The Neurobiological Underpinnings of Depression-Related Maternal Behavior Deficits

THE NEUROBIOLOGICAL UNDERPINNINGS OF DEPRESSION-RELATED MATERNAL BEHAVIOR DEFICITS

A Dissertation Presented

By

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“It is true
I was created in you.
It is also true
That you were created for me.
I owned your voice.
It was shaped and tuned to soothe me.
Your arms were molded
Into a cradle to hold me, to rock me.
The scent of your body was the air
Perfumed for me to breathe.
Mother,
During those early, dearest days
I did not dream that you had
A large life which included me,
For I had a life
Which was only you.

Time passed steadily and drew us apart.
I was unwilling.
I feared if I let you go
You would leave me eternally.
You smiled at my fears, saying
I could not stay in your lap forever.
That one day you would have to stand
And where would I be?
You smiled again.
I did not.
Without warning you left me,
But you returned immediately.
You left again and returned,
I admit, quickly,
But relief did not rest with me easily.
You left again, but again returned.
You left again, but again returned.
Each time you reentered my world
You brought assurance.
Slowly I gained confidence.

You thought you know me,
But I did know you,
You thought you were watching me,
But I did hold you securely in my sight,
Recording every moment,
Memorizing your smiles, tracing your frowns.
In your absence
I rehearsed you,
The way you had of singing
On a breeze,
While a sob lay
At the root of your song.

The way you posed your head
So that the light could caress your face
When you put your fingers on my hand
And your hand on my arm,
I was blessed with a sense of health,
Of strength and very good fortune.

You were always
the heart of happiness to me,
Bringing nougats of glee,
Sweets of open laughter.

I loved you even during the years
When you knew nothing
And I knew everything, I loved you still.
Condescendingly of course,
From my high perch
Of teenage wisdom.
I spoke sharply of you, often
Because you were slow to understand.
I grew older and
Was stunned to find
How much knowledge you had gleaned.
And so quickly.

Mother, I have learned enough now
To know I have learned nearly nothing.
On this day
When mothers are being honored,
Let me thank you
That my selfishness, ignorance, and mockery
Did not bring you to
Discard me like a broken doll
Which had lost its favor.
I thank you that
You still find something in me
To cherish, to admire and to love.

I thank you, Mother.
I love you.”

- Mother, A Cradle to Hold Me
By Maya Angelou

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ABSTRACT

THE NEUROBIOLOGICAL UNDERPINNINGS OF DEPRESSION-RELATED MATERNAL BEHAVIOR DEFICITS

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Maternal caregiving is a dynamic process that requires extensive cognitive, motivational, and affective processing. World-wide, approximately 17% of mothers are diagnosed with postpartum depression yearly (Wang et al., 2021). Untreated, mothers with postpartum depression experience deficits in cognition, motivation, affect, and parenting (Arteche et al., 2011; Dix and Meunier, 2009; Lovejoy et al., 2000). Although postpartum depression is related to compromised parenting, to date, few studies have examined the neurobiological mechanisms by which maternal behavior is compromised in postpartum depression (Field, 2010; Murray et al., 1996). This dissertation aims to examine how depression neurobiologically disrupts parenting abilities. These studies utilize the Wistar-Kyoto (WKY) animal model of depression and control Sprague-Dawley (SD) rats, which differ dramatically in their cognitive, motivational, affective, and parenting abilities. Through behavioral, hormonal, neurochemical, and neurogenetic analyses this body of work identified that depression-related caregiving deficits of WKY mothers are associated with substantial dysfunction of mesocorticolimbic brain circuitry. Findings from these experiments provide a new perspective on how cognitive and motivational components of caregiving behavior may be neurobiologically disrupted in mothers with depression.

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INTRODUCTION

1.1 General Background and Significance

There is a wide variation of caregiving approaches across mammalian species that lead to the successful rearing of offspring (Bales, 2017). Human mothers provide their infants with nutrients, shelter, protection, warmth, socialization, and education. This extensive care requires the coordination of cognitive (attention, working memory, decision making), motivational (seeking and effort related functions), and affective (reduced anxiety, and increased in-group attachment, and increased out-group aggression) processes. These neurobiological processes allow mothers to successfully caregive and rear viable and thriving offspring. When cognitive, motivational, and affective processes are at a deficit, as is common in depressive disorders, maternal and offspring health and wellbeing becomes at risk. For example, mothers experiencing postpartum depression show decreased caregiving sensitivity, responsiveness, and synchrony (Brummelte and Galea, 2016). Offspring of mothers with postpartum depression are more likely to exhibit cognitive and emotional dysfunction, and in more severe cases, disruption to physical development (Beck, 1998; Grace et al., 2003; Murray et al., 1996; Slomian et al., 2019). This dissertation research aims to reduce the substantial gap in knowledge regarding the neurobiological mechanisms by which maternal behavior is specifically disrupted when maternal cognition, motivation, and affect are at a deficit.

1.2 The maternal caregiving network

1.2.1 Overview of the maternal caregiving circuitry: Coordinated neural networks underlying cognitive, motivational, and affective processes are primed by hormones of pregnancy to facilitate maternal behavior at parturition (Pereira and Ferreira, 2016). The maternal brain remains flexible across the postpartum period, enabling mothers to sensitively attune their caregiving behaviors toward the developmental needs of their offspring (Pereira, 2016). These processes are fundamental to fostering healthy maternal caregiving, and consequently, healthy development of offspring. Evidence indicates that sensitive maternal responses require the joint activity of a distributed network of brain structures, including the medial preoptic area (mPOA), medial prefrontal cortex (mPFC), the nucleus accumbens (NA), and its dopaminergic input from the ventral tegmental area (VTA) (Lonstein et al., 2003; Numan, 1988; Numan et al., 2005; Pereira and Ferreira, 2016). The involvement of these brain regions in maternal cognitive, motivational, and affective processes is outlined below.

1.2.2 The mPOA & the orchestration of maternal behavior: Offspring are not static stimuli. At birth, infants need high levels of care to aid in their thermoregulation, nutrition, cleanliness, shelter, learning, and socialization. Across development, offspring gain independence and require a different set of caregiving behaviors from their mothers. Overall, offspring display a repertoire of intricate and ever-changing proximate and distal cues from birth throughout development that mothers must learn to both respond and adapt to. This dynamic attunement of caregiving behavior is possible because of the mPOA (Pereira and Morrell, 2009).

The mPOA has critical reciprocal connections with somatosensory cortices, the mPFC, mesolimbic dopaminergic circuitry, and oxytocinergic pathways, making it a central director of maternal responsiveness. The mPOA is necessary for both the onset and early expression of maternal behavior across mammalian species (Kalinichev et al., 2000; Numan, 1974; Numan et al., 1977). The mPOA is one of primary sites of neural action of estradiol during pregnancy (Numan et al., 1977). Transient chemogenetic inactivation of the mPOA removes the ability of early postpartum rat mothers to address the acute physiological needs of their pups (Copelas et al., 2021; Pizarro-Colon et al., 2021). Furthermore, transient pharmacological inactivation of the mPOA has revealed that the mPOA facilitates caregiving behaviors in the early postpartum period and inhibits caregiving by the late postpartum period (Pereira and Morrell, 2009). Thus, the mPOA, through its breadth of neural connections, allows for the sensitive attunement of caregiving behaviors that are most appropriate for the developmental and acute needs of offspring. The mPOA interfaces with cognitive, motivational, and affective processes to optimize this maternal caregiving attunement.

1.2.3 Cognition: Motherhood requires a refocused set of cognitive abilities. Pregnancy itself has been associated with cognitive deficits such as decreased memory performance, slowed information processing, and decreased executive control (Groot et al., 2006; Henry and Rendell, 2007). While these cognitive changes may be viewed as detrimental, they are in fact a reflection of the immense adaptive neuroplasticity occurring in the brain across pregnancy. These changes ultimately prime maternal cognitive circuitry for efficiently and effectively taking on the cognitive load that new mothers assume at the start of the postpartum period. Mothers rely on their executive functioning to attend to,

learn, and remember how to interact with offspring-related stimuli. For example, mothers with higher levels of impulse control and cognitive flexibility have been shown to be more maternally responsive (Lovic et al., 2011; Lovic and Fleming, 2003).

The medial prefrontal cortex (mPFC) is the center of maternal cognitive and executive function, including appraisal, rule learning, decision making, and goal-directed behavior. Inactivation of the entire mPFC has been shown to eliminate the expression of retrievals of pups to a nest (Febo et al., 2010). Within the mPFC resides the infralimbic cortex (ILC), which biases maternal seeking behavior toward prioritizing offspring care (Pereira and Morrell, 2020). Targeted transient inactivation of the ILC reverses this effect such that mother rats will lose their preference for offspring associated environments in a conditioned place preference (CPP) test (Pereira and Morrell, 2020).

The ILC has important reciprocal connections with central components of the mesolimbic dopaminergic system (the VTA and NA) as well as with the mPOA (Azuma and Chiba, 1996; Hurley et al., 1991; Simerly and Swanson, 1988; Wood et al., 2019). The IL connections with the mPOA likely aid with the sensitivity of maternal responsiveness to the acute and developmental needs of the offspring.

1.2.4 Motivation: Parenting requires mothers to utilize an exceptional number of resources to care for their offspring. This investment of time, attention, effort, and energy is possible because of an intense neurobiological drive mothers have to caregive. The experience of pregnancy alters maternal motivational neural circuitry to facilitate this prioritization. The hormonally primed mPOA has been suggested to regulate appetitive aspects of motivated maternal behavior via estradiol- and oxytocin-mediated activation of the VTA dopamine input into the NA (Numan et al., 2005; Numan and Stolzenberg, 2009).

Disrupting DA action in the mPOA-NA-VTA circuitry disrupts maternal retrievals but leaves nursing behavior intact (Numan and Stolzenberg, 2009), suggesting that this network is involved in directing active goal-directed components of maternal behavior.

Mothers find their offspring highly rewarding. For mother rats, pups can be used as reinforcement in an operant conditioning paradigm. Lesioning the mPOA significantly decreases bar-press rates for pup reinforcement (Lee et al., 2000). This tremendous maternal motivational drive works to the extent that mothers will choose to seek out their needy offspring over otherwise highly salient cocaine (Pereira and Morrell, 2011). Additionally, transient inactivation of the maternal VTA removes pup preference (Seip and Morrell, 2009). Finally, in response to their own infant's smiling and crying faces, human mothers with secure infant attachment show increased mPOA and VTA activity (Lenzi et al., 2009; Strathearn et al., 2009, 2008).

1.2.5 Affect: Maintaining a solid attachment between mother and offspring is essential for healthy offspring development and long-term wellbeing. A mother-offspring bond is founded in the expression and exchange of emotions – particularly, of love. Affective states are challenging to study given the internal and subjective nature of emotions. Despite the challenges of studying affective states, research has revealed the important roles of oxytocin, limbic, and cortical brain regions in facilitating maternal affective processes.

Oxytocin activity facilitates maternal social and affective behaviors. Oxytocin is released from the periventricular nucleus (PVN) of the hypothalamus to the mPOA, NA, VTA, amygdala, cortex, and posterior pituitary (Brownstein et al., 1980; Sofroniew, 1983; Swanson and Sawchenko, 1983). Oxytocin has recently been shown to increase the

neurobiological salience of pup calls and increase subsequent caregiving responses to these calls in mothers (Marlin et al., 2015). Additionally, in the midbrain dorsal raphe, the major source of forebrain serotonin, knocking down oxytocin receptors leads to decreased nursing, increased aggression, and increased despair-like behavior in a forced swim test (Grieb et al., 2021). Oxytocin also facilitates mother-infant bonding. The close physical proximity and somatosensory stimulation between mother and offspring during breastfeeding has been shown to increase oxytocin release, maternal positive affect, and attachment between mother and offspring (Bosch and Neumann, 2012).

Infants can directly increase their own nurturance by encouraging a positive affect from their caregivers. Offspring with high baby schema (round features, small nose, stout extremities, etc.) are perceived as "cute" by adults – this perception of cuteness increases attention toward infants and motivation to caregive (Glocker et al., 2009; Senese et al., 2013). Mothers and offspring are also strongly influenced by each other's moods, an essential factor in maternal sensitivity and empathy (Laranjo et al., 2008). This ability to dynamically attune to and regulate each other's emotional states is robust, persisting from birth through adulthood (Yaniv et al., 2021).

1.3 Approach

1.3.1 Modeling Parenting Disturbances: Deficits in cognitive, motivational, and affective processes as seen in neuropsychiatric disorders, especially depressive disorders, can culminate in dysfunctional caregiving abilities in mothers suffering with these disorders (Pereira and Ferreira, 2015). To date, few studies have examined the neurobiological mechanisms by which maternal behavior is compromised in depression.

Neuroscientists have taken diverse approaches to study aspects of the neurobiology of depression and postpartum neuropsychiatric disorders, including postpartum depression. The goal of these studies is typically to understand the etiology of depression-related symptoms, but not to explicitly understand how caregiving behavior becomes disrupted. Using animal models, neuroscientists can observe complex social behaviors, induce depressive phenotypes, investigate underlying neurobiology, and evaluate depression treatment efficacy in highly controlled environments. Several excellent reviews of approaches to modeling general depressive disorders (Gururajan et al., 2019; Krishnan and Nestler, 2011; Pollak et al., 2010), as well as postpartum depression, are available (Brummelte and Galea, 2016; Pawluski et al., 2021, 2017; Payne and Maguire, 2019)

The primary method used to induce depression-like behavior in rodents is to introduce stress, a predominate risk factor for depressive disorders (Swendsen and Mazure, 2000). This can be accomplished either environmentally via chronic mild stress or gestational restraint stress, or chemically via corticosterone injection (Brummelte and Galea, 2010; Leuner et al., 2014; Workman et al., 2013). Another approach is to look at phenotype-first models of depression – models that naturally show heritable depression-like behavior without manipulation. One such model is the Wistar-Kyoto (WKY) rat strain.

To best determine how disruption to cognition, motivation, and affect lead to dysfunction in maternal caregiving abilities, I utilized the WKY rat strain. WKY rats were originally derived from the Wistar strain as a control strain for spontaneously hypertensive rats. Through behavioral observations, it became clear that WKY males and females showed high depression-like behaviors (Armario et al., 1995; Lahmame et al., 1997; Rittenhouse and López-Rubalcava, 2002; Solberg et al., 2001) compared with control

strains, such as Sprague-Dawley (SD) rats. WKY rats do not require environmental or chemical manipulation to bring about their depression-like phenotype. Prior studies from the Pereira lab have demonstrated that WKY females show decreased cognitive flexibility, effort-related functions, anhedonia, and especially, decreased maternal responsiveness (Pereira et al., 2014). These are cognitive, motivational, and parenting deficits that recapitulate major clinical features of postpartum depression (American Psychiatric Association, 2013). Additionally, human depression is a highly heterogenous disorder with many endophenotypes. WKY mothers show individual variance in the extent of their depressive phenotypes that further strengthens the validity of this model in mirroring core symptoms of human depressive disorders. This variation will be discussed in Chapter III.

1.4 Dissertation Research Strategy:

There is a substantial gap in our knowledge regarding the mechanisms by which parenting is impacted by disruption to cognition, motivation, and affect associated with depressive symptoms. The objective of this dissertation research is to examine the neurochemical and molecular mechanisms by which depression disrupts parenting abilities. The overarching hypothesis is that parenting disturbances associated with a depressive phenotype are mediated by altered hormonal activity, monoamine neurotransmission, and gene expression within mesocorticolimbic brain loci involved in cognitive, motivational, and affective features of depression.

This dissertation is composed of three studies. The first set of experiments (Chapter II) used behavioral and neurochemical approaches to determine whether changes in monoamine levels within discrete mesocorticolimbic structures modulate maternal caregiving behaviors across the postpartum period. Levels of the monoamine

neurotransmitters dopamine, noradrenaline, and serotonin, as well as their major metabolites, were measured within the mPFC, striatum, NA, and mPOA of SD and WKY mothers at early (postpartum days [PPD] 7-8), late (PPD15-16), and weaning (PPD25) postpartum stages.

The second set of experiments (Chapter III) examined whether altered levels of estradiol and progesterone mediate parenting deficits associated with a depressive phenotype. For these experiments, I measured serum levels of estradiol and progesterone as well as mPOA estrogen and oxytocin receptor expression in early postpartum WKY and SD mothers.

The final set of studies (Chapter IV) determined potential molecular mechanisms that contribute to parenting disturbances associated with a depressive phenotype in new mothers. These experiments used next-generation RNA-sequencing (RNAseq) to conduct a full transcriptome analysis of the mPFC, NA, and the mPOA in WKY and SD mothers. I conducted differential gene expression, ontology, and co-expression network analyses to identify candidate genes associated with parenting deficits.

CHAPTER II

DEPRESSION-RELATED DISTURBANCES IN RAT MATERNAL BEHAVIOR ARE ASSOCIATED WITH ALTERED MONOAMINE LEVELS WITHIN MESOCORTICOLIMBIC STRUCTURES.

This study was published in the Journal of Neuroendocrinology in June 2019. The text version of the manuscript is below, and the published version can be found at DOI: 10.1111/jne.12766.

2.1 Abstract

The ability of mothers to sensitively attune their maternal responses to the needs of their developing young is fundamental to a healthy mother-young relationship. The biological mechanisms that govern how mothers adjust caregiving to the dynamic changes in the demands of the young remains an open question. In the present study, we examined whether changes in monoamine levels within discrete mesocorticolimbic structures involved in cognitive and motivational processes key to parenting, modulate this flexibility in caregiving across the postpartum period. The present study used Wistar-Kyoto (WKY) animal model of depression and control Sprague-Dawley (SD) rats, which differ dramatically in their cognitive, motivational, and parenting performance. Levels of the monoamine neurotransmitters, dopamine, noradrenaline, and serotonin, and their major metabolites, were measured within the medial prefrontal cortex, striatum, nucleus accumbens, and medial preoptic area of SD and WKY mothers at early (PPD7-8), late (PPD15-16) and weaning (PPD25) postpartum stages using high-performance liquid

chromatography with electrochemical detection. Consistent with our prior work, we find that caregiving of SD mothers declined as the postpartum period progresses. Relative to nulliparous females, early postpartum mothers had lower intracellular concentrations of monoamines, as well as lower noradrenaline turnover, and elevated serotonin turnover within most structures. Postpartum behavioral trajectories subsequently corresponded with a progressive increase in all three monoamine levels within multiple structures. Compared to SD mothers, WKY mothers were inconsistent and disorganized in caring for their offspring and exhibit profound deficits in maternal behavior. Additionally, WKY mothers had generally lower levels of all three monoamines, as well as different patterns of change across the postpartum period compared to SD mothers, suggesting dysfunctional central monoamine pathways in WKY mothers as they transition and experience motherhood. Together, results suggest a role for monoamines at multiple mesocorticolimbic structures in modulating caregiving behaviors attuned to the changing needs of the young.

2.2 Introduction

Mothers must adjust their caregiving interactions to match the dynamic changes in their young's physiological and developmental needs in the context of everyday interactions. This ability of mothers to link sensory cues with the underlying needs of their young, and appropriately respond to them is a critical aspect of mother-young interactions fundamental to the healthy socio-emotional developmental outcome in the young (Bowlby, 1983; Tronick, 2007). Parenting recruits a distributed network of cortical, striatal, and limbic structures (Lonstein et al., 2015a, 2015b; Numan and Insel, 2003). Recent research has shown that many of these structures and connections undergo significant plasticity

across the postpartum period not only in preparation for the arrival of the young but also in response to the social experience of interaction with them (Brunton and Russell, 2008; Kim et al., 2010; Pereira and Morrell, 2011, 2009). However, relatively little is known about how the maternal circuitry modulates this experiential- and context-dependent flexibility in caregiving behavior.

Given the dramatic re-organization of the maternal brain, it is not surprising that motherhood is accompanied by elevated risk for neuropsychiatric disorders, including postpartum depression and anxiety. Depression in new mothers is associated with cognitive and motivational deficits, and, more specifically, disturbances in parenting associated with reduced maternal sensitivity to their infant's cues and signals (Arteche et al., 2011; Dix and Meunier, 2009; Lovejoy et al., 2000). Although the clinical literature consistently relates postpartum depression to compromised parenting, to date, few studies have examined the neurobiological mechanisms by which maternal behavior is compromised in postpartum depression (Field, 2010; Murray et al., 1996). A substantial body of work from both clinical and animal studies implicates a role for altered monoamine neurotransmission, particularly dopamine dysfunction at discrete mesocorticolimbic structures, in the pathophysiology of the cognitive and motivational symptoms of depression (Chaudhury et al., 2012; Dunlop and Nemeroff, 2007; Nestler, 2015; Pawluski et al., 2017; Salamone et al., 2006). Significantly, these cognitive and motivational processes affected by depression, including attention, behavioral flexibility, decision-making, and effort-related processes, are key to parenting (Barrett and Fleming, 2011; Pereira and Ferreira, 2016). In parallel, compelling evidence indicates that the mesocorticolimbic monoamine systems facilitate early aspects of mother-infant

attachment, social memory, and motivation in rats and humans (Lonstein, 2018; Numan and Stolzenberg, 2009; Sekiyama et al., 2013; Smith et al., 2013; Thomas and Palmiter, 1997). Interference with dopaminergic, noradrenergic, and/or serotonergic signaling severely impacts the onset and early expression of maternal behavior (Angoa-Pérez et al., 2014; Cox et al., 2011; Curry et al., 2013; Gemmel et al., 2016; Keer and behavior, 1999; Li, 2015; Numan and Stolzenberg, 2009; Pawluski et al., 2019; Smith et al., 2012; Thomas and Palmiter, 1997). However, little is known about whether (and how) these monoaminergic systems change throughout the postpartum period to support parenting.

Here we examined whether the ongoing ability of mothers to sensitively respond to their young is associated with changes in the activity of monoamine systems across the postpartum period, both under healthy conditions and in the context of cognitive and motivational dysfunctions associated with a depressive phenotype. To address this aim, this study uses the Wistar-Kyoto (WKY) rat strain, which exhibits a unique behavioral phenotype relative to the Sprague-Dawley (SD) normative strain that supports their characterization as an animal model of endogenous depression (Holl et al., 2018; Paré, 1989; Tejani-Butt et al., 2003; Willner and Belzung, 2015). Studies from our lab further demonstrate that WKY postpartum females exhibit behavioral traits that recapitulate the major clinical features of depression in new mothers, including the cognitive, motivational and parenting disturbances (Farrar et al., 2016; Pereira et al., 2014; Winokur et al., 2016). In addition, WKY rats also display many characteristics related to increased vulnerability to anxiety (Cominski et al., 2014; Servatius et al., 2008); this is an important feature because a significant proportion of women with postpartum depression also experience anxiety (O'Hara and McCabe, 2013; Research and Organization, 2009). Tissue levels of

monoamine neurotransmitters and their major metabolites were determined in discrete mesocorticolimbic brain regions implicated in cognitive and motivational processes of SD and WKY mothers during early (EPP), late (LPP) and weaning (WPP) stages of the postpartum period using high-performance liquid chromatography with electrochemical detection (HPLC-ED). These regions were the medial prefrontal cortex (mPFC), the striatum, the nucleus accumbens (NA), and the medial preoptic area (mPOA). This approach measures primarily intracellular stores of monoamines and their metabolites, but does not necessarily describe the entirety of monoaminergic neurotransmission. Still, tissue concentrations of neurotransmitters and metabolites provide an estimate of processes related to steady-state neurotransmitter synthesis and storage, as well as aspects of enzymatic degradation. Gathering these data on dopamine, noradrenaline and serotonin, within multiple interconnected brain regions, offers a critical starting point to direct future work toward elucidating the neurobiological mechanisms by which cognitive and motivational dysfunctions can impact parenting abilities.

2.3 Materials and Methods

2.3.1 – Animals: Primiparous postpartum Wistar-Kyoto (WKY) and Sprague-Dawley (SD) female rats (purchased from Charles River Laboratories, Kingston, NY), bred in our laboratory at the University of Massachusetts Research Animal Facility, were used in this study. The SD rat is an outbred rat strain that our lab and many others commonly use to study parenting, and has been one of the most widely used comparator rat strains for the WKY strain. Animals were maintained in a temperature-controlled room (21 ± 1 °C) on a 12-hr light/dark cycle (lights on at 7:00 AM) with *ad libitum* access to water, rat chow

(5058 PicoLab® Diet, LabDiet, Brentwood, MO) and sunflowers seeds (5LP8 PicoLab® Sunflower, LabDiet, Brentwood, MI). Animals were habituated to the facility for 2 weeks prior to mating. Before giving birth, pregnant females were housed in individual cages lined with fresh Sani-Chips® bedding (P.J. Murphy Forest Products Corp, Montville, NJ) and containing Eco-Bedding as nest-building material (Fibercore LLC, Cleveland, Ohio). Postpartum females remained with their pups for 24h after parturition, without interruption. On postpartum day 1 (PPD1; birth=day 0), litters were culled to 8 pups per mother (3-5 males and 3-5 females). All procedures were performed in compliance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals, and with the approval of the Institutional Animal Care and Use Committee at University of Massachusetts Amherst.

2.3.2 – Study Design: SD and WKY postpartum females were either examined for fine-grained analyses of maternal behavior across the postpartum period (i.e., daily home-cage observations and maternal behavior tests across 25 days), or sacrificed at either the EPP (PPD7-8), LPP (PPD15-16) or WPP (PPD25) stage for tissue analysis of monoamine neurotransmitters and their major metabolites. In addition, SD and WKY virgin females were sacrificed for tissue analysis of monoamines. Virgin females' estrous cycles were monitored daily using vaginal smears. Females cycling regularly (i.e., showing at least two consecutive cycles) were sacrificed on a day of diestrus.

2.3.3. – Maternal Behavior Test*:* Maternal behavior was studied in the female's home cage as previously described (Pereira and Ferreira, 2006; Pereira and Morrell, 2009). SD and WKY females were tested on PPD7-8 (n=11 and n=10, respectively) or PPD14-15 (n=9 and n=9, respectively), following a 20-min mother-litter separation. The floor of the cage was divided into four equal compartments by a 5-cm high Plexiglas insert to give the

mother enough time, especially during the late postpartum testing when pups are highly mobile, to retrieve and group pups to the nest. The insert delays but not prevents the pups from grouping together in the nest. After the separation, the eight pups were scattered in the home cage opposite to the female's nest (40W x 50L x 20H cm). The number of the following maternal behavioral components was continuously recorded for 30 min: retrievals of the pups into the nest, mouthings (oral repositioning of the pups into the nest), full body and anogenital lickings, and nest building. In addition, the total duration of huddling behaviors, including lying in contact with pups and hovering over the pups in the nest while actively performing other behaviors (i.e. licking of pups or self-grooming), and the nursing posture kyphosis, a quiescent upright crouching over pups, were recorded(Stern and Johnson, 1990). Total time in contact with pups was the summed durations of huddling plus nursing behaviors. Also, the latency (i.e. time from the introduction of the pups to the first behavioral response by the female) to begin retrieving pups, to reunion of the entire litter into the nest as well as to begin hovering over and nursing was registered. Only those postpartum females that retrieved each of their eight pups into the nest were considered to have grouped the litter. The latency to begin hovering over or nursing the pups was the first occurrence of a bout of each behavior ≥2min in duration. A latency of 1800 s was given for any category of behavior that was not initiated (or completed, i.e. reunion of the litter) within the 30-min observation period. Other behaviors recorded included general exploration (insert crosses and rearings), self-grooming and eating/drinking.

2.3.4 – Maternal Behavior Daily Observations: In order to evaluate whether the observed differences in caregiving responses between SD and WKY mothers were driven by the testing procedure, undisrupted home-cage maternal behavior of a separate cohort of

SD (n=7) and WKY (n=6) mothers was systematically scored across the entire postpartum period. The observation procedure was adapted from work of others examining natural variations in maternal behavior(Champagne et al., 2003a). The behavior of each postpartum female in her home cage was observed and scored daily for three 60-min observation periods between PPD1 and PPD25. Behavioral observations occurred at regular times each day, with two periods during the light (08:00 and 12:00) and one period during the dark (17:00) phases of the light/dark cycle (12h light/dark cycle, lights on at 04:00). During the dark phase, a dim red light was used to permit observations. Within each observation period, the mother's behavior was accounted for every 4 minutes, resulting in 48 observations per day and a total of 1,200 observations across the 25 postpartum days observed (16 observations per period x 3 periods per day x 25 days = 1200 observations per mother). Behaviors registered included mother off pups, mother carrying pup, mother licking any pup (both body and anogenital licking were included), mother hovering over pups, mother nursing pups, nest building, self-grooming, eating and drinking. Frequency of each behavior was calculated as the number of observations that behavior was observed divided by the total number of observations across PPD1-8, PPD9-16 or PPD17-25.

2.3.5 – Offspring Physical and Neurobiological Developmental Milestones: In order to investigate whether the differences in parenting observed between SD and WKY mothers are driven by differences in pup's characteristics, separate SD and WKY litters were assessed systematically for physical and neurobiological developmental milestones over the entire postnatal period (PND1-25). Two male and two female pups per mother were randomly selected. Pups were marked with a unique identification number using a

nontoxic indelible marker. Starting on PND1 and during the entire postnatal growth period, each of these four pups were weighed daily, and then examined for emergence of physical maturation landmarks and reflexes. Pups were reunited with their mothers immediately after examination. The rest of the litter remained always with their mother, except for the 20-min period prior to maternal behavior testing on PPD7-8 and PPD15-16 (details below). Physical landmarks included pinnae detachment (unfolding of ears), incisor eruption, eye opening (bilateral), ear opening (bilateral), and fur development (full coat of fur). In addition, pups were evaluated for the following neurological reflexes:

***Forelimb Palmar Grasp*:** Each pup was held by the scruff of its neck. Each forepaw was stroked gently with the rounded edge of forceps, and the presence or absence of grasping was recorded. Forelimb palmar grasp was evaluated daily until the pup didn't grasp for two consecutive days.

***Surface Righting Reflex*:** Each pup was placed on its back and then released. The time taken for the pup to right itself to prone position (with all four paws in contact with the surface) was recorded. Each pup was evaluated in 3 x 30s trials per day. Surface righting was evaluated daily until the pup could right itself within 10s for two consecutive trials.

***Negative Geotaxis*:** Each pup was placed facing downwards on a 30° inclined plane. The time taken by the pup to rotate a full 180° and face up the incline was recorded. Each pup was given 3 x 60s trials per day beginning on PND6. The negative geotaxis test was repeated daily until the pup could reposition facing upwards within 10s for two consecutive trials.

To control for the putative impact of daily handling of select pups for the assessment of developmental milestones on maternal behavior, a comparison of the two

cohorts of SD and WKY mothers was conducted. A 30-min maternal behavior test was conducted, as described in "Maternal Behavior Test", on PPD7-8 and PPD15-16 for SD and WKY mothers participating in the observation cohort. Additionally, undisturbed home-cage observations were performed, as described in "Maternal Behavior Daily Observations", on PPD7-8 and PPD15-16 for SD and WKY mothers that participated in the testing cohort.

2.3.6 – Tissue Processing and Monoamine Analysis: Separate groups of SD and WKY females on diestrus, or on PPD 7-8, 15-16, or 25 were sacrificed for tissue collection. Each female was decapitated, and each brain was rapidly extracted and immediately frozen on a freezing stage microtome. Bilateral tissue punches (1 mm diameter) were dissected from 1 mm-thick tissue sections from the prelimbic (PrL) and infralimbic (IL) medial prefrontal, and the orbitofrontal (OFC) cortices, the nucleus accumbens (NA) core and shell, the dorsomedial (DMS), dorsolateral (DLS) and ventrolateral (VLS) striatum, and the medial preoptic area (mPOA) (see Fig. 1). Tissue samples were placed in pre-weighed 1.5 ml tubes and stored at −80°C until neurochemical analysis. Tissue samples were weighed, homogenized in 0.1 N perchloric acid + 1 mM EDTA-2Na at 20 µl/mg, and centrifuged at 16,000 rcf for 15 min at 4°C through a 0.45µm filter. The resulting supernatants were assayed for monoamine neurotransmitters and their major metabolites, including dopamine and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA); noradrenaline and its metabolite normetanephrine (NM), and serotonin and its metabolite 5-hydroxyindole acetic acid (5-HIAA), using high-performance liquid chromatography with electrochemical analysis (HPLC-ED; HTEC-500, Amuza, San Diego, CA, USA). An Eicompak SC-30DS separation column (Amuza,

San Diego, CA, USA) was used with circulating mobile phase consisting of 0.1 M citrate-acetate buffer, pH 3.5, 0.1 mM EDTA-2Na, 1.2 mM sodium octane sulfonate (SOS), and 20% (v/v) methanol. The electrochemical detector included a graphite electrode set at a voltage of 750 mV vs. Ag/AgCl reference analytical electrode. The flow rate of the mobile phase was set to 350 µl/min. Sample chromatograms were analyzed based on calibration standard solutions (10^{-6} to 10^{-9} standards) containing dopamine, DOPAC, HVA, serotonin, 5-HIAA, noradrenaline and NM (Sigma-Aldrich, St. Louis, MO, USA) using the Envision EPC-700 chromatography software (Amuza, San Diego, CA, USA). Monoamines and metabolites are presented as pg/mg of tissue. Ratio of metabolite(s)/neurotransmitter was calculated as an index of metabolism, with dopamine turnover indicated by DOPAC+HVA/dopamine, noradrenaline turnover indicated by NM/noradrenaline indicate, and serotonin turnover indicated by 5-HIAA/serotonin.

2.3.7 – Statistics: Results are expressed as the mean ± standard error of the mean (SEM). Behavioral data were analyzed using linear mixed models, with strain as the between-subjects factor and postpartum stage as the within-subject factor. Pup characteristics, including physical milestones and reflex acquisition are expressed as the cumulative number of days required for first appearance, and were analyzed using linear mixed models with strain and sex as between-subjects factors. Behavioral categorical data were analyzed using the Fisher's exact test. Data were considered significant when $p<0.05$. Neurochemical data were analyzed using two-way multivariate analysis of variance (MANOVA), with postpartum stage and strain as between-subjects factors, and brain region as the within-subject factor, carried out for each neurotransmitter, metabolites or turnover ratio. Significant effects were first followed up with univariate ANOVAs per

structure and then with Tukey's HSD post hoc tests. Neurochemical pairwise comparisons were considered significant when $p<0.01$. Pearson's correlation coefficients were used to examine correlations between intracellular levels of dopamine, noradrenaline or serotonin across structures. Correlograms, showing Pearson's correlation coefficients, were generated using the R package "corrplot"(Wei and Simko, 2017). All data were analyzed using SPSS version 25 software (SPSS Inc., Chicago, IL).

2.4 Results

Analysis of mothers' behavior across the postpartum period, either during 30-min maternal behavior tests or undisrupted home-cage observation sessions, similarly confirmed previous reports that social interaction with developing pups results in a progressive decline of maternal behaviors across the postpartum period(Grota and Ader, 1974; Reisbick et al., 1975; Wansaw et al., 2008) (Fig. 2).

2.4.1 – WKY mothers exhibited severe deficits in active caregiving during the 30-min behavioral test. There was a main effect of strain and stage, as well as a significant strain x postpartum stage interaction for all active components of maternal behavior during the 30-min maternal behavior test (Retrieving: Strain $F_{(1,35)}=33.6$ $p<0.001$ $\eta_p^2=0.49$; Stage $F_{(1,35)}=49.2$ $p<0.001$ $\eta_p^2=0.58$; Strain x Stage $F_{(1,35)}=8.5$ $p=0.006$ $\eta_p^2=0.2$; Mouthing: Strain $F_{(1,35)}=16.4$ $p<0.001$ $\eta_p^2=0.32$; Stage $F_{(1,35)}=20.4$ $p<0.001$ $\eta_p^2=0.37$; Strain x Stage $F_{(1,35)}=5.7$ $p=0.023$ $\eta_p^2=0.14$; Corporal Licking: Strain $F_{(1,35)}=73.9$ $p<0.001$ $\eta_p^2=0.68$; Stage $F_{(1,35)}=34.9$ $p<0.001$ $\eta_p^2=0.5$; Strain x Stage $F_{(1,35)}=23.9$ $p<0.001$ $\eta_p^2=0.41$; Anogenital Licking: Strain $F_{(1,35)}=51.4$ $p<0.001$ $\eta_p^2=0.59$; Stage $F_{(1,35)}=28.5$ $p<0.001$ $\eta_p^2=0.45$; Strain x Stage $F_{(1,35)}=13.9$ $p=0.001$ $\eta_p^2=0.29$; Nest Building: Strain $F_{(1,35)}=13.3$ $p=0.001$ $\eta_p^2=0.28$;

Stage $F_{(1,35)}$=35.6 p<0.001 η_p^2=0.50; Strain x Stage $F_{(1,35)}$=8.1 p=0.007 η_p^2=0.19). Tests of simple main effects indicated WKY females consistently exhibited fewer retrievals, mouthings, and corporal and anogenital lickings than SD mothers across postpartum (p<0.05; Fig. 2a), this strain difference in caregiving was more pronounced in early vs. late postpartum.

There were significant strain, stage, and strain x stage interaction effects for latency to group the entire litter in the nest (Strain $F_{(1,35)}$=26.8 p<0.001 η_p^2=0.43; Stage $F_{(1,35)}$=28.6 p<0.001 η_p^2=0.45; $F_{(1,35)}$=8.3 p=0.007 η_p^2=0.19), and significant main effects of strain and stage on latency to first retrieval (Strain $F_{(1,35)}$=7.1 p=0.01 η_p^2=0.17 Stage $F_{(1,35)}$=15.6 p<0.001 η_p^2=0.31). As shown in Fig. 2b, PPD7-8 WKY females took longer to retrieve their first pup and to group the entire litter compared to SD mothers. In fact, only 2 of 10 EPP WKY mothers completed retrieving and grouping the pups into the nest; 6 of 10 retrieved ≤3 pups of their 8-pup litter, and the remaining 2 did not retrieve any pups. In contrast, all 11 EPP SD females retrieved and grouped all pups into the nest (2/10 and 11/11, respectively; Fisher's exact test, p<0.05). As expected, during late postpartum the majority of mothers did not group their pups into the nest, with 6 of 9 SD females retrieving at least 1 pup and only 1 of 9 WKY females doing so. No differences were found in the latency to retrieve or group pups between PPD15-16 SD and WKY mothers (Fig. 2b).

2.4.2 – WKY females spent less time with their pups. There was a significant main effect of strain on the latency to hover over ($F_{(1, 35)}$=18.7 p<0.001 η_p^2=0.35), and nurse (Strain $F_{(1, 35)}$=9.8 p=0.03 η_p^2=0.22), with WKY mothers taking significantly more time to hover over and nurse their litters than SD mothers, regardless of stage (Latency to begin hovering over (s): SD_{EPP}=303.5±47.4 versus WKY_{EPP}=867.4±186.6 and

SD_{LPP}=304.0±46.1 versus WKY_{LPP}=1000.9±226.4; Latency to begin nursing (s): SD_{EPP}=863.6±74.5 versus WKY_{EPP}=1369.0±123.7 and SD_{LPP}=993.3±133.5 versus WKY_{LPP}=1261.9 ±163.6; $p<0.05$).

In addition, there were significant main effects of strain and postpartum stage on the duration of hovering over (Strain $F_{(1,35)}$=24.2 $p<0.001$ η_p^2=0.41, Stage $F_{(1,35)}$=6.6 p=0.014 η_p^2=0.16), and a significant effect of strain for nursing ($F_{(1,35)}$=22.4 $p<0.001$ η_p^2=0.39) and total time in contact ($F_{(1,35)}$=30.1 $p<0.001$ η_p^2=0.46). Tests of simple main effects indicated that WKY mothers spent less time in contact with their litter during the 30-min test, including hovering over and nursing, than SD mothers, regardless of stage (Duration of hover over (s): SD_{EPP}=804.2±77.5 versus WKY_{EPP}=426.3±88.9 and SD_{LPP}=605.4±66.7 versus WKY_{LPP}=231.4 ±69.4; Duration of nursing (s): SD_{EPP}=676.3±66.1 versus WKY_{EPP}=243.1±73.5 and SD_{LPP}=706.3±80.9 versus WKY_{LPP}=401.4 ±108.7; $p<0.05$).

Home cage activity, including general exploration and self-grooming was not different between SD and WKY mothers across postpartum stages [Crossing: Strain $F_{(1,35)}$=0.7 p=ns η_p^2=0.01, Stage $F_{(1,35)}$=3.8 p=ns η_p^2=0.09 and Strain x Stage $F_{(1,35)}$=0.2 p=ns η_p^2=0.00; Rearing: Strain $F_{(1,35)}$=0.1 p=ns η_p^2=0.00, Stage $F_{(1,35)}$=0.0 p=ns η_p^2=0.00 and Strain x Stage $F_{(1,35)}$=0.3 p=ns η_p^2=0.01; Self-grooming: Strain $F_{(1,35)}$=0.1 p=ns η_p^2=0.00, Stage $F_{(1,35)}$=1.3 p=ns η_p^2=0.04 and Strain x Stage $F_{(1,35)}$=0.02 p=ns η_p^2=0.00) (data not shown).

2.4.3 – Confirmation of distinct maternal phenotypes between SD and WKY mothers via undisrupted home-cage observations. Consistent with the results above and previous analyses of change in maternal behavior across the postpartum period, daily home

cage observations indicated significant main effects of strain and postpartum stage on active caregiving, nest building, nursing, and time off pups, as well as a significant strain x postpartum stage interaction on the frequency of active caregiving [Active Caregiving: Strain $F_{(1,11)}$=34.3 p<0.001 η_p^2=0.76, Stage $F_{(2,22)}$=65.8 p<0.001 η_p^2=0.86, Strain x Stage: $F_{(2,22)}$=13.4 p<0.001 η_p^2=0.55; Nest Building: Strain $F_{(1,11)}$=4.9 p=0.04 η_p^2=0.31, Stage $F_{(2,22)}$=11.2 p<0.001 η_p^2=0.51, Strain x Stage: $F_{(2,22)}$=2.2 p=ns η_p^2=0.17; Nursing: Strain $F_{(1,11)}$=9.6 p=0.01 η_p^2=0.47, Stage $F_{(2,22)}$= 25.0 p<0.001 η_p^2=0.69, Strain x Stage: $F_{(2,22)}$=0.86 p=ns η_p^2=0.07; Time off Pups: Strain $F_{(1,11)}$=5.3, p=0.04, η_p^2=0.33, Stage $F_{(2,22)}$= 58.5 p<0.001 η_p^2=0.84, Strain x Stage: $F_{(2,22)}$=2.6 p=ns η_p^2=0.19]. SD mothers displayed higher frequency of active caregiving and nursing behaviors than WKY mothers across the first 16 postpartum days (p<0.001, Fig. 2e,f). There were no differences in active caregiving and nursing between SD and WKY mothers at PPD18-25. Tests of simple main effects indicated that the decline in active caregiving from early to late postpartum was more pronounced in SD than WKY mothers. Moreover, comparison of fold change in active caregiving across the postpartum period between strains revealed that WKY mothers showed a significantly smaller fold decrease in caregiving across the postpartum period than SD mothers (t(11)=2.59, p=0.025). In contrast, WKY mothers displayed higher frequency of nest building and time away from the pups (mostly resting) than SD mothers at PPD1-8 (p<0.001; Fig. 2e,f). Such differences in nest building and time off pups were no longer evident at PPD9-16 or PPD17-25, respectively. There were no significant differences in the frequency of self-grooming, eating, or drinking between SD and WKY mothers throughout the postpartum period (data not shown).

2.4.4 – Deficits in WKY maternal behavior are not due to testing conditions. The comparison of maternal behavior between the two cohorts of mothers did not reveal significant differences in any of the maternal components for either strain, as evaluated by observation or testing, suggesting that testing differences, including pup handling, did not impact ongoing maternal behavior (p=ns; Student's t-tests; data not shown).

2.4.5 – WKY offspring develop normally. No significant differences were found in a comparison of postnatal growth and developmental milestones between SD and WKY offspring. Growth gain across postnatal period did not differ between strains (p=ns, data not shown). In addition, development of physical milestones (pinna detachment, fur development, eruption of incisor teeth, ear and eye opening) and neurological reflexes (palmar grasp, righting reflex, and negative geotaxis) during the postnatal period did not differ between SD and WKY offspring (all ps=ns; Table 1).

2.4.6 – WKY mothers exhibit an altered pattern of change in monoamine neurotransmitters and their turnover ratios across the postpartum period. MANOVAs and follow-up ANOVAS per structure revealed significant differences in the intracellular levels of all three monoamines and their metabolites in most of the structures examined between strains as they transition to and experience motherhood.

Transition to Motherhood (Virgin vs Early Postpartum). Intracellular dopamine levels and turnover ([DOPAC+HVA] / [dopamine]) were similar within all structures of SD and WKY females from virgin to EPP stages, except for the PRL and NAShell that exhibited a reduction in dopamine levels only in EPP SD mothers ($p<0.01$) (Fig. 4 and Table 2). The levels of intracellular noradrenaline and serotonin were generally lower in EPP SD mothers compared to SD virgins in most structures, especially in the NA and

mPOA ($p<0.01$)(Fig. 4A , 5A). In contrast, WKY females showed either no change or an increase in noradrenaline and serotonin levels from virgin to EPP stages. EPP SD mothers exhibited a significant increase in serotonin turnover ([5-HIAA]/[5-HT]) in the OFC, DLS, NACore, NAShell, and mPOA relative to the virgin stage ($p<0.01$), whereas WKY mothers did not (Fig. 5B and Table 2). Noradrenaline turnover ([NM] / [Noradrenaline]) was lower in almost every structure of SD mothers, aside from the NAShell and the mPOA, and in all structures of WKY mothers compared to the virgin stage ($p<0.01$) (Fig. 4B and Table 2).

In addition to exhibiting an altered pattern of change in monoamine systems from the virgin to EPP stages, EPP WKY mothers also showed differences in monoamine levels and/or their turnover ratios compared to EPP SD mothers. Specifically, dopamine levels were higher in the DLS, VLS and mPOA of EPP SD mothers than in WKY mothers ($p<0.01$) (Fig. 3A and Table 2). In addition, dopamine turnover was lower in the VLS and higher in the PRL of EPP SD mothers compared to WKY mothers ($p<0.01$) (Fig. 3B and Table 2). The tissue levels of noradrenaline and serotonin were similar within most structures between SD and WKY EPP groups, except for the DMS and the NACore that had higher levels of noradrenaline and/or serotonin in WKY mothers compared to SD mothers ($p<0.01$) (Fig. 4A, 5A and Table 2). Noradrenaline turnover in all structures was similar between SD and WKY mothers. In contrast, serotonin turnover was higher in the DLS, DMS, NACore and NAShell of EPP SD mothers than in WKY mothers ($p<0.01$) (Fig. 4B, 5B and Table 2).

Experience of Motherhood (Early vs Late vs Weaning Postpartum). The levels of dopamine remained relatively similar in most regions between stages, except for the DMS, where levels increased, and the OFC and the DLS, where levels decreased in LPP and/or

WPP vs EPP SD mothers. WKY mothers in general exhibited a similar profile to SD mothers, except that DA levels decreased in the DMS, and increased in the PRL of WKY mothers from EPP to WPP. Dopamine turnover decreased in the IL and DMS, and increased in the mPOA from EPP to LPP and in the OFC from LPP to WPP, and across all stages in the NAShell of SD mothers (all ps<0.01) (Fig.3B and Table 2). In contrast, dopamine turnover was higher in the DMS, VLS, NACore, and NAShell of WKY mothers with the progression of the postpartum period (Fig.3B and Table 2). The levels of noradrenaline increased in the OFC, DMS and VLS, and decreased in the IL of SD mothers from EPP to LPP and WPP ($p<0.01$). In contrast, WKY mothers exhibited a different pattern of change in noradrenaline across the postpartum period. Noradrenaline levels decreased in the NACore and NAShell from EPP to LPP and WPP, and increased in the DLS and IL from LPP to WPP ($p<0.01$) (Fig. 4A and Table 2). Noradrenaline turnover did not change across postpartum stages for SD or WKY mothers (Fig. 4B and Table 2). The levels of serotonin increased in the DLS, DMS, and NACore, and decreased in the IL of SD mothers across the postpartum period ($p<0.01$) (Fig. 5A). In contrast, WKY mothers showed a decrease in intracellular serotonin levels in the PRL and NACore, and an increase in serotonin levels in the IL and mPOA across the postpartum period ($p<0.01$) (Fig. 5A). Serotonin turnover decreased in the OFC, IL, DLS, DMS, NACore, NAShell and mPOA of LPP or WPP SD mothers ($p<0.01$), whereas the opposite was true for WKY mothers. Specifically, serotonin turnover in these structures increased to a peak during LPP in most structures, and then decreased from LPP to WPP in WKY mothers ($p<0.01$) (Fig. 5B, Table 2).

In addition to trajectory differences, WKY mothers also exhibited altered levels of all three monoamines and/or their turnover ratios within discrete mesocorticolimbic structures compared to SD mothers. For instance, WKY mothers consistently had lower dopamine levels and higher DA turnover in almost all striatal structures evaluated than SD mothers throughout the postpartum period (DLS, DMS, VLS, and NACore; all $p<0.01$). In addition, WPP WKY mothers had significantly lower dopamine turnover in the NAShell and mPOA compared to WPP SD mothers ($p<0.01$) (Fig. 3B and Table 2). WPP WKY mothers were also found to have significantly higher noradrenaline levels in the IL and the DLS, and lower levels in the DMS compared to WPP SD mothers ($p<0.01$) (Figure 4A). Noradrenaline turnover was higher in the mPOA of WPP WKY females than WPP SD mothers ($p<0.01$) (Figure 4B and Table 2). Furthermore, the levels of serotonin were higher in the mPOA and lower in the NACore and VLS of WKY mothers than SD mothers ($p<0.01$) (Figure 5A). WKY mothers also had higher serotonin turnover ratios in the PRL, DLS, DMS, NAShell and the mPOA during LPP, and in the VLS during WPP compared to SD mothers ($p<0.01$) (Fig. 5B and Table 2).

2.4.7 – Monoamine level covariance across the maternal brain. As shown in Figure 6, relative to the virgin stage, EPP SD mothers' dopamine and noradrenaline levels were more strongly correlated across nodes of the maternal circuitry, as measured by Pearson's correlation coefficients. In contrast, EPP WKY females' monoamine levels was weakly correlated across the circuitry. Additionally, there was a decline in the number of correlations as the postpartum period progressed for all monoamine systems in SD mothers. This pattern was not apparent in WKY mothers, as the number of correlations remained similar for all monoamine systems across all postpartum stages.

2.5 Discussion

The present study examined changes in maternal behavior and in the activity of monoamine systems across the postpartum period, both under healthy conditions and in the context of a depressive phenotype. Consistent with prior work, present results find different patterns of maternal care at different postpartum stages. Moreover, these behavioral trajectories across the postpartum period correspond with distinct patterns of change in all monoamine levels within multiple mesocorticolimbic structures. WKY mothers exhibit deficits in their caregiving behavior across the postpartum period compared to the control SD mothers. Concomitantly, WKY mothers have lower intracellular levels of all monoamines, as well as different patterns of change in their monoamine pathways as they transition across postpartum, compared to SD mothers. Taken together, our data suggest a relationship between monoamine systems and maternal responsiveness to offspring cues across the postpartum period. Moreover, disturbances in parenting associated with a depressive phenotype may be related to underlying alterations in monoamine systems within various mesocorticolimbic brain regions.

Consistent with previous studies, we find variations in maternal behavior across the postpartum period; frequencies and durations of active caregiving and nursing are elevated during the early postpartum period, and subsequently decline across the postpartum period (Grota and Ader, 1974; Pereira and Morrell, 2009; Reisbick et al., 1975; Wansaw et al., 2008). Early postpartum SD mothers spend most of their time in contact with their young and exhibit robust expression of active caregiving behaviors. As the postpartum period progresses, SD mothers reduce their time in contact with their young and decrease their

active caregiving when with them. This change in maternal behavior parallels the developmental needs of the pups.

In the present study, we find that WKY mothers exhibit severe deficits in parenting, not just quantitatively, but also qualitatively. For instance, in the maternal behavior test, upon reunion with their pups following a separation period, EPP SD mothers avidly care for their young, directing most of their behavior toward their pups, with little display of non-maternal activities such as self-grooming, resting, and feeding. In contrast, WKY females respond to being reunited with their pups following separation with minimal active caregiving, spending most of the test time away from their young. This is consistent with prior studies showing deficient maternal behavior in the WKY strain (Ahmadiyeh et al., 2004; Cierpial et al., 1987). Moreover, indicative of the magnitude of the deficit in active caregiving of WKY mothers is the fact that their behavior is similar to that exhibited by SD females treated with DA receptor antagonists (Pereira et al., 2011; Pereira and Ferreira, 2006), suggesting that deficits in mesolimbic dopamine might underlie the deficits in active caregiving of WKY mothers. In addition, WKY mother are more abrupt and disorganized during interactions with their pups than SD mothers - often retrieving pups by body parts (i.e., limbs, side, belly) other than the typical nape of the neck. Furthermore, WKY mothers exhibit frequent interruptions of mother-young contact time, resulting in fragmented patterns of maternal behavior. A similar fragmentation of maternal behavior is evident in mother rats following functional interference of the mPFC (Afonso et al., 2007; Febo et al., 2010; Pereira and Morrell, 2011), suggesting that disorganized aspects of WKY mothers' caregiving behavior might suggest mPFC dysfunction. In support, results from our lab demonstrate that WKY mothers exhibit performance deficits on the reversal and

extradimensional shift discrimination components of the attentional set-shifting task, indicative of impaired cognitive flexibility (Pereira et al., 2014). In addition, WKY mothers exhibit less change in active caregiving (i.e., smaller fold decrease) across the postpartum period compared to the characteristic pattern of SD mothers, highlighting their reduced maternal sensitivity.

These striking differences in parenting between SD and WKY mothers during the 30-min maternal behavior test are also evident during the extensive 25-day undisrupted observations in the home cage, confirming that the deficits of WKY mothers are not driven by stress due to handling associated with the testing procedures. In addition, behavioral differences in caregiving between SD and WKY mothers are not the result of interacting with offspring with differential development trajectories, as WKY offspring gain weight and reach physical and neurological reflex developmental milestones similarly to SD offspring. Daily testing of select pups did not affect their physical development, as their non-handled siblings were observed to achieve physical landmarks on the same days (i.e., pinnae detachment, incisor eruption eye opening, etc.). In addition, daily testing of selected pups did not affect the mothers' caregiving behaviors, thus demonstrating lack of handling effects of our procedure(Raineki et al., 2010). In general, our developmental data for SD and WKY male and female pups was similar to that reported by others (Breit et al., 2019; Cierpial and McCarty, 1987; Maurissen et al., 2000; Wallace et al., 2010). This result is consistent with clinical studies derived from populations in high-income countries indicating no impact of postpartum depression on child growth (i.e., wasting, stunting or weight for age) (Parsons et al., 2012; Santos et al., 2010). Nonetheless, SD and WKY pups are likely neurobiologically different, as they grow to behave differently. In this sense, we

have found decreased isolation-induced ultrasonic vocalizations in WKY pups compared to SD pups, potentially indicative of decreased emotionality (Winokur et al., 2016). Noteworthy, our initial cross-fostering studies suggest that WKY mothers continue to show deficits in maternal behavior despite the strain of the pups (Pereira et al., 2014); this has also been supported by other studies assessing WKYs cross-fostering SHP pups (Ahmadiyeh et al., 2004). Taken together, results suggest neurobiological differences in processing sociosensory cues between strains. Further analysis, including deep phenotyping, is needed to discriminate whether altered USVs and/or other characteristics of WKY pups also underlie (more subtle) aspects of behavioral deficits of WKY mothers. It is important to note that despite these severe deficits in parenting, most WKY mothers raise their litters to weaning. Thus, results suggest that WKY mothers exhibit a distinct maternal phenotype characterized by fragmented care that lacks sensitivity and recapitulates aspects of parenting by depressed human mothers(Arteche et al., 2011; Dix and Meunier, 2009; Lovejoy et al., 2000). Such compromised mother-young interactions have been shown to produce atypical socioemotional development and pathology in offspring (Baram et al., 2012; Curley and Champagne, 2016).

Consistent with prior work (Avraham et al., 2017; Desan et al., 1988; Lonstein et al., 2003), our results show that the transition to motherhood in SD females is accompanied by significant changes in intracellular levels of monoamines and their turnovers within many mesocorticolimbic structures. Relative to nulliparous females, early postpartum mothers had lower intracellular concentrations of monoamines, as well as lower noradrenaline turnover, and elevated serotonin turnover within most structures examined. These results are consistent with prior work comparing mPOA intracellular tissues levels,

as well as release dynamics of monoamines within NA between virgin and early postpartum rodents (Afonso et al., 2007; Avraham et al., 2017; Lonstein et al., 2003). Additional findings not detected previously might be related to the fact that more structures (and discrete subregions) and/or different postpartum days were sampled than in previous work, and due to the increased sensitivity of the HPLC protocol used for simultaneous tissue analysis of monoamines. While extracellular measures are needed to fully understand the functional status of the monoamines systems, it is tempting to speculate that these findings suggest reduced basal monoaminergic tone during the early postpartum period within key mesocorticolimbic structures, which has been proposed in the maternal and other contexts as a mechanism to maximize signal-to-noise ratio (Afonso et al., 2007; Hasselmo et al., 1997; Lőrincz and Adamantidis, 2017). A lower monoaminergic tone within relevant nodes of the maternal circuitry during the early postpartum period might play a role in facilitating the flow of social information across multiple brain regions. For instance, improved signal-to-noise ratio in mPOA neurons, which project to the VTA, NA, and mPFC, could amplify behaviorally-relevant pup-related cues and signals, and thus promote sensitive parenting. In line with this idea, presentation of pups with increased needs ameliorates the deficits of blocking DA signaling on early postpartum expression of maternal behavior (Pereira and Ferreira, 2006).

In contrast, progression across the postpartum period was characterized by significant changes in intracellular levels of monoamines and their turnovers, but varied on a structure-by-structure basis, as highlighted in the following examples. Within striatal structures, progression across the postpartum period was characterize by a pattern of increase in intracellular dopamine and serotonin levels, along with a decrease in their

turnover within the DMS and NACore. Both the DMS and NACore are regions that participate in goal-directed behaviors (Balleine et al., 2007; Balleine and O'Doherty, 2010), and thus results suggest that monoamines within these structures may modulate aspects of sensitive caregiving across the postpartum period. Substantial evidence suggests that the DLS encodes behavioral habits in a familiar sensory context (Alloway et al., 2017; Balleine et al., 2009) and therefore increases in DLS basal serotonin neurotransmission observed from EPP to LPP might underlie habitual aspects of maternal behavior. In addition, our prior work demonstrated a progressive waning of the necessary role of the IL in maternal behavior(Pereira and Morrell, 2009) and thus it is tempting to speculate that the present results, showing decreased monoamine levels within the IL during LPP, may represent aspects of this transition. Collectively, these results are consistent with reorganization of the maternal circuitry across the postpartum period, as exemplified above, and future studies will investigate the functional relationship between parenting and monoamine activity within these identified structures.

Consistent with previous studies in WKY male rats(Scholl et al., 2010), our results show that WKY virgin females consistently demonstrate lower levels of monoamines and higher turnovers within many striatal structures, compared to SD virgins. WKY males have been shown to exhibit altered monoaminergic functioning in a variety of brain regions when compared to SD controls (Jiao et al., 2003; Pearson et al., 2006; Tejani-Butt et al., 1994). Our results confirm and expand upon prior ones suggesting that these discrete differences in monoamine levels likely represent neurobiological factors underlying the depressive phenotype of the WKY strain.

Our results suggest disruption in the normal function of the central monoamines in WKY females during the transition to motherhood and throughout the postpartum period. Specifically, WKY mothers show altered levels of monoamines and their turnovers within several structures, especially the DLS, DMS, VLS, NACore, and mPOA. Moreover, WKY mothers have notable variations in their profile of change across the postpartum period, distinct from SD mothers. This is further exemplified by the differences in monoamine level covariance across maternal nodes between strains. These results suggest that initial monoamine-related alterations underlying behavioral deficits in early postpartum WKY mothers may have pervasive effects that linger throughout the postpartum period. One potential explanation underlying this possibility might involve strain differences in the neuroendocrine events of pregnancy and parturition that prime the maternal circuitry. In this sense, about 10% of our WKY mothers lose their litters within a few days following parturition, which suggests complications in the establishment of nursing, including impairments with lactation (i.e., delayed onset and/or insufficient milk volume) and/or suboptimal nursing behaviors and experiences of WKY mothers (and/or their newborn pups). Considering that maternal behavior has an epigenetic impact on offspring estrogen receptor alpha expression (Champagne and Curley, 2008), it is tempting to speculate that as a consequence of receiving deficient caregiving (with reduced licking), WKY females may have reduced sensitivity to estrogen during pregnancy and parturition, resulting in an impaired onset of maternal behavior. This in turn likely impacts ongoing epigenetic mechanisms mediating transcriptional changes in the maternal brain that coordinate further adjustments across the postpartum period (Stolzenberg and Champagne, 2016). Future work should explore this possibility.

Surprisingly, no differences were found between SD and WKY mothers in cortical monoamine levels or their turnover across the postpartum period. However, studies in our lab have indicated that WKY mothers exhibit deficits in cognitive flexibility (Pereira et al., 2014), which is dependent on mPFC monoamine function (Birrell and Brown, 2000). Deficits in cognitive flexibility and fragmented maternal behaviors exhibited by WKY mothers strongly suggest altered cortical function, consistent with the notion that other aspects of monoamine neurotransmission not detected by our method (such as synaptic release) might underlie the above strain differences. In addition, the degree to which differences in intracellular concentrations of monoamine neurotransmitters and their metabolites contribute to the behavioral phenotype of WKY mothers remains unclear.

Taken together, our results suggest that variations in monoamine neurotransmitters within discrete mesocorticolimbic structures might mediate adaptations of the maternal brain circuitry underlying maternal behavior across the postpartum period. Alterations to the normal functioning of monoamine systems result in parenting deficits that can compromise the healthy progression of maternal care. Future studies examining monoamine release dynamics and postsynaptic signaling are necessary to ultimately elucidate the functional role of monoaminergic neurotransmission as it relates to maternal phenotype. Identifying the neural basis that yield such complex behavior in mothers is crucial to understanding the mechanisms of depression, anxiety, and other neuropsychiatric disorders that most impact parenting.

2.6 Figures

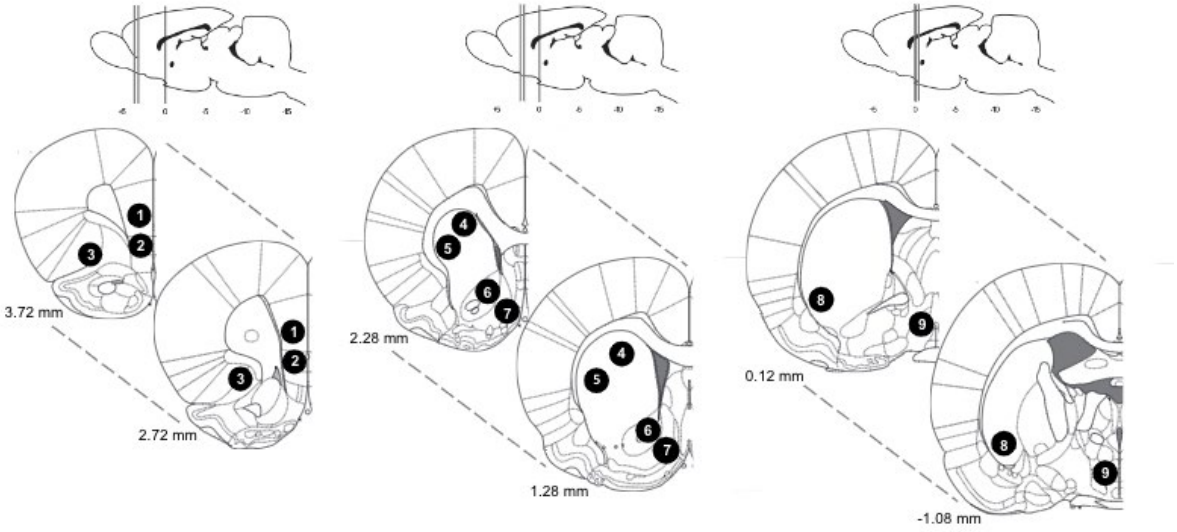


Figure 1. Schematic representation of rat brain sagittal and coronal view of 1mm slices used for punch microdissection. Circles represent tissue-punch locations of the (1) PRL, (2) IL, (3) OFC, (4) DMS, (5) DLS, (6) NACore, (7) NAShell, (8) VLS, and (9) mPOA. Illustrations are adapted from the atlas of (Paxinos and Watson, 2009). Numbers beside each plate indicate the distance from Bregma in millimeters.

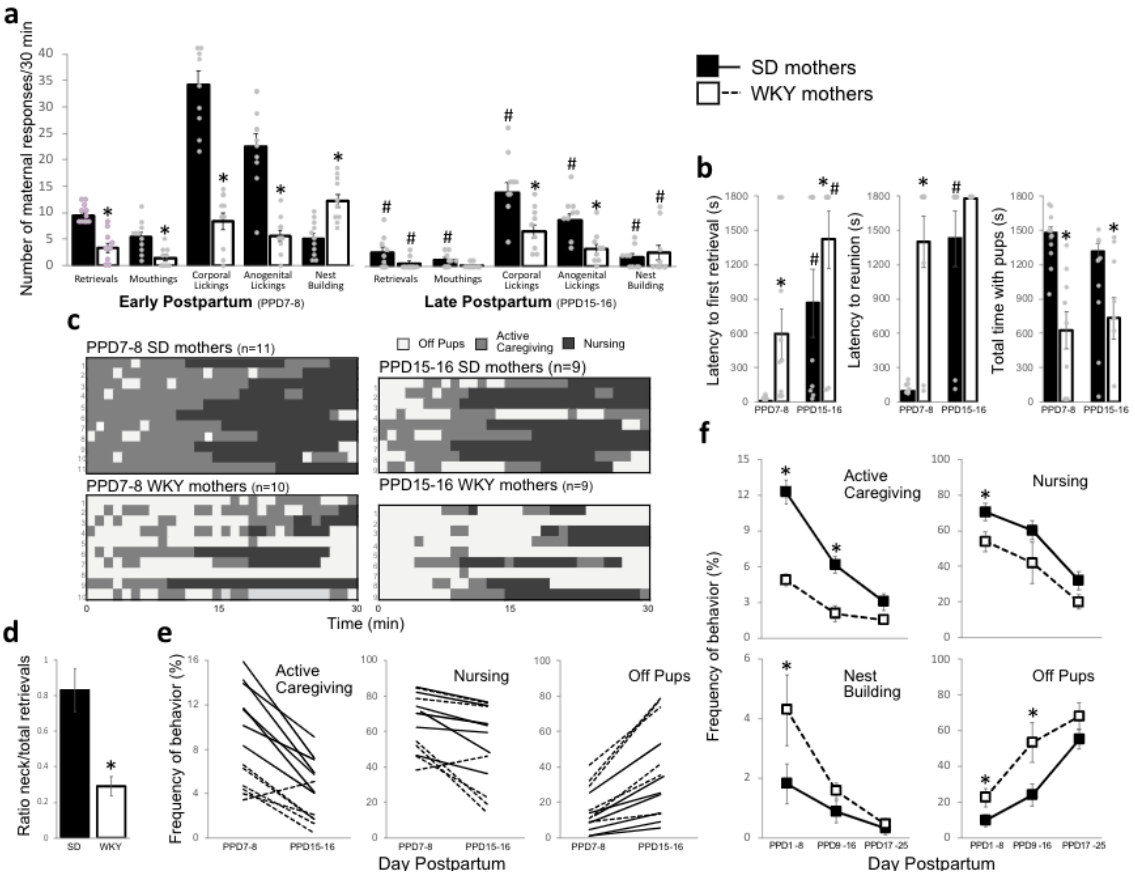


Figure 2. WKY mothers exhibit severe deficits in maternal behavior compared to SD mothers. SD and WKY females were evaluated in a 30-min maternal behavior test on early PPD7-8 (n=11 and n=10, respectively) or late PPD14-15 (n=9 and n=9, respectively). **a**, Number of active maternal responses over the behavioral test. **b**, Latency to first retrieval and reunion of the litter, and duration of total time with pups over the maternal behavior test. Gray dots represent values from individual mothers. **c**, Activity of SD and WKY mothers during the maternal behavior test. Each line depicts one mother's activity during 30 1-minute epochs. Individual blocks are color-coded to represent the mother's activity during each epoch. **d**, Ratio of retrievals over total number of retrievals by EPP SD and WKY mothers during the maternal behavior test. **e**, Frequency of undisrupted home cage observations in which SD (n=7) and WKY (n=6) mothers were observed displaying active

caregiving, nest building, or nursing, or being away from their pups just considering at PPD7-8 or PPD14-15 or **f**, across PPD1-8, PPD9-16, and PPD17-25. Data is expressed as mean ± SEM. *Significant difference in responding relative to SD mothers (between-strain comparison). #Significant difference in responding across postpartum (within-strain comparison)

Table 1.
Developmental milestones in SD and WKY male and female pups.

		Physical Milestones					Neurological Reflexes		
		Ears Unfolding	Incisor Irruption	Ear Opening	Eye Opening	Fur Development	Palmar Grasp	Righting Reflex	Negative Geotaxis
SD	F	3.0 ± 0.2	9.3 ± 0.3	13.1 ± 0.4	14.8 ± 0.3	12.2 ± 0.2	3.0 ± 0.3	4.2 ± 0.2	13.1 ± 0.7
	M	2.9 ± 0.2	9.4 ± 0.4	12.7 ± 0.4	15.1 ± 0.2	12.0 ± 0.2	2.8 ± 0.2	4.3 ± 0.4	12.8 ± 0.6
WKY	F	3.1 ± 0.2	9.8 ± 0.4	13.7 ± 0.4	14.9 ± 0.5	12.1 ± 0.3	3.1 ± 0.2	4.7 ± 0.4	13.4 ± 0.7
	M	3.0 ± 0.4	9.9 ± 0.3	13.0 ± 0.3	14.8 ± 0.5	12.2 ± 0.3	3.0 ± 0.2	4.4 ± 0.4	12.9 ± 0.6

Data are expressed as means ± SEM PND at which pups show each milestone; 2 males and 2 females per litter were included n=12-14 pups per sex/strain. F, female pups and M, male pups.

Table 2.
Monoamine metabolites 5-HIAA, DOPAC, HVA and NM within mesocorticolimbic structures of SD and WKY virgin and postpartum female rats.

		SD Females				WKY Females			
		Virgin	EPP	LPP	WPP	Virgin	EPP	LPP	WPP
Structure									
DLS	5-HIAA	25.1 ± 5.3	25.5 ± 2.6^{c}	35.3 ± 3.5	28.2 ± 3.7	22.2 ± 3.0^{B,C,D}	26.8 ± 4.1	41.1 ± 4.1	25.6 ± 1.9
	DOPAC	3819.8 ± 2248.4	1462.1 ± 76.7	1327.6 ± 128.8	1414.8 ± 105.4	1327.7 ± 146	1660.8 ± 200.6	1417.9 ± 139.4	1516.6 ± 46.3
	HVA	600.9 ± 38.8	745.4 ± 27.2^{c,d}	584 ± 50.3	485.3 ± 27.4	625.3 ± 50.9	859.4 ± 82.2^{A,C}	635.2 ± 55.8	773.3 ± 22.2*
	NM	193.1 ± 61.3	120.6 ± 9.7	99.6 ± 17.2	122.1 ± 18.7	447 ± 132.3^{B,C,D,*}	180 ± 27.7	118.2 ± 17.1	236.2 ± 47.3*
DMS	5-HIAA	25.5 ± 3.1	23.8 ± 2.5	29.2 ± 3.3	40.2 ± 4.7	22.7 ± 3.0	29.6 ± 3.0	29.2 ± 5.4	30.0 ± 3.0
	DOPAC	1139.1 ± 33.6	1385.4 ± 98.1	1284.4 ± 126.3	1510.5 ± 100.9	1542.6 ± 222.1	1608.3 ± 83.6	1201.6 ± 163.8	1579.3 ± 80.6
	HVA	860.8 ± 181.5^{b,c,d}	492.7 ± 22.0	431.9 ± 36.0	455.9 ± 33.5	538.0 ± 85.8*	622.5 ± 39.7^{C}	432.8 ± 50.0	572.0 ± 29.0
	NM	1381.8 ± 675.0^{b,c,d}	167.1 ± 39.1	89.3 ± 10.2	101.4 ± 13.0	461.9 ± 91.4*	143.2 ± 16.9	73.8 ± 9.1	243.3 ± 52.5
ILC	5-HIAA	22.3 ± 4.2	30.6 ± 1.8	29.5 ± 3.7	23.0 ± 2.9	38.2 ± 11.4	31.7 ± 1.5	32.1 ± 5.3	26.4 ± 5.4
	DOPAC	149.0 ± 36.0	87.6 ± 16.4	132.3 ± 15.2	106.9 ± 13.8	97.3 ± 18.0	81.3 ± 14.3	177.7 ± 49.7^{A,B}	114.9 ± 20.8
	HVA	144.4 ± 33.1^{b,c,d}	87.9 ± 16.2	71.8 ± 6.5	56.5 ± 9.4	129.4 ± 28.3^{B,C}	59.3 ± 9.3	58.4 ± 8.6	83 ± 16.2
	NM	687.9 ± 241.7^{b,c,d}	43.3 ± 10.2	46.4 ± 8.5	48.6 ± 14.5	323.6 ± 108.7^{B,C,D,*}	36.2 ± 8.9	35.9 ± 11.5	105.1 ± 23.6
mPOA	5-HIAA	26.6 ± 4.8	34.2 ± 3.4^{c,d}	23.6 ± 2.3	23.5 ± 2.3	10.8 ± 1.8^{B,C,D}	35.4 ± 3.0	41.1 ± 5.1	24.0 ± 2.2^{BC}
	DOPAC	328.8 ± 82.1	255.2 ± 32.3	280.4 ± 32.7	335.3 ± 49	171.8 ± 32.9*	163.2 ± 19.3	311.4 ± 45.2	232.7 ± 20.2
	HVA	124.4 ± 44.6	89.3 ± 9.4	95.6 ± 12.1	113.1 ± 13.6	97.7 ± 19.5	80.6 ± 11.2	101.7 ± 13.5	108.5 ± 10.5
	NM	187.6 ± 53.3^{b,c,d}	59.2 ± 13.0	52.2 ± 11.1	69.5 ± 16.3	387.3 ± 134.6^{B,C,D,*}	57.0 ± 12.3	36.3 ± 6.2^{D}	145.7 ± 38.3
NACore	5-HIAA	14.7 ± 2.4^{c,d}	21.6 ± 1.9^{d}	26.8 ± 2.9	30.0 ± 4.5	13.0 ± 2.3	22.9 ± 1.8	17.6 ± 1.0	19.4 ± 2.2
	DOPAC	1472.3 ± 167.8	1707.7 ± 118.4	328.8 ± 82.1	1629.5 ± 187.1	1454.3 ± 202.8	1565.9 ± 180.7	1290.9 ± 99.5*	1423.7 ± 140.2
	HVA	705.8 ± 147.0^{b,c,d}	468 ± 29.6^{d}	431.3 ± 30.4	336.8 ± 29.4	402.8 ± 30.4*	358.5 ± 21.6*	236.5 ± 6.7^{A,B,*}	350.7 ± 44.8
	NM	1328.8 ± 483.2^{b,c,d}	180.7 ± 47.4^{c}	112.8 ± 15.5	180.6 ± 46.1	487.3 ± 119.4^{B,C,*}	167.7 ± 16.7	80.4 ± 13.8	234.6 ± 60.9
NAShell	5-HIAA	27.1 ± 6.2	34.7 ± 2.7	26.7 ± 2.9	28.4 ± 4.7	9.5 ± 2.7^{B,C,D}	27.3 ± 3.1	32.9 ± 4.3	37.4 ± 4.6
	DOPAC	4584.9 ± 2307.8^{b,c,d}	699.7 ± 90.3	1159.1 ± 121.9	1046.0 ± 162.0	1152.5 ± 67.7*	676.5 ± 63.6	724.9 ± 64.2	987.1 ± 172.3
	HVA	341.5 ± 76.5^{b,d}	210.6 ± 25.8	283.9 ± 37.4	217.3 ± 28.0	276.7 ± 17.5	246.4 ± 44.5	196 ± 12.1^{D}	315.4 ± 46.6
	NM	414.1 ± 228.6^{b,c,d}	97.6 ± 27.8	108.2 ± 25.2	73.1 ± 14.8	413.1 ± 119.1^{B,C,D}	74.2 ± 18.9	33.2 ± 6.9	163.5 ± 37.4
PRL	5-HIAA	18.8 ± 2.3	22.9 ± 1.2	19.1 ± 2.3	18.3 ± 2.5	23.3 ± 3.5	20.5 ± 2.3	27.2 ± 4.1	16.8 ± 2.8
	DOPAC	148.7 ± 22.0	94.6 ± 10.9	136.3 ± 17.0	103.2 ± 11.0	128.3 ± 19.1	127.5 ± 14.2	103.4 ± 14.1	110.4 ± 16.7
	HVA	85.5 ± 9.8^{d}	69.2 ± 8.0	65.7 ± 6.3	51.8 ± 6.1	91.4 ± 19.0^{C,D}	93.5 ± 9.6^{C,D}	52.7 ± 5.2	61.0 ± 9.9
	NM	265.9±140.9^{b,c,d}	29.6 ± 3.5	35.5 ± 4.5	42.9 ± 14.4	282.6 ± 92.6^{B,C,D}	53.0 ± 4.1	18.4 ± 1.8	31.8 ± 5.1
OFC	*5-HIAA*	19.0 ± 4.9	28.4 ± 1.8	39.9 ± 9.2^{a,d}	17.9 ± 2.7	11.9 ± 1.5^{B,C}	33.4 ± 4.4	30.3 ± 2.2	24.0 ± 3.9
	DOPAC	448.9 ± 186.1^{b,c,d}	123.4 ± 33.3	207.6 ± 34.0	105.5 ± 13.3	207.1 ± 32.1*	257.8 ± 73.2^{C}	79.6 ± 12.2	140.0 ± 50.0
	HVA	163.2 ± 39.3^{d}	110.2 ± 22.3	115.2 ± 15.4	73.6 ± 7.5	203.2 ± 49.6^{C,D}	189.3 ± 31.3^{C,D,*}	68.0 ± 9.6	116.0 ± 22.2
	NM	255.6 ± 128.6^{b,c,d}	53.0 ± 14.2	56.4 ± 15.0	48.0 ± 13.0	360.3 ± 153.9^{B,C,D}	76.0 ± 16.1	22.9 ± 9.2	125.4 ± 19.4
VLS	5-HIAA	37.4 ± 6.9	34.6 ± 2.6	31.2 ± 3.0	30.3 ± 3.3	18.8 ± 3.7	34.0 ± 4.3	35.8 ± 6.3	23.3 ± 2.3
	DOPAC	853.0 ± 63.3^{d}	1072.9 ± 81.8	1065.5 ± 82.6	1163.6 ± 97.5	931.6 ± 84.0	1087.7 ± 100.6	864.4 ± 89.7	1280.4 ± 58.6^{AC}
	HVA	854.1 ± 125.1^{b,c,d}	527.7 ± 28.3	454.3 ± 35.6	447.1 ± 33.7	476.8 ± 51.8*	613.5 ± 50.2^{C}	422.8 ± 40.9	508.2 ± 10.4
	NM	1298.2 ± 586.1^{b,c,d}	125.0 ± 18.9	77.0 ± 9.7	126.3 ± 31.0	314.9 ± 64.4*	97.8 ± 9.5	53.8 ± 6.7	151.7 ± 9.5

Data are expressed as means ± SEM pg/mg of tissue. Superscript letter indicates significant difference between stages (within strain comparison). *denotes significant difference relative to the corresponding SD group (between strains comparison). $p<0.05$.

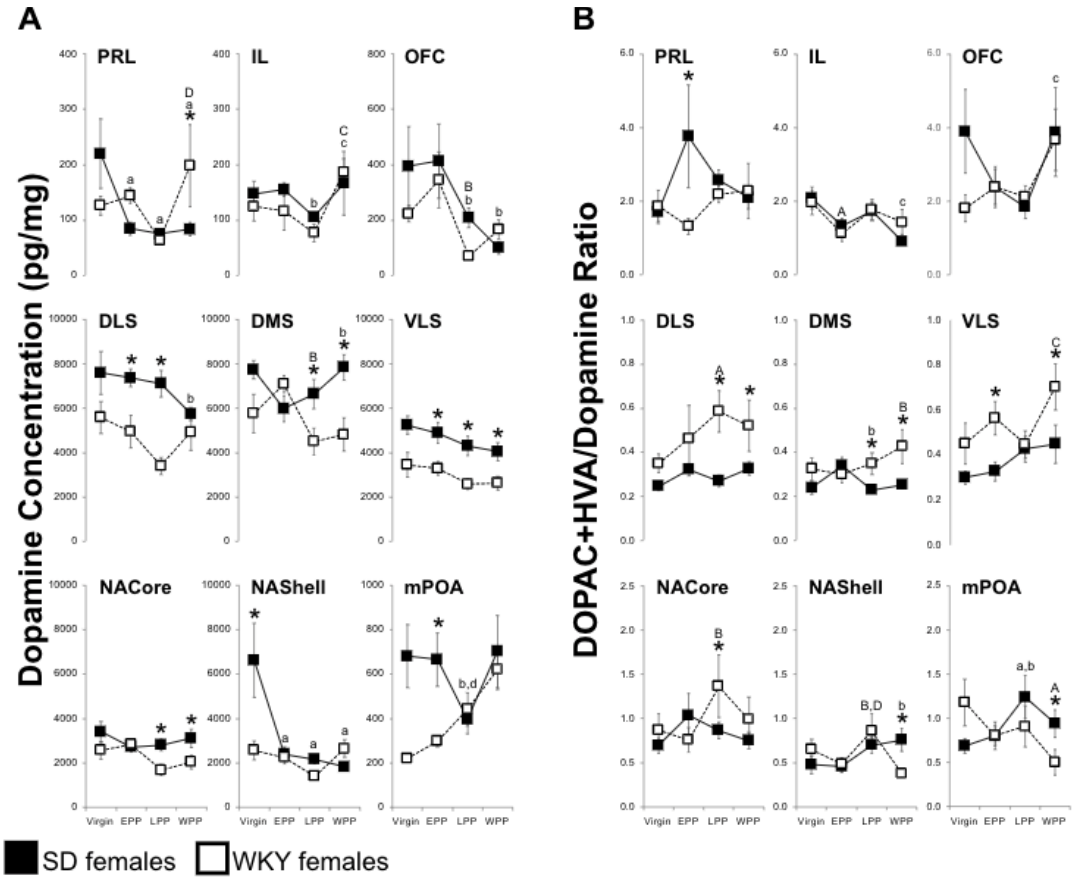


Figure 3. Dopamine and dopamine turnover. Measures (mean ± SEM) of intracellular dopamine (A), and dopamine turnover (B) within mesocorticolimbic brain regions of SD and WKY virgin (n=7 and n=7, respectively) and postpartum (PPD7-8 n=15 and n=13, PPD15-16 n=17 and n=12, PPD25 n=18 and n=9, respectively) female rats. *Significant difference relative to SD strain. Within strain significant difference between stages is indicated with letters (SD/WKY: a/A virgins, b/B EPP, c/C LPP, and d/D WPP). $p<0.01$.

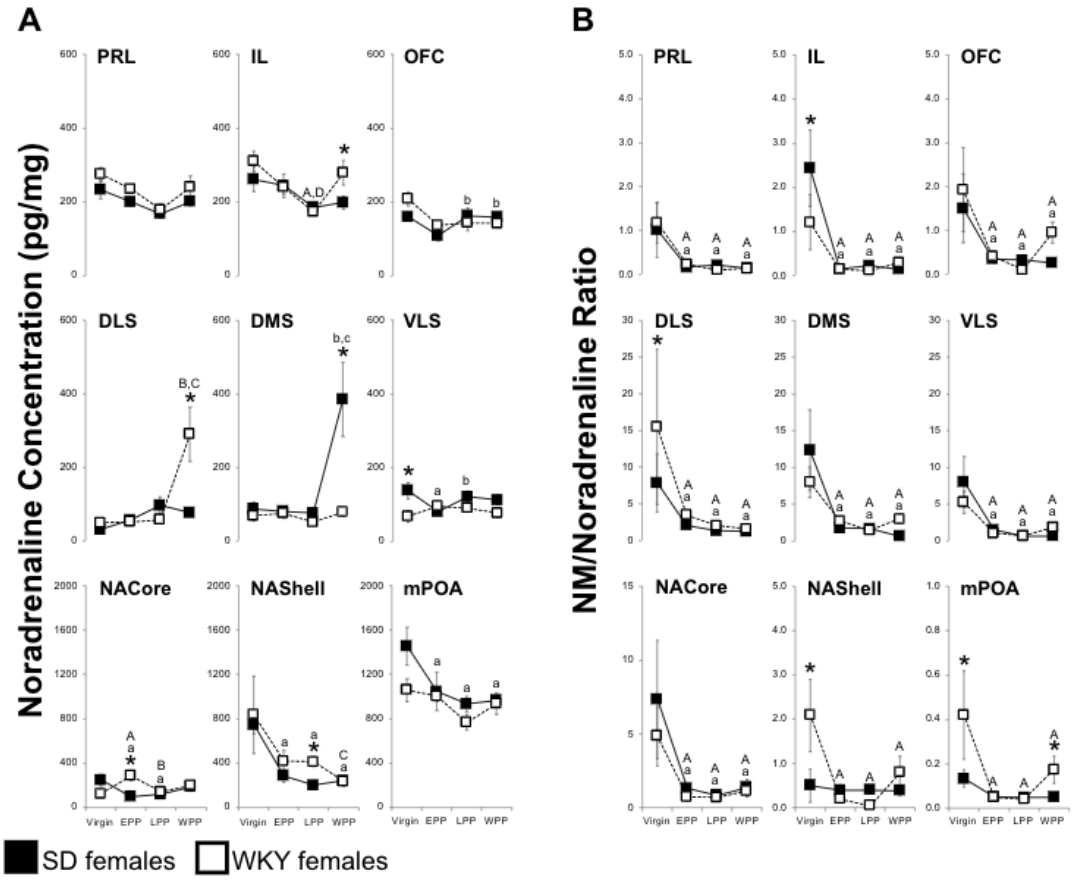


Figure 4. Noradrenaline and noradrenaline turnover. Measures (mean ± SEM) of intracellular noradrenaline (A), and noradrenaline turnover (B) within mesocorticolimbic brain regions of SD and WKY virgin (n=7 and n=7, respectively) and postpartum (PPD7-8 n=15 and n=13, PPD15-16 n=17 and n=12, PPD25 n=18 and n=9, respectively) female rats. *Significant difference relative to SD strain. Within strain significant difference between stages is indicated with letters (SD/WKY: a/A virgins, b/B EPP, c/C LPP, and d/D WPP). $p<0.01$.

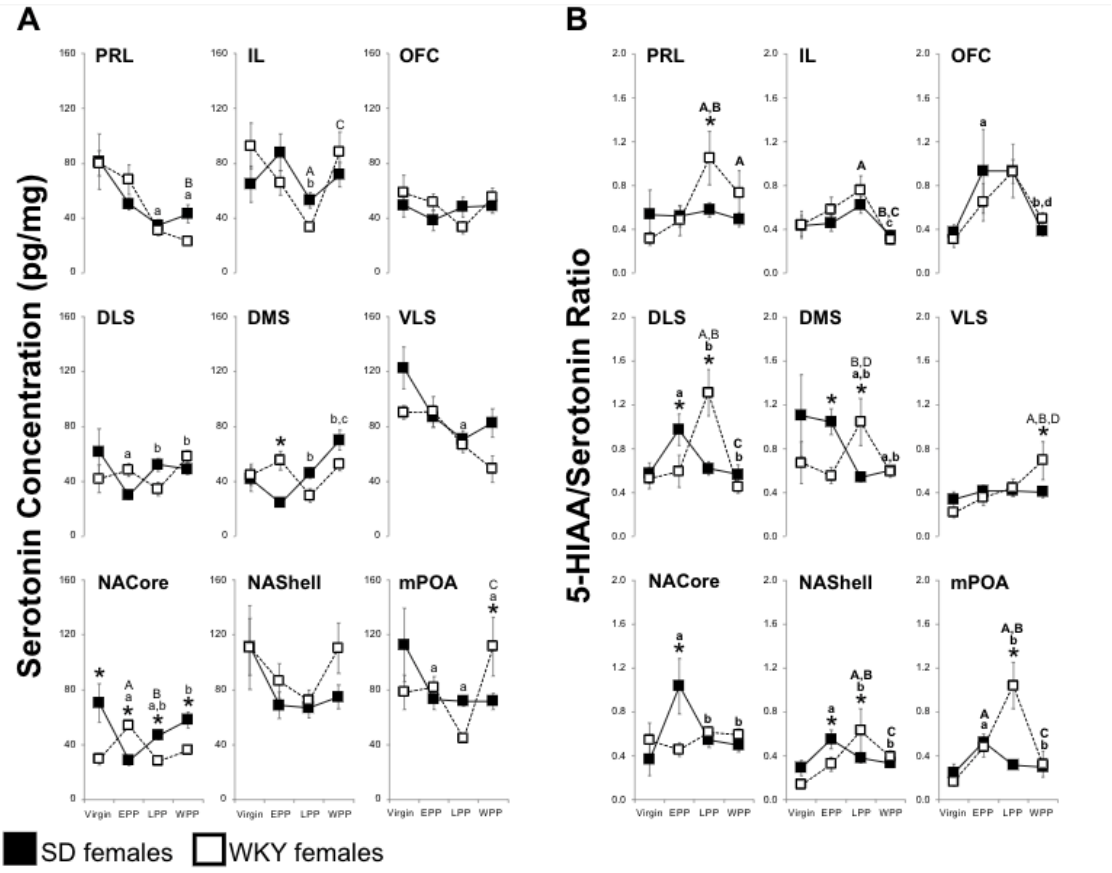


Figure 5. Serotonin and serotonin turnover. Measures (mean ± SEM) of intracellular serotonin (A), and serotonin turnover (B) within mesocorticolimbic brain regions of SD and WKY virgin (n=7 and n=7, respectively) and postpartum (PPD7-8 n=15 and n=13, PPD15-16 n=17 and n=12, PPD25 n=18 and n=9, respectively) female rats. *Significant difference relative to SD strain. Within strain significant difference between stages is indicated with letters (SD/WKY: a/A virgins, b/B EPP, c/C LPP, and d/D WPP). $p<0.01$.

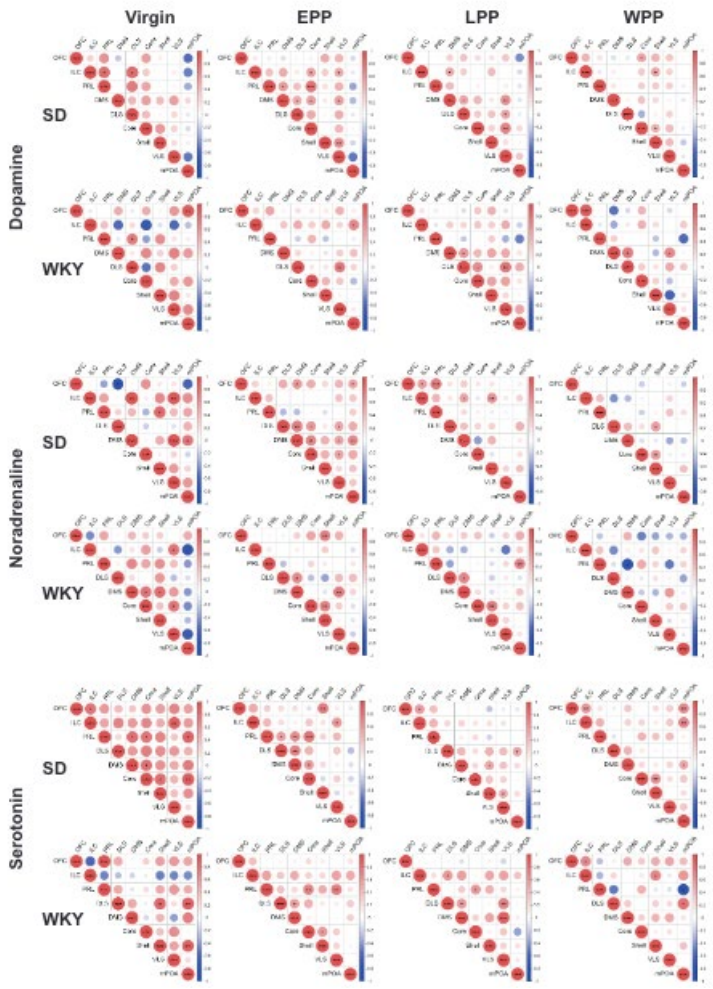


Figure 6. Monoamine level covariance across maternally-relevant brain regions in SD and WKY females across the postpartum period. All correlograms show pair-wise comparisons (Pearson's correlation coefficients) of dopamine, noradrenaline, or serotonin levels across mesocorticolimbic brain regions. Red color represent positive correlations and Blue represents negative correlations. Size of circles is proportional to the absolute value of the correlation, and color intensity indicates the correlation coefficient as specified by the color bar. Correlograms were generated using the data visualization R package "corrplot"[49]. Color version available online.

CHAPTER III

CAREGIVING DEFICITS ASSOCIATED WITH INCREASED EARLY POSTPARTUM PROGESTERONE AND DIFFERENTIAL ACTIVATION OF THE MPOA

3.1 Abstract

Estradiol and progesterone play a large role in establishing the onset of maternal behavior. When peripartum levels of these hormones are altered, maternal behavior is disrupted. This study aimed to determine whether caregiving deficits observed in the Wistar-Kyoto (WKY) rat model of depression are related to disrupted early postpartum hormonal signaling. Serum estradiol and progesterone were evaluated, and medial preoptic area (mPOA) cFos, oxytocin, and estradiol receptor alpha expression were measured. Results indicate that WKY caregiving deficits are associated with elevated serum progesterone levels in the early postpartum period. Preliminary IHC trends suggest that the WKY mPOA has elevated expression of cFos and lower ERα+ cFos cells, indicating that cells in the WKY mPOA may be differentially recruited. Taken together, the present findings suggest that elevated progesterone and differential activation of the early postpartum mPOA may contribute to the caregiving deficits of WKY mothers.

3.2 Introduction

Across pregnancy, wide networks of cortical, striatal, and limbic brain structures are recruited to prepare the maternal brain and body for motherhood; this is largely prompted by changing hormonal signals from the ovaries and placenta to the brain

(Brunton and Russell, 2010, 2008). In rats, 17β-estradiol (E2) levels increase gradually across pregnancy up to parturition, while progesterone (P) levels rise sharply during early pregnancy and continue to increase across pregnancy with levels consistently above estradiol levels before falling just prior to parturition. These hormonal changes throughout pregnancy upregulate hormone receptor levels, including estrogen receptor (ER) expression, in key maternal brain structures, such as the medial preoptic area (mPOA). These changes are necessary for the rapid onset (Siegel and Rosenblatt, 1978) and the ongoing expression of maternal behavior at birth (Champagne et al., 2003b).

Ample evidence indicates that changes in E2 and P occurring at the time of birth and just after birth trigger the onset of maternal behavior at parturition (Terkel and Rosenblatt, 1968, 1972). The natural or hormonally simulated termination of pregnancy produces an immediate onset of maternal behavior (Siegel and Rosenblatt, 1978). Estradiol benzoate implants into the mPOA of pregnancy-terminated primigravid rats (by combination of hysterectomy and ovariectomy on day 16 of gestation) accelerate the onset of maternal behavior (Numan et al., 1977). Moreover, pregnancy hormones increase ER distribution and binding in the mPOA, and high levels of nuclear ERs in the mPOA are necessary for the cellular and molecular changes that prompt the maternal brain to respond to infant stimuli (Giordano et al., 1989).

In addition to the role of hormones in timing the onset of maternal behavior, hormonal activity across pregnancy influences the expression of maternal behavior after parturition. Specifically, gestational E2 and P and the estrogen-to-progesterone ratio (E2:P) have been linked to maternal responsiveness to offspring in human and non-human primate mothers (Fleming et al., 1997; Glynn et al., 2016; Maestripieri and Wallen, 1995;

Maestripieri and Zehr, 1998). Human mothers who express higher maternal responsivity and feelings of attachment to their infants in the early postpartum period had an increase in E2:P from early to late pregnancy. In contrast, those who express lower responsivity and feelings of attachment had a decrease in E2:P over this same period (Fleming et al., 1997; Glynn et al., 2016).

Postpartum ER expression and E2 sensitivity in the mPOA have also been associated with maternal caregiving in rats, rabbits, and sheep (Champagne et al., 2003b; González-Mariscal, 2001; Meurisse et al., 2005). The ER subtype ERα, has particularly been implicated in individual differences in caregiving. Variations in maternal behavior have been associated with differences in ERα in the postpartum mPOA of rats (Champagne et al., 2006, 2003b) and are transmitted across generations. Such heritable variations in maternal behavior have been associated with methylation status of the ERα1b promoter (Champagne et al., 2006). Specifically, female offspring of mothers that exhibit decreased pup licking provide similarly low pup licking to their young and show decreased ERα expression in the mPOA (Champagne et al., 2006). Taken together, prior research illustrates the pivotal role that E2 and P play in the onset and ongoing expression of maternal behavior at parturition.

Caregiving deficits are common in mothers with postpartum depression. WKY mothers, a genetic model of depression-like behavior, show caregiving deficits related to their depressive phenotype. These deficits were previously characterized by our lab (Winokur et al., 2019). This prior research suggests that the transition to motherhood is disrupted in WKY mothers, as reflected by their atypical monoaminergic neurotransmitter profiles. As illustrated in the literature reviewed above, E2 and P, as well as related mPOA

activity, play a substantial role in this transition to motherhood. Thus, it is hypothesized that circulating hormones and subsequent mPOA activation may be disrupted in WKY mothers, leading to their caregiving deficits.

The goal of the current study was to determine whether peripartum alterations in circulating E2, P, and/or ERα expression in the mPOA underlie the depression-related parenting deficits observed in WKY mothers. To this aim, this study evaluated serum hormone concentrations and mPOA expression of ERα and oxytocin (Oxt). In addition, expression of cFos, a marker for neuronal activation, was quantified to identify mPOA neurons containing ERα that were recruited during offspring interactions. It was hypothesized that parenting disturbances in early postpartum WKY mothers are associated with altered hormonal levels and activation of mPOA cells containing ERα.

3.3 Methods

3.3.1 – Subjects: SD and WKY females were maintained on a 12-hr light/dark cycle (lights on at 7AM) with *ad libitum* access to rat chow, sunflower seeds, and water. All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at UMass Amherst. All mating occurred in-house, and all subjects were first-time mothers. All behavioral testing occurred during the light cycle.

3.3.2 – Maternal Behavior Test: Early postpartum (PPD 7-8) SD and WKY mothers were assessed for their maternal behavior during a 30-min maternal behavior test. Following a 2-hr mother-litter separation, 8 pups were scattered in the maternal home cage. The frequency, duration, and latency of maternal caregiving behaviors were then recorded continuously for 30-mins by an experimenter, as described previously (Winokur et al.,

2019). Other non-maternal behaviors recorded included general exploration (zone crosses and rearings), self-grooming, eating, and drinking. Active caregiving scores were calculated as cumulative frequency of corporal and anogenital lickings plus mouthings and nest building. Huddling duration was calculated as the cumulative duration of time spent in contact with offspring, either while hovering over or nursing. Behavior was also categorized into 1-min bins based on whether moms were away from their offspring, actively caregiving, or nursing.

3.3.3 – Blood Serum Sample Collection: Blood samples (3-4mL per subject) were collected via cardiac puncture 60-mins after maternal behavior testing, directly preceding perfusion. Blood samples were allowed to clot at room temperature for 45-mins, and then centrifuged at 2,000xg for 10-mins. The upper serum layer was extracted and stored at -80°C until processing for E2 and P measurements.

3.3.4 – Hormone Assays: Serum levels of E2 and P were evaluated using Enzyme-linked Immunosorbent Assay (ELISA) kits from Cayman Chemical (Item No. 501890 and 582601, respectively). Serum samples were first purified according to the manufacturer's instructions. Manufacturer instructions were followed to run the assay.

3.3.5 – Brain Tissue Preparation: Brains were transcardially perfused with 4°C 0.9% saline followed by 4% paraformaldehyde (PFA). Brains were then extracted, stored in 4% PFA, and cryoprotected with 15% and 30% sucrose solution at 4°C. Three series of 40µm-thick cross sections per animal were sliced on a cryostat, and stored in cryoprotectant at –20°C until immunohistochemistry analysis.

3.3.6 – Immunohistochemistry (IHC): Anatomically-matched sections containing the mPOA (-0.24 and -0.36 mm from Bregma; (Paxinos and Watson, 2009)),

were used for IHC analysis. Floating brain sections were washed 3 times with 0.3% Triton X-100 (PBST) for 20-mins each, blocked with 10% normal donkey serum for 1-hr at room temperature, and then incubated with primary antibodies overnight at 4°C. After washing in PBST 3 times for 20-mins each, sections were incubated with secondary antibodies for 2-hrs at room temperature. Primary antibodies were guinea pig anti-cFos (SySy 226005, 1:1000), mouse anti-ERα (Santa Cruz Biotechnology sc-8005, 1:1000), and rabbit anti-oxytocin (Immunostar 20068, 1:1000). Secondary antibodies were Alexa Fluor donkey anti-rabbit 488 (115-545-003), donkey anti-mouse 594 (715-585-150), and donkey anti-guinea pig 647 (706-605-148), all 1:800, Jackson ImmunoResearch. Floating sections were then washed twice in PBST and once in PBS for 20-mins. Sections were then mounted on gelatin-coated slides and cover slipped in Vectashield mounting medium with DAPI (Vector Laboratories).

3.3.7 – Imaging and cell count: Slides were imaged on a CrestV2 with 2xTIRF Nikon Ti2 stand confocal microscope (UMass IALS Light Microscopy Core Facility) at 20x magnification. Images were stitched together using NIS Elements 5.3 software. cFos-, ERα- and Oxt-expressing cells counts within several mPOA subregions were measured using a supervised semi-automatic image counting tool on NIS Elements 5.3. mPOA subregions were identified on fluorescent images according to Paxinos and Watson atlas (2009) and included the medial preoptic area (MPA), the medial preoptic nucleus medial (MPOM), medial preoptic nucleus lateral (MPOL) and ventromedial preoptic nucleus (VMPO).

3.3.8 – Statistics: Behavioral, hormonal, and histological data is expressed as mean ± SEM and was analyzed using independent t-tests. A significance level of $p<0.05$ was

used for all comparisons. Statistical analysis was performed using SPSS software (IBM, v28.0).

3.4 Results

3.4.1 – WKY mothers show caregiving deficits. Consistent with previous results (Winokur et al., 2019), WKY mothers showed severe caregiving deficits (Figure 1).

***i.* WKY mothers exhibited decreased active components of maternal behavior.** Compared to SD (n=10) mothers, WKY (n=8) mothers showed decreased composite active caregiving frequency ($t(16)=2.106$, $p=0.026$, $d=0.999$); this was due to their significantly decreased corporal ($t(16)=2.403$, $p=0.014$, $d=1.14$) and anogenital ($t(11.869)=2.508$, $p=0.014$, $d=1.087$) lickings (Figure 1A). Other active components of maternal caregiving (mouthings and nest building) were not significantly different between strains. As shown in Figure 1B, the number of 1-min active care bouts was decreased in WKY mothers ($t(16)=2.065$, $p=0.028$, $d=0.979$). The number of away and nursing bouts did not differ between strains, nor did the number of transitions between the three categories of care phases. Strains did not differ in non-maternal behaviors, including self-grooming, rearings, drinking, and eating.

***ii.* WKY mothers spent less time with their offspring.** Compared to SD mothers, WKY mothers spent less time huddling with their offspring ($t(16)=2.006$, $p=0.026$, $d=0.952$; Figure 1C). The separate hovering over and nursing durations were not significantly different between strains.

3.4.2 – WKY mothers had heightened serum progesterone. In addition to maternal behavior, serum E2 and P concentrations were examined to determine if strain phenotype is related to circulating hormone levels. On average, WKY (n=8) mothers had

5.68 times greater serum P concentrations compared to SD (n=9) mothers (t(7.169)=-3.093, p=0.011, *d*=-1.503; Figure 2B). No significant difference was found in serum estradiol concentrations between strains (Figure 2A). Subjects that had both E2 and P measured were used to evaluate the ratio between E2 and P. A trend suggests that the ratio between E2 and P may be lower in WKY (n=8) compared to SD (n=6) mothers (t(12)=1.562, p=0.072, *d*=0.844; Figure 2C).

3.4.3 – Progesterone is negatively correlated with huddling. Pearson correlations were run to evaluate whether serum hormone levels correlated with aspects of maternal behavior. Serum P concentrations were significantly negatively correlated with time huddling (r = -0.495, n=17, p=0.043); higher P correlated with less time huddling (Figure 2E). P levels did not correlate with active components of maternal behavior (Figure 2D), and no significant correlations were found between E2 levels and maternal behavior.

3.4.4 – mPOA pattern of activation may differ between WKY and SD mothers. Preliminary ICC data suggests higher levels of cFos expression in the mPOA of WKY (n=5) compared to SD (n=5) mothers (t(8)=-1.796, p=0.055, *d*=-1.136; Figure 3A). The proportion of cFos cells positive for ERα (ERα+ cFos) out of the total cFos cell population trends toward being lower in WKY mothers (t(8)=1.468, p=0.090, *d*=0.928; Figure 3B). Preliminary evaluation of mPOA subregions (Figure 3C) indicates that WKY (n=5) mothers have significantly lower Oxt expression within the MPA compared to that of SD (n=7) mothers (t(6)=2.209, p=0.035, *d*=1.078; Figure 3F). No other significant differences were found in cFos or ERα+ cFos expression within the mPOA as a whole or as divided into subregions (Figures 3D & 3E).

3.5 Discussion

This study revealed that early postpartum WKY mothers have higher P compared to SD mothers, as well as a trend toward elevated mPOA cFos expression and decreased ERα+ cFos expression. Additionally, there is a negative correlation between time huddling and P levels. Together, the results from this study suggest that the deficits in maternal behavior of early postpartum WKY mothers are associated with elevated circulating P as well as altered mPOA activation in response to offspring.

One main result that we obtained is that WKY P levels are elevated in the early postpartum period compared to that of SD mothers. The noted differences in P could be reflective of a longer-term developmental disruption that occurred during gestation or could be a sign of more acute disrupted physiological processes. In rats, the sharp decline of P at parturition is necessary for the timing onset of maternal behavior. In the day or two leading up to parturition when P begins to decline, maternal responsiveness begins to increase in unmanipulated primiparous rats (Pepe and Rothchild, 1974; Slotnick et al., 1973). In P-implanted ovariectomized and hysterectomized (OH) late-pregnant rats, the latency for the onset of maternal behavior toward foster young is significantly longer compared to that of control OH rats (Bridges et al., 1978). Additionally, administration of P in OH late-pregnant rats inhibits estradiol benzoate facilitation of maternal behavior onset (Numan, 1978). In sum, disrupting the decline of P in parturient females puts their maternal responsiveness at a deficit. Since WKY mothers were found to have elevated P in the early postpartum period, it is conceivable that their P did not decline adequately at parturition, resulting in a disruption to maternal behavior. It is also possible that the heightened early postpartum P levels are indicative of elevated P signaling across gestation.

However, a study utilizing WKYs as controls for research on spontaneously hypertensive heart failure rats determined that across gestation WKYs have serum P concentration that did not differ from SD rats on matched gestational days (Sharkey et al., 2005). Elevated P in early postpartum is thus more likely a sign that P did not decline as expected at the time of parturition for WKY mothers. Future research is needed to evaluate WKY P levels across gestation and at parturition.

The mechanism underlying elevated P in early postpartum WKY mothers requires further investigation. In early pregnancy, P is produced by the corpus luteum in the ovaries. By late pregnancy P production has fully transferred to the placenta. Placental production of P is largely regulated by estrogens, cholesterol availability, and 3β-hydroxysteroid dehydrogenase enzymatic activity (Byrns, 2014). After birth and the delivery of the placenta, P levels drop and the production of P does not resume until ovulation recommences, which ensues between 16 hours to 4 days after parturition in rats (Blandau and Soderwall, 1941). Recommencement of ovarian activity after the anovulatory period is largely mediated by estrogen-induced activation of the hypothalamic-pituitary-gonadal (HPG) axis, starting with the production of gonadotropin releasing hormone from the hypothalamus (Ying et al., 1973). Since E2 was not elevated in WKY serum, it is unlikely that overstimulation of the HPG axis is responsible for increased WKY P production in the ovaries; although, increased neuronal-derived E2 may cause increased WKY HPG activity. Future studies should evaluate whether brain production of E2 may be elevated in early postpartum WKYs.

As an alternative possible mechanism, increased WKY P may relate to increased production of P within the adrenal glands. While the adrenal glands primarily produce

glucocorticoids and mineralocorticoids, progesterone is also secreted by the adrenal gland in small quantities (Short, 1960). This production is mediated by the activity of adrenocorticotropic hormone (ATCH; Heap et al., 1966). Rats who experienced acute stress were found to have elevated adrenal P content, and rats who experienced chronic mild stress showed elevated adrenal progesterone secretion compared to ovarian progesterone secretion (Fajer et al., 1971). WKY rats are hyper-responsive to stress and males have been shown to have related elevated corticosterone and ACTH levels (Solberg et al., 2001). Thus, upon the stress of parturition and caregiving, WKYs may secrete elevated adrenal gland-derived P. Although unlikely to be fully responsible for the observed elevated WKY P levels, future studies should evaluate early postpartum WKY corticosterone, ACTH, and adrenal gland P production as a possible explanation for the observed elevated WKY P.

Elevated P in the early postpartum period has been associated with increased incidence of PPD. For example, treating women with norethisterone enanthate, a synthetic progestogen, within 48 hours of delivery significantly increased new mothers' risk for developing PPD as well as the severity of their depression scores (Dennis et al., 2008). This suggests that elevated P disrupts the depression-related cognitive, motivational, and affective processes that are key to parenting, which would explain why these deficits are also reflected within the WKY behavioral repertoire.

Interestingly, allopregnanolone, a neurosteroid synthesized from P, has recently been approved by the FDA as a treatment for postpartum depression. Clinical studies in postpartum women have revealed that intravenous treatment with allopregnanolone (Brexanolone), administered for the first 60-hrs after giving birth, has robust and lasting

antidepressant effects (Kanes et al., 2017). Allopregnanolone levels rise and fall in synchrony with P across pregnancy and parturition (Luisi et al., 2000; Nappi et al., 2001). Allopregnanolone acts as a positive allosteric modulator of the γ-aminobutyric acid A ($GABA_A$) receptor, exerting an inhibitory effect on the circuits that it acts upon (Hosie et al., 2006; Schumacher et al., 2014). It is theorized that disrupted $GABA_A$ receptor plasticity to changing levels of allopregnanolone at parturition may contribute to postpartum depression onset (Maguire and Mody, 2008). Ongoing research is working to determine the specific mechanism underlying how allopregnanolone, via $GABA_A$ receptors, influence postpartum depression (for review, see Walton and Maguire, 2019). It is possible that WKY turnover between P and allopregnanolone is disrupted, altering $GABA_A$ receptor activity and, consequently, neural inhibition. Future studies should evaluate the neurobiological components of allopregnanolone activity in early postpartum WKY mothers, including synthesizing enzyme and GABAergic activity within the mPOA.

When P levels rise during ovulation, body temperature in humans and rats also rises (Rothchild, 1969). While progestins have been found to increase body temperature, estrogens have been found to decrease body temperature (Charkoudian and Stachenfeld, 2016). It is therefore possible that WKY maternal body temperature is elevated in response to their elevated early postpartum P, which could explain their fragmented huddling behaviors. Future studies should examine WKY body temperatures in the early postpartum period to evaluate this possibility.

Another result from the current study is that cFos expression in the mPOA was increased in WKY compared to SD mothers, indicating a differential activation of the mPOA between strains. In addition, dual labeling of these cFos cells with ERα further

suggests that different neuronal populations are recruited in WKY versus SD mothers. In lactating female rats, tactile pup stimulation has been found to increase expression of ERα+ cFos cells in the mPOA (Lonstein et al., 2000). Since WKY mothers spend less time huddling with their young, it follows that they would receive less tactile stimulation and consequently less activation of ERα+ cells within the mPOA. The decreased ERα+ cFos cells in combination with increased cFos+ cells in the WKY mPOA may more generally represent larger scale changes in mPOA representation of somatosensory pup signals and/or corresponding caregiving decisions, which may underly the observed disturbances in parenting in WKY mothers.

The mPOA receives converging sensory information from all sensory modalities (Beach and Jaynes, 1956; Numan and Numan, 1995), and postpartum plasticity in sensory cortices is necessary for appropriate expression of maternal behavior. For example, mother mice have increased neural representation of offspring vocalizations compared to virgin females within the auditory cortex, even before maternal experience with pups (Liu et al., 2006; Schiavo et al., 2020). It is possible that WKY mothers do not have proper representation of pup-related cues within their auditory cortices, resulting in a non-specific, and potentially over-activated, mPOA. With non-specific activation, the communication to behavioral output networks may also be disrupted, which could explain WKY mothers' lack of sensitivity to offspring signals (Winokur et al., 2019; Anderson et al., 2021). Completion of ongoing ICC analysis would verify differential recruitment of neurons in the mPOA, including those containing ERα in WKY and SD mothers. If confirmed, follow up studies should further phenotype these cFos+ cells, by examining their inputs and outputs via cell-type specific circuit tracing strategies.

Taken together, results from the present study suggest that the depression-related parenting deficits observed in WKY mothers are related to disrupted circulating hormones and differential mPOA activation in the early postpartum period. Specifically, we discovered that WKY mothers have elevated early postpartum levels of P, which correlates with their decreased huddling behavior. Trends suggest that ERα+ cells within the mPOA are differentially recruited in early postpartum WKY mothers, which may reflect a lack of proper representation of offspring-related sensory signals, leading to disrupted WKY caregiving responses. The completion of ICC analyses as well further investigation into adrenal gland production of P, allopregnanolone activity, and body temperature, are essential to detail the specific relationship between E2, P, and WKY parenting dysfunction within the early postpartum period.

3.6 Figures

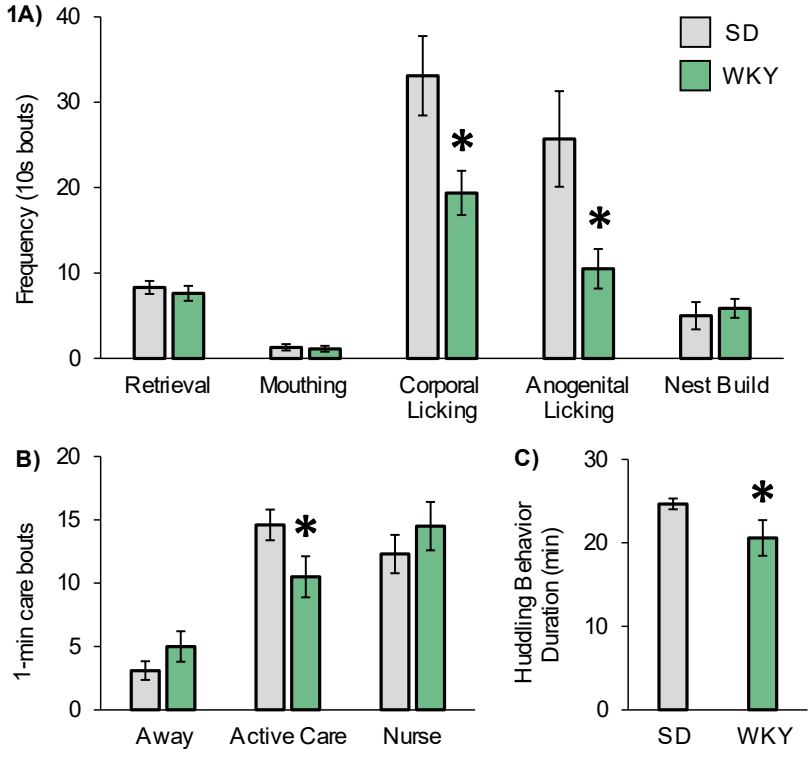


Figure 1. WKY mothers display caregiving deficits. In a 30-min observation of maternal behavior after a 2-hr mother-litter separation, WKY mothers reunited with their young exhibited significantly lower frequencies of corporal and anogenital lickings compared to SD mothers **(A)**. Over the course of the observation, WKY mothers also displayed significantly fewer 1-min active care bouts **(B)**, and spend significantly less time huddling with offspring **(C)**. *$p<0.05$. Bar graphs expressed as mean ± SEM.

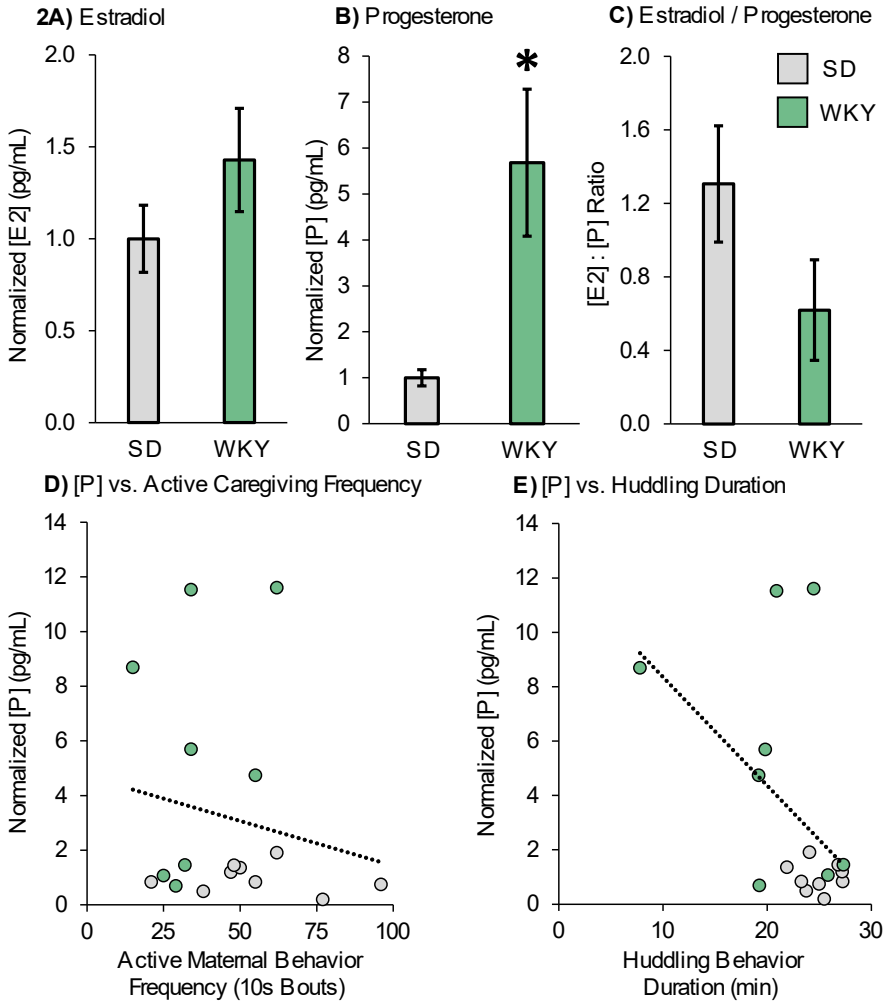


Figure 2. WKY mothers exhibited elevated progesterone concentrations, which negatively correlate with huddling duration. WKY's serum concentrations of E2 **(A)** and P **(B)** were normalized to the average SD serum levels. WKY mothers had significantly elevated P and a trend toward lower E2:P ratio **(C).** P concentrations did not correlate with frequency of active maternal behavior (**D)** but did significantly negatively correlate with the duration of huddling behaviors **(E)**. *$p<0.05$. Bar graphs expressed as mean ± SEM.

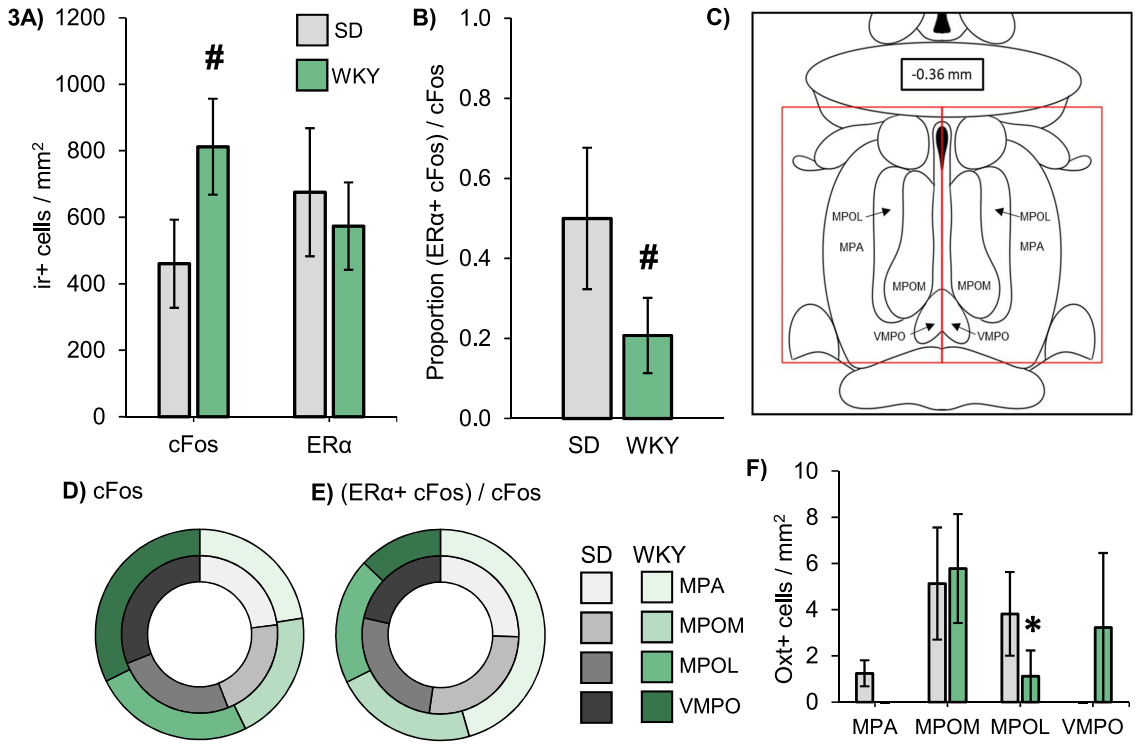


Figure 3. ICC analysis suggests differential mPOA neuronal recruitment in WKY mothers. Within the mPOA, cFos expression trended toward being higher in WKY mothers, whereas ERα expression was not different between strains **(A)**. The proportion of ERα-positive cFos cells out of the population of mPOA cFos-positive cells trended toward being lower in WKY mothers **(B).** MPOL oxytocin (Oxt) was significantly lower in WKY mothers **(F).** Distribution of cFos **(D)** and ERα-positive cFos **(E)** cells did not vary significantly between mPOA subregions**.** Diagram depicts layout of mPOA subregions **(C).** *$p<0.05$, #$p > 0.05$, but less than 0.09. ir+ indicates number of positive immunoreactive cells. Bar graphs expressed as mean ± SEM.

CHAPTER IV

GENETIC NETWORK ANALYSIS REVEALS CANDIDATE GENES WITHIN MESOCORTICOLIMBIC BRAIN REGIONS INVOLVED IN REGULATING DEPRESSION-RELATED MATERNAL BEHAVIOR DEFICITS

4.1 Abstract

Postpartum depression is one of the most prevalent debilitating health problems that affects millions of mothers and their babies worldwide. Depression in new mothers is associated with cognitive and motivational deficits, and more specifically, disturbances in parenting, with life-long adverse outcomes for their children. However, the molecular mechanisms by which postpartum depression impact parenting abilities are currently unclear. The present study aimed to gain novel insight into the molecular basis of depression that most impact parenting. To this aim, we used postpartum Wistar-Kyoto (WKY) rats, who, compared to Sprague-Dawley (SD) controls, demonstrate cognitive, motivational, and parenting deficits representative of postpartum depression symptomatology. Following maternal behavior phenotyping, RNA sequencing (RNAseq) in brain regions that are relevant to depression and parenting, including the nucleus accumbens (NA), the medial prefrontal cortex (mPFC), and the medial preoptic area (mPOA) of SD and WKY early postpartum females was performed to identify expression networks underlying deficits in parenting associated with depressive phenotype. Analysis of trait severity narrowed down the mPOA candidate genes to DEGs mediating severity of parenting disturbances. Bioinformatic analyses utilizing mPOA, NA and mPFC transcriptional profiles suggests networks of co-regulated genes associated with

depressive-like parenting disturbances. Together, results from this study provide important insights into transcriptional mechanism of parenting disturbances associated with postpartum depression and contribute to bettering the understanding of the pathophysiology of depression and its impact on parenting.

4.2 Introduction

Across pregnancy and the postpartum period, the maternal brain undergoes immense neuroplasticity in preparation for its new maternal role. Circulating hormones of pregnancy, including estradiol and progesterone, prime the brain for the onset of maternal behavior (Brunton and Russell, 2010, 2008; Siegel and Rosenblatt, 1978). These hormones induce alterations in gene expression within brain regions critical for maternal caregiving, including the medial preoptic area (mPOA), the medial prefrontal cortex (mPFC), and the nucleus accumbens (NA). In mice, several hundred genes within this maternal brain circuitry have been found to undergo significant changes in expression levels during the transition to motherhood (Gammie et al., 2016; Ray et al., 2016). Understanding the functional role of these maternal-relevant genes is pivotal to understanding how caregiving is established and maintained in new mothers.

Not all mothers exhibit sensitive caregiving abilities. Approximately 17% of mothers are diagnosed with postpartum depression yearly around the world (Wang et al., 2021). Mothers with untreated postpartum depression show deficits in cognition, motivation, and affect, as well as severe disturbances in parenting (Arteche et al., 2011; Dix and Meunier, 2009; Lovejoy et al., 2000). Being raised by a mother with PPD creates

a substantially increased risk for adverse offspring outcomes, including increased vulnerability to depression (Murray et al., 1996; Wachs et al., 2009).

There is a substantial gap in our knowledge regarding the molecular mechanisms by which parenting is compromised by depressive symptoms. While studies have evaluated brain expression of single target genes in the context of caregiving, few have evaluated gene expression within the maternal brain in the context of depression-related caregiving deficits. It is probable that the depressive-like phenotypic features that predict parenting disturbances in new mothers result from altered gene expression in brain loci that are involved in cognitive and motivational processes of parenting. The present study used the Wistar-Kyoto (WKY) rat strain because they exhibit a behavioral phenotype during the postpartum period relative to control Sprague-Dawley (SD) rats, that is reflective of the core symptoms of postpartum depression in human mothers, including cognitive, motivational, and parenting disturbances (Winokur et al., 2019).

This study aimed to gain unbiased insight into all possible transcriptional networks that may be implicated in driving depression-related maternal behavior deficits. I used next-generation RNA-sequencing (RNAseq) methods to examine whether these deficits are associated with altered transcriptome profiles in structures associated with depression and parenting. These regions are the infralimbic cortex (IL) of the mPFC, involved in executive function and cognitive flexibility, the nucleus accumbens shell (NAShell), involved in regulation of effort-related functions, and the medial preoptic area (mPOA), involved in maternal behavior.

This genome-wide analysis provides lists of candidate genes that correlate with behavioral phenotypes of interest. We can begin to understand potential ways in which

identified candidate genes may play a role in altering neurobiological functioning through gene ontology and network level analyses. The approach taken in this study ultimately provides a valuable framework for future analysis into the mechanistic role of identified candidate genes within these neural circuits in the context of caregiving abilities.

4.3 Methods

I. Establishing Maternal Phenotype

4.3.1 – Animals: Age-matched nulliparous SD and WKY females were maintained on a 12-hour light/dark cycle (lights on at 7AM) with ad libitum access to rat chow, sunflower seeds, and water. All mating occurred in-house. Before giving birth, pregnant females were housed in individual cages containing woodchip bedding and shredded paper towels as nest-building material. Postpartum females remained with their pups for 24 h after parturition, without interruption. On postpartum day (PPD) 1 (birth = day 0), litters were culled to four male and four female pups per mother. All behavioral testing occurred during the animals' light cycle. All experiments followed were approved by the UMass Institutional Animal Care and Use Committee.

4.3.2 – Experimental Design: Although both strains are derived from the Wistar strain, SD and WKY likely have differences in their genetic makeup. Genes that are differentially expressed between the two strains may not solely be those genes driving deficits in parenting performance. To address the potential confound of strain differences, the following four groups were used to allow for identification of phenotype-relevant genes: SD (control), SD_{WKY} (SDs raised by WKY mothers), WKY_{LS} (low severity WKY) and WKY_{HS} (high severity WKY). The SD (n=10) and SD_{WKY} (N=9) groups were

composed of SD females who were raised by either SD or WKY mothers, respectively. Each SD_{WKY} female was cross fostered from PPD1 through weaning (PPD25) by a WKY new mother, who raised the SD_{WKY} along with her other WKY offspring. All WKYs (n=19) were raised by WKY mothers. After weaning, SD, SD_{WKY} and WKY females were housed in groups and left undisturbed until mating at ~90 days of age. SD and WKY females were then mated and then evaluated during the postpartum period for their parenting abilities. Following the maternal behavior test, WKY mothers were classified into two groups, characterized by low (WKY_{LS}) or high (WKY_{HS}) severity caregiving deficits. These classifications were determined by a median split of cumulative active caregiving responses as well as total time spent huddling (hovering and nursing) during their maternal behavior test (Fig. 2). The four groups exhibit marked variation in their maternal behaviors, with SD_{WKY} mothers showing deficits in maternal behavior comparable to that of WKY_{LS} mothers (see 4.2.3).

4.3.3 – Maternal Behavior Testing: Early postpartum (PPD 7-8) WKY and SD females were assessed for their maternal behavior toward pups during a 30-min test. Following a 20-min mother-litter separation, pups were scattered in the maternal home cage, and the frequency, duration, and latency of the mothers' caregiving behaviors were recorded continuously for 30-mins as described previously (Winokur et al., 2019). Other behaviors recorded included general exploration (zone crosses and rearings), self-grooming, and eating or drinking. SD_{WKY} maternal behavior toward their own biological SD litter was evaluated in this study.

4.3.4 – Statistical Analysis: Behavioral data is expressed as means ± standard errors of the means (SEM) and was analyzed by between-groups analysis of variance

(ANOVA) with follow-up analysis of significant main effects and interactions when appropriate. In all cases an effect was considered significant if $p<0.05$.

II. Transcriptome Analysis via RNAseq

4.3.5 – Tissue Collection: Behaviorally representative females from each group were selected for RNAseq analysis. Thirty min after the end of the maternal behavior testing, females were decapitated following CO_2 exposure, and their brains removed rapidly and sliced into 1 mm-thick cross sections using a freezing microtome. Bilateral punches were taken from IL cortex, NAShell and the mPOA using 1 mm tissue puncher (Harvard Apparatus BS4 62-0052), with reference to (Paxinos and Watson, 2009) rat brain atlas (Fig.1). Samples were stored in a -80 freezer until further processing.

4.3.6 – RNA Extraction: Total RNA was extracted from tissue samples with TriZol reagent (Invitrogen) and purified using the RNeasy Lipid Tissue Mini Kit (Qiagen 74804), following the kit's protocol. All RNA samples used to prepare libraries had a concentration >100 ng/ul of purified RNA (obtained with Qubit fluorometer), and RIN values >7 (obtained with Agilent Bioanalyzer). Samples were stored in a -80°C freezer.

4.3.7 – Library Preparation: SD and WKY female mPOA, NAShell, and IL libraries were prepared using the TruSeq RNA Library Preparation Kit (Illumina RS-122-2001/2) and were sequenced on an Illumina Hi-seq machine with 50-nt single-end reads in the UMass Amherst IALS core facility Genomics Resource Laboratory.

4.3.8 – RNAseq Alignment: RNAseq reads were aligned to the rat reference genome (Rattus norvegicus UCSC rn5) using and Tophat2 (2.1.0)/Bowtie (2.2.6) software (Trapnell et al., 2009). The average mapping rate was 91.4%.

III. Bioinformatic Analyses

4.3.9 – DESeq2 differential gene expression analysis: Differential gene expression analysis was performed using DESeq2 (V1.1.0) to determine differentially expressed genes (DEGs) within each brain region (Love et al. 2014). DESeq2 analyses determined lists of DEGs in each region, comparing SD vs WKY, SD vs WKY_{HS}, SD vs WKY_{LS}, and SD vs SD_{WKY}.

4.3.10 – Planned Comparisons: Lists of DEGs identified via DESeq2 were compared to each other in order to determine phenotype-related candidate genes (Fig. 3). Those DEGs that overlapped between SD vs SD_{WKY} and SD vs WKY_{HS} represent genes associated with depression-related maternal behavior deficits (referred to as the "parenting deficit" list). DEGs unique to SD vs WKY_{HS} when compared to SD versus WKY_{LS} DEGs represent genes related to severity of depression-related maternal behavior deficits (referred to as the "deficit severity" list). All DEGs evaluated met the criteria of Fold Change (FC) $\geq |1.3|$. DEGs used in the DAVID analysis additionally had an FDR < 0.05.

4.3.11 – General functional enrichment analyses: Functional annotation clustering was conducted using The Database for Annotation Visualization and Integrated Discovery (DAVID) version 6.8 (Huang et al., 2009a, 2009b). Enrichr (Chen et al., 2013; Kuleshov et al., 2016; Xie et al., 2021) was used to determine the enrichment of KEGG pathways. Significant enrichment of a KEGG pathway was considered as an adjusted $p<0.05$. The CDC HuGE Navigator Phenopedia (Yu et al., 2008) was utilized to obtain a list of genes that have been previously related to postpartum depression. A list of genes related to the transition to motherhood created by Gammie et al. (2016) was also utilized.

Simple overlaps between postpartum depression, motherhood transition lists, and DEG sets were generated using Venny v2.1 (Oliveros, 2017-2015). The statistical significance and hypergeometric probability of the overlap between gene sets was performed using the overlap tool on nemates.org/MA/progs/overlap_stats.html. Representations factors (RFs) >1 indicates that more than the number of expected genes overlapped between sets, and RFs <1 indicate that fewer genes overlapped than expected.

4.3.12 – Ranked functional enrichment analysis: Lists of DEGs from each region, unique to our phenotypes of interest and with a log2(FC) of ≥|1.3|, were run using UCSanDiego and the Broad Institute's Gene Set Enrichment Analysis (GSEA) software (Mootha et al., 2003; Subramanian et al., 2005). DEG lists were ranked using log2(fold change) values. All gene sets were run against the C5 gene set, containing 14765 gene sets annotated by the same gene ontology term (C5.all.v7.2.symbols.gmt). C5 gene sets include those that have root gene ontologies (GO) belonging to biological processes, cellular component, or molecular function, as well as human phenotype (HP) ontology (those related to specific human diseases). Each GSEA was run using 1000 permutations, and excluded gene sets larger than 500 or smaller than 5. C5 gene ontology gene sets were considered enriched in our DEG lists if the FDR q-value was <0.05. The normalized enrichment score (NES) indicates the correlations between gene sets and the DEG data. Enriched gene sets with negative NES are those that are downregulated; a positive NES are those sets that are upregulated.

4.3.13 – Multiscale Embedded Gene Co-Expression Network Analysis (MEGENA): MEGENA (Song and Zhang, 2015) was used to construct a gene co-expression network, considering mPOA gene expression from all groups. Detailed

methodology is outlined by Song and Zhang (2015). Briefly, MEGENA first determines gene pairs with a significant correlation (FDR < 0.05) and uses these values to construct a planar fitted network (PFN). Next, multiscale clustering is performed on the PFN, uncovering the hierarchy of parent and child modules. MEGENA then identifies driver genes (highly connected hub genes) within each module. Sunburst plots were created using the R package sunburstR to visualize DEG enrichment and gene ontology processes of individual modules.

4.3.14 – Between-Region Gene Co-Expression Networks: Log2(FC) of DEGs with FC ≥|1.3| were compared between mPOA-IL and mPOA-NAShell to determine the relatedness between DEGs within these regions between groups. Log2(FC) of all genes were correlated between all regions to determine general co-activation of genes between regions and groups. Pearson's correlations were run to determine correlation coefficients of each comparison.

4.4 Results

Analysis of maternal behavior in the early postpartum period (PPD 7-8) during 30-minute maternal behavior tests confirmed our prior published results that WKY mothers exhibit deficits in maternal caregiving compared to SD mothers (Winokur et al., 2019).

I. Active Caregiving

There was a main effect of group for all active components of maternal behavior, with WKY_{HS}, WKY_{LS}, and SD_{WKY} mothers exhibiting deficits in active caregiving compared to SD mothers (Fig. 4A; Retrieval: $F_{(3,34)}$=13.69 p<0.001 η_p^2=0.547; Mouthing: $F_{(3,34)}$=3.57 p=0.024 η_p^2=0.24; Corporal Licking: $F_{(3,34)}$=82.46 p<0.001 η_p^2=0.879;

Anogenital Licking: $F_{(3,34)}$=47.895 p<0.001 η_p^2=0.809; and Nest Building: $F_{(3,34)}$=3.889.46 p=0.017 η_p^2=0.256).

4.4.1 – WKY_{HS} mothers exhibited severe active caregiving deficits. WKY_{HS} mothers displayed significantly lower retrievals, corporal, and anogenital lickings compared to SD mothers (all p<0.001). When analyzing patterns of 1-min caregiving bouts across a 30-min observation, WKY_{HS} mothers were found to spend significantly less time active caregiving compared to SD mothers (p<0.001). Instead of active caregiving or nursing, WKY_{HS} mothers spent the majority of their test time away from their young, significantly contrasting SD mothers, who instead spent the majority of their time providing active care (Away: 26 of 30 vs 7 of 30, p<0.001).

4.4.2 – SD_{WKY} and WKY_{LS} mothers exhibited moderate active caregiving deficits. Both SD_{WKY} and WKY_{LS} mothers displayed significantly lower corporal and anogenital lickings compared to SD mothers (all p<0.001). While SD_{WKY} and WKY_{LS} mothers did show deficient lickings compared to SD, their frequency was not as depleted as that of WKY_{HS} mothers (SD_{WKY} and WKY_{LS} vs WKY_{HS} both p<0.001). Additionally, SD_{WKY} mothers displayed increased retrievals compared to SD mothers (p<0.001).

II. Huddling

There was a main effect of group for the time spent providing care in direct physical contact with their young (Fig. 4B; Hover Over: $F_{(3,34)}$=19.75 p<0.001 η_p^2=0.635; Nursing: $F_{(3,34)}$=12.26 p<0.001 η_p^2=0.520).

4.4.3 - WKY_{HS} mothers spent little time huddling their offspring. WKY_{HS} mothers demonstrated significantly lower hover over and nursing durations compared to SD mothers (p=0.032 and p<0.001, respectively). WKY_{HS} mothers did not nurse their

offspring during the 30-min observation, which significantly contrasted the nursing behavior of all other groups ($p<0.001$).

4.4.4 – SD_{WKY} and WKY_{LS} mothers did not exhibit extensive huddled care deficits. SD_{WKY} and WKY_{LS} mothers showed no significant differences in nursing durations compared to SD mothers. Their nursing durations were also significantly higher than that of WKY_{HS} mothers (both $p<0.001$). WKY_{LS} mothers did exhibit significantly lower hover over durations compared to SD mothers ($p=0.032$), whereas SD_{WKY} mothers did not. Both SD_{WKY} and WKY_{LS} mothers displayed higher hover durations compared to WKY_{HS} mothers (both $p<0.001$).

III. Differential Gene Expression

RNAseq was used to identify candidate genes in SD, SD_{WKY}, WKY_{HS}, and WKY_{LS} that may underlie depression-related caregiving deficits and the severity of those deficits. Sets of relevant DEGs per region of interest can be found in Supplemental Table 1.

4.4.5 – Differences in cell membrane function associated with caregiving deficits. Functional annotation clustering using DAVID (Huang et al., 2009b, 2009a) revealed that, across the mPOA, IL, and NAShell, caregiving deficit-related DEGs are associated with cell membrane function. Of genes related to caregiving deficits, Ltb (FC=2.13, FDR<0.001) and Mocos (FC=-3.14, FDR<0.001) within the mPOA had the largest absolute fold change. Of genes related to severity, Rbp1 (FC=2.60, FDR<0.001) and Pcdha10 (FC=-2.00, FDR<0.001) within the IL had the largest absolute fold change.

4.4.6 – IL deficit severity DEGs associated with neurophysiology-related functions. Functional annotation analysis of IL deficit severity related DEGs via DAVID

suggests that these DEGs are related to synapse function, steroid metabolism, and voltage-gated ion channels (calcium and potassium related).

4.4.7 – Overlap in expression of genes related to postpartum depression and the transition to motherhood. DEG sets were compared to lists of genes that have been previously recognized as being meaningful in postpartum depression or the transition into motherhood. Interestingly, significant overlap was found between deficit severity-related DEG sets and motherhood transition-related genes in the IL ($p=0.003$, RF=1.3) and NAShell ($p<0.0001$, RF=2.1). IL deficit-related genes were also significantly related to motherhood transition-related genes ($p=0.039$, RF=3.1). Only the mPOA deficit severity-related DEG set significantly overlapped with postpartum depression-related genes ($p=0.037$, RF=6.5).

4.4.8 – Functional Annotation Clustering of DEGs via GSEA and KEGG enrichment analyses. To further characterize the observed DEG lists, we examined enrichment of KEGG and gene ontologies. GSEA was conducted on all DEG lists against gene ontology (GO) sets (C5.all.v7.2.symbols.gmt) to determine which functional gene groups are significantly (FDR q-val < 0.05) enriched in each region for both lists of DEGs (See Supplemental Table 2 for all GSEA results). The GSEA considers the rank in which DEGs fall in a list using log2(FC) as the rank indicator, which allows the analysis to output the up or down regulation of each cluster. The KEGG enrichment analysis was run against all DEGs from both lists of DEGs within all three regions (See Supplemental Table 3 for all KEGG results). This analysis reveals significant enrichment (adjusted $p<0.05$) of pathways that represent knowledge of molecular interaction, reaction, and relation

networks, and it does not consider rank. Together, the results below begin to reveal the functional relationships between DEGs.

mPOA: KEGG enrichment analysis for the severity mPOA DEG list revealed neuroactive ligand-receptor interaction as the only significantly enriched pathway (adjusted p=0.007). This pathway included notable genes such as muscarinic acetylcholine receptors 2 and 3, 5-hydroxytryptamine receptor 1A (5-HT1A), a subregion of the $GABA_A$ receptor, and oxytocin.

IL: 152 GO clusters were significantly enriched for IL deficit severity-related DEGs. Ion and cation transport, vesicular transport, regulation of morphogenesis, axon development, and potassium channel activity were the most common themes among enriched GO sets. Genes enriched in these sets were primarily downregulated. KEGG enrichment analysis for IL severity DEGs revealed 40 significantly enriched pathways. Of note are the calcium signaling; neuroactive ligand-receptor interaction; axon guidance; glutamatergic, cholinergic, and GABAergic synapses; and cocaine and morphine addiction pathways.

NAShell: 12 GO clusters were significantly enriched for NAShell severity related DEGs. Of these enriched GO sets, synapse signaling is the most common theme. Genes enriched in these sets were downregulated. KEGG enrichment analysis for the NAShell severity DEGs revealed 48 significantly enriched pathways. Of note are the oxytocin, calcium, cGMP-PKG, and MAPK signaling; serotonergic synapse; and long-term potentiation pathways.

4.4.9 – Co-expression analysis reveals mPOA gene networks underlying caregiving deficits. While functional enrichment analyses provide a useful window into

the role of DEGs, we utilized a multi-scale embedded gene co-expression network analysis (MEGENA) to determine the broad scale correlations and relatedness of all genes, considering their raw (rather than relative) expression (Fig. 5). The mPOA was focused upon for its central role in coordinating maternal behaviors. Utilizing this transcriptome-wide co-expression analysis, we identified 294 parent modules containing 660 child modules. Module parent p1 was the largest parent module, containing 33 children modules. Within p1, child module sizes ranged from 39 to 2418 nodes. The largest child module, c22, contained 139 hub genes with significantly enriched KEGG pathways for mRNA surveillance (PPP1CB, UPF2, PPP2CA, PNN, PPP2R1A, UPF3B; adjusted p=0.01), AMPK signaling (RAB2A, PPP2CA, RAB10, PPP2R1A, RPS6KB2, PIK3R3; adjusted p=0.02), and dopaminergic synapses (PPP1CB, PPP2CA, ATF6B, PPP2R1A, GNB2, GNAI3; adjusted p=0.02). The hub gene with the largest number of connections (34 total) in c22 was PNPT1, encoding for an enzyme involved in import of RNA into the mitochondria (Vedrenee et al., 2012). PNPT1 was not significantly differently expressed between groups or regions. Oxytocin (OXT) and arginine vasopressin (AVP) were identified as hub genes within parent module p16, having 9 and 13 connections, respectively. AVP was also a p1 hub gene, with 21 connections. AVP expression did not significantly differ between groups or regions. OXT expression between regions and groups was approaching a significant interaction ($F_{(6,40)}$=2.287, p=0.054, η_p^2=0.55). Within the mPOA, WKY_{HS} mothers had significantly higher OXT expression compared to that of SD (p<0.001), SD_{WKY} (p<0.001), and WKY_{LS} (p=0.041) mothers. WKY_{LS} mothers showed significantly higher mPOA OXT expression compared to SD (p=0.027) and SD_{WKY} (p=0.029) mothers.

4.4.10 – Co-activation between the mPOA, IL, and NAShell. To explore transcriptional synchrony, we compared gene lists between pairs of brain regions. First, Pearson's correlations between the Log2(FC) of all genes were evaluated between all regions and all groups in order to determine whether brain regions per group show coordinating activity. There were significant positive correlations between all regions, across all groups ($p<0.05–0.001$, depending on comparison). Thus, there was a baseline co-activation between networks, suggesting expected connectivity between regions. Following this, we looked for co-activation between brain regions based on parenting deficit and deficit severity lists between the mPOA and IL (cognition-related maternal circuitry), and the mPOA and NAShell (effort-related maternal circuitry). There were significant correlations between regions for both DEG sets. The expression of parenting deficit- ($r=0.473$, $p<0.001$, $N=97$) and severity- ($r=0.800$, $p<0.001$, $N=35$) related genes was significantly correlated between the mPOA and NAShell. Parenting deficit- ($r=0.765$, $p<0.001$, $N=41$) and severity- ($r=0.629$, $p<0.001$, $N=284$) related gene expression was also significantly correlated between the mPOA and the IL. Of note, gene ontology analysis of concordant (similarly up- or down-regulated) fold change revealed that synapse assembly is significantly represented ($p<0.001$) for co-upregulated severity-related DEGs (CDH1; RYK; SLITRK6; PCDHB5; PCDHB9) between the mPOA and IL. Additionally, between the mPOA and NAShell, concordant up-regulated parenting deficit-related DEGs were significantly related to response to axon injury (P2RY12; SOD1; $p<0.00001$).

4.5 Discussion

This study utilized of the natural variation of maternal behavior phenotypes within and between rat strains along with next-generation full transcriptome sequencing of key maternal brain regions to identify candidate key drivers of depression-related maternal behavior deficits, and the severity of those deficits. We discovered numerous candidate genes and biological processes of interest which ultimately serve as an extensive basis for future investigation into the neurobiology of depression-related caregiving deficits.

In this study, we built upon our previous phenotyping of the WKY rat model of depression (Winokur et al., 2019) by characterizing the naturally occurring caregiving variance observed in WKY mothers. While WKY mothers show significantly decreased maternal responsiveness overall, WKY_{HS} mothers display the greatest disruption to their caregiving abilities compared to SD mothers. There is a substantial amount of natural variation in caregiving behaviors from individual mother to mother. This can be seen in humans (Klahr and Burt, 2014; Power, 2013) as well as rats (Champagne et al., 2003b). Similarly, depressive disorders house within them several endophenotypes (Goldstein et al., 2014; Webb et al., 2016). Behavioral diversity is challenging to represent in animal research, where we optimize our models by inbreeding and selecting for robust behavioral phenotypes. The use of WKY_{HS} and SD_{WKY} mothers in this study serves to recognize that there is variation in the caregiving deficits associated with depression, and that these variations may derive from different genetic roots. Thus, in addition to providing a useful control for potential strain-related differences, these groups nicely represent the spectrum of caregiving that one would expect to see in human mothers with depression-related caregiving deficits.

Broadly, this study determined that many genes are differentially expressed within the maternal brain of mothers with depression-related caregiving deficits. We focused on the IL, NA, and the mPOA because of their known role in cognition, motivation, and parenting (Pereira and Ferreira, 2015). Together, our analysis revealed that differences in gene expression within key maternal brain regions is associated with depression-related caregiving deficits, as well as the severity of those deficits.

OXT was one gene that consistently was found to be relevant to caregiving deficits within the mPOA and NAShell. Oxytocin expression upregulation was associated with caregiving deficits, which contrasts its recognized positive role in maternal behavior. Our results in the mPOA are consistent with a recently published study using a related animal model of depression (Luo et al., 2020). Oxytocin signaling has been shown to be important for social interactions and maternal behavior (Bridges, 2015). Increased expression of oxytocin receptors in the mPOA has been shown to correlate with high pup licking, and administration of an oxytocin receptor antagonist into the mPOA reduced such high levels of pup licking (Champagne et al., 2001). Future studies should experimentally validate the role of oxytocin in the mPOA and NAShell in depression-related caregiving deficits.

Additional hub genes identified in the MEGENA analysis are also of interest to examine further. Of particular interest or those genes related to dopaminergic signaling (PPP1CB, PPP2CA, ATF6B, PPP2R1A, GNB2, and GNAI3), as dopamine activity has been shown by our lab to be disrupted in WKY mothers (Winokur et al., 2019). Future research could evaluate the role of these genes in depression-related caregiving deficits through a combination of CRISPR gene deletion or upregulation via small activating RNAs, followed by behavioral analyses.

While all brain regions showed differential gene expression, the IL appeared to be the most changed based on deficit severity. The IL is involved in cognitive processes key to parenting, and functional inactivation of the IL in SD mothers severely disrupts organizational aspects of caregiving behavior (Pereira and Morrell, 2020), recapitulating aspects of WKY deficits. The identified GO biological processes and KEGG pathways were related to synaptic transmission, including synaptic signaling and neuroactive ligand-receptor interaction. These processes and pathways influence synaptic maturation and maintenance of synapses. Decreased expression of synapse-related genes and loss of synapses have been reported in the prefrontal cortex of depressed patients (Kang et al., 2012). Future research should evaluate synaptic physiology within the IL to determine whether this may be drive aspects of depression-related maternal behavior deficits.

In conclusion, we identified candidate genes and processes that are altered in key maternal brain regions in association with depression-related caregiving deficits. These results further support a key role for the mPOA, IL, and NAShell network dysfunction in caregiving deficits. Furthermore, this study provides an extensive foundation to further explore how depression influences maternal behavior at the molecular level.

4.6 Figures

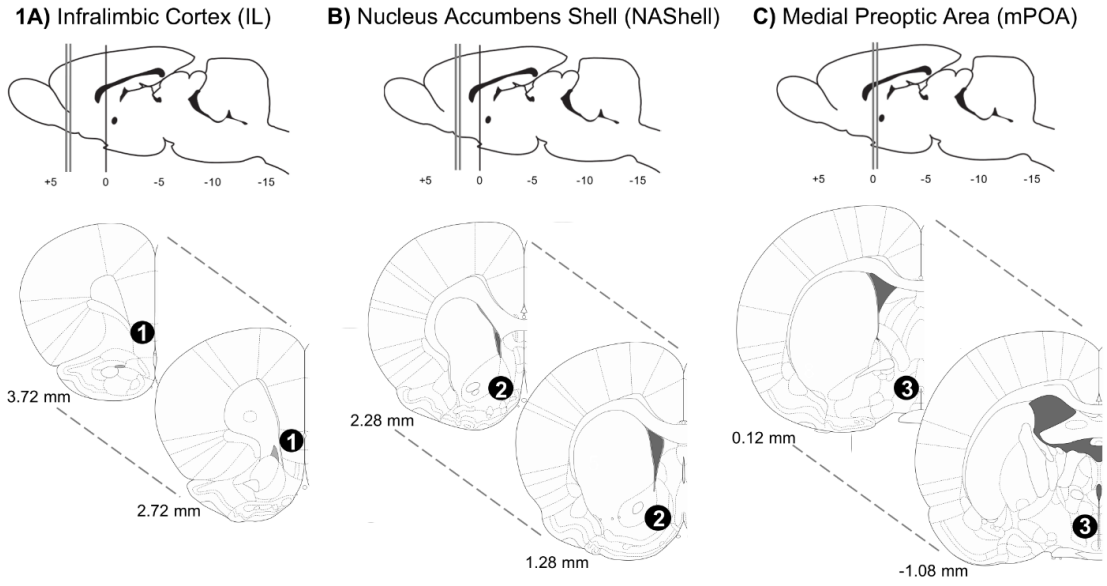


Figure 1. Tissue used for RNAseq analysis. Fresh frozen brains were extracted after maternal behavior testing and sliced into 1mm-thick cross sections using a freezing microtome. Bilateral 1mm tissue punches were taken from IL cortex (1A), NAShell (1B), and the mPOA (1C). Anatomy was determined with reference to the rat brain atlas (Paxinos and Watson, 2009).

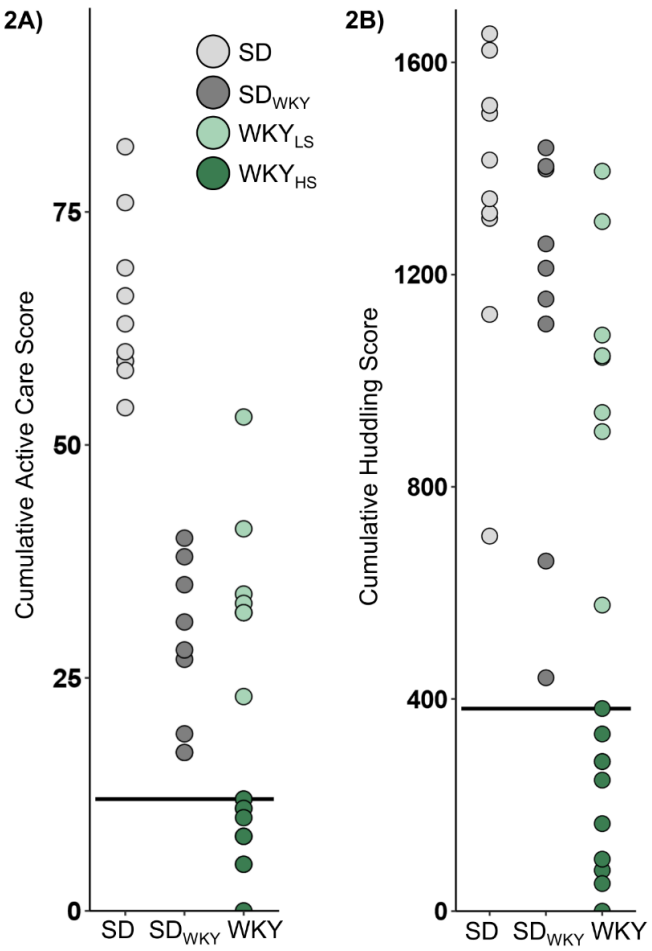


Figure 2. Median split of maternal behavior. A median split of cumulative active care score (anogenital and corporal licking frequency; 2A) as well as cumulative huddling score (total duration hovering plus duration nursing, in seconds; 2B) was utilized to determine caregiving deficit phenotypes of WKY mothers. Those WKY mothers who fell at or below the median scores (black line) were labeled as high severity (WKY_{HS}), and those who fell above the median were labeled as low severity mothers (WKY_{LS}).

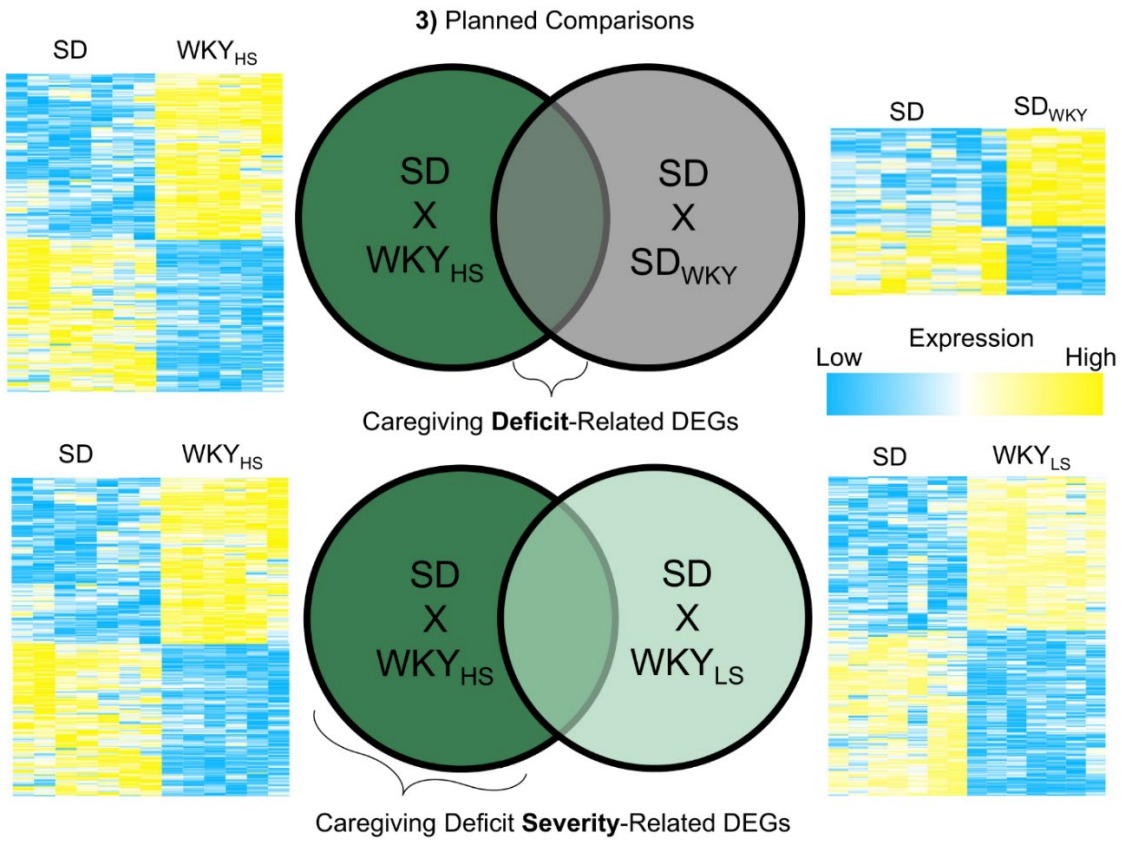
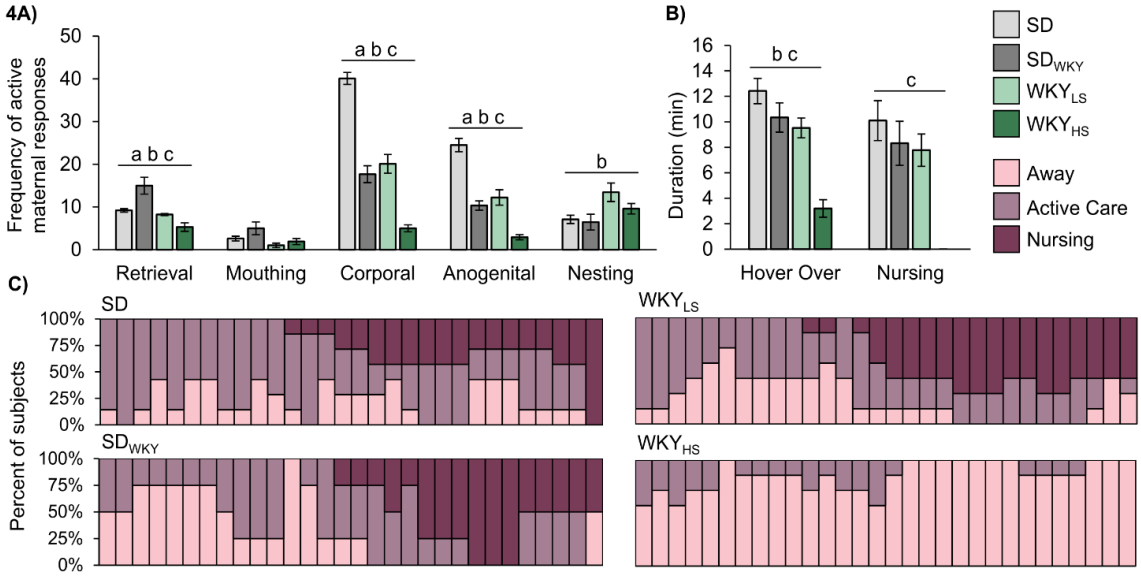


Figure 3. Planned comparisons for RNAseq analysis. In order to determine which genes may underlie caregiving deficits as well as the severity of those deficits, lists of DEGs identified in DESeq2 were compared to each other. Overlap between SD x WKY_{HS} DEGs and SD x SD_{WKY} DEGs represent genes associated with depression-related caregiving. DEGs unique to SD x WKY_{HS} when compared to SD x WKY_{LS} DEGs represent genes related to severity of depression-related maternal behavior deficits. Representative heat maps of DEGs from DESeq2 analyses are shown above to illustrate the large number of DEGs that each comparison is drawing from. Each column represents one subjects, and every row is an individual DEG. Color represents whether the DEG has relatively higher or lower expression in the respective comparisons.


4A)
Frequency of active maternal responses
50
40
30
20
10
0
a b c
a b c
a b c
b
Retrieval
Mouthing
Corporal
Anogenital
Nesting
B)
Duration (min)
16
14
12
10
8
6
4
2
0
b c
c
Hover Over
Nursing
SD
SDWKY
WKYLS
WKYHS
Away
Active Care
Nursing
C)
Percent of subjects
SD
WKYLS
SDWKY
WKYHS
100%
75%
50%
25%
0%

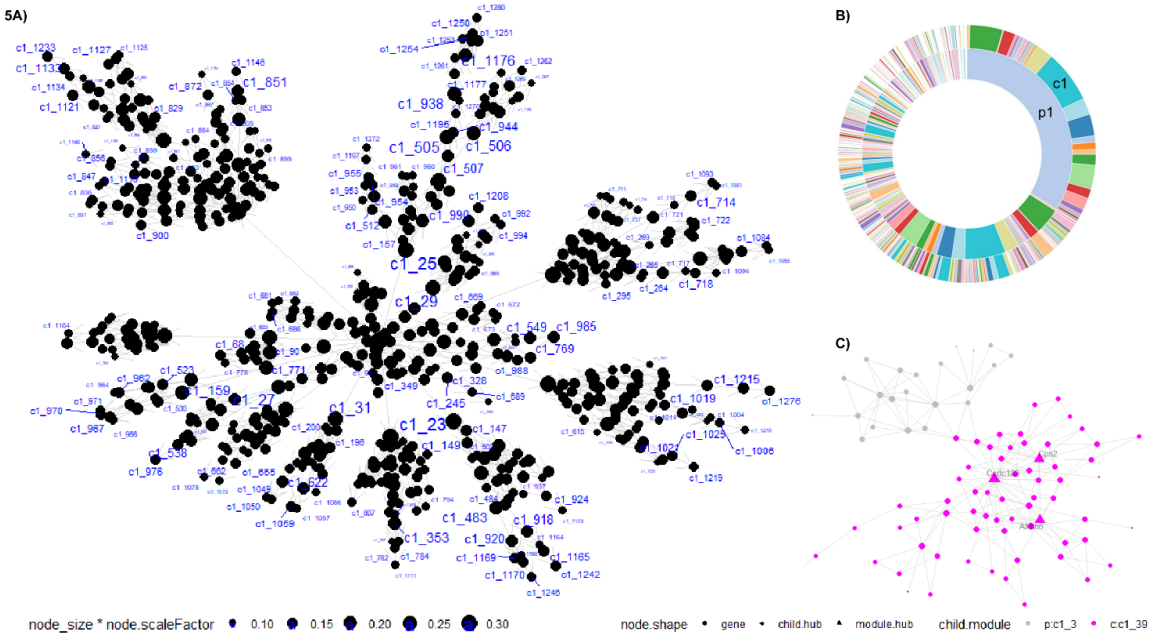
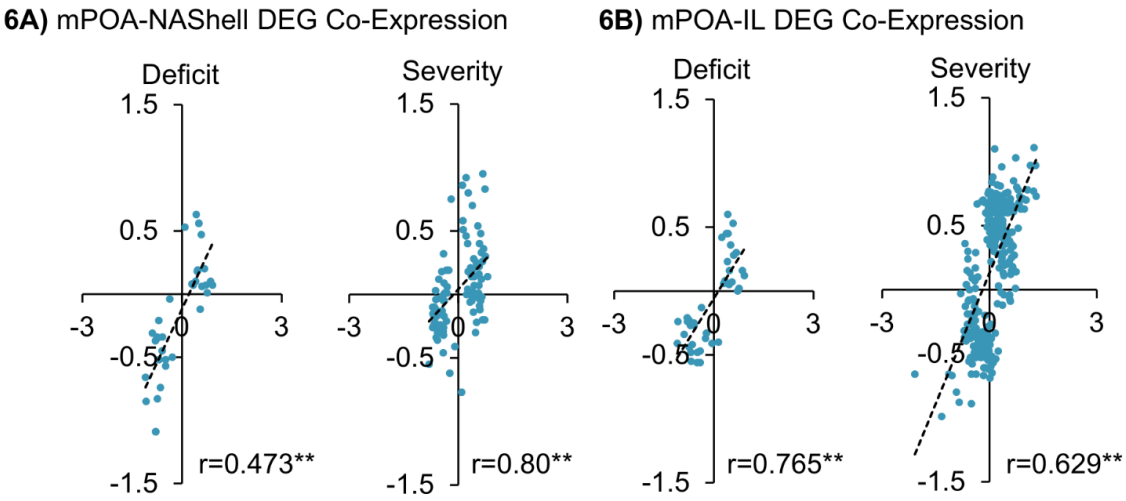


Figure 5. MEGENA analysis of gene co-expression. MEGENA analysis revealed 294 parent modules containing 660 child modules related to caregiving deficits **(A).** Of these modules, parent module p1 and child module c1 were the largest **(B).** Example gene network layout and relatedness between hubs, parent, and child modules is illustrated in **C**.


6A) mPOA-NAShell DEG Co-Expression
Deficit
Severity
1.5
0.5
-0.5
-1.5
-3
0
3
r=0.473**
r=0.80**
6B) mPOA-IL DEG Co-Expression
Deficit
Severity
r=0.765**
r=0.629**

CHAPTER V

CONCLUSION

In this dissertation, I used an array of molecular, neurochemical, and behavioral tools to examine the neurobiology of depression-related maternal behavior deficits in WKY rats. Findings from these experiments provide a new perspective on how cognitive and motivational components of caregiving behavior may be neurobiologically disrupted in mothers with depression.

To this aim, I compared the neurobiology and maternal behavior phenotypes of WKY postpartum rats, an animal model of depression-like behavior, relative to SD control rats. Through extensively characterizing the maternal phenotype of WKY rats, I determined that WKY mothers show decreased maternal responsiveness and disorganized patterns of caregiving. More specifically, WKY mothers are abrupt and disorganized during interactions with their offspring, show minimal active caregiving behaviors, and spend less time spent nursing and huddling their young. In addition, WKY mothers exhibit less change in their caregiving behavior across the postpartum period. However, they do provide sufficient caregiving and their offspring develop and reach milestones normally. These patterns of disrupted caregiving behaviors in WKY mothers are similar to that of human mothers suffering from postpartum depression (Barrett and Fleming, 2011; Lovejoy et al., 2000)

I further demonstrated that not all WKY mothers behave the same. WKY mothers show significant maternal behavior deficits, as illustrated across all chapters of my dissertation. However, a subset of WKY mothers display caregiving deficits that are much

more severe than others. The group of WKY mothers that showed the highest severity caregiving deficits (WKY_{HS}) spent the majority of the maternal behavior test time away from their young, and any caregiving that they displayed took place in short sporadic bouts. Identifying this high severity WKY group allowed for important within-strain comparisons to determine not only candidate genes underlying depression-related maternal behavior deficits, but also what may neurobiologically drive the severity of such deficits. This work began based on the behavioral face validity of the WKY rat strain as a model that shows robust depression-like behavior. Via deep examination, I identified that WKY mothers show deficits in caregiving that mirrors that of human mothers with depression, further validating its use as a model of depression-related caregiving deficits.

The results from this study suggest that depression-related maternal behavior deficits may stem from disrupted hormonal signaling, either at parturition or shortly thereafter. Specifically, I found that depression-related caregiving deficits are associated with elevated circulating progesterone levels and differential activation of mPOA cells (Chapter III). In addition, I determined that mesocorticolimbic monoamine neurotransmitter levels are disrupted in WKY mothers in the transition to and across the postpartum period (Chapter II). Finally, I demonstrated that gene expression profiles differ across the mPOA, IL, and NAShell in mothers with depression-related caregiving deficits (Chapter IV). Furthermore, I identified several candidate genes that may underlie the depression-related dysfunction of maternal cognitive, motivational, and parenting processes (Chapter IV).

One of the most striking findings from these studies was that progesterone levels were greatly elevated in WKY mothers in the early postpartum period. Progesterone and

allopregnanolone can act as modulators for monoaminergic systems. For example, progesterone (along with estradiol) has been found to increase cellular resilience and decrease cell death of serotonin neurons in macaques (Bethea et al., 2009). Furthermore, progesterone seems to have a bimodal faciliatory followed by inhibitory impact on dopaminergic neuron activity within the rat striatum (Dluzen and Ramirez, 1984). Progesterone treatment in female rats was also found to elevate hypothalamic noradrenaline turnover and decrease hypothalamic serotonin concentrations (Renner et al., 1987) Together, this suggests that the elevated WKY progesterone levels may disrupt typical monoamine activity, synthesis, and metabolism. This is supported by Chapter II, in which I found that mesocorticolimbic monoamine concentrations and turnover ratios were largely different in WKY mothers in the early postpartum period compared to SD mothers.

It is important to note that the conclusions drawn across this dissertation are largely correlational. To determine whether these findings have causal relationships with WKY caregiving deficits, several follow-up studies should be conducted. One avenue of exploration would be to determine whether actions of elevated progesterone in the brains of WKY mothers underlie the behavioral differences. The Pereira lab is currently preparing to conduct a follow-up IHC analysis to determine the expression and activation of progesterone receptors in the WKY brain. Alternatively, the effects of postpartum ovariectomy and/or administration of progesterone antagonists (antiprogestins) or progesterone receptor modulators (PRMs) on maternal behavior of WKY mothers could be examined to determine if WKY caregiving deficits could be ameliorated by decreasing brain progesterone activity. Prior research from the Pereira lab has determined that activation of the entire mPOA seems to ameliorate WKY maternal behavior deficits

(Anderson et al. 2021). This knowledge combined with the newfound notion that WKY mothers have differential mPOA activation of ERα positive cells leads nicely to another important follow-up study. Selectively activating ERα positive cells using chemogenetic or optogenetic tools within the mPOA would reveal whether we see similar amelioration of maternal behavior deficits. If these cells are found to be functionally relevant, we could further determine their input and output through circuit tracing using cell-type-specific viral tracing strategies. This approach has similarly been used to determine the role of mPOA galanin neurons in parental behavior (Wu et al., 2014).

In sum, this dissertation research revealed that depression-related caregiving deficits are associated with large-scale dysfunction of the maternal mesocorticolimbic circuitry. This research has opened many avenues to investigate further in order to paint a picture for how impairments in cognitive and motivational processes, as seen in depression, neurobiologically may disrupt maternal caregiving behaviors. This work is a pivotal steppingstone on the path toward better understanding maternal behavior, the maternal brain, and neuropsychiatric disorders that disrupt parenting. Future studies should aim to determine the mechanisms by which these factors work together to influence caregiving, and how they become disrupted in mothers with depression. It is critical to continue research in this field so that the scientific community can come together to support mothers who are struggling with neuropsychiatric disorders - ultimately bettering the health of mothers and their families.

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