I, Nicholas J Jury, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Neuroscience/Medical Science Scholars Interdisciplinary.

It is entitled:
Alterations in Peripheral and Central Serotonin Physiologies during Lactation: Relevance to Mood during the Postpartum Period

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Alterations in Peripheral and Central Serotonin Physiologies during Lactation: Relevance to Mood during the Postpartum

A dissertation submitted to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

In the Neuroscience / Medical Science Scholars Interdisciplinary 2012

by

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GENERAL ABSTRACT

The discovery of a novel serotonergic system within the mammary gland has led us to investigate central and peripheral serotonergic systems during lactation in an intact, non-depressed phenotype mouse model. During lactation we observed significant changes in both central and peripheral serotonergic physiologies. Platelet serotonin (5-HT) was elevated in lactating dams when compared to virgin animals. 5-HT immunostaining was significantly lower in the dorsal raphe of lactating mice than nulliparous mice. These findings are what led us to probe the serotonergic systems with a selective serotonin reuptake inhibitor (SSRI), citalopram. Lactating, nulliparous, and postpartum/non-lactating mice were all given a subchronic treatment regimen with SSRI and then subjected to behavioral tasks to assess mood-related behavior. Strikingly, only lactating dams responded to SSRI treatment with an improvement of mood-related behaviors. Locomotor and home-cage activity tasks indicated that these changes in behavior were specific to mood-related behaviors, and not due to an alteration of sensorimotor function. The novel findings from these studies are that lactating mice exhibit an elevated mood-related behavioral phenotype and respond to SSRI treatment that is ineffective in non-lactating mice. The current study shows that there is an interaction between lactation status and responsiveness to SSRIs, which has important implications for the treatment of postpartum depression (PPD).

The immunostaining and behavioral results provide evidence of the potential enhancement of serotonergic activity into the projection fields of the brains of lactating mice. Using Palkovits micropunch technique we determined that there were changes in 5-HT and 5-HIAA activity within the posterior basolateral amygdala (PBLA), a brain
region that may participate in mood regulation, of lactating mice. Furthermore, treatment with SSRI significantly reduced 5-HIAA content and increased 5-HT content within the PBLA of lactating dams. These results support the hypothesis that there is altered serotonergic activity within limbic projection fields. Again these changes were induced only within brains of lactating mice.

The elevations in platelet 5-HT that were observed in lactating mice from our initial study led us to hypothesize that the upregulated serotonergic system within the mammary gland was responsible. Using a mammary specific knockout and overexpression mouse model of tryptophan hydroxylase, the rate limiting enzyme in 5-HT biosynthesis, we demonstrated that the mammary gland is the source of the elevated platelet 5-HT observed in lactating mice.

Taken together these findings have significant clinical implications for the treatment of PPD. The clinical literature often does not address the breastfeeding (lactation) status of women when reporting or conducting studies in PPD. These data show that lactation is important and that there should be renewed interest in researching the interaction between breastfeeding and responsiveness to SSRI treatment during the postpartum period.
ACKNOWLEDGMENTS

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I am deeply indebted to my dissertation committee members, Jim Eliassen, Jim Herman, Nelson Horseman, and Kim Seroogy for all of their scientific guidance and advice. Thank you for challenging me, providing excellent experimental design critiques, and helpful career advice.

I would also like to thank my fellow lab members, Mike Murawsky, Heather Christensen, and Cheryl Minges for all of their help assisting me with experiments and technical questions. Thanks for your friendship and making the lab a fun place to work no matter what time of day.

I would like to thank Deb Cummins for her moral support. Thank you for always going above and beyond what was in your job description. It is because of you that I was able to keep my sanity through this whole process.
I thank my family because I could not have made it this far without your love and support. Thank you mom, dad, Brian, Aunt Lynn, Grandma and Grandpa Cox, and Adam for being my support and strength. Thanks for always believing in me and encouraging me to pursue my dreams. Thank you for listening to me, even when you did not completely understand what I was talking about.

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<td>5-HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>11βHSD1</td>
<td>11β-hydroxysteroid dehydrogenase 1</td>
</tr>
<tr>
<td>11βHSD2</td>
<td>11β-hydroxysteroid dehydrogenase 2</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>APGN</td>
<td>Allopregnanolone</td>
</tr>
<tr>
<td>BB</td>
<td>Baby blues</td>
</tr>
<tr>
<td>BDI</td>
<td>Beck Depression Inventory</td>
</tr>
<tr>
<td>CBM</td>
<td>Cognitive-behavioral model</td>
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<tr>
<td>CBT</td>
<td>Cognitive-behavioral therapy</td>
</tr>
<tr>
<td>CORT</td>
<td>Corticosterone</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>E</td>
<td>Estrogens</td>
</tr>
<tr>
<td>EPDS</td>
<td>Edinburgh Postnatal Depression Scale</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotrophin</td>
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<tr>
<td>HIP</td>
<td>Hippocampus</td>
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<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
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<td>HYP</td>
<td>Hypothalamus</td>
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<td>IM</td>
<td>Interpersonal model</td>
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<td>Definition</td>
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<tr>
<td>IPT</td>
<td>Interpersonal psychotherapy</td>
</tr>
<tr>
<td>MDD</td>
<td>Major depressive disorder</td>
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<tr>
<td>MG</td>
<td>Mammary gland</td>
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<tr>
<td>PBLA</td>
<td>Posterior basolateral amygdala</td>
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<td>P</td>
<td>Progesterone</td>
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<td>PPD</td>
<td>Postpartum depression</td>
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<tr>
<td>PRL</td>
<td>Prolactin</td>
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<td>SERT</td>
<td>Serotonin transporter</td>
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<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
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<td>TCA</td>
<td>Tricyclic antidepressants</td>
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<td>TCM</td>
<td>Transactional conflict model</td>
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<td>TIDA</td>
<td>Tubero-infundibular dopamine</td>
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<td>TPH</td>
<td>Tryptophan hydroxylase</td>
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<td>TRP</td>
<td>Tryptophan</td>
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<tr>
<td>VLPAG</td>
<td>Ventrolateral periaqueductal grey</td>
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CHAPTER I

General Introduction and Literature Review


**Postpartum Depression: A Public Health Concern**

**Definition, Incidence and Prevalence**

Postpartum depression (PPD) is defined as a major depressive disorder (MDD) that occurs four weeks to six months following parturition (Amaya-Jackson et al., 2000; Meltzer-Brody, 2011). PPD is a major public health concern with approximately 10-15% of women being diagnosed annually (Stuart et al., 1998; Gavin et al., 2005; Gaynes et al., 2005; Dietz et al., 2007). Recent estimates indicate that 7% of women experience a major depressive episode within the first three months following child birth (Segre et al., 2007), however when minor episodes of depression are included the prevalence rate climbs to 20% (Gavin et al., 2005). Some studies suggest that PPD is not more common than the normal incidence of MDD in women (Philipps and O’Hara, 1991; O’Hara et al., 1991a), but one very large epidemiological study suggests that there is an increased incidence of depression during the postpartum period (Vesga-Lopez et al., 2008). PPD is the leading cause of maternal morbidity and mortality worldwide (Lindahl et al., 2005).

The postpartum period is a time when there is an increased risk for the development or recurrence (Wisner et al., 2004) of mood and anxiety disorders (Cox et al., 1993). Depression is more common among women and specifically women of childbearing age than men (Weissman and Olfson, 1995). Premenopausal women who have previously been diagnosed with MDD or other affective disorders are more susceptible and much more likely to be diagnosed with PPD (Munk-Olsen et al., 2006; Dossett, 2008). The healthcare costs to mothers diagnosed with PPD are 20% higher
than non-PPD mothers, and the costs are highest among mothers who have a prolonged bout of PPD (Petrou et al., 2002). The economic impact of depression in the United States is estimated to cost $30 to $50 billion in lost productivity and direct medical costs annually (Robinson et al., 2005; Gjerdingen and Yawn, 2007). PPD has contributed to the rise in healthcare costs in the United States for decades. It was only recently that the United States Congress enacted the Melanie Blocker Stokes Mothers Act in 2010. This act is a subsection of the Patient Protection and Affordable Care Act and federally mandates initiatives to expand research and education of PPD (Meltzer-Brody, 2011).

Presentation, Assessment and Diagnosis

Patients diagnosed with PPD present with a majority of classically identified symptoms: depressed mood, anhedonia, sleep and appetite disturbance, impaired concentration, psychomotor disturbance, lethargy, feelings of worthlessness or guilt, and suicidal ideation (Meltzer-Brody et al., 2011). Suicidal thoughts are the least common symptoms among women with PPD, but more common among women with postpartum psychosis (Lindahl et al., 2005). Women who experience symptoms of negative mood, crying, anxiety and confusion within a week of delivery are said to have the baby blues (BB). The blues are considered to be a mild form of dysphoria and usually are alleviated two weeks after parturition (O'Hara et al., 1991a). The symptoms of BB are less severe than PPD and resolve spontaneously without any treatment. It is estimated that 50-80% of women who deliver experience symptoms of BB (Henshaw,
2003). Even though BB are common among women who deliver children, it is still debated whether or not BB are a risk factor for PPD (Bloch et al., 2005).

The primary method for identifying and assessing patients with PPD is through answers to written questionnaires. These are usually administered by a physician, but can also be administered by a nurse or other healthcare professional. The two most common questionnaires are the Edinburgh Postnatal Depression Scale (EPDS) and Beck Depression Inventory (BDI). EPDS is a ten item questionnaire that patients are given. The questions target symptoms of depression and anxiety from the previous week. Each question is scored based on a point value system and patients who accrue a minimal threshold score of twelve points or more are determined to be “depressed” (Cox et al., 1987). BDI has more questions to evaluate depression and anxiety than EPDS, but the questions are similar. If the patient accrues more than ten points from the BDI then a diagnosis of mild depression is assigned (Beck et al., 1961). The scores are further divided into bins whereby 10-18 points indicate mild depression, 19-29 points indicate moderate depression, and 30-63 points indicate severe depression (Beck et al., 1961; Beck and Gable, 2001). Even though these two questionnaires are a great tool for screening and diagnosing women with PPD, the EPDS and BDI are not fool-proof and some inconsistencies have been identified. In one study an EPDS score of greater than ten was able to successfully identify women with PPD in the fourth week following parturition 60% of the time (Peindl et al., 2004). In other studies there was much greater variability in the determination of a depression diagnosis that was most likely due to inconsistencies in the minimum threshold scores (Gjerdingen and Yawn, 2007). The fact that different questionnaires have more than one type of depression
classification (e.g. mild, moderate, and severe) could explain why one screening test may successfully detect PPD, but others may not. The lack of standardization of the minimal threshold scores for the diagnosis of PPD in these questionnaires poses a problem to physicians and healthcare workers. The standardization of the scores could decrease the amount of false positive or false negative identifications of PPD. New screening methods for PPD would greatly improve the assessment and screening of women during the postpartum period (Fergerson et al., 2002).

Mother and Child Outcomes

Mothers afflicted with PPD have been reported to have more frequent visits to healthcare providers and complain of more health issues in general. This increase in frequent visits to healthcare providers in turn leads to a decrease in work productivity due to the mother taking more time off from work. Women with PPD also complain of enhanced difficulty in completing common everyday tasks (Gjerdingen et al., 2009). The depressive symptoms of PPD also can put a strain on marital and family relationships. Male spouses of women with PPD are more likely to become depressed (Burke, 2003).

The relationship between untreated maternal depression and negative infant outcomes is well established. Depression during the postpartum period has been linked to both short-term and long-term cognitive, emotional, and behavioral abnormalities in children (Murray and Cooper, 1993; Grace et al., 2003; Hay et al., 2008). There is also an increased risk of childhood psychiatric disorders in children born to mothers with PPD (Goodman et al., 2011). Women experiencing PPD are more
likely to express negative emotions and respond less to signals from infants (Murray et al., 1993; Field, 2010). Mothers diagnosed with PPD are less likely to have positive parenting interactions with the child such as reading, singing, and storytelling (Paulson et al., 2006). Often these same women can be withdrawn due to the stress of the infant crying, or hostile towards the infant (Lovejoy et al., 2000; Martins and Gaffan, 2000). The lack of positive interaction between the mother and her child may have implications on the cognitive development of the infant into adolescence (Sharp et al., 1995; Hay and Kumar, 1995; Hay et al., 2008). Women diagnosed with PPD are less likely to breastfeed their infants and are more likely to terminate breastfeeding prematurely (Ip et al., 2007; Dennis and McQueen, 2009). Studies have been conducted that have linked successful breastfeeding and normal neurodevelopment of the infant into adolescence (Lucas et al., 1992).

Treatment of PPD

Previously women who had been diagnosed with PPD were not normally included in treatment studies along with other patients who had been diagnosed with non-postpartum depression, MDD (O’Hara et al., 2009). It was thought that PPD and other forms of depression were functionally similar and therefore could easily be treated using the same approaches. The postpartum period is often stressful with the mother having to adjust to a new social role, sleep deprivation, changes to her social support structure, and interpersonal dynamics (O’Hara et al., 2009; Meltzer-Brody, 2011). These factors make treating women diagnosed with PPD much different than treating non-postpartum MDD. Within the last two decades the amount of research focusing on
depression during the postpartum period has more than doubled with a plethora of clinical studies having been conducted (Dietz et al., 2007; O’Hara et al., 2009; Pearlstein et al., 2009; Ng et al., 2010; Meltzer-Brody, 2011). These studies have attempted to identify the most efficacious and safe therapies for the alleviation of PPD symptoms.

Primary treatments for PPD include psychotherapy, pharmacological treatment with antidepressants, and a combination of both psychotherapy and antidepressant treatment (Pearlstein et al., 2008; Meltzer-Brody, 2011). The two most common types of psychotherapy that are currently used to treat PPD are interpersonal psychotherapy (IPT) and cognitive-behavioral therapy (CBT). IPT focuses primarily on the changes to a woman’s social and interpersonal interactions that occur after parturition. The primary goal of IPT is to enhance the mother’s social support network by helping her reach out to family and friends (O’Hara et al., 2000). Randomized controlled studies found that women diagnosed with PPD and that also participated in IPT sessions exhibited a reduction in depression and an enhancement in social adjustment when compared to control subjects (O’Hara et al., 2000; Klier et al., 2001; Clark et al., 2003; Stuart, 2012).

CBT is another common treatment for PPD and it focuses on identification and modification of negative thoughts. CBT equips the mother with problem solving skills to handle stressful situations. Ultimately, CBT promotes positive thoughts and behavior designed to allow a mother to cope with depressive symptoms and feelings (Rush et al., 1981; Dennis and Hodnett, 2007). Many studies have been conducted with CBT and have determined that CBT is a superior treatment for PPD (Appleby et al., 1997). However, a recent study determined that IPT was more efficacious in the treatment of
PPD than CBT (Sockol et al., 2011). Pharmacologic treatment with antidepressants is the most common and most utilized form of treatment for PPD when compared to IPT and CBT. Selective serotonin reuptake inhibitors (SSRIs) are the most popular treatment for depression, and are seen as a first line of defense in the treatment of PPD (Yonkers, 2003; Pearlstein et al., 2008). Despite the fact that only a few, first time, randomized controlled studies have been conducted with SSRIs as the primary treatment for PPD, SSRIs remain the most widely prescribed antidepressant medications for women diagnosed with PPD (Appleby et al., 1997; Wisner et al., 2006; Pearlstein et al., 2008; Ng et al., 2010). Tricyclic antidepressants (TCAs) have also been prescribed and have successfully treated PPD. One study determined that pharmacological treatment with TCAs was just as effective as treatment with SSRIs for the alleviation of PPD symptoms (Wisner et al., 2006). Combination therapy that utilizes pharmacological drug treatment and psychotherapy has been shown to be successful in treating PPD as well (Appleby et al., 1997; Misri et al., 2004). However, there is not enough evidence to demonstrate that any one of the three aforementioned treatments is more effective than the other for the successful treatment of PPD symptoms (Sockol et al., 2011). Even though there is evidence to suggest that pharmacological treatment of PPD with SSRIs is safe (Wisner et al., 2006; Fortinguerra et al., 2009) many women are still hesitant to breastfeed while taking these medications (Chabrol et al., 2004).

Clinical Studies: Proposed Models of PPD Etiology
Despite the abundance of clinical studies that have been conducted, the etiology and pathophysiology of PPD are currently not known. This is most likely due to the fact that there are many different models that are used to describe the etiology of PPD. The three most widely referenced models of the etiology of PPD are the cognitive-behavioral model (CBM; O’Hara et al., 1982), the interpersonal model (IM; Cutrona and Troutman, 1986), and the transactional conflict model (TCM; Hayes et al., 2000).

The CBM states that the onset of PPD is due to psychological vulnerabilities after experiencing a stressful life event such as childbirth. This model also predicts that women who have had a previous bout of MDD are more likely to develop symptoms of PPD following pregnancy. O’Hara and colleagues conducted a study where a selected population of 170 women who had experienced stressful life events and symptoms of MDD prior to pregnancy would be more likely to experience symptoms of PPD (O’Hara et al., 1982). The results demonstrated that cognitive-behavioral vulnerabilities that were assessed prenatally were able to predict the onset of PPD (O’Hara et al., 1982). Although the study found a significant difference between those women that developed symptoms of PPD, the cognitive-behavioral vulnerabilities only accounted for 4% of the variation in the incidence of PPD. However, when life stresses and social adjustments were included in the analysis they accounted for 40% of the variation in the number of subjects who developed PPD. In a follow-up study O’Hara and colleagues determined that both negative cognitive deficits and a history of stressful life events were risk factors for the development of PPD (O’Hara and Swain, 1996). The CBM successfully identified some of the risk factors that were used to identify women at risk for PPD. The CBM is inconsistent in that it predicts that early treatment interventions prior to
pregnancy, or during should prevent symptoms of PPD, but this has not been demonstrated. Even though some of the risk factors identified by the CBM can be treated using a prophylactic approach, there are conflicting studies about the effectiveness of treating at risk individuals for PPD. One study found that treating women during the postpartum period was more advantageous than treating prior to parturition (Dennis and Creedy, 2004). Other studies found that treatment with CBT, IPT, and/or pharmacological treatment with antidepressants prevented the development of PPD symptoms (Zlotnick et al., 2001; Wisner et al., 2004; Cho et al., 2008). The discrepancies between these studies indicate that further research is needed to investigate the underlying etiology of PPD. It also indicates that more research needs to be conducted into which risk factors are more predictive of the development of PPD.

The IM suggests that a woman’s social support network and her role transitioning into being a mother are related to the onset of PPD (Cutrona and Troutman, 1986; Beck, 2002). Studies conducted using this model were able to successfully identify a link between a mother’s perceived feelings about her social support network and the severity of ensuing PPD symptoms following parturition (Cutrona and Troutman, 1986). The perceived feelings about the adequacy of the mother’s social support network and her parenting role contributed to 55% of the variance in PPD symptoms (Cutrona and Troutman, 1986). The use of IPT to treat PPD according to this model has been relatively successful with mood improvements noted after several sessions (O’Hara et al., 2000; O’Hara, 2009).

The TCM was recently proposed by Hayes and colleagues, and it states that the etiology of PPD is due to an intersection of the dysfunction of the psychobiological
mechanisms in the brain and Western culture of social isolation following childbirth (Hayes et al., 2000). The TCM states that the medical and family practices that are common in Western cultures are also the primary triggers in causing the appropriate changes to physiological and neurophysiological mechanisms leading to PPD. The pressure of mothers to return to work and the social isolation during the postpartum period that is frequent in Western cultures may drive the etiology of PPD according to this particular model (Hayes et al., 2000). In other cultures the family and extended-family are much more involved with the delivery process and the care for a newborn child. The TCM attempts to combine the results from both the CBM and IM in trying to explain the etiology of PPD. Many aspects of the TCM have not been tested and the treatment implications of this particular model are not exactly clear. The obvious flaw in the TCM’s explanation of the etiology of PPD is that women from non-Western cultures would have a lower incidence rate of PPD, but this has not been demonstrated (Cox et al., 1993; Gavin et al., 2005).

The existing clinical models of the etiology of PPD account for a significant amount of variance in the literature. However, of the models that have been tested, the strongest predictor of PPD was depression during pregnancy, rather than cognitive vulnerabilities or lack of social support, as the models suggest (O’Hara and Swain, 1996). Each model of PPD has distinct treatment implications. The best treatment for women based on these various models has not been tested fully and requires further investigation. For example, it is yet to be determined whether women with PPD would benefit more from CBT, IPT, or pharmacologic treatment with antidepressants. Additional studies need to be conducted in order to improve treatment selection and
efficacy for women with PPD. Furthermore, better models of the etiology of PPD need to be pursued so that the underlying cause and pathophysiology of PPD can be identified. Once a better model of the etiology has been determined the treatment options should be clearer in treating the symptoms of PPD.

**Hormonal Changes during Pregnancy and Lactation**

*Pregnancy*

The mean duration of human pregnancy lasts 266 days (38 weeks), however the gestational age can range from 37 to 42 weeks post ovulation. In contrast, the duration of gestation the rodent (e.g. mice or rats) averages from 18 to 22 days (Crawley, 2000). The human pregnancy is broken up into three trimesters and the child is usually delivered at the end of the third trimester. The analogous third trimester in a rodent occurs after parturition outside of the womb (Crawley, 2000).

Upon the fertilization of the oocyte by the sperm, the zygote moves down the fallopian tube into the uterus where it can begin the process of implantation through the epithelium of the uterus and into the decidualized endometrium (Smith and Lau, 2010). Progesterone (P) produced by the mother inhibits the movement of the conceptus down the uterus and stimulates the growth of the endometrial lining, thus promoting its implantation. The blastocyst releases human chorionic gonadotrophin (hCG) and this signals to the brain of the mother that the fertilized egg has successfully implanted within the uterus (Smith and Lau, 2010). hCG continues to be released from the developing conceptus and replaces the function of maternal luteinizing hormone (LH).
Instead of LH stimulating the corpus luteum in the ovary, the continued release of hCG provides a constant stimulation to the corpus luteum. This action increases P production, release, and signaling to the mother. Both hCG and P inhibit the ovarian cycle and regulate the hold of the conceptus in the uterus (Smith and Lau, 2010). Estrogen (E) and P increase dramatically during the first trimester to levels much higher than during the menstrual cycle. P reduces the motility of the uterus, and prevents contractions. These actions ensure that labor is not initiated prematurely and that the developing embryo remains in the decidualized endometrium of the uterus (Boron and Boulpaep, 2011). Plasma concentrations of E continue to climb into the third trimester and are one hundred times higher than those prior to pregnancy (Boron and Boulpaep, 2011). P concentrations are also one hundred times higher during the third trimester than those during the pre-follicular phase of the menstrual cycle (Boron and Boulpaep, 2011). The E and P concentrations are maintained primarily by the maternal-placental unit. Both the mother’s ovaries and the placenta provide the elevated levels of E and P. The ovarian hormones remain at these elevated concentrations until dropping suddenly after parturition where they return to pre-follicular levels (Boron and Boulpaep, 2011). Many have hypothesized that the precipitous withdrawal of the ovarian hormones after child birth may explain the underlying cause of PPD. The hormone withdrawal hypothesis and its attributes will be addressed later.

During the later part of the third trimester small and inconsistent contractions within the uterus begin. These contractions continue and become more forceful and rhythmic leading to the thinning of the cervix (Boron and Boulpaep, 2011). The contractions are induced by the action of oxytocin binding its cognate receptors within
the cervix. E increases the number of oxytocin receptors within the myometrium and decidual tissue during pregnancy, thus increasing the responsiveness of the tissue to oxytocin. Furthermore, prostaglandins and E enhance the number of gap junctions within the cervix allowing for more uniform and rhythmic contractions to occur (Boron and Boulpaep, 2011). The contractions continue until the fetus and placenta are ejected from the mother and parturition has occurred.

Lactation

The mammary gland (MG) is the functional secretory unit responsible for breastfeeding an infant after delivery. During puberty E and P promote the development of the breast through lobuloalveolar and ductal growth. The alveoli within the MG are organized into lobules that each drain into ductules and groups of twenty ductules drain into the lactiferous duct (Boron and Boulpaep, 2011). The elevated concentrations of E and P during pregnancy play an important role in the development of the gland in preparation for lactogenesis. These hormones promote the branching of the ducts to become larger and more extensive prior to parturition. Prolactin (PRL), the principle prolactogenic hormone, is present at increasing concentrations at the end of pregnancy and also promotes the final development of the MG for the production of milk. In pregnant women circulating PRL increase 15-fold during mid-pregnancy (Gaynes et al., 2005). PRL released from the anterior pituitary is obligatory in the production of milk within the mammary gland and fertility (Horesman and Gregerson, 2005). Early in rat pregnancy PRL is secreted in surges in the afternoon and night and has a luteotrophic role. Its secretion is suppressed in mid-pregnancy, when
placental lactogenic hormone secretion predominates (Brunton and Russell, 2008). The ejection of the milk is initiated by the presence of a suckling stimulus to the nipple and a subsequent release of oxytocin from the posterior pituitary (PP). Oxytocin is released from the PP in rapid pulses that quickly lead to milk let-down. The MG responds to high plasma concentrations of oxytocin and rapidly desensitizes to oxytocin. This makes a pulsatile release of oxytocin obligatory for milk ejection (Leng and Brown, 1997; Soldo et al., 2004). Oxytocin causes contraction of smooth muscles within the MG and milk ejection follows (Leng et al., 2005). The sucking stimulus causes the release of both PRL and oxytocin in a coupled manner, and oxytocin has been hypothesized to be a PRL releasing factor due to its ability to reach the anterior pituitary (Horsman and Gregerson, 2005). PRL and oxytocin continue to remain elevated daily with the presence of a suckling stimulus, but eventually these hormones are reduced upon weaning of the infant (Leng et al., 2005).

Neuroendocrine Induced Changes to Brain Function, Structure, and Behavior during Pregnancy and Lactation

The multiple neuroendocrine changes that occur throughout pregnancy not only allow for the successful development and delivery of the infant, but they are also crucial in establishing the appropriate neural circuitry that maternal behavior depends upon. The continued exposure of the brain to hormones (e.g. oxytocin and PRL) during lactation is elicited by the external stimulation of suckling during breastfeeding and social bonding (Brunton and Russell, 2008). It seems reasonable to predict that the
possible dysfunction or inhibition of any of the neuroendocrine processes involved in the remodeling of the brain neural circuitry may play a role in the etiology of PPD.

**Attenuation of the HPA axis during Pregnancy**

The hormonal environment that is maintained throughout pregnancy aids in the proper development of the conceptus. It is important that certain hormonal responses be kept in check to protect the fetus. For example, if the fetus is exposed to increasing concentrations of glucocorticoids, a class of steroid hormones, it can increase the risk of developing cardiovascular, metabolic, and affective disorders later on in life (Levitt et al., 2000; Welberg and Seckl, 2001; Barker, 2002). Both the mother and the developing fetus have mechanisms in place to limit the exposure to glucocorticoids. The fetal defense mechanism consists of an increase in placental 11β-hydroxysteroid dehydrogenase 2 (11βHSD2), an enzyme that metabolizes corticosterone to its inactive form, 11-dehydrocorticosterone (Brunton and Russell, 2008). The placental expression of 11βHSD2 acts as an effective barrier to prevent the fetus from being exposed to glucocorticoids. However, this is only a second line of defense for preventing exposure. The primary mechanism to prevent the conceptus from being exposed to glucocorticoids is through a suppression of the maternal hypothalamic-pituitary-adrenal (HPA) axis (Brunton and Russell, 2008).

Many studies have been conducted in rodents and humans that have characterized the suppression of the HPA axis activity during pregnancy. During pregnancy there is a decrease in neuronal activity in brain regions that modulate stress
and anxiety (da Costa et al., 1996; Wartella et al., 2003). One study found that expression of Fos decreased in the hypothalamus (HYP), medial amygdala, lateral septum, hippocampus (HIP), and basolateral amygdala during pregnancy (da Costa et al., 1996; Wartella et al., 2003). The expression of Corticotrophin-releasing hormone (CRH) during pregnancy decreases dramatically in the parvocellular region of the paraventricular nucleus of the hypothalamus (Johnstone et al., 2000). Decreases in the expression of the CRH receptor were also determined within the HYP (da Costa et al., 2001). Furthermore, studies conducted in humans and rats have demonstrated that the amount of adrenocorticotropic hormone (ACTH) that is released from the anterior pituitary during pregnancy is much lower than in nulliparous subjects (Carr et al., 1981; Waddell et al., 1994). Studies have consistently shown that both physical and psychological stressors elicit an attenuated response, lower amounts of both ACTH and corticosterone (CORT), from the HPA axis that persists throughout pregnancy and lactation until weaning (Schulte et al., 1990; Hartikainen-Sorri et al., 1991; Windle et al., 1997; Neumann et al., 1998; Wigger et al., 1999). In nulliparous animals the HPA axis is under a rapid negative feedback control by glucocorticoids, but this is most likely not the only reason why the HPA axis is hyporesponsive during pregnancy. Adrenalecotomized pregnant rats that were injected with CORT failed to have a further inhibition of ACTH release, indicating that other mechanisms must be responsible for the continued suppression of the HPA axis during pregnancy (Johnstone et al., 2000). During the middle to late pregnancy periods the expression of 11β hydroxysteroid dehydrogenase 1 (11βHSD1) increases in the paraventricular nucleus and the anterior pituitary (Johnstone et al., 2000). 11βHSD1 metabolizes the inert 11-
dehydroxycorticosterone into corticosterone, thus increasing the intracellular concentrations available within the brain and inhibiting CRH release. The evidence suggests a delayed negative feedback inhibition of the HPA axis that is not dependent upon peripheral glucocorticoids (Brunton and Russell, 2008).

Some evidence suggests that the HPA axis is unresponsive to increasing concentrations of peripheral glucocorticoids (Johnstone et al., 2000; Brunton and Russell, 2008) and that this is responsible for a delayed feedback inhibition, but other studies suggest that additional mechanisms may play a role. The presence of sex steroids and their neuroactive metabolites throughout pregnancy make them prime candidates for modulating the reduction of HPA axis activity. In the rat, blood concentrations of E and P peak during the last week of pregnancy and this suggests a role for them in the inhibiting the HPA axis (Shaikh et al., 1971, 1975; Concas et al., 1998). However, this action is not directly modulated by E and P, but rather a neuroactive steroid metabolite of P, allopregnanolone (APGN; Douglas et al., 2000).

The conversion of P into its neuroactive metabolite APGN occurs through a two step enzymatic process. P is converted into 5α-dihydroprogesterone by 5α-reductase and 5α-dihydroprogesterone is further metabolized to its neuroactive form, APGN, by 3α-hydroxysteroid dehydrogenase (Brunton and Russell, 2008). APGN concentrations within the brain are continually elevated during pregnancy as a consequence of the increased P secretion and the expression of both 5α-reductase and 3α-hydroxysteroid within the brain (Concas et al., 1998). Studies conducted in rodents have demonstrated that APGN is a strong activator of γ-aminobutyric acid (GABA) via its allosteric modulation of postsynaptic GABA_A receptors (Brunton and Russell, 2008). It is believed
that APGN prolongs the amount of time that GABA_A receptors remain open, thus, keeping the postsynaptic cell in an inhibitory hyperpolarized state. The inhibitory action of APGN within the HYP could provide the appropriate stimulus for dampening the activity of the HPA axis (Brussaard et al., 1999). A couple of studies conducted in non-pregnant female and male rats found that administering APGN reduced the stress-induced responsiveness of the HPA axis (Patchev et al., 1996; Brunton et al., 2004). Moreover, the application of finasteride, a 5α-reductase inhibitor, reversed the hyporesponsive HPA axis because elevated levels of ACTH were restored after stress induction (Concas et al., 1998; Brunton et al., 2004; de Brito Faturi et al., 2006). These studies provided further evidence that another mechanism may play a role in the inhibition of the HPA axis during pregnancy.

Anxiolytic Effects of Pregnancy

The dampening of the HPA axis during pregnancy is beneficial to the developing fetus and it has a profound effect on the behavior of the mother. Studies conducted in rodents have demonstrated that during gestation behavioral tasks that are indicative of anxiety decrease dramatically (Macbeth and Luine, 2010). Macbeth and colleagues established that pregnant female rats that were either in early or late gestation exhibited an increase in exploratory behavior when compared to nulliparous controls in the elevated plus maze (Macbeth et al., 2008). Another study using the elevated plus maze did not show the same decrease in anxiety-related behavior during early gestation, but it did suggest a decrease in anxiety related-behavior during late gestation (de Brito Faturi et al., 2006). Another group investigating anxiety-related behavior demonstrated that
pregnant rats exhibit a greater preference for the more brightly lit white chamber than the darker chamber during the black-and-white paradigm (Zuluaga et al., 2005). This behavior is thought to be indicative of a less anxious phenotype because rodents that exhibit a more anxious phenotype do not usually spend a majority of their time exploring brightly lit spaces (Crawley and Goodwin, 1980; Blumstein and Crawley, 1983; Costall et al., 1989).

Alterations to Hippocampal Structure during Pregnancy

During pregnancy the fluctuations of the various ovarian hormones and glucocorticoids have led many to hypothesize that there is a rewiring of the mother’s brain that enhances connections between existing neurons. Many believe that these actions cause an unprecedented amount of neural plasticity (Numan, 2007; Macbeth and Luine, 2010). In female human subjects the brain is reduced in volume and returns to its preconception size following parturition (Oatridge et al., 2002). In rats the volume of the HIP decreases during gestation (Galea et al., 2000), but the overall cortical thickness increases during gestation (Hamilton et al., 1977). Furthermore, other scientific consortiums have demonstrated that there are increases in dendritic spine densities within the hippocampal CA1 pyramidal cells of late primiparous pregnant rats when compared to nulliparous controls (Kinsley et al., 2006). E and P were most likely responsible for these changes in dendritic spine densities in the CA1 region of the HIP. Nulliparous rats that were treated with E and P through a pregnancy-like regimen exhibited these same increases in dendritic spine densities within the CA1 region of the HIP (Kinsley et al., 2006). Pawluski and colleagues also saw these same increases in
spine densities within the CA1 region of multiparous dams when compared to nulliparous rats (Pawluski and Galea, 2006). Clearly the actions of the ovarian steroids have a dramatic impact on the structure of the HIP from early to late pregnancy. With the exception of the study by Oatridge and colleagues, there are few studies that have investigated the changes that occur to the HIP in pregnant human subjects.

_Improved Cognition and Memory during Gestation in Rodents_

The studies conducted in pregnant rodents demonstrate that there are changes in hippocampal structure and these results support the theory that there may be an enhancement of cognition and memory during pregnancy in rodents. Since the CA1 region of the HIP has been shown to be important in spatial and temporary cues in memory (Kesner et al., 2004), the increase in dendritic spine densities may facilitate synaptic function. This theory was supported by studies that established an enhancement of spatial memory in pregnant dams as evidenced by an increased amount of exploration of a novel object location during the novel object placement task (Ennaceur et al., 1997; Macbeth et al., 2008). The novel object placement task consists of placing two novel objects into the cage with the animal and allowing exploration. The objects are then removed and only one of the objects is placed back into the cage in a different location. If the rodent spends more time exploring the familiar object in the novel location, then the animal is considered to have better spatial memory (Ennaceur et al., 1997). Investigational studies conducted in rats determined that nulliparous females spent equal amounts of time exploring the novel and familiar object locations, whereas, primiparous and multiparous pregnant females spent a majority of time
exploring the novel object locations (Macbeth et al., 2008). Moreover, multiparous pregnant dams exhibited an increase in correct responses during the radial-arm maze task indicating an improvement in learning behavior during pregnancy (Kinsley et al., 1999).

Another common procedure for assessing memory in a rodent is the Morris water maze. It specifically measures behavior that is applicable to spatial working memory. A platform is hidden in a swimming container filled with water and rats are placed into the container. The rats are allowed to swim to locate the hidden platform until it is reached and the rat uses the platform to escape the water (Morris, 1984). The same animals are tested daily and the platform location is moved and the animal must use exterior visual cues to help identify the location of the platform. The length of time it takes the animal to find the submerged platform is directly correlated with the working spatial memory (Morris et al., 1982; Morris, 1984). Pregnant rats exhibited shorter latencies to discovery of the platform and shorter path lengths when compared to their nulliparous counterparts during the Morris water maze (Galea et al., 2000; Macbeth et al., 2008). When the platform was moved to a different location the pregnant dams located it faster than did nulliparous controls (Bodensteiner et al., 2006).

**Impaired Cognition and Memory during Pregnancy in Human Subjects**

The improvement of cognition and mood during gestation that has been observed in rodent behavioral studies does not seem to correlate well with human studies of behavior during pregnancy. There is anecdotal evidence that some women have been described as having “pregnancy brain.” This stereotype describes pregnant
women as inattentive, forgetful, and unable to focus on basic tasks (Macbeth and Luine, 2010). Several reports have demonstrated that pregnant women have a decreased performance on basic memory tasks. Both implicit and visual memory have been shown to be impaired in pregnant human subjects (Silber et al., 1990; Brindle et al., 1991; Sharp et al., 1993; Keenan et al., 1998; Buckwalter et al., 1999; de Groot et al., 2006). Working memory was also determined to be impaired in pregnant women when compared to their non-pregnant counterparts (Casey et al., 1999; Janes et al., 1999). In addition women who are pregnant often self report that they have a hard time remembering details about specific pieces of information when compared to nulliparous control human subjects (Brindle et al., 1991; Sharp et al., 1993; Casey et al., 1999; Janes et al., 1999; Crawley et al., 2003). Multiparous women who were surveyed also felt that their memory was more impaired than before they were pregnant (Casey et al., 1999).

In contrast, the recognition memory of primiparous and multiparous mothers during pregnancy was determined to be either maintained, or in some cases enhanced when compared to nulliparous controls (Silber et al., 1990; Brindle et al., 1991; Sharp et al., 1993; Mickes et al., 2008). Also, some argue that the impairments in memory and cognition that were measured during some of these tests used very subjective measures, whereas, other studies that used more objective measures of memory did not find the same deficits during pregnancy (Workman et al., 2012). Other groups have utilized more objective laboratory measures that require more effort with respect to processing. They demonstrated using that there were relatively minor impairments in both executive function and working memory (Cutler et al., 2011). Another study
conducted a meta-analysis and also determined that the impairments to cognition and memory were minor to negligible during pregnancy (Henry and Rendell, 2007).

The cognition and memory studies conducted in human subjects are in stark contrast to the behavioral studies conducted in rodents, and this demonstrates an extreme disconnection between the human condition and the animal models. Both humans and rodents exhibit the same pattern in the fluctuations of ovarian hormones and glucocorticoids following parturition, but this does not necessarily correlate to the same changes in behavior. However, the conflicting data in the literature regarding cognition and memory during pregnancy also presents a problem in trying to reconcile whether or not the animal models are predictive of the human condition. There is much more data available about changes to brain structures and their effects on behavior in the rodent during pregnancy, but this same information is scant in humans.

*Regulation of Oxytocin Release during Late Pregnancy and Lactation*

The neuropeptide, oxytocin, is produced by neurons in the paraventricular nucleus of the HYP and the supraoptic nuclei (Leng et al., 2005). The magnocellular neurons from the paraventricular nucleus and supraoptic nuclei project to the posterior pituitary whereby oxytocin is released in a pulsatile fashion from the posterior pituitary into the general circulation (Jiang and Wakerley, 1995). The axons of the magnocellular neurons converge into the posterior pituitary and a couple thousand neurosecretory endings hold the stores of oxytocin where they can be readily released into the circulation (Nordmann, 1977). Oxytocin is only released when there is a burst of firing by oxytocin neurons, and this burst firing acts a coupling mechanism between all of the
oxytocin neurons (Moos et al., 1991). This action allows for the pulsatile release of oxytocin. The continuous release of oxytocin is maintained via a positive feedback loop, whereby oxytocin that is released dendritically and presynaptically within the paraventricular nucleus and supraoptic nuclei thus, enhancing the firing rate of magnocellular neurons (Russell et al., 2003). Oxytocin is responsible for the induction of smooth muscle contractions in the uterus during delivery of the fetus. Furthermore, oxytocin is essential for lactation as it allows for ejection of milk from the mammary gland (Brunton and Russell, 2008). If oxytocin is released too early it could prematurely commence contractions of the uterus and cause a miscarriage. Thus, regulation of oxytocin release is obligatory during gestation.

The nucleus tractus solitarii neurons that arise from the brainstem project rostrally and synapse directly onto the apical dendrites of magnocellular neurons. This tract contains noradrenergic inputs into the magnocellular neurons and when stimulated can elicit a robust firing of magnocellular neurons and a pulsatile release of oxytocin stores into the blood from the posterior pituitary (Meddle et al., 2000; Wang and Hatton, 2004).

Endogenous opioids and neurosteroids act at different targets on noradrenergic neurons of the nucleus tractus solitarii to dampen the activity and neurotransmission to the magnocellular neurons (Douglas et al., 1993; Leng et al., 1997). The source of endogenous opioids that is thought to inhibit oxytocin release during pregnancy is the nucleus tractus solitarii because of its direct connection to the supraoptic nuclei and paraventricular nucleus (Leng et al., 2005; Brunton and Russell, 2008). The endogenous opioids enkephalin and dynorphin are both synthesized and released from
the noradrenergic neurons of the nucleus tractus solitarii (Bronstein et al., 1992; Ceccatelli et al., 1992). Enkephalin and dynorphin both bind to μ-opioid receptors and have an inhibitory effect on presynaptic vesicle release in neurons. Both enkephalin and dynorphin bind to μ-opioid receptors on the presynaptic terminals of noradrenergic nucleus tractus solitarii neurons that synapse onto magnocellular neurons. This action decreases the release of noradrenaline and reduces the firing rate of oxytocin containing magnocellular neurons (Brunton et al., 2006). The neurosteroid, APGN, also has an inhibitory effect on noradrenergic nucleus tractus solitarii neurons because it modulates GABA_A channels to remain in the open position longer. Furthermore, APGN increases the number of GABA_A channels present on magnocellular neurons within the paraventricular nucleus and supraoptic nuclei to increases the inhibitory input (Koksma et al., 2005). APGN sensitizes the nucleus tractus solitarii neurons to opioid inhibition by increasing the dephosphorylated, inactive state, of adrenergic receptors on nucleus tractus solitarii neurons (Brussaard and Herbison, 2000). The GABA_A channels remain open longer and keep the cell membranes of magnocellular neurons hyperpolarized longer, thus, inhibiting their ability to fire action potentials (Koksma et al., 2005). Fewer action potentials prevent the magnocellular neurons from firing the bursting action potentials that are needed to couple other magnocellular neurons and allow effective and pulsatile release of oxytocin. During pregnancy APGN, enkephalin, dynorphin all interact within the paraventricular nucleus and supraoptic nuclei to suppress the neuronal activity of magnocellular neurons and prevent the release of oxytocin (Brunton and Russell, 2008).
During parturition the concentration of circulating APGN decreases as a consequence of the reduction in progesterone. The decrease of plasma APGN lowers the GABA inhibition acting on magnocellular neurons within the paraventricular nucleus and supraoptic nuclei. Also, there is an increase in the phosphorylated state of GABA$_A$ receptors on magnocellular neurons, thus decreasing their sensitivity to GABA (Koskma et al., 2003; Koskma et al., 2005). The somato-dendritic release of oxytocin from magnocellular neurons begins to drive the autoregulatory positive feedback loop that will increase the amount of oxytocin being released (Richard et al., 1991). There is also an increase in glutamatergic tone that further releases the inhibition by GABA in magnocellular neurons (Leng et al., 1997; Wang and Hatton, 2007).

Due to the decrease in GABA and endogenous opioid inhibition of noradrenergic nucleus tractus solitarii neurons at parturition the release of oxytocin may commence upon the moment of parturition. The pulsatile release of oxytocin assists in the rhythmicity of smooth muscle contractions within the uterus (Boron and Boulpaep, 2011). These contractions continue until the fetus has been delivered out of the mother’s womb. The surges in oxytocin continue in response to a suckling stimulus from the mother’s nipple that is relayed via mechanoreceptors (Boron and Boulpaep, 2011). The oxytocin neurons are said to be more excitable following parturition and this allows oxytocin to be released with more ease (Teruyama and Armstrong, 2002).

*Regulation of PRL Release during Late Pregnancy and Lactation*

Prolactin is essential for the production and stimulation of milk secretion, and during pregnancy it aids in the development of the MG alveoli for milk production.
In pregnant human subjects, circulating PRL increases by 15-fold (Gaynes et al., 2005). During gestation in the rat, PRL is secreted in surges in the afternoon and night and has a luteotrophic role. Its secretion is suppressed in mid-pregnancy, when PL secretion predominates (Mann and Bridges, 2001). In the night before parturition, PRL secretion surges. Both PRL and oxytocin act in the brain to elicit maternal behavior (Mann and Bridges, 2001). PRL also acts within the paraventricular nucleus to cause a hyporesponsiveness of the HPA-axis to stressors (Torner et al., 2002). PRL and PL enter the brain through the ventral hypothalamus, where the blood–brain barrier is deficient, and through the choroid plexus (Brunton and Russell, 2008).

PRL secretion from the anterior pituitary is primarily regulated through tonic inhibition by DA that is secreted from tubero-infundibular dopamine (TIDA) neurons in the arcuate nucleus of the hypothalamus. During pregnancy the high levels of E increase the production of the short and, especially, the long isoforms of the PRL receptor in the choroid plexus, thus, allowing the entry of PRL into the brain (Augustine et al., 2003; Pi et al., 2003).

The long-form of the PRL receptor is expressed on TIDA neurons and when activated the receptor enhances the expression of the gene for tyrosine hydroxylase, the rate limiting step for DA biosynthesis (Ma et al., 2005). Thus, PRL upregulates DA biosynthesis and indirectly inhibits its own secretion. PRL opposes DA inhibition of tyrosine hydroxylase by inducing phosphorylation of the enzyme by protein kinases (Ma et al., 2005). Between mid-pregnancy and parturition, PL suppresses the production and secretion of maternal PRL (Lee and Voogt, 1999).
The continued exposure of the TIDA neurons to PRL causes a reduction in PRL sensitivity due to an increase in the expression of genes for suppressor of cytokine signaling proteins (Anderson et al., 2006). This action causes a final resetting of the negative feedback of TIDA neurons and permits the increase in PRL release from the anterior pituitary. The continued biosynthesis and secretion of PRL is obligatory for lactation and the induction of maternal behavior (Brunton and Russell, 2008).

Increasing estrogen (E) levels and decreasing progesterone (P) levels might regulate the resetting of the negative feedback of TIDA neurons, through E and P receptors on TIDA neurons (Andrews, 2005). TIDA neurons are inhibited by endogenous opioids in late pregnancy, and this inhibition facilitates the pre-term PRL surge (Andrews and Grattan, 2002). In nulliparous rodents few TIDA neurons co-express enkephalin, but in pregnancy they all express enkephalin, which is most likely due to the combined actions of PRL and P (Merchenthaler et al., 1995). It is still unclear whether P acting through allopregnanolone (APGN) has an inhibitory effect on PRL release (Brunton and Russell, 2008). It seems reasonable to speculate that APGN could induce the same inhibitory action through the modulation of GABA_A receptors, thus, prevent the DA neurons from inhibiting PRL secretion. Enkephalin is released from TIDA neurons during pregnancy and has a stimulatory role; however it might act in an auto-inhibitory manner similar to the inhibitory mechanism that has been proposed for noradrenergic nucleus tractus solitarii neuron terminals in the paraventricular nucleus in late pregnancy (Brunton et al., 2005). During gestation at approximately day 19 in the pregnant rat, endogenous opioids inhibit PRL secretion. Studies conducted in rats demonstrated that naloxone, an endogenous opioid receptor antagonist, increased
PRL secretion in rats that received a pretreatment of mifepristone, a P receptor antagonist (Soaje et al., 2006). These results suggest that P has a stimulatory action on TIDA neurons that can be blocked to by simulating P withdrawal at the end of pregnancy (Soaje et al., 2006). Furthermore, naloxone had no effect on the TIDA neuron activity at this same time point. It is possible that an increase PRL secretion could have occurred by the reversal of opioid inhibition of a putative PRL releasing-factor (Soaje et al., 2006). Another possibility could be that these same neurons could have been magnocellular or oxytocin neurons because they were inhibited by endogenous opioids in late pregnancy (Douglas et al., 1995) and due to the fact that oxytocin release has a further stimulatory effect on PRL secretion (Egli et al., 2006). All of the data indicate that PRL biosynthesis and secretion in the rat are regulated differently in early, mid and late pregnancy (Brunton and Russell, 2008). In late pregnancy, DA synthesis in TIDA neurons is desensitized to stimulation by PRL and a surge of PRL secretion follows shortly prior to parturition. The most likely reason for this desensitization is the fact that P decreases precipitously prior to parturition (Brunton and Russell, 2008). The action of endogenous opioids on PRL secretion changes from inhibitory to excitatory at during late gestation in the rat. This seems to suggest the possibly of a switch from predominant inhibition of oxytocin neurons to inhibition of TIDA neurons (Brunton and Russell, 2008).

Regulation of Neural Pathways Involved in Maternal Behavior during Pregnancy and Lactation

The prolonged exposure of the maternal brain to ovarian steroids and endogenous opioids during pregnancy and lactation has profound effects on the neural
plasticity, circuitry, and behavior during the postpartum period (Numan, 2006; Brunton and Russell, 2008; Macbeth and Luine, 2010). The numerous changes to the structure and function of several brain regions, and the initial exposure to offspring are thought to be the essential underpinnings for proper maternal behavior. Indeed virgin female rodents who are repeatedly exposed to pups over a several days have an induction of maternal behavior (Brunton and Russell, 2008). Some investigators have found evidence for the order of exposure to these steroids and endogenous opioids during pregnancy that are obligatory for the expression of maternal behavior. The evidence suggests a priming mechanism that occurs when several brain regions are cyclically exposed to high concentrations of estrogens, PRL, placental lactogen, and P (Mann and Bridges, 2001; Bridges and Hayes, 2005).

Just prior to parturition, female rodents exhibit a non-maternal phenotype towards pup exposure. This non-maternal behavior is mediated primarily through the olfactory bulb projections to the medial amygdala, anterior HYP, and periaqueductal grey (Fleming et al., 1980; Bridges et al., 1999; Sukikara et al., 2006). During pregnancy the medial preoptic area and ventral bed nucleus of the stria terminalis, brain regions that have been found in rodents to have a significant role in the expression of maternal behavior (Byrnes et al., 2000; Byrnes and Bridges, 2000), are activated immediately following parturition (Brunton and Russell, 2008). A series of rodent studies suggest that these regions are crucial in overriding a normally xenophobic response to pup odor that is mediated by the olfactory bulb projections to the anterior HYP and periaqueductal grey regions (Bridges et al., 1999; Sukikara et al., 2006). The activation of these brain regions are thought to induce responsiveness of the mother to
tactile and olfactory stimuli from the pups. Also, excitatory glutamate neuron projections from the medial preoptic area and ventral bed nucleus of the stria terminalis to mesolimbic DA neurons in the ventral tegmental area cause the maternal behavior to be more rewarding via activation of the reward circuitry in the nucleus accumbens (Kinsley and Bridges, 1990; Numan, 2006). Specifically, the DA projections from the ventral tegmental area to the nucleus accumbens inhibit GABA output to the ventral pallidum (Numan et al., 2005). The medial preoptic area appears to provide the excitatory input to reward circuitry pathways that are important in the retrieval of pups by the dam. During the postpartum period the inhibitory control of the medial preoptic area via endogenous opioids is abrogated through the decline of ovarian steroids, and thus, maternal behavior is encouraged by the activation of the reward pathway (Byrnes et al., 2000; Byrnes and Bridges, 2000).

During late gestation, P levels precipitously decline along with a concurrent increase in E levels. Studies in rodents have demonstrated that these two fluctuations in both P and E can activate the neurons within the medial preoptic area (Sheehan and Numan, 2002). Furthermore, exposure to pups during the postpartum period has been demonstrated to activate this same brain region in rodents (Mattson and Morrell, 2005; Numan, 2006). Following parturition, oxytocin also plays a major role in the expression of maternal behavior with actions at multiple brain regions: medial preoptic area, nucleus accumbens, ventral tegmental area, olfactory bulb, lateral septum, bed nucleus of the stria terminalis, paraventricular nucleus, and amygdala (Francis et al., 2000).

It is well known that oxytocin neurons undergo major morphological (Theodosis et al., 1981, Hatton et al., 1992; Theodosis and Poulain, 1992) and biochemical
reorganization following pregnancy and just prior to lactation (Jirikowski et al., 1989; Brooks et al., 1990; Insel, 1990). However, Jin and colleagues were curious to know exactly what the impact of knocking out the release central oxytocin would have on maternal behavior. Genetically engineered mice were generated with a centrally specific knock-out of oxytocin and they discovered that there was a lack of maternal behavior in postpartum mice (Jin et al., 2007). Central injections of oxytocin within the brains of the knock-out mice rescued the maternal behavior phenotype thus, providing further support for an important rapid action of central oxytocin release (Jin et al., 2007). Furthermore, injections of oxytocin into the ventricular spaces of virgin rats elicited maternal behavior even though these same animals had not been parous (Pedersen and Prange, 1979).

Oxytocin receptor mRNA is upregulated in mid-pregnancy in the lateral septum, amygdala, and medial preoptic area (Young et al., 1997) and during parturition in the amygdala, bed nucleus of the stria terminalis, medial preoptic area, olfactory bulb, and ventral medial hypothalamus (VMH; Young et al., 1997; Meddle et al., 2007) and this causes more receptor availability to mediate oxytocin action and explains the enhanced oxytocin receptor binding that has been previously observed at parturition (Insel, 1986). Although P withdrawal might explain the upregulation of oxytocin receptor expression in the lateral septum, a general mechanism of regulation has not been identified (Insel, 1986; Young et al., 1997). The observed increases in oxytocin receptor density are most likely due to the action of E during gestation. These oxytocin receptor increases are correlated with the oxytocin dependent intensity of maternal behavior that the postpartum dams exhibit (Champagne et al., 2001).
Oxytocin is not the only hormone that is responsible for the successful expression of maternal behavior. PRL and placental lactogenic hormone have also been demonstrated to contribute to the successful evolution of maternal behavior (Mann and Bridges, 2001). There is evidence to suggest that oxytocin is a pro-releasing factor for PRL, and thus, these two hormones have been studied intensely with regard to maternal behavior (Siegel and Rosenblatt, 1975; Bridges et al., 1978, 1984, 1985, 1996; Pedersen and Prange, 1979). Heterozygous PRL receptor-knockout mice have major deficits in maternal behavior, demonstrating the essential role of PRL and/or placental lactogenic hormone in the initiation of maternal behavior (Lucas et al., 1998). The many neuroendocrine changes that occur in the brain of pregnant rats allow for the establishment of maternal behavior and disappear quickly unless they are reinforced by pup exposure directly after parturition (Numan, 2006).

**Proposed Etiologies of PPD: Human and Animal Studies**

**Hormone Withdrawal Hypothesis of PPD**

The etiology of PPD is currently not known, however some clinical studies have suggested that the rapid decline of E and P to prefollicular levels after parturition (Keenan et al., 1998) may lead to PPD (Arpels, J. 1996; Abou-Saleh et al., 1998; Hendrick et al., 1998; Nonacs and Cohen, 1998). However, in these clinical studies there were no significant or consistent differences in the hormone levels between the women who were diagnosed as having developed PPD and women who were not (Hendrick et al., 1998; Bloch et al., 2005). Some animal studies using rats have shown that giving E can alleviate depressive symptoms and withdrawing it can elicit the
symptoms (Galea et al., 2001; Molina-Hernandez and Tellez-Alcantara, 2001; Stoffel and Craft, 2004; Suda et al., 2008). The problem with these animal studies was that the animals never were parous and did not deliver pups. The studies only simulated gestation by giving predetermined concentrations of E and P to nulliparous overiectomized animals. The studies were inconsistent in the actual concentrations of E and P being given to the rodents. Furthermore, two studies treated for a duration that was consistent with rodent gestation (Galea et al., 2001; Stoffel and Craft, 2004), whereas, another study treated for what was a similar duration to human pregnancy (Suda et al., 2008). The studies did not produce similar results with behavioral testing yielding much different outcomes.

The hormone withdrawal hypothesis has a solid biological basis, since the ovarian hormones are integral in their effects on rewiring neural circuitry prior to parturition, but many of the rodent studies were inherently flawed because using a hormone-simulated pseudopregnancy is not the same thing as an actual pregnancy itself. The studies did not demonstrate the difference between the pharmacology and the physiology of this very complex system that is the gestational period.

*HPA axis Dysregulation may be Linked to PPD*

Some clinical evidence has demonstrated that HPA axis dysfunction may play a role in the onset of PPD symptoms (Okano and Nomura, 1992; Harris et al., 1996). These studies noted a higher level of salivary cortisol that correlated with higher depression mood scores. However, several other studies found no evidence of a correlation between cortisol levels in saliva and women who were diagnosed as PPD.
(Feksi et al., 1984; Harris et al., 1989, 1994; O’Hara et al., 1991a, b; Magiaku et al., 1996; Abou-Saleh et al., 1998; Buckwalter et al., 1999). The function of the HPA axis normalizes at approximately 12 weeks postpartum (Mastorakos and Ilias, 2003). In contrast, other studies have found that individuals afflicted with a non-postpartum type of MDD exhibit a similar dysregulation of the HPA axis with increases in basal concentrations of plasma cortisol. One study found that women who were diagnosed with PPD exhibited elevated cortisol levels, which could then be suppressed by dexamethasone treatment (Bloch et al., 2003). In one recent study, for women with PPD there was no association between ACTH and cortisol levels in response to a stress test, whereas among non-depressed control women, there was a more regulated association with cortisol levels rising following the increase in ACTH (Jolley et al., 2007).

Some evidence suggests that higher cortisol levels at the end of pregnancy are associated with increased blues symptoms (Handley et al., 1980). In a rodent study postpartum animals were given high concentrations of CORT via injection and the behavioral and neurobiological outcomes were consistent with a more depressed phenotype (Brummelte et al., 2006). The dams exhibited increases in hippocampal dendritic pruning, and decreases in hippocampal cell proliferation (Brummelte et al., 2006). However, it remains unclear whether HPA axis dysregulation contributes to the onset of PPD or occurs as a consequence of PPD.

**Breastfeeding Status and Symptoms of PPD**

In the aforementioned clinical studies the breastfeeding status was neither reported, nor controlled. Since PRL and oxytocin, in the blood and in the brain, are
elevated in response to suckling and have been shown to have profound effects on mood and behavior, it is important to control for breastfeeding status when studying PPD. Clinical studies that have considered breastfeeding in PPD have produced conflicting results. Some reports have shown that women who breastfeed do not present with symptoms of PPD (McCoy et al., 2006; Abou-Saleh et al., 1998). However, another study found that 83% of women who experienced symptoms of PPD reported that the symptoms began prior to cessation of breastfeeding (Misri et al., 1997). In human clinical studies it is almost impossible to control for exclusive breastfeeding and assure that equal lactation frequency and duration is occurring among all subjects. It is striking the paucity of studies that have examined whether or not breastfeeding status has any bearing on the etiology, or treatment of PPD.

**The Role of Serotonin (5-HT) in Mood Regulation and PPD**

Serotonin (5-HT) is a monoamine neurotransmitter derived from the essential amino acid tryptophan (TRP) and has been shown to play a role in mood modulation (Siever et al., 1991). The first step in the biosynthesis of 5-HT is the conversion of TRP to 5-hydroxytryptophan by the rate limiting enzyme, TRP hydroxylase (TPH). Next, 5-hydroxyTRP is converted to 5-hydroxytryptamine, 5-HT, by amino acid decarboxylase (Grahame-Smith, 1964). 5-HT is very labile and can easily be converted to its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), by monoamine oxidase, or it can be converted to melatonin by the sequential actions of serotonin-N-acetyltransferase and hydroxyindole-O-methyltransferase within the pineal gland (Struder and Weicker, 2001). Two isoforms of TPH exist: (1) TPH1 is a non-neuronal form that is expressed in
the enterochromaffin cells of the gut and the pineal gland, (2) TPH2 is expressed within the raphe nuclei and myenteric plexus (Walther et al., 2003; Walther and Bader, 2003). The majority of 5-HT that is made within the body is found in the enterochromaffin cells of the gut (Mossner and Lesch, 1998). Blood platelets store the excess amounts of 5-HT from the enterochromaffin cells and have a role in the clotting mechanism (Struder and Weicker, 2001; Cote et al., 2003).

Serotonergic neurotransmission within the central nervous system occurs in a stereotypical chemical synapse, where 5-HT is released from the presynaptic neuron and then diffuses across a synaptic cleft to bind to its appropriate receptor on the postsynaptic neuron. 5-HT is synthesized within the terminal of the presynaptic neuron and packaged into synaptic (secretory) vesicles by vesicular monoamine transporter 2 (Johnson, 1988). The primary mechanism of terminating serotonergic neurotransmission is through reuptake of 5-HT by the serotonin transporter (SERT) back into the presynaptic terminal (Blakely et al., 1991). Some have proposed that decreases in extracellular concentrations of 5-HT or decreased serotonergic neurotransmission could result in depressive symptoms (Murphy et al., 1978, Krishnan and Nestler, 2008). This hypothesis was supported with the use of selective serotonin reuptake inhibitors (SSRIs) to effectively block SERT, maintaining elevated extracellular concentrations of 5-HT and prolonging serotonergic neurotransmission (Nestler et al., 2002). Even though SSRIs are effective in treating depression, the exact mechanism(s) by which they alleviate symptoms are not known. Although SERT blockade occurs rather rapidly after the administration of a SSRI it often takes several weeks of treatment prior to the alleviation of the depressive symptoms in human subjects. A
direct measure of extracellular levels of 5-HT in limbic brain regions in human patients is not possible. Instead platelet 5-HT levels are commonly measured as a peripheral index of the amount of SERT blockade that is occurring. The SERT isoform that is present on the terminal axons of neurons in the brain is the same as that located on platelets (Lesch et al., 1993). Patients that have been administered a SSRI have exhibited decreases in platelet 5-HT levels, which would indicate an effective blockade of the SERT protein (Karege et al., 1994).

Serotonergic Dysfunction and Altered TRP Metabolism may Contribute to Symptoms of PPD

The pathophysiology of PPD remains unclear, but evidence is accruing that serotonergic dysfunction may be involved (Doornbos et al., 2008; Misri and Kendrick, 2007; Dennis and Stewart, 2004; Newport et al., 2004; Appleby et al., 1997; Karege et al., 1994; Hannah et al., 1991; 1992a,b). These clinical studies indicate that changes in both peripheral 5-HT and changes in TRP metabolism may play a role in patients diagnosed as having PPD. Also, platelet binding studies from collected blood indicate that there are changes to SERT on platelets that correlate with depression. However, most of these data are only correlational and do not point to cause, which underscores the importance of investigating 5-HT function during the postpartum period. Figure 1.1 shows a rapid decline in plasma TRP levels during pregnancy and then a recovery following parturition. Those women diagnosed with PPD do not exhibit a return to baseline plasma TRP levels. 5-HT biosynthesis in the brain is dependent upon the amount of available TRP. This is due to the fact that TRP is an essential amino acid that is restricted to dietary intake (Fernstrom and Wurtman, 1972). Plasma TRP levels
in healthy female subjects recover after parturition and remain elevated above baseline until returning to levels similar to non-pregnant subjects. Since plasma TRP levels ultimately determine brain 5-HT levels and have been correlated to mood scores, it makes it an important parameter for understanding what perturbations might occur during the postpartum to contribute to PPD (Fernstrom and Wurtman, 1972). One clinical study measured plasma TRP and calculated a brain TRP availability index as a measure of the amount of TRP that was available to the brain (Bailara et al., 2006). The study determined that there was a positive correlation between the decrease in the brain TRP availability index and BB symptoms in women who were three days postpartum (Bailara et al., 2006).

Multiple research groups have described changes to various brain regions involved in mood regulation in postpartum depressed women who underwent functional MRI assessment. A study conducted by Moses-Kolko and colleagues determined that there were reductions in 5-HT 1a receptor, an important modulator of presynaptic 5-HT release activity, in brain regions of women that were diagnosed with PPD (Moses-Kolko et al., 2008). This same research group determined that there were reductions in the activity and connectivity of the dorsomedial prefrontal cortex to the dorsomedial amygdala (Moses-Kolko et al., 2010). These studies further support the hypothesis that serotonergic dysfunction may play a role in the presentation of PPD symptoms.

**Conclusions and State of the PPD Research Field**

Despite the large body of PPD literature that exists, the etiology and pathophysiology of PPD are still not known. The many neuroendocrine changes that
occur during pregnancy and the postpartum make the task of identifying the underlying cause of PPD even harder. Many basic research studies that have used animals have attempted to behaviorally phenotype animals that have had their E and P levels simulated with pseudopregnancy hormone treatments. Unfortunately, these studies have not produced consistent results and have inherent design flaws that could impede the progress of uncovering the main causes of PPD. Furthermore, many clinical studies have failed to detect a difference in a wide range of biochemical measures in PPD women. The ability to correlate depressed mood scores with a biochemical measure is crucial in determining a candidate hormone that could be contributing to the underlying cause of PPD. In fact, identifying a candidate hormone and attempting to generate a conditional knock-out animal might be more helpful than trying to simulate pregnancy in a nulliparous animal.

Other clinical studies have attempted to find a genetic single nucleotide polymorphism (SNP) that could explain the molecular underpinnings that could ultimately lead to PPD. A couple of research groups have characterized specific variations in SNPs of the SERT gene and correlated them to mood disorders (Coyle et al., 2000; Canli and Lesch, 2007). One clinical study was able to successfully predict the onset of PPD symptoms within the first eight weeks in women who possessed a certain polymorphic region on the SERT gene (Binder et al., 2010). However, these genetic SNPs are only correlative and do not directly point to a causative pathophysiology.

There are many hypotheses on what could be contributing to the etiology and pathophysiology of PPD. Understanding the normal baseline physiology of mood
regulation during the postpartum period could help to discover its causes. Numerous studies have reported that there is serotonergic dysfunction in human clinical studies of women who have been diagnosed with PPD. The clinical studies that determined that the availability of TRP, the substrate for 5-HT, is limited in those individuals with PPD further warrants more research into the possible serotonergic dysfunction that may underlie the symptoms of PPD (Figure 1.1). Furthermore, many women are prescribed SSRIs as a first line pharmacotherapy for treating the symptoms of PPD. Yet, we know so little about the baseline serotonergic physiology during the postpartum period. One of the best ways to address what is occurring to serotonergic physiology during the postpartum period is to use animal models. Obviously, animal models are not perfect, but one of the biggest advantages to using rodents to study the postpartum period is that the suckling stimulus can be controlled for during lactation. As mentioned before, one of the biggest experimental design flaws in attempting to study PPD in women is the fact that either the breastfeeding status is not reported, or not controlled. This is not a problem in rodent mammals where pups can be culled at a certain time point to ensure equal nursing and suckling stimulus. The study of the baseline changes to serotonergic physiology in rodent mammals during the postpartum period is warranted due to the fact that few if any studies have addressed this particular aspect of the postpartum period during lactation.

**Dissertation Specific Aims**

Given all of the data demonstrating that serotonergic dysfunction may play a role in PPD, the studies within this dissertation were designed to measure the baseline changes in serotonergic physiology that occur during within the postpartum period. The
central hypothesis for these studies was that the state of lactation is a period of altered serotonergic physiology, and that this altered physiology contributes to changes in mood during the postpartum. The serotonergic systems were probed using SSRIs to determine if these changes to baseline serotonergic physiologies could have application to mood during the postpartum period. In chapter 2 behavioral and biochemical measurements determined that there were alterations to both central and peripheral serotonergic physiologies during lactation. In addition, they also demonstrated that there was an interaction between lactation and responsiveness to treatment with SSRIs. Chapter 3 examined the specific aim that there are changes in serotonergic activity within limbic projection fields, and that SSRI treatment enhances this activity. Chapter 4 examined the specific aim that the mammary gland is the source of elevated platelet serotonin that occurs during lactation. The results from these experiments attempt to address the huge gaps in the literature with regard to lactation status and mood regulation, and have significant clinical implications for the understanding of the treatment of PPD.
Figure 1.1 Plasma tryptophan levels measured from non-pregnant (NP), pregnant, and postpartum women.

Schematic adapted from Bailara et al., 2006; Kohl et al., 2005; Schrocksnadel et al., 1996, 2006
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CHAPTER II

Alterations in serotonin physiology, affective behavior and responsiveness to selective serotonin reuptake inhibitors during lactation

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ABSTRACT

The physiology of mood regulation in the postpartum is poorly understood despite the fact that postpartum depression is a common pathology. Serotonergic mechanisms and their dysfunction are widely presumed to be involved, which has led us to investigate whether lactation induces changes in peripheral or central serotonin physiology and related affective behaviors. Platelet serotonin levels were significantly higher in lactating mice than in nulliparous females. Lactating mice also exhibited significantly lower immunoreactive serotonin in the dorsal raphe compared with nulliparous controls. Affective behavior was assessed in lactating dams and non-lactating females ten days postpartum, as well as in nulliparous controls. To probe the activity of central serotonin, animals were treated for the preceding five days with a selective serotonin reuptake inhibitor (SSRI) or vehicle. Lactating mice exhibited behavioral evidence consistent with “elevated mood” compared to non-lactating females as evidenced by lower baseline immobility time in the forced swim test and fewer marbles buried in the marble burying test. SSRI treatment changed these behaviors in lactating mice with further reductions in immobility time and decreased marble burying. In contrast, the same regimen of SSRI treatment had no effect on these behaviors in either non-lactating postpartum or nulliparous females. Decreased platelet serotonin following SSRI treatment also corresponded with lactation in both mice and women. Our findings demonstrate changes in both peripheral and central serotonergic systems associated with lactation, independent of parity. They also demonstrate a significant interaction of lactation and responsiveness to SSRI treatment, which has important implications in the treatment of postpartum depression.
**INTRODUCTION**

Mood alterations are commonly experienced by women during the postpartum, and postpartum depression (PPD) adversely affects not only the mother, but also disrupts bonding and the health of the child (Dossett, 2008). The relationships between untreated maternal depression and negative infant outcomes, even through adolescence, are well established (Halligan et al., 2007; Hay et al., 2008; Pearlstein et al., 2008). PPD, (defined strictly in the psychiatric nomenclature as a major depressive disorder with a specifier of postpartum onset within 1 month after childbirth) affects 10-20% of women who give birth (Dietz et al., 2007; O’Hara et al., 1996).

Despite the obvious importance of PPD, it is important to reflect on the fact that most women do not experience PPD (80-90%). Few studies have assessed postpartum mood along an open-ended scale, but one which did observed that the most consistent finding was that “the highest ratings were always on the happiness scale, and that the mean rating on this scale was over 50 every day” (with 50 being “normal” mood) (Kendell et al., 1981). From a biological perspective, it is an evolutionary imperative that female mammals cope with the physiological stresses of pregnancy, parturition, and lactation without suffering the debilitations inherent with PPD. Despite this biological perspective, attention naturally focuses on PPD as a disorder, and several high-profile papers have suggested specific mechanisms of PPD based on animal studies (Maguire and Mody, 2008; Brummelte and Galea, 2010). However, these studies have often not applied standard behavioral tests for “mood and affect”, but instead have highlighted extreme cases of disrupted stereotypic maternal behaviors (nursing, nesting, infanticide, etc.) (Maguire and Mody, 2008; Brummelte and Galea, 2010).
The control of mood in general, and the etiology of depressive disorders in particular, are not completely understood. However, substantial evidence has accrued that serotonergic systems play a central role (Delgado et al., 1991; Owens and Nemeroﬀ, 1994; Newport et al., 2004; Doornbos et al., 2009). Genetic variants in components of the serotonergic system have been correlated with depression (see review Lohoff, 2010). Altered function of the serotonin transporter (SERT) or tryptophan hydroxylase (TPH) has been found in PPD subjects (Hannah et al., 1992; Newport et al., 2004; Doornbos et al., 2008). Levels of 5-HT and its major metabolite, 5-HIAA, are significantly lower in the cerebrospinal fluid of depressed patients and in brain tissue of suicide victims (van Praag, 1984; Nordstrom and Asberg, 1992). Reduced availability of the 5-HT precursor, tryptophan, has also been found in depressed patients (Cowen et al., 1989). Moreover, selective serotonin reuptake inhibitors (SSRIs) are the first line of pharmacotherapy in PPD and relieve depressive symptoms in most of these patients (Yonkers, 2003; Pearlstein et al., 2008).

To address the regulation of mood in the postpartum, we have investigated changes in peripheral and central 5-HT physiology in lactating mice and examined the effect(s) of SSRI treatment on affective state, as measured by depression-related and anxiety-related behaviors. The present studies have compared these variables among normal lactating dams and non-lactating females without experimentally induced depression. Herein we demonstrate changes in both peripheral and central 5-HT levels during lactation. The data also demonstrate a strikingly different responsiveness to SSRI treatment in lactating dams compared with non-lactating females. Clinical
implications of these findings are analyzed in comparison with data from human subjects.

**MATERIALS AND METHODS**

**Animals and Reagents**

C57Bl/6J mice (age 3-6 months) were used in these studies. The initial analysis of immunoreactive 5-HT was performed on brains from lactating dams (day 10 postpartum; see below) and age-matched virgin females. For subsequent studies involving behavioral analyses, all females were housed with a stud male for at least two weeks, checked for vaginal plugs, then separated and housed individually on a 12h:12h light:dark cycle with water and standard lab chow available *ad libitum*. Mated mice that did not produce a litter were assigned to the “nulliparous” groups. Mice that did become pregnant and deliver pups had their litters culled to six pups on day 1 postpartum (day of parturition = day 0) to normalize the suckling stimulus, and these constituted the “lactating” groups. In behavioral experiment 1, a third group of dams was included (“postpartum-nonlactating”) which were mice that had their entire litters removed immediately after delivery (postpartum day 0). All procedures were reviewed and approved by the University of Cincinnati’s Institution for Animal Care and Use Committee.

Rat polyclonal anti-5-HT and biotinylated rabbit anti-rat antibodies were purchased from Chemicon International (Billerica, MA) and Vectastain® *Elite ABC* immunoperoxidase system from Vector Laboratories Ltd (Burlingame, CA). Serotonin RIA Fast Track kits were purchased from Rocky Mountain Diagnostics (Colorado
Springs, CO). Citalopram was purchased from Tocris Biosciences (Ellisville, MO). Fluoxetine and all other chemicals, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO).

**Serotonin Immunohistochemistry**

Animals were deeply anesthetized with an i.p. injection of ketamine-xylazine (90mg/kg and 10mg/kg, respectively) 10 minutes prior to collection of whole blood serum via cardiac puncture (see “Blood collection and assays” below). Animals then underwent transcardial perfusion with 10mL of 0.01M phosphate buffered saline (PBS) followed by 20mL of 4% paraformaldehyde (PFA). Brains were dissected free, post-fixed overnight in 4% PFA, then stored in 30% sucrose at 4°C until they sank. Brains were sectioned on a freezing microtome in 30 μm coronal slices between -4.84 Bregma and -5.02 Bregma, according to a mouse brain stereotaxic atlas (Paxinos and Franklin, 2001).

Brain slices were immunostained for 5-HT as free-floating sections, after which they were mounted on slides. Briefly, sections were rinsed with ice-cold 0.01 M PBS (5 min, 3x), then incubated for 60 minutes in blocking buffer (0.01 M PBS containing 3% rabbit serum, 2% BSA, and 0.4% Triton X-100), followed by additional washes (0.01 M PBS; 5 min, 3x). Sections were then incubated 16 hours (room temp) with rat monoclonal anti-5-HT (1:200 in 0.01 M PBS + 0.4% Triton X-100). Following rinses in ice-cold 0.01 M PBS (5 min, 3x), sections were incubated for 60 min (room temp) with biotinylated rabbit anti-rat IgG (1:5000 in 0.01 M PBS + 0.4% Triton X-100). Sections were then processed according to manufacturer’s instructions, using the Vectastain® Elite ABC immunoperoxidase system and Ag/Ab complexes were visualized with Ni²⁺-
DAB enzyme substrate. Brain slices from virgin and lactating mice were exposed to the Ni$^{2+}$-DAB solution for identical periods of time (5 min). Negative controls omitted the primary antibody from the 16-hr incubation.

Immunostained sections from three or four brains per group were analyzed using the open source NIH Image software on a Macintosh computer. The number of stained cell bodies in a defined field of the dorsal raphe were documented for each section. The optical densities (background subtracted) of the perikarya were also recorded and averaged for each section.

**Experimental Designs**

*Preliminary Behavioral Experiment.* Citalopram dose and duration used in the experiments described below were based on published literature, which has used male mice almost exclusively. Thus, a preliminary study was conducted to confirm the efficacy of this sub-chronic SSRI treatment. Male mice received daily i.p. injections of citalopram, (5mg/kg/day) or vehicle (0.9% saline) between 08:00h and 09:30h for 5 days. Each animal was subjected to the forced swim test (FST) thirty minutes after the final injection.

*Behavioral Experiment 1.* Using the FST as an assay of depressive behavior, three groups of female mice were tested: Group 1, nulliparous; Group 2, lactating; and Group 3, postpartum-nonlactating (see Figure 2.1A). On day 6 postpartum, dams of Groups 2 and 3, and age-matched nulliparous females (Group 1) began receiving daily i.p. injections of SSRI (citalopram, 5mg/kg/day) or vehicle (0.9% saline). Injections were administered between 08:00h and 09:30h for 5 days. Thirty minutes after the fifth
injection (day 10 postpartum, a period of peak lactation), each animal was subjected to the FST.

**Behavioral Experiment 2.** The effect of SSRI treatment was tested again, using the marble burying test (MBT), a behavioral assay of anxiety. In addition, several other behaviors were analyzed to determine if differences were specific to affective behavior. Only lactating dams and age-matched nulliparous females were used in this study (see Figure 2.1B), and all animals received daily i.p. injections of citalopram (5mg/kg/day) or vehicle for five days. As in experiment 1, injections in the lactating dams began on day 6 postpartum. Between the fourth and fifth injection, home cage activity was monitored for 24 hours. Thirty minutes after the last injection, each animal was assessed with the MBT and then tested for motor performance using a rotarod.

**Behavioral Tests**

**Forced Swim Test.** The FST was always administered between 09:00h and 11:30h. Mice were placed in a swim tank (height = 30cm, diameter = 20cm) containing water (25°C ± 1°C) at a height of 20cm for a total of six minutes. All sessions were recorded on video and scored by two independent observers who were blinded to the treatments. The total time of immobility was recorded. The first two minutes were not used in the determination of the total immobility time as per Porsolt et al. (1977). A mouse was deemed to be immobile when it was floating and making only minor movements to keep its head above water. Tanks were emptied and rinsed clean after each animal.

**Home Cage Activity.** In behavioral experiment 2, locomotor activity within the home cage was monitored for 24 hours beginning 30 minutes after the penultimate injection of
vehicle or citalopram. The home cage was placed in a SmartFrame stainless steel rack (Hamilton-Kinder Scientific Company, Poway, CA) and infrared photobeam interruption recorded both vertical and horizontal movements. Data were collected and analyzed using HMM100 MotorMonitor software and the total numbers of basic movements were broken into 60-minute time intervals.

**Marble Burying Task.** For the marble-burying test (MBT) in experiment 2, each test mouse was placed into a larger, novel cage with a 5cm layer of sawdust on top of the bedding. In the lactating group, each dam was transferred to the novel cage without their pups. Mice were allowed to acclimate in the novel cage for 30 minutes after which 20 clean, transparent glass marbles (1.5 cm diameter) were placed on top of the sawdust, in five rows of four marbles each, with the marbles spaced equally apart. At the end of 20 minutes, the number of buried marbles (at least two-thirds covered with sawdust) was recorded (Njung’e and Hardley, 1991a, b). This design was chosen following a preliminary experiment in which the effects of novel cage versus separation from pups on marble burying behavior in lactating dams was assessed. In that experiment, marbles were either introduced into the home cage (pups remaining with some dams and removed from others), or in a novel cage setting (some dams having been transferred with their pups and others without their pups).

**Motor Performance Task** Motor performance was determined using a rotarod. Mice were placed on a stationary drum (~62 mm diameter) that then began to revolve at a constant speed. The total amount of time the mouse remained on the rotarod was recorded during each of three trials administered 30 minutes apart. The first (acclimation) trial used a speed of 16 rpm for 120 seconds, or until the mouse failed to
remain on the revolving drum. The second and third trials used a speed of 20 rpm for up to 300 seconds (Dawson et al., 2004).

**Blood Collection and Assays**

Twenty minutes after the FST (Experiment 1) or rotarod test (Experiment 2), animals were sacrificed and trunk blood collected. These blood samples and those collected by cardiac puncture from perfused animals, were allowed to clot overnight at 4°C to release serotonin (5-HT) from platelets. Serum was separated by centrifugation at 12,000 rpm for 15 minutes at 4°C, and stored at -80°C until assayed. 5-HT levels were determined in duplicate using a commercial RIA kit according to the manufacturer’s instructions.

**Data Analysis**

Data obtained in each experiment were analyzed using either one-way or two-way analysis of variance (ANOVA) followed by Bonferonni post hoc test for comparison of the means. In some experiments when only two means were compared, a two-tailed Student’s t-test (unpaired) or Mann Whitney test was used. All data shown are presented as the mean ± standard error of the mean (SEM). All experiments were designed to achieve a statistical power of 80 percent. Means were considered to be significantly different when \( p<0.05 \).
RESULTS

Serotonin in the dorsal raphe and serum

The levels of 5-HT in the rostral aspects of the dorsal raphe nucleus (DRN), the region of the cell bodies for the ascending tracts of serotonergic neurons, were determined by immunohistochemical staining, examples of which are presented in Figures 2.2, A and B. In all sections of this region, immunoreactive 5-HT was noticeably less in the lactating mice as compared to virgin mice, even without quantitative analysis. The differences were particularly evident in the lateral “wings” of the DRN – the ventrolateral aspect of the periaqueductal gray (VLPAG). Image analysis of the staining indicated that both the number of stained cells and the intensity (over background) of staining were significantly reduced in the DRN of lactating mice (Figures 2.2, C and D).

The levels of 5-HT measured in serum were significantly elevated in lactating mice compared with virgin female mice (Figure 2.3). Nearly all circulating 5-HT is sequestered in the dense granules of the platelets and is released into the serum upon clotting (da Prada and Picotti, 1979). Thus, we will refer to this measurement as “platelet 5-HT”.

Behavioral Experiment 1

Male mice treated with citalopram by injection for five days (5mg/kg/day), showed a significant (p<0.05) antidepressant effect in the FST (reduced immobility time) (Figure 2.10A), as has been widely reported in the literature. In female mice subjected to the FST there was a very highly significant main effect of lactation state (Figure 2.4), such that the lactating females overall had lower immobility times than either of the non-
lactating groups [two-way ANOVA, F(2,93)=11.23; p<0.001]. In response to citalopram the lactating females showed a significant antidepressant effect with decrease immobility time (p<0.05, compared with vehicle-treated). However, neither of the non-lactating groups (nulliparous or pup-deprived) responded to citalopram (Figure 2.4). Nulliparous female mice tested at a higher dose of citalopram (30mg/kg/day, 10 days) also showed no response to citalopram in the FST (Figure 2.10). Similarly, non-lactating females were insensitive to a second antidepressant SSRI, fluoxetine, while lactating females responded to fluoxetine with a significant antidepressant effect in the FST (Figure 2.11).

Behavioral Experiment 2.

To verify the differential responsiveness of lactating and non-lactating to SSRIs, and also to determine if these responses are specific to affective behavior, multiple behaviors were assessed following five days of citalopram treatment (see Figure 2.1B).

Affective behavior was assessed using the MBT, a test of behavioral anxiety. Separation of the dams from their pups during the MBT caused elevated anxiety-like behavior (more marbles buried) (Huot et al., 2004; Michaels and Holtzman, 2006; Kosten and Kehoe, 2010), which was independent of whether they remained in the home cage, or were moved to a novel cage (Figure 2.12). Lactating mice kept with their pups buried so few marbles (1.2 ± 0.7, with several dams burying no marbles at all) that an effect of SSRI treatment would be impossible to measure. Thus we chose to test citalopram on dams moved to a novel cage without their pups (Figure 2.5). Similar to results in the FST, non-lactating females were insensitive to citalopram (five days, 5mg/kg/day) in the MBT. In contrast, lactating females experienced a very highly
significant \(p<0.0001\) reduction in anxiety-like behavior in response to citalopram (Figure 5). Notably, the marble-burying activity of citalopram-treated lactating females in the absence of their pups was similar to that in mothers with their pups present (Figure 2.5).

SSRI treatment did not significantly alter locomotor activity measured during the 24 hour period between the 4\textsuperscript{th} and 5\textsuperscript{th} injection in either lactating or nulliparous mice. Not surprisingly, lactating dams had significantly less overall home-cage activity than nulliparous mice (Figure 2.6, \textit{inset}). Since rodents are nocturnal animals, the home cage activity was divided into light and dark phases and reanalyzed. SSRI treatment did not alter locomotor activity in either lactating or nulliparous controls during the light or dark phases of the cycle (Figure 2.6). As expected, nulliparous animals exhibited an increase in locomotor activity during the dark phase. However, lactating dams (day 9-10 postpartum) did not exhibit any significant changes in locomotor activity between the dark and light phases (Figure 2.6).

SSRI treatment also had no effect on motor skill performance as assessed by the rotarod task in either lactating mice (day 10 postpartum) or nulliparous mice (Figure 2.7). The data obtained from the rotarod task were analyzed using the Friedman test followed by a Dunn's post-hoc analysis, which indicated that both lactating and nulliparous mice spent significantly more time on the rotarod during Trial 3 than Trial 1 for both vehicle and citalopram (5mg/kg/day) treatment groups (Lactating = \(p<0.01\); Nulliparous \(p<0.05\); Figure 2.7).

Circulating 5-HT levels were also analyzed in the mice from the behavioral experiments. As found in the mice used for the immunohistochemistry study, lactating
mice (vehicle-treated controls) again had significantly elevated levels of platelet 5-HT (Figure 2.8). Platelet 5-HT was not significantly different between nulliparous and postpartum-nonlactating female mice. In addition, citalopram treatment significantly reduced platelet 5-HT concentration in lactating mice \( (p<0.01; \text{Figure 2.8}) \), but had no effect on platelet 5-HT in either the nulliparous or postpartum-nonlactating groups. Two-way ANOVA (drug treatment X lactation state) of these data determined there were significant main effects of both drug treatment \([F(1,88)=10.75; p<0.01]\) and lactation state \([F(2,88)=6.079; p<0.01]\), but the interaction between the two factors was not quite significant \([F(2,88)=2.507; p=0.08]\).

The platelet 5-HT response to SSRI treatment, expressed as a percent reduction, was substantially greater in lactating female mice compared with postpartum females from whom pups had been removed (Figure 2.9A). Using published data derived from human females (Epperson, et al., 2001), we reanalyzed the results to determine whether nursing frequency affected response to SSRI. In these data, there was an overall negative relationship between the number of breast feedings per day and platelet 5-HT levels \( (R^2 = 0.22 \text{ with } p=0.088) \) (Figure 2.9C). Comparing exclusive breast feeders with all non-exclusive breast feeders, the exclusively breastfeeding mothers experienced a significantly greater decline in platelet 5-HT in response to SSRI treatment (Figure 2.9B).

**Discussion**

In 2004 a novel serotonergic biosynthetic system in the mammary gland was identified and found to be highly upregulated during late pregnancy and lactation.
(Matsuda et al., 2004). This discovery provides a new context in which to consider whether serotonergic systems are altered in the post-partum, and ultimately whether the peripheral and central serotonergic systems influence one another during this time. This paper presents our initial examination of these serotonin systems in the lactating animal, using a selective SSRI with which to probe the behavioral responsiveness of the central serotonin system.

The present studies have produced several novel findings. First, and perhaps most significantly, lactating mice (day 10 postpartum, a time of peak lactation) exhibit behavioral responsiveness to SSRI treatments that have no measurable effect on non-lactating female mice. Second, lactating mice have significantly elevated platelet 5-HT stores when compared to non-lactating females. Moreover, SSRI treatment significantly reduces platelet 5-HT stores in lactating dams, while having no effect on those of non-lactating females. Third, immunoreactive 5-HT is significantly decreased in the dorsal raphe of lactating mice compared with nulliparous females.

The elevated platelet 5-HT storage pools in lactating dams is striking, being up to 40% greater than that in nulliparous mice. The majority of serotonin in the body is produced by the enterochromaffin cells in the mucosa of the gut (Barter and Pearse, 1955) where it regulates peristaltic and secretory reflexes (Grider et al., 1996; Cooke, 2000). Serotonin produced in the gut enters the bloodstream where it is rapidly transported into platelets via SERT (Lesch et al., 1993) and stored in dense-core granules (da Prada and Picotti, 1979). The 5-HT is released upon platelet activation, and functions in vasoconstriction and thrombosis. High circulating 5-HT is found in certain pathologies, such as carcinoid tumors, but lactation appears to be a unique
physiological state in which circulating 5-HT levels are elevated. Platelet 5-HT levels do not differ between sexes or among healthy people of varying age (with the exception of newborns) (Flachaire et al., 1990). Also, there is no circadian rhythm in platelet 5-HT content despite a small circadian rhythm in plasma tryptophan (Eynard et al., 1993). It is conceivable that the elevated 5-HT during lactation is traceable to 5-HT synthesis in the mammary glands (Matsuda et al., 2004), but it is also the case that the intestine of female mammals undergoes marked mucosal hyperplasia during lactation (Hammond, 1997). Thus, elevated platelet 5-HT during lactation may be derived from multiple sources.

5-HT itself cannot cross the blood brain barrier, necessitating its synthesis within the brain from a limited substrate – tryptophan. Tryptophan is an essential amino acid, so its plasma levels are determined by the balance between dietary intake and metabolic removal. Tryptophan transport into the brain occurs via a carrier for which it must compete with all other large neutral amino acids (LNAAs; tryptophan, valine, leucine, isoleucine, phenylalamine, and tyrosine). Thus, uptake and levels of tryptophan in the brain depend on the ratio of tryptophan to other LNAAs in plasma (Fernstrom and Wurtman, 1972; Perez-Cruet et al., 1974). Increased metabolism of tryptophan in the periphery may reduce the amount available to be transported into the brain and converted to 5-HT, particularly if metabolism of the other neutral amino acids is not similarly increased. The demonstration that tryptophan restriction can result in decreased 5-HT levels in the brain has been done directly in experimental animals (Fernstrom and Wurtman, 1972) and indirectly (5-HT metabolites in CSF) in humans.
(Carpenter et al., 1998; Moreno et al., 2000). Our findings here should prompt renewed attention to the role of tryptophan metabolism in lactating females.

The markedly lower 5-HT immunostaining in the dorsal raphe nuclei (DRN) of lactating dams as compared with nulliparous controls clearly indicates a change in central 5-HT activity. The vast majority of cell bodies of the central serotonergic system are located within the brainstem and caudal midbrain. Of particular interest are the cell bodies contained within the DRN, as these are the origins of the major ascending serotonergic pathways to the forebrain, including numerous limbic structures. However, the immunocytochemical data are only a snapshot of the levels of 5-HT in the cell bodies. The level of stored neurotransmitter is a function of the synthesis rate and transport/release rates. Either decreased synthesis or increased transport and release at nerve endings, without a comparable adjustment in the other, would result in depletion of storage pools in the DRN of lactating mice. Consequently, the quantification of 5-HT neurons in the DRN must be interpreted in light of other evidence. The predicted effects on affective behaviors are very different in the cases of reduced 5-HT synthesis or increased 5-HT transport and release.

Decreased synthesis, as may occur with reduced transport of tryptophan into the brain, would lead to a reduction in 5-HT neurotransmission, with concomitant increases in depression-like and anxiety-like behaviors. In contrast, transport and increased release of 5-HT would be expected to reduce depressive-like behavior. Indeed, such alterations in affective behavior can be induced by changing central 5-HT levels through experimental manipulation of dietary tryptophan. The results of the behavioral experiments indicate that the decreased immunostaining for 5-HT in the brains of
lactating mice is not likely to be due to a reduction in 5-HT synthesis. First of all, the lactating mice exhibited a trend of decreased immobility in the FST as compared with non-lactating mice (Figure 2.4). In addition, lactating mice buried fewer marbles than non-lactating mice – so few that we were required to introduce the stress of separation from pups in order to test for an anxiolytic effect of the SSRI treatment (see Figure 2.5 and Figure 2.12). Taken together, these data indicate that lactating mice have an “elevated mood” as compared to non-lactating female mice. Such a change in affective behavior would be predicted if 5-HT release from ascending serotonergic neurons were increased in the lactating mice.

The rate-limiting enzyme in 5-HT synthesis, tryptophan hydroxylase (TPH), is not normally saturated with tryptophan, so increasing the levels of this substrate can increase 5-HT synthesis in the brain in both rodents (Fernstrom and Wurtman, 1971) and humans (Young and Leyton, 2002). L-tryptophan intake has been shown to significantly reduce depressive symptoms in patients in a placebo-controlled, double-blind study (Thomson et al., 1982). Conversely, restriction of tryptophan in the diet can decrease brain 5-HT levels. In rodents, reduction in central 5-HT levels due to tryptophan depletion is associated with depressive-like behavior (increased immobility in the FST) and increased anxiety-related behavior (more time spent in corner areas during the open field test) (Blokland et al., 2002). In humans, tryptophan depletion also results in “lower mood” in healthy subjects and triggers depressive episodes in patients with previous affective disorders, a response not observed with depletion of other essential amino acids (Young and Leyton, 2002).
The reduced 5-HT staining in the DRN of lactating mice was particularly striking in the lateral aspects of the DRN and VLPAG. Serotonergic neurons arising from these regions project to a distributed system involved in physiological and behavioral responses associated with stress and anxiety (Lowry et al., 2008). Retrograde labeling and double-staining experiments have demonstrated that the serotonergic neurons projecting to the amygdala (Kiyasova et al., 2011) and lateral septum (Köhler et al., 1982) are concentrated at the level shown in Figures 2.2, A and B (Bregma -4.48). Both the amygdala and the lateral septum are key components in the brain’s emotional circuitry that modulates affective behavior and stress responses (Aggleton, 1992; Singewald et al., 2011). Increased serotonergic neurotransmission to these regions could be expected to reduce depressive-like or anxiety-related behavior.

Lactating dams not only exhibited baseline affective behavior consistent with an elevated mood compared with nonlactating females, but they also exhibited a greatly increased responsiveness to SSRI treatments. This was true with both the subchronic administration of citalopram (5mg/kg/day for 5 days) and the acute administration of fluoxetine (single injection of 40mg/kg). While both block 5-HT uptake via SERT, the two SSRIs differ in structure, metabolism, and pharmacokinetics (Baumann et al., 1995). Neither SSRI altered affective behavior in nulliparous female mice. Even longer treatment with a much higher dose of citalopram (30mg/kg/day for 10 days) did not change this lack of response in the nulliparous females.

Also not responsive to SSRI treatment were female mice that had gone through a full pregnancy but had their pups removed and were not lactating. These data indicate that it is the state of lactation and/or the presence of the pups, but not parity per se, that
underlies the change in responsiveness to SSRIs. This does not necessarily mean that pregnancy is not required to prepare the central circuitry for the alterations observed in the lactating female. The brain undergoes dramatic changes that often begin during pregnancy in preparation for parturition and lactation. The supraoptic and paraventricular nuclei, sites of oxytocin cell bodies, undergo extensive neuro-glial remodeling (Theodosis et al., 2002). Also during lactation, the tuberoinfundibular dopaminergic (TIDA) neurons that normally inhibit prolactin release, begin to express enkephalin, which stimulates prolactin (Merchenthaler, 1993). The notable point is that even if changes were initiated by pregnancy, the enhanced responsiveness to SSRI clearly requires the active state of lactation.

We do not propose that 5-HT acts alone in regulating affective behavior and mood during lactation. Numerous other neurohormones and transmitters alter emotion, many of which undergo dramatic changes during pregnancy and lactation. Obvious candidates during lactation include prolactin and oxytocin, the endocrine secretions of which are required for milk production and milk let-down in the mammary gland. Receptors for both hormones are present in a number of brain regions, including those associated with affective behavior, such as the amygdala and lateral septum (Bale et al., 2001; Bakowskaa and Morrell, 1997). Both hormones regulate behaviors associated with reproduction, such as pair-bonding and parenting (Insel and Hulihan, 1995; Bridges et al., 1990). In addition, oxytocin has been reported to have antidepressant and anxiolytic activities (Arletti and Bertolini, 1987; McCarthy et al., 1996). One study found anxiolytic actions of oxytocin only in pregnant and lactating rats, and not in virgin females (Neumann et al, 2000). Intracerebroventricular
administration of antisense oligonucleotides against the prolactin receptor was found to increase anxiety-related behavior in lactating rats as well as impair maternal behavior (Torner et al., 2002). 5-HT serves as a mediator in the neuroendocrine reflexes that result in the secretion of oxytocin and prolactin, including suckling-induced and stress-induced release (Kordon et al., 1973; Moos and Richard, 1983; Jørgensen et al., 1992). Thus, either or both of these two hormones may be involved in the differential behavioral responsiveness to SSRIs in lactating and nonlactating mice that we report here.

In addition to being exclusive to lactating dams, the effect of SSRI treatment was specific for measures of ‘emotional behavior’. The FST is considered a measure of depressive behavior. Treatments or psychological states that elevate mood result in decreased time of immobility. The MBT is considered a measure of anxiety-related behavior and treatments that reduce anxiety in humans result in a decrease in the number of marbles buried. Both of these behavioral tests are commonly used in the pharmaceutical industry for screening antidepressants and anxiolytics (Njung’e and Hardley, 1991a; Cryan et al., 2005; Nicolas et al., 2006).

Serotonin is involved in numerous central functions including motor skills, learning and memory, sleep-wakefulness cycles, and others. SSRI treatment had no effect on motor skill, as determined in the rotord test. This test also involves some learning over the three trials and SSRI treatment did not alter the rate at which motor performance improved in any of the animals (Figure 2.7). Spontaneous locomotor activity in the home cage also was unaffected by SSRI in both the nulliparous and lactating mice. The absence of a circadian rhythm in home cage activity in lactating
mice was both surprising and novel (Figure 2.6). Of the few studies we could find in which circadian rhythms were assessed in lactating animals, even fewer monitored spontaneous activity. Both nocturnal rats and hamsters (Kittrell and Satinoff, 1988; Scribner and Wynne-Edwards, 1994) have a significant reduction in the difference between the levels of spontaneous activity during the light and dark periods in lactating animals as compared with virgin or pregnant animals. Yet a significant rhythm is still present. The diurnal Nile grass rat also exhibits a circadian rhythm in activity during lactation which is even greater than that of virgin females but less than that of pregnant females (Schrader et al., 2009). Although we expected a dampening of the difference in cage activity between the light and dark periods in lactating mice, due to time spent nursing in the nest, we were surprised at the total absence of a rhythm. By day 10 of lactation, pups are more than 70% of their weight at weaning (data not shown) and should well tolerate the dam spending time outside the nest. Whether this arhythmicity in cage activity of lactating C57Bl6 mice is characteristic of other non-maternal behaviors and/or vegetative functions (e.g., hormone rhythms) would require further investigation.

As with the measures of affective behavior, only lactating dams had a significant decrease in platelet 5-HT stores in response to SSRI treatment. In the clinical literature, various parameters of platelet 5-HT have been examined as a potential peripheral marker of SSRI efficacy. This has arisen from the search for a biochemical marker that would have greater discriminatory power than the subjective rating scales used for measuring depression. The concentration of platelet 5-HT has consistently appeared useful as a marker in data from multiple laboratories. There is a single gene for SERT
and the transporter expressed in platelets is the same as that expressed in neurons (Lesch et al., 1993), so SSRI treatment blocks uptake of 5-HT into platelets as well. Because platelets do not contain 5-HT biosynthetic enzymes, blocking SERT results in a gradual loss of platelet serotonin stores. It has been demonstrated by independent laboratories that the magnitude of SSRI effect on platelet 5-HT directly correlates with their efficacy in alleviating depressive symptoms as determined by standard psychometric tools such as the Hamilton Depression Rating Scale (Maurer-Spurej et al., 2007; Axelson et al., 2005). Correlation of SSRI-induced decrease in platelet 5-HT levels with effects on affective behavior was also seen in the present mouse experiments. Citalopram treatment significantly reduced platelet 5-HT concentration only in lactating dams (Figure 2.8), and the lactating mice was the only group in which citalopram (or fluoxetine) significantly altered mood as assessed by the FST and MBT (Figures 2.4 and 2.5).

Any potential relationship between breastfeeding and maternal mood has not been definitively established. Although most studies have found a positive correlation between breastfeeding and alleviation of PPD symptoms (Misri et al., 1997; Fergerson et al., 2002; McCoy et al., 2006), a few studies have found a higher incidence of PPD among breastfeeding women (Alder and Cox, 1983; Alder and Bancroft, 1988). A recent qualitative systematic review of the literature found that most of these studies have methodological limits and weaknesses that equivocate conclusions about how breastfeeding influences maternal depressive symptomatology (Dennis and McQueen, 2009). However, the authors did conclude that there is unequivocal evidence that PPD negatively influences infant-feeding outcomes.
To our knowledge, no studies of the interaction of lactation and efficacy of SSRIs have been done. In animal models, studies on the efficacy of antidepressant treatment have been done primarily in males and, to a much lesser extent, in virgin females. The few studies that have used lactating animals have not compared their responses to those of non-lactating females. The clinical literature also has not addressed whether breastfeeding can alter responsiveness to SSRIs. Most clinical studies on the response to SSRIs (and other types of antidepressants) in PPD patients do not even report the breastfeeding status of the subjects. Those studies that have examined SSRI use in lactating mothers have been concerned with risk assessment for negative outcomes in babies exposed to the medications during breastfeeding. Current research focuses on two aspects of antidepressant use during breastfeeding: 1) how much of the medication passes into breast milk, and 2) does the medication affect the infant? One such study, however, measured platelet [5-HT] as evidence of SSRI effect in mother-infant pairs and presented the disaggregated data in tabular form (Epperson et al, 2001). Since the focus was the possible transfer of the SSRI (sertraline) to the infant, the number of breastfeedings per day for each mother-infant pair was also reported. We have used the published data from that small study (14 subjects) to compare the change in platelet [5-HT] following SSRI treatment in mothers who breastfed exclusively to that in mothers who supplemented with bottle feedings of formula (Figure 2.9B). SSRI treatment reduced platelet 5-HT concentrations to a greater extent in exclusive breastfeeders (7-8 nursing bouts/day) than in mixed feeders (1-2 to 6-7 nursing bouts/day). This is remarkably similar to our mouse data (Figure 2.9A). Unlike the human data, we did not have paired pre- and post-treatment measures of platelet [5-HT] in the mice. However,
if expressed as a percentage of values from vehicle-treated controls, citalopram treatment reduced platelet [5-HT] in lactating mice significantly more than in postpartum-nonlactating females. When the human data is plotted with the decrease in platelet [5-HT] as a function of number of breastfeedings per day, linear regression analysis produces $R^2 = 0.22$ with $p=0.088$. This does not quite reach significance based on our criterion ($p<0.05$). However, it should be noted that this was a very small clinical study in which there was substantial variation in numerous parameters, such as the duration of SSRI treatment (ranged from 6 to 16 weeks) and the dose of SSRI (all but one patient began treatment at 50 mg/day, then increased dose as needed up to 200 mg/day). Notable is the fact that most of the exclusive breastfeeders (4 out of 6) remained at the low dose.

The clinical implications of these findings are significant. It is clear that untreated maternal depression has a highly negative impact on the development of the child, while breastfeeding is recognized as having a highly positive effect on child health and development. Yet, mothers with PPD often believe that they must make a choice between treatment and breastfeeding (Kendall-Tackett and Hale, 2010). It may be that combined treatment and breastfeeding has the greatest benefit for both mother and child. Moreover, if the state of lactation does increase sensitivity to the antidepressant actions of SSRIs, then it is possible that lower doses of drug may be effective in treating PPD in many patients. What remains clear is that clinical studies on the potential interactions of lactation and SSRI efficacy are very much needed.
Acknowledgments

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FIGURE 2.1 Schemas for experiments assessing behavior after multiple days of citalopram treatment.

A. Behavioral Experiment 1 in which three groups were studied: 1) nulliparous females; 2) lactating mice; and 3) postpartum-nonlactating mice. Only the FST was administered in this study. B. Behavioral Experiment 2 examined only two groups: 1) nulliparous females and 2) lactating mice. This study included multiple “control” behavioral tests and an alternate test of mood assessment – the marble burying test (MBT)
FIGURE 2.2 Serotonin immunostaining of the dorsal raphe nucleus (DRN) immediately caudal to oculomotor nucleus. (Bregma -4.48 mm).

Representative examples of sections processed for 5-HT immunostaining and visualized with Ni²⁺-DAB (blue-black stain) from a virgin female mouse (A) and a lactating mouse (B). C. The average optical density of cell bodies (background-subtracted) in the field. D. The average number of stained cells in the field. Aq, Aqueduct of Sylvius; DRD, dorsal raphe-dorsal part; DRV, dorsal raphe-ventral part; VLPAG, ventrolateral aspect of the periaqueductal gray; mlf, medial longitudinal fasciculus. *p<0.05, **p<0.01 (n=4-5 per group)
FIGURE 2.3 Serum levels of serotonin in virgin female and lactating C57Bl/6J mice.

Trunk blood was allowed to clot overnight, releasing platelet 5-HT stores. Lactating mice had significantly higher levels of platelet 5-HT than virgin females. *p<0.05 determined by Student’s t-test (virgin, n=30; lactating, n=24).
FIGURE 2.4 In the FST, lactating mice respond to regimen of citalopram treatment that has no effect on nulliparous or postpartum-nonlactating female mice.

Mice received daily injections of vehicle or citalopram (5mg/kg/day, i.p.) for 5 days. Total immobility time (excluding the first two minutes of the test). *p<0.05 compared to vehicle-treated lactating mice; ***p<0.001 overall effect of lactation determined by 2-way ANOVA (n=7-30 per group).
FIGURE 2.5 In the marble-burying test, lactating mice respond to citalopram treatment that has no effect on nulliparous mice.

All mice were moved to a novel cage for testing. Lactating mice were tested while separated from their pups. *p<0.05 compared to vehicle-treated nulliparous mice. ***p<0.001 compared to vehicle-treated lactating group (n=5-10 per group).
FIGURE 2.6 Citalopram has no effect on home cage activity of either nulliparous or lactating mice.

Activity was monitored over a 24-h period and data is shown for light and dark periods separately or for entire 24-h period (Inset). Citalopram treatment did not affect home cage activity in any of the groups. Note significant increase in activity of nulliparous animals during the dark period. *p<0.05, **p<0.01 activity in dark vs. corresponding light period. ***p<0.001 lactating vs. corresponding nulliparous group (n=8-10 per group).
FIGURE 2.7  Citalopram has no effect on motor performance in either nulliparous or lactating mice.

The time spend on the rotarod in each of 3 trials is shown. All animals demonstrated improved ability to stay on the rotarod with each subsequent trial. *Inset:* Means of the slopes for each animal’s performance (time over trial number, n=8-10 per group).
FIGURE 2.8 Platelet 5-HT from female mice at various reproductive states, following five days of treatment with vehicle or SSRI (citalopram, 5mg/kg/day).

All mice were age-matched and mated. Blood was collected from postpartum-nonlactating and lactating animals on day 10 postpartum. *p<0.05 compared to vehicle-treated nulliparous group; **p<0.01 compared to vehicle-treated lactating group. Two-way ANOVA analysis indicated significant main effects of both drug treatment [F(1,88)=10.75; p<0.01] and lactation state [F(2,88)=6.079; p<0.01], and not quite significant interaction between lactation state and drug treatment [F(2,88)=2.507; p=0.08 (n=12-20 per group).
FIGURE 2.9 Interaction of lactation and response to SSRI.

A. Platelet levels of 5-HT in mice following 5 days of treatment with citalopram, expressed as a percentage of mean levels in the appropriate vehicle-treated group (postpartum nonlactating or lactating, n=12-16 per group); ***p<0.001. B. Platelet levels of 5-HT in women following treatment with sertraline, expressed as a percentage of their pre-treatment levels (“residual 5-HT levels”); *p=0.056. C. Residual platelet 5-HT levels as a function of breastfeeding frequency (#/day). Subjects were diagnosed with postpartum depression and ultimately treated with the following doses of sertraline (mg/day): 200 (black square); 150 (black circle); 100 (gray circles); 50 (white circles); 25 (white square). Linear regression produced an R²=0.222 with p=0.088. Data from exclusive breastfeeders are contained within the dashed-line box. All human data was obtained from Epperson et al., 2001 (n=14 women).
FIGURE 2.10 Citalopram treatment reduces total immobility during FST in male C57Bl6 mice, but not in nulliparous female mice.

A. Male mice respond to regimen of citalopram treatment that has no effect on nulliparous or postpartum-nonlactating female mice (5 days @ 5mg/kg/day, i.p.; see Figure 2.4). **p=0.012 compared with vehicle-treated male mice (n=15 per group). B. Nulliparous female mice do not respond to increased dose and duration of citalopram. Mice received daily injections of vehicle or citalopram (30mg/kg/day, i.p.) for 10 days (n=6 per group).
FIGURE 2.11 Lactating mice, but not nulliparous female mice, respond to acute fluoxetine treatment in the FST.

All mice received a single injection of vehicle or fluoxetine (Flx; 10 or 40 mg/kg) and subjected to FST 90 minutes later. **A.** Acute SSRI reduced total immobility time in the FST. **p<0.01** compared vs. vehicle-treated. Linear regression analysis of reduced immobility with increased dose of Flx showed significance (F=10.83, p=0.0025, n=9-14 per group). **B.** Acute SSRI treatment (fluoxetine, 40mg/kg) had no effect on total immobility time nulliparous female C57/Bl6 mice (n=11 per group).
FIGURE 2.12 Separation from pups increases marble-burying activity of lactating female mice.

The number of marbles buried by lactating mice with pups or separated from their pups was measured either in their home cage or a novel cage. ***p<0.001 vs. respective “with pups” group (n=5-8 per group).
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CHAPTER III

Enhanced Serotonergic Activity in Limbic Projection Fields may Underlie Altered Responsiveness to Selective Serotonin Reuptake Inhibitors during Lactation
ABSTRACT

Selective serotonin (5-HT) reuptake inhibitors (SSRI) are the first line of pharmacotherapy in postpartum depression (PPD). Potential interactions of lactation and SSRIs have not been addressed and clinical research on breastfeeding mothers who take SSRIs has focused entirely on the transfer of medication to the infant via breast milk. In light of these issues, we are investigating the effects of SSRIs in female mice during lactation, a state in which we have found significant changes in peripheral 5-HT levels and an enhanced responsiveness to SSRIs in tests of affective behavior. In the current study, we examined the levels of 5-HT and its major metabolite, 5-HIAA, in various nuclei of the brains of lactating C57Bl/6J mice and non-lactating females ten days postpartum, as well as in nulliparous controls. These groups were further divided into vehicle- and SSRI-treated (citalopram, 5mg/kg/day, for five days). 5-HT content in the ventrolateral periaqueductal gray (VLPAG), a site of serotonergic cell bodies, was significantly lower in lactating mice compared with nulliparous controls (16 ± 4 vs. 70 ± 18 pg/µg protein, respectively, p<0.05) but was unaffected by SSRI treatment. In the posterior basolateral amygdala (PBLA), a limbic serotonergic projection site, SSRI treatment did alter 5-HT and 5-HIAA content, but only in lactating dams. 5-HT content was increased (181 ± 54 vs. 320 ± 53 pg/µg protein, vehicle vs. SSRI, p<0.05) and 5-HIAA content was decreased (3.63 ± 1.42 vs. 0.94 ± 0.50 ng/µg protein, vehicle vs. SSRI, p<0.05), changes that would be consistent with an elevated rate of 5-HT release in the PBLA of lactating mice compared with non-lactating females. These findings provide further evidence of a significant interaction between lactation and SSRI actions in brain regions associated with emotions and affective behavior.
INTRODUCTION

The etiology and pathophysiology of PPD are not currently known, but evidence is mounting that serotonergic dysfunction may play a role (Doornbos et al., 2009, 2008; Misri and Kendrick, 2007; Dennis and Stewart, 2004; Newport et al., 2004; Appleby et al., 1997; Karege et al., 1994; Hannah et al., 1991; 1992a, b). SSRIs have been able to successfully treat and alleviate symptoms of PPD. Despite the fact that SSRIs are the most popularly prescribed medication for the pharmacological treatment of PPD (Wisner et al., 2006; Pearlstein et al., 2009; Ng et al., 2010), no clinical or animal studies have investigated the potential interaction between breastfeeding status (lactation) and SSRI responsiveness during the postpartum period. Furthermore, most of the clinical studies that have been conducted in postpartum depressed women have focused solely on the transmission of SSRIs into breast milk, and exposure of the infant (Epperson et al., 2001).

Previous studies in our laboratory have demonstrated that there are dramatic changes to central and peripheral serotonergic physiologies during lactation (see Chapter 2; Jury et al., 2012, submitted). In lactating (day 10 postpartum) dams we have observed significant reductions in both the number of cell bodies immunostained for 5-HT and the 5-HT immunostaining intensity within the dorsal raphe nuclei (DRN) when compared to age-matched nulliparous mice. The serotonergic cell bodies within the DRN are the principle site of 5-HT synthesis. Nascent 5-HT in the DRN is packaged into presynaptic vesicles that are then transported to terminal projection fields and released. The serotonergic projections are numerous and innervate a plethora of forebrain and limbic brain structures (Cooper et al., 2003). The amount of 5-HT that can
readily be measured within the perikaryia is dependent upon a balance between the rate of synthesis and the quantity of 5-HT presynaptic vesicles being transported and released into the projection fields (Cooper et al., 2003). The decreased 5-HT immunostaining that was quantified from brain slices of lactating (day 10 postpartum) mice is suggestive of two possible biochemical outcomes: 1) that there is a decrease in the synthesis of 5-HT within the DRN cell bodies, or 2) that there is an increase in transport and release of 5-HT into the projection fields. Either of these two outcomes would be predicted to have a dramatic impact on mood. A decrease in synthesis of 5-HT would mean that less 5-HT would be available for release and be predictive of a more “depressed” mood-related behavioral phenotype. Whereas, an increase in the release of 5-HT into projection fields would be indicative of a more “non-depressed” mood-related behavioral phenotype (Asberg et al., 1976a, b; Owens and Nemeroff, 1994; Lucki, 1998).

We have demonstrated that lactating (day 10 postpartum) mice exhibit an “elevated” and “non-depressed” mood-related behavioral phenotype as determined by a lower baseline immobility time during the FST, and a reduction in marble burying activity during the MBT. Moreover, we have also demonstrated that these same lactating mice respond to SSRI treatment that is ineffective in non-lactating mice (see Chapter 2; Jury et al., 2012, submitted). These results were obtained by probing the central serotonergic system with SSRIs. These data strongly suggest that there are alterations in central serotonergic physiology during lactation that may mediate these mood-related behavioral phenotypes and increased responsiveness to SSRIs. Our hypothesis was
that lactating mice exhibit a baseline enhancement of serotonergic activity in limbic brain structures, and that the application of a SSRI further elevates this activity.

To test this hypothesis we decided to measure 5-HT from the perikaryia of the DRN [e.g. dorsal raphe dorsalis (DRD), ventrolateral periaqueductal grey (VLPAG)] from vehicle and citalopram (5mg/kg/day) treated nulliparous and lactating mice that were administered the FST. We also chose to measure 5-HT and 5-HIAA from other brain regions that have been thought to be implicated in mood-related behaviors [e.g. hippocampus (CA3 region), paraventricular nucleus of the hypothalamus (PVN), and the posterior basolateral amygdala (PBLA)]. We measured 5-HT and 5-HIAA levels from the brains of vehicle and citalopram (5mg/kg/day) treated nulliparous, postpartum/non-lactating, and lactating mice. The DRD was selected because of its rostral projections to brain regions thought to be involved in stress and anxiety-related behaviors, and physiological responses to threats (Lowry et al., 2005). The VLPAG, which consists of the “lateral wings” of the DRN, was selected because the greatest change in immunostaining for 5-HT in lactating mice was observed here (see Chapter 2, Jury et al., 2012). The cell bodies from the VLPAG project caudally to the dorsolateral periaqueductal grey (DLPAG) and this region has been implicated in fight-or-flight and panic-related behaviors (Stezhka and Lovick, 1997; Bandler et al., 2000). Since 5-HT has been shown to modulate anxiety- and panic-like behaviors through the VLPAG (Johnson et al., 2004) we decided to conduct an experiment whereby nulliparous and lactating (day 10 postpartum) mice treated for 5 days with vehicle or citalopram (5mg/kg/day) would be subjected to a very loud noise to measure the acoustic startle reflex (ASR) without a prepulse inhibition (PPI), and also to an ASR with PPI. The ASR
and PPI behavioral tasks have a wide range of application, but we specifically chose to apply the test as a measurement of the animal’s evoked response to an anxiogenic/fearful stimulus (e.g. a very loud noise).

**MATERIALS AND METHODS**

**Animals and Reagents**

C57Bl/6J female mice (age 3-6 months) were used in these studies (Jackson Laboratories). All females were housed with a stud male for at least two weeks, checked for vaginal plugs, then separated and housed individually on a 12h:12h light:dark cycle with water and standard lab chow available *ad libitum*. Mated mice that did not produce a litter were assigned to the “nulliparous” groups. Mice that did become pregnant and deliver pups had their litters culled to six pups on day 1 postpartum (day of parturition = day 0) to normalize the suckling stimulus, and these constituted the “lactating” groups. A third group of dams was included (“postpartum-nonlactating”) which were mice that had their entire litters removed immediately after delivery (postpartum day 0). All procedures were reviewed and approved by the University of Cincinnati’s Institutional Animal Care and Use Committee.

5-HT and corticosterone (CORT) radioimmunoassay (RIA) Fast Track kits, and 5-HIAA enzyme-linked immunosorbent assay (ELISA) kits were purchased from Rocky Mountain Diagnostics (Colorado Springs, CO). Citalopram was purchased from Tocris Biosciences (Ellisville, MO). The Bradford protein quantification kit was obtained from Thermo Scientific/Pierce Protein Biology Products (Rockford, IL).
Drug Treatment

The same animals that were administered the FST in behavioral experiment 1 and the MBT in behavioral experiment 2, Group 1, nulliparous; Group 2, lactating; and Group 3, postpartum-nonlactating (see Chapter 2, Figure 2.1) were used in the current study. The frozen brains from these animals were used in determining brain 5-HT and 5-HIAA levels. Since the FST is considered a stressor, CORT levels were quantified using RIA from the serum of these animals. Lactating, and age-matched nulliparous mice were used in ASR and PPI protocols (postpartum/non-lactating mice, Group 3, were not used in these behavioral tasks). On day 6 postpartum, dams of Groups 2 and 3, and age-matched nulliparous females (Group 1) began receiving daily i.p. injections of SSRI (citalopram, 5mg/kg/day) or vehicle (0.9% saline). Injections were administered between 08:00h and 09:30h for 5 days. Thirty minutes after the fifth injection (day 10 postpartum, a period of peak lactation), each animal was subjected to ASR with or without PPI, FST or MBT.

Acoustic Startle Reflex and Prepulse Inhibition

The behavioral testing was conducted over two consecutive days with both vehicle and citalopram (5mg/kg/day) treated lactating and nulliparous groups being equally distributed among the two days of testing. Acoustic startle reflex (ASR) and ASR inhibition by prepulse (PPI) were measured in a SR Lab test chamber (San Diego Instruments, San Diego, CA). The test chamber was calibrated using the manufacturer's guidelines and sensitivity was regularly checked using a calibrated oscillation device to ensure consistent sensitive readings. At the start of each test, mice were placed in acrylic cylindrical tubes (12.6 cm in length× 3.8 cm internal diameter) mounted on
platforms equipped with ultrasensitive motion sensors inside a sound attenuating test chamber. Motion sensors recorded ASR responses as a change in voltage as arbitrary units that were digitized and stored by the computer controlling the delivery of white noise acoustic stimuli through loudspeakers. Background white noise was set at 70 dB. The testing paradigm used was similar to those previously reported (de Jong et al., 2006; Vorhees et al., 2011). Each test session consisted of a 5 min acclimation period followed by a $5 \times 5$ Latin square sequence of trials of 5 different types: no stimulus, startle signal with no prepulse, startle signal with prepulse 5 dB above background (75 dB), startle signal with prepulse 10 dB above background (80 dB), and startle signal with prepulse 15 dB above background (85 dB). Each animal received each trial type once in each of the 5 orders, and the entire Latin square sequence was repeated. Each animal received each trial type/order twice. ASR amplitude was averaged for each trial (1-10) and then graphed chronologically for linear regression analysis for slope comparison. PPI was defined as $[1-(\text{ASR amplitude with prepulse}/\text{ASR amplitude with no prepulse})] \times 100$ (Flood et al., 2007). PPI from each treatment group were averaged together for analysis. The intertrial intervals were 10-20 s with randomized spacing. The interstimulus interval was 100 ms (measured from prepulse onset to startle signal onset). The startle signal was a 120 dB mixed frequency, white noise burst that lasted for 20 ms. The recording window was 100 ms and prepulses lasted for 20 ms. The entire testing procedure including the 5 minute acclimation period lasted approximately 25 minutes. Stimulus intensity was measured using a Quest sound level meter (SPL scale) with the meter placed in the test chamber in the center of the test stage with the door closed and the microphone directed upward toward the ceiling-mounted speaker.
Response amplitude (\(V_{\text{max}} = \) maximum voltage change within the recording window) was recorded in units of voltage change (mV). The response output amplifier was set to a value of 6.0 for all testing sessions. Test chambers were cleaned with 70% ethanol between animals.

**Blood Collection and Assays**

Twenty minutes after the FST animals were sacrificed and whole brains were removed and trunk blood collected. Brains were immediately frozen on dry ice (-78ºC) and then later stored at -80ºC until sectioning. Whole blood was allowed to clot overnight at 4ºC to release 5-HT from platelets. Serum was separated by centrifugation at 12,000 rpm for 15 minutes at 4ºC, and stored at -80ºC until assayed. Blood 5-HT and CORT levels were determined in duplicate using a commercial RIA kit according to the manufacturer’s instructions.

**Brain Sectioning**

Frozen brains and glass slides were placed inside a pre-cooled (-13ºC) cryostat and allowed to come to temperature after 30 minutes of incubation. Whole brains were glued to cryostat chucks using tissue freezing medium (Triangle Biomedical Sciences, Durham, NC). The brains were placed and oriented on the chuck so that coronal sections would be obtained. The freezing medium was allowed to harden at -13ºC for 30 minutes. Upon the freezing medium becoming firm the cryostat chuck was moved and mounted into the slicing holder and tightened. Just prior to sectioning the brain, the cryostat temperature was adjusted to -8ºC and the blade was allowed to come to temperature after 15 minutes of incubation. Brains were then sectioned at -8ºC in a
coronal orientation proceeding from rostral to caudal brain regions. Sections were sliced in order to contain the following brain regions: posterior basolateral amygdala (PBLA), dorsal raphe dosalis (DRD), the CA3 subdivision of the hippocampus (HIP), paraventricular nucleus (PVN), and ventrolateral periaqueductal grey (VLPAG).

Predetermined coordinates and visible landmarks from a mouse brain stereotaxic atlas were used to assist in slicing brain sections (Paxinos and Franklin, 2001). The following coordinates were used for slicing brain sections to ensure isolation of the brain regions of interest: PBLA and HIP = Bregma -2.70mm to -2.92mm, DRD and VLPAG = Bregma -4.48mm to -4.96mm, and PVN = Bregma -0.70mm to -1.22mm (Paxinos and Franklin, 2001). Sections were sliced at a thickness of 240µm and were placed onto pre-cooled glass slides.

**Palkovits Micropunches**

Microdissection of discrete brain regions of serotonergic perikarya and projection fields was performed using a modified Palkovits micropunch technique (Palkovits, 1973). A pre-cooled aluminum jig/block, continuously supplied with dry ice (-78 °C), was used as holder for the glass slides containing the 240µm brain sections. Glass slides were removed from the cryostat and immediately placed onto the aluminum jig/block prior to isolation of micropunched brain regions. Using the mouse brain stereotaxic atlas (Paxinos and Franklin, 2001), brain perikarya and projection fields were micropunched with a steel sample corer with an inner diameter of 0.5mm (Fine Scientific Tools, Foster City, CA). The steel sample corer was cooled on dry ice prior to each punch being removed. Upon removal of the brain region of interest, the cored micropunch was then expelled into a microcentrifuge tube that was on dry ice (-78 °C).
All brain micropunches were stored at -80 °C until extraction of 5-HT and its metabolite, 5-HIAA was performed.

**Extraction and Quantification of 5-HT and 5-HIAA from Brain Micropunches**

5-HT and 5-HIAA were extracted from brain micropunches using a modification of an existing protocol (Shankaran and Gudelsky, 1999). Briefly, 25 µL of ice cold 0.2N perchloric acid (PCA) was added to the tube of each micropunch sample. Each sample tube was sonicated in an ice-water mixture using a Branson ultrasonic disintegrator (VWR Scientific, Leicestershire, UK) with a 20 kHz continuous pulse for 10 seconds. Each sample was spun in a microcentrifuge at 12,000 rpm for 5 minutes at 4°C. 10 µL of supernatant was immediately transferred to a clean test tube for determination of 5-HT via RIA and the remaining 10 µL of supernatant was transferred to a clean microcentrifuge tube for determination of 5-HIAA via ELISA. The pellet was resuspended in 25 µL of 1.0N sodium hydroxide and stored at -20°C until its use in a Bradford assay to correct (5-HT and 5-HIAA measurements) for protein content. All samples were assayed in duplicate and according to the manufactures' instructions for RIA, ELISA and Bradford protocols.

**Data Analysis**

Data obtained in each experiment were analyzed using a two-way analysis of variance (ANOVA) followed by Bonferonni post hoc test for comparison of the means. The slopes of ASR amplitudes without PPI were compared using a linear regression analysis. All data shown are presented as the mean ± standard error of the mean.
(SEM). All experiments were designed to achieve a statistical power of 80 percent. Means were considered to be significantly different when $p<0.05$.

**RESULTS**

**5-HT Content within the Serotonergic Perikarya – Dorsal Raphe Nuclei**

Brain micropunches from the midline DRD and VLPAG, sub-regions of the DRN, were analyzed for 5-HT and 5-HIAA. Five days of citalopram (5mg/kg/day) treatment did not alter 5-HT content within the DRD (Figure 3.1). No significant differences in 5-HT content were observed between nulliparous and lactating female mice (Figure 3.1). Lactating dams exhibited a decrease in 5-HT content within the VLPAG sub-region of the DRN when compared to age-matched nulliparous mice ($p<0.05$; Figure 3.2). There was an overall effect of lactation status on 5-HT content within the VLPAG region of the DRN, as revealed by two-way ANOVA [$F(1,32)=7.55; p=0.0098$] (Figure 3.2). Drug treatment did not alter 5-HT content in the VLPAG among nulliparous or lactating female mice.

**5-HT and 5-HIAA Content within the Basolateral Amygdala**

Five day treatment with citalopram (5mg/kg/day) significantly increased the 5-HT content within the PBLA of lactating dams ($p<0.05$) when compared to vehicle-treated lactating mice (Figure 3.3A). Two-way ANOVA revealed a significant overall effect of drug treatment [$F(1,32)=4.97; p=0.0328$] and lactation status [$F(2,32)=6.43; p=0.0045$] on 5-HT content within the PBLA (Figure 3.3A). Citalopram treatment had no
effect on the 5-HT content within the PBLA of both nulliparous and postpartum/non-lactating mice.

Subchronic treatment with citalopram (5mg/kg/day) for five days significantly decreased the 5-HIAA content within the PBLA of lactating dams \( (p<0.05) \) when compared to vehicle-treated controls (Figure 3.3B). Two-way ANOVA determined that there was a trend \( [F(2,33) = 3.09; p=0.0588] \) towards an interaction between drug treatment and lactation status on 5-HIAA content (Figure 3.3B). Drug treatment did not significantly alter 5-HIAA content in either nulliparous or postpartum/non-lactating mice.

5-HT and 5-HIAA Content within the Paraventricular Nucleus of the Hypothalamus

Five day treatment with citalopram (5mg/kg/day) did not change 5-HT content among any of the groups (Figure 3.4A). However, two-way ANOVA did reveal a significant overall effect of parity on the 5-HT content within the PVN \( [F(2,33) = 3.60, p=0.0385] \) (Figure 3.4A). No differences in 5-HIAA content were observed among any of the groups in the study, and five days of citalopram (5mg/kg/day) treatment had no bearing on 5-HIAA either (Figure 3.4B).

5-HT and 5-HIAA Content within the Hippocampus

No differences in 5-HT or 5-HIAA content were observed within the CA3 sub-region of the HIP (Figure 3.5). Furthermore, drug treatment had no effect on 5-HT or 5-HIAA content in any of the groups in the study (Figure 3.5).

Corticosterone Quantification following FST

Blood samples were collected from mice 20 minutes after administration of the FST and analyzed for CORT via RIA. Animals were treated for 5 days with vehicle or
citalopram (5mg/kg/day) prior to administration of the behavioral task. Lactating mice exhibited an overall decrease in blood CORT levels as revealed by two-way ANOVA \[F(2,72) = 23.88, p<0.0001\] (Figure 3.6). Furthermore, vehicle-treated lactating dams had an attenuated release of CORT when compared to vehicle treated nulliparous \((p<0.001)\) and vehicle treated postpartum/non-lactating controls \((p<0.05;\) Figure 3.6).

**Acoustic Startle Reflex without Prepulse**

The ASR without prepulse was measured in nulliparous mice treated with vehicle or citalopram (5mg/kg/day) for 5 days. ASR amplitude was significantly higher in SSRI-treated nulliparous mice during the first trial \((p<0.01)\) (Figure 3.7). There was an overall effect of drug treatment on ASR amplitude as determined by two-way ANOVA \(F(1,190) = 6.947; p=0.0249\). Linear regression analysis determined that the slopes of the two lines representing the ASR amplitudes were significantly different (vehicle: \(-12.69 \pm 3.747\) vs. SSRI: \(-25.07 \pm 4.783;\) \(F(1,236)=4.154; p=0.04263\)) (Figure 3.7).

ASR without prepulse was measured in day 10 postpartum lactating mice treated with vehicle or citalopram (5mg/kg/day) for 5 days. ASR amplitude was significantly higher in SSRI-treated mice \((p<0.05)\) when compared to vehicle-treated lactating mice during trial number 8 (Figure 3.8). There was a trend towards an effect of drug treatment on ASR amplitude \(F(1,228)=4.307; p=0.0601\) as determined by two-way ANOVA (Figure 3.8). Linear regression analysis revealed that the slopes of the two lines representing the ASR amplitudes were not significantly different between vehicle and citalopram (5mg/kg/day) treated lactating mice \(F(1,276)=2.678; p=0.1029\) (Figure 3.8).
**Prepulse Inhibition of the Acoustic Startle Reflex**

The PPI of the ASR was measured in nulliparous and lactating (day 10 postpartum) mice that were treated with vehicle or citalopram (5mg/kg/day) for 5 days. SSRI-treated nulliparous mice exhibited an enhanced PPI of ASR when compared to vehicle-treated nulliparous mice exposed to the 75dB prepulse ($p<0.05$; Figure 3.9). The drug treatment only altered PPI of the ASR at the 75dB prepulse in nulliparous mice. Subchronic SSRI treatment did not significantly alter PPI of ASR in lactating mice, however there was a trend towards an increase in the 75dB exposed prepulse group ($p=0.0544$; Figure 3.9). There were overall effects of drug treatment [$F(1,66)=24.95$; $p<0.0001$] and lactation status [$F(5,66)=6.087$; $p<0.001$] on the PPI of ASR as determined by two-way ANOVA (Figure 3.9). There was no significant interaction between drug treatment and lactation status.

**DISCUSSION**

Our initial findings that lactating (day 10 postpartum) mice exhibited “elevated” and “non-depressed” mood-related behavioral phenotypes and responded to SSRI treatment that was ineffective in non-lactating mice (see Chapter 2; Jury et al., 2012, submitted) warranted the investigation of the potential changes to serotonergic activity within limbic projection fields of these same animals. Furthermore, the reduction in 5-HT immunostaining within the VLPAG of the DRN in lactating dams necessitated us to measure ASR and PPI because of the proposed role of the VLPAG in the modulation of panic-like behavioral responses.
5-HT content was measured from the DRD of the DRN in nulliparous and lactating (day 10 postpartum) mice that were treated for 5 days with vehicle and citalopram (5mg/kg/day). No differences in the amount of 5-HT was measured between any of the groups (Figure 3.1). This was not surprising since the original immunostaining was not that different in the DRD region when comparing brain sections from nulliparous and lactating mice (see Chapter 2, Jury et al., 2012, submitted). 5-HT content within the VLPAG was significantly lower in vehicle treated lactating animals than in age-matched vehicle treated nulliparous controls ($p<0.05$; Figure 3.2). Furthermore, there was a significant effect of lactation on the overall level of 5-HT content within the VLPAG as determined by two-way ANOVA ($p<0.01$; Figure 3.2). These results verify the immunostaining results that were obtained previously, but they still leave the question about whether this is due to a decrease in synthesis or an increase in transport and release. The changes in 5-HT content from the VLPAG of the DRN support other evidence that there are changes that inhibit this region and allow for the presentation and maintenance of maternal behavior by rodents (Sukikara et al., 2006). The changes in 5-HT content in the DRN are suggestive of an increase in 5-HT release into the projection fields. We did not directly measure release, but one research group found that there is an increase in the firing rate of neurons within the DRN during postpartum (Klink et al., 2002).

Few studies have investigated the role of the posterior basolateral amygdala (PBLA) with regards to “mood-like” behaviors. The PBLA is part of the basolateral amygdaloid complex and this particular brain region has been demonstrated to be involved with the regulation of mood- and anxiety-related behaviors (Bandler et al.,
Furthermore, efferents from the DRD project rostrally to the amygdala (Lowry et al., 2008) making it a key brain region to measure serotonergic activity. Lactating dams treated with SSRI for 5 days exhibited an increase in 5-HT content and a decrease in 5-HIAA content within the PBLA when compared to vehicle-treated lactating dams \( (p<0.05); \text{Figure } 3.3 \). Also, SSRI treatment significantly elevated 5-HT content in lactating mice when compared to postpartum/non-lactating mice treated with SSRI \( (p<0.05); \text{Figure } 3.3A \). There were significant effects of SSRI treatment \( [F(1,32) = 4.97; \ p=0.0328] \) and lactation status \( [F(2,32) = 6.43; \ p=0.0045] \) on 5-HT content within the PBLA (Figure 3.3A). There was a trend towards an interaction between drug treatment and lactation status \( [F(2,33) = 3.09; \ p=0.0588] \) (Figure 3.3B) on the 5-HIAA content in the PBLA. These results suggest that there is an enhancement of serotonergic activity within the PBLA of lactating dams, and that this activity is further elevated by treatment with SSRIs. The trend towards an increase in 5-HIAA content (Figure 3.3B) in the PBLA of lactating dams would indicate that more 5-HT is being released, and then being taken back up by serotonin transporter (SERT) and metabolized to 5-HIAA. This is further validated because application of the SSRI decreases the 5-HIAA content in lactating mice (Figure 3.3B), which would be expected because SSRI block SERT, and then less 5-HT is taken back up and exposed to monoamine oxidase and metabolized to 5-HIAA. Finally, the fact that more 5-HT is readily detectable with the application of the SSRI further suggests that there is more serotonergic activity in this particular projection field (Figure 3.3A). This is because the SSRI would be blocking SERT and less 5-HT would be getting exposed to monoamine oxidase, thus protecting it from being metabolized.
SSRI treatment had no effect on the 5-HT or 5-HIAA content within the paraventricular nucleus of the hypothalamus among any of the groups (PVN; Figure 3.4). However, there was an overall effect of parity on the 5-HT content within the PVN of postpartum/non-lactating and lactating mice \([F(2,33) = 3.60, p=0.0385]\) (Figure 3.4A). The magnocellular neurons of the PVN are involved in the release of oxytocin and there are significant changes to its neuronal circuitry during late pregnancy (Theodosis, 2002). These results support the fact that there is an effect of parity on 5-HT content within the PVN of both postpartum/non-lactating and lactating animals (Figure 3.4A). However, the data do not support an alteration of serotonergic activity in this particular brain region because there were no changes in 5-HIAA content observed (Figure 3.4B).

No differences in 5-HT or 5-HIAA content were measured within the CA3 sub-region of the hippocampus. There is evidence that this brain region is important in the formation of new memories, spatial recognition memory, and procedural memory (Marr, 1971; Cravens et al., 2006; Pittenger et al., 2006; Hunsaker et al., 2007). In our previous study, lactating and nulliparous mice that were treated with vehicle or citalopram (5mg/kg/day) for 5 days were subjected to a motor performance task on a rotarod. Neither drug treatment or lactation status altered the ability of mice to remain on the revolving rotarod (see Chapter 2; Jury et al., 2012, submitted). Furthermore, mice exhibited improvements by remaining on the rotarod longer during each subsequent trial. Thus, the ability of the animals to learn and remember how to perform on the rotarod task demonstrates that there were no obvious changes in learning or memory. Although this particular brain region is highly involved with many different processes, we did not expect to see any changes in serotonergic activity.
Mice that were administered the FST also had blood samples collected 20 minutes from the beginning of the behavioral test. SSRI treatment had no effect on corticosterone (CORT) release (Figure 3.6) following administration of the FST. Hesketh and colleagues conducted a similar experiment and demonstrated that chronic treatment with citalopram did not significantly alter CORT release following FST in nulliparous rats (Hesketh et al., 2005). Lactating mice exhibited a decrease in CORT release as determined by two-way ANOVA \([F(2,72) = 23.88, p<0.0001]\) (Figure 3.6). These results support other data that have shown that there is a general dampening of the HPA-axis in response to stressful stimuli during lactation (Neumman et al., 1998, 2000; Donner et al., 2007).

The ASR without PPI was administered to animals and the results were interpreted as a response to a fearful stimulus (e.g. a very loud noise, 120 dB). Nulliparous mice treated with SSRI exhibited a significant decrease in ASR amplitude when compared to vehicle treated nulliparous mice during the first trial of the testing procedure \((p<0.01)\). Furthermore, nulliparous mice that were treated for 5 days with citalopram (5mg/kg/day) exhibited an attenuated habituation to the ASR when compared to vehicle treated mice, as demonstrated by the change in slopes between the two ASR amplitudes (Figure 3.7). Lactating mice treated with SSRI did not have a significant attenuation of habituation to the ASR, except for trial number 8 (Figure 3.8). No significant effect of drug treatment was observed when comparing SSRI and vehicle treated lactating dams (Figure 3.8). In contrast to these data, lactating mice exhibited an attenuation of PPI of the ASR as determined by an effect of lactation on PPI \((p<0.001; \text{Figure } 3.9)\). SSRI treated nulliparous mice exhibited an enhancement of the
PPI of ASR at the 75 dB prepusle when compared to their vehicle treated controls (Figure 3.9). The results from the ASR without PPI indicate that lactating dams exhibit a more attenuated panic-like response. This response is not enhanced by application of citalopram, but it is in nulliparous mice (Figure 3.9).

Studies conducted in the mouse strain, C57BL/6J, have demonstrated that agents that enhance serotonergic function usually decrease PPI, and attenuate habituation to ASR (Dulawa et al., 1997, 2000). However, we did not see these same results as nulliparous mice treated for 5 days with citalopram (5mg/kg/day) exhibited an enhancement of the PPI, and a decrease in habituation to the ASR (Figure 3.7). There are many conflicting studies that have been conducted with testing of agonists and antagonists of serotonergic function prior to administration of the ASR or PPI. The two aforementioned studies treated with these agents acutely prior to testing, whereas we treated subchronically for 5 days prior to testing.

These behavioral results from the ASR and PPI have direct application to the changes in 5-HT content within the VLPAG region of the DRN. The neurons within the VLPAG region project caudally to the DLPAG, a region that has been implicated in sympathoexcitatory responses, such as panic-like behavior (Johnson et al., 2004). The VLPAG is hypothesized to inhibit the actions of DLPAG and prevent the panic-like response to a fearful stimulus. The decrease in the 5-HT content in the VLPAG is suggestive of an increase in 5-HT release into the DLPAG region, thus, inhibiting the panic-like response in lactating dams (Johnson et al., 2004).

Taken together these results support the hypothesis that there is an increase in serotonergic activity, perhaps even release, within limbic projection fields of lactating
dams. The behavioral data from our previous studies demonstrates that there is an increased responsiveness to SSRI treatment during lactation, and that parity alone is not sufficient to induce this responsiveness. These findings along with the brain biochemical measures suggest that the enhanced serotonergic activity that occurs in the PBLA of lactating (day 10 lactating) mice may play a role in the increased responsiveness to SSRIs as demonstrated in mood-related behavioral tasks.
Figure 3.1 5-HT content measured from the dorsal raphe dorsalis (DRD) of the dorsal raphe nuclei (DRN; Bregma -4.48mm).

5-HT content did not differ between nulliparous or lactating mice. 5 day citalopram (5mg/kg/day) treatment had no effect on 5-HT content in either group (n=10-13 per group).
Figure 3.2  5-HT content measured from the ventrolateral periaqueductal grey (VLPAG) of the dorsal raphe nuclei (DRN; Bregma -4.48mm).

Drug treatment had no effect on 5-HT content in nulliparous or lactating mice. Lactating animals exhibited a decrease in 5-HT content when compared to age-matched nulliparous controls (*p<0.05). There was a significant effect of lactation on 5-HT content as determined by two-way ANOVA, (**p<0.01) (n=7-12 per group).
PBLA

Figure 3.3 Altered serotonergic activity within the posterior basolateral amygdala (PBLA) of lactating mice (Bregma -2.70mm).

**A.** 5-HT content measured from the PBLA of animals treated with vehicle or citalopram (5mg/kg/day) for 5 days. *p<0.05 compared to vehicle treated lactating mice. **p=0.0045 overall effect of lactation, and *p=0.0328 overall effect of drug treatment determined by two-way ANOVA. Drug treatment significantly elevated 5-HT content in lactating mice when compared to nulliparous and postpartum/non-lactating controls (*p<0.05).**

**B.** 5-HIAA content measured from the PBLA of animals treated with vehicle or citalopram (5mg/kg/day) for 5 days. *p<0.05 compared to vehicle treated lactating mice. Two-way ANOVA indicated p=0.0588 for interaction between drug treatment and lactation state (n=5-8 per group).
Figure 3.4 5-HT and 5-HIAA content measured from the paraventricular nucleus of the hypothalamus (PVN; Bregma -0.70mm).

A. 5-HT content measured from the PVN of animals treated with vehicle or citalopram (5mg/kg/day) for 5 days. *p=0.0385 overall effect of parity on 5-HT content determined by two-way ANOVA. B. 5-HIAA content measured from the PVN of animals treated with vehicle or citalopram (5mg/kg/day) for 5 days. No significant alterations in 5-HIAA content were observed (n=5-9 per group).
Figure 3.5  5-HT and 5-HIAA content measured from the CA3 sub-region of the hippocampus (HIP; Bregma -2.70mm).

A.  5-HT content measured from the CA3 sub-region of the HIP of animals treated with vehicle or citalopram (5mg/kg/day) for 5 days.  B.  5-HIAA content measured from the CA3 sub-region of the HIP of animals treated with vehicle or citalopram (5mg/kg/day) for 5 days (n=5-7 per group).
Figure 3.6  Lactating dams exhibit attenuated serum corticosterone (CORT) release following exposure to the FST.

Serum CORT measured twenty minutes after FST administration from animals treated for 5 days with vehicle or citalopram (5mg/kg/day). There was an overall effect of lactation status on CORT release as determined by two-way ANOVA, \( ***p<0.0001 \). Vehicle-treated lactating dams exhibited a decrease in CORT release when compared to vehicle-treated nulliparous mice (\( ^{a}***p<0.001 \)) and vehicle-treated postpartum/non-lactating mice (\( ^{b}p<0.05 \)). Lactating dams treated with citalopram exhibited attenuated CORT levels when compared to citalopram-treated nulliparous mice (\( ^{c}***p<0.001 \)), and a trend towards a decrease with respect to citalopram-treated postpartum/non-lactating mice (\( p=0.0683 \)) (n=6-21 per group).
Figure 3.7 Acoustic startle reflex (ASR) amplitude measured in nulliparous mice.

Acoustic startle response without prepulse measured in nulliparous mice treated with vehicle (black circles connected with dashed lines) or citalopram (grey squares connected with solid lines) (5mg/kg/day) for 5 days. ASR amplitude was significantly higher in SSRI-treated mice during the first trial (**p<0.01). There was an overall effect of drug treatment on ASR amplitude as determined by two-way ANOVA (*p=0.0249).

Linear regression analysis determined that the slopes of the two lines representing the ASR amplitudes were significantly different (vehicle: $\pm 3.747$ vs. SSRI: $\pm 4.783$; *p=0.04263) (n=6 per group).
Figure 3.8 Acoustic startle reflex (ASR) amplitude measured in lactating (day 10 postpartum) mice.

Acoustic startle response without prepulse measured in lactating mice treated with vehicle (black circles connected with dashed lines) or citalopram (grey squares connected with solid lines) (5mg/kg/day) for 5 days. ASR amplitude was significantly higher in SSRI-treated mice during trial number eight (*p<0.05). There was a trend towards an effect of drug treatment on ASR amplitude (p=0.0601) as determined by two-way ANOVA. Linear regression analysis determined that the slopes of the two lines representing the ASR amplitudes were not significantly different (n=6-8 per group).
Figure 3.9 Prepulse inhibition (PPI) of the acoustic startle reflex (ASR) measured in nulliparous and lactating (day 10 postpartum) mice.

PPI of the ASR measured in lactating (day 10 postpartum) and nulliparous mice treated with vehicle or citalopram (5mg/kg/day) for 5 days. SSRI-treated nulliparous mice exhibited an enhanced PPI of ASR when compared to nulliparous vehicle-treated mice exposed to the 75dB prepulse (*p<0.05). SSRI treatment did not significantly alter PPI of ASR in lactating mice, however there was a trend towards an increase in the 75dB exposed prepulse group (p=0.0544). There were overall effects of drug treatment (***p<0.0001) and lactation status (***p<0.001) on the PPI of ASR as determined by two-way ANOVA. There was no interaction between drug treatment and lactation status (n=6-8 per group).
REFERENCES


CHAPTER IV

Manipulation of the TPH1 Gene within the Mammary Gland Alters Blood Serotonin, and Behavior during Lactation
The recent discovery of a novel serotonergic biosynthetic pathway within the mammary gland (MG) and its upregulation during lactation (Matsuda et al., 2004), have led us to investigate the potential changes to central and peripheral 5-HT physiology during lactation. Previously, we have shown that lactating dams have elevated levels of platelet 5-HT when compared with both nulliparous and postpartum/non-lactating mice. Lactating mice also exhibit a dramatic decrease in 5-HT content within the dorsal raphe nuclei, the site of serotonergic neuronal cell bodies. These changes in the lactating animals raise two important questions: 1) is the MG the source of the elevated platelet stores of 5-HT; and 2) does this increased peripheral 5-HT biosynthesis reduce the availability of tryptophan (TRP), the substrate for 5-HT biosynthesis, leading to decreased 5-HT synthesis in the brain and changes in affective behavior?

We have addressed these questions using lactating mice that were genetically manipulated to have a mammary-specific deletion or overexpression of tryptophan hydroxylase 1 (TPH1), the rate-limiting biosynthetic enzyme for 5-HT. Deleting TPH1 only in the MG abrogated the increased platelet 5-HT levels, while TPH1 overexpression in the MG resulted in platelet 5-HT levels above those measured in control mice. Overexpression of TPH1 within the MG was accomplished by knocking out LRP5, a known TPH1 inhibitor. There were no measurable differences in total circulating TRP among the three groups of mice (WT, MG LRP5−/−, and MG TPH1−/−) that were used in these studies. However, the mice overexpressing TPH1 within the MG exhibited an increase in immobility time during the forced swim test (FST). These
data demonstrate that the mammary 5-HT system, upregulated during lactation, exerts effects beyond the gland itself.

**INTRODUCTION**

In our initial studies we observed that lactating (day 10 postpartum) wild type C57BL/6J mice had a significant increase in platelet 5-HT concentrations when compared to age-matched nulliparous controls (see Chapter 2; Jury et al., 2012, submitted). TPH1 expression and 5-HT production within the mammary gland (MG) are at their highest levels during pregnancy and lactation (Matsuda et al., 2004). These results suggest that the 5-HT biosynthetic system within the MG is responsible for the production of additional 5-HT that is observed in these mice. Since elevated 5-HT has only been associated with a pathology (enteric carcinoma), determining the source of this excess 5-HT during a physiological state is important.

The serotonergic system, utilizing the TPH1 enzyme, within the MG requires tryptophan (TRP) and so does the brain. Since TRP is an essential amino acid, its availability is limited to what is circulating within the blood. These changes in serotonergic physiology and the fact that the MG 5-HT system is highly upregulated and induced during lactation raises two important questions: 1) Does the MG 5-HT system contribute to the elevated 5-HT levels that were measured in the blood of lactating animals? 2) Is the availability of TRP, the substrate for 5-HT biosynthesis, to the brain being reduced by an increased peripheral demand for TRP by the MG?

To address these questions we have utilized two transgenic mouse lines where TPH1 expression has been selectively manipulated in the mammary gland: either
knocked out (MG TPH1-/-) or overexpressed by knocking out LRP5 (MG LRP5 -/-), a
TPH1 inhibitor (Yadav et al., 2008). We obtained two strains in which either the TPH1
or the LRP5 gene is flanked with two loxP (FL/FL) sites globally. Transgenic mice
homozygous for the floxed genes were crossed with mice that express CRE
recombinase under the control of the whey acid protein (WAP) promoter (Feng et al.,
2007). During late pregnancy and lactation, production of WAP is expected to drive
expression of CRE within the mammary epithelial cells and cause gene deletion of
either TPH1 or LRP5. Abolishing TPH1 from the MG is not expected to inhibit the ability
of the gland to lactate as evidenced by successful lactation in mice with a global
knockout of TPH1 (Walther et al., 2003; Matsuda et al., 2004). The global knockout of
LRP5 by others did not have any detrimental effects on lactation (Yadav et al., 2008).

TRP availability to the brain is crucial in the ability of the brain to synthesize 5-HT
since 5-HT does not cross the blood brain barrier (Fernstrom and Wurtman, 1972).
Ultimately blood TRP levels dictate brain 5-HT levels, and restricting TRP can
significantly reduce biosynthesis of 5-HT within the brain (Fernstrom and Wurtman,
1972). The reduction of 5-HT synthesis could lead to depleted 5-HT stores available for
release and this could lead to a more “depressed” mood-related behavior phenotype. In
the current study we decided to administer the FST and MBT as measures of “mood”
and “anxiety” related behavioral phenotypes. These experiments were important
because other clinical studies have demonstrated that there might be a limited
availability of TRP to the brains of patients presenting with postpartum mood
perturbations (see Chapter 1, Figure 1.1). The problem with these studies was that
most of them did not control for, or even report, the breastfeeding status of the mother.
In the current study we investigated the importance of the novel MG 5-HT system and its potential effects on platelet 5-HT and mood during lactation.

**MATERIALS AND METHODS**

**Animals and Reagents**

Female mice (age 3-6 months) homozygous for loxP sites (FL/FL) flanking the LRP5 or TPH1 gene were used as control animals throughout the study. We did not detect any measurable biochemical or behavioral differences when comparing LRP5 FL/FL female mice to TPH1 FL/FL female mice (data not shown). These animals are therefore referred to as wild type (WT) throughout the paper and the data were pooled together for these two groups. Female mice (age 3-6 months) that were homozygous for loxP sites (e.g. LRP5 FL/FL, TPH1 FL/FL) were crossed with mice that had expression of CRE recombinase that was under the control of a whey acid protein (WAP) promoter (gift of Susan Waltz, University of Cincinnati). WAP is produced during late pregnancy and lactation, thus the expression of CRE was specific to MG epithelial cells.

Lactating animals that had the WAP-CRE promoter present and that were homozygous for the loxP sites flanking LRP5 or TPH1 genes are referred to as MG LRP5 -/- or MG TPH1 -/- throughout the paper. In collaboration with Laura Hernandez, Ph.D., Dhruv Amratia, and Nelson Horseman, Ph.D. at the University of Cincinnati, these animals were successfully characterized. The mRNA expression levels of TPH1 within the MG were determined using quantitative real-time polymerase chain reaction
(qRT-PCR). TPH1 expression measured from mammary tissue of lactating mice (multiparous) with the LRP5 gene knocked out exhibited increases in relative TPH1 expression when compared to WT lactating dams (Lauran Hernandez and Dhruv Amratia, data not shown). The TPH1 expression levels were significantly reduced in mammary tissue from lactating (multiparous) mice with the TPH1 gene knocked out (data not shown).

Three females (3-6 months of age; WT, MG LRP5 FL/FL + WAP-CRE, and MG TPH1 FL/FL + WAP-CRE) were harem mated with a stud male. Female mice were allowed to go through one full-term pregnancy, and then pups were removed on the day of delivery of the first litter. Previous studies that have utilized the WAP-CRE promoter have demonstrated that after one full-round of pregnancy and lactation there were still intact homozygous loxP (FL/FL) alleles present within mammary epithelial cells (Feng et al., 2007). Indeed others have determined that WAP itself is not fully induced through only one pregnancy (Robinson et al., 1995). Mice that delivered litters continued to be housed with a stud male and checked for vaginal plugs for at least two weeks. Upon visible confirmation of the second pregnancy females were separated and housed individually on a 12h:12h light:dark cycle with water and standard lab chow available ad libitum. Litters were culled to six pups on day 2 postpartum (day of delivery = day 0 postpartum) of the second litter. On day 10 postpartum of the second litter, lactating dams (WT, MG LRP5 -/-, and MG TPH1 -/-) were administered the marble bury task (MBT) followed by the forced swim test (FST).

In a separate experiment, nulliparous female mice (age 3-6 months) that had a global knockout of the TPH1 gene were used to assess behavioral despair in the FST.
All procedures were reviewed and approved by the University of Cincinnati’s Institution for Animal Care and Use Committee.

5-HT radioimmunoassay (RIA) Fast Track kits, and tryptophan (TRP) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Rocky Mountain Diagnostics (Colorado Springs, CO). The fluorometric DNA quantification kit was obtained from Thermo Scientific/Pierce Protein Biology Products (Rockford, IL).

**Marble Burying Task**

For the marble-burying task (MBT) each test mouse was placed into a larger, novel cage without pups present. Mice were allowed to acclimate in the novel cage for 30 minutes after which 20 clean, transparent glass marbles (1.5 cm diameter) were placed on top of the bedding, in five rows of four marbles each, with the marbles spaced equally apart. At the end of 20 minutes, the number of buried marbles (at least two-thirds covered with bedding) was recorded (Njung’è and Hardley, 1991a, b). The MBT was administered prior to the FST.

**Forced Swim Test**

Immediately following the MBT, the FST was always administered between 09:00h and 11:30h. Mice were placed in a swim tank (height = 30cm, diameter = 20cm) containing water (25°C ± 1°C) at a height of 20cm for a total of six minutes. All sessions were recorded on video and scored by two independent observers who were blinded to the treatments. The total time of immobility was recorded. The first two minutes were not used in the determination of the total immobility time as per Porsolt et al. (1977). A mouse was deemed to be immobile when it was floating and making only minor
movements to keep its head above water. Tanks were emptied and rinsed clean after each animal.

**Blood Collection and Assays**

Twenty minutes after the FST animals were sacrificed and the left 4th MG and duodenum were removed and trunk blood collected. Mammary and duodenal tissues were immediately frozen on dry ice (-78°C), and then later stored at -80°C until they were extracted for determination of 5-HT. Whole blood was allowed to clot overnight at 4°C to release 5-HT from platelets. Serum was separated by centrifugation at 12,000 rpm for 15 minutes at 4°C, and stored at -80°C until assayed. Blood 5-HT and levels were determined in duplicate using a commercial RIA kit according to the manufacturer’s instructions. Blood TRP levels were assessed in duplicate using a commercial ELISA kit according to the manufacture’s instructions.

**MG and Duodenum Tissue 5-HT Extraction Protocol**

Thawed MG and duodenal tissues were washed twice in 200μL of ice cold 0.01M Phosphate Buffered Saline (PBS) to remove any excess blood from tissues. PBS was then aspirated to remove any excess fluid from tissue. Each piece of tissue was placed into a clean glass homogenizer and 200μL of ice cold 0.2N perchloric acid was added. Tissues were homogenized using a Glas-Col tissue homogenizer. The tissue was fully homogenized by making eight complete and full iterations (pushing the pestle down, and then bringing it up). The homogenate was then transferred to a clean microcentrifuge tube, and then spun in a microcentrifuge for ten minutes at 2,000 x g at 4°C. The middle layer of the supernatant from MG homogenate was removed as the
upper layer contained significant amounts of fat. The entire supernatant of the duodenal homogenate was removed. A total of 25μL of each supernatant were used for determination of 5-HT via RIA. The remaining pellet was frozen at -20°C until assayed for the quantitation of DNA via fluorometric assay according the manufacturer’s instructions. All 5-HT measurements from MG and duodenal homogenates were corrected for the amount of DNA contained within each sample. DNA was measured instead of protein because the MG is significantly more fatty tissue than the duodenum.

Data Analysis

Data obtained in each experiment were analyzed using a one-way analysis of variance (ANOVA) followed by Bonferonni post hoc test for comparison of the means. In one experiment the Student’s t-test was employed for comparison of the means. All data shown are presented as the mean ± standard error of the mean (SEM). All experiments were designed to achieve a statistical power of 80 percent. Means were considered to be significantly different when p<0.05.

RESULTS

5-HT Measured from MG and Duodenal Tissues

5-HT content was measured from homogenates of the MG and duodenum of WT, MG LRP5 -/-, and MG TPH1 -/- multiparous lactating mice. One-way ANOVA was significant [F(2,23)=18.75; p<0.0001] (Figure 4.1A) and Bonferonni post hoc analysis indicated that 5-HT content was much higher in lactating (day 10 postpartum) dams with
a selective mammary specific knockout of the LRP5 gene ($p<0.01$; Figure 4.1A). There was a significant reduction in 5-HT content within the MG of MG TPH1 -/- lactating dams when compared to wild type lactating dams ($p<0.05$) and MG LRP5 -/- lactating dams ($p<0.0001$; Figure 4.1A). 5-HT content within the duodenum did not differ between any of the groups (Figure 4.1B).

**Serum 5-HT from Genetically Manipulated Mice**

As we have previously reported, lactating WT mice had significantly higher levels of platelet 5-HT than their non-lactating counterparts ($p<0.05$ determined by Student’s t-test, Figure 4.2). When the TPH1 gene was deleted from all tissues ("global knock-out"), platelet 5-HT was significantly lower as compared with non-lactating WT mice. Moreover, there was no difference in platelet 5-HT between non-lactating and lactating mice in this global TPH1 knock-out strain (Figure 4.2). One-way ANOVA was significant [$F(2,39)=12.69; p<0.0001$] and Bonferonni post hoc analysis indicated that serum 5-HT was significantly elevated in MG LRP5 -/- lactating dams ($p<0.05$; Figure 4.3). There was a significant reduction in circulating serum 5-HT in MG TPH1 -/- lactating mice when compared to WT ($p<0.01$) and MG LRP5 -/- ($p<0.0001$; Figure 4.3) lactating.

**Serum Tryptophan**

No measurable changes in serum TRP were determined from any of the groups. Manipulation of the LRP5 or TPH1 genes had no effects on serum TRP (Figure 4.4).

**Behavioral Tests**

A one-way ANOVA was not quite significant [$F(2,30)=2.739; p=0.0819$] for the number of marbles buried among the different genotypes of the lactating mice (Figure
Bonferonni post hoc analysis revealed that there was a trend towards a decrease in marble burying behavior in MG TPH1 -/- lactating dams when compared to both WT (p=0.0543) and MG LRP5 -/- (p=0.0544; Figure 4.5).

MG LRP5 -/- lactating mice exhibited greater immobility time during the FST when compared to WT lactating mice (p<0.01; Figure 4.6). Nulliparous mice with a global knock out of the TPH1 (-/-) gene exhibited a reduction in immobility time during the FST when compared to nulliparous homozygous WT (TPH1 +/+ ) and heterozygous TPH1 (+/-) mice (p<0.01; Figure 4.7) as determined by Bonferonni post hoc analysis following a significant one-way ANOVA [F(2,17)=12.48; p=0.0006] (Figure 4.7).

**DISCUSSION**

The measurement of blood 5-HT from WT and mammary transgenic animals demonstrated that the MG plays a pivotal role in the contribution to the elevated 5-HT levels that are observed during lactation. As hypothesized, knocking out TPH1 within the MG caused a decrease in platelet 5-HT levels when compared to both WT and MG LRP5 -/- lactating mice (Figure 4.3). Furthermore, lactating mice overexpressing TPH1 (MG LRP5 -/-) within the MG, via knocking out its inhibitor LRP5, exhibited an increase in platelet 5-HT when compared to WT lactating dams (Figure 4.3). The majority of 5-HT in the body is produced within the enterochromaffin cells of the gut (Barter and Pearse, 1955). Even though there is a significant amount of gut hyperplasia during lactation (Hammond, 1997), these results support the notion that the MG alone is responsible for the changes in platelet 5-HT observed during lactation. Measurement of 5-HT from the duodenum of transgenic and WT animals did not reveal any changes in
5-HT content (Figure 4.1). Thus, manipulating the expression of TPH1 within the mammary gland had no effect on 5-HT production within the gut. However, further studies using gut specific TPH1 knockouts would be helpful in determining if the gut does play a significant role in the elevated platelet 5-HT during lactation.

Serum TRP levels were not significantly altered in any of the groups that were assessed (Figure 4.4). There were behavioral effects of manipulating TPH1 within the MG during lactation, which are perhaps suggestive of altered TRP availability. The MG LRP5 -/- lactating mice exhibited a more "depressed" mood-related behavioral phenotype as evidenced by an increase in immobility time during the FST when compared to WT lactating dams (Figure 4.6). Also, there was a trend towards an anxiolytic effect in the MG TPH1 -/- lactating dams when compared to WT and MG LRP5 -/- mice. The behavioral data support a reduction in TRP availability to the brain in the MG LRP5 -/- lactating animals, and increase availability in MG TPH1 -/- lactating animals. Nulliparous animals that have are homozygous for a knockout of the TPH1 gene (global knockout) exhibit a decrease in immobility time during the FST (Figure 4.7). This would certainly support the evidence that knocking out the TPH1 gene within the periphery would make more TRP available to the brain, and thus, a “happier” and less “depressed” mood related behavioral phenotype. Taken together more studies need to be conducted in these animals to determine if manipulating the MG serotonergic system has effects on other mood-related behaviors. Furthermore, another study where plasma is collected from these same lactating mammary transgenic animals would be helpful. Using platelet measures of TRP would help to
ascertain the total amount of TRP and the amount of free versus bound TRP within the platelets.

We have demonstrated that the MG and its serotonergic system have a profound impact upon the physiology of platelet 5-HT and perhaps mood-related behavior during lactation in mice. These results further demonstrate the importance of reporting breastfeeding status from clinical studies that are investigating mood during the postpartum period.
Figure 4.1 5-HT content measured from mammary and duodenal tissue of lactating mice with a mammary-specific knockout of LRP5 or TPH1.

A. 5-HT content measured from the mammary gland. One-way ANOVA was significant ($p<0.0001$) and Bonferroni post hoc analysis indicated that 5-HT content was much higher ($a^p<0.01$) in lactating dams with a selective mammary-specific knockout of the LRP5 gene. There was a significant reduction in 5-HT content within the mammary gland of lactating dams with a mammary-specific knockout of the TPH1 gene when compared to wild type lactating dams ($b^p<0.05$) and lactating dams that had a mammary specific knockout of the LRP5 gene ($c^p<0.0001$) (n=8 per group). B. 5-HT content measured from the duodenal tissues of lactating animals that had a mammary-specific knockout of the either the LRP5 or TPH1 gene. No differences were determined between any of the groups with respect to knocking out either gene (n=5-8 per group).
Figure 4.2 Platelet serotonin concentrations in virgin and lactating C57Bl/6J female mice of wild type (WT) strain or with global deletion of the TPH1 gene.

Trunk blood was allowed to clot overnight, releasing platelet 5-HT stores. Lactating WT mice had significantly higher levels of platelet 5-HT than their virgin counterparts. *p<0.05 determined by Student’s t-test. Platelet 5-HT in TPH1-KO mice was significantly lower than in WT and there was no difference between virgin and lactating (n=24-30 per group).
Figure 4.3  Serum 5-HT measured from lactating mice with a mammary-specific knockout of LRP5 or TPH1.

One-way ANOVA was significant (p<0.0001) and Bonferonni post hoc analysis indicated that serum 5-HT was significantly elevated (a p<0.05) in lactating dams with a selective mammary specific knockout of the LRP5 gene. There was a significant reduction in serum 5-HT from lactating mice with a mammary specific knockout of the TPH1 gene when compared to wild type lactating dams (b p<0.01) and lactating dams that had a mammary specific knockout of the LRP5 gene (c p<0.0001) (n=10-20 per group).
Figure 4.4  Serum tryptophan measured from lactating mice with a mammary specific-knockout of LRP5 or TPH1.

Tryptophan was measured using an ELISA. Knocking out LRP5 or TPH1 selectively within the mammary gland had no effect on serum tryptophan levels (n=9-15 per group).
Figure 4.5 Marble burying task (MBT) administered to lactating mice with a mammary-specific knockout of LRP5 or TPH1.

One-way ANOVA was not quite significant ($p=0.0819$) for the number of marbles buried with respect to genotype. Bonferroni post hoc analysis revealed that there was a trend towards a decrease in marble burying behavior in lactating dams with a selective knockout of the TPH1 gene within the mammary gland ($p=0.0543$) when compared to both wild type and mammary specific LRP5 knockout lactating mice (n=8-15 per group).
Figure 4.6 Forced Swim Test (FST) administered to lactating mice with a mammary-specific knockout of LRP5.

Lactating mice with a mammary specific selective knockout of the LRP5 gene exhibited greater immobility time during the FST when compared to WT lactating mice (**p<0.01) (n=10 per group).
Figure 4.7 Forced Swim Test (FST) administered to nulliparous mice with a global knock out of TPH1.

Nulliparous mice with a global knock out of the TPH1 gene exhibited lower immobility time during the FST when compared to both WT (homozygous) and heterozygous TPH1 mice (\(**p<0.01\)) (n=6 per group).
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CHAPTER V

Clinical Implications and Future Directions
Summary of Dissertation Experiments

In this dissertation I have presented novel discoveries that have implications in the treatment of PPD. I have demonstrated that there is an interaction between lactation and SSRI responsiveness in mice. Also, I have shown that there are striking changes to baseline mood-related behavioral phenotypes and serotonergic activity within limbic brain structures during lactation. Furthermore, I demonstrated that knocking out the mammary serotonergic system within the mammary gland completely abrogated the increase in platelet 5-HT that had previously been observed in wild type lactating dams. I also presented evidence that manipulating the serotonergic system within the mammary gland may play a role in anxiety- and mood-related behaviors.

New Preliminary Data: Validation of Behavioral Studies

All of the experiments that I conducted were done in exclusively in mice. Mice are a great tool for assessing a whole host of mood- and anxiety-related behaviors and the effects of centrally acting agents that can cause an alteration in these same behaviors. Moreover, they are crucial in creating a knockout or overexpression of a particular gene of interest. Many studies that have been conducted in mice have led to the discovery of a multitude of different physiological processes that do occur in humans. However, not every experimental outcome that is determined within a mouse has direct translation to humans, or even to rodents that are higher on the hierarchical tree.
To convince myself that these data were not simply an *in vivo* artifact in mice, I decided to use the same testing procedures for the marble bury task (MBT) and the forced swim test (FST) in Sprague Dawley rats. Rats were first used by Porsolt and colleagues in the seminal finding that swimming rats in a tank could be used as a behavioral test of despair in the assessment of the effectiveness of antidepressant drugs (Porsolt et al., 1977). Lactating (day 14 postpartum) and age-matched nulliparous female Sprague Dawley rats (3-6 months of age) were treated with vehicle or citalopram (5mg/kg/day) for 14 days prior to administration of the MBT followed by the FST. The chronic (14 day) treatment regimen and citalopram (5mg/kg/day) dose were selected based upon previous literature (Reneric and Lucki, 1998; Lucki et al., 2001; Craft et al., 2010). Lactating rats exhibited an elevation in marble burying behavior, and this behavior was abrogated with chronic SSRI treatment (*p*<0.001; Figure 5.1). Furthermore, there was a significant effect of drug treatment (*p*<0.05; Figure 5.1) and an interaction between drug treatment and lactation status (*p*<0.01; Figure 5.1) on marble burying behavior. Nulliparous rats did not respond to a chronic 14 day treatment with SSRI as evidenced by a lack of a change in marble burying behavior (Figure 5.1). In the FST neither lactating nor nulliparous rats that were treated chronically with SSRI responded to the treatment with a corresponding decrease in immobility (Figure 5.2). However, vehicle- and SSRI-treated lactating rats exhibited decreases in immobility when compared to nulliparous rats in general (Figure 5.2). Two-way ANOVA revealed a significant effect of lactation on the time spent immobile (*p*<0.0001; Figure 5.2).
These preliminary results support the previous findings in mice that SSRI treatment can have an anxiolytic effect only within lactating dams. Even though the lactating rats did not respond to SSRI treatment with a decrease in immobility time during the FST, the effect of lactation still remains. Taken together these results provide evidence that lactation still has some of the same effects upon anxiety- and mood-related behaviors in a different rodent species.

**Future Directions: What do we want to know?**

The results from my basic research projects have provided behavioral and biochemical evidence of an interaction between lactation and SSRI treatment in rodent models. Moreover, postpartum/non-lactating controls demonstrated that parity alone is not necessarily responsible for these changes in SSRI responsiveness. These results raise two important questions: 1) what are the mechanisms that mediate this enhancement of SSRI responsiveness during lactation and 2) what are the potential clinical applications that can be gleaned from these results? These two questions are very broad and it would take numerous experiments to answer them. However, I will outline a couple of key experiments in both rodents and humans that would be crucial in advancing this research project and making it relevant to the human condition of postpartum depression (PPD). Furthermore, the current understanding of how SSRIs exert their therapeutic effects is still not clear, and these studies would potentially aid in our understanding of their mechanism of action.
Proposed Future Experiments in Rodents

Understanding the underlying mechanism(s) of the enhanced responsiveness to SSRI treatment during lactation in rodents is warranted. I have demonstrated that the changes in serotonergic physiologies correlate with an improvement in mood-related behaviors. I have also demonstrated that there are changes in 5-HT activity in the limbic projection fields of the amygdala, a brain region that has been implicated in mood-related behaviors (LeDoux, 2000; Phelps, 2004). However, I did not directly measure the release of 5-HT into the amygdala of mice. Previous studies have shown that a decrease in 5-HT release within the amygdala can enhance the anxiety- and panic-like behavior in animal behavioral tasks (Graeff and Zangrossi, 2010). Furthermore, studies in rodents have demonstrated that an increase in 5-HT release into the amygdala can attenuate the anxiety-related behaviors via chronic treatment with SSRIs (Graeff et al., 1998). The results from my behavioral experiments (specifically the decrease in marble burying behavior in SSRI treated lactating mice, see Chapter 2) suggest a connection between the increased 5-HT activity within the posterior basolateral amygdala (PBLA; see Chapter 3) and the increased responsiveness to the SSRIs within the lactating mice. This suggests that there is an increase release of 5-HT within the PBLA of lactating dams because they were the only group that responded to the SSRI treatment during the MBT.

To determine whether the release of 5-HT is being altered within the PBLA I would conduct future studies in mice (e.g. nulliparous, postpartum/non-lactating, and lactating) using the same five-day treatment regimen of citalopram (5mg/kg/day) in combination with an inhibitor of 5-HT synthesis (e.g. NSD-1015) to determine if the
actual amount of 5-HT release differs between any of the groups of mice with respect to endocrine status. This would be determined by measuring the levels of 5-HT within PBLA after animals have been given the inhibitor (e.g. NSD-1015), or vehicle. Mice that exhibit a decrease in 5-HT levels within the PBLA upon application of the SSRI when compared to vehicle-treated mice would be considered to have a greater amount of release within the PBLA. Also, I would measure levels of 5-HT within the dorsal raphe nuclei (DRN) to determine if there was a corresponding decrease in 5-HT levels in the DRN. A decrease in 5-HT levels within the DRN after administration of the SSRI and the 5-HT synthesis inhibitor would be expected. The comparison of the decrease in 5-HT content within the DRN and the PBLA between vehicle- and SSRI-treated groups would determine the extent to which depletion is occurring, and thus, if release is greater into the PBLA.

Another experiment that would be conducted to assess the potential mechanism of the increased responsiveness to SSRI treatment would be to determine if oxytocin is having a modulatory role. Oxytocin has been shown to be a hormone that can suppress stress responses in animal behavioral tasks, and has been shown to be released during a “feel good moment” in humans (Neumann et al., 2000; Carter et al., 2007). Emiliano and colleagues have confirmed that the 5-HT transporter (SERT) is present within the nerve fibers of the supraoptic nucleus and the paraventricular nucleus of the hypothalamus (Emiliano et al., 2007). Also, serotonergic neurons innervate these brain regions extensively. Since oxytocin is synthesized and released from these brain regions enhancing blockade of SERT within these regions could have implications for an increase in oxytocin release. One study demonstrated the potential for an
enhancement of oxytocin release with chronic SSRI treatment, and observed an increase in detectable oxytocin (Uvnas-Moberg et al., 1999). The pulsatile release of oxytocin is obligatory for milk ejection from the breast during lactation (Leng and Brown, 1997; Soldo et al., 2004) and has even been considered a co-release factor for prolactin (Horseman and Gregerson, 2005).

Obviously a knockout model of oxytocin would not be useful in this particular case because the ability to lactate would not occur since oxytocin has been shown to be crucial for milk ejection (Leng and Brown, 1997; Soldo et al., 2004). However, giving an acute intracerebroventricular injection of antisense oligonucleotides against SERT within the SON and PVN of lactating dams would be a way to assess the activity of 5-HT within this region and determine if it was having any modulatory effects on the responsiveness to the SSRI treatment. Lactating mice that are treated with the subchronic treatment of citalopram (5mg/kg/day) would then either be treated with scrambled antisense oligonucleotides, or ones specific to the SERT mRNA a day prior to the behavioral testing. The same behavioral tasks would be administered to the two different oligonucleotide antisense treatment groups. This would be a simple way to assess if 5-HT function within the SON and PVN is having an effect upon the behavior specific to the oxytocin release pathway.

Proposed Future Clinical Studies

Understanding the reason(s) why lactating mice are more responsive to a sub-threshold dose of SSRI treatment has obvious clinical implications for the treatment of
PPD. Even though there is evidence to suggest that pharmacological treatment of PPD with SSRIs is safe (Wisner et al., 2006; Fortinguerra et al., 2009) many women are still hesitant to breastfeed while taking these medications (Chabrol et al., 2004). Often many women see breastfeeding and the treatment of PPD with SSRIs as two completely separate things (Kendall-Tackett and Hale, 2010).

The published literature on breastfeeding and pharmacological treatment of PPD with SSRIs focuses mainly on the transport of the drug into the breast milk and exposure of the infant. There have been several reports that have supported the notion that breastfeeding prevents symptoms of PPD (Misri et al., 1997; Fergerson et al., 2002; Mezzacappa and Katkin, 2002; McCoy et al., 2006), but there have also been reports that contradict these finding and provide evidence of the development of PPD symptoms before weaning occurs (Alder & Cox, 1983; Alder and Bancroft, 1988). However, the majority of the clinical literature investigating symptoms of PPD does not control or report the breastfeeding status of the subjects being investigated (Schrocksnadel et al., 1996; Schrocksnadel et al., 2003; Kohl et al., 2005; Bailara et al., 2006; Schrocksnadel et al., 2006; Doornbos et al., 2008; Dennis and McQueen, 2009; Doornbos et al., 2009).

The increase responsiveness to SSRI treatment in lactating mice, and that fact that numerous clinical studies have not addressed exclusive breastfeeding in their investigations, warrant a clinical study to be conducted in PPD women who are exclusively breastfeeding or not. The possible interactions between SSRI responsiveness and breastfeeding status have not been addressed and a clinical study would help answer the question of whether or not breastfeeding can enhance the
therapeutic effects of SSRIs. A very small clinical study conducted by Epperson and colleagues presented clinical data that listed not only the breastfeeding status, but also the frequency of breastfeeding (Epperson et al., 2001). They were measuring amounts of sertraline, an SSRI, and serotonin (5-HT) in both the blood from the mother and the blood of the child to determine exposure of the child to sertraline. They presented disaggregated 5-HT data indicating the amount of serotonin transporter (SERT) blockade within the blood. This disaggregated data was reanalyzed using the breastfeeding status and the blood sample that was taken before sertraline treatment was begun. The data were analyzed to determine the decrease in platelet 5-HT, an accepted peripheral measure of SSRI efficacy in humans (Karege et al., 1994; Axelson et al., 2005; Maurer-Spurej et al., 2007). The exclusive breastfeeders exhibited a decrease in platelet 5-HT levels when compared to samples that were taken before sertraline treatment began. However, this was not significant based on our criterion ($p<0.10$) in the exclusive breastfeeders and not the mixed feeders, who used bottle feeding to supplement (see Chapter 2; Jury et al., 2012, submitted). These data agree with our original findings that lactating mice respond to SSRI treatment with a decrease in platelet 5-HT levels, but nulliparous and postpartum/non-lactating mice do not. However, Epperson and colleagues did not assess mood scored in the women who were treated to ascertain if there was a difference in responsiveness to the SSRI (Epperson et al., 2001).

Clinical studies must be conducted to ascertain if breastfeeding in humans enhances the responsiveness to SSRIs. The study should include women who are diagnosed with PPD and are either exclusively breastfeeding or exclusively bottle
feeding. The assessment should include measurement of mood by Hamilton Depression Scale questionnaires before and after treatment with the SSRI begins. Furthermore, a blood sample should be collected prior to treatment with SSRI and four weeks after treatment with the SSRI has begun. The levels of platelet 5-HT should be assessed as a peripheral marker of SSRI effectiveness, and compared to the Hamilton Depression mood scores as a partial determinant of whether or not the exclusive breastfeeders exhibit an increased responsiveness to SSRI treatment. Furthermore, the measure of other neuroendocrine factors (e.g. cortisol, oxytocin, and prolactin) should be measured to determine if any differences in the blood levels of these could explain the potentially altered responsiveness to SSRI treatment. The study would be best if it could include several different groups within the study in addition to the placebo group. At least three different doses of the SSRI should be given to three respective groups to determine not only if the responsiveness is greater in exclusive breastfeeding women, but also to determine if the effects of lactation can enhance the responsiveness of the SSRI in women at higher and lower doses. Ultimately these clinical studies would be beneficial in determining if breastfeeding women will have an enhanced responsiveness to SSRI treatment by alleviation of PPD symptoms.
Figure 5.1 Marble burying task (MBT) assessed in nulliparous and lactating (day 14 postpartum) female rats.

The total number of marbles that were buried by rats were counted at the end of the behavioral task. Two-way ANOVA indicated that there was a main effect of drug treatment (*p=0.0254), and an interaction between drug treatment and lactation status (**p=0.0053). Post hoc analysis indicated that lactating rats treated with citalopram (5mg/kg/day) for 14 days exhibited a significant decrease in marble burying behavior when compared to vehicle-treated controls (**p<0.001) (n=7-9 per group).
Figure 5.2 Forced Swim Test (FST) assessed in nulliparous and lactating (day 14 postpartum) female rats.

The total amount of time spent immobile was measured while excluding the first two minutes (Porsolt et al., 1977). Two-way ANOVA revealed that there was a significant effect of lactation on time spent immobile (***p<0.0001). Chronic (14 days) drug treatment did not significantly alter immobility time in either nulliparous, or lactating (day 14 postpartum) (n=7-9 per group).
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