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Effects of maternal immune activation and repeated maternal separation on postpartum behaviors in the female rat offspring

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EFFECTS OF MATERNAL IMMUNE ACTIVATION AND REPEATED MATERNAL SEPARATION ON POSTPARTUM BEHAVIORS IN THE FEMALE RAT OFFSPRING

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Early life stress can induce persistent brain and behavioral alterations. As a lifetime history of clinical symptoms similar to those caused by early adversities may predict postpartum dysfunctions, these stressors likely contribute to their etiology. Postpartum neuropsychiatric disorders (e.g. postpartum depression, anxiety and depression) are costly, yet due to the complex neuronal reorganization during this period, insights into how early adversities-induced CNS functional changes affect postpartum processes remain limited, especially under multiple stressors. Thus, there is a need to determine postpartum functions altered by early stress, in order to increase understandings of risks associated with postpartum maladaptations. Accordingly, this work was designed to assess early stress-induced behavioral and neuronal changes in postpartum female rats, using pre- and postnatal stressors independently and concurrently. We hypothesized that pre- and/or postnatal insults would disrupt postpartum cognitive and affective regulations, maternal behaviors, and neuronal functions. Females exposed to maternal immune activation (MIA) *in utero* and/or repeated maternal separation (RMS) in the early postnatal period were assessed for maternal performance in postpartum. Prepulse inhibition (PPI) of acoustic startle

response (ASR), forced swim test (FST), sucrose preference, fear potentiated startle (FPS), and conditioned avoidance response (CAR) were also tested in both dams and virgin littermates to assess various psychological functions. In neuronal functions, c-Fos expression following FPS, and amphetamine-, phencyclidine- (PCP), nicotine-, and 2,5-dimethoxy-4-iodoamphetamine-induced hyperlocomotion were examined. Results show that MIA reduced nest building in mother rats, as well as their PPI and CAR performance. MIA also increased dorsal medial preoptic area and dorsal periaqueductal grey c-Fos following FPS. In addition, virgin offspring exposed to MIA also showed reduced struggling behavior in the FST and increased basolateral and medial amygdala c-Fos following FPS. RMS reduced nest building, ASR, FPS, and amphetamine- and PCP-induced hyperlocomotion, and increased dentate gyrus c-Fos following FPS. MIA and RMS were antagonistic in maternal behaviors and ASR, and otherwise showed little interactive effects. Overall, these results indicate that early environmental stressors could have long-term impacts on postpartum functions, including maternal behavior and performances in various behavioral tests. This impact is also influenced by reproductive experiences.

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LIST OF ABBREVIATIONS

5-HIAAA	5-hydroxyindoleacetic acid
5HT	Serotonin
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ASR	Acoustic startle response
BLA	Basolateral amygdala
BSTd	Bed nucleus of stria terminalis, dorsal portion
BSTv	Bed nucleus of stria terminalis, ventral portion
CAR	Conditioned avoidance response
CeA	Central amygdala
Cg	Cingulate cortex
CRF	Corticotropine releasing factor
CS	Conditioned stimulus
CS-	Conditioned stimulus present
CS+	Conditioned stimulus absent
Ctrl	Control
DA	Dopamine
DG	Dentate gyrus
DLSt	Dorsolateral striatum
DMH	Dorsomedial hypothalamus
DMSt	Dorsomedial striatum
DR	Dorsal raphe

EPM	Elevated plus maze
F0	Parent generation
F1	Offspring generation
FPS	Fear potentiated startle
FST	Forced swim test
G	Gestation day
GABA	Gamma-aminobutyric acid
h	Hour(s)
IES	Inescapable shock
IL-	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
IR	Immunoreactive/immunoreactivity
LG-ABN	Lick/groom-arched back nursing behavior
LH	Lateral habenula
LI	Latent inhibition
LS	Lateral septum
LSD	Least significant difference
MeA	Medial amygdala
MIA	Maternal immune activation
min	Minute(s)
mmd	Minimum mean difference
mPFC	Medial prefrontal cortex
mPOAd	Medial preoptic area, dorsal portion

mPOAv	Medial preoptic area, ventral portion
mRNA	Messenger ribonucleic acid
ms	Millisecond(s)
MS	Maternal separation
MS	Medial septum
NAc	Nucleus accumbens core
NAs	Nucleus accumbens shell
NES	Novel environmental stress
NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
NaOH	Sodium hydroxide
OFT	Open field test
PAGd	Periaqueductal grey, dorsal portion
PAGv	Periaqueductal grey, ventral portion
PBS	Phostphate buffered saline
PCP	Phencyclidine
PFA	Paraformaldehyde
PND	Postnatal day(s)
polyI:C	Polyinosinic:polycytidylic acid
PPD	Postpartum day(s)
PPI	Prepulse inhibition
PV	Paraventricular nucleus of thalamus
RMS	Repeated maternal separation
RS	Restraint stress

s	Second(s)
s.c.	Subcutaneous
SAL	Saline
SEM	Standard error of mean
SPT	Sucrose preference test
US	Unconditioned stimulus
v.	Versus
VMH	Ventromedial hypothalamus
VMSt	Ventromedial striatum
VTA	Ventral tegmental area

CHAPTER 1

INTRODUCTION

Preface

It is well known that early life environmental stress can result in persistent brain and behavioral alterations (Teicher *et al*, 2003). Literature clearly establishes significant impairments in cognitive and emotional processes, as well as neuronal functions, in adults exposed to early life adversities (Maccari *et al*, 2014). In particular, many studies have elucidated the impacts of early environmental stress on the mental and physical conditions of adult females (Davis and Pfaff, 2014). However, so far little work has focused on how these changes affect the conditions in postpartum females. For example, it is unclear whether early environmental insults can cause distinct deficits in motherhood through interactions with natural changes associated with the reproductive processes. In addition, whether postpartum behaviors are disrupted by the early stress-induced cognitive and affective alterations evident in adult females also remain uncertain.

The postpartum period is characterized by dramatic changes at the molecular, cellular and behavioral levels to prepare females for motherhood (Brunton and Russell, 2008). If these adaptations are disrupted by environmental factors, proper postpartum cognitive, affective and brain functions may be compromised, resulting in postpartum neuropsychiatric disorders. Approximately 5–12% of mothers display postpartum

anxiety (Andersson *et al*, 2006), 5-25% postpartum depression (Beck, 2006), and 0.1% postpartum psychosis (Jones *et al*, 2008). Postpartum dysfunctions may result not only in reduced individual productivity, but also heavy societal burden (Homish *et al*, 2004). Most critically, these symptoms may lead to deficient maternal care (Arteche *et al*, 2011; Murray *et al*, 2010). This can potentially expose offspring to stressful environments during infancy, extending the transgenerational effect of adverse early life experiences (Champagne, 2008; Meaney, 2001).

Amongst various predictors of postpartum disturbances, such as poor social support (Milgrom *et al*, 2008), stressful life events and low socioeconomic status (O'Hara and Swain, 1996), the single largest risk factor is a lifetime history of clinical symptoms (Harlow *et al*, 2007; Sit and Wisner, 2009). Consequently, if early environmental stressors induce long-term affective and cognitive dysregulations before pregnancy, it is possible that they may also contribute to postpartum abnormalities.

Despite these known detrimental effects of stress-induced maladaptive brain and behavioral changes in motherhood, due to the complex neuronal reorganization during this period, insight into how early stress-induced deficits affect postpartum brain and psychological functions remains limited. Thus, there is a need to determine postpartum functions altered by early stress, in order to increase understandings of risks associated with postpartum maladaptations. Here, we examined the role of stressful conditions during early life (both prenatal and postnatal) in postpartum functions, potentially

contributing insights into the underlying pathology associated with irregular postpartum processes.

The remainder of this chapter provides additional background information regarding consequences associated with early environmental traumas, as well as current knowledge of healthy and abnormal postpartum adaptations. The subsequent chapters detail the experimental procedures and results, providing further rationales, aims, and hypotheses within each section. Finally, a chapter discussing implications of the major findings and potential future directions concludes this dissertation.

Early life stress negatively affects behavioral development and may alter maternal behaviors

Stress during the perinatal period (humans: 20-22 weeks of gestation to 7-28 days after birth [Nguyen and Wilcox, 2005]) produces various behavioral and physiological impairments evident in adulthood. For example, a history of child abuse is associated with lower basal cortisol levels, increased stress-induced adrenocorticotropic hormone, and blunted stress responsiveness in adults (Carpenter *et al*, 2007; Heim *et al*, 2008). However, as early trauma varies greatly across human subjects, animal studies offer the advantage of isolating specific variables.

Rodent models of early insults may be broadly categorized as either prenatal or postnatal. Prenatal stressors are those experienced *in utero*, and include gestational social and environment stress (i.e. gestational corticosterone administration, social intruder,

physical restraint), nutritional deficiency, obstetrics complications, and immune activation (Meyer and Feldon, 2010). Conversely, early postnatal stressors are those experienced immediately following birth, mainly through poor maternal care via postpartum stress of the mother (i.e. postpartum corticosterone administration, social intruders, chronic mild stress, maternal separation [Maccari *et al*, 201]). In general, early environment adversities in rodents have been shown to cause alterations in learning and memory (Kosten *et al*, 2012), attentional processing (Wolff and Bilkey, 2008), emotional regulation (Gleason *et al*, 2011; Heim *et al*, 2008), as well as neuronal changes such as reduced hippocampal volume (Schmitz *et al*, 2002), impaired neurogenesis (Korosi *et al*, 2012), and increased corticosterone response to stress (Aisa *et al*, 2007). Consequently, if early environmental stress induces long-term affective and cognitive dysregulations, this may lead to abnormal postpartum behaviors. However, while many impacts of early life adversities on adult virgin females are known (Davis and Pfaff, 2014), few have investigated the outcomes for females during the postpartum period. Because of the unique behavioral and neuronal changes that occur during the postpartum period, it is unclear whether early stressors cause distinct deficits in maternal behavior. To examine this, we must first understand normal postpartum adaptations.

Rodent mothers (dams) display robust maternal behaviors

In order to examine maternal behaviors in rodents, a brief overview of the most archetypal displays is provided. Rodent dams exhibit robust maternal behaviors upon

parturition (Myers *et al*, 1989), and represent ideal subjects for understanding the transition to motherhood. This is especially dramatic as virgin rat females are naturally aversive to pups (Meaney, 2001). Similar to humans, maternal responsiveness is primed through sex hormonal changes and maintained through offspring interactions (Fleming and Corter, 1988). In general, the most recognizable maternal behaviors that rodents spontaneously execute may be characterized as follows: 1) nest building – construction of a nesting area for pups through gathering of bedding and nesting materials; 2) pup retrieval – using the mouth of pick up a pup that is away from the nest area and returning it to the litter; 3) pup licking and grooming – as new born pups cannot defecate spontaneously, dams provide this vital function through licking of a pup's anogenital area to stimulate reflexive urination and defecation (Brouette-Lahlou *et al*, 1992); and 4) nursing. Nursing behavior may be further differentiated by the energy expenditure required (Myers *et al*, 1989). In passive nursing, mothers are simply present within the nest area, sometimes resting on her side, allowing pups to freely latch on for suckling. A slightly more active form is "blanket nursing," where a mother situates herself atop the entire litter. The most proactive behavior, termed "arched back nursing," demands much more energy expenditure as a mother displays kyphosis, arching over the litter with her legs stretched to ensure ample space under her abdomen for pups.

Maternal behaviors are controlled by the hypothalamic and limbic circuitry

Aspects of brain circuitry, transmitter function and neuroendocrine control related to maternal behavior have been identified through rodent studies (Brunton and Russell, 2008). The medial preoptic area (mPOA) of the hypothalamus is by far the most crucial brain area for maternal behavior. To this extent, Numan has contributed a large body of literature to our understanding of the mPOA's role in maternal behavior. In some of the earliest work, Numan and colleagues showed that not only was the mPOA required for the onset of maternal behavior, but its function is dependent upon the actions of estrogen. Using hysterectomized and ovariectomized rats, researchers showed that estradiol infusions into the mPOA significantly improved maternal behavior in pup retrieval (Numan *et al*, 1977). They further showed that while pup interactions and displays of active maternal care increased Fos expression in the mPOA and ventral bed nucleus of the stria terminalis (BSTv) – even after olfactory bulbectomy and thelectomy – exposure to pup cues alone was insufficient (Numan and Numan, 1995), indicating that mPOA and BSTv function in generating the physical expression of maternal care.

Building upon the identification of mPOA as the central component of maternal response regulation, Numan and colleagues found that projections of the mPOA to the ventral tegmental area (VTA) participated in goal directed maternal behaviors, such as pup retrieval and nest building (Numan and Nagle, 1983; Numan and Smith, 1984), implicating reward circuitry modulation in the transition to motherhood. In addition, the mPOA and BSTv efferent neurons projecting to the ventromedial hypothalamus

(VMH), lateral septum, medial hypothalamus, and periaqueductal grey (PAG) are also activated during displays of maternal behavior (Numan and Numan, 1997). Subsequently, these regions became known as part of the "maternal circuitry."

Maternal care is a highly motivated behavior, requiring the inhibition of aversive response to pup odor and enhancement of the incentive salience of pup cues (Olazábal *et al*, 2013). Synthesizing results from circuitry studies, the current view regarding maternal motivation states that the mPOA, facilitated by hormonal fluctuations, overrides the defensive response activated by the VMH-medial PAG pathway, and generates pup-directed behavior via stimulation of the VTA dopaminergic neurons and activation of the ventral pallidum (Numan and Stolzenberg, 2009).

Following these work, the roles of other brain regions in maternal behavior continued to be revealed. For instance, Lonstein and colleagues established the function of caudal PAG in processing pup suckling stimuli and expressing kyphosis (Lonstein and Stern, 1997a, 1997b; Lonstein *et al*, 1998). In addition, Li and Fleming ascertained that the nucleus accumbens shell is required for pup retrieval performance as well as maternal memory consolidation (Li and Fleming, 2003a, 2003b).

Beyond the neural circuitry, dendritic morphological modifications in brain regions critical for cognitive functions, including the medial prefrontal cortex and hippocampus, along with neurogenic alterations in the subventricular zone of the lateral ventricles, have also been noted in lactating females (Hillerer *et al*, 2014). However, the

association between these modifications and postpartum cognitive rearrangements is thus far speculative, and more work is necessary to verify these hypotheses.

In terms of neurotransmitter functions, systems involved in motivation and emotionality have been implicated. The mesolimbic dopaminergic system was suggested to play a role in maternal pup retrieval behavior, and infusion of a dopamine (DA) D1 receptor antagonist into the nucleus accumbens significantly disrupted pup retrieval performance (Numan *et al*, 2005). Recently, we also demonstrated the involvement of the serotonergic (5HT) system in the maternal behavior. In the study, systemic administration of the $5HT_{2C}$ agonist, MK212, severely disrupted all aspects of maternal behavior, including pup retrieval, pup licking, nursing, and nest building (Chen *et al*, 2014). Other transmitters, including endogenous opioids and GABA, have also been linked to the regulation of maternal behavior (Byrnes and Bridges, 2000; Byrnes *et al*, 2000; Lonstein and De Vries, 2000). While the roles of the cholinergic and glutamatergic systems have been less explored, it is well known that one important neurohormone associated with maternal behavior, oxytocin, facilitates maternal bonding in sheep through inducing the release of these transmitters into the olfactory bulb (Dwyer, 2014). It has long been known that oxytocin mediates maternal behavior, capable of inducing maternal behavior in virgin females (Pedersen *et al*, 1982), and that estrogen exerts its influence through upregulation of oxytocin receptors in numerous limbic and hypothalamic structures, including the mPOA (Patchev *et al*, 1993).

Through comparing licking, groom and arched back nursing behavior (LG-ABN) across generations, Champagne and Meaney demonstrated that the "mothering-begets-mothering" phenomenon, where female offspring of high LG-ABN mothers also display high LG-ABN (Meaney, 2001), is regulated through epigenetic changes in estrogen-inducible oxytocin receptor density in the mPOA, BST, central amygdala, and LS (Champagne *et al*, 2001; Francis *et al*, 2000). This neuronal mechanism is now widely accepted as the basis for the intergenerational transmission of maternal behavior.

Postpartum brain adaptations lead to changes in psychological functions

One of the most interesting phenomena associated with pregnancy, proverbially termed the "pregnant brain," is the altered cognition in females. While some reports have suggested these impairments as self-perceived changes (Crawley *et al*, 2003; Janes *et al*, 1999), evidence points to observable deficits. For example, deficits in verbal recall memory has been consistently demonstrated in postpartum women (Buckwalter *et al*, 1999; Glynn, 2010; de Groot *et al*, 2006).

In rodents, studies examining postpartum cognitive changes have focused on spatial memory. An early study by Kinsley *et al*, 1999 showed that multiparous Sprague-Dawley rat dams significantly outperformed nulliparous females in the radial arm maze. Interestingly, pup-sensitized virgins also performed similarly to multiparous dams. The notion that maternal experience may enhance spatial memory was supported by

Pawluski *et al*, 2006b showing that primiparous dams also made fewer errors in the radial arm maze compared to virgins.

Surprisingly, Pawluski and colleagues also showed a positive correlation between LG-ABN and the number of reference memory errors (i.e. entry into the incorrect arm of the radial maze). This contradicted Kinsley's results suggestive of pup interaction as a mechanism of improved spatial memory. However Lambert *et al*, 2005's findings that dams with pups removed at parturition performed worse in the dry land maze compared to mothers allowed to care for their young indicated that pup interaction and reproductive experience likely work in tandem to increase spatial memory in dams. Overall, rodent studies clearly support a postpartum enhancement in spatial memory, though the specific mechanisms remain to be elucidated.

In rodents, spatial memory seems to be preferentially augmented postpartum, while non-spatial memory is unaffected (Lemaire *et al*, 2006). This has led to a theory for the evolutionary underpinnings of maternal adaptation. It is believed that since offspring survival is dependent upon successful foraging behavior of dams, increased spatial awareness offers a behavioral economics advantage (Kinsley *et al*, 2015). Corroborating evidence showed that mothers are faster at capturing and subduing preys, even after inhibition of olfactory, auditory, or somatosensory functions (Kinsley *et al*, 2014). Similarly, auditory plasticity in postpartum dams demonstrated enhanced auditory cortex lateral inhibition during pup ultrasonic vocalizations (USV) stimulation, as well as selective suppression of spontaneous activities in USV responsive neurons

(Lin *et al*, 2013). These results suggest that postpartum cognitive reorganization enhances maternal behaviors advantageous for the survival of the offspring.

Unfortunately, so far few groups have expanded upon this model to examine whether sensory filtering mechanisms beyond USV may be affected. A study by Byrnes *et al*, 2007 suggested reduced gating functions postpartum in the prepulse inhibition (PPI) task, which assesses the ability to filter sensory information (Swerdlow *et al*, 2008), though more work is necessary to support this conclusion.

In addition to changes in cognition, robust postpartum anxiolysis is seen in both humans and rodents. In humans, elevated mood, increased calmness and reduced stress-induced cortisol were seen immediately post breast-feeding infants (Heinrichs *et al*, 2013). In rodents, Hard and Hansen, 1985 showed similar results in reduced novelty-induced freezing behavior by postpartum females tested with pups present, but not in the absence of pups, suggesting reduced anxiety in mothers require pup exposure. Other studies have also shown reduced freezing, increased entry into the center region, and increased exploratory rearing behavior in the OFT in postpartum females (Wartella *et al*, 2003). In addition, Love *et al*, 2005 demonstrated the persistence of reproductive experience induced anxiolysis in primiparous females with increased time spent in the open arms of the elevated plus maze (EPM) at 22 months of age.

Besides unconditioned stress paradigms (i.e. a novel environment, OFT, or EPM that exploit rodent's innate aversion to novel, open, bright and elevated environments [Ohl *et al*, 2001]), conditioned fear also indicated reduced anxiety postpartum. Pairing an

aversive unconditioned stimulus (i.e. noise) with a conditioned, previously neutral conditioned stimulus (CS, i.e. white light), postpartum dams displayed reduced freezing during a CS-only test session compared to virgins (Rima *et al*, 2009).

The reduction in fear and anxiety seen in postpartum rodents is also thought to contribute to the parallel increase in maternal aggression (Lonstein and Gammie, 2002). In mice, maternal aggression performance on a male intruder test inversely correlated with the total time spent in the light compartment of a light dark box (Maestripieri and D'Amato, 1991). While maternal aggression is extensively documented in rodent studies, less is known about the behavior in humans (Campbell, 2013). Thus, the clinical implication of increased postpartum aggression remains uncertain.

In contrast to aggression, due to the societal ramifications associated with postpartum depression, postpartum mood regulation is extensively studied in both humans and rodent models. As mentioned, women often perceive elevated mood following breast feeding (Heinrichs *et al*, 2013). In rodent models, offspring-exposure was also shown to be necessary for mood regulation. In Pawluski *et al*, 2009, primiparous dams with pups removed at parturition spent significantly more time immobile in the forced swim test (FST), while pup-sensitized virgin exhibited similar performance as dams permitted to raise their young. In general, however, healthy mother rats do not display increased depressive-like behavior (i.e. increased immobility in the FST) compared to nulliparous counterparts. This is also supported by findings in the sucrose preference test (SPT, Navarre *et al*, 2010). One interesting finding by Craft *et*

al, 2010, however, suggested that while time spent immobile did not differ between virgins and dams in the FST, time spent diving was significantly reduced in mothers. As reduced diving may signify decreased active stress-coping behavior (Singewald *et al*, 2011), the implications of this result require further exploration.

In summary, healthy postpartum adaptations require the participation of neural structures within the hypothalamus and limbic system, in conjunctions with transmitter systems involved in motivation and mood regulation. These changes result in enhanced cognitive and emotional functions supportive of maternal motivation and increase the chance of offspring survival.

Peripartum physiological and psychological disturbances may lead to postpartum disorders

In humans, a significant portion of women are afflicted by postpartum psychiatric disorders (Andersson *et al*, 2006; Beck, 2006; Jones *et al*, 2008). Epidemiological evidence suggested a seven-fold increase in psychiatric admissions for women through the first three months of childbirth (Munk-Olsen *et al*, 2006). Importantly, postpartum mental disorders may reduce maternal responsiveness toward the infant (Arteche *et al*, 2011), resulting in decreased care and even aversion toward the young (Adamakos *et al*, 1986). To understand the etiology of postpartum mental illness, basic researchers have developed an array of preclinical models. Of these, the most notable is gestational stress application, based on human evidence that peripartum stress poses a large risk for postnatal psychiatric disorders (Robertson *et al*, 2004).

Maestripieri *et al*, 1991 first demonstrated the effect of peripartum stress with two different forms of gestational stressors – a daily 2 h restraint stress (RS) and a novel environment stress (NES) from gestation days (G) 4 to 14. Although the NES did not affect postpartum anxiety in the light-dark box, RS reduced the amount of time postpartum dams spent in the light portion of the chamber, indicating increased anxiety. RS dams also displayed increased pup-licking, which, when interpreted in conjunction with the increased anxiety seen in the light-dark box, might represent an anxious, or "helicopter," parenting style as seen in some women with postpartum anxiety (Bridges *et al*, 2008). In addition, RS has also been shown to induce depressive like behaviors. For example, O'Mahony *et al*, 2006 saw increased immobility in the FST on postpartum day 23 in G 14 to 21 RS mothers. Similarly, 1 h daily restraint from G 10 to 20 also increased immobility in the FST at an earlier postpartum time (postpartum day 4, [Smith, 2004]).

Interestingly, using chronic ultra-mild stress (i.e. daily 1 to 2 h presentations of cage tilts, confined space, paired housing, soiled cage, difficulty to access food, and reversal of light cycle), Pardon *et al*, 2000 identified a dissociation between defensive behavior and pup-directed maternal care. When presented with male intruders, dams not only exhibited a dramatic passive response, but also a perplexing display suggestive of deficits in behavioral flexibility. During the test session, stressed dams repeatedly retrieved scattered pups back to the nest location occupied by the male intruder. This result provided valuable considerations for women that might behave in ways unintentionally harmful to the infant despite having strong maternal responsiveness.

Other physical manipulations have used psychosocial stressors to induce depression-like behaviors in dams (Nephew and Bridges, 2011). For instance, daily 1 h exposure to a male intruders from postpartum days 2 to 16 caused a significant reduction saccharine preference (Carini *et al*, 2013). Daily 3 h separations between dams and pups also increased immobility in the FST (Boccia *et al*, 2007).

Postpartum is a period of high hormonal fluctuation. As such, some models have used of hormone regimens to simulate pregnancy, marking the subsequent sudden withdrawal of hormones as the postpartum period. Using this model, Suda *et al*, 2008 found that ovariectomized rats with 21 days of estradiol and progesterone supplement followed by 7 days of withdrawal showed a significant increase in escape latency in an escape learning task following an inescapable shock (IES) procedure, indicative of increased "helplessness" display. These females also spent significantly less time in the open arms of the EPM. However, it is important to note that no behavioral differences were found in the OFT, and the hormone withdrawn females actually displayed decreased immobility and increased struggling behavior in the FST. While a similar hormone simulation design did show decreased preference in the SPT in females experiencing hormone withdrawal (Navarre *et al*, 2010), one must keep in mind these discrepancies between assays. Although different preclinical tasks may be designed with the same target variable in mind (i.e. FST and SPT for depressed state, EPM and OFT for anxiety), the behavioral measures sometimes leave room for interpretation and require multiple convergent results to confirm a hypothesis.

One other peripartum hormone manipulation utilizes corticosterone administration during or immediately after pregnancy, which has been shown to produces depression-like behavior in the FST (Brummelte and Galea, 2010; Brummelte *et al*, 2006).

Early life stress affects maternal behavior in the female offspring

In addition to peripartum stressors, as early life stress can cause life-long behavioral dysfunctions, they may also contribute to postpartum disorders. However, available evidence regarding the effect of early environmental hardships on postpartum functions is limited. Deficits in maternal care have been shown in female offspring that experienced difficult early postnatal environment, such as in conditions where novel male intruders were repeatedly presented (Carini and Nephew, 2013; Murgatroyd and Nephew, 2013), or where offspring were subjected to various durations of separation from the mother in early life (Boccia and Pedersen, 2001; Gonzalez *et al*, 2001; Lovic *et al*, 2001). Some prenatal stress studies using gestational restraint stress (Bosch *et al*, 2007) or *in utero* lipopolysaccharide exposure (Soto *et al*, 2013) have also shown alterations. Importantly, however, no study has examined the effects of multiple stressors on postpartum outcomes.

In particular, the studies within this work employed two specific animal models of early life stress – maternal immune activation (MIA) and repeated maternal separation (RMS). As such, procedural considerations for the two will be described in

detail. MIA and RMS are ideal for investigating the effects of early environmental stress on postpartum behaviors as both produce well-documented behavioral and molecular deficits (Meyer, 2014; Nishi *et al*, 2014). The two are also complementary since one alters the fetal milieu (i.e. MIA) while the other affects the postnatal environment (i.e. RMS).

Effects of maternal immune activation are dose- and time- dependent

Maternal immune activation (MIA) is a well-studied model of neurodevelopmental psychiatric disorders that gained much popularity in the recent decade (Meyer and Feldon, 2010). These infections may activate the mother's immune system, increasing cytokine expressions, disrupting offspring neurodevelopment, and causing abnormal phenotypes (Meyer and Feldon, 2009; Smith *et al*, 2007). Preclinical studies show that *in utero* exposure to immunostimulants results in adult offspring that display specific histological and behavioral abnormalities relevant to those seen in human patients with neuropsychiatric disorders such as schizophrenia and autism. Some of these include increased prefrontal cortical pyramidal cell density (Fatemi *et al*, 1999), abnormal latent inhibition (LI, i.e. the ability to ignore inconsequential stimulus [Meyer *et al*, 2010; Zuckerman and Weiner, 2003; Zuckerman *et al*, 2003]), learning deficits (Meyer *et al*, 2006a; Shi *et al*, 2003a; Zuckerman and Weiner, 2005), disrupted sensorimotor gating (Howland *et al*, 2012; Wolff and Bilkey, 2008), poor working memory (Ozawa *et al*, 2006), increased amphetamine-induced hyperlocomotion

(Vorhees *et al*, 2012; Zager *et al*, 2012), and reduced hippocampal neurogenesis (Bernstein *et al*, 2012).

MIA can be achieved with the administration of the viral mimetic polyinosinic:polycytidilic acid (polyI:C), a synthetic double-stranded RNA (Meyer *et al*, 2009). Often, this is applied during the rat gestational period corresponding to the human second trimester (Clancy *et al*, 2001), a time point important for neural migration and differentiation (de Graaf-Peters and Hadders-Algra, 2006). However, current studies employing polyI:C induction of MIA vary widely in administration regimen.

Studies have shown that the activation of cytokines mediates the behavioral alterations induced by polyI:C. For example, in pregnant mice concurrently injected with both IL-6 neurtralizing antibodies and polyI:C on G 12.5, offspring displayed a reversal of behavioral deficits seen with polyI:C injections alone (Smith *et al*, 2007). However, as polyI:C exerts a dose dependent effect on sickness behavior and immune reactivity, it is critical to choose a dose sufficient for the activation of cytokines and subsequent offspring behavioral changes without inducing pregnancy loss (Arad *et al*, 2005; Cunningham *et al*, 2007; Fortier *et al*, 2007; Meyer, 2014; Meyer *et al*, 2005). In addition, gestational time point studies have also indicated that precise domains of deficits may be associated with specific timing of the prenatal insult (Fortier *et al*, 2007; Li *et al*, 2009; Meyer *et al*, 2006b, 2008). For example, Meyer and colleagues saw that a 5 mg/kg intravenous (i.v.) polyI:C injection on G 9, but not G 17, decreased central area exploration in the OFT, and disrupted PPI in adult offspring (Meyer *et al*, 2006b, 2008).

In contrast, G 17 MIA, but not G 9, decreased reversal learning and Morris water maze performance in adult male offspring.

Within current literature, single and multiple administrations of polyI:C between G 12 and G 15, have repeatedly demonstrated the ability to alter offspring brain and behavioral functions. For example, Zuckerman *et al*, 2003 showed disrupted LI in rats exposed to 4.0 mg/kg polyI:C (i.v.) on G 15. The same study also identified increased hippocampal cell loss within this population. In Ozawa *et al*, 2006, daily injections of 5.0 mg/kg polyI:C (i.p.) from G 12 to 17 in mice induced a disruption in novel object recognition, and a decrease in striatal DA D_2-like receptor density. Wolff and Bilkey, 2008 further described PPI deficits in offspring exposed to 4.0 mg/kg polyI:C (i.v.) on G 15. In addition, Shi *et al*, 2009 demonstrated abnormal cerebellar Purkinje and granule cell migrations in offspring exposed to 20 mg/kg polyI:C (i.p.) on G 12.5.

In a preliminary study, we found that a combination of 4.0 mg/kg polyI:C administration on G 13 (i.v.) and 6.0 mg/kg on G 15 delayed conditioned avoidance response (CAR, i.e. avoiding a shock that is preceded by a light-cue) acquisition in male adolescent offspring (Fig. 1). Accordingly, this regimen was chosen for the following studies.

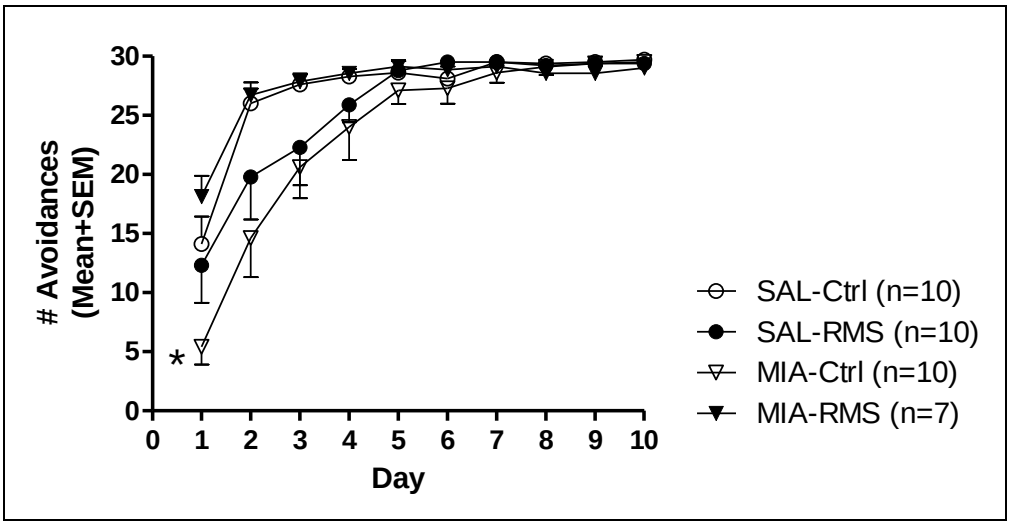

Figure 1. *Maternal immune activation disrupts conditioned avoidance response acquisition.* Juvenile male rats (postnatal days 45 to 54) exposed to maternal immune activation *in utero* (MIA-Ctrl, 4.0 mg/kg polyI:C on gestation [G] day 13 and 6.0 mg/kg polyI:C on G 15) exhibited deficits in conditioned avoidance response learning. **p*<0.01 compared to control animals (SAL-Ctrl) over 10 days of testing.

Effects of repeated maternal separation are duration-dependent

Maternal separation (MS) is an ecologically valid and clinically relevant model of early life stress (Nishi *et al*, 2013), and is believed to disrupt the offspring stress hyporesponsive state and prevent proper neurogenesis (Rosenfeld *et al*, 1991). As in MIA, adults exposed to early life separation show disruptions in learning (Aisa *et al*, 2007), anxiety regulation (Li *et al*, 2013), HPA axis stress response (Nishi *et al*, 2014), and hippocampal neurogenesis (Mirescu *et al*, 2004). Similarly, procedural parameters of MS also differ across studies (Bailoo *et al*, 2014). In particular, while it is generally agreed upon that MS alters brain and behavioral development, the duration of separation seems to play a critical role in the direction and type of consequences (Vetulani, 2013).

One of the most common MS manipulations involves the separation of pups from dams for 3 h daily throughout the first two postnatal weeks (i.e. postnatal days [PND] 2 to 14). This procedure has been shown to increase stress-induced corticosterone in adult males and decrease the duration of open arm exploration in the EPM in adult females (Kalinichev *et al*, 2002). Other disturbances in adult males subjected to this procedure include: decreased dentate gyrus cell proliferation (Mirescu *et al*, 2004); increased male aggression and basal plasma corticosterone levels (Veenema *et al*, 2006); increased immobility in the forced swim test (Lajud *et al*, 2012); and increased acoustic startle response habituation following repeated stimuli presentation (Lehmann *et al*, 2000a).

Other MS designs have also shown altered adult functioning. For example, in Kao *et al*, 2012, daily 1 h separation from PND 2 to 9 was sufficient to reduce fear-potentiated startle in adult female offspring. In addition, Hofer, 1976 demonstrated that a single 24 h maternal deprivation episode at PND 14 was capable of disrupting the normal sleep cycle. Similarly, Oomen *et al*, 2010 showed that 24 h maternal deprivation on PND 3 significantly reduced adult neurogenesis, disrupted spatial learning in the Morris water maze, and enhanced fear-associated memory. While the above mentioned manipulations seemed to affect the offspring adversely, daily 15 min MS from PND 2 to 14 led to decreased basal corticotropin-releasing factor (CRF) mRNA and hormone levels, diminished stress-induced median eminence CRF turnover, lower stress-induced corticosterone, reduced frontal cortex serotonin and metabolite (i.e. 5-HIAA), and

increased glucocorticoid mRNA (Francis *et al*, 1999; Plotsky and Meaney, 1993; Smythe *et al*, 1994).

In a second preliminary study, we exposed offspring to nine days of repeated MS (RMS): 3 h daily from PPD 2 to 4, then 3 h every other day from PPD 6 to 10, and once again 3 h daily from PPD 12 to 14. This design allowed us to assess F0 generation maternal behavior using the pup retrieval test on days without the MS manipulation (i.e. PPD 5, 7, 9, and 11), preventing RMS from confounding behavioral results when it is carried out in close temporal proximity (Lonstein, 2005; Smith and Lonstein, 2008). In addition, we found that these male adult offspring displayed decreased nicotine-induced hyperlocomotion, indicating altered cholinergic functions at the behavioral level (Fig. 2). Thus, this procedure was chosen for the current experiments.

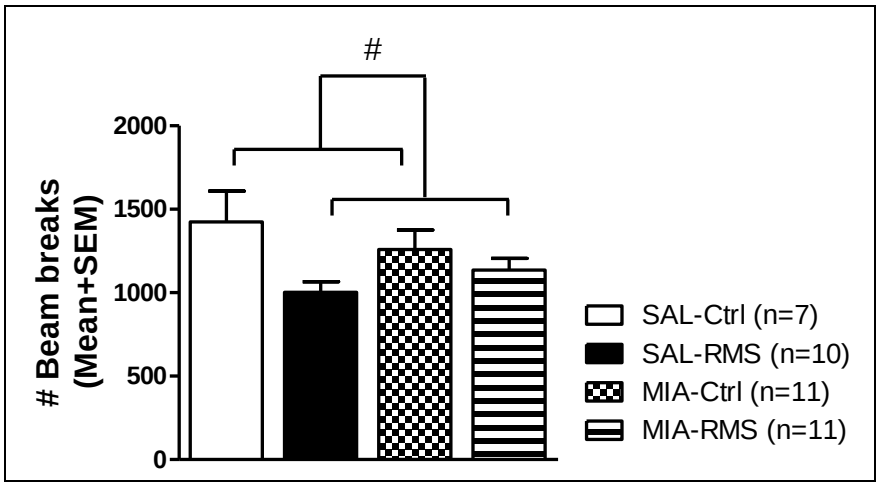

Figure 2. *Repeated maternal separation alters nicotine sensitivity.* Adult male rats exposed to repeated maternal separation (RMS, 3 hours each on postnatal days 2 - 4, 6, 8, 10, 12 - 14) exhibited decreased nicotine-induced hyperlocomotion relative to non-RMS animals (Ctrl). #$p<0.05$ between all RMS versus all Ctrl animals.

Multiple early stressors may synergistically disrupt adulthood functions

In our studies, MIA and RMS were assessed independently and in combination. The use of these temporally distinct stressors allowed not only for comparing the postpartum effects of the two, but also for understanding their interactions. This is critical as previous work has demonstrated synergistic effects of *in utero* and adolescent environmental insults (Giovanoli *et al*, 2013). Curiously, Lehmann *et al*, 2000b found that, contrary to their expectation of RMS exacerbating the deficits induced by prenatal stress, some phenotypes showed antagonistic outcomes. For instance, offspring born from gestationally restrained mothers exhibited PPI deficits, which was attenuated by RMS. Conversely, while RMS actually improved LI, prenatal stress abolished the enhancement.

Intriguingly, in recent years a theoretical framework surrounding stress as a trigger for environmental adaptation has emerged. The concept promotes the perspective of phenotypes induced by prenatal stress as "adaptive" to the presumed postnatal environment based on the information received *in utero*, albeit they may often be detrimental in a normal, non-aversive environment (Lee and Goto, 2013). A complementary hypothesis also suggests that when stressors are experienced at distinct time points, a mild stress in early life may "prime" one for similar stressors in later life, thus promoting resiliency (Daskalakis *et al*, 2013; Karatsoreos and McEwen, 2013).

Remaining issues

After a review of current understandings regarding early life adversities, postpartum modifications and disorders, and the potential contribution of early stress to maladaptive postpartum processes, some issues remain. First, while some studies have examined the effects of RMS on the maternal behavior of the offspring, the effects of MIA on maternal behavior is currently unknown. Second, it is unclear whether multiple stressors (i.e. MIA and RMS) would act in synergy and augment maternal care deficits, or antagonize each other and lead to resiliency. Third, as RMS is known to induce behavioral alterations in both the mother and offspring, the maternal behavior of RMS offspring is likely shaped by both the RMS manipulation as well as the quality of maternal care. However, studies of RMS offspring maternal behavior have not examined the behaviors of the parental generation dams. Thus, it is unclear whether the RMS manipulation independently poses risks for postpartum deficits. Fourth, as MIA and RMS both cause behavioral alterations in adult offspring, it is unclear whether postpartum MIA and RMS females would show the same deficits as virgins. To address these, the following experiments were designed to use MIA and RMS as stressors that simulate human early life adversities, in order to examine behavioral and neuronal disruptions during the postpartum period. The stressors were tested both independently and in combination. We hypothesized that postpartum females exposed to early stress would display behavioral and neuronal abnormalities that disrupt not only maternal behavior, but also the associated cognitive and affective processes.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Facility

The animal facility colony rooms maintained a 12h light/dark condition (light on between 0630 and 1830 h). Room temperature was maintained at 22 ± 1°C with a relative humidity of 45-60%. Food (2019 Teklad Global 19% Protein Extruded Rodent Diet) and water was available *ad libitum* throughout the entire experiment. All experiments were run during the light cycle with the exception of the sucrose preference test, which was conducted overnight. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln.

Parent (F0) generation

Figure 3 illustrates the procedural manipulations for the parent and offspring generation animals. All animals used were Sprague-Dawley rat dams from Charles River (Portage, MI) and their offspring. Parental generation (F0) Sprague-Dawley dams (Gestation day [G] 6 on arrival) were single housed in 48.3 × 26.7 × 20.3 cm transparent polycarbonate cages with TEK-Fresh cellulose bedding covering the cage floor and left undisturbed in the animal facility colony room upon arrival.

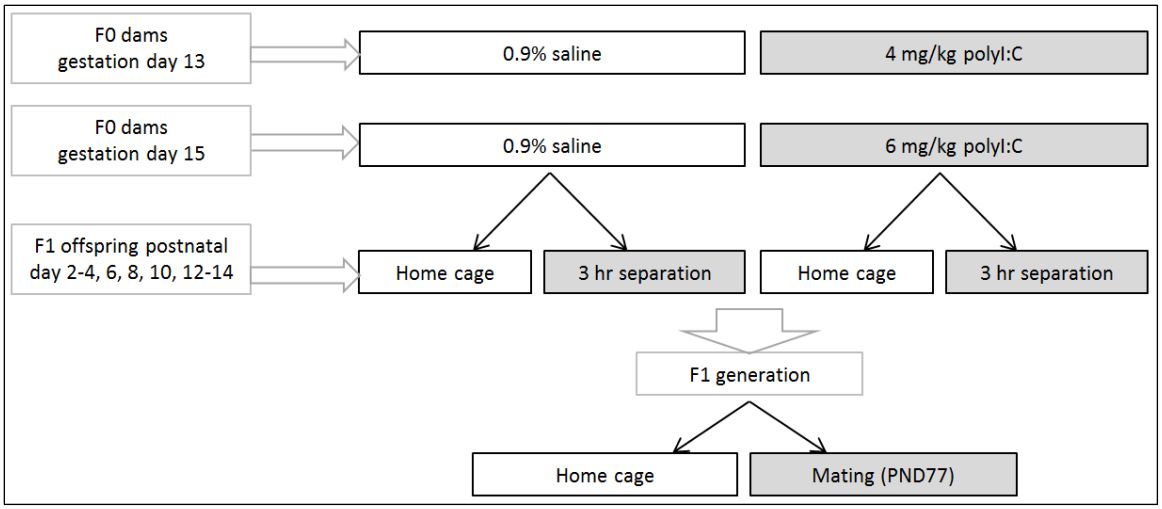

Figure 3. *Procedural manipulations for the generation of offspring test subjects.*

F0 parturition and culling. Beginning two days prior to expected parturition (G 21), dams were monitored twice daily. When new pups were found during the morning spot check (0900 h), parturition was considered to be the evening prior and the day of the spot check is considered postpartum day (PPD) 1. When new pups were found during the afternoon spot check (1600 h), parturition was considered to be the same day as the spot check, noted as PPD 0. Once pups were found, paper towel strips were placed in the cage as nesting material. On PPD 1, the numbers and weights of male and female pups were recorded. As litter size difference has been previously cited as a possible confounding variable in experimental designs, offspring (F1) were culled to 5 males and 5 females wherever possible according to standard recommended practice (Agnish and Keller, 1997; Chahoud and Paumgartten, 2009). When less than 5 pups per sex were available, litters were culled to a total of 10 pups irrespective of sex.

Maternal immune activation

F0 dams received either 0.9% saline (SAL) on both G 13 and G 15 for the control group or polyI:C (Sigma-Aldrich, St. Louis, MO) dissolved in 0.9% saline on both G 13 (4 mg/kg) and G 15 (6 mg/kg) for the maternal immune activation (MIA) group. To administer saline or polyI:C, pregnant dams were anesthetized with 3% isoflurane (Fisher Scientific, Denver, CO) in 98% O_2 and given a single i.v. injection at the tail vein. Weights of pregnant dams were recorded both immediately prior to and one day post saline or drug administration. Unless otherwise specified, all drug injections in the experiments were administered at 1.0 ml/kg.

Repeated maternal separation

Half of the F0 generation dams and corresponding litters from each prenatal treatment (SAL v. MIA) underwent repeated maternal separation (RMS) 3 h daily from PPD 2 to 4, then 3 h every other day from PPD 6 to 10, and 3 h daily again from PPD 12 to 14, resulting in nine days of separation treatment throughout the first two postnatal weeks for pups (i.e. PND 2, 3, 4, 6, 8, 10, 12, 13, 14). During separation, pups were removed from home cages and placed as entire litters in new, clean cages housed in a quiet testing room. Cages were set atop heating pads set to 100°F to maintain normal body temperature (Stammers, 1926). Dams remained in home cages in a different testing room than the pups. After 3 h, pups were replaced to home cages with dams and

returned to the colony room. Dams and litters that were not subjected to RMS (i.e., control group [Ctrl]) remained in the colony room throughout this period.

Pup retrieval test

F0 maternal behavior was assessed on PPD 5, 7, 9 and 11 using the standard pup-retrieval test (Hahn and Lavooy, 2005). Pups in the entire litter were removed from the home cage for 10 s and the nest was destroyed. They were then promptly returned to the cage away from the mother, and the mother's behavior was video recorded for 10 min. The following behaviors were analyzed by experimenters blinded to the treatment conditions: 1) Latency in s to first pup retrieval back to the original nest location; 2) % time spent crouching over pups, as a general indication of nursing; 3) % time spent nest-building, represented by dams gathering paper towel strips or bedding around the nest area; 4) % time spent pup licking and grooming. Inter-rater reliability was determined via Pearson's correlation. Videos were analyzed using the JWatcher program (version 1.0, http://www.jwatcher.ucla.edu). A score of 600 s was assigned to dams who did not display retrieval behavior during the entire 10 m duration.

Offspring (F1) Generation

From the prenatal and postnatal treatments, four groups of F1 generation subjects were created: control animals that did not experience any environmental stress (SAL-Ctrl); animals exposed to only RMS (SAL-RMS); animals exposed to only MIA (MIA-Ctrl); and animals exposed to both environmental stressors (MIA-RMS). F1

generation rat pups remained with dams until weaning (P21), upon when they were separated and housed two per cage with littermates of the same sex in 182 × 50 × 188.1 cm transparent polysulfone individually ventilated cages. Females were handled for approximately 2 min and weighed once weekly for five weeks starting on PND 40 (i.e. PND 40, 47, 54, 61, 68) and otherwise left undisturbed. Males were used in separate experiments. Virgin females were housed two per cage with littermates throughout all experiments, which has been shown to have no effect on behavioral outcomes (Byrnes *et al*, 2007; Lonstein, 2005; Neumann *et al*, 2000; Pereira *et al*, 2005). The estrus cycle of virgin females were not monitored. While it has been shown that phases of estrus may play a role in certain behaviors, including elevated plus maze performance (Marcondes *et al*, 2001), latent inhibition (Quinlan *et al*, 2010), and prepulse inhibition (Koch, 1998), it has also been shown to be unrelated to others, such as the forced swim test (Craft *et al*, 2010). Furthermore, the estrus cycle in rats spans a short duration of four days (Baran *et al*, 2009), and it has been argued that the non-synchronization of estrus among female rats prevents this factor to act as a confound. The random distribution of animals thus alleviates this factor to serve as an intermediate variable (Parra *et al*, 1999).

F1 mating, parturition and culling. On PND 77, one or two females from each litter were mated. Only a maximum of two animals per litter were used to minimize litter effects (Zorrilla, 1997). Females randomly chosen from each litter for mating were single housed in 48.3 × 26.7 × 20.3 cm transparent polycarbonate cages, and an adult male (PND 68-90 on arrival; Charles River, Portland, MI) was placed in each cage and remained with the female partner for seven days to ensure successful pregnancy (Baran

et al, 2009). Upon removal of the male partners, females were left undisturbed until parturition. The same parturition monitoring procedure for F0 dams was employed to determine PPD 0, and nesting material (paper towel strips) was provided as soon as pups were found. On PPD 1, numbers of male and female pups were recorded and pups weighed as described in the F0 procedure. Offspring were then culled to 5 males and 5 females wherever possible. When an insufficient number of pups were available, pups from other dams were supplemented to complete the 5 males and 5 females litter.

Dependent measures and statistical analysis

Specific dependent measures are described in further details throughout each experiment (see Chapters 3, 4 and 5). In general, dependent measures consisted of various behavioral outputs in Experiments 1 and 3: specific criteria of maternal behavior, % prepulse inhibition, acoustic startle response, startle response habituation, specific criteria of forced swim behavior, sucrose consumption, drug-induced hyperlocomotion, and avoidance and escape behaviors in the two-way shuttle box chambers. Dependent measures in Experiment 2 are the numbers of c-Fos immunoreactive cells identified in individual brain regions.

All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). All data were expressed as mean± standard error of mean (SEM), except for correlational analyses, which used individual values from each subject. Specific analyses are described in further details throughout each experiment (see Chapters 3, 4 and 5). In general, a three-way analysis of covariance (ANCOVA) was

employed. The three between-groups factors included the prenatal stress manipulation (MIA versus SAL), the postnatal stress manipulation (RMS versus control [Ctrl]), and reproductive experience (nulliparous versus primiparous). All F1 generation analyses also included the covariate of F0 maternal behavior in order to control for the moderating effects of individual differences in parental generation behavior. Details of the identification and utilization of the proxy variable for the F0 maternal behavior covariate is described in further details in Chapter 3 (see *F0 maternal behavior* of the *Results* section). In procedures where multiple test sessions were conducted, the types or numbers of sessions were included as within-groups factors using repeated measures three-way ANCOVA. Post hoc analyses were conducted using either Bonferroni tests for between-groups effects or least significant difference (LSD) minimum mean difference (mmd) pairwise comparisons for within-groups effects. For all analyses, p=0.05 was considered statistically significant.

CHAPTER 3

EXPERIMENT 1

BEHAVIORAL INVESTIGATIONS OF COGNITIVE, AFFECTIVE, AND MATERNAL FUNCTIONS IN POSTPARTUM FEMALES EXPOSED TO EARLY ENVIRONMENTAL STRESS

Introduction

This study examined the maternal behavior, cognitive function, and affective regulation of adult females exposed to MIA *in utero* and/or RMS during infancy. The design was aimed at three specific questions: 1) Does early environmental stress disrupt female maternal behavior in adulthood? 2) Are cognitive and affective processes altered in adult females exposed to early environmental stressors? 3) Does reproductive experience influence cognitive and affective functions of females exposed to early environmental stress differentially than healthy females?

The general approach was to expose females to stress *in utero* and/or during early infancy, and mate these females in adulthood. Postpartum maternal behavior was then assessed, followed by a battery of behavioral tests for these primiparous dams as well as their age-matched nulliparous littermates. These included baseline acoustic startle response (ASR) of prepulse inhibition (PPI) for examination of pre-attentive cognition, pup-present ASR of PPI for pup cues-induced alterations, forced swim test (FST) for depressive symptoms and general motivation, sucrose preference test (SPT) for hedonic

state, and fear potentiated startle (FPS) for anxiety and stress response. Serial testing is regularly carried out in both behavioral and pharmacological preclinical experiments for between-groups comparisons (Aguggia *et al*, 2013; Li *et al*, 2013; Malkova *et al*, 2012; Qin *et al*, 2013). While this reduces the number of animals required to carry out the extensive studies at hand, there is a potential risk for carry-over effects. This may limit our data interpretation, and we were cautious in stating over-reaching conclusions. Figure 4 details the procedural timeline for each behavioral assessment.

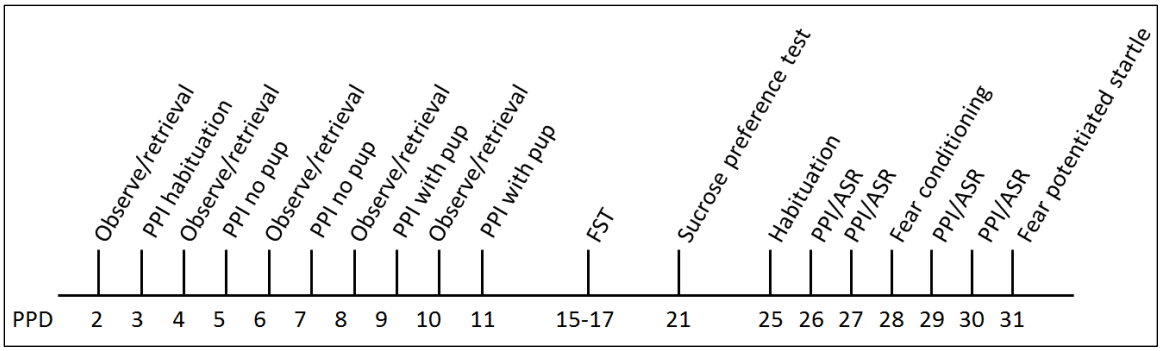

Figure 4. *Procedural timeline of behavioral assessments conducted in Experiment 1.* Time schedule is indicated by postpartum (PPD) days of the F1 primiparous subjects. F1 virgin females are age matched littermates.

The FPS paradigm utilized a fear conditioning procedure within the PPI chambers, pairing a conditioned stimulus (CS, light) with an unconditioned stimulus (US, shock). This allowed us to record changes in ASR and PPI performance in an FPS challenge test following fear conditioning, using both CS-present and CS-absent trials. We have previously demonstrated effective conditioning using this method through comparison of conditioned versus unconditioned groups (unpublished). In the FPS challenge test, animals that underwent the fear conditioning session displayed an

increase in ASR during CS-present trials, while animals that did not undergo the CS-US association training showed no changes in ASR during CS-present trials.

In F1 dams exposed to early stress, we hypothesized a disruption in maternal care, deficit in both baseline and pup cues-influenced ASR of PPI, decreased general motivation in the FST, increased anhedonic state in the SPT, and increased stress response in the FPS.

Apparatus

Prepulse inhibition of acoustic startle response apparatus. The PPI test was performed using six Startle Monitor Systems (Kinder Scientific, Julian, CA). Each system, controlled by a PC, was housed in a compact sound attenuation cabinet (36 cm wide × 28 cm deep × 50 cm high). A speaker (diameter: 11 cm) mounted on the cabinet's ceiling was used to generate acoustic stimuli (70 dB - 105 dB). The startle response was measured by a piezoelectric sensing platform on the floor, which was calibrated daily. During testing, rats were placed in a rectangular box made of transparent Plexiglas (19 cm wide × 9.8 cm deep × 14.6 cm high) with an adjustable ceiling positioned atop the box, providing only limited restraint while prohibiting ambulation.

Forced swim test apparatus. The apparatus consists of 4 vertical cylindrical polycarbonate tubes (height 40 cm × diameter 20 cm) filled to 30 cm with 25° C tap water to ensure the hind paws of the animals could not touch the floor.

Procedures

Baseline maternal behavior observation. Baseline maternal behavior observation was conducted with animals in their home cages, inside the animal colony. Observation procedure was adapted from Myers *et al*, 1989. Observations were conducted twice on PPD 2, 4, 6, 8 and 10, once during 0900 and once during 1500 h, by observers blinded to the treatment conditions. All efforts were made to ensure minimal disturbance to the cages and animals at least 1 h prior to the observation and throughout the observation session, which lasted approximately 20 min. During a session, the behaviors occurring within a litter were observed for approximately 5 s, and the behaviors were recorded on a checklist. Each litter was observed 10 times, with approximately a 2 min gap in between each observation. The behaviors recorded include: 1) Dam location (in or out of nest); 2) Dam eyes position (open or closed); 3) Dam-pup contact (in contact with any pup or more than half of the litter); 4) Dam licking/grooming pups; 5) Dam carrying pups; 6) Dam moving inanimate objects (nest building or moving non-nesting material); 7) Dam self-care behaviors (eating, drinking, self-grooming, resting away from pups); 8) Dam observation of the environment (rearing/positioning upright on hindpaws or sniffing with head raised); 9) Dam nursing (arched back with legs extended over pups; blanket-nursing with body positioned over pups, without creating a space underneath the chest and abdomen for pups; passive nursing with dams laying on the side and pups attached). Data were analyzed as the total number of observations made for each behavior throughout the 10 observation trials within each session.

Pup retrieval test. The pup retrieval test for the F1 dams followed the same procedures as those of F0 dams described in Chapter 2 (see Chapter 2 *Maternal pup retrieval test* section). Due to technical errors, only data from the first three retrieval test sessions were analyzed (i.e. PPD 2, 4 and 6).

Acoustic startle response, prepulse inhibition, and fear potentiated startle. ASR and PPI procedures were adapted from Culm and Hammer, 2004. The PPI session lasted approximately 18 min and began with a 5 min period of 70 dB background noise (which continued throughout the duration of the session) followed by four different trial types: PULSE ALONE trials and three types of PREPULSE+PULSE trials, which consisted of a 20 ms 75, 78, or 82 dB prepulse (5, 8, and 12 dB above background) followed 100 ms later by a 105 dB pulse (40 ms). Each session was divided into 4 blocks. Blocks 1 and 4 were identical, each consisting of 4 PULSE ALONE trials. Blocks 2 and 3 were also identical and each consisted of 8 PULSE ALONE trials and 5 of each PREPULSE+PULSE trial type. A total of 54 trials were presented during each test session. Trials within each block were presented in a pseudorandom order and were separated by a variable intertrial interval averaging 15 s (ranging from 9 to 21 s). Startle magnitude was defined as the maximum force (measured in Newtons) applied by the rat to the startle apparatus recorded over a period of 100 ms beginning at the onset of the pulse stimulus. Between each stimulus trial, 100 ms of activity was recorded when no stimulus was present. These trials were called NOSTIM trials and were not included in the calculation of intertrial intervals. Startle habituation was calculated as the % reduction in startle magnitude of the last four pulse-only trials compared to the first four pulse-only trials within the session. Startle

responses from testing blocks 2 and 3 were used to calculate percent prepulse inhibition (% PPI) for each acoustic prepulse trial type:

$$\%\ \mathrm{PPI} = 100 - [\left(\frac{\text{average startle response to PREPULSE + PULSE trials}}{\text{average startle response to PULSE alone trials}}\right) \times 100]$$

Prior to the first ASR and PPI testing, animals were allowed to habituate to the startle chambers for 20 min on PPD 3, with the 70 dB background noise present throughout the entire habituation session. The first two sessions of ASR and PPI (PPD 5 and 7) were assessed under baseline conditions, and the second two sessions (PPD 9 and 11) were assessed with pups present in the chambers. Two pups, one male and female, aged PND 9 in session 3 and PND 11 in session 4, were placed in transparent containers attached to the sides of the restrainers. Test subjects were thus exposed to pup cues, i.e. olfactory, auditory, and visual cues, without physical interactions with the pups.

The FPS test consisted of the following sessions: one habituation session on PPD 25, two pre-conditioning ASR and PPI sessions on PPD 26 and 27, one CS-US conditioning session on PPD 28, two more post-conditioning ASR and PPI sessions on PPD 29 and 30, and a FPS challenge test on PPD 31. All procedures between PPD 25 and 31 were conducted under the pup-absent condition. The fear conditioning procedure was adapted from Walker and Davis (Walker and Davis, 2002; Walker *et al*, 2002; Zhao *et al*, 2009) and consisted of 10 CS (3.7 s light)-US (0.6 mA 0.5 s footshock) paired trials with 4 min intertrial intervals. The FPS challenge test consisted of 40 CS-present (CS+) and 40 CS-absent (CS-) trials, both with 10 PULSE-ALONE trials and 10 trials of each of the three PREPULSE+PULSE type, presented in a random fashion.

Forced swim test. The FST procedure was adopted from Aguggia *et al*, 2013 and Boccia *et al*, 2007. On days of testing, animals were first habituated to the test environment for 30 min. They were then placed gently into the water-filled cylindrical tubes and their behaviors were video recorded for 15 min on day 1 (PPD 15), 10 min on day 2 (PPD 16) and 5 min on day 3 (PPD 17). Animals were immediately removed from the water after testing, patted dry with clean towels, and returned to their home cages. Videos were analyzed using Forced Swim Scan (CleverSys, Inc., Reston, VA) and the following parameters were measured: 1) % time spent struggling (% time with upward-directed movements of all 4 paws); 2) % time spent swimming (% time with horizontal movements throughout the test chamber); and 3) % time spent immobile (% time with only minimal movements necessary to remain afloat with head above the water surface). Due to technical errors, only data from half of the test subjects were analyzed.

Sucrose preference test. SPT procedure was modified from (Maniam and Morris, 2010) and (Carini and Nephew, 2013). On PPD 21, post-weaning of pups, SPT was carried out using a two-bottle choice design. Two pre-weighed bottles, one filled with water and the other 2.5% sucrose, was placed into each cage, with the location of the bottles (i.e. right or left side of the cage) randomly assigned to eliminate the side bias confound. Bottles remained in the cages from 1730 of PPD 21 to 0930 of PPD 22. Following the test session, bottles were removed and weighed once more, and sucrose preference was determined as the ratio of the volume of sucrose taken over total fluid intake.

Dependent measures and statistical analysis

F0 weight change following polyI:C injections was measured using analysis of variance (ANOVA, between-subjects factor: prenatal treatment [SAL/MIA]). F0 maternal behavior was assessed via repeated measures two-way ANOVA (between-subjects factors: prenatal treatment [SAL/MIA] and postnatal treatment [Ctrl/RMS]; within-subjects factor: sessions). The inclusion of prenatal treatment as a factor for F0 maternal behavior allowed us to examine potential changes in maternal care as a result of alterations in F1 pup behavior following *in utero* MIA. All analyses of F1 females included the factor of F0 maternal care as a covariate (see *F0 maternal behavior* section). F1 % survival from culling to weaning was analyzed using a two-way analysis of covariance (ANCOVA, between-subjects factors: SAL/MIA, Ctrl/RMS; covariate F0 maternal care). % sucrose consumption and ASR habituation on the FPS test day were conducted using a three-way ANCOVA (between-subjects factors: SAL/MIA, Ctrl/RMS, reproductive experience [nulliparous/primiparous]; covariate: F0 maternal care). Baseline maternal observation, pup retrieval test, ASR, PPI, FST and the FPS challenge test were all analyzed using repeated measures three-way ANCOVA (between-subjects factors: SAL/MIA, Ctrl/RMS, nulli/primiparous; within-subjects factor: sessions). % PPI was averaged across the three prepulse levels. All data were expressed as mean± SEM. Post hoc analyses were conducted using either Bonferroni tests for between-groups effects or LSD mmd pairwise comparisons for within-groups effects. For all analyses, p=0.05 was considered statistically significant.

Results

Parent (F0) generation. In weight gain post initial polyI:C administration, MIA significantly decreased F0 dams weight gain in separate replication analyses (Fig. 5; Replication 1: $F(1,20)=4.485$, $p=0.048$; Replication 2: $F(1,19)=7.665$, $p=0.013$). However, due to replications differences, the overall effect was not significant (Fig. 5). In pup retrieval, both MIA and RMS alone significantly increased F0 % time crouching compared to MIA-RMS (Fig. 6a; MIA × RMS interaction, $F(1,37)=9.694$, $p=0.004$; post hoc MIA-Ctrl v. MIA-RMS, $p=0.014$; SAL-RMS v. MIA-RMS, $p=0.021$). The F0 % time crouching averaged across test sessions was thus chosen as a covariate for subsequent analyses to control for maternal care as a factor (termed "F0 maternal care"). The MIA × RMS interaction held true in average % time crouching, $F(1,37)=9.173$, $p=0.004$. Finally, in % F1 pup survival, MIA and RMS significantly increased pup survival (Fig. 7; MIA × RMS interaction, $F(1,36)=10.139$, $p=0.003$; post hoc SAL-Ctrl v. SAL-RMS, $p=0.048$; SAL-Ctrl v. MIA-Ctrl, $p=0.032$).

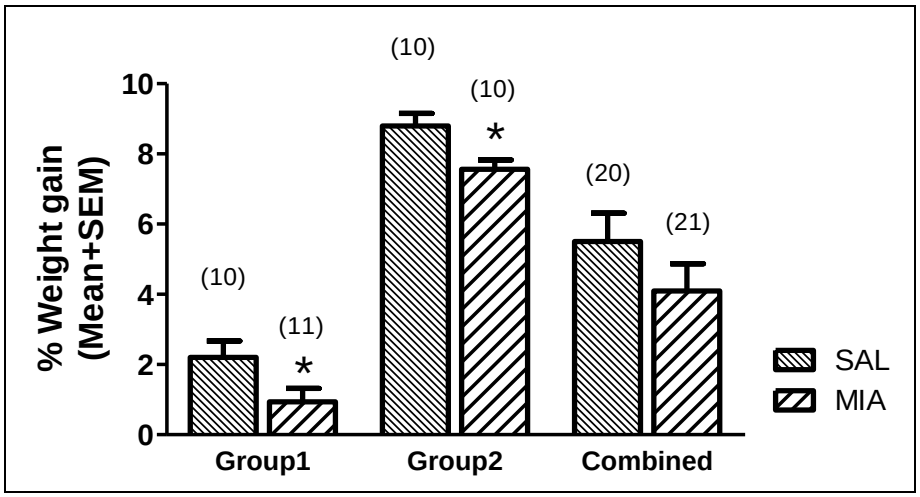

Figure 5. *Parent generation dams weight changes one day post saline or polyI:C injections.* 4.0 mg/kg polyI:C on gestation (G) day 13 was sufficient to induce significantly lower weight gains in pregnant dams on G 14 compared to saline when data for individual replications were analyzed independently. However, when combined, the difference was not statistically significant. Parenthesis denotes n value for each condition. *$p<0.05$ compared to SAL.

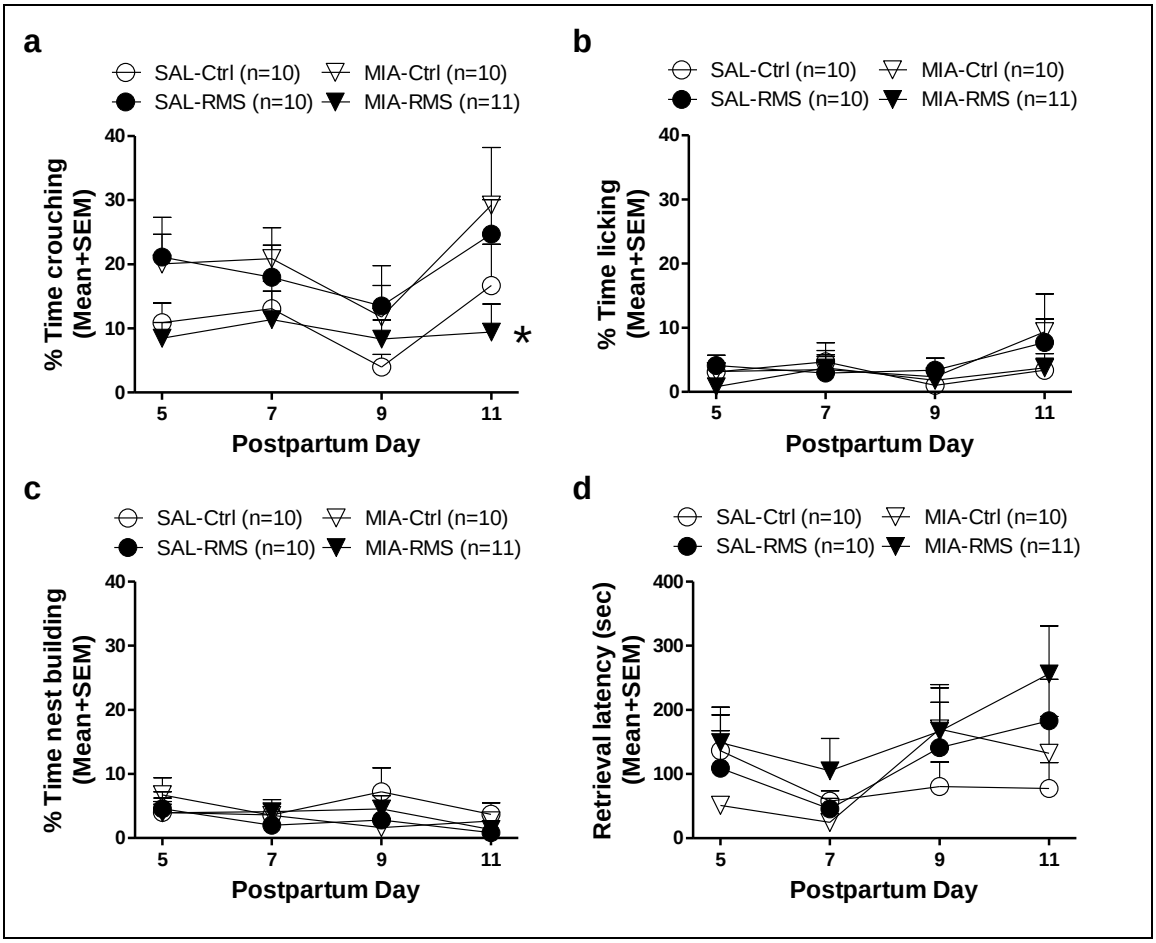

Figure 6. *Maternal immune activation and repeated maternal separation alter maternal behavior of parent (F0) generation dams.* % time spent crouching/nursing pups (a), licking/grooming pups (b), nest building (c), and retrieval latency (d). F0 dams with offspring that were either exposed to only maternal immune activation (MIA-Ctrl) *in utero* or repeated maternal separation (SAL-RMS) during infancy exhibited significantly higher % time spent in crouching/nursing behavior relative to F0 dams with offspring that were exposed to the combination of MIA-RMS (a). *$p<0.05$ relative to the MIA-Ctrl and SAL-RMS groups across days of testing.

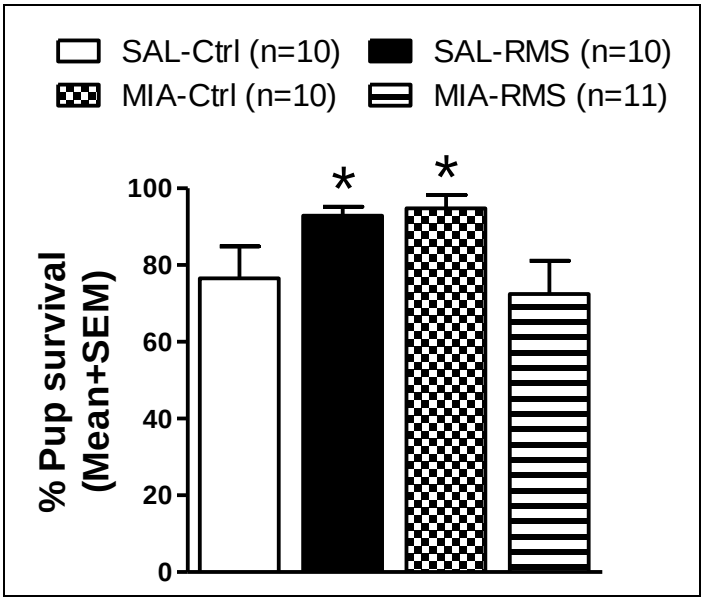

Figure 7. *Maternal immune activation and repeated maternal separation alter offspring (F1) survival.* F1 animals exposed either to only repeated maternal separation (RMS) or maternal immune activation (MIA) exhibited higher overall survival rate from day of culling (postnatal day [PND] 1) to weaning (PND 21). *p<0.020 relative to both the control (SAL-Ctrl) and combined stress (MIA-RMS) groups.

Effect of maternal immune activation on F1 offspring. In pup retrieval, MIA significantly decreased the % time nest building (Fig. 8c; MIA × RMS interaction, $F(1,32)=7.087$, $p=0.012$; post hoc SAL-Ctrl v. MIA-Ctrl, $p=0.011$). In baseline PPI, MIA disrupted average % PPI (Fig. 9b; main effect of MIA, $F(1,64)=4.496$, $p=0.038$). In FST, MIA reduced % time struggle in virgin females on session 3 (Fig. 10e, Session × MIA × Reproductive experience interaction, $F(2,33)=4.710$, $p=0.016$; post hoc SAL-nulliparous v. MIA-nulliparous in session 3, $p=0.004$). In the FPS procedure, MIA significantly reduced ASR across the pre- and post-fear conditioning sessions (Fig. 11a; MIA × RMS interaction, $F(1,64)=11.525$, $p=0.001$; post hoc SAL-Ctrl v. MIA-Ctrl, $p=0.031$). Finally, in the FPS challenge test, MIA significantly reduced startle magnitude in virgins during CS+ trials (Fig. 12a; Trial type × MIA × RMS × Reproductive experience interaction, $F(1,64)=5.397$, $p=0.023$; post hoc SAL-nulliparous v. MIA-nulliparous in CS+, $p=0.005$).

Effect of maternal separation on F1 offspring. In pup retrieval, RMS significantly reduce % time nest building (Fig. 8c; MIA × RMS interaction, $F(1,32)=7.087$, $p=0.012$; post hoc SAL-Ctrl v. SAL-RMS, $p=0.004$). In baseline ASR, RMS reduced startle magnitude (Fig. 9a; MIA × RMS interaction, $F(1,64)=10.037$, $p=0.002$; post hoc SAL-Ctrl v. SAL-RMS, $p=0.021$). In the FPS procedure, RMS reduced ASR across the pre- and post-fear conditioning sessions (Fig. 11a; MIA × RMS interaction, $F(1,64)=11.525$, $p=0.001$; post hoc SAL-Ctrl v. SAL-RMS, $p=0.001$). Finally, in the FPS challenge test, RMS significantly reduced startle magnitude during both CS- and CS+ trials in mothers, and during CS+ trials in virgins as well (Fig. 12a,b; Trial type × MIA × RMS × Reproductive experience four-way interaction, $F(1,64)=5.397$, $p=0.023$; SAL-Ctrl primiparous v. SAL-RMS primiparous in CS-, $p=0.020$; SAL-Ctrl primiparous v. SAL-RMS primiparous in CS+, $p=0.048$; post hoc SAL-Ctrl nulliparous v. SAL-RMS nulliparous in CS+, $p=0.001$).

Effect of reproductive experience on F1 offspring. In baseline PPI, postpartum females had significantly higher average % PPI in session 2 (Fig. 9b; Session × Reproductive experience interaction, $F(3,62)=4.320$, $p=0.008$; post hoc nulliparous v. primiparous in session 2, $p=0.002$), suggesting that reproductive experience significantly increased average % PPI. In FST, reproductive experience reduced % time struggle in session 3 (Fig. 10e,f; Session × MIA × Reproductive experience interaction, $F(2,33)=4.710$, $p=0.016$; post hoc SAL-nulliparous v. SAL-primiparous, $p=0.002$). In SPT, reproductive experience decreased sucrose preference (Fig. 13; $F(1,64)=6.433$, $p=0.014$). In the FPS procedure, reproductive experience significantly increased % ASR habituation across the pre- and post-conditioning sessions (Fig. 11b; main effect of reproductive experience, $F(1,64)=20.612$, $p<0.001$). Finally, reproductive experience also increased average % PPI across the pre- and post-conditioning sessions (Fig. 11c; main effect of reproductive experience, $F(1,64)=4.259$, $p=0.043$).

Overall within-subjects effects. In baseline maternal behavior observations, due to the low number of observations made across the majority of behaviors, only data for arched back and blanket nursing were analyzed (Fig. 14). There was a main effect of time of day (AM and PM), $F(1,32)=9.850$, $p=0.004$, with less nursing behaviors observed overall during the PM sessions. In baseline ASR, there was a main effect of session, $F(3,62)=3.952$, $p=0.012$ (Fig. 9a). Pairwise comparisons (LSD mmd = 1.462) showed that ASR was lower in the pup-present sessions compared to the pup-absent sessions. In the FST, all animals displayed a significant main effect of session in % immobile and % swimming across sessions, $F(2,33)=30.307$, $p<0.001$ and $F(2,33)=29.049$, $p<0.001$, respectively (Fig. 10a-d). In the FPS procedure, ASR across the pre- and post-conditioning sessions showed a main effect of session, $F(3,62)=4.696$, $p=0.005$ (Fig. 11a). Pairwise comparisons (LSD mmd = 1.051) showed that ASR was significantly higher immediately post-fear conditioning. Likewise, % ASR habituation also indicated a main effect of session, $F(3,26)=4.808$, $p=0.004$ (Fig. 11b). Pairwise comparisons (LSD mmd = 24.355) showed that % ASR habituation was also significantly lower immediately post-fear conditioning. Finally, during the FPS challenge test, there was a main effect of trial type, with higher ASR during CS+ trials, $F(1,64)=48.638$, $p<0.001$ (Fig. 12a,b).

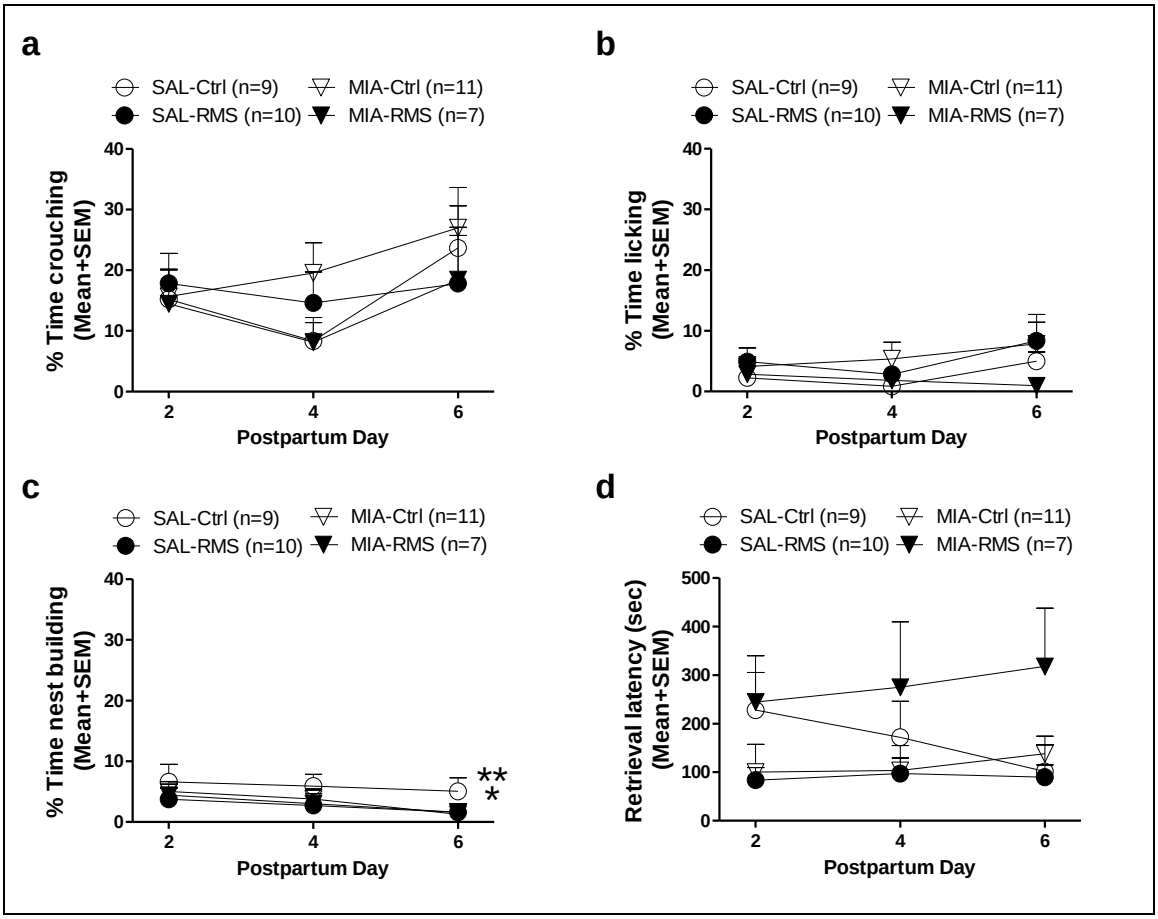

Figure 8. *Maternal immune activation and repeated maternal separation alter maternal behavior of offspring (F1) generation dams.* % time spent crouching/nursing pups (a), licking/grooming pups (b), nest building (c), and retrieval latency (d). F1 dams exposed either to only maternal immune activation (MIA) *in utero* or repeated maternal separation (RMS) during infancy exhibited significantly less % time spent nest building compared to control (SAL-Ctrl) animals (c). *p<0.05 relative to the MIA-Ctrl group and **p<0.01 relative to the SAL-RMS group.

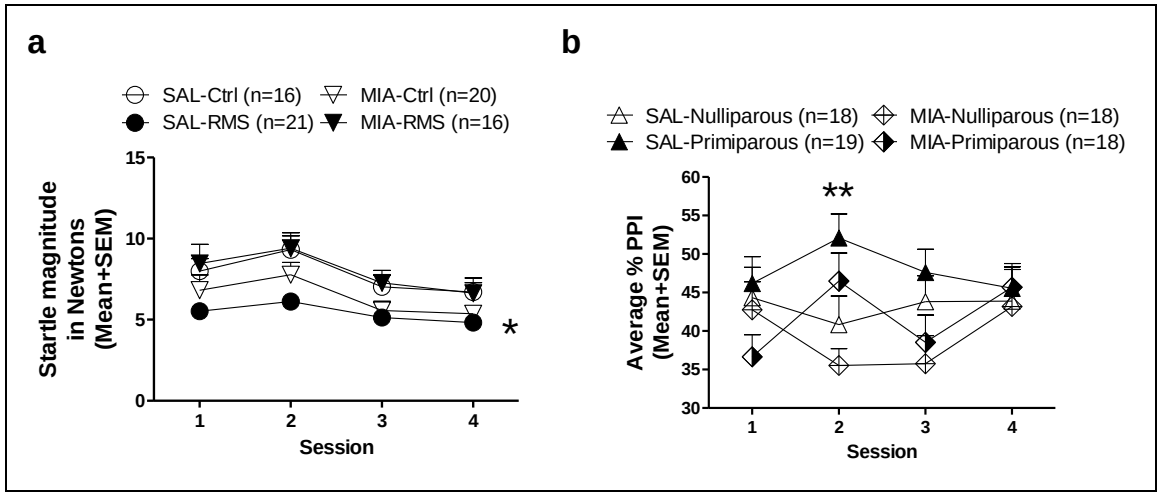

Figure 9. *Maternal immune activation, repeated maternal separation, and reproductive experience alter acoustic startle response of prepulse inhibition.* Acoustic startle response (ASR) of F1 adult females with reproductive experiences combined (a). Average % prepulse inhibition (PPI) of F1 adult females with postnatal stress conditions combined (b). ASR was significantly lower for all F1 adult females in the pup-present sessions 3 and 4 (postpartum days [PPD] 9 and 11) compared to pup-absent sessions 1 and 2 (PPD 5 and 7). F1 adult females subjected to repeated maternal separation alone (SAL-RMS) also displayed significantly lower ASR relative to control (SAL-Ctrl) and combined stressor (MIA-RMS) females, irrespective of reproductive experience (a). In contrast, both reproductive experience and *in utero* stress affected average % PPI, with F1 primiparous females exhibiting higher average % PPI in session 2 (PPD 7) compared to nulliparous females, and all MIA females exhibiting lower average % PPI relative to non-MIA females across the four test sessions (b). *$p<0.05$ relative to both the SAL-Ctrl and MIA-RMS groups across test sessions. **$p<0.01$ between primiparous and nulliparous females comparison in session 2.

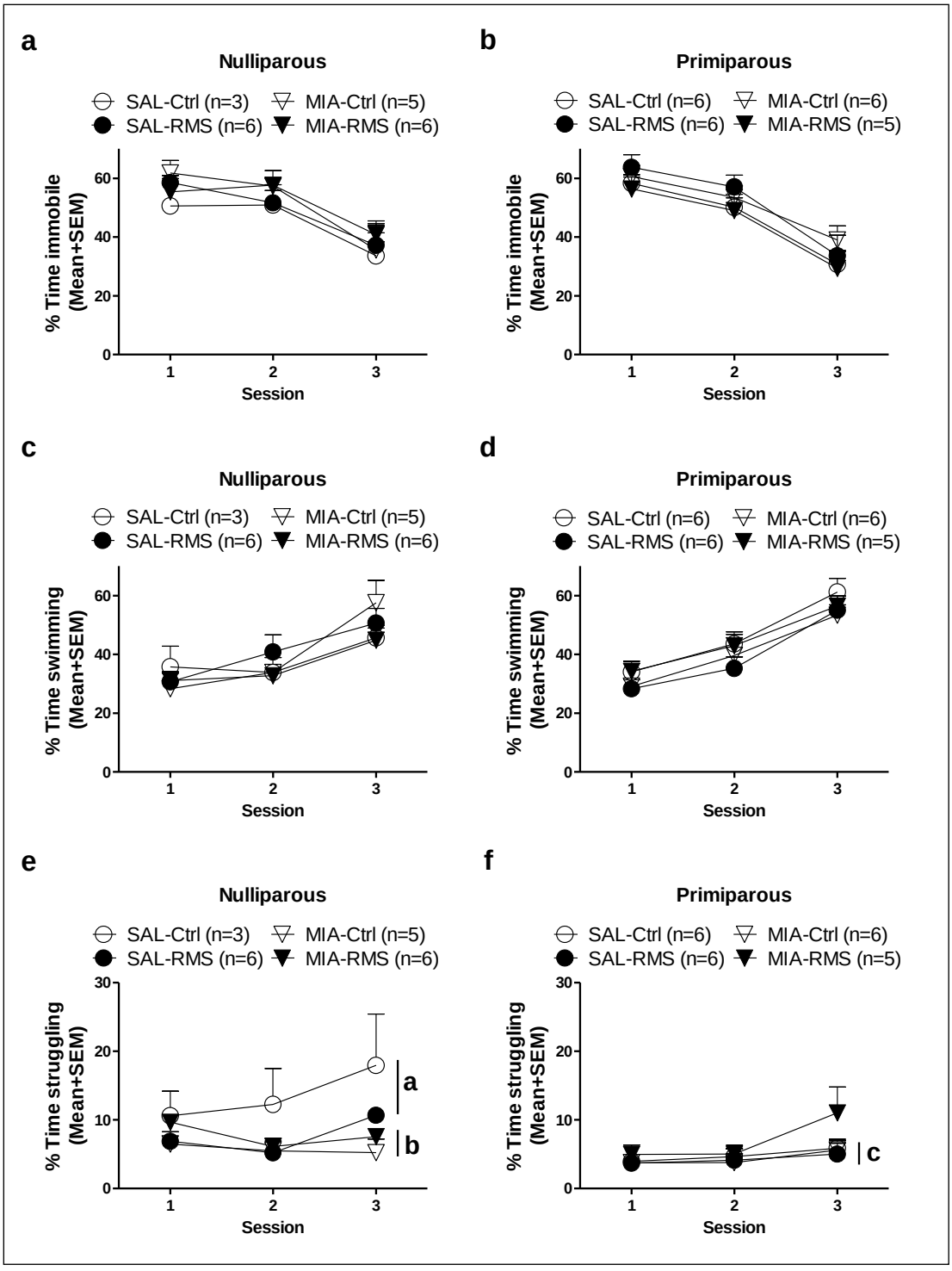

Figure 10. *Maternal immune activation and reproductive experience alter active struggling behavior in the forced swim test.* % time spent immobile for nulliparous (a) and primiparous (b) females from all four early environmental treatments. % time spent swimming for nulliparous (c) and primiparous (d) females. % time spent struggling for nulliparous (c) and primiparous (d) females. All non-MIA nulliparous females (SAL) exhibited significantly more % time spent struggling compared to MIA nulliparous females as well as SAL primiparous females across the three test sessions (c,d). $p<0.01$ between ***a,b*** (all SAL-nulliparous versus all MIA-nulliparous) and ***a,c*** (all SAL-nulliparous versus all SAL-primiparous) comparisons.

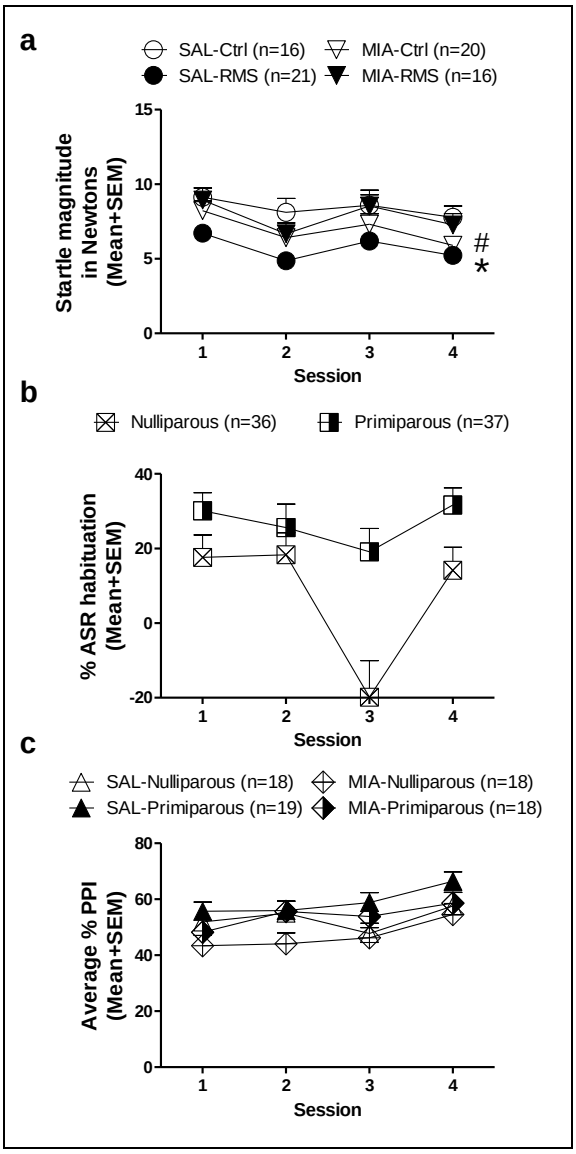

Figure 11. *Maternal immune activation, repeated maternal separation, and reproductive experience alter acoustic startle response, % acoustic startle response habituation, and average % prepulse inhibition.* Acoustic startle response (ASR) of F1 females from each early environment treatments groups with reproductive experience combined (a). % ASR habituation of F1 adult virgin and primiparous females with all early environment conditions combined (b). Average % prepulse inhibition (PPI) of F1 adult females with repeated maternal separation (RMS) conditions combined (c). ASR was significantly lower for all F1 adult females in session 2 (second baseline session prior to fear conditioning) compared to session 1, higher in session 3 (first baseline session post fear conditioning) compared to session 2, and again lower in session 4 (second baseline session post fear conditioning) compared to session 3. F1 adult females subjected to RMS only (SAL-RMS) exhibited significantly lower ASR compared to both control (SAL-Ctrl) and combined stressor females (MIA-RMS) irrespective of reproductive experience across the four test sessions, and MIA only (MIA-Ctrl) females also showed lower ASR compared to SAL-Ctrl females irrespective of reproductive experience across the four test sessions (a). % ASR habituation was significantly lower in session 3 relative to sessions 1, 2, and 4, and all primiparous females displayed higher % ASR habituation relative to all virgin females across the four test sessions (b). All primiparous females also exhibited higher average % PPI compared to all nulliparous females across the four test sessions (c). *p<0.01 relative to SAL-Ctrl and MIA-RMS groups. #p<0.05 relative to the SAL-Ctrl group.

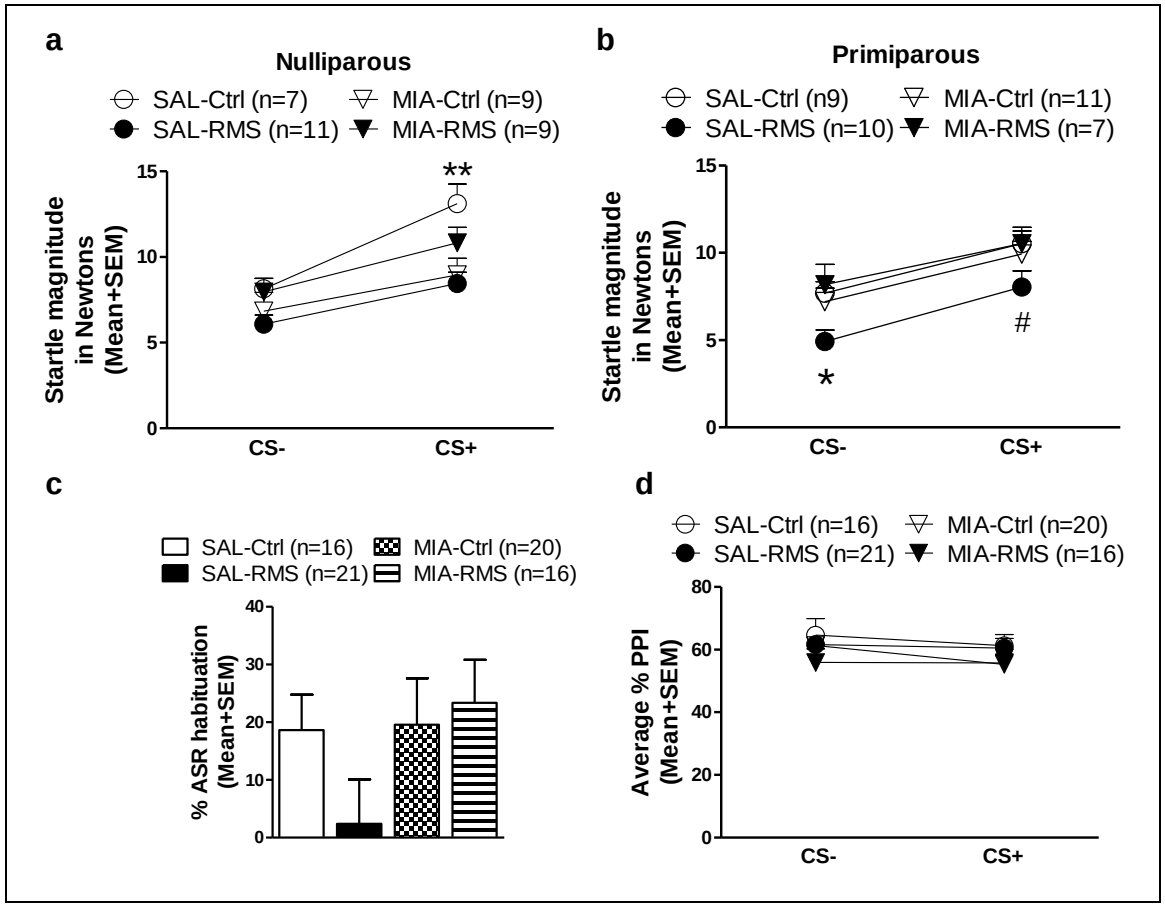

Figure 12. *Maternal immune activation, repeated maternal separation, and reproductive experience alter fear potentiated startle response.* Acoustic startle response (ASR) of nulliparous (a) and primiparous (b) F1 females from each early environment treatment groups. % ASR habituation of each treatment condition with reproductive experience combined (c). Average % prepulse inhibition (PPI) of each treatment condition with reproductive experience combined (d). All F1 females displayed significantly higher ASR during conditioned stimulus (i.e. light stimulus)-present (CS+) trials compared to CS-absent (CS-) trials. F1 virgins subjected either to only repeated maternal separation (SAL-RMS) or maternal immune activation (MIA-Ctrl) showed significantly lower ASR relative to control (SAL-Ctrl) virgins during CS+ trials (a). In addition, F1 SAL-RMS primiparous females exhibited significantly lower ASR compared to SAL-Ctrl and combined stressor (MIA-RMS) primiparous females during CS- trials. SAL-RMS primiparous females also showed significantly lower ASR compared to SAL-Ctrl primiparous animals during CS+ trials (b). *p<0.05 for RMS-Ctrl relative to SAL-Ctrl and MIA-RMS groups. #p<0.05 for RMS-Ctrl relative to SAL-Ctrl. **p<0.01 for SAL-Ctrl relative to SAL-RMS and MIA-Ctrl groups.

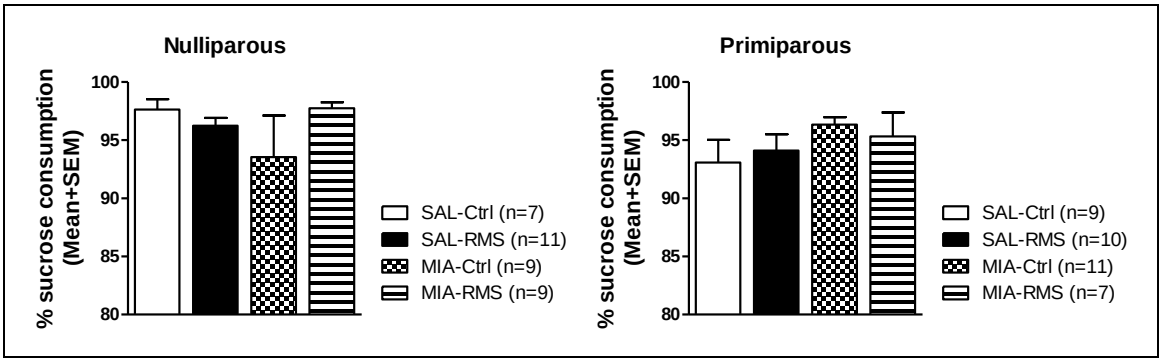

Figure 13. *Reproductive experience alters anhedonic state.* % sucrose consumption of nulliparous and primiparous females from all four early environmental treatments. There was a main effect of reproductive experience irrespective of early environmental treatment, p=0.014.

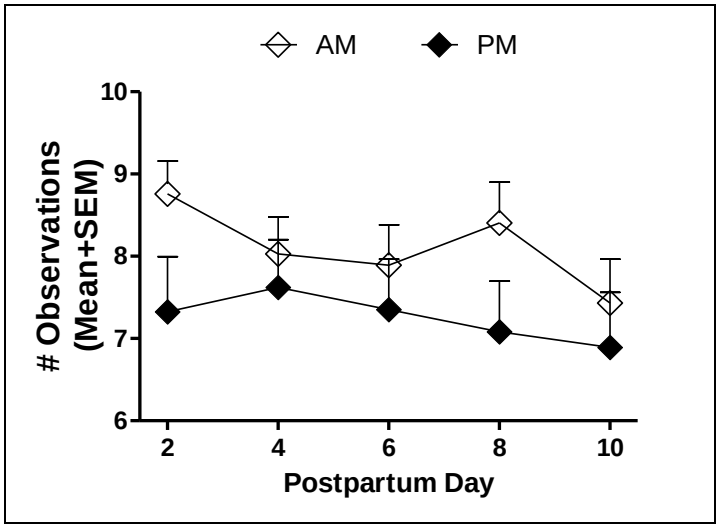

Figure 14. *Baseline maternal nursing behavior is dependent on the time of day.* The number of observations made for all nursing behaviors (i.e. both arched back and blanket nursing) during the AM period was significantly higher in F1 dams from all early environment conditions compared to the PM period. p=0.004 between AM and PM sessions comparison across test days.

Summary

In the parental generation, F0 dams subjected to MIA only or RMS only displayed higher % time crouching compared to F0 dams of MIA-RMS offspring, suggesting that changes in dams' behavior or their F1 pup behaviors influenced their maternal care. Interestingly, pup survival rates were higher in MIA- and RMS-only offspring, implicating a compensatory mechanism that improved the outcomes of these animals.

For F1 females, MIA significantly reduced nest building, suggesting a deficit in maternal behavior. In addition, MIA disrupted sensory motor gating (i.e. baseline average % PPI), reduced active stress-coping in virgins (i.e. % struggle in FST), and reduced fear potentiated startle in virgins as well (i.e. startle magnitude in FPS challenge CS+ trials). Similar to MIA, RMS also significantly reduced nest building. In addition, RMS reduced baseline anxiety (i.e. baseline and pre- and post- fear conditioning ASR), as well as fear potentiated startle (i.e. startle magnitude in FPS challenge CS+ trials).

Overall, reproductive experience enhanced sensory motor gating (i.e. baseline and pre- and post-fear conditioning average % PPI), reduced active stress-coping behavior (i.e. % struggle in FST), lowered hedonic state (i.e. sucrose preference), and increased habituation to an anxiogenic stimulus (i.e. % ASR habituation pre- and post-fear conditioning).

CHAPTER 4

EXPERIMENT 2

NEUROANATOMICAL SUBSTRATES ASSOCIATED WITH FEAR POTENTIATED STARTLE RESPONSE IN POSTPARTUM FEMALES EXPOSED TO EARLY ENVIRONMENTAL STRESS

Introduction

This study used c-Fos as a biomarker for neuronal activity and examined the relevant neural circuitry underlying early stress-induced behavioral changes. We examined the c-Fos expressions of adult females exposed to MIA *in utero* and/or RMS during infancy following the FPS response task. The design was aimed at two specific questions: 1) Does early environmental stress alter female brain activation following an anxiogenic procedure in adulthood? 2) Does reproductive experience influence brain activation of females exposed to early environmental stress following an anxiogenic procedure differentially than healthy females?

The general approach was to examine c-Fos immunoreactivity (IR) following the FPS test in adult virgin and primiparous females exposed to stress *in utero* and/or during early infancy. c-Fos is an immediate early gene that acts as a marker for recent cell activity (Morgan and Curran, 1991; Sagar *et al*, 1988). In particular, increased regional c-Fos signals has been observed following anxiogenic procedures in postpartum rats (Smith and Lonstein, 2008). In measuring c-Fos expression of females exposed to early

stress following the FPS response task, we hypothesized elevated c-Fos immunoreactivity in a regionally selective, reproductive experience dependent manner, indicative of increased stress responsivity.

Procedures

Tissue collection. 30 min following FPS testing, all animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p., Sigma-Aldrich, St. Louis, MO) and transcardially perfused with 0.02 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA, pH 7.4) in 0.1 M PBS. Brains were then harvested, post-fixed in 4% PFA overnight and cryoprotected in 30% sucrose for 72 hours and stored at -80°C until processing. Prior to immunohistochemistry, serial coronal sections (40 μm) were cut on a cryostat and collected in 0.02 M PBS.

Immunohistochemistry. The c-Fos immunohistochemistry procedure was modified from previous protocols established in our laboratory (Zhao and Li, 2010; Zhao *et al*, 2012). All steps were performed on ice. Free floating sections were incubated for 30 min in 1.5% hydrogen peroxide and 50% methanol for endogenous peroxidase inhibition, followed by three 10 min wash buffer rinses (0.05% normal goat serum [NGS] and 0.3% Triton x-100 in 0.02 M PBS). Sections were then incubated in blocking solution (10% NGS and 0.3% Triton x-100 in 0.02 M PBS) for 1 h, then transferred to rabbit polyclonal anti-c-Fos (1:3,000 dilution; sc-52, Santa-Cruz Biotechnology, Dallas, TX) in 0.02 M PBS containing 1% NGS, 0.3% Triton x-100 and 1% blocking reagent (Roche Diagnostics, Indianapolis, IN). Following a 24 h incubation in the primary antibody, sections were

rinsed three time in wash buffer, 10 min each, and incubated in biotinylated goat anti-rabbit secondary antibody (1:200 dilution, Vector Laboratories, Burlingame, CA) in 0.02 M PBS containing 1% NGS for 2 h. They were then rinsed three more times for 10 min each in 0.02 M PBS and incubated in avidin-biotinylated enzyme complex (1:200 dilution, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) for 1 h. 3,3'-diaminobenzidine (DAB peroxidase substrate, Vector Laboratories, Burlingame, CA) was used to reveal c-Fos labeling. Post staining, sections were mounted onto slides, air dried, dehydrated in a graded series of diluted ethanol, cleared in xylene, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). Normal horse serum substitution for primary antibody was used as a negative control, and no immunostained was visualized in these sections.

Image capture and cell counting. Photomicrographs of sections were captured with an INFINITY lite digital camera (Canada) equipped with an Olympus CX41RF microscope (Japan) using a 10x objective lens. Cell counting was conducted by an experimenter blinded to the treatment conditions. Images were processed using ImageJ (National Institutes of Health, USA). Each image was cropped to a 300 × 300 μm^2 unit area with the target region of interest in the center and background eliminated. The number of c-Fos IR cells was counted unilaterally in four serial sections from comparable anatomical levels. c-Fos immunoreactivity was determined by a constant threshold of size and staining intensity. Only clearly labeled, darkly stained nuclei were included (see sample micrographs in Fig. 17), and number of cells obtained from each slice in a given brain region per rat was averaged. The brain regions analyzed included

those involved in cognitive processing, motor function, motivation, learning, emotion regulation and maternal behavior (Fig. 15). Levels of brain slices, based on Paxinos and Watson, 2007, were: Bregma +3.00 mm (medial prefrontal cortex [mPFC]); Bregma +1.92 mm (dorsolateral striatum [DLSt], nucleus accumbens core [NAc], nucleus accumbens shell [NAs]); Bregma +1.44 mm (lateral septum [LS], medial septum [MS], dorsomedial striatum [DMSt]); Bregma -0.24 mm (cingulate cortex [Cg], bed nucleus of stria terminalis dorsal and ventral portions [BSTd/BSTv], medial preoptic area dorsal and ventral portions [mPOAd/mPOAv], ventromedial striatum [VMSt]); Bregma -2.92 mm (dentate gyrus [DG], lateral habenula [LH], paraventricular nucleus of the thalamus [PV], dorsomedial hypothalamus [DMH], ventromedial hypothalamus [VMH], basolateral amygdala [BLA], medial amygdala [MeA], central amygdala [CeA]); Bregma -6.24 (ventral tegmental area [VTA]); and Bregma -7.92 (periaqueductal grey dorsal and ventral portions [PAGd/PAGv], dorsal raphe [DR]).

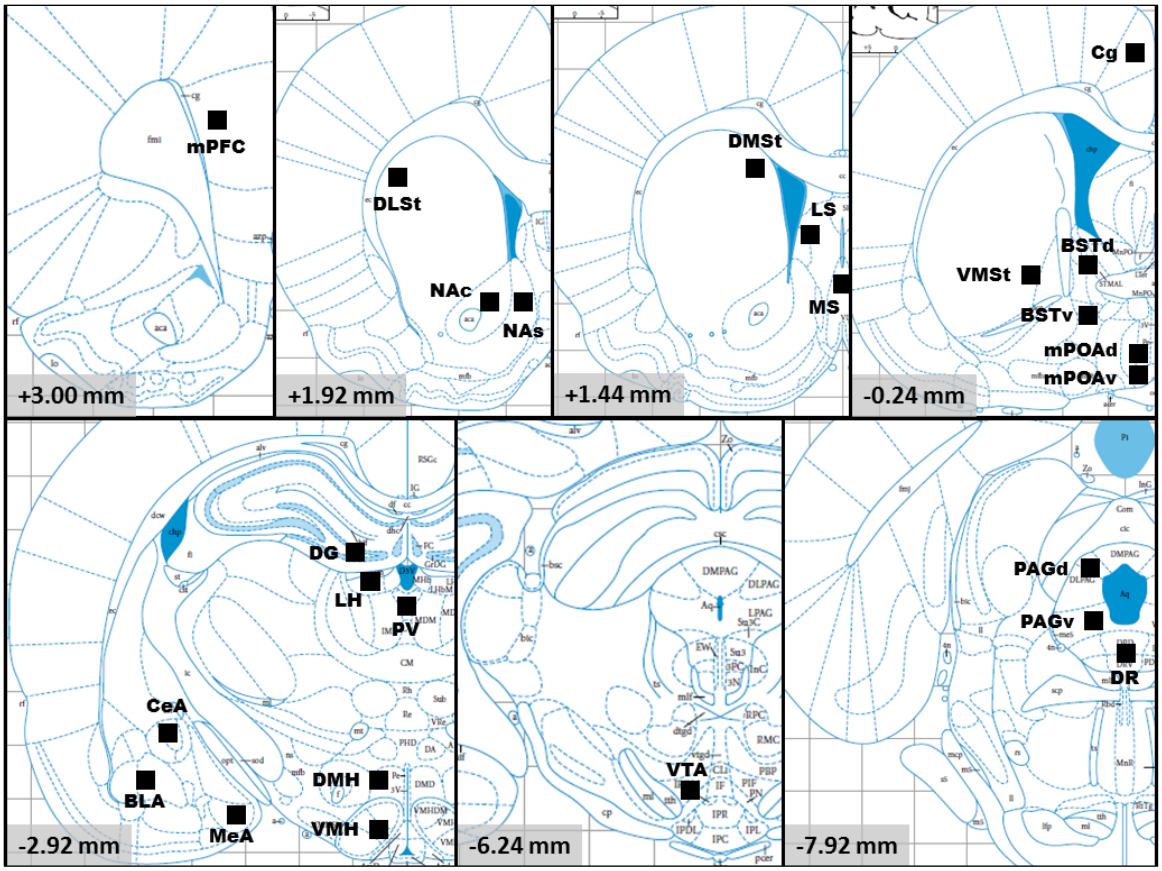

Figure 15. *Schematic representations of brain regions analyzed for c-Fos immunoreactivitiy.* Distance from Bregma (rostral: +, caudal: -) is indicated in mm unit. Drawings were modified from the altas of Paxinos and Watson, 2007. BLA, basolateral amygdala; BSTd, bed nucleus of stria terminalis, dorsal portion; BSTv, bed nucleus of stria terminalis, ventral portion; CeA, central amygdala; Cg, cingulate cortex; DG, dentate gyrus; DLSt, dorsolateral striatum; DMH, dorsomedial hypothalamus; DMSt, dorsomedial striatum; DR, dorsal raphe; LH, lateral habenula; LS, lateral septum; MeA, medial amygdala; mPFC, medial prefrontal cortex; mPOAd, medial preoptic area, dorsal portion; mPOAv, medial preoptic area, ventral portion; MS, medial septum; NAc, nucleus accumbens core; NAs, nucleus accumbens shell; PAGd, periaqueductal grey, dorsal portion; PAGv, periaqueductal grey, ventral portion; PV, paraventricular nucleus of thalamus; VMH, ventromedial hypothalamus; VMSt, ventromedial striatum; VTA, ventral tegmental area.

Dependent measures and statistical analysis

Overall difference in c-Fos expression was analyzed via repeated measures three-way ANCOVA (between-subjects factors: SAL/MIA, Ctrl/RMS, reproductive experience; within-subjects factor: regions; covariate: F0 maternal care). Data for individual brain

regions was also using univariate three-way ANCOVA (between-subjects factors: SAL/MIA, Ctrl/RMS, reproductive experience; covariate: F0 maternal care). All data were expressed as mean± SEM. Post hoc analyses were conducted using Bonferroni-adjusted comparisons tests. For all analyses, p=0.05 was considered statistically significant.

Results

Overall c-Fos immunoreactivity. Figure 16 depict sample micrographs of c-Fos immunohistochemistry. Table 1 summarizes the number of c-Fos IR cells (mean ± SEM) for each individual treatment group across the brain regions analyzed. As shown, there were significant regional differences in c-Fos IR comparisons. Repeated measures three-way ANCOVA revealed a significant main effect of region, $F(27,30)=6.921$, $p<0.001$.

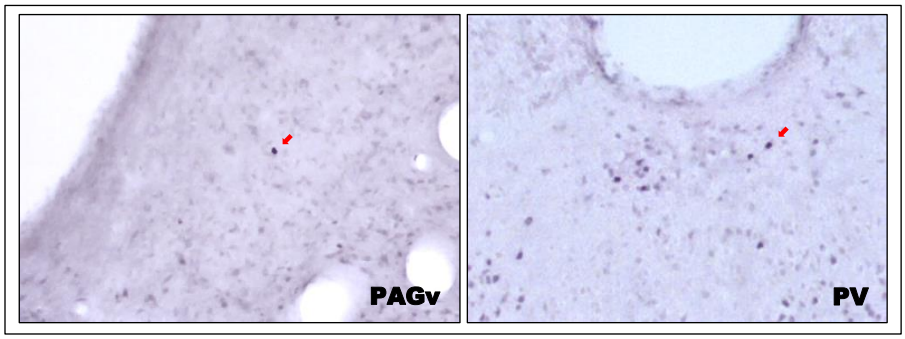

Figure 16. *Sample micrograph of c-Fos immunohistochemistry.* Sample images from PAGv and PV. Only clearly labeled, darkly stained nuclei (indicated by the red arrow) were included in the cell count analysis.

	SAL				MIA					
	CTRL		RMS		CTRL		RMS			
	Nulliparous	Primiparous	Nulliparous	Primiparous	Nulliparous	Primiparous	Nulliparous	Primiparous	*F*(1,63)	*p*
Regions with MIA main effect										
mPOAd	2.86 (.88)	7.33 (1.57)	4.73 (1.07)	5.90 (1.54)	10.51 (3.11)	6.52 (1.06)	7.97 (2.12)	7.86 (2.62)	5.491	0.022
PAGd	3.87 (.73)	4.00 (.53)	3.77 (.58)	3.24 (.72)	4.41 (.89)	5.09 (.89)	6.89 (1.51)	5.07 (.94)	6.034	0.017
Regions with RMS main effect										
DG	1.86 (.29)	1.64 (.25)	2.73 (.58)	1.95 (.41)	1.39 (.40)	1.48 (.38)	3.20 (.66)	1.86 (.19)	5.247	0.025
Regions with MIA x parity interaction										
BLA	3.54 (.65)	4.50 (1.00)	3.28 (.31)	4.28 (.60)	4.22 (.90)	3.14 (.54)	5.81 (1.45)	4.11 (.83)	4.013	0.049
MeA	4.36 (1.13)	9.64 (1.32)	6.47 (1.41)	8.33 (1.37)	7.25 (1.72)	9.25 (2.93)	14.84 (3.13)	8.07 (1.71)	4.777	0.033
PV	3.68 (1.02)	6.28 (.96)	5.95 (1.02)	6.55 (1.27)	7.97 (2.18)	3.80 (.75)	8.81 (1.90)	8.07 (2.17)	5.09	0.028
Other regions										
BSTv	9.04 (2.50)	10.19 (1.74)	7.31 (1.61)	8.91 (1.58)	8.53 (2.88)	7.98 (1.32)	9.78 (1.51)	8.04 (1.42)		
BSTd	0.57 (.35)	0.78 (.25)	0.56 (.22)	0.63 (.18)	1.15 (.49)	0.43 (.14)	0.58 (.22)	0.36 (.07)		
CeA	4.25 (.69)	4.81 (.73)	3.91 (.73)	4.65 (.94)	6.67 (1.66)	4.07 (.82)	6.07 (1.09)	5.32 (1.11)		
Cg	6.18 (1.59)	9.14 (2.30)	6.32 (.81)	6.26 (1.14)	7.95 (1.15)	8.99 (1.57)	11.61 (2.41)	8.00 (1.38)		
DLSt	0.79 (.34)	1.11 (.27)	0.61 (.14)	1.03 (.34)	0.78 (.66)	1.19 (.37)	0.61 (.22)	0.98 (.61)		
DMH	5.25 (1.37)	8.92 (1.62)	6.23 (.97)	6.53 (1.25)	5.31 (1.09)	6.77 (1.01)	9.71 (1.75)	7.64 (1.63)		
DMSt	0.83 (.15)	1.69 (.32)	1.00 (.22)	1.95 (.63)	1.53 (.92)	2.14 (.65)	1.28 (.43)	1.07 (.44)		
DR	1.74 (.53)	1.97 (.76)	0.84 (.25)	1.57 (.53)	2.22 (.29)	1.32 (.36)	2.90 (.74)	2.11 (.75)		
LH	2.25 (.55)	3.72 (.97)	2.39 (.48)	2.65 (.56)	4.22 (1.55)	1.86 (.46)	4.76 (1.75)	5.11 (1.25)		
LS	2.14 (.48)	3.47 (1.08)	3.07 (.41)	4.43 (1.77)	2.08 (.42)	4.32 (1.06)	3.72 (1.19)	3.25 (.75)		
mPFC	4.14 (.63)	12.03 (3.35)	9.25 (1.67)	9.79 (2.68)	10.25 (2.76)	9.05 (1.74)	13.83 (3.33)	10.31 (2.26)		
mPOAv	4.75 (1.65)	7.53 (1.28)	5.00 (1.15)	5.76 (.92)	5.90 (1.89)	6.32 (1.21)	6.56 (.94)	5.11 (1.65)		
MS	4.29 (.53)	5.14 (.93)	4.23 (.59)	5.43 (1.35)	5.11 (1.49)	5.16 (.81)	5.64 (.95)	5.25 (.70)		
NAc	4.89 (1.54)	6.14 (1.46)	2.89 (.43)	4.13 (1.01)	3.99 (1.23)	5.26 (1.00)	5.25 (1.24)	3.95 (1.17)		
NAs	4.11 (1.70)	4.92 (1.28)	2.39 (.55)	3.25 (.72)	3.21 (1.06)	3.28 (.73)	4.81 (1.25)	3.00 (.73)		
PAGv	3.18 (.90)	4.17 (1.08)	3.30 (.98)	3.00 (.81)	3.14 (.62)	3.61 (.80)	5.17 (.78)	5.32 (.85)		
PV	3.68 (1.02)	6.28 (.96)	5.95 (1.02)	6.55 (1.27)	7.97 (2.18)	3.80 (.75)	8.81 (1.90)	8.07 (2.17)		
VMH	5.71 (2.13)	9.89 (2.01)	6.41 (1.43)	7.05 (1.49)	6.58 (1.50)	6.70 (1.26)	7.70 (1.71)	8.18 (1.59)		
VMSt	0.71 (.43)	1.44 (.41)	0.72 (.22)	1.78 (.61)	1.08 (.58)	0.98 (.25)	1.86 (.50)	1.14 (.49)		
VTA	1.43 (.34)	2.69 (.53)	2.30 (.49)	2.40 (.60)	2.41 (.66)	2.02 (.53)	4.36 (1.08)	2.75 (.97)		

Table 1 *Number (±SEM) of c-Fos immunoreactive cells in individual brain regions.* Statistical results (F and *p* values) are indicated for regions with significant main effects and interactions.

Effect of maternal immune activation on F1 offspring. MIA significantly increased c-Fos IR in the mPOAd and PAGd (Table 1, F and *p* values shown in the right most columns of the table) in both nulliparous and primiparous females. MIA also significantly increased c-Fos expression in the BLA, MeA, and PV in virgin females (Fig. 17a,c,e; see Table 1 for F and *p* values; post hoc SAL-nulliparous v. MIA-nulliparous for BLA, p=0.047; MeA, p=0.005; PV, p=0.007).

Effect of repeated maternal separation on F1 offspring. RMS significantly increased c-Fos IR in the DG (see Table 1 for F and *p* values).

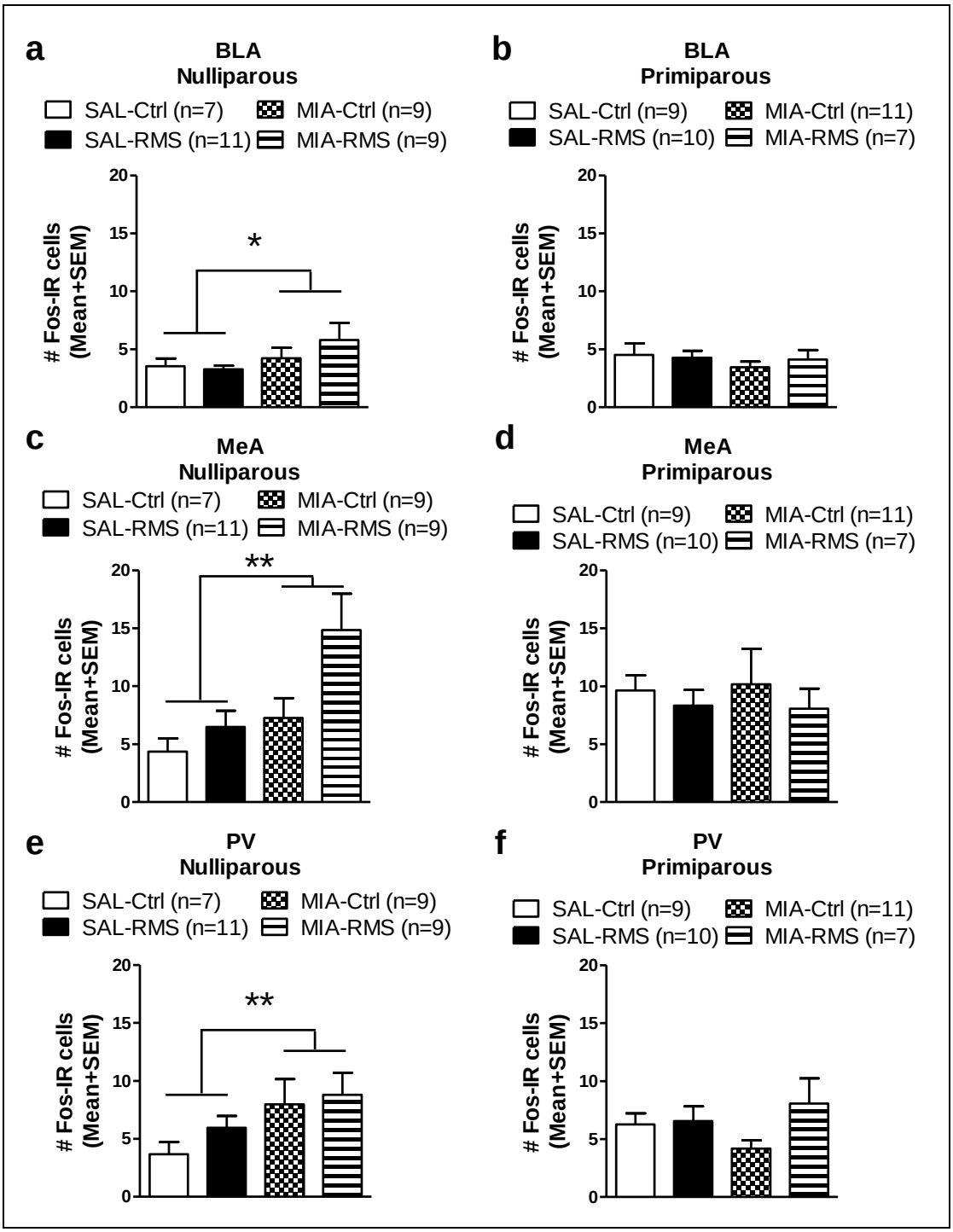

Figure 17. *Maternal immune activation and reproductive experience alter fear potentiated startle-induced c-Fos expression in the basolateral amygdala, medial amygdala, and paraventricular nucleus of the thalamus.* Number of c-Fos immunoreactive (IR) cells in the basolateral amygdala (BLA) of F1 nulliparous (a) and primiparous (b) females from all early environment treatment groups. Number of c-Fos IR cells in the medial amygdala (MeA) of virgin (c) and primiparous (d) females. c-Fos expression in the paraventricular nucleus of thalamus (PV) of virgin (e) and primiparous (f) females. All virgin F1 females exposed to maternal immune activation (MIA) had significantly higher numbers of c-Fos IR cells in the BLA, MeA, and PV compared to non-MIA exposed virgin females (SAL) (a,c,e). (f). *p<0.05 and **p<0.01 between comparison groups.

Summary

Following the FPS challenge test, MIA induced higher levels of c-Fos expression in the dorsal medial preoptic area and dorsal periaqueductal grey. In addition, in nulliparous females only, MIA also showed higher c-Fos IR in the basolateral and medial amygdala and the paraventricular nucleus of the thalamus. In contrast, RMS showed only an increase in c-Fos expression in the dentate gyrus.

CHAPTER 5

EXPERIMENT 3

PHARMACOLOGICAL AND BEHAVIORAL INVESTIGATIONS OF RECEPTOR FUNCTIONS AND LEARNING PROCESSES IN POSTPARTUM FEMALES EXPOSED TO EARLY ENVIRONMENTAL STRESS

Introduction

This study examined the brain receptor functions and associative learning processes of adult females exposed to MIA *in utero* and/or RMS during infancy. The design was aimed at three specific questions: 1) Does early environmental stress alter brain receptor functions in adult females? 2) Are learning processes altered in adult females exposed to early environmental stressors? 3) Does reproductive experience influence brain receptor functions and learning processes of females exposed to early environmental stress differentially than healthy females? The specific transmitter systems of interest included the dopaminergic, glutamatergic, nicotinic cholinergic, and serotonergic systems.

The general approach was to record locomotor activities following various agonist and antagonist injections. Amphetamine, as a dopaminergic transmission enhancer, increases locomotion in subjects with hyperdopaminergic function (Chou *et al*, 2015b). Conversely, phencyclidine (PCP) is an NMDA receptor antagonist, and increased PCP-induced hyperlocomotion may be indicative of hypoglutamatergic

function (Chou *et al*, 2015a). In addition, nicotine, a nicotinic acetylcholine receptor agonist, is known to induce acute hyperlocomotion and increased locomotor activity may represent increased nicotinic cholinergic function (Cohen *et al*, 1991; Welzl *et al*, 1988). Finally, as a selective serotonin receptor 2 agonist, DOI has also been shown to enhance basal locomotion, and increased DOI-hyperlocomotion is indicative to hyperserotonergic function as well (Zaniewska *et al*, 2009).

In addition to receptor functions, we also examined learning processes. Using a conditioned stimulus (CS, light) paired with an unavoidable unconditioned stimulus (US, shock) followed by a CS-avoidable US task, we assessed the avoidance response as a measure of the subject's ability to evaluate stimulus associations (Bolles, 1970; Crawford and Masterson).

In examining receptor functional changes, we hypothesized an increase in amphetamine-, phencyclidine- and nicotine-induce hyperlocomotion, and a decrease in DOI-induced hyperlocomotion in females exposed to early environmental stress. We also hypothesized an attenuation of these alterations by reproductive experiences. Furthermore, we hypothesized a deficit in associative learning in females exposed to early environmental stress based on our preliminary work, and the attenuation of this disruption by reproductive experience.

Apparatus

Locomotor activity monitoring apparatus. Sixteen 48.3 cm × 26.7 cm × 20.3 cm transparent polycarbonate cages housed in a quiet room served as locomotor monitoring chambers. Each cage was similar to the home cages, but equipped with a row of 6 photocell beams (7.8 cm between two adjacent photobeams) placed 3.2 cm above the floor of the cage. A computer with recording software (Aero Apparatus Sixbeam Locomotor System v1.4) was used to detect the disruption of the photocell beams and recorded the number of beam breaks.

Two-way avoidance conditioning apparatus. Eight identical two-way shuttle boxes (Med Associates, St. Albans, VT) were used. Each box (64 cm W x 24 cm D x 30 cm H from grid floor) was housed in a ventilated, sound-insulated isolation cubicle (96.52 cm W × 35.56 cm D × 63.5 cm H). Each box was divided into two equal-sized compartments by a partition with an arch style doorway (15 cm H × 9 cm D at base) and a barrier (4 cm H from grid floor) so that rats had to jump in order to cross from one compartment to the other. The grid floor consisted of 40 stainless-steel rods measuring 0.48 cm in diameter spaced 1.6 cm apart center to center, through which a scrambled footshock (unconditioned stimulus [US], 0.8mA, maximum duration: 5 s) was delivered by a constant current shock generator (Model ENV-410B) and scrambler (Model ENV-412). The rat location and crossings between compartments were monitored by a set of 16 photobeams (ENV-256-8P) affixed at the bottom of the box (3.5 cm above the grid floor). Illumination was provided by two houselights mounted at the top of each compartment.

The conditioned stimulus (CS; i.e. 76 dB white noise) was produced by a speaker (ENV 224 AMX) mounted on the ceiling of the cubicle, centered above the shuttle box. Background noise (~74 dB) was provided by a ventilation fan affixed at the top corner of each isolation cubicle. All training and testing procedures were controlled by Med Associates programs running on a computer.

Drugs and choice of doses

The injection solutions of amphetamine (1.5 mg/kg, s.c.; Sigma-Aldrich, St. Louis, MO), phencyclidine (PCP, 2.0 mg/kg, s.c.; gift from National Institute on Drug Abuse Chemical Synthesis and Drug Supply Program), 2,5-dimethoxy-4-iodo-amphetamine (DOI, 2.5 mg/kg, s.c.; RBI-Sigma, Natick, MA), and (–)-Nicotine hydrogen tartrate (0.4 mg/kg, s.c.; Sigma-Aldrich, St. Louis, MO) were obtained by mixing the drugs with 0.9% saline. Nicotine was further adjusted to pH 7.2 with diluted sodium hydroxide (NaOH) solution. All doses were chosen based on our previous work showing drug efficacy without induction of severe stereotypy (Charntikov *et al*, 2012; Li *et al*, 2012; Sun *et al*, 2009; Swalve *et al*, 2015a, 2015b; Zhao and Li, 2010). All drugs were all administered at 1.0 ml/kg.

Procedures

Figure 18 details the experimental timeline. All test subjects were F1 healthy female adults or those subjected to MIA *in utero* and/or RMS postnatally. Females were either tested on the postpartum days indicated or at the same age for virgin littermates.

One day prior to the first hyperlocomotion testing (PPD 17 or age matched nulliparous females), animals were habituated to the locomotion monitoring chambers for 30 min.

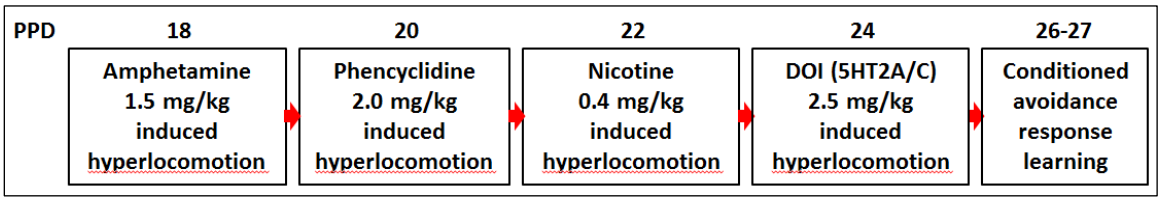

Figure 18. *Procedural timeline of behavioral assessments conducted in Experiment 3.* Time schedule is indicated by postpartum (PPD) days of the F1 primiparous subjects. F1 virgin females are age matched littermates.

Amphetamine-induced hyperlocomotion. The hyperlocomotion procedures were modified from protocols previously published by our group (Sun *et al*, 2009, 2010). All rats were injected with 0.9% saline and placed into the activity monitoring boxes, and their locomotor activities were recorded for 30 min. At the end of the 30 min period, rats were taken out, injected with amphetamine, and immediately placed back into the boxes for another 90 min. Locomotor activity (number of photobeam breaks) was measured and recorded in 5 min intervals throughout the entire 120 min testing session.

Phencyclidine-induced hyperlocomotion. PCP-induced hyperlocomotion followed the same procedure as that for amphetamine, with the exception that animals were injected with PCP at the end of the 30 min saline period.

Nicotine-induced hyperlocomotion. Nicotine-induced hyperlocomotion followed the same procedures as those for amphetamine and PCP, with the exception that animals were injected with nicotine at the end of the 30 min saline period.

DOI-induced hyperlocomotion. The DOI-induced hyperlocomotion procedure was modified from previously established locomotion test protocols by our group (Gao and Li, 2013; Sun *et al*, 2009, 2010). All animals were injected with DOI and immediately placed into the test chambers, and their locomotor activities were measured in 5 min intervals for 120 min.

Reinforcement attenuation and conditioned avoidance response. The procedure consisted of two phases: reinforcement attenuation and avoidance conditioning. The reinforcement attenuation procedure used the two-way shuttle chambers, attempting to reduce the reinforcement efficacy of the CS by selectively decreasing the shuttling response to the CS (Li *et al*, 2007). During the 30 trial session, each trial began with the presentation of a 76 dB white noise for 10 s, followed by a continuous scrambled footshock delivery (0.8 mA, 1 s) on the grid floor irrespective of the subject's motoric response (i.e. both a shuttling response or no response to the CS would result in the rat receiving the shock). One day later, the conditioned avoidance response (CAR) procedure was performed as previously described (Qiao *et al*, 2013). There were a total of 30 trials. Each trial began with the presentation of a 76 dB white noise for 10 s, followed by a continuous scrambled footshock (0.8 mA, 5 s) on the grid floor. If the rat moved from one compartment into the adjacent one within the 10 s sound presentation (recorded as "avoidance" behavior), the shock was not triggered and the sound was terminated. Intertrial intervals varied randomly between 30 and 60 s in both sessions. Number of avoidances in the CAR test day was recorded in 5-trial bins.

Dependent measures and statistical analysis

Locomotor activities (measured as the number of photobeam breaks) and CAR (measured as the number of avoidance in each 5-trial bin) were analyzed using repeated measures three-way ANCOVA (between-subjects factors: SAL/MIA, Ctrl/RMS, nulli/primiparous; within-subjects factor: time or trial bins; covariate: F0 maternal care). All data were expressed as mean± SEM. Post hoc analyses were conducted using either Bonferroni tests for between-groups effects or LSD mmd pairwise comparisons for within-groups effects. For all analyses, p=0.05 was considered statistically significant.

Results

Effect of maternal immune activation. In the 90-min PCP session, MIA nulliparous females exhibited significantly higher PCP-induced hyperlocomotion than MIA primiparous females (Fig. 20c; MIA × Reproductive experience interaction, $F(1,64)=4.096$, $p=0.047$; post hoc MIA-nulliparous v. MIA-primiparous, $p=0.016$). In addition, MIA caused a deficit in conditioned avoidance response that is evident in trials 20 to 25 (Fig. 23; Bin × MIA interaction, $F(5,56)=3.837$, $p=0.005$; post hoc SAL v. MIA in trial 20 to 25, $p=0.012$).

Effect of repeated maternal separation. In the 90-min amphetamine session, RMS reduced amphetamine-induced hyperlocomotion (Fig. 19c; MIA × RMS interaction, $F(1,64)=6.665$, $p=0.012$; post hoc SAL-Ctrl v. SAL-RMS, $p=0.008$). In the 90-min PCP session, RMS also significantly reduced PCP-induced hyperlocomotion (Fig. 20b; Time × RMS interaction, $F(17,48)=1.904$, $p=0.041$; post hoc RMS v. Ctrl for 30-, 35-, 40-, 65-min time point, $p=0.024$; 35-min, $p=0.046$). In the 90-min nicotine session, RMS alone significantly decreased nicotine-induced hyperlocomotion compared to MIA-RMS (Fig. 21c; $F(1,64)=5.916$, $p=0.018$; post hoc SAL-RMS v. MIA-RMS, $p=0.002$).

Effect of reproductive experience. In all three 30-min saline sessions (i.e. before amphetamine, PCP, and nicotine testing), reproductive experience significantly reduced baseline locomotion (Fig. 19a, 20a, 21a; $F(1,64)=28.718$, 19.732, 16.468 by session order, $p<0.001$ for all). In the 90-min amphetamine session, reproductive experience decreased amphetamine-induced hyperlocomotion compared to virgins at the start of the session, but increased hyperlocomotion at the end of the session (Fig. 19b; Time × Reproductive experience interaction, $F(17,48)=6.024$, $p<0.001$; post hoc primiparous v. nulliparous for 5-min time point, $p=0.009$; 10-min, $p<0.001$; 70-min, $p=0.048$; 75-min, $p=0.024$; 85-min, $p=0.044$). In the 90-min nicotine session, reproductive experience increased nicotine-induced hyperlocomotion (Fig. 21b; main effect of reproductive experience, $F(1,64)=7.044$, $p=0.010$; Time × Reproductive experience interaction, $F(17,48)=2.163$, $p=0.019$; post hoc primiparous v. nulliparous for 5-, 10-, 15-min time point, $p<0.001$; 20-min, $p=0.031$; 60-min, $p=0.005$; 90-min, $p=0.036$). Finally, in the 120-min DOI session, reproductive experience significantly decreased DOI-induced hyperlocomotion (Fig. 22; main effect of reproductive experience, $F(1,64)=14.941$, $p<0.001$).

Overall within-subjects effects. In the 30 conditioned avoidance response trials, there was a main effect of bins, $F(5,56)=6.093$, $p<0.001$ (Fig. 23). Pairwise comparisons (LSD mmd = 0.856) indicated a significantly higher number of avoidances in trials 20 to 25 and 25 to 30 compared to the first 15 trials.

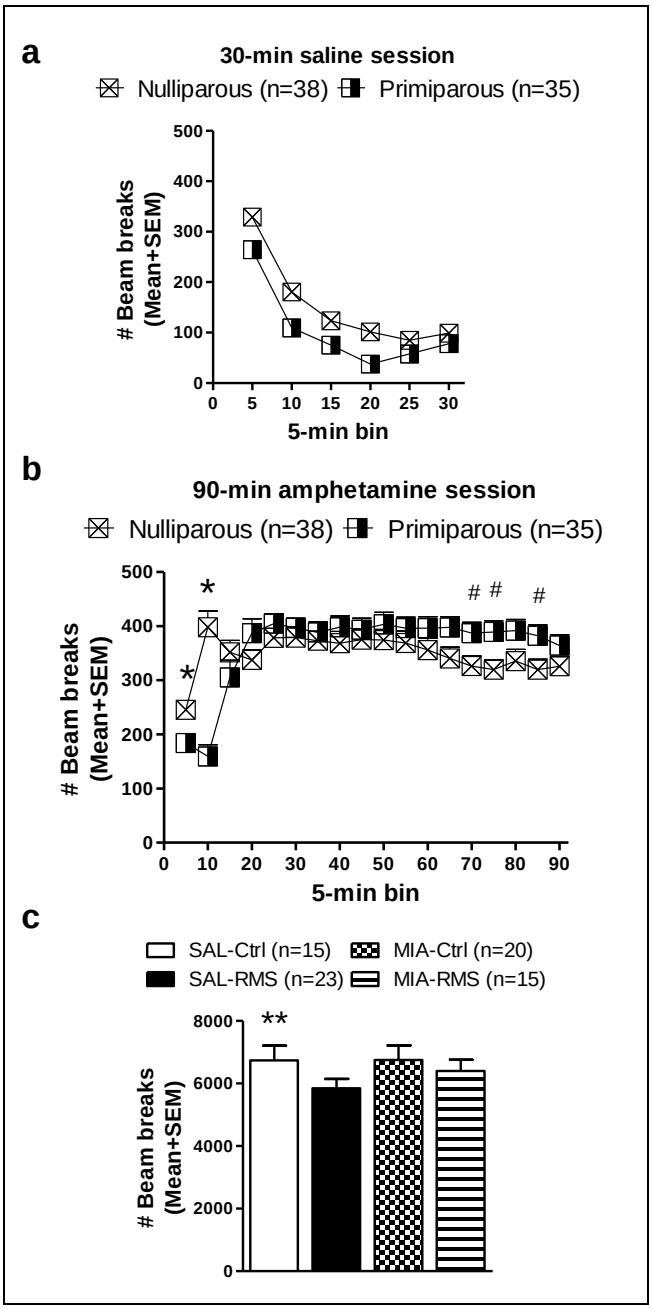

Figure 19. *Repeated maternal separation and reproductive experience alter amphetamine-induced hyperlocomotion.* Number photobeam breaks of F1 virgin and primiparous females recorded in 5 min intervals during the 30-min saline session with all early environmental treatments combined (a). Beam breaks of virgins and postpartum dams in 5 min intervals during the 90-min amphetamine session with all early environmental treatments combined (b). Total number of beam breaks during the 90-min amphetamine session for each early environmental conditions with reproductive experience combined (c). During the 30-min saline session, overall locomotion was significantly higher in virgins compared to mothers (a). Virgins also exhibited higher locomotion compared to postpartum dams at time points 5 and 10, and lower locomotion at 70, 75 and 85 min post amphetamine injection (b). Irrespective of reproductive experience, F1 adult females exposed to repeated maternal separation alone (SAL-RMS) made significantly fewer number of total beam breaks during the 90-min amphetamine session compared to control (SAL-Ctrl) females (c). *p<0.01 and #p<0.05 between reproductive experience comparison. **p<0.005 relative to the SAL-RMS group.

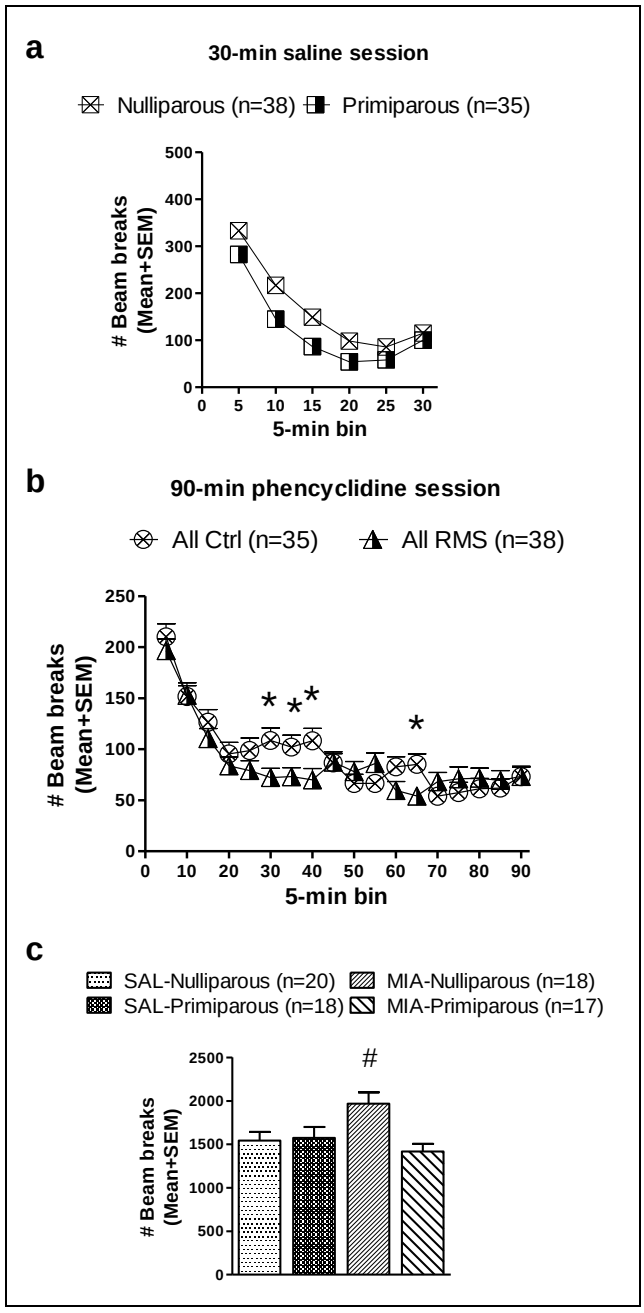

Figure 20. *Maternal immune activation, repeated maternal separation, and reproductive experience alter phencyclidine-induced hyperlocomotion.* Number photobeam breaks of F1 virgin and primiparous females recorded in 5 min intervals during the 30-min saline session with all early environmental treatments combined (a). Beam breaks of animals subjected to repeated maternal separation (RMS) or control (Ctrl) in 5 min intervals during during the 90-min phencyclidine (PCP) session with all prenatal stress and reproductive experience combined (b). Total number of photobeam breaks in the 90-min PCP session for all animals with RMS conditions combined (c). During the 30-min saline session, overall locomotion was significantly higher in virgins compared to mothers (a). RMS females, irrespective of reproductive experience or prenatal conditions, made significantly fewer beam breaks at time points 30, 35, 40, and 65 min post PCP injection relative to non-RMS animals (b). MIA virgins exhibited higher total number of PCP-induced photobeam breaks relative to MIA primiparous females (c). *p<0.05 relative to Ctrl females. #p<0.05 relative to the MIA-primiparous group.

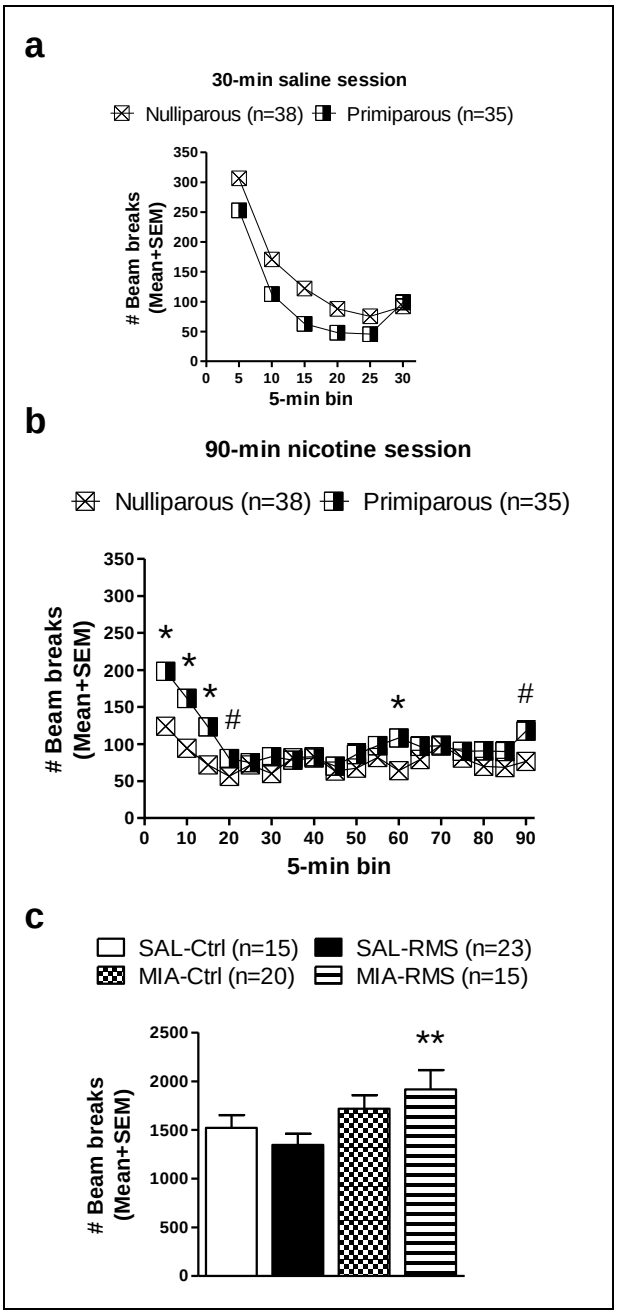

Figure 21. *Maternal immune activation, repeated maternal separation and reproductive experience alter nicotine-induced hyperlocomotion.* Number photobeam breaks of F1 virgin and primiparous females recorded in 5 min intervals during the 30-min saline session with all early environmental treatments combined (a). Beam breaks of virgins and postpartum dams in 5 min intervals during the 90-min nicotine session with all early environmental treatments combined (b). Total number of beam breaks during the 90-min nicotine session for each early environmental conditions with reproductive experience combined (c). During the 30-min saline session, overall locomotion was significantly higher in virgins compared to mothers (a). In contrast, virgins exhibited lower locomotion compared to postpartum dams at time points 5, 10, 15, 20, 60, and 90 min post nicotine injection (b). Irrespective of reproductive experience, F1 adult females exposed to repeated maternal separation alone (SAL-RMS) displayed significantly lower total nicotine-induced locomotion compared to combined stressor (MIA-RMS) females (c). #$p<0.05$ and *$p<0.01$ between comparison groups. **$p<0.005$ relative to the MIA-RMS group.

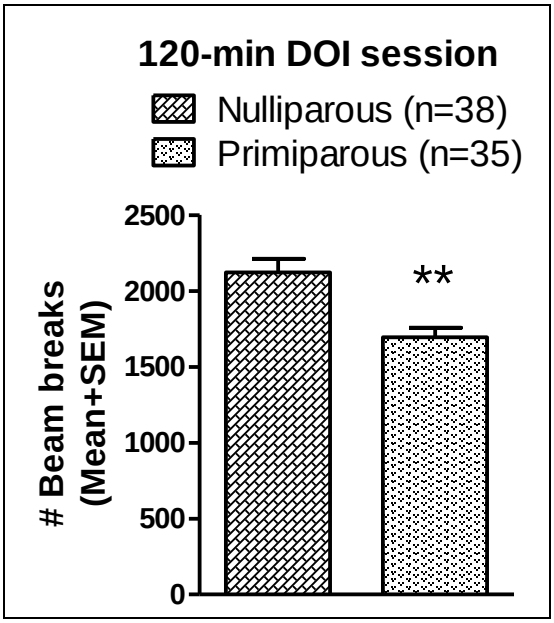

Figure 22. *Reproductive experience alters DOI-induced hyperlocomotion.* Total number of photobeam breaks during the 120-min DOI session showed significantly lower locomotion in F1 primiparous females compared to virgins, combining all early environment treatment groups. ** $p<0.001$.

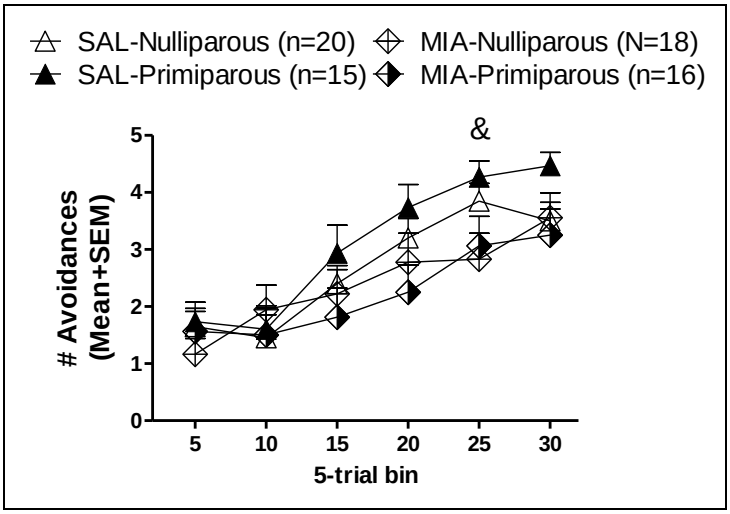

Figure 23. *Maternal immune activation alters conditioned avoidance response.* Number of avoidances (a) and escapes (b), recorded in 5-trial bins, are represented with repeated maternal separation (RMS) conditions combined. During trials 20 to 25, all F1 animals exposed to maternal immune activation (MIA) displayed significantly lower numbers of avoidances compared to non-MIA females (a). &$p<0.05$ between all SAL and all MIA animals.

Summary

For F1 females, results from this study show that MIA increased PCP-induced hyperlocomotion in virgin females compared to mothers. In contrast, RMS significantly decreased amphetamine- and PCP-induced hyperlocomotion, as well as reduced nicotine-induced hyperlocomotion relative to MIA-RMS females.

Overall, reproductive experience suppressed baseline locomotor activities, delayed the onset of amphetamine-induced hyperlocomotion, increased nicotine-induced hyperlocomotion, and reduced DOI-induced hyperlocomotion.

CHAPTER 6

GENERAL DISCUSSION

Introduction

The present study examined the effects of maternal immune activation (MIA) and repeated maternal separation (RMS) on behavioral and neuronal processes during the postpartum period. The goal of the design aimed to address four main issues: 1) The knowledge gap in the effects of MIA on postpartum functions; 2) The lack of information regarding effects of multiple early stressors on postpartum adaptations; 3) The unclarified distinction between contributions of parental care quality and maternal separation experience on postpartum behaviors of the female offspring; 4) The lack of nulliparous and primiparous population comparisons in females exposed to early life adversities to elucidate reproductive experience dependent alterations.

In our hypotheses, we reasoned that since MIA has been shown to disrupt adulthood functions, it would also interfere with postpartum behaviors. Considering previous work indicating an unmasking of MIA deficits following adolescent stress exposure (Deslauriers *et al*, 2013; Giovanoli *et al*, 2013), we also believed that the combination of MIA and RMS would synergistically exacerbate deficits seen in each stressors independently. In addition, through examination of parental (F0) generation maternal care toward the offspring (F1), we predicted that when the care quality is controlled as a covariate, the RMS experience would reveal unique contributions in

inducing maternal behavior, cognitive, affective, and neuronal deficits in F1 adult females. Finally, due to the multitude hormonal and structural modifications during the postpartum period, we expected certain abnormalities associated with MIA and RMS to manifest differentially based on reproductive experience.

Table 2 summarizes the results from the current study. While results from the experiments varied in the extent of substantiation, in general, we believe they were supportive of our hypotheses. We showed that MIA disrupted specific aspects of maternal behavior in F1 female offspring, that RMS produced behavioral deficits in postpartum offspring even after controlling for the F0 parent care variable, and that some disruptions associated with MIA were reproductive experience dependent. Interestingly, the results indicated an unexpected antagonistic interaction between MIA and RMS in maternal behavior, as well as a lack of interaction in the majority of the assessments.

	Parent (F0) generation		Offspring (F1) generation			
	MIA	RMS	MIA		RMS	
			Virgin	Mother	Virgin	Mother
Maternal behavior						
Nursing	↑*	↑*	-	↔	-	↔
Lick/grooming	↔	↔	-	↔	-	↔
Nest building	↔	↔	-	↓	-	↓
Retrieval latency	↔	↔	-	↔	-	↔
Pre-attentive cognitive function						
Prepulse inhibition	-	-	↓	↓	↔	↔
Learning processes						
Conditioned avoidance	-	-	↓	↓	↔	↔
Depressive-like behavior						
Immobility (forced swim)	-	-	↔	↔	↔	↔
Struggle (forced swim)	-	-	↓	↔	↔	↔
Sucrose preference	-	-	↔	↔	↔	↔
Anxiety-like behavior						
Baseline acoustic startle	-	-	↔	↔	↓	↓
Fear potentiated startle	-	-	↓	↔	↓	↓
Transmitter function						
Amphetamine	-	-	↔	↔	↓	↓
Phencyclidine	-	-	↔	↓**	↓	↓
Nicotine	-	-	↔	↔	↓*	↓*
DOI	-	-	↔	↔	↔	↔
c-Fos expression post fear potentiated startle						
Thalams						
Paraventricular nucleus	-	-	↑	↔	↔	↔
Hypothalamus						
Dorsal medial preoptic area	-	-	↑	↑	↔	↔
Amygdala						
Basolateral amygdala	-	-	↑	↔	↔	↔
Medial amygdala	-	-	↑	↔	↔	↔
Hippocampus						
Dentate gyrus	-	-	↔	↔	↑	↑
Periaqueductal grey (PAG)						
Dorsal PAG	-	-	↑	↑	↔	↔

Table 2 *Comparison of the effects of maternal immune activation (MIA) and repeated maternal separation (RMS) in F0 and F1 females.* Behavioral and brain alterations of the MIA and RMS models in parent (F0) dams and offspring (F1) nulli- and primiparous females are compared to age-matched control subjects within the same reproductive experience conditions (exceptions: *compared to MIA-RMS combined; **compared to MIA virgins).↑, increase; ↓, decrease; ↔, no significant effect; –, no data available.

Parent (F0) generation

MIA increased nursing behavior in F0 dams. Our results revealed a surprising effect of MIA on F0 maternal care, namely an increase in nursing behavior as indicated by the duration spent crouching atop F1 pups. The result trended toward significance relative to controls, and was significantly different compared to combined stress subjects. To our knowledge, this is the first study that examined the effect of gestational polyI:C administration on the maternal behavior of the F0 dams.

Previous work suggest that exposure to immostimulants postpartum may acutely induce sickness behavior and disrupt maternal care (Aubert *et al*, 1997), thus it is possible that the altered behavioral and brain functions seen here in the F0 dams were due to gestational weight loss and immune activation.

Alternatively, It is possible that the increase in nursing behavior seen in postpartum F0 mothers is triggered by abnormal behaviors of the F1 pups. Results from previous studies suggest that MIA produces deficits in the offspring that is evident in infancy (Malkova *et al*, 2012). Specifically, MIA pups emit less ultrasonic vocalizations (USV) during the early postnatal period (Chou *et al*, 2015b). This may seem counterintuitive at first, as maternal motivation is maintained by pup related sensory information postpartum (Numan, 2007), and studies have shown auditory adaptations postpartum specifically for enhanced saliency of pup USV (Lin *et al*, 2013). However, our recent study also suggested an age dependent alteration in pup USV that is reduced in MIA during the first postnatal week compared to control offspring, but increased during

the second postnatal week (Dunaevsky, unpublished). This in turn supports the current result of F0 nursing behavior increasing toward the later observation sessions.

RMS increased nursing behavior in F0 dams. In F0 RMS dams, we also saw an increase in nursing behavior. This is in line with previous work showing that 4 h daily RMS from postpartum day (PPD) 1 to 13 significantly increased active nursing behavior in F0 dams (Macrí *et al*, 2004). It is likely that the increased nursing behavior is the result of increased anxiety. While we did not asses F0 dams anxiety, others have found that RMS reduces the duration of time spent in the open arms of the elevated plus maze (EPM, [Maniam and Morris, 2010], but see Kalinichev *et al*, 2000). As suggested previously, in some women, postpartum anxiety may be manifested as "helicopter" parenting, resulting in increased offspring-direct behavior (Bridges *et al*, 2008).

Combined MIA and RMS normalized maternal care in F0 dams. Surprisingly, the increased nursing behavior in MIA and RMS F0 dams was attenuated by the combined treatment of MIA and RMS, suggesting an antagonistic effect between the two stressors. Interestingly, this is similar to the outcomes of combined MIA and RMS in various assays for F1 subjects also. The nursing behavior in F0 dams may be the result of an interaction between the aforementioned anxiety induced by RMS and the altered behaviors of MIA pups. However, more in depth examination is required to clarify this result.

Offspring (F1) generation

Effect of maternal immune activation

MIA reduced nest building behavior of offspring (F1) generation dams. This is the first study showing that *in utero* exposure to polyI:C produces deficits in maternal care of the female offspring. Our results showed that F1 MIA dams exhibited significantly less nest building behavior, indicative of decreased pup-directed activities. In a previous study using lipopolysaccharide (LPS) administration on gestation day (G) 9.5 in mice, Soto *et al*, 2013 showed a deficit in maternal care displayed by F1 MIA females on PPD 6. Similar to the current study, no differences were seen between control and MIA subjects in duration spent crouching/nursing, licking/grooming, and latency to first pup retrieval. However, in that study, F1 dams took a significantly longer time to complete full retrieval of the entire litter. Thus, it seems that MIA disrupts offspring maternal behavior and reduces maternal motivation.

MIA disrupted PPI and CAR in female offspring irrespective of reproductive experience. Our results of MIA cognitive deficits in prepulse inhibition (PPI) and conditioned avoidance response (CAR) were evident in both nulliparous and primiparous F1 MIA females. Current literature regarding the effect of MIA on PPI and learning have focused on males, while the results of sex-dependent effects are less clear (Meyer and Feldon, 2010). In general, MIA PPI and learning deficits in males have been reproduced across studies (Ozawa *et al*, 2006; Wolff and Bilkey, 2008; Zuckerman and Weiner, 2005; Zuckerman *et al*, 2003). In sex differences, one prior study examining polyI:C induced

PPI alterations found increased PPI in MIA females, contrary to the decrease seen in the current study and by others in male subjects (Vorhees *et al*, 2015). However, one distinction in that previous study is the separation of MIA offspring into groups whose mother showed a significantly large decrease in weight gain following polyI:C treatment, and those with a less substantial reduction. In this analysis, the results showed a PPI increase only in females from the high loss group. Thus, while MIA associated sex dependent alterations in cognitive functions still require further study, our result here support those seen by others and in our preliminary study in males, namely that MIA produces disruptions in pre-attentive sensory motor gating functions and learning. Here, we further established that cognitive deficits in MIA females are independent of reproductive experience.

MIA reduced FST struggling behavior and FPS in nulliparous offspring. The current study is the first to examine the effect of MIA on female offspring affective functions across reproductive experience. So far, few studies have examined the effect of *in utero* polyI:C exposure on depressive- and anxiety-like behaviors, mainly in male offspring. These have shown increased immobility in the forced swim test (FST), indicative of increased behavioral despair (Depino, 2015; Khan *et al*, 2014). In the current study, we found no increase in immobility by MIA in the FST. However, we did identify a reduction in struggle behavior in MIA virgins, which is suggestive of reduced active stress-coping (Singewald *et al*, 2011). Surprisingly, we also saw decreased acoustic startle following fear conditioning in virgin females, which may indicate reduced anxiety. While this was somewhat unexpected, the previously mentioned study by Vorhees and

colleagues also showed a reduction in acoustic startle in MIA females. However, studies in MIA male offspring have found anxiety-related behaviors in the EPM, open field test (OFT), light-dark box, and fear conditioning (Depino, 2015; Yee *et al*, 2012). As such, conclusions regarding *in utero* polyI:C's effect on female affective processes require further investigation. Interestingly, effects of MIA on depressive- and anxiety-like behaviors were only seen in nulliparous females, suggesting a possible modulation of emotion and mood regulation by postpartum adaptations. While this is mere speculation based on the novel results seen here, it is in agreement with our hypothesis that certain early stress-induced deficits may be modulated by the myriad of brain alterations during the postpartum period. Indeed, brain regional activations and transmitter functions also seemed to be reproductive experience dependent in our MIA results.

MIA increased FPS induced c-Fos expression in the mPOAd and PAGd, as well as PV, BLA, and MeA in nulliparous offspring. While nulliparous MIA females exhibited significantly lower startle in the FPS challenge relative to control subjects, they actually showed increased FPS induced c-Fos expression in the PV, BLA, and MeA. Previous studies have shown that the FPS response is mediated by BLA/MeA projections to the PAGd, and increase activities within these regions should correspond to an increase in startle response (Zhao *et al*, 2009). Similarly, a recent study identified a central role of the thalamic PV during both fear conditioning and fear memory expression (Penzo *et al*, 2015). Taking the evidence from BLA, MeA, PAGd, and PV into account, it would seem our results from the c-Fos expression study contradicted those from the FPS challenge

test. However, we also saw an increase in FPS induced c-Fos expression in the mPOAd of MIA offspring irrespective of reproductive experience, suggesting possible alterations in regions regulating both motivation and aversion. The exact relationship between the c-Fos and FPS results in MIA offspring is unclear beyond the parallel of reproductive experience dependence. More work is needed to clarify these results.

MIA increased PCP-induced hyperlocomotion in nulliparous offspring. Similar to previous findings in males (Shi *et al*, 2003b; Zuckerman and Weiner, 2005), our results indicated an increase in phencyclidine (PCP)-induced hyperlocomotion in MIA offspring. However, the main effect of MIA only approached significance, perhaps due to the attenuation of increased PCP sensitivity in primiparous offspring. In fact, primiparous MIA subjects showed similar total PCP-induced hyperlocomotion compared to controls. PCP is an NMDA receptor antagonist, and the increased PCP-induced hyperlocomotion seen in virgin MIA may be attributed to the reduction in glutamatergic synapses (Coiro *et al*, 2015), resulting in enhanced suppression of glutamatergic transmission. In addition, while PCP is an NMDA receptor antagonist, it also acts as a partial agonist for DA D_2 receptors, and its locomotor effect is suggested to be a result of actions on both transmitter systems (Kapur and Seeman, 2002). This has important implications for the current result, as decreased DA D_2 receptor sensitivity in postpartum dams has been reported previously (Byrnes *et al*, 2007). Thus, it is possible that the attenuation of increased PCP-induced hyperlocomotion seen in primiparous MIA offspring may be due to postpartum modifications of the dopaminergic system. However, it is important to note that amphetamine-induced hyperlocomotion showed

no significant differences between MIA and control subjects, which is contrary to most findings (Meyer *et al*, 2010b; Zager *et al*, 2012; Zuckerman *et al*, 2003). In addition, the amphetamine challenge was administered prior to the PCP challenge, and we cannot avoid the possibility that this may have caused an interactive effect (Costa *et al*, 2015).

MIA induced postpartum disruptions are clinically relevant. Our study has revealed important novel findings regarding the effect of MIA on female offspring postpartum. We demonstrated that *in utero* MIA exposure effectively disrupted maternal behavior of postpartum offspring. In addition, we found a congruent result of cognitive deficits that persists to postpartum. Furthermore, we showed evidence that the influence of MIA on female offspring affective regulation and brain and transmitter functions may be dependent on reproductive experience.

MIA is regarded as an epidemiologically driven, clinically relevant model of neurodevelopmental psychiatric disorders (Meyer *et al*, 2009). In particular, the behavioral abnormalities seen in MIA offspring are relevant to symptoms associated with schizophrenia and autism in humans (Patterson, 2009). This is of interest, since women with schizophrenia are at an increased risk for postpartum psychiatric admissions, yet the effect of these postpartum episodes on maternal and ancillary functions is not well understood (Jones *et al*, 2014). Thus, in addition to providing a general understanding of the effects of prenatal environmental stress on postpartum processes, MIA may also serve as a more specific model for investigating the postpartum alterations seen in women with neurodevelopmental psychiatric disorders.

Effect of repeated maternal separation

RMS disrupted maternal behavior of F1 dams. In the RMS F1 postpartum dams, similar to the results in MIA, we saw a disruption in maternal behavior. This is in line with previous studies examining F1 maternal behavior following separation from F0 dams during the early postnatal period (Gonzalez *et al*, 2001; Lovic *et al*, 2001). In Gonzalez and colleague's work, however, only female offspring reared artificially with minimal tactile stimulation showed a significant reduction in time spent performing maternal behaviors, while females reared artificially but received maximum tactile stimulation exhibited levels of maternal behaviors comparable to normally reared subjects. This result led the authors to suggest that the maternal care experience exerts a stronger influence on offspring maternal behavior. However, in the current work we saw that maternal behavior of the F1 dams is significantly disrupted even after controlling for the F0 maternal care factor. This is supported by previous studies in male offspring demonstrating a dissociation between the effect of the separation itself and the alterations in F0 maternal care on offspring development (Macrí *et al*, 2004; Macrì *et al*, 2008). In these male offspring studies, the authors analyzed groups of F0 dams and litters subjected to daily brief separation (15 min), long separation (3 h), and normal rearing. Both brief and long separation significantly increased F0 nursing relative to normal rearing, with no differences between the two separation groups. However, in adult offspring, the brief separation experience significantly reduced both anxiety-related behavior (i.e. novelty induced suppression of drinking) and physiological

response to stress (i.e. restraint stress induced increase in plasma corticosterone). This indicated a unique contribution of the separation manipulation itself on offspring behavior, independent of the maternal care component. Here, we demonstrated that F1 dams displayed reduced nest building behavior after controlling for maternal care.

RMS reduced ASR and FPS in female offspring irrespective of reproductive experience.

Our affective assessment indicated a reduction in baseline and stress-induced anxiety-related behavior in F1 RMS females. This effect was independent of reproductive experience, with both nulliparous and primiparous RMS subjects exhibiting significantly lower baseline and fear potentiated ASR. While various studies have suggested that early separation stress increases anxiety and stress response in adulthood (Aisa *et al*, 2007; Plotsky and Meaney, 1993; Vetulani, 2013), there are some cases showing lack of anxiety enhancement in males (Hulshof *et al*, 2011; Lajud *et al*, 2012), or alternative results in females (Tata, 2012). In general, RMS seems to exert a smaller effect on anxiety-related behaviors in adult female offspring, which also corresponds to a smaller neuroendocrine response following stress manipulations in RMS females compared to males (Kalinichev *et al*, 2002; Wigger and Neumann, 1999). In RMS F1 postpartum studies, both Lovic and Bocca saw no difference in EPM performance between RMS and control offspring postpartum. Gonzalez, on the other hand, reported increased time spent in center region of the OFT in postpartum females artificially reared with minimum tactile stimulation. In addition, several studies have indicated an increase in center exploratory activities in the OFT in both male and female RMS offspring (Anier *et al*, 2014; Kundakovic *et al*, 2013; Marmendal *et al*, 2006), suggesting that while the

decreased anxiety seen in our study may be in line with some previous work, there may be procedural, strain, and sex-dependent considerations in RMS induced anxiety alterations that require more in depth review.

RMS increase FPS induced c-Fos expression in the DG. c-Fos expression following the FPS challenge saw an increase within the DG of RMS females irrespective of reproductive experience. The DG is a unique region within the hippocampus that supports adult neurogenesis (Cameron and McKay, 2001), and early life stress, including maternal separation, has been implicated in the suppression of this function (Hulshof *et al*, 2011; Korosi *et al*, 2012; Lajud *et al*, 2012). The mechanism for early stress induced suppression of neurogenesis is suggested to be due to the reduction in glucocorticoid receptor expression, leading to impairments in the negative feedback of stress response and overexposure of corticosteroid (Loi *et al*, 2014; Oitzl *et al*, 2010). While it is unclear the association between increased DG c-Fos activity and the reduced startle seen in RMS females during the FPS challenge, it is possible that alterations in glucocorticoid receptor mediated functions may play a role in the increased DG activation.

RMS decreased amphetamine-, PCP-, and nicotine-induced hyperlocomotion. In the current study, we saw a decrease in amphetamine- and PCP-induced hyperlocomotion in RMS F1 females relative to control subjects. The effect was irrespective of reproductive experience. Previous studies have indicated a reduction in DA D2 receptor expression in the nucleus accumbens and hippocampus of RMS offspring (Li *et al*, 2013), as well as an attenuation in restraint stress induced increase of DA as seen in control

subjects (Jahng *et al*, 2010). Our results from amphetamine and PCP testing are consistent with these findings of reduced dopaminergic functions, which may in part explain the deficits in maternal behaviors seen in F1 RMS dams (Numan *et al*, 2005).

In addition, our results also demonstrated a reduction in nicotine-induced hyperlocomotion in RMS females. While few studies have investigated the effect of RMS on cholinergic functions, a previous report suggested that acetylcholine potentiated calcium release was abolished in the prefrontal cortex of RMS rats (Proulx *et al*, 2014). Although the study targeted a different cholinergic receptor subtype, our results implicate the disruption in cholinergic transmission as a possible mechanism for altered behaviors in these offspring.

RMS induced postpartum disruptions are clinically relevant. In the current study, we confirmed previous findings that RMS reduces maternal behavior in F1 postpartum offspring. This was accompanied by a decrease in anxiety-related behaviors, an increase in anxiogenic stimulus induced DG activity, and a disruption in dopaminergic and cholinergic systems functions.

RMS is regarded as an ecologically valid model of parental neglect, and previous studies have pointed to an increase in depressive-like behaviors in exposed offspring (Vetulani, 2013). While results from this set of experiments provided mixed conclusions regarding the effect of RMS on emotional regulation in relation to other studies, they suggest altered psychological and brain processes in RMS F1 adult females that are evident in both nulliparous and primiparous offspring. In contrast to the reproductive

experience dependent variations seen in MIA subjects, this seems to play a less prominent role in the modulation of RMS induced deficits. Over all, the current work corroborates other evidence pointing to RMS having a life-long impact on brain and behavioral functions. In addition, our results promote the benefit of using this model to expand our understanding of the effects of early postnatal stress on postpartum processes, based on the epidemiologically driven hypothesis that postpartum disorders may be stress triggered episodes that are present in antenatally diagnosed females.

Combined effect of maternal immune activation and repeated maternal separation

An important issue addressed within the current design is the consequence of experiencing multiple early life insults. Contrary to our hypothesis, we did not see a synergistic effect of combined MIA and RMS treatments in exacerbating any deficit. In fact, to our surprise, we actually found antagonistic effects in certain processes. For instance, in maternal behavior, the nest building disruption in MIA- and RMS-only offspring was attenuated by combined treatments. This was also true for the alteration in ASR. In other assays, results from the combined MIA-RMS offspring mostly reflected the MIA effect. In PPI, for example, both MIA-Ctrl and MIA-RMS offspring displayed reduced PPI. Similarly, in amphetamine- and nicotine-induced hyperlocomotion and the FST, MIA-RMS subjects exhibited phenotypes more closely aligned with those of MIA-Ctrl animals.

Interestingly, MIA induced PPI deficits has been shown to be highly sensitive to different factors, such as prepulse intensity, MIA timing, and test age (Basta-Kaim *et al*, 2012; Meyer *et al*, 2006b; Wolff and Bilkey, 2010). In Giovanoli *et al*, 2013, neither MIA on G 9 nor adolescent variable unpredictable stress alone was sufficient to induce PPI disruptions, but when the two were applied in the same individual, abnormalities in not only PPI, but also EPM and amphetamine- and MK-801-induced hyperlocomotions were revealed. Similarly, using MIA on G 12 and adolescent restraint stress, Deslauriers *et al*, 2013 saw a reduction in PPI only in subjects that were exposed to both manipulations. In contrast, results here demonstrated that MIA on G 13 and 15 was able to decrease PPI, irrespective of postnatal manipulations. It is possible that our MIA regimen produced brain alterations sufficient to manifest and maintain the abnormal PPI phenotype independent of later life experiences.

The idea that multiple stressors may interact to produce detrimental effects has also been investigated by others using RMS. The combination of RMS with adolescent chronic variable stress was shown to decrease exploratory behavior in males, which was not evident in animals from either single treatment groups (Renard *et al*, 2007). RMS was also found to interact with adolescent corticosterone treatment, increasing DA D_3 receptor mRNA expression only in animals exposed to both stressors (Deslauriers *et al*, 2013). However, others have also found that in certain conditions, subjects treated with multiple insults preferentially exhibit the effect of a single factor. For example, combined prenatal stress (gestational restraint) and RMS caused a reduction in novelty induced hyperlocomotion, which was similar to individuals only stressed prenatally, but not

RMS only subjects, reflecting the prenatal stressor's dominant phenotype in the anxiogenic task (Lehmann *et al*, 2000b). Conversely, the same two-hit subjects did not display PPI deficits, which were evident in animals stressed only prenatally, but not those with only RMS, indicating RMS's stronger influence in this cognitive assessment. In a different study, RMS disrupted the normal increase in performance over time in male offspring tested for PPI during both adolescence and adulthood, an effect obscured by the additional stress of CAR training during adolescence, which by itself did not alter the change in PPI performance from adolescence to adulthood (Chen *et al*, 2011).

The current findings indicated several interesting characteristics regarding postpartum vulnerability conferred by *in utero* MIA. First, MIA did not seem to interact with RMS to produce cumulative stress effects. Second, the MIA stress inoculation may have resulted in changes adaptive to the RMS stress in terms of maternal behavior (Daskalakis *et al*, 2013). Finally, in other processes, MIA induced alterations seemed to produce ceiling effects and masked the RMS phenotypes (Lee and Goto, 2013).

Impact of reproductive experience on the effects of MIA and RMS in F1 offspring

Comparing the effects of reproductive experience across all subjects, we saw enhanced in PPI, reduced struggling in the FST, decreased sucrose consumption, and increased ASR habituation in all primiparous females.

Our result of reduced struggling in the FST parallels that of Craft *et al*, 2010 showing a decrease in diving behavior in postpartum dams despite a lack of difference in immobility compared to virgin subjects(Craft *et al*, 2010). While both of these may be indicative of reduced active stress-coping behavior (Singewald *et al*, 2011), they may not be directly due to a depressive-like state, and more work is necessary to deduce the psychological components that distinguish behavioral despair (i.e. immobility) and active coping (i.e. struggling/diving).

While studies by others have shown that primiparous dams do not show depressive-like symptoms in the FST and SPT (Craft *et al*, 2010; Navarre *et al*, 2010), here we found a decrease in sucrose consumption in postpartum F1 females. This may be explained by the timing of the procedure. In the current study, SPT was conducted on PPD 21, immediately after the weaning of pups. It has been shown in both humans and rodents that contact with offspring is necessary for the antidepressive effects of motherhood (Heinrichs *et al*, 2013; Pawluski *et al*, 2009). It is possible that the reduction in sucrose preference reflects the decreased hedonic state following separations from pups.

Regarding postpartum anxiolysis, while some studies have indicated the prerequisite of recent infant contact (Lonstein, 2005; Smith and Lonstein, 2008), others have shown that reproductive experience confers a lasting effect and the reduced anxiety persists long after weaning (Love *et al*, 2005; Wartella *et al*, 2003). Our result in ASR habituation is more supportive of the latter finding, with dams exhibiting higher

rates of habituation to an anxiogenic stimulus (i.e. 105 dB white noise) when tested post weaning.

Finally, in sensory motor gating, our results contradicted those by Byrnes *et al*, 2007 showing reduced PPI in postpartum dams on both PPD 2 and 14. However, as results of PPI are sensitive to testing parameters, this may explain the differences seen here (Swerdlow *et al*, 2008). Byrnes and colleagues employed a much stronger pulse (i.e. 120 dB compared to 105 dB in the current study) such that the prepulse and pulse intensity difference is much larger (i.e. 35 dB difference compared to 23, 27, and 30 dB differences in the current study). In addition, all animals in that study were given saline injections, a treatment that may be mildly stressful (Renard *et al*, 2007). It is possible that the PPI results in postpartum females may actually reflect an increased sensitivity in the modulation of cognitive processes by emotional states. We believe our result is supportive of this hypothesis, as the increase in PPI performance was evident in dams throughout all phases of testing, except during the FPS challenge session, when primiparous and nulliparous subjects displayed similar levels of PPI.

Limitations of the current study

While this work provided important insights into the effects of early stress on postpartum behaviors, some limitations should be noted when interpreting the results. First, it is well known that postpartum brain circuitry changes as a function of time and stimulus presentation (i.e. intensity of pup cues, [Olazábal *et al*, 2013]). As such, it is

possible that behaviors at different postpartum time points may actually represent mechanistically distinct processes. Second, as differences in stress procedures may produce variable outcomes (Daskalakis *et al*, 2011, 2012; Meyer *et al*, 2006a), the generalizability of the current results must await further supporting evidence using alternative stress procedures (e.g. other gestational time points for polyI:C treatment, RMS of different durations, etc.). Finally, we must constantly keep in mind that serial testing and dosage selections in behavioral and pharmacological testing can always influence results, and we cannot ignore the possibility that some results may represent interactive effects of multiple test variables.

Future work should expand upon current findings through investigating the longitudinal effects of MIA and RMS in postpartum females across the postpartum period. In addition, the generalizability of early stress-induced postpartum alterations should be assessed with alternative manipulations (e.g. restraint stress, chronic ultra-mild stress). Furthermore, reversal of deficits through targeting the implicated neuronal and transmitter systems may aid in future development of treatments.

Conclusion

We believe this set of experiments provided novel results that answered the important questions at hand. Maternal immune activation, as a prenatal stressor, altered brain and behavioral processes of female offspring in adulthood and postpartum.

Repeated maternal separation, as a postnatal stressor, also produced various changes in both maternal behavior and other psychological functions. The two stressors seemed to antagonize each other in regulating maternal behavior, resulting in a generally normal maternal phenotype in offspring exposed to both insults. In other functions, the two showed relatively low levels of interactive effects. Finally, some of the alterations following early life stress seemed to be reproductive experience dependent. These findings have significant clinical implications. They point to the importance of life long mental health maintenance in improving the pregnancy and postpartum outcomes of women. Furthermore, they indicate the critical need of additional peripartum support for this at risk population as they transition into motherhood.

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