in extremely nonphysiological conditions involving the removal of peripheral sources of serotonin and infusing serotonin's precursor for neuronal conversion to serotonin (Nakatani et al., 2008). While these peripheral patterns do not preclude similar postpartum changes in central serotonin, postpartum mice have elevated peripheral serotonin in the face of reduced DR

serotonin compared to cycling nulliparae (Jury et al., 2015). Still, it is possible that serotonin from the DR does control postpartum anxiety but that the remaining serotonergic DR neurons were sufficient for this role, especially because over 50% of the serotonergic neurons in the DRlw were left intact.

New mothers have increased serotonin turnover in efferents of the DR that are directly involved in maternal behavior, including the medial preoptic area and the bed nucleus of the stria terminalis (Lonstein et al., 2003; Smith et al., 2013) and this neurochemical change could promote the initiation of postpartum behaviors. Not only is the serotonergic DR sensitive to hormones that initiate postpartum behaviors, but thereafter it could help mediate the sensory control of postpartum behavior by tactile input from the young. It is possible that DR serotonin does this to affect crouching (Stern & Johnson, 1990; Lonstein et al., 1997) and/or maternal aggression (Svare & Gandelman, 1976; Stern and Kounie 1993). This explanation may be slightly simplistic, or not universal for all postpartum neurobehavioral systems, because such tactile input is also required for low postpartum anxiety (Lonstein, 2005) and lesioning the serotonergic DR did not increase the low postpartum anxiety in this experiment. Although it is often suggested that low anxiety disinhibits maternal aggression (Lonstein, 2001), postpartum rats bred for high anxiety are more aggressive than postpartum rats bred for low anxiety (Bosch et al., 2005), suggesting that these two suites of behavior can be independently controlled. Here, we showed that a serotonergic DR lesion reduced maternal aggression despite low anxiety in both control and lesioned dams, which further suggests separate control of postpartum anxiety and aggression.

Serotonin is a classic neuromodulator (Agnati et al., 1995; Bunin & Wightman, 1999; Rubio-Casillas et al., 2015) that regulates both excitatory and inhibitory signaling (Cirana, 2006),

so it is well suited to instill behavioral flexibility in a changing environment (Hurley et al., 2004; Kiser et al., 2012). Developing young provide a constantly changing environment for their mothers, who must alter their behavioral responses continuously and synchronously with their young while still meeting their own needs. The postpartum decline in maternal caregiving and aggression begins during the second week after parturition (Grota & Ader, 1969; Moltz & Robbins, 1965; Rosenblatt & Lehrman, 1963; Caughey et al., 2011). Conversely, anxiety-like behavior is low during the first week postpartum, but rises back to virgin levels by the end of the second week after parturition (Lonstein, 2007). Alongside these reversals in postpartum behavior, there is a reduction in DR serotonin synthesis and metabolism (Holschbach & Lonstein, in prep), further suggesting that serotonin might play a role in temporal changes in postpartum behavior. Killing serotonergic neurons in the DR prevented the postpartum alterations in nursing postures that occurs across time and may generally interfere with dams' continuous, sensitive, behavioral adjustments that optimize development of the young.

Acknowledgments

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CHAPTER 4: General discussion

Maternal female mammals undergo tremendous behavioral and physiological modifications to promote the survival and wellbeing of their offspring (Hillerer et al., 2012; Sisk et al., 2013; Lonstein, Pereira et al., 2014). To support these profound changes, new mothers have extensive neuroplasticity, including the generation of new cells and death of existing cells in the SVZ, DG, bed nucleus of the stria terminalis, nucleus accumbens, and prefrontal cortex (Galea et al., 2006; Pawluski & Galea, 2007; Akbari et al., 2007; Pawluski et al., 2009; Levy et al., 2011; Larsen & Grattan, 2012; Erbil et al., 2014). I proposed that such plasticity may also occur in the DR because previous research indicated increased neural activity in the DR of new mothers (Klink et al., 2002; Lin et al., 1998) and because the very small literature on serotonin's involvement in postpartum behavior and physiology is compelling. Studies of the effects of serotonin-specific DR lesions have suggested that the DR was critical for successful rearing of newborns (Barofsky et al., 1983) but the behavioral impacts on mothers was largely unknown. Although genetic and pharmacological manipulations interfering with neural serotonin systems impair maternal behaviors, including caregiving and aggression (Zhao et al., 2009; Zhao et al., 2010; DeAlmeida & Lucion, 1994; Veiga et al., 2005; DeAlmeida et al., 2005; DeAlmeida et al., 2006; Lonstein & Gammie, 2008; Veiga et al., 2011), involvement of the DR and its serotonin content in postpartum behavior was obscured by superficial or sometimes even ethologically flawed measurements of behavior in the two previous lesion studies (Barofsky et al., 1983; Yurino et al., 2001), as discussed in Chapter 3. Given the importance of serotonin in governing many aspects of postpartum behavior and physiology, plasticity within the DR may be particularly important in new mothers. In this dissertation I showed that motherhood altered

newborn cell survival, cell death, and neurochemical function in the DR and that serotonin-specific DR lesions prevented normal adjustments in nursing across the postpartum period, reduced pup licking, and reduced maternal aggression toward an intruder. These findings were very exciting given the dearth of knowledge about the DR's involvement in postpartum behaviors.

Neuroplasticity in the peripartum DR

I hypothesized that reproduction and maternal experience sculpt the DR to support neurochemical changes that are critical for the control of postpartum behavior and physiology. The first step in testing this hypothesis required the demonstration of plasticity within the DR. Of the many forms of brain plasticity, cytogenesis and cell death represent mechanisms that could support profound changes in chemical functioning of the DR. I measured cell proliferation and survival by injecting BrdU, a thymidine analogue that is permanently incorporated into dividing cells, into adult female rats with a controlled reproductive history. I found that DR cells born during the first week postpartum were less likely to survive than those born during late pregnancy. Previous research has indicated that prolactin is responsible for the high SVZ neurogenesis, and that corticosterone is responsible for the low DG neurogenesis, in new mothers (Shingo et al., 2003; Larsen & Grattan, 2010; Leuner et al., 2007). We were not able to identify the endocrine factor(s) responsible for our effect on DR cell survival, but it is almost certainly not prolactin, which increases cytogenesis and is high in postpartum, lactating females. We also determined that corticosterone is not responsible, because we showed that adrenalectomy had no effect on postpartum DR cytogenesis. We did find that removing offspring immediately after parturition increased newborn cell survival in the DR of postpartum

rats, and it is possible that a subsequent increase in gonadal hormones partially mediated this effect, as discussed in Chapter 2.

Alternatively, it is possible that these social interactions caused a local reduction in some anti-apoptotic, pro-neurogenic factor. A very good candidate is BDNF, which increases newborn cell survival (for review, see Salgado et al., 2015). In support of a role for BDNF, the postpartum reduction of DG cell proliferation is prevented by exercise (Kim et al., 2012), and exercise increases serum BDNF in postpartum women (Veiga et al., 2011). The DR also has high expression of the neurotrophic factor, Bcl-2 (Vinet et al., 2011). If interacting with pups reduces BDNF or Bcl-2 in the DR of postpartum females, administering exogenous neurotrophic factors could prevent the late postpartum reductions in newborn cell survival and increase in cell death. It is intriguing to consider that this manipulation may result in an inappropriately prolonged maintenance of early postpartum behaviors, and prevent their reversion back to a prepartum state that occurs as the pups develop. This could be tested in future experiments.

Another important path for future research would be to complete a demonstration of neurogenesis—the birth, survival, differentiation, and functional integration of new neurons-within the maternal DR. This dissertation demonstrated that new cells were born in the DR of adult female rats, and that they survived for at least 12 days, by which time more than half of the newborn cells had differentiated into neurons (indicated by NeuN labeling). In addition to neuronal phenotype determination, establishing functional integration is necessary to demonstrate the full process of neurogenesis (e.g. Bonfanti & Perretto, 2011). If postpartum DR plasticity were not affected by presence of the offspring, we would have concluded that the new cells were probably not functionally relevant because nearly every postpartum behavioral and physiological adaptation requires contact with the young. Demonstrating that these forms of

plasticity depended on maternal contact with offspring was a crucial first step to suggest that the neuroplasticity observed in postpartum females could be functionally significant, but we have not yet tested whether the newborn DR cells were synaptically integrated. Integration of pubertally born neurons in the hypothalamus of male hamsters was demonstrated using immediate early gene expression in response to sexual encounters (Mohr & Sisk, 2013). Future studies outside the realm of this dissertation should explore the functional integration of new neurons in the maternal DR, perhaps by measuring immediate early gene expression in newborn neurons in response to a reunion with young pups. If new neurons are functionally integrated into the maternal DR, determining the chemical phenotype of these neurons would be the next step in determining their role. New DR neurons could be make a variety of neurochemicals, but two of the most likely phenotypes would be serotonergic or GABAergic. Many adult-born olfactory neurons that proliferate in the SVZ lining the lateral ventricles migrate to the olfactory bulb and develop into GABAergic neurons (for review, see Sakamoto et al., 2014). Many DR neurons are GABAergic (Day et al., 2004) and these GABAergic neurons respond to serotonin receptor modulators and control activity of serotonergic neurons (Boothman & Sharp, 2005). Instead of playing this modulatory role, newborn DR neurons could directly contribute to serotonergic function of the DR by becoming serotonergic neurons themselves. Newborn serotonergic neurons isolated from fetal tissue can survive in the adult brain (Zhou & Azmitia, 1990; Ueda et al., 1996; Daszuta et al., 1988) and endogenous newborn cells differentiate into dopaminergic neurons in the substantia nigra (Zhao et al., 2003; Zhao et al., 2009) so it is clear that the adult midbrain can support integration of new monoaminergic neurons.

Although we have not yet demonstrated functional integration of the newborn DR cells, the timing of alterations in newborn cell survival in the DR suggest that altered DR

neuroplasticity may facilitate postpartum modulation of mothers' behavior eventually leading to the weaning of offspring. The highest levels of newborn cell survival occur when maternal caregiving and aggression are high and anxiety is low, whereas the lowest levels occur as these behaviors return to virgin levels. Given that reductions in DR cytogenesis and serotonin synthesis/metabolism occurred concurrently with reductions in postpartum caregiving and aggression and increases in anxiety, reduced serotonin from the DR may play a critical role in postpartum adjustments in behavior as offspring develop.

Serotonin synthesis and metabolism in the maternal DR

In addition to neuroplasticity, I hypothesized that mothering would influence neurochemical function of the DR, so we measured numerous aspects of the serotonin synthetic and metabolic pathway. Although it seems that postpartum mice have lower serotonin immunoreactivity in the DR compared to nulliparae (Jury et al., 2015), we found via Western blotting that postpartum rats had equivalent TPH2 compared to nulliparae, and HPLC and IHC revealed a nearly identical nonsignificant difference in serotonin content in the DR across reproductive state. Jury and colleagues noted that their difference in immunoreactivity was due to a loss of serotonin-immunoreactivity in the DRlw of postpartum mice (Jury et al., 2015), but if subregion specific measures were taken in this study, they were not described. In our experiments, tissue punches analyzed by Western blot or HPLC did not include the entire DRlw, and this could have contributed to the discrepancy, but we did do microscopic analyses of serotonin immunoreactivity in each subregion of the DR and found no effect of reproductive state in any subregion. Moreover, rostrocaudal levels analyzed in mice were not described so direct comparisons are difficult to make. Finally, it is possible that the discrepancy is due to they

days of sacrifice. Whereas we compared diestrus virgins to PPD 8 and PPD19 rats, Jury and colleagues compared virgins (estrous state unknown) to PPD 10 mice. As discussed above, ovarian hormones regulate activity of serotonergic neurons, so stage of the estrous cycle in the virgin mice could have increased DR serotonin above diestrus levels and contributed to the differences in results between the studies. Despite equivalent levels of serotonin and TPH2 in virgin, PPD 8 and PPD 19 rats, the early postpartum increase and late postpartum decrease in serotonin synthesis/metabolism within the DR could lead to altered serotonergic innervation or release at terminal sites of the raphe nuclei within the forebrain and midbrain. In support of this, early postpartum rats have higher serotonin turnover in the medial preoptic area, bed nucleus of the stria terminalis, and the hippocampus (Lonstein et al., 2003; Smith et al., 2013; Desan et al., 1988; Glaser et al., 1992) compared to nulliparae.

Brain regions involved in the perception of touch, which is the most important determinant of postpartum behavior, also receive serotonin from the DR (Kirifides et al., 2001; Simpson et al., 2003; Sheikhkanloui-Milan et al., 2010). Serotonin from the DR supports plasticity in the somatosensory cortex of adult female rats after spinal injury (Ganzer et al., 2013). If serotonin is similarly involved in postpartum plasticity in the somatosensory cortex of new mothers, this could be one way serotonin promotes postpartum behavioral adaptations. Increased ventrum representation in the somatosensory cortex of new mothers (Xerri et al., 1994; Rosselet et al., 2006; Rosselet et al., 2008) makes new mothers more sensitive to suckling and non-suckling contact from the offspring. SSRI treatment increases extracellular serotonin and reduces tactile pain (e.g. Leventhal et al., 2003) and multiparous women report more pain during nursing than women nursing their first infant (Holdcroft et al., 2003). Multiparous postpartum rats spend less time being maternal and have higher hippocampal cell survival compared to

primiparous postpartum rats (Pawluski & Galea, 2007), suggesting that differences in neuroplasticity could contribute to differences in nursing pain and maternal behavior. These alterations somatosensory perception across reproductive state and parity could affect anxiety, aggression, and caregiving through the serotonin system because serotonin is involved in both touch (Arora & Chopra, 2013; Nagakura et al., 2009; Hole & Berge, 1981; Kulkarney & Robert, 1982) and socioemotional behavior (Donner & Handa, 2009; Charoenphandhu et al., 2011; Hiroi et al., 2011; Ferreira et al., 2000; Zhao & Li, 2010; da Veiga et al., 2011; Chen et al., 2014). Thus, neurochemical function of the DR could mediate the effects of tactile input from the pups on postpartum behavior and physiology.

If so, serotonin-specific lesions of the DR would be expected to be particularly devastating in new mothers. Although we did not find any effect of lesioning the DR on postpartum anxiety, lesioning the DR of postpartum rats interfered with numerous social behaviors. Lesioning the serotonergic DR prevented postpartum adaptations in nursing such that instead of reducing the frequency of kyphosis over time, DR lesioned rats maintained high levels of this nursing posture. Conversely, lesioned dams almost never used the more passive, supine nursing posture that becomes more common as lactation progresses (Stern and Levine, 1973). Consistent with these findings is our recent finding that TPH2 expression in the DR of intact postpartum rats was positively correlated with supine nursing postures in an experiment that is not included in this dissertation (Holschbach et al., *in prep*). Serotonin increases kyphosis in crayfish that adopt this posture as a sign of aggression (e.g. Tierney & Mangiamele, 2001) so such a relationship may be specific to nursing rather than posture in general.

In addition to its effects on nursing postures, lesioning the DR reduced pup licking, an important component of maternal caregiving in rodents that may lead to differences in

socioemotional behavior of offspring (Starr-Phillips & Beery, 2014; Ragan et al., 2015). Previous reports have indicated that peripheral injections of serotonin receptor antagonists also reduce maternal licking (Zhao et al., 2009; Zhao et al., 2010), and our data more specifically implicate DR serotonin in this caregiving behavior. Finally, lesioning the serotonergic DR of postpartum rats reduced maternal aggression. This result seems at odds with most reports of the role of serotonin in maternal aggression or other forms of territorial aggression (for reviews, see Oliver & Mos, 1992; Carrillo et al., 2009), but there are notable exceptions. For example, adaptive levels of aggression are positively correlated with central serotonin in male rats (Van der Vegt et al., 2003) and mice lacking the enzyme that metabolizes serotonin are more aggressive in various paradigms, including an intruder test (Vishnivetskaya et al., 2011). Moreover, increasing synaptic serotonin via SSRI increases maternal aggression (Johns et al., 2005) and infusing serotonin-2A/2C receptor agonists into the central amygdala also increases maternal aggression (Almeida et al., 2005). These inconsistent findings demonstrate the complexity of the serotonergic system and the need for more thorough examination of its role in not only aggression but also many other behavioral and physiological processes. Some of the complexity is due to the structure of the system itself: serotonergic projections are ubiquitous throughout the brain, which expresses at least 14 different types of serotonin receptors (for review, see McCorvy & Roth, 2015)

We also predicted that lesioning the DR would reduce lactation because the suckling-induced prolactin and oxytocin surges necessary for milk production and release rely on DR serotonergic efferents to the hypothalamus (Crosignani et al., 1979; Moos and Richard, 1983; Bodnar et al., 2009). The suckling-induced prolactin surge is reduced by infusion of glutamate receptor antagonists into the DR in rats (Bodnar et al., 2009) and completely prevented by either

blockade of serotonin synthesis (Kordon et al., 1973), lesions of serotonergic innervations of the hypothalamus (Bodnar et al., 2002), or hypothalamic infusions of a serotonin receptor antagonist in rats (Gallo et al., 1975). Despite these findings, we found that pups fed by DR-lesioned dams gained as much weight each day as pups fed by control females. Some factors that might explain this finding include that: 1) litters were alternated daily between lesioned and control dams, which would prevent any additive effect of continuous feeding with a lesioned dam, 2) litter weight gain was measured after a full day of feeding rather than after a brief feeding challenge so if the lesion affected temporal control of milk letdown and ejection, we probably would not have seen such an effect, and 3) lesioned dams showed more kyphosis than control dams, which might have helped the pups ingest more milk than they would have otherwise because milk letdown is most readily elicited when dams are in kyphosis compared to other nursing postures (Lonstein et al., 1998). We also expected that lesioning the DR would impair pup retrieval because serotonin receptor antagonists increase the latency to retrieve pups (Zhao & Li, 2009). During the first retrieval test, lesioned animals seemed to take longer to retrieve their litters than control dams, but this was not statistically significant and the retrieval latencies of lesioned and control dams were matched in the second and third tests. It is possible that using multiple retrieval tests gave DR lesioned dams enough practice to overcome any deficit caused by reducing DR serotonin, in which case one test at the end of the experiment when lesions are fully established might have revealed a role of DR serotonin in pup retrievals. In fact, one practice retrieval almost completely prevented retrieval deficits after perioral anesthesia in postpartum rats (Stern & Kolunie, 1998). An ongoing experiment is testing the role of DR serotonin in establishing postpartum behaviors by performing similar lesions during pregnancy before the initiation of maternal behaviors and will test retrieval only once to avoid this consideration.

Perhaps the most surprising result was that lesioning the serotonergic DR of new mothers did not affect postpartum anxiety. The Anti-SERT-Saporin infusions performed in this dissertation targeted the DRv for many reasons, including that in nulliparous animals this subregion is more often associated with anxiety-like behavior whereas the DRlw is more often associated with panic (Paul et al., 2014; Johnson et al., 2004; Lowry et al., 2008). As a result, the lesions were least effective in the DRlw, and it is possible that this subregion is more closely linked with anxiety in postpartum females than nonreproductive or male rodents. We previously showed that exposing postpartum rats to the elevated plus maze activates serotonergic and nonserotonergic DR neurons and that the number of activated serotonergic neurons in the DRlw tended to be positively correlated with open arm exploration (Holschbach, Smith, & Lonstein, *unpublished data*). Moreover, we found that TPH2 expression in the MR was positively correlated with open arm exploration in intact postpartum rats (Holschbach, Grieb, & Lonstein, *in prep*). Perhaps eliminating serotonin from one of these two sites would be more effective in increasing postpartum anxiety.

This dissertation provides insight into the unique nature of the peripartum serotonergic DR and increases our understanding of the role of DR serotonin in maternal behavior. Because the serotonergic system is a main target of pharmaceutical interventions for emotion and mood disorders, which when untreated can be devastating for postpartum women and their infants (Lonstein, 2007; Lonstein et al., 2014; Grigoriadis et al., 2013), it was essential to know how manipulations of the serotonergic system in new mothers affected their maternal behavior. Postpartum mice and women are more sensitive than nonpostpartum females to the effects of SSRIs (Jury et al., 2015) and it is possible that the neuroplasticity and neurochemical changes discovered here contribute to this increased sensitivity. I hope that this work

demonstrates the importance of further research into the impact of serotonin signaling for mother-infant behavioral interactions and bonding.

The discoveries of neuroplasticity and concomitant changes in serotonin synthesis/metabolism within the DR represent a new avenue for experiments involving serotonin-sensitive processes. Serotonin from the DR is critical in many behavioral and physiological processes that are not unique to new mothers and these forms of plasticity should be assessed in other models during future studies. For example, the onset of puberty requires serotonin (Monroy et al., 2003; Ayala et al., 1998) and reproductive senescence reduces serotonin (Bethea et al., 20011; Carretti et al., 2007) so DR plasticity may be similarly involved in these reproductive milestones. Even outside the realm of reproduction, DR serotonin enhances many aspects of reward processing (for review, see Luo et al., 2015; Kranz et al., 2010) and restores some Alzheimer's disease-related pathologies (for review, see Trillo et al., 2013). I hope that these first demonstrations of neuroplasticity-associated enhancements and reductions in DR serotonin synthesis/metabolism open the door for future studies looking at DR plasticity involvement in development, aging, learning, and neurodegenerative disorders.

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