EFFECTS OF MATERNAL PRECONCEPTION STRESS ON BRAIN AND BEHAVIOR IN MALE AND FEMALE OFFSPRING

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DEDICATION

For my husband, whose support and belief in my ability has never wavered.

ABSTRACT

Brain and behavior are shaped by experience. Stress during the preconception period is a new topic of interest, as it involves both prospective parents. The current study examined how maternal preconception stress impacts the development of offspring brain and behavior throughout the lifespan. Methods mirror those used in studies on paternal preconception stress, which allowed direct comparison between the effects on offspring when preconception stress is experienced by each parent. Female rats experienced stress for 27 days immediately prior to mating, and offspring completed a battery of behavioral tests beginning in early life and continuing into adulthood. Offspring brain was examined at weaning and in adulthood. Major findings include increased anxiety-like behavior, impaired working memory, deficits in fine motor control, decreased body weight, and reduced cortical thickness. Male offspring were disproportionately affected. Maternal preconception stress affects offspring similarly to stress at other times, but less severely than paternal preconception stress.

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5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
5-HT1A	Serotonin receptor
5-HTT	Serotonin transporter
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
AID	Agranular insular dorsal cortex
AVP	Arginine vasopressin
BDNF	Brain-derived neurotrophic factor
CA1	Hippocampal area
Cg3	Cingulate cortex
COMT	Catechol-O-methyltransferase
COR	Corticosterone
CRH	Corticotrophin-releasing hormone
CUS	Chronic unpredictable stress
DA	Dopamine
DAT	Dopamine transporter
DNA	Deoxyribonucleic acid
DOPAC	Dihydroxyphenyl acetic acid
Fr1	Frontal area 1
FST	Forced swim task
G	Gestational day
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
HPC	Hippocampus
IL	Infralimbic
LO	Lateral orbital
mPFC	Medial prefrontal cortex
MPS	Maternal preconception stress
MR	Mineralocorticoid receptor
MWT	Morris water task
NAc	Nucleus accumbens
NE	Norepinephrine
NET	Norepinephrine transporter
P	Post-natal day
Par1	Parietal cortex
PFC	Prefrontal cortex
PGC	Primordial germ cell
PL	Prelimbic
POMC	Proopiomelanocortin
PPS	Paternal preconception stress

PS	Prenatal stress
PVN	Paraventricular nucleus
RNA	Ribonucleic acid
SERT	Serotonin transporter
SNP	Single-nucleotide polymorphism

Chapter 1

General Introduction

The developing animal is shaped by its experiences; the trajectory of development is determined by the interplay between genetics and the environment (Johnston & Edwards, 2002). Ongoing research over the past several decades has elucidated many of the factors that influence development, including negative experiences such as early brain injury, drug exposure, environmental deprivation, and stress, as well as positive experiences such as tactile stimulation, diet supplementation, and environmental enrichment. Stress is particularly well studied and has been examined during many life stages, from during the preconception period in potential mothers and fathers, to prenatally, to during adulthood and aging. Hans Selye is considered the founder of stress research. He first described the stress response in 1936, when he described the "alarm reaction" observed in rodents injected with "impure extracts" from cattle ovaries (Selye, 1973). Excessive stress impacts development through the over-activation of the stress response, which puts the body in a state of readiness so that it can effectively deal with the stressor. Chronic activation of the stress response has detrimental effects on the brain and, subsequently, behavior throughout life, and the impact is exacerbated during times of rapid growth.

This thesis contributes to our understanding of how stress impacts development by examining chronic maternal stress during the preconception period, the time immediately preceding conception, using an animal model (Long-Evans rats). Female rats were exposed to a consistent stressor prior to mating with stress-naïve males, and the brain and behavior of male and female offspring were examined throughout life. Chosen methods

exactly mirrored those used in recent research on paternal preconception stress, allowing direct comparisons. What follows is a brief overview of the stress response in mammals, a summary of the major findings of the consequences of stress throughout development, the current knowledge regarding maternal and paternal preconception stress, and a description of the proposed mechanism of preconception stress, epigenetic modification to the germline.

1.1. The Stress Response

Stress is a common occurrence that serves an adaptive function of alerting us when a situation may be dangerous or otherwise aversive. Stress can be broadly defined as a disruption in homeostasis which induces an automatic, bodily attempt to restore balance (Johnson et al., 1992; Tsigos & Chrousos, 2002). This automatic reaction is the physiological stress response, which involves activation of the sympathetic nervous system to prepare the body for dealing with the perceived threat. The neural circuit involved in the stress response comprises the hypothalamic-pituitary-adrenal (HPA) axis (Tsigos & Chrousos, 2002; Griffiths & Hunter, 2014).

1.1.1. The HPA Axis

The HPA axis is a circuit that includes, not surprisingly, the hypothalamus, the pituitary gland, and the adrenal glands, and is responsible for the release of glucocorticoids that ready the body for dealing with stressors (Figure 1.1 from Griffiths & Hunter, 2014). In response to stress, a population of corticotrophin-releasing hormone (CRH) neurons in the central amygdala trigger increased firing in the locus coeruleus, which increases levels of norepinephrine (NE; Francis & Meaney, 1999). NE stimulates

the paraventricular nucleus (PVN) of the hypothalamus to release CRH and arginine-vasopressin (AVP) into the pituitary portal. CRH and AVP trigger the production of proopiomelanocortin (POMC) in the anterior pituitary, which is converted to adrenocorticotropic hormone (ACTH) and released into the blood. ACTH causes the adrenal glands to produce corticosteroids (corticosterone (COR) in rats and cortisol in humans), which bind to glucocorticoid and mineralocorticoid receptors (GR and MR) that are located throughout the brain. Corticosteroids can also act as transcription factors, thereby controlling gene expression (Lupien, McEwan, Gunnar, & Heim, 2009). Corticosteroids exert negative feedback on the hypothalamus, which turns off the stress response under normal conditions.

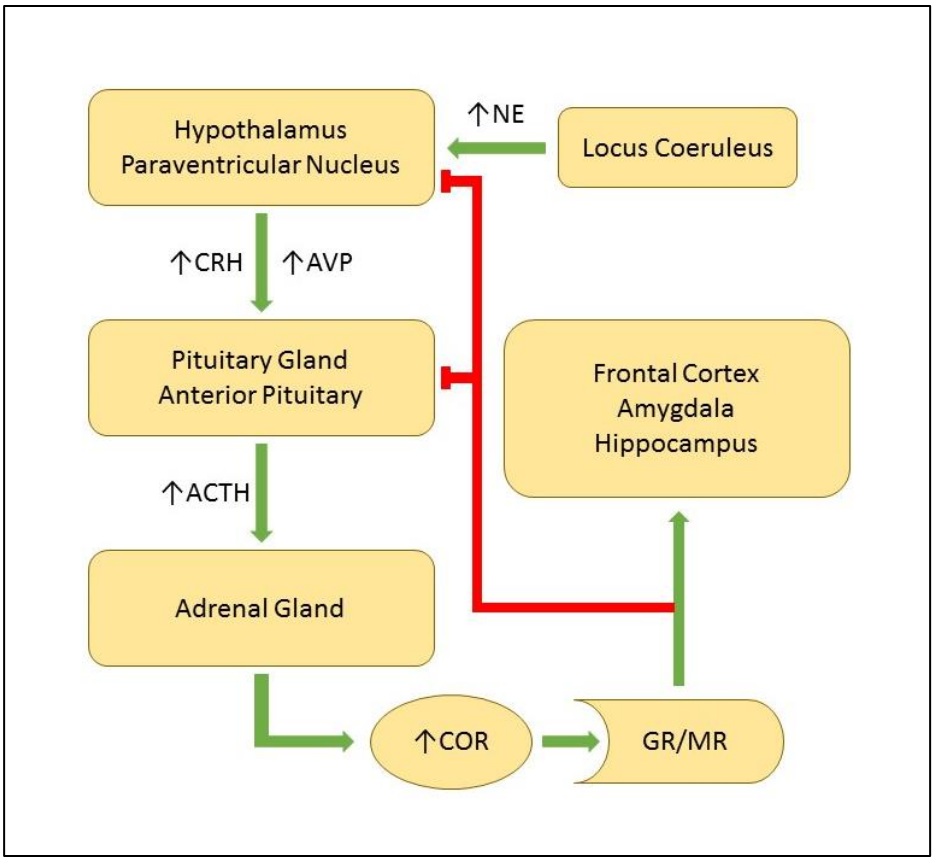

Figure 1.1: The HPA-axis and interacting brain structures. Norepinephrinergic neurons in the locus coeruleus increase NE levels in the PVN of the hypothalamus. CRH and AVP

levels are subsequently increased and activate the anterior pituitary to release ACTH into the bloodstream. ACTH travels to the adrenal glands and increases levels of circulating COR, which binds to GRs and MRs throughout the brain, including the frontal cortex, amygdala, and hippocampus. COR binding exerts negative feedback on the PVN and anterior pituitary (Adapted from Griffiths & Hunter, 2014).

1.2. Consequences of Stress Throughout Development

Typical functioning of the stress response is beneficial and helps to ensure survival. Normal levels of glucocorticoids are critical for normal development and brain maturation (Lupien et al., 2009). However, dysregulation or over-activation of the response is harmful and is associated with the development of several pathologies. Excessive stress during times of rapid growth is especially detrimental because the developing system is more vulnerable to perturbations. Three such developmental time periods that are well-studied with respect to the effects of excessive stress are gestation, neonatal life, and adolescence. Lupien and colleagues (2009) provide an excellent review of stress throughout the lifespan using animal and human studies.

1.2.1. Prenatal

Research into the consequences of gestational stress on offspring development originated with the study by L.W. Sontag in 1941 (Weinstock, 2001). Since then, innumerable studies have been completed examining the effects of prenatal stress (PS) on postnatal development. PS has been associated with a wide range of developmental abnormalities, including physical (e.g. birth weight) and mental (e.g. attention deficit hyperactivity disorder [ADHD], depression) problems.

Stress during pregnancy drastically increases the level of maternal corticosteroids that pass through the placenta, which programs the development of the fetal HPA axis. In

reviews of PS literature, many studies report that offspring exhibit increased basal levels of COR and show heightened responsiveness to stress (Glover, O'Connor, & O'Donnell, 2010). Such changes in HPA axis activity likely underlie the changes in behavior and brain that are observed in prenatally stressed offspring. PS also decreases gestational weight gain in dams and the birth weight of male and female offspring (Van den Hove et al., 2006). Furthermore, PS effects offspring brain weight at postnatal day (P)21, dependent on the intensity of the stressor. Mild stress results in decreased brain weight in male and female offspring compared to controls. However, severe stress causes decreased brain weight in males but increased brain weight in females (Mychasiuk et al., 2011).

PS affects how dams interact with their offspring. Meaney and colleagues have extensively studied how maternal care impacts offspring development and found that stress during gestation alters maternal care, but only in dams that are the high licking/grooming phenotype (Champagne & Meaney, 2006). In this study, female rats gave birth to one litter and were observed for their frequency of licking/grooming, then classified as high or low licking/grooming. Once these litters were weaned, the same females were mated a second time and exposed to PS during the final week of gestation. High licking/grooming dams exposed to PS showed significant deficits in maternal care towards their second litter, whereas low licking/grooming dams did not. Interestingly, when the same dams were bred a third time and not exposed to any additional PS, the deficit prevailed. Studies that examine the effects of PS on offspring often use cross-fostering to control for changes in maternal care, meaning that the litters of prenatally stressed dams are reared by stress-naïve dams.

1.2.1.1. Prenatal stress effects on behavioral development. Chronic and severe PS is associated with a number of behavioral aberrations in offspring. Marta Weinstock has published several reviews on this topic and cites articles that describe increased depressive symptomology, anhedonia, anxiety, impairments in learning and memory, altered male sexual behavior, and impaired social interaction and play (Weinstock, 2001, 2007, 2008). The three most commonly reported behavioral impairments observed in adult animals exposed to PS are depression/anxiety, increased drug-seeking behavior, and deficits in learning and memory (Lupien et al., 2009).

Weanling offspring (P21) of prenatally stressed dams display delays in behavioral development compared to control offspring, in an intensity-dependent manner. Mild PS (i.e. elevated platform stress for 10min twice daily from G12-16) stunts sensorimotor development in male and female offspring; compared to controls, stress offspring spend less time rotated above the horizontal plane in negative geotaxis on both P9 and P10. Interestingly, male and female offspring of dams exposed to severe PS (i.e. elevated platform stress for 30min twice daily from G12-16) perform equally well as control offspring on P9, but fail to show any improvement on P10. These findings suggest that how mild and severe PS influences offspring is inherently different; mild stress lowers basal sensorimotor ability but does not impair development, whereas severe stress inhibits sensorimotor development (Mychasiuk et al., 2011).

1.2.1.2. Prenatal stress effects on brain development. Behavioral and cognitive alterations induced by PS are accompanied by changes in brain morphology, including in the hippocampus (HPC), amygdala, corpus callosum, neocortex, cerebellum, and hypothalamus. The HPC is particularly vulnerable and consistently shows a PS-induced

decrease in neurogenesis, GR density, volume, and synaptic density (Charil et al., 2010). Microscopic changes to dendritic architecture are observed in the prefrontal cortex (PFC) and HPC of weanling rats whose dam was exposed to direct PS or indirect prenatal bystander stress (Mychasiuk, Gibb, & Kolb, 2012; Mychasiuk, Gibb, & Kolb, 2011). Changes in neuron morphology are also observed in the HPC of adult rats exposed to prenatal restraint stress during the latter half of gestation (Bock et al., 2015). PS also induces changes in DNA methylation, a mechanism involved in gene expression (discussed in detail in section 1.4.). Mild PS results in increased methylation in the hippocampus and frontal cortex, whereas severe PS causes a decrease in methylation in these same regions (Mychasiuk et al., 2011). PS during the third week of gestation decreases cell proliferation in P1 offspring in the HPC, olfactory bulb, subventricular zone, and cerebellum (Van den Hove et al., 2006).

1.2.1.3. Sex differences. Sex differences are apparent in many aspects of brain and behavior. Not surprisingly then, differences between male and female offspring emerge in how each is affected by PS. In general, female offspring are more greatly impacted. Dendritic architecture in PFC and HPC is more affected by both direct PS and indirect prenatal bystander stress in females compared to males (Mychasiuk, Gibb, & Kolb, 2012; Mychasuik, Gibb, & Kolb, 2011). When considering behavior, depressive- and anxiety-like symptoms are more readily observed in female offspring of prenatally stressed dams, although male offspring are more likely to display deficits in learning and memory (Weinstock, 2007). Although the trend is towards a greater impact on female offspring, many studies have reported the contrary so this finding should be interpreted

with caution. Factors such as the timing and intensity of the stress are important to consider (Bock et al., 2015).

1.2.2. Neonatal

Many studies on neonatal stress use maternal separation as the stressor. Short-term maternal separation (e.g. 15min) enhances maternal care upon reunion of pups and dam, does not appear to have long-term negative effects on offspring development, and has a "hardening" effect by decreasing stress responsiveness in adulthood. Decreased stress responsiveness may manifest as resiliency in certain situations, but a degree of stress is conducive to survival in many cases, so these offspring may not be better off in the long run. Prolonged bouts of maternal separation (i.e. 3hrs) increases stress reactivity in adulthood, and both pups and dams are apprehensive upon reunion (Vetulani, 2013).

Neonatal stress impairs adult hippocampal neurogenesis, and results in associated deficits in learning and memory throughout life. Hippocampal neurogenesis is the process by which progenitor cells in the hippocampus, especially the dentate gyrus, are differentiated into neurons and incorporated into existing hippocampal networks. The dentate gyrus develops during the first two weeks postnatally in rodents, making this process particularly sensitive to neonatal stress. Aberrations in hippocampal neurogenesis result in memory impairments, as well as emotional dysregulation and addiction (Korosi et al., 2012; Hays et al., 2012). Unpredictable stress during the second week postnatal (i.e. 0.5mA hindlimb shock for 1s from P8-P12) increases adult anxiety-like symptomology and produces concomitant changes in gene expression in the amygdala (Sarro, Sullivan, & Barr, 2014).

1.2.3. Adolescent

The brain during adolescence is continuing to undergo change; white matter volume steadily increases, suggesting more efficient communication between regions, and grey matter volume reaches its peak then begins to decline due to widespread synaptic pruning (Buwalda, Geerdink, Vidal, & Koolhaas, 2011). The pre-pubertal brain is more sensitive to COR than the adult brain, and regions that are continuing development into adolescence (e.g. HPC, PFC, amygdala) are especially vulnerable (Romeo and McEwan, 2006). Peri-pubertal psychogenic stress (i.e. exposure to predator odor followed by elevated platform) decreases anxiety-like symptomology and increases risk-taking and novelty-seeking during late adolescence, but does not affect depressive-like behaviors or HPA axis activity (Toledo-Rodriguez & Sandi, 2011). Adolescent social stress (i.e. resident-intruder paradigm) increases social anxiety in adult male rats, assessed as the amount of time spent interacting with an unfamiliar male; the authors did not test female rats (Vidal et al., 2007). Additionally, adolescent rats exposed to variable social stress, including isolation, crowding, and subordination, show inhibited behavioral sensitization to amphetamine, accompanied by decreased levels of dopamine (DA) receptor D1R in the caudate putamen (Kabbaj, Isgor, Watson, & Akil, 2002; Kabbaj & Isgor, 2007).

1.2.4. Summary

Stress during many stages of development has a negative impact on offspring brain and behavior, and the effects are typically long-term and prevail into adulthood. HPA axis activity is commonly impaired and animals show increases in anxiety- and depressive-like behaviors, as well as deficits in learning and memory. Furthermore,

changes appear in brain structure, plasticity, and biology. Although not discussed here, stress during adulthood and aging continues to have detrimental effects on brain and behavior (Lupien et al., 2009).

1.3. Preconception Stress

Within the last decade, research interests have shifted towards the preconception period; that is, the time that immediately precedes mating and conception. Converse to prenatal experiences, preconception experiences pertain to both parents, which opens up many new interesting avenues for scientific inquiry. Stress is one experience that has been examined in the preconception period in both prospective mothers and fathers. Initial results indicate that preconception stress appears to have similar influences on offspring development as stress during other developmental stages. This finding is interesting considering the mechanisms through which preconception stress, and stress at other times, impacts offspring are necessarily different. As described, excessive stress impacts brain and behavior due to over-activation of the stress response and abnormally high levels of corticosteroids. This sort of direct influence is impossible in the case of preconception stress; instead, a proposed mechanism is epigenetic – preconception stress may induce epigenetic modifications in the parental germline, which are transmitted to the offspring and have the potential to influence development. What follows is a description of the research completed on both maternal and paternal preconception stress to date. The following section provides a discussion of how this experience may be inherited epigenetically.

1.3.1. Maternal Preconception Stress

1.3.1.1. Animal studies. To date, all animal studies examining maternal preconception stress (MPS) have used a chronic unpredictable stressor (CUS). This type of stressor subjects the females to a pseudo-randomly selected stressor each day of the paradigm, typically three weeks. Examples of CUS include cage rocking, lights on overnight, elevated temperature (40°C), cold swim (4°C), restraint, and food and water deprivation (e.g. Huang et al., 2012). Completed studies have examined offspring of preconception stressed dams at several ages, including fetal (Huang et al., 2012), pubertal (Huang et al., 2013), and early adulthood (P60; Huang et al., 2010; Li et al., 2010; Bock et al. 2016). Both behavioral and neurobiological parameters indicate that CUS prior to conception has aversive effects on offspring development throughout the lifespan.

CUS prior to conception results in a significant increase in maternal COR and CRH relative to control dams, both immediately following the stress procedure and following delivery (Huang et al., 2013). A similar increase in COR and CRH is observed in the fetal and pubertal offspring of CUS dams compared to control-offspring (Huang et al., 2012; Huang et al., 2013). Furthermore, fetal CUS-offspring have decreased body weight compared to controls, although brain weight is unaffected (Huang et al., 2012).

CUS causes disruptions in neurotransmitter systems, notably the serotonergic and dopaminergic systems. Levels of serotonin (5-HT) were increased in the HPC and hypothalamus in fetal CUS-offspring relative to controls, accompanied by a decrease in the ratio of 5-hydroxyindoleacetic acid (5-HIAA, the metabolite of 5-HT) to 5-HT. Levels of serotonin receptor (5-HT1A) and transporter (SERT) were also decreased in the HPC, and both the HPC and hypothalamus, respectively (Huang et al., 2012). Interestingly, in

adulthood (P60), 5-HT is decreased in the hypothalamus and unaffected by CUS in the HPC (Li et al., 2010).

The serotonergic system is largely involved in mood disorders and decreased levels of 5-HT have been associated with major depressive disorder and suicidal actions. 5-HIAA is used as a proxy for 5-HT activity – the higher the levels of 5-HIAA, the more serotonergic activity is inferred (Mann, 1999). Therefore, a decreasing ratio of 5-HIAA:5-HT may be indicative of depressive symptomology, although the authors did not directly measure depressive behaviors (Huang et al., 2012). However, in a separate study, pubertal CUS-offspring did display depressive-like behaviors when assessed in the forced swim task (FST; Huang et al., 2013). Serotonin also plays an important role in HPA axis activity by triggering a cascade of events that leads to the release of COR from the adrenal cortex (Mann, 1999).

The dopaminergic system is also disrupted by CUS prior to pregnancy. In a similar fashion as the serotonergic system, the metabolite of DA, dihydroxyphenyl acetic acid (DOPAC), is less abundant in the medial PFC (mPFC) of pubertal CUS-offspring compared to control-offspring, as is the ratio of DOPAC to DA. Furthermore, expression of dopamine transporter (DAT), as well as norepinephrine transporter (NET) and catechol-O-methyltransferase (COMT), was also reduced in CUS-offspring (Huang et al., 2013).

CUS also induces changes in neuron structure. Young-adult offspring (P60) of dams exposed to CUS for one week, two weeks prior to mating, showed alterations in neuron size and complexity in the prelimbic (PL) and infralimbic (IL) cortices, and spine density and dendritic length in the anterior cingulate cortex (Bock et al., 2016).

Specifically, CUS increased these measures relative to controls. Interestingly, changes in the anterior cingulate cortex were restricted to the left hemisphere of male offspring.

Fewer studies have examined the effects of MPS on offspring behavior. So far, CUS-offspring display increased depressive-symptomology compared to control-offspring based on increased time spent passively floating in the FST (Huang et al., 2013). CUS-offspring also show memory impairments, based on deficits in learning the location of the hidden platform in the Morris Water Task (MWT) and in remembering the previous platform location in probe trials (Huang et al., 2010; Li et al., 2010).

1.3.1.2. Human studies. Retrospective human studies show correlations between MPS and birth outcomes. In a population-based study of over three million Swedish-born infants between 1973 and 2008, maternal preconception bereavement stress (i.e. the death of a first-degree relative) during the six months immediately preceding conception significantly predicted infant mortality; interestingly, there was no association between prenatal bereavement stress and infant mortality (Class et al., 2013). This peculiar finding may suggest that the fetus and/or pregnancy hormones play a role in maintaining maternal health as a means of ensuring fetal survival, which definitely has adaptive value. In a similar large-scale study, however, these authors found no relationships between preconception bereavement stress and offspring psychopathology in childhood or adulthood (Class et al., 2014).

In a review of articles examining obstetric outcomes following stress, Witt and colleagues found a consistent association between preconception stress and small for gestational age infants and preterm births. Unfortunately, all of the studies included in the review reported that the mother experienced both preconception and PS, making it

difficult to infer the contribution of the preconception experience. There were no articles that isolated preconception stress included in the review, likely a reflection of the difficulty of controlling such variables in human studies (Witt et al., 2014).

These authors also found associations between poor maternal mental health during the preconception period (self-reported) and adverse pregnancy outcomes. Specifically, women who reported having fair to poor mental health prior to pregnancy were more likely to experience pregnancy complications (40% greater risk), have a non-live birth, or birth a low-weight infant. Women who reported poor mental health made up 8.9% of non-live births, but only 5.7% of live births. Women with poor mental health gave birth to 12.7% of low-birth weight infants, 5.7% of normal birth-weight infants, and only 1.2% of high birth-weight infants (Witt et al., 2012).

1.3.2. Paternal Preconception Stress

With the shift towards studying the preconception period came the ability to study how prospective fathers influence their offspring via their experiences prior to conception. Spermatogenesis, the generation of new sperm cells, is an ongoing process that lasts approximately 48 days in rats. Paternal experiences can induce epigenetic modifications in the sperm which are then transmitted to the offspring. A few studies in our lab have begun to examine how paternal preconception stress (PPS) impacts offspring development (Harker et al., 2015; Mychasiuk et al., 2013). These studies exposed male rats to a predictable chronic stressor, elevated platform stress, immediately prior to mating with naïve females. The development of early brain, behavior, and the epigenome were all affected by the paternal experience.

Golgi analyses of neurons in five brain regions (agranular insular dorsal cortex (AID), cingulate cortex (Cg3), hippocampus (CA1), nucleus accumbens (NAc), and parietal cortex (Par1)) revealed changes in both neuron morphology and connectivity throughout the brain in stressed-offspring compared to non-stressed-offspring (Harker et al., 2015). There is also an effect of sex, with female offspring experiencing changes in 48% of measures, and male offspring showing changes in 29%. The most consistent changes are observed in AID, where both male and female offspring have decreased dendritic branching and length and a marginal decrease in spine density. Most measures in which a change is observed show a decrease relative to controls, with the exception of the nucleus accumbens and apical dendritic branching in Cg3.

Early behavioral development is impaired in offspring of preconception stressed fathers. When tested in the negative geotaxis task, stress-offspring perform significantly worse compared to controls on P9, but equally well on P10. This finding suggests that PPS delayed offspring sensorimotor development (Mychasiuk et al., 2013).

Analysis of global DNA methylation reveals that PPS impacts offspring in a manner that is both region- and sex-dependent. In the hippocampus, DNA methylation is significantly increased in both male and female offspring of stressed fathers. However, in the frontal cortex, only female offspring show a decrease in methylation (Mychasiuk et al., 2013).

Taken together, the current studies examining PPS demonstrate several interesting findings. First, paternal experience is transmitted to offspring, likely through epigenetic mechanisms, and in a manner that may or may not be similar to how maternal stress, whether preconception or prenatal, influences offspring. Second, as with other forms of stress, the effects of paternal preconception stress are sexually-dimorphic.

1.4. Epigenetic Modification to the Germline

As discussed above, PS has a direct effect on offspring development; stress activates the maternal HPA axis, resulting in increased circulating CRH and, subsequently, COR, which passes through the placenta (Charil et al., 2010). The mechanism behind the transmission of preconception stress is likely less direct and is proposed to occur through epigenetic modifications to the germline. Epigenetics, a term proposed by Conrad Waddington in the mid-1900's (Noble, 2015), is the study of experience-dependent non-DNA sequence alterations in the genome, including DNA methylation, histone modifications (e.g. acetylation, phosphorylation, methylation), and non-coding RNAs (Griffiths & Hunter, 2014). Such alterations make sections of the genome more or less accessible during transcription, and essentially up- or down-regulate the expression of certain genes, respectively. Epigenetic modification is an essential component of development. All of the cells in our bodies share the same DNA, and epigenetic regulation of gene expression allows the differentiation of different tissues by turning off some genes and turning on others (Keverne, Pfaff, & Tabansky, 2015).

Since 2010, a collection of studies has questioned how a preconception parental experience, such as stress, may be transmitted to offspring through epigenetic inheritance (Bale, 2015). During development, primordial germ cells (PGCs) undergo global DNA demethylation to "reset" the genome for subsequent generations (Lees-Murdock & Walsh, 2008; Hsu, Clark, & Chen, 2015). However, a recent study by Tang et al. identified a collection of demethylation-resistant genes, which the authors speculate may be the substrate for transgenerational epigenetic inheritance (Tang et al., 2015). Interestingly, many of these "escapee" genes are expressed in brain tissue, and may

provide a link between preconception experiences and the observed increase in neurological disorders (Tang et al., 2015).

With a potential mechanism in hand, some researchers have begun searching for specific epigenetic alterations that coincide with preconception stress. Franklin et al. (2010) exposed male mice to unpredictable maternal separation during the first two weeks of life and found increased depressive-like behaviors in adulthood and changes in DNA methylation in the germline. These males were then bred, and males of the next generation also showed methylation changes in both the germline and the brain. A similar study with rats found that subjecting adult males to chronic swim stress for three consecutive weeks prior to mating led to increased anxiety and COR levels in the offspring, and hypermethylation of the hippocampal NR3C1 promotor, which codes for GR, resulting in decreased levels of this receptor. Additionally, male offspring were more affected than females (Niknazar et al., 2017).

There is also evidence to suggest that sperm microRNAs may be responsible for transmitting paternal experience. Rodgers et al. subjected male mice to six weeks of chronic unpredictable stress and later found significantly increased levels of nine sperm microRNAs in the offspring (Rodgers et al., 2013). These authors then injected single-cell zygotes with a concoction of these nine microRNAs and the developing offspring displayed the same phenotype as the offspring of the stressed fathers, indicating the important role that sperm microRNAs play in the transmission of paternal stress (Rodgers et al., 2015).

Little is known about how epigenetic modifications in the oocyte may influence post-fertilization development. To my knowledge, there are no studies that examine if and how mature oocytes undergo epigenetic modification as a result of stress. Recently,

however, Stewart et al. (2016) have developed a technique to study histone modification and DNA methylation in developing mouse oocytes. Their technique circumvents traditional issues surrounding studying the epigenetics of oocytes, such as the difficulty in collecting a sufficient number of cells for analysis. These authors collected embryonic day (E)18.5 oocytes, the point at which minimal DNA methylation is present following reprogramming, and P10 oocytes, the age at which *de novo* methylation begins. This new technique for oocyte collection could hopefully be applied to mature oocytes to reveal how methylation is affected by preconception experiences.

Following fertilization, a process known as "maternal-to-zygotic transition" (MZT) takes place in which maternal proteins and RNAs contained in the oocyte are deactivated and transcription of zygotic DNA commences (Fraser & Lin, 2016; Wasson et al., 2016). One maternal protein, the lysine demethylase KDM1A, plays an essential role in embryogenesis; deletion of the *kdm1a* gene in mouse oocytes results in embryonic arrest (Wasson et al., 2016). These same authors also showed that reduced levels of KDM1A results in behavioral impairments in adult offspring, such as stereotypical behaviors including excessive digging and food grinding, and increased anxiety. This study demonstrates that changes in maternal gene expression in the oocyte can result in behavioral changes in the offspring.

It is important here to carefully define what is meant by the phrase "transgenerational epigenetic inheritance" to avoid confusion. In order for there to be transgenerational inheritance, germline epigenetic marks must be passed on to a subsequent generation that had no direct exposure to the actual experience. In the case of maternal preconception stress, the female experiencing the stressor (F0 generation) and her germline (F1 generation) are directly exposed to the stress. The F2 generation would

be the earliest subject of inquiry to study transgenerational epigenetic inheritance (Skinner, 2011). Therefore, in the current research and in all the research on maternal preconception CUS discussed above, transgenerational epigenetic inheritance is not a possibility; the experience was neither transgenerational or inherited. Rather, stress-induced epigenetic modifications to the germline that may shape the development of the yet-to-be conceived offspring is one proposed mechanism.

1.5. Current Research

1.5.1. Goals of the Current Research

Given our current knowledge of the effects of MPS on offspring development, the goal of the current research is to provide a more complete picture of how offspring are affected by such an experience. Although effective at inducing a physiological stress response, the commonly used CUS paradigm is not necessarily translatable to human experience. Chronic stress is undoubtedly prevalent in the human population; however, most humans in developed areas of the world are not subjected to "random" stressors on a daily basis. Furthermore, many of the individual stressors included in the CUS paradigm are undeniably harsh and could only be relatable to human populations inhabiting impoverished or war-torn areas of the world. While the effects of such extreme experiences on offspring development are important, the current research aims to elucidate how a consistent, mild stressor may influence development, and in this way provide results that are more translatable to human experience in developed areas. Most humans experience predictable chronic stress, such as financial or work-related worries or

relationship problems. The current research mimics this type of stress in an animal model by subjecting female rats to a consistent daily stressor.

Current research into MPS has begun to gain an understanding of the lifelong effects of this preconception experience by electing to examine offspring at various ages, including fetal, pubertal, and early adult. The current research examined the *same* offspring at three different ages – neonatal, adolescent, and early adult. This allowed us to make direct observations regarding how a single experience can have varying effects depending on the age of examination. Also, although most studies in this area study both male and female offspring, select studies only examine male offspring due to the complications introduced by the female estrous cycle. The current research examined both male and female offspring.

Finally, the current research provided the very first opportunity to directly compare the effects that a single experience (i.e. stress) can have on offspring development when it is experienced by either the prospective mother or father. This is because the current research used methods that exactly mirror those used in the studies on PPS discussed above (Harker et al., 2015; Mychasiuk et al., 2013). By controlling all other factors, including timing and length of the stress paradigm, the type of stressor, and timing and nature of offspring testing, the only factor being differentially manipulated between the maternal and paternal experiments is the subject of the stress. It has long been assumed that the mother is the main, if not sole, determinate in developmental health. In recent years, research into the influence of fathers has become more prevalent, but it remains impossible to directly compare the influence of both parents. Therefore, the current research has important practical implications that have the potential to change our approach to preconception care.

1.5.2. Overview of the Current Research

Adult female rats experienced chronic predictable stress for 27 days, as in other studies that have used this paradigm (Harker et al., 2015; Mychasiuk et al., 2013; Mychasiuk et al., 2011; Muhammad et al., 2012; Mychasiuk et al. 2012), prior to mating with stress-naïve males. Maternal care was assessed on P12 and P16 to monitor any stress-induced changes in how dams interact with their pups. PS negatively impacts maternal care, but it is not currently known if MPS does as well. Given there is a three-week "wash-out" period (i.e. gestation) between the last stress-exposure and the birth of pups, it is likely that MPS dams will act normally towards their litters. Pups completed early behavioral testing beginning on P9. Negative geotaxis (P9 and P10) required pups to correct their orientation when placed facing downwards on an inclined plane and demonstrates the development of sensorimotor ability (Patin et al., 2004). Open field (P10-13 and P15) assessed early exploratory behavior and motor development.

Offspring completed two behavioral tests in adolescence; activity box to evaluate generalized activity and elevated plus maze (EPM). EPM is consistently shown to be a reliable indicator of anxiety in an animal model (Walf & Frye, 2007). Once in adulthood, animals completed activity box and EPM again, as well as Novel Object Recognition (NOR) and Whishaw Tray Reaching. NOR is a working memory task that required the animal to recall which object is novel in a given context; test paradigms typically include two contexts and two objects types, and animals spend time in each context first with two identical objects, then with one of each object (Antunes & Biala, 2012). Animals that perform well at this task spend more time attending to (e.g. sniffing) the novel object. Whishaw tray reaching assessed skilled forelimb movements; animals must reach though the bars of their holding cage and grasp food pieces contained in a tray outside the bars.

Food must be successfully grasped and held during consumption, or it will be lost through the wire floor of the cage (Whishaw, 1996).

Offspring were euthanized at two ages, weanling (P21) and adulthood (P100). A subset of offspring sacrificed at each age was perfused and prepared for Golgi-Cox staining; Golgi-Cox data will not be discussed in the present thesis. Remaining offspring at each age were prepared for Cresyl Violet staining. Cresyl Violet is a Nissl stain that allows quantification of neuron density, as well as measurements of cortical thickness and volumetric estimates of various subcortical structures. Cortical thickness was measured at several points throughout the brain, including medial, central, and lateral cortex. Thalamic area measured at anterior and posterior locations will be used as a proxy for thalamic volume.

1.5.3. Research Questions and Hypotheses

Based on the goals of the current research, this thesis aims to answer three main questions:

1) How does predictable, chronic maternal stress prior to conception influence the development of offspring brain and behavior throughout the lifespan?

2) Are male and female offspring differentially affected by maternal preconception stress?

3) How do the effects of maternal preconception stress compare with what we currently know about the effects of paternal preconception stress?

I predict widespread changes in brain and behavioral development in the offspring of preconception stressed mothers. Specifically, I anticipate stress-offspring to show delays in behavioral development, increased anxiety, decreased exploration of open

fields, impaired learning and memory, and deficits in fine motor control. Regarding brain development, I suspect that stress-offspring will show decreased cortical thickness and thalamic volume. I predict that aberrations in stress-offspring will be present throughout the lifespan and that there will be a sex difference, with female offspring being more affected overall. Finally, I predict that the offspring of preconception stressed fathers will show greater impairments than the offspring of preconception stressed mothers. Although the female germline is susceptible to epigenetic reprogramming, I anticipate that the nature of spermatogenesis will result in sperm that are more vulnerable to experience-dependent changes in gene expression when compared to oocytes.

1.5.4. Organization of Thesis

Chapter 2 provides a description of the general methods used in this thesis, such as the stress paradigm and mating techniques. Chapter 3 discusses the effects of MPS on the development of offspring behavior throughout the lifespan (i.e. early life, adolescence, adulthood). Chapter 4 is concerned with the effects of MPS on offspring brain. The final chapter is a general discussion of the major findings and their implications in the field of stress research.

Chapter 2

General Methods

2.1. Animals

All procedures were conducted in accordance with the Canadian Council of Animal Care and were approved by the University of Lethbridge Animal Care and Use committee. Three rounds of breeding were required to obtain the necessary number of litters for statistical analysis. Twenty Long-Evans females (8 MPS and 12 control) were bred with 14 Long-Evans males across the three rounds, resulting in six successful MPS pregnancies and four successful control pregnancies. In total, among six maternal stress litters and four control litters there were 80 MPS pups and 45 control pups. Animals were given food and water ad libitum and were maintained on a 12-h light/dark schedule (lights on from 07:30 to 19:30) in a temperature-controlled (21°) breeding room.

2.2. Stress Paradigm

All female rats were pair-housed with a female in the same condition for the length of the stress paradigm. Females in the MPS condition (n = 8) experienced elevated platform stress daily for 27 consecutive days immediately prior to mating. For 30min twice a day (09:00 and 15:00), females were individually placed on a 1m high Plexiglas® platform with a surface measuring 21cm x 21cm in a brightly lit room (Figure 2.1). If the rat jumped or fell from the platform, it was promptly replaced. Overt signs of distress, such as frequent urination and defecation, were observed regularly. Females in the control condition (n = 12) were removed from their home cages and brought to a separate room where they were left undisturbed with their cagemate following the same schedule as the

stress paradigm; this was done to ensure control females and stressed females received the same amount of handling each day. All females were weighed daily in the morning.

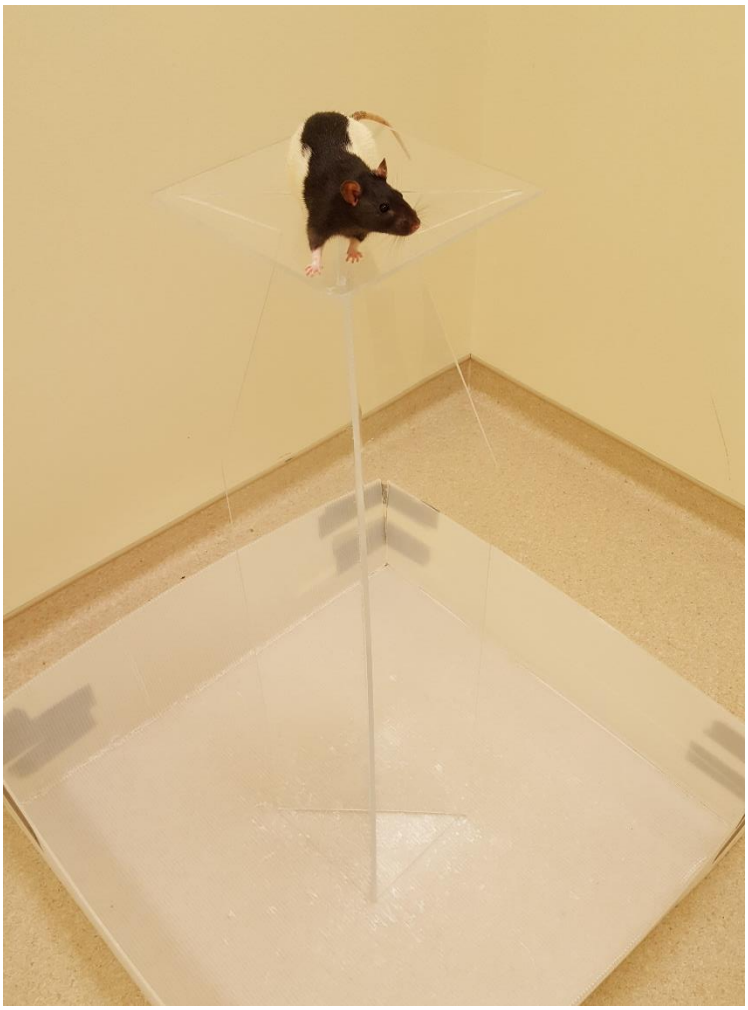

Figure 2.1. Elevated platform stress. Adult female rats were placed on a 1m high platform with a surface of 21cm x 21cm for 30min twice daily for 27 consecutive days immediately prior to mating.

2.3. Breeding and Gestation

Following the last day of the stress paradigm, all females were paired with a stress-naïve male Long-Evans rat and left to mate for one week. Males were then removed and females were pair-housed with the same cagemate as during the stress paradigm, and daily weighing commenced to monitor pregnancy. Females remained pair-

housed until a weight gain of ≥100g was achieved or 21 days had passed since mating began, at which time they were singly housed until parturition. Dams and their litters were left undisturbed until post-natal day (P)7, aside from cage cleaning twice weekly. Pups were weaned on P21.

2.4. Statistical Analysis

All statistical analysis was done with IBM SPSS 23 for Mac. Prior to weaning, hierarchical linear mixed models were used to group animals by litter and account for litter effects. Litter was included as a random factor and condition and sex as fixed factors. Post-weaning, two-way ANOVAs with condition and sex as factors were used in behavioral analysis. For anatomical analyses, three-way ANOVAs with condition, sex, and hemisphere were used. Graphs and tables were constructed using Excel 2016 (Microsoft) for Windows.

Chapter 3

Maternal Preconception Stress: Effects on Offspring Behavior

3.1. Methods

3.1.1. Maternal Care

Maternal care was recorded on P12 and P16 for 15min at 09:00 and 16:00. Recording occurred in the housing room in the home cage of each dam. Home cages were placed in the same location during each filming session and left for 5min prior to filming to allow dams and litter to settle following displacement. Videos were scored for time spent doing each of the following behaviors: 1) being in non-contact with pups; 2) licking and grooming pups; 3) arch-back nursing; 4) blanket-posture nursing; 5) passive-posture nursing; and 6) unspecified contact, including carrying.

3.1.2. Negative Geotaxis

Pups were tested in negative geotaxis on P9 and P10. Each pup was individually placed facing downwards in the center of a 40° incline covered in rubber mesh to provide grip, and replaced each time it fell. Pups were filmed for 1min on each day. Videos were scored for the length of time rotated above the horizontal plane and for the number of falls from the platform.

3.1.3. Open Field

Pups were tested in the open field on P10-13 and P15. Pups were individually placed in the center of a Plexiglas® box measuring 10in x 15in. The floor of the open

field was divided into 150 1in x 1in squares (Figure 3.1). Pups were filmed for 1min while they explored the open field. The open field was cleaned with virkon between pups. Videos were scored for the number of novel squares and total squares entered by either front paw.

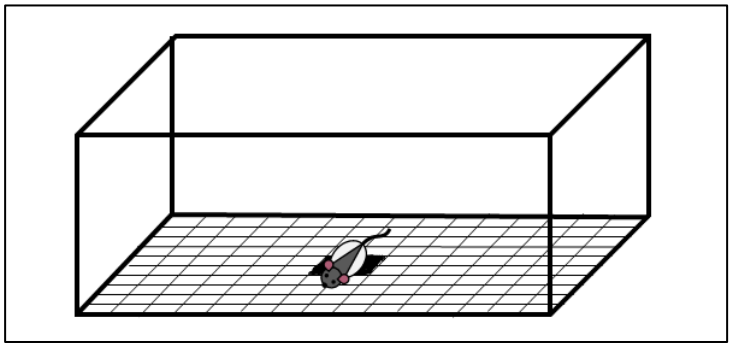

Figure 3.1: Open Field. Pups were tested in the Open Field on P10-13 and P15. The apparatus was a Plexiglas® box measuring 10in x 15in with a grid of 150 1in x 1in squares on the floor.

3.1.4. Activity Box

Animals were tested in Activity Box in adolescence (P35) and early adulthood (P65). Activity was recorded using the Versamax Animal Activity Monitor system (Accuscan Instruments, Inc.). Rats were placed individually in a clear Plexiglas box (42cm x 42cm x 30cm) nestled in the monitoring system. Activity was recorded for 10min as a series of 10 1min samples. Software recorded the amount of horizontal activity, the total time spent moving, and the time spent in each the perimeter and center of the box. Boxes were cleaned with virkon between animals. Activity was summed across the 10 1min samples to provide one data point per animal per measure.

3.1.5. Elevated Plus Maze

Offspring were tested in elevated plus maze in adolescence (P36) and early adulthood (P66). The apparatus was constructed of black opaque Plexiglas®, was 1m tall, and had two arms with no walls and two arms with walls 40cm high. All arms were 40cm long and 10cm wide (Figure 3.2). The distal 20cm of each open arm was considered the "titanic zone", and was marked with a white tick so that it was clear when the rats crossed this point. Each rat was placed in the maze with head and shoulders in the center square and body on an open arm. Animals were left undisturbed and recorded for 5 minutes. The maze was cleaned with virkon between animals. Videos were scored for the length of time the rat spent in each area (open arm, titanic zone, closed arm, center square), the number of entries into each area, and the latency to a closed arm upon first being placed on the maze. Rats were considered to have entered an area once the shoulders passed the boundary (i.e. once fur color changed from black to white).

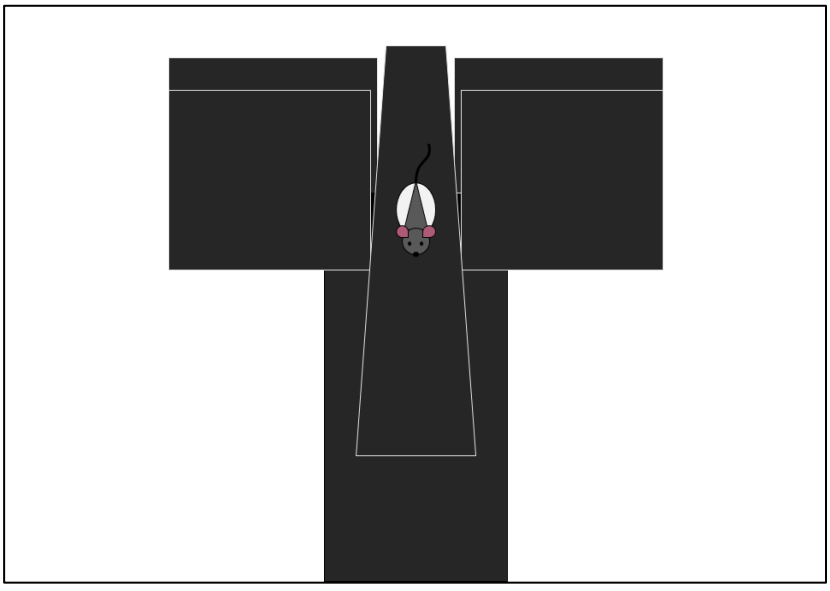

Figure 3.2: Elevated Plus Maze. Offspring were tested on P35 and P65. The apparatus was constructed on black Plexiglas® and had two "open" arms and two "closed" arms. Each arm was 40cm long and 10cm wide; the apparatus was 1m above the floor.

3.1.6. Novel Object Recognition

Offspring were tested for novel object recognition in early adulthood (~P70). Over the course of 3 days, rats were habituated to the two different contexts (Day 1 and Day 2), then exposed to distinct objects in each context and tested for working memory (Day 3). Context 1 (C1) was a square blue container in a dimly light room, and Context 2 (C2) was a circular orange container in a brightly lit room. During Habituation, there were no objects in either container and each rat spent 10min per context. On Day 3, rats spent 5min per context, but objects were now present. In C1, there were two of Object 1 (O1), which was a clear colorless cube, and in C2, there were two of Object 2 (O2), a blue irregularly-shaped object of similar size (Familiarization). Following 5min in each context, rats rested for 5min, and were then tested, again for 5min. For the test, each rat was placed in whichever context it experienced first, with half of rats being tested in C1 and half in C2. Each context now had one of each object; rats were placed in the container equidistant from each object (Test Phase; Figure 3.3). Rats were filmed for 5min. Videos were scored for the length of time the rat spent attending to either object; this included sniffing and active exploration of the object, and excluded staring in the direction of the object and standing on the object.

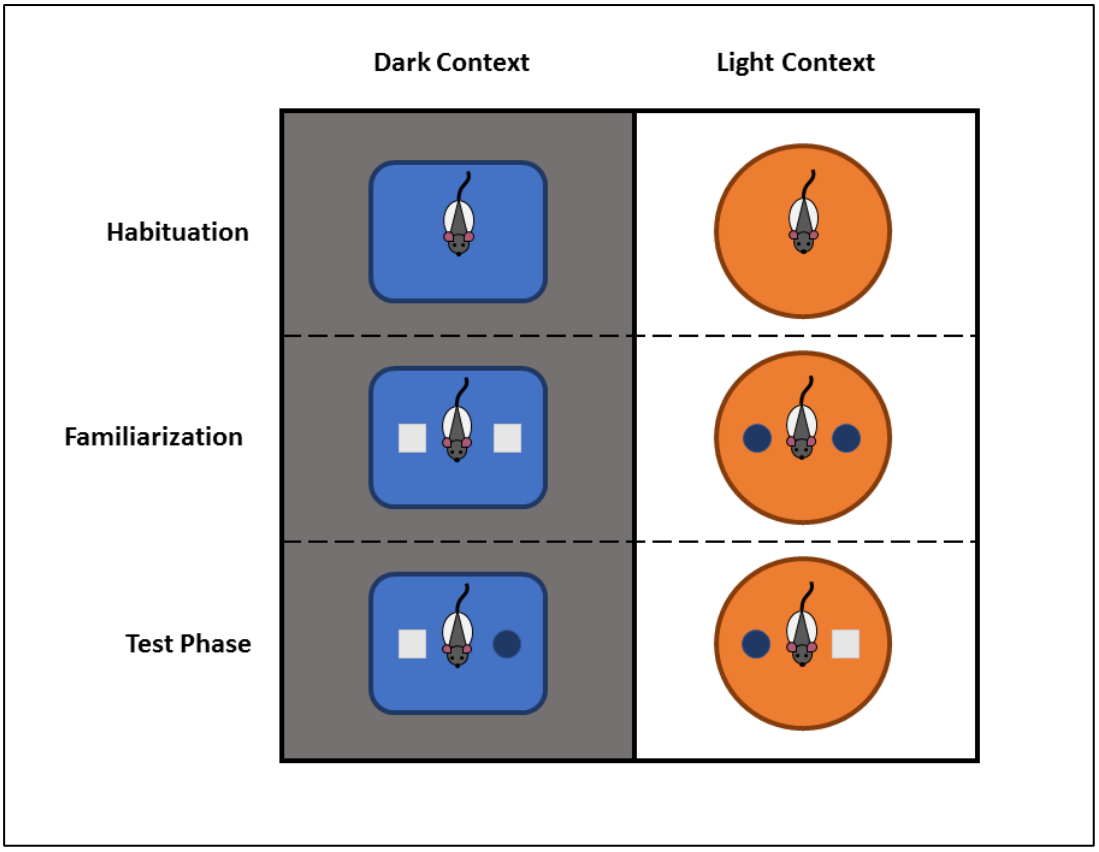

Figure 3.3: Novel Object Recognition. Animals completed a 3-day paradigm to test working memory that consisted of habituation, familiarization, and testing in two distinct contexts.

3.1.7. Whishaw Tray Reaching

Offspring were tested in Whishaw Tray Reaching beginning in early adulthood (~P75). A week of food restriction preceded training to motivate rats to reach for a food reward. Rats were allowed to lose no more than 10% of their starting body weight. Rats remained food restricted for the duration of training and testing. Rats were trained for 30min daily for 14 days. The testing apparatus was a Plexiglas® cage with wire mesh floor and 3mm wide bars spaced 10mm apart along one wall; the three other walls were solid Plexiglas®. Outside of the bars there was a food tray 5cm wide and 7mm deep that contained food pieces approximately 5mm in size (Figure 3.4). Rats were trained to reach

through the bars, grab a piece of food, and bring it to their mouths. Any dropped food was lost through the wire floor. Rats were discouraged from obtaining food using the mouth or tongue with a gentle tap on the nose. Following the last day of training, rats were placed in the cages as usual and filmed for 10min. Videos were scored for the number of grasp attempts made with either paw, and the number of attempts that resulted in successfully consuming the food (hits).

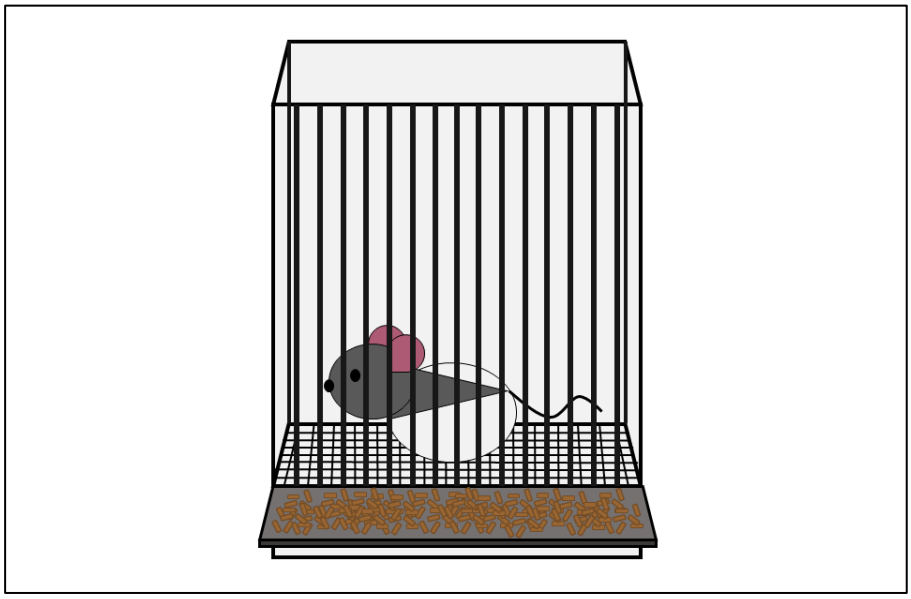

Figure 3.4: Whishaw Tray Reaching. Animals were trained to reach through the bars to grasp food pieces and bring them to their mouths without dropping through the wire floor.

3.2. Results

Of the eight MPS-dams and 12 control-dams, six and four successfully conceived and gave birth to litters, respectively. Of note is the particularly low success rate for control-dams, a mere 33%. Maternal age was the most likely contributor to this finding; some females were older than others and, having never given birth, were unable to conceive, as was the case for the two MPS-dams that did not become pregnant.

3.2.1. Maternal Weight

Preconception stressed-dams and control-dams did not differ with respect to the percent of initial body weight lost between the first and final day of the stress paradigm ($F(1,9) = 0.988$, $p = 0.35$; Figure 3.5). MPS-dams lost approximately 5.2% (±2.8%) of their initial weight and control-dams lost 0.8% (±3.4%).

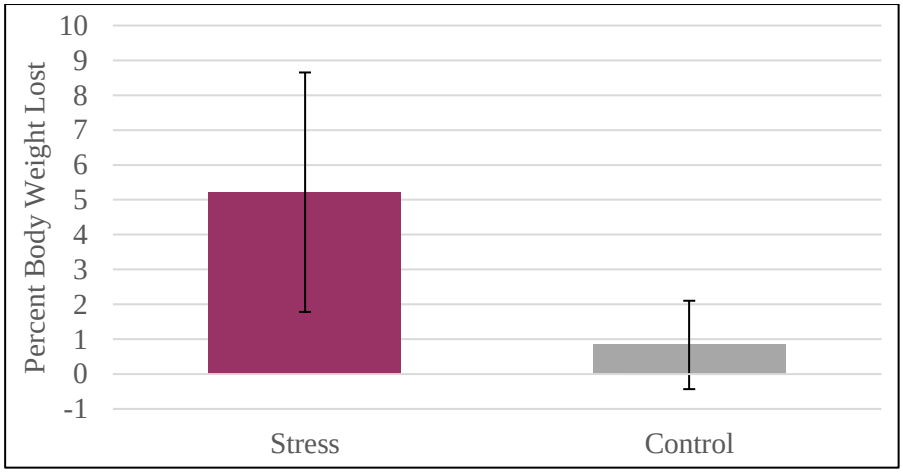

Figure 3.5: Percent of initial body weight lost during stress paradigm by MPS- and control-dams; there was no significance difference. Error bars = SE.

3.2.2. Litter Characteristics

Litter size did not differ between MPS- and control-dams ($F(1,9) = 1.424$, $p = 0.27$; Figure 3.6). MPS-dams had 13.5 (±1.3) pups and control-dams had 11.25 (±1.1) pups. Sex ratio also did not differ ($F(1,9) = 0.066$, $p = 0.80$); MPS-litters contained 48.2% (±3.7%) male pups, and control-litters 51.2% (±13.4%) males. However, there was more variability in litter composition for control-litters than MPS-litters. Control-litters had a range of 22.2% - 80.0% males, whereas MPS-litters were more balanced, with 40.0% - 62.5% males.

Figure 3.6: Number of pups per litter born to MPS- and control-dams. Error bars = SE.

3.2.3. Maternal Care

MPS- and control-dams provided the same level of maternal care towards their pups in terms of nursing ($F(1,13) = 0.341$, $p = 0.57$ on P12; $F(1,17) = 0.000$, $p = 0.99$ on P16), licking and grooming ($F(1,13) = 0.055$, $p = 0.82$ on P12; $F(1,17) = 0.693$, $p = 0.42$ on P16), and being in non-contact ($F(1,13) = 0.002$, $p = 0.96$ on P12; $F(1,17) = 0.138$, $p = 0.72$ on P16) with pups on both P12 and P16 (Figure 3.7).

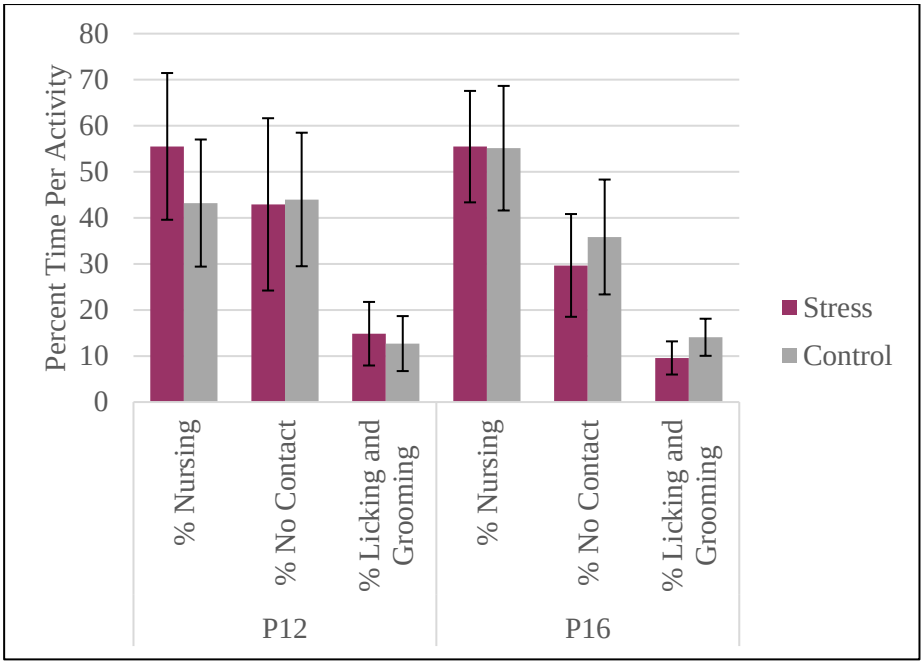

Figure 3.7: Maternal care. Percentage of time dams spent doing each activity with pups on P12 and P16. AM and PM data have been combined. There were no significant differences. Error bars = SE.

3.2.4. Early Behavior

Ten litters were included in the analyses of early behavior, comprised of six MPS-litters and four control-litters. There were 125 pups in total, 80 MPS-offspring (39 males and 41 females) and 45 control-offspring (23 males and 22 females). Hierarchical linear models were used in the analysis of early behavior to group pups by litter and account for litter effects; degrees of freedom may have a decimal point for this reason.

3.2.4.1. Negative geotaxis. MPS did not impact performance in negative geotaxis on P9 or P10 in either male or female offspring. MPS- and control-offspring spent the same amount of time rotated above the horizontal plane on each day tested (P9 $F(1,60.2) = 0.700$, $p = 0.41$; P10 $F(1,57.4) = 0.371$, $p = 0.55$). Similarly, male and female

offspring did not differ on either day (P9 $F(1,119.3) = 0.000$, $p = 0.99$; P10 $F(1,118.9) = 0.021$, $p = 0.89$; Figure 3.8a).

There were also no differences in the number of falls from the platform on either day between MPS- and control-offspring (P9 $F(1,39.2) = 0.537$, $p = 0.47$; P10 $F(1,51.5) = 0.841$, $p = 0.36$) or between male and female offspring (P9 $F(1,117.6) = 0.322$, $p = 0.57$; P10 $F(1,118.5) = 0.000$, $p = 0.99$; Figure 3.8b).

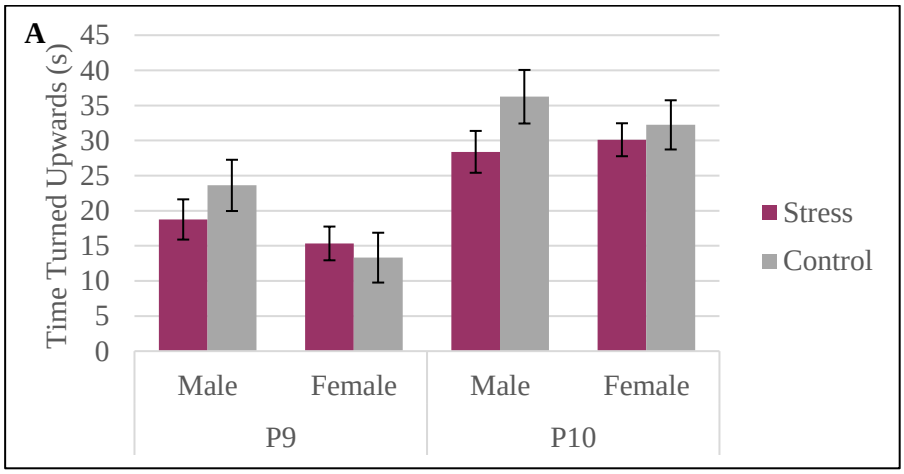

Figure 3.8a: Negative Geotaxis. Mean time in seconds spent rotated above the horizontal plane; there were no significant differences between MPS- and control-offspring or male and female offspring on either day tested. Error bars = SE.

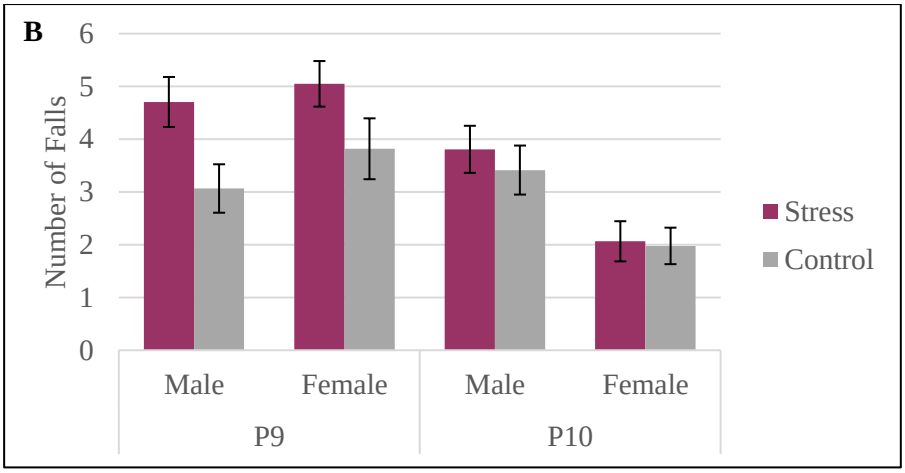

Figure 3.8b: Negative Geotaxis. Mean number of falls from the platform; there was no difference between MPS- and control-offspring or between male and female offspring. Error bars = SE.

3.2.4.2. Open field. A significant main effect of MPS was present for number of novel squares on P11; MPS-offspring entered fewer novel squares than control-offspring ($F(1,44.519) = 5.464$, $p = 0.02$). There were no main effects of sex or significant interactions (Table 3.1; Figure 3.9).

Table 3.1: Summary of Open Field results. Only one significant effect was apparent. ↓ = decrease in MPS-offspring relative to control-offspring. --- = no change. * = $p < 0.05$.

		Condition		Sex		Interaction
		Change	*p*	Change	*p*	*p*
P10	Novel	---	0.41	---	0.84	0.77
	Total	---	0.258	---	0.053	0.073
P11	Novel	↓	0.024 *	---	0.394	0.082
	Total	---	0.724	---	0.873	0.853
P12	Novel	---	0.576	---	0.89	0.991
	Total	---	0.082	---	0.381	0.137
P13	Novel	---	0.745	---	0.736	0.984
	Total	---	0.09	---	0.772	0.255
P15	Novel	---	0.469	---	0.074	0.171
	Total	---	0.624	---	0.311	0.902

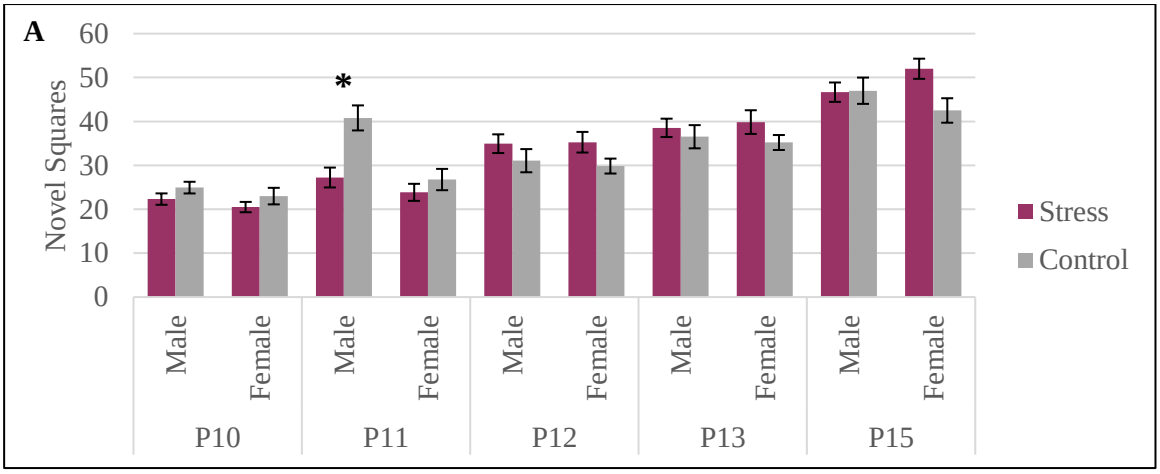

Figure 3.9a: Open Field. Number of novel squares entered by male and female offspring; MPS-males entered fewer novel squares than control-males on P11. Error bars = SE. * = $p < 0.05$.

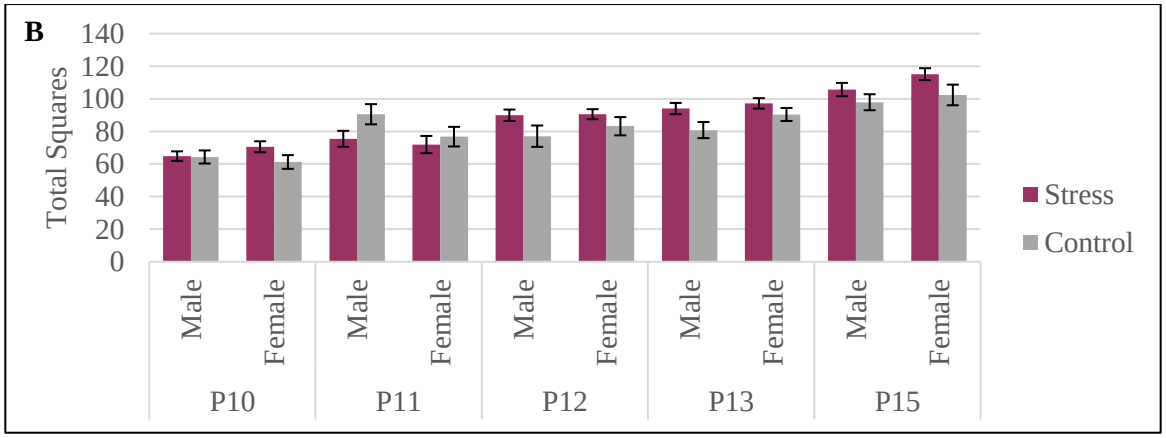

Figure 3.9b: Open Field. Number of total squares entered; there were no significant differences. Error bars = SE. ***** = $p < 0.05$.

3.2.5. Adolescent Behavior

Eighty offspring were included in the analyses for adolescent behavior, comprised of 53 MPS-offspring (28 male and 25 female) and 27 control-offspring (14 male and 13 female).

3.2.5.1. Activity box. MPS did not impact overall activity in the Activity Box on P35 in male or female offspring ($F(1,79) = 0.081$, $p = 0.78$; Figure 3.10a), or the length of time spent moving ($F(1, 79) = 0.042$, $p = 0.84$; Figure 3.10b). However, MPS did affect the length of time spent along the perimeter of the box relative to the center, but only in male offspring. MPS-males spent more time along the perimeter, and therefore less time in the center, compared to control-males ($F(1,41) = 4.499$, $p = 0.04$). MPS- and control-females did not differ ($F(1,37) = 0.284$, $p = 0.60$; Figure 3.10c-d).

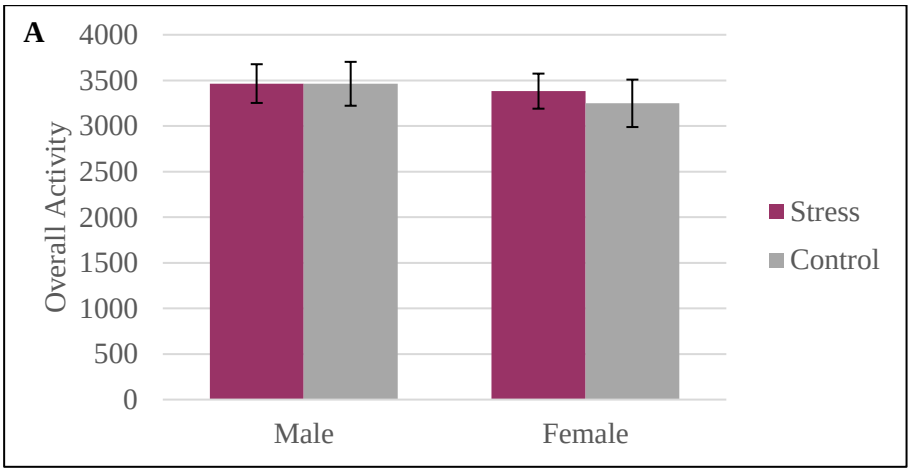

Figure 3.10a: P35 Activity Box. Overall horizontal activity; there were no significant main effects or interactions. Error bars = SE.

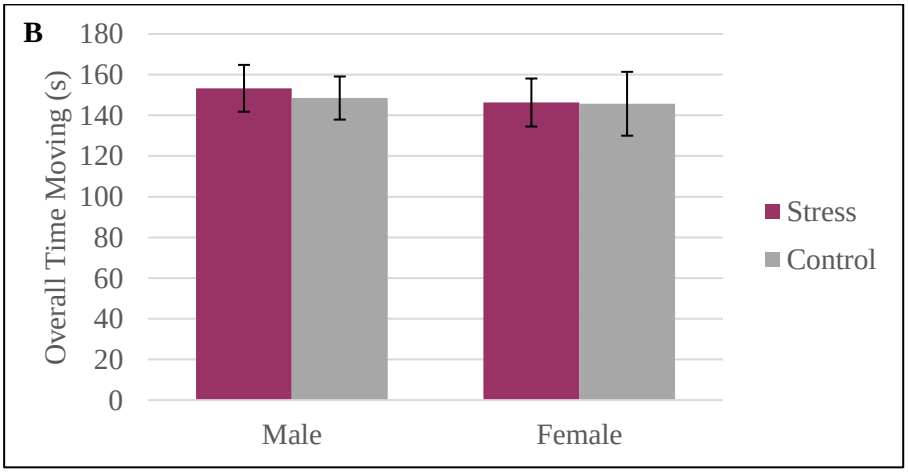

Figure 3.10b: P35 Activity Box. Total time spent moving in the box; there were no significant main effects or interactions. Error bars = SE.

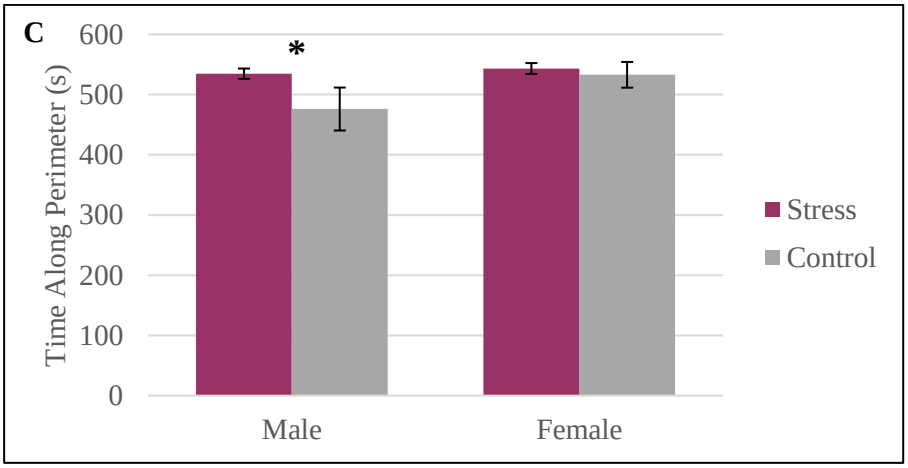

Figure 3.10c: P35 Activity Box. Time spent in the perimeter zone of the box; male MPS-offspring spent more time along the perimeter than control-males. Error bars = SE. * = $p < 0.05$.

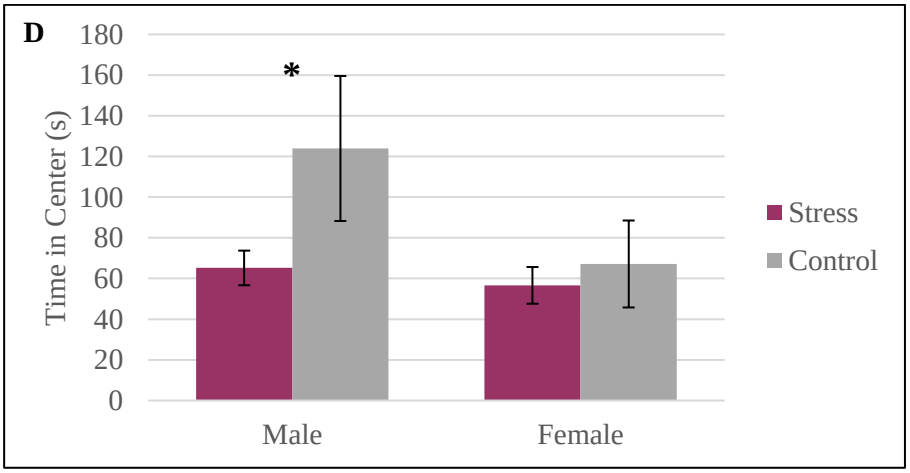

Figure 3.10d: P35 Activity Box. Time spent in the center of the box; male MPS-offspring spent less time in the center than control-males. Error bars = SE. ***** = $p < 0.05$.

3.2.5.2. Elevated plus maze. MPS- and control-offspring did not differ with respect to length of time spent in each area of the maze (closed arm, open arm, or titanic zone) on P36. However, MPS-offspring entered both the closed arms and the titanic zone fewer times than control-offspring ($F(1,79) = 11.989$, $p = 0.001$ and $F(1,79) = 4.269$, $p = 0.04$ respectively; Figure 3.11a-b). There was also a Condition X Sex interaction for latency to enter a closed arm ($F(1,79) = 4.184$, $p = 0.04$); male MPS- and control-

offspring did not differ (F(1,41) = 0.460, p = 0.50), but MPS-females entered a closed arm significantly faster than control-females F(1,36) = 4.824, p = 0.04; Figure 3.11c).

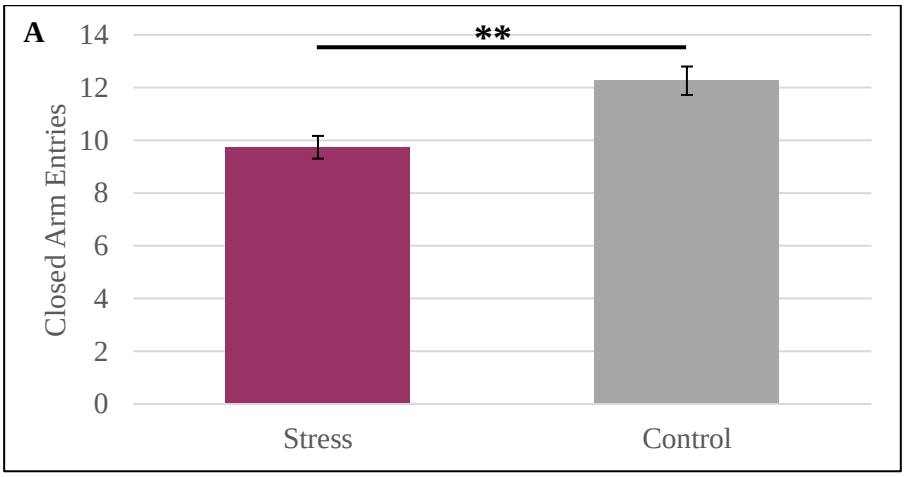

Figure 3.11a: P36 Elevated Plus Maze. Number of closed arm entries; control-offspring entered the closed arms significantly more times than MPS-offspring. Error bars = SE. ** = $p < 0.01$.

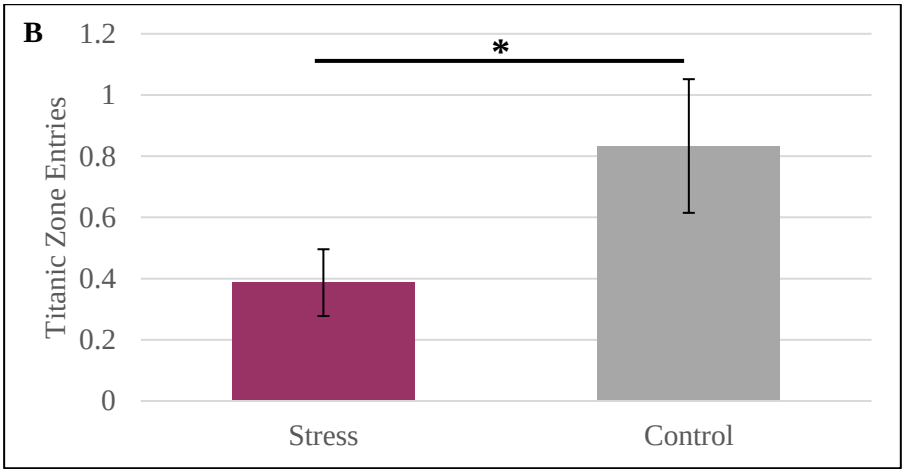

Figure 3.11b: P36 Elevated Plus Maze. Number of titanic zone entries; control-offspring entered the titanic zone significantly more times than MPS-offspring. Error bars = SE. * = $p < 0.05$.

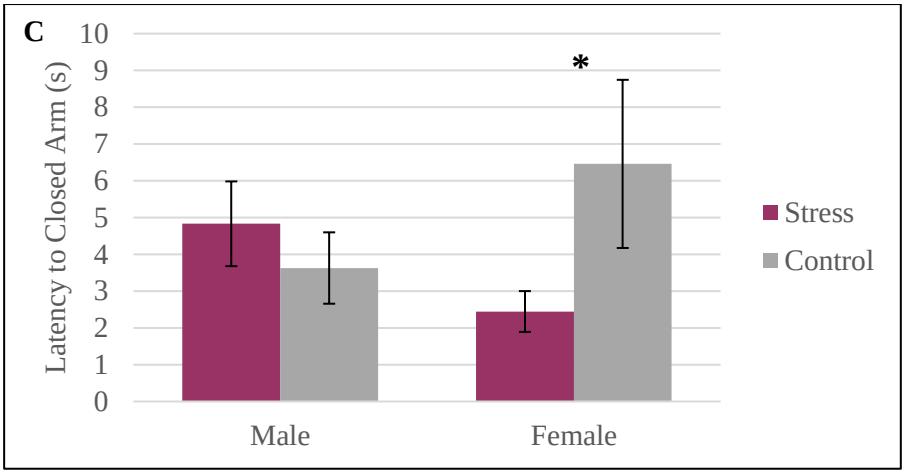

Figure 3.11c: P36 Elevated Plus Maze. Latency to enter to closed arm; MPS-females took significantly less time to enter a closed arm compared to control-females. Error bars = SE. * = $p < 0.05$.

3.2.6. Adult Behavior

Seventy-eight offspring were included in the analysis of adult behavior, 53 MPS-offspring (28 male and 25 female) and 25 control-offspring (14 male and 11 female).

3.2.6.1. Activity box. There were no significant main effects or interactions for horizontal activity at P65. MPS- and control-offspring were equally active in adulthood ($F(1,77) = 0.266$, $p = 0.61$), as were male and female offspring ($F(1,77) = 2.328$, $p = 0.13$; Figure 3.12).

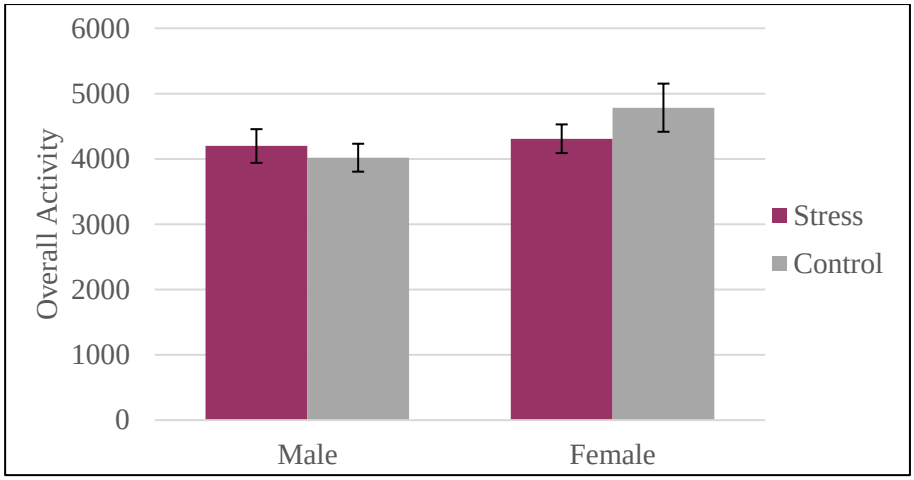

Figure 3.12: P65 Activity Box. There were no significant main effects or interaction for the level of horizontal activity. Error bars = SE.

3.2.6.2. Elevated plus maze. MPS- and control-offspring spent an equal length of time in each area of the maze (closed arm, open arm, titanic zone) on P66. There were significant main effects of Sex for time in open arms (female > male; $F(1,77) = 10.706$, $p = 0.002$; Figure 3.13a), time in the titanic zone (female > male; $F(1,67) = 4.073$, $p = 0.05$; Figure 3.13c), and the number of open arm entries (female > male; $F(1,77) = 6.568$, $p = 0.01$, Figure 3.13b) and titanic zone entries (female > male; $F(1,77) = 9.418$, $p = 0.003$; Figure 3.13d).

The effects of MPS on the number of entries into open arms and the titanic zone were sexually dimorphic. Male MPS-offspring entered each of these areas fewer times that control-males ($F(1,41) = 5.06$, $p = 0.03$ and $F(1,41) = 7.17$, $p = 0.01$, respectively). Female offspring were unaffected by MPS ($F(1,35) = 0.384$, $p = 0.54$ and $F(1,35) = 0.246$, $p = 0.62$, respectively). Furthermore, there was a trend towards MPS-males spending more time in the closed arm compared to control-males ($F(1,41) = 3.706$, $p = 0.06$), but this was not observed in female offspring ($F(1,35) = 0.256$, $p = 0.62$; Figure 3.13e).

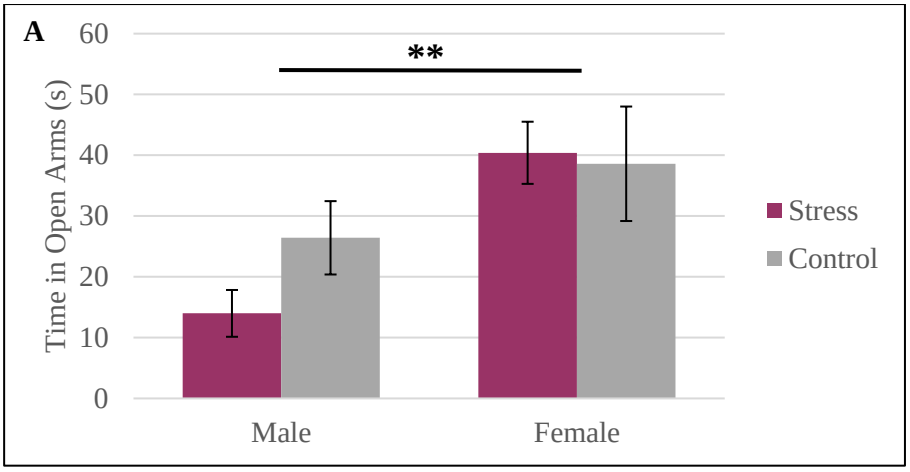

Figure 3.13a: P66 Elevated Plus Maze. Time spent in the open arms; females spent more time than males. Error bars = SE. ** = $p < 0.01$.

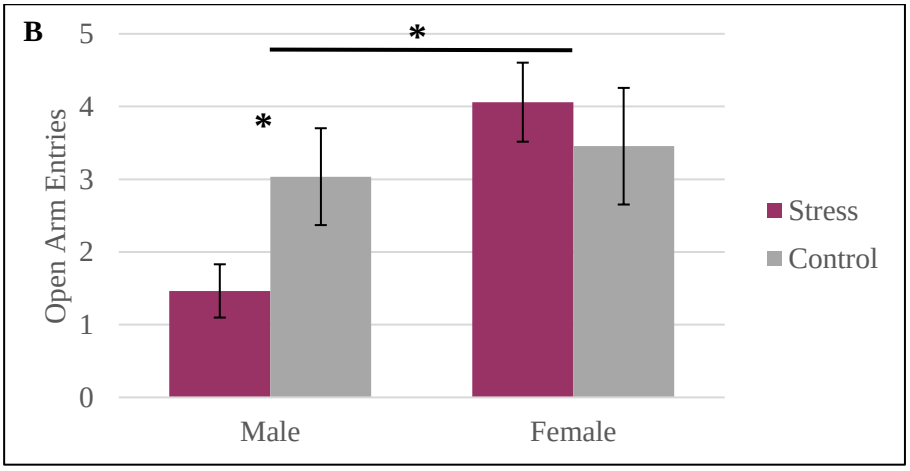

Figure 3.13b: P66 Elevated Plus Maze. Number of entries into the open arms; MPS-males entered fewer time than control-males, and males fewer times than female. Error bars = SE. * = $p < 0.05$.

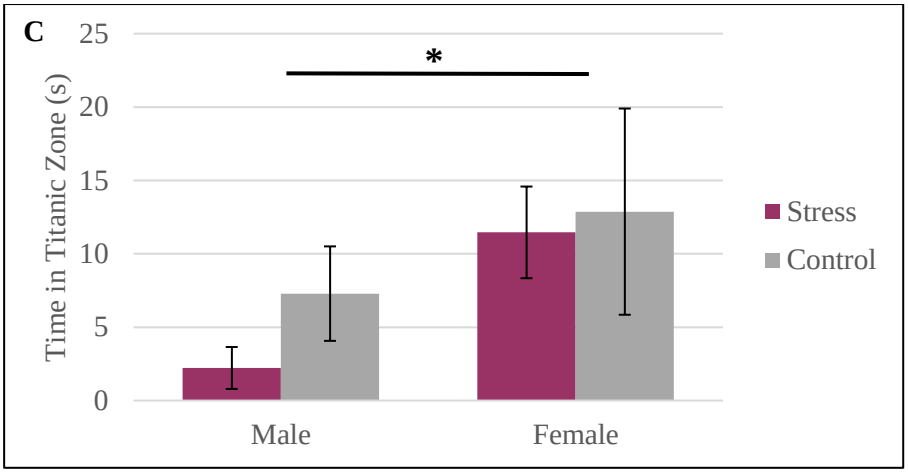

Figure 3.13c: P66 Elevated Plus Maze. Time spent in the titanic zone; females spent more time than males. Error bars = SE. * = $p < 0.05$.

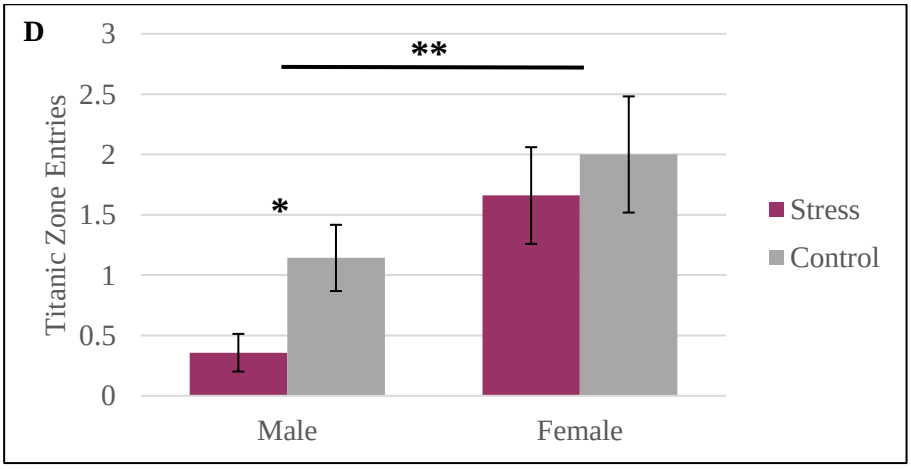

Figure 3.13d: P66 Elevated Plus Maze. Number of entries into the titanic zone; MPS-males entered fewer times than control-males, and males fewer times than females. Error bars = SE. * = $p < 0.05$. ** = $p < 0.01$.

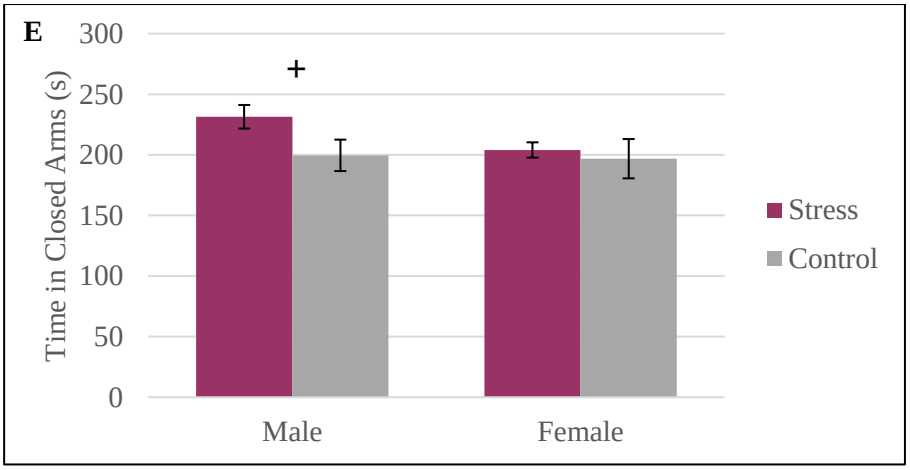

Figure 3.13e: P66 Elevated Plus Maze. Time spent in the closed arms; there was a trend towards MPS-males spending more time that control-males. Error bars = SE. + = p = 0.06.

3.2.6.3. Novel object recognition. There was a main effect of MPS on time spent attending to the novel object ($F(1,76) = 4.468$, $p = 0.04$). Only male offspring were affected; MPS-males spent less time attending to the novel object than control-males ($F(1,40) = 6.794$, $p = 0.01$), and the effect was limited to the dark context ($F(1,19) = 6.016$, $p = 0.03$ for dark; $F(1,20) = 0.548$, $p = 0.47$ for light). Female offspring showed no significant difference in either context ($F(1,19) = 0.086$, $p = 0.77$ for dark; $F(1,15) = 0.355$, $p = 0.56$ for dark; Figure 3.14).

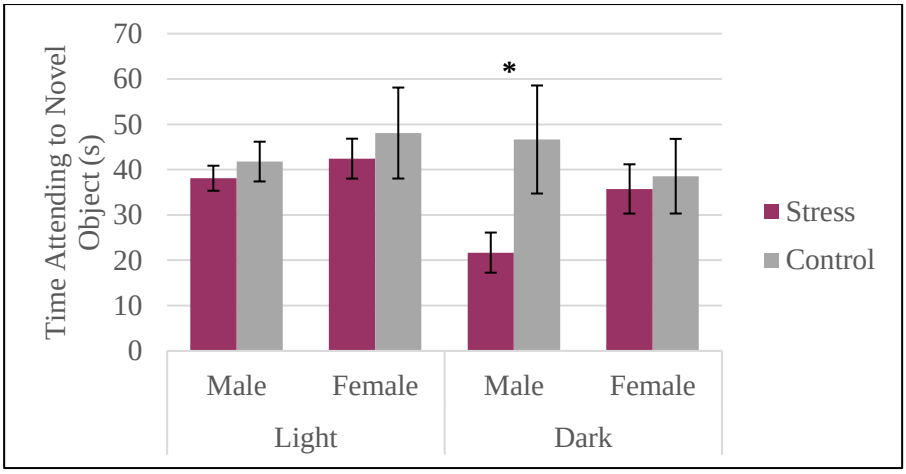

Figure 3.14: Novel Object Recognition. Male MPS-offspring spent less time attending to the novel object than control-males in the dark context only. There were no significant differences in female offspring, or in the light context. Error bars = SE. ***** = $p < 0.05$.

3.2.6.4. Whishaw tray reaching. MPS-offspring were significantly impaired at skilled forelimb reaching relative to control-offspring ($F(1,77) = 14.467$, $p < 0.001$; Figure 3.15). Further analysis revealed that only male MPS-offspring were impaired ($F(1,41) = 16.784$, $p < 0.001$ for males; $F(1,35) = 1.937$, $p = 0.17$ for females). Male and female offspring did not differ ($F(1,77) = 1.181$, $p = 0.28$), nor was there a Condition x Sex interaction ($F(1,77) = 3.158$, $p = 0.08$).

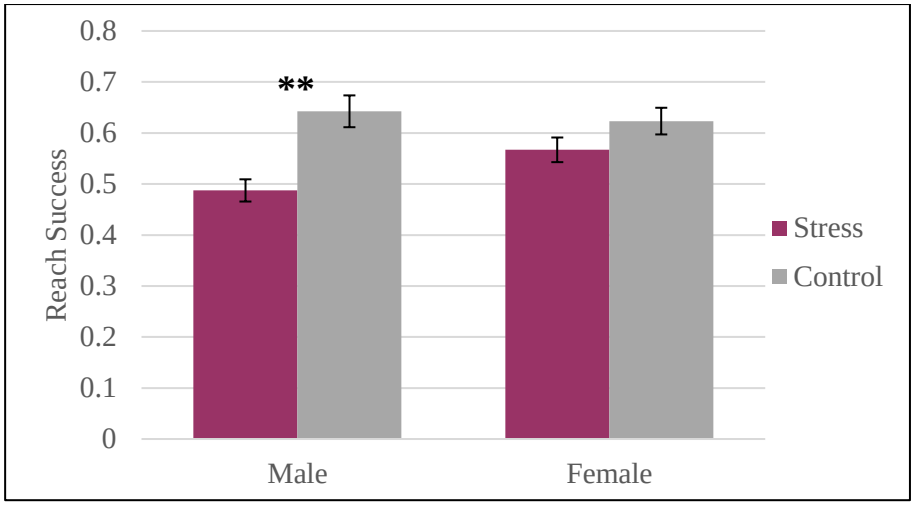

Figure 3.15: Whishaw Tray Reaching. Male MPS-offspring were significantly impaired at reaching compared to male control-offspring; females were unaffected. Error bars = SE. ** = $p < 0.01$.

3.3. Discussion

MPS dams demonstrated signs of distress while on the elevated platform, including weight loss, frequent defecation and urination, and jumps from the platform. However, weight change in MPS- and control-dams between the first and final day of the paradigm was not significantly different, contrary to the anticipated result and the observed result in males exposed to PPS (Harker et al., 2015). This may be due to the weight of the control-dams remaining stable; control males in the PPS study gained weight during the course of the stress paradigm. Litter size was unaffected by MPS, as was the case with PPS (Harker et al., 2015).

3.3.1. MPS Impacts Offspring Development Without Affecting Maternal Care

There were no MPS-induced changes in maternal care observed in the present study; MPS-dams and control-dams acted identically towards their litters on P12 and P16. This research used a truncated sampling method to assess maternal care; dams and litters

were recorded twice daily for 15min on two days. Michael Meaney and colleagues, who have completed extensive studies on maternal care, typically record dam-litter interactions over six to eight days in five, 72-minute recording sessions spread evenly over 24hrs. The present study opted for a condensed sampling schedule because how MPS affects maternal care was not the main question of interest, but rather an unavoidable confound. To attribute any observed differences in the offspring of MPS-dams to the preconception stress experience, I had to verify that MPS-dams did not act differently towards their litters than control-dams, given that variations in maternal care affect offspring development (Caldji, Diorio, & Meaney, 2000; Champagne, Francis, Mar, & Meaney, 2003). If differences were observed in maternal care, then differences observed in offspring could have been behaviorally transmitted. Fortunately for the present study, maternal care was unaffected by the preconception stress experience. Therefore, any changes observed in offspring can reasonably be attributed to the indirect influence of MPS.

3.3.2. MPS Increases Anxiety-Like Behavior in Offspring

MPS-offspring showed increased anxiety compared to control offspring, assessed by activity box in adolescence, and the elevated plus maze in adolescence and adulthood. Although MPS- and control-offspring showed no differences in overall activity, adolescent MPS-offspring spent more time along the perimeter of the activity boxes relative to the center. Rats naturally display positive thigmotaxis, meaning that they prefer to be in close proximity to objects or walls. Open spaces pose a greater threat to an animal against predators; therefore, positive thigmotaxis serves an adaptive function (Harris, D'Eath, & Healy, 2009). Thigmotactic behavior can be used as an indicator of anxiety;

the more anxious an animal, the more inclined it will be to shield itself from predators, so the more time it will spend away from open spaces. In activity box, MPS-offspring displayed increased positive thigmotaxis relative to control-offspring, suggesting increased anxiety and a propensity to avoid open spaces.

In elevated plus maze, anxiety is associated with increased time in the closed arms relative to the open arms; time in the titanic zone is associated with low-level anxiety. MPS did not influence how much time animals spend in any area of the maze. However, MPS did influence the number of entries into the different arms. Adolescent and adult MPS-offspring entered the titanic zone fewer times than control-offspring, implying an MPS-induced increase in anxiety (Walf & Frye, 2007). In line with this interpretation, adult MPS-offspring also entered the open arms fewer times than controls. Interestingly, adolescent MPS-offspring entered the closed arms fewer times than controls. This finding has two potential interpretations. Overly-anxious animals may be inclined to enter a closed arm and refuse to leave, giving them a low number of closed arm entries. Conversely, anxious animals may spend their time in the maze running from one closed arm to another, giving them a high number of entries. Given that the other findings from elevated plus maze and from activity box suggest increased anxiety, I am inclined to accept the first explanation. One further finding is that female MPS-offspring were faster to enter a closed arm upon being placed on the maze than control-females, again suggesting increased anxiety. Control animals spent more time investigating their new surroundings before seeking shelter in a closed arm.

Increased anxiety is a typical and potentially the most robust outcome associated with developmental stress (Weinstock, 2008; Lupien et al., 2009), especially in female progeny (Weinstock, 2007). To my knowledge, there have been no studies examining

how MPS predisposes offspring to display anxious behaviors. One study has demonstrated increased depressive-like symptoms (Huang et al., 2013). Interestingly, PPS appears to reduce anxiety in young (Mychasiuk et al., 2013) and adolescent offspring, whereas adult offspring show the typical pattern of increased anxious behavior (Harker, 2015).

3.3.3. MPS Impairs Motor Control in Offspring Dependent on Age

MPS-offspring demonstrated impaired motor control in adulthood, but no impairments were present in pre-weanling rats, suggesting that the impact of MPS on motor development is delayed, or that MPS only impairs complex, fine motor control. MPS- and control-offspring performed identically in Negative Geotaxis and P9 and P10. Therefore, MPS did not affect sensorimotor development in offspring. This finding can be contrasted with PPS, in which PPS-offspring were impaired in Negative Geotaxis relative to controls on P9, but performed equally well on P10 (Mychasiuk et al., 2013). PS also induces impairments in sensorimotor development in male and female offspring relative to controls (Mychasiuk et al., 2011). It is unclear why sensorimotor development was resistant to MPS-induced aberrations in the current study. Offspring born to dams stressed while pregnant experience elevated levels of COR while in utero, which directly disrupts the development of the fetal HPA axis. Conversely, MPS-offspring are only present during the stressor as the maternal germline; therefore, the shaping of the offspring HPA-axis is not directly influenced by the stressor. It is possible that MPS-dams continue to have elevated COR levels into gestation, even after the stress has subsided, but that this level of COR is not sufficient to induce impairments in early development. PPS potentially has a more substantial impact on early offspring development than MPS due

to epigenetic changes in sperm (Rodgers et al., 2013) that may be more readily induced epigenetic changes in the oocyte. Further research into the extent to which the epigenome of the oocyte is influenced by experience is necessary.

MPS-offspring were impaired at skilled reaching in adulthood compared to control-offspring. Offspring of PPS-sires are similarly impaired (Harker, 2015). Furthermore, third generation offspring of prenatally stressed dams show fine motor control deficiencies as assessed through skilled walking (McCreary, Erickson, & Metz, 2016). Increased COR contributes to motor impairments induced by stress, but is unable to explain the effect in its entirety. Female rats given supplemental COR show forelimb motor deficits, but no deficits in hindlimb control; conversely, females exposed to stress show impairments in both forelimb and hindlimb coordination. Stress-induced anxiety appears to also contribute to motor impairments; motor ability is restored in stressed rats given an anxiolytic (Metz, Jadavji, & Smith, 2005).

3.3.4. MPS Impairs Working Memory

MPS-offspring demonstrated deficits in identifying the novel object in a context-specific manner. Stress impairs memory function due to increased levels of glucocorticoids acting on GRs and MRs; specifically, memory formation is enhanced, whereas retrieval is hindered (Wolf et al., 2016). NOR assesses working memory – animals are exposed to two distinct objects in different contexts, then are assessed for their ability to recall which object was present in a given context during the test phase. Working memory depends on the PFC (McEwen & Morrison, 2013); acute stress impairs the ability of PFC neurons to retain information regarding past spiking activity, which is associated with working memory impairments (Devilbiss, Jerison, & Berridge, 2012).

Restraint stress impairs working memory, accompanied by increased COR levels and decreased BDNF levels (Radahmadi et al., 2015). Vargas-López and colleagues (2015) found similar results following restraint stress experienced for 4hr and ending 30min prior to testing. These authors confirmed the role of COR by administering COR to a subset of animals and observed a memory impairment. However, they propose that the impairment was due to increased arousal and novelty avoidance, rather than a deficit in memory. In the current research, it is unlikely that novelty avoidance explains our findings; it is more probable that offspring had increased COR levels and decreased BDNF, as has been found in studies on maternal preconception chronic unpredictable stress (CUS; Huang et al., 2010).

3.3.5. The Effects of MPS on Behavior are Sexually Dimorphic

Male offspring were more greatly affected by MPS than female offspring. On the majority of the measures in which a significant difference was found between MPS- and control-offspring, further analyses revealed that only male offspring were affected. On two measures (P36 elevated plus maze entries into titanic zone and closed arms) both male and female offspring were impacted. There was only one measure on which only female offspring were affected (P36 elevated plus maze, latency to closed arm). Overall, female offspring showed very few MPS-induced changes in behavioral development.

Male and female offspring are well-documented to respond differently to developmental stress (Weinstock, 2007). Typically, females show a greater propensity to develop emotional disorders, such as depression and anxiety, and males are more likely to display learning and memory deficits (Glover & Hill, 2012; Weinstock, 2007). A curious finding of the present study is that male offspring showed many more signs of increased

anxiety than female offspring. Second generation offspring of prenatally stressed dams (i.e. prenatal stress experienced by the grandmothers) showed a similar pattern; only male offspring demonstrated increased anxiety, whereas female offspring were unaffected. This study relied on the elevated plus maze and the light-dark box to assess anxiety, and males showed anxiety-like behavior on both measures (Grundwald & Brunton, 2015). Similarly, PPS induces anxiety-like behaviors that disproportionately affect male offspring. Adult males of PPS-sires spend more time in the closed arms of elevated plus maze relative to control-males, and females are unaffected (Harker, 2015). However, anxiety is decreased in male offspring early in development. In open field (P10-13 and P15), male PPS-pups spend more time in the center of the open field compared to male control-pups (Mychasiuk et al., 2013).

Taken together, these results suggest that offspring of MPS-dams develop anxiety-like behaviors in a pattern more similar to second generation PS-offspring and PPS-offspring than to PS-offspring or animals exposed to stress at other times throughout the lifespan. One conclusion that can be drawn is that female offspring are vulnerable to direct stress experiences, whereas male offspring are vulnerable to indirect stress experiences. The development of anxiety disorders has both a genetic component and hormonal component. Attempts to identify the genetic basis of anxiety are inconclusive, but candidate genes include COMT, serotonin transporter 5-HTT, and brain-derived neurotrophic factor (BDNF). Single-nucleotide polymorphisms (SNPs) in each of these genes have been associated with anxiety disorders, although results are difficult to replicate (McGrath et al., 2012). Testosterone has a protective function against anxiety, explaining in part why males are much less likely to development anxiety disorders than

females; gonadectomized males show a greatly increased prevalence of anxiety disorders that is reversed by testosterone replacement (McHenry et al., 2014).

The proposed mechanism of MPS is epigenetic rather than genetic. Therefore, SNPs in the above-mentioned genes could not explain the increased anxiety-like behaviors observed in male MPS-offspring. However, two of the proposed candidate genes have reduced expression in offspring exposed to maternal CUS prior to pregnancy (COMT Huang et al., 2013; BDNF Huang et al., 2010; Radahmadi et al., 2015), and a different serotonin transporter gene is also reduced (SERT Huang et al., 2012). MPS-induced epigenetic modification of these genes may play a role in the development of anxiety in offspring.

The present study did not assess how MPS impacts testosterone levels during development. Given that only male offspring show increased anxiety, and the close relationship between testosterone and anxiety, it is possible that MPS-males have diminished testosterone levels compared to control-males. Further studies should investigate this relationship.

A sex difference is also apparent in the development of fine motor skills. Only MPS-males were impaired in Whishaw tray reaching relative to controls. Conversely, both male and female offspring of PPS-sires were impaired. Although neither study measured COR levels in offspring, both studies found increased anxiety-like behaviors that may help explain reaching impairments (Metz et al., 2005). In the current study, anxiety was increased only in male offspring, and only males showed deficits in reaching ability. It is reasonable to speculate that COR levels were only elevated in males as well. This may also explain why only male offspring displayed a deficit in working memory.

3.4. Conclusion

Chronic stress prior to pregnancy impaired offspring behavior in a sex-dependent manner. Male MPS-offspring were more anxious, had impaired working memory, and showed deficits in fine motor control compared to control males. MPS impacted behavior throughout the lifespan. Maternal care between MPS- and control-dams did not differ, meaning that changes in offspring behavior were not behaviorally transmitted. The following chapter will focus on structural changes in offspring brain that accompany the behavioral alterations.

Chapter 4

Maternal Preconception Stress: Effects on Offspring Brain

4.1. Methods

4.1.1. Perfusion and Staining

Two males and two females per litter were perfused at P21, and remaining offspring at ~P100. Animals were perfused for Golgi-Cox staining (perfusant of 0.9% saline) or Cresyl Violet staining (0.9% saline followed by 4% paraformaldehyde (PFA)). Animals were administered an i.p. injection of pentobarbital sodium, intracardially perfused, decapitated, then brains promptly removed and placed in Golgi-Cox solution or 4% PFA in 30% sucrose (destined for Cresyl Violet staining). Brains prepared for Golgi-Cox staining are not included in the present thesis and will be discussed elsewhere. The following section refers only to Cresyl Violet-stained tissue.

Brains were kept in 4% PFA in 30% sucrose until sectioning; at least three days. Brains were mounted on cryostat stages and placed in -80°C for approximately 20min. Brains were then sectioned at 50µm using a cryostat and placed on 1 %gelatin/0.2 %chromium-coated slides. Sections air-dried overnight then were stained with 1% Cresyl Violet, cleared, and coverslipped with Permount.

4.1.2. Anatomical Measures

4.1.2.1. Cortical thickness. Cortical thickness was measured using a Carl Zeiss Jena slide viewer, magnified 17.5x. Thickness was measured at six different planes

beginning in PFC and continuing posteriorly until the final appearance of the hippocampus (Bregma coordinates 2.70mm, 2.20mm, -0.26mm, -2.12mm, -3.60mm, and -6.30mm). In the first plane anteriorly, thickness was measured at five locations (frontal area 1 (Fr1), agranular insular dorsal (AID) cortex, lateral orbital (LO) cortex, prelimbic (PL) cortex, and infralimbic (IL) cortex). For the posterior five planes, thickness was measured at three locations (medial, central, lateral). Measurements were taken from the left and right hemisphere.

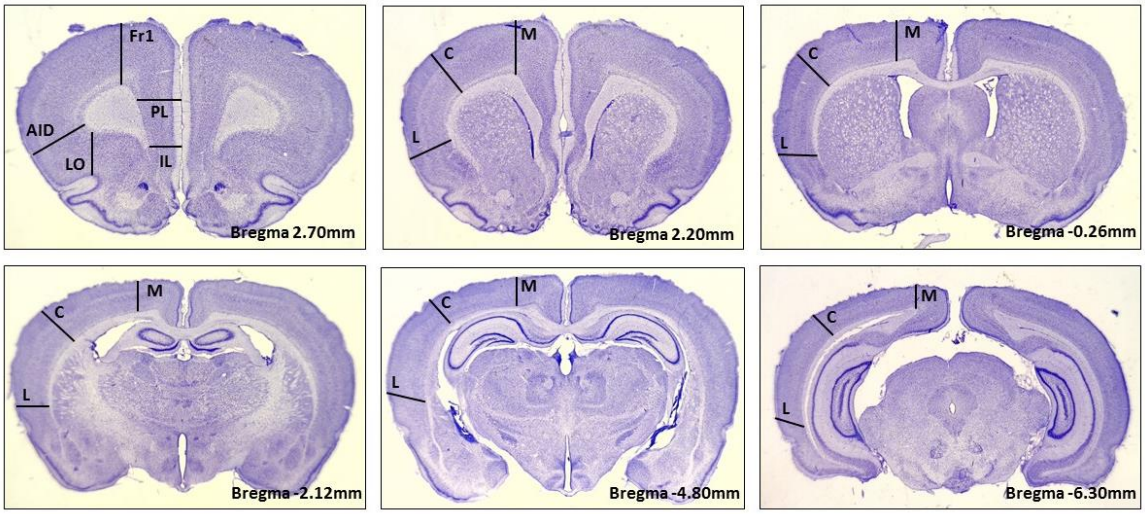

Figure 4.1: Cortical thickness. Thickness was measured in the left and right hemisphere at each site indicated in six sections.

4.1.2.2. Thalamic area. Thalamic area at two locations was used as a proxy for thalamic volume, one location located anteriorly (Bregma -2.12mm; Figure 4.2a) and one posteriorly (Bregma -4.80mm; Figure 4.2b). Brain sections were photographed using DinoCapture 2.0 software (Dino-Lite) and areas measured using ImageJ. Each area was measured in triplicate and the average used as the data point.

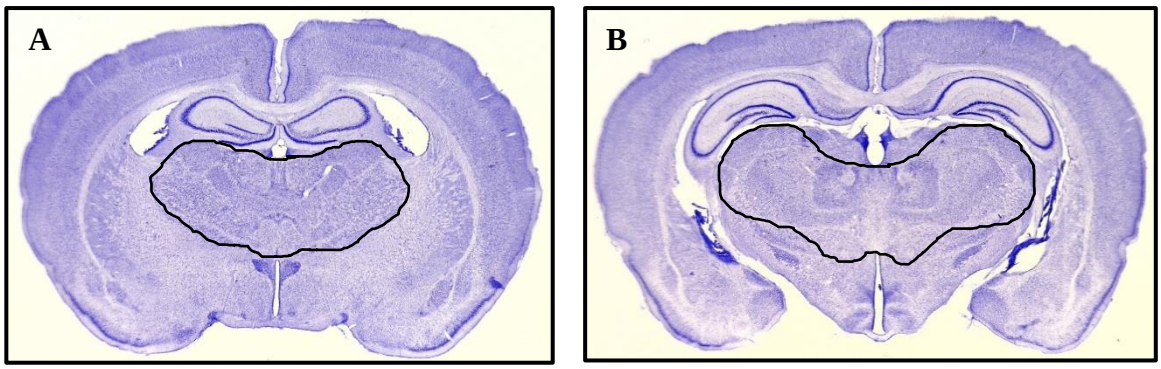

Figure 4.2: Contours of anterior **(A)** and posterior **(B)** thalamus used to assess thalamic area.

4.2. Results

Forty-five offspring were sacrificed on P21 from 10 litters (six MPS and four control), 27 from MPS-dams and 18 from control-dams. Of these, 19 were prepared for Cresyl Violet staining and were included in the following analyses. The remaining offspring were prepared for Golgi-Cox staining and will not be discussed here.

In adulthood, 21 offspring from eight litters (six MPS and two control) were sacrificed and prepared for Cresyl Violet staining, comprised of 17 MPS-offspring (eight male and nine female) and four control-offspring (two male and two female). Remaining adult offspring were sacrificed and prepared for Golgi-Cox staining and will not be discussed. There was no effect of hemisphere at either age, so right and left measurements were combined where applicable.

4.2.1. Offspring Weight

4.2.1.1. P21. MPS-offspring were significantly lighter than control-offspring on P21 ($F(1,41) = 8.739$, $p = 0.005$). Further analysis revealed that male offspring exposed to

MPS were lighter than control-males ($F(1,20) = 6.144$, $p = 0.02$), but female body weight was unaffected ($F(1,20) = 2.613$, $p = 0.12$). Unsurprisingly, male offspring were heavier than female offspring regardless of condition ($F(1,41) = 10.424$, $p = 0.003$; Figure 4.3a).

Brain weight was unaffected by MPS ($F(1,41) = 1.238$, $p = 0.27$), although male offspring had significantly heavier brains than female offspring ($F(1,41) = 23.491$, $p < 0.001$). There was no significant interaction between condition and sex ($F(1,41) = 0.103$, $p = 0.75$; Figure 4.3b).

Relative brain size was increased in male MPS-offspring relative to control males ($F(1,20) = 8.224$, $p = 0.01$). Female offspring were unaffected by MPS with respect to relative brain weight ($F(1,20) = 3.398$, $p = 0.08$; Figure 4.3c).

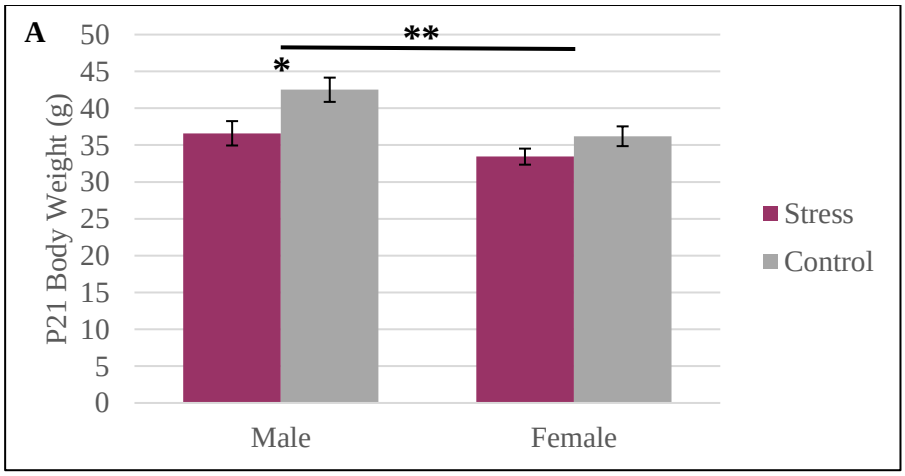

Figure 4.3a: P21 Body Weight; MPS-males were significantly lighter than control-males, but females were unaffected. Error bars = SE. * = $p < 0.05$. ** = $p < 0.01$.

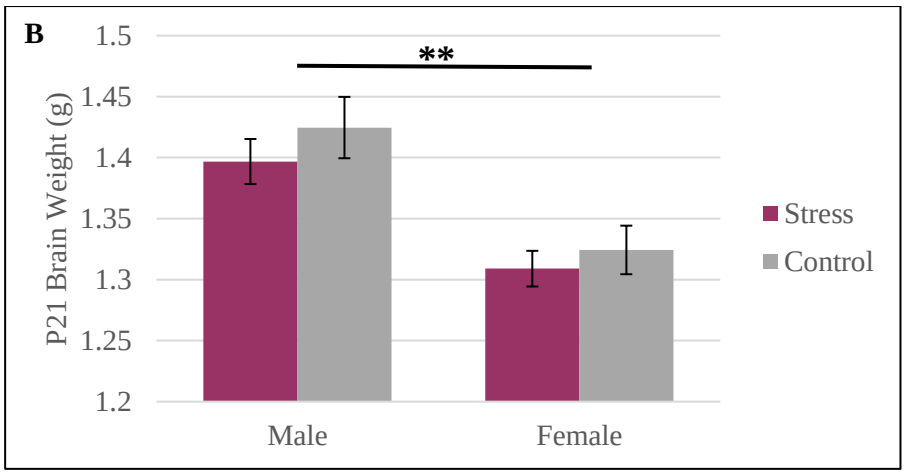

Figure 4.3b: P21 Brain Weight; there were no significant effects of MPS on brain weight in P21 offspring. Error bars = SE. ** = $p < 0.01$.

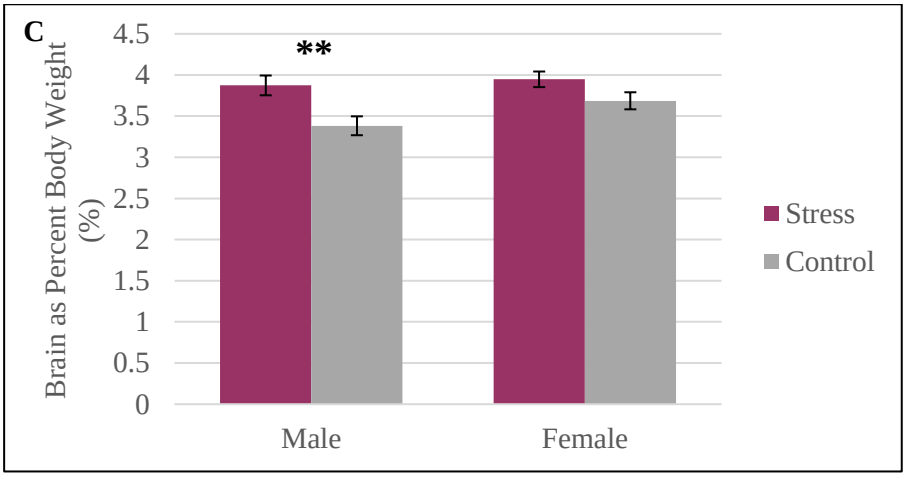

Figure 4.3c: P21 Brain as percent of body weight; Male MPS-offspring had larger relative brain size than control-males, but females were unaffected. Error bars = SE. **** = $p < 0.01$.

4.2.1.2. Adulthood. Adult offspring of MPS-dams were significantly lighter than control-offspring ($F(1,76) = 18.486$, $p < 0.001$). There was also a Condition X Sex interaction; MPS-males were significantly lighter than control-males ($F(1,40) = 17.864$, $p < 0.001$), but females were unaffected ($F(1,35) = 2.869$, $p = 0.10$; Figure 4.4a). Males were significantly heavier than females regardless of condition ($F(1,77) = 443.008$, $p < 0.001$).

Female MPS-offspring had larger brains than control-females ($F(1,35) = 5.585$, $p = 0.02$); male offspring brain weight was unaffected ($F(1,41) = 0.350$, $p = 0.56$). Male offspring had larger brains that female offspring ($F(1,77) = 28.213$, $p < 0.001$; Figure 4.4b).

Both male and female MPS-offspring had larger relative brain size compared to control-offspring ($F(1,76) = 14.888$, $p < 0.001$). Female offspring had larger relative brain size than males ($F(1,76) = 216.121$, $p < 0.001$; Figure 4.4c). There was no Condition X Sex interaction ($F(1,77) = 0.039$, $p = 0.84$).

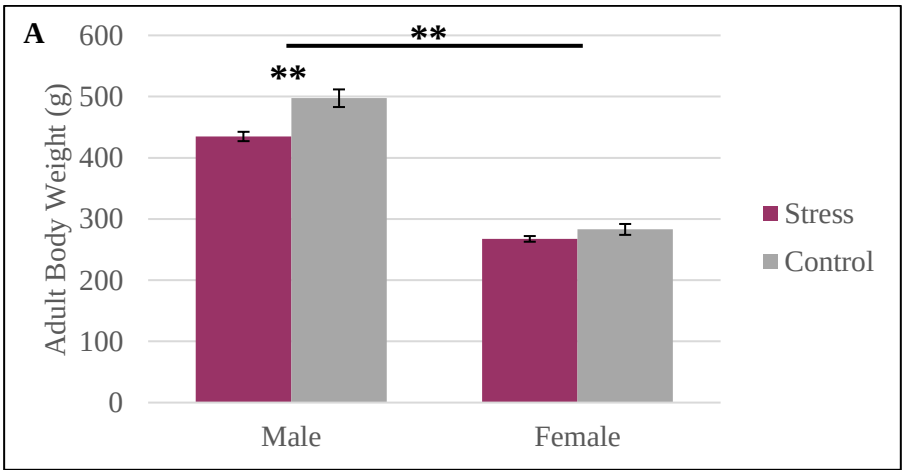

Figure 4.4a: Adult Body Weight; MPS-males were significantly lighter than control-males. Error bars = SE. ** = $p < 0.01$.

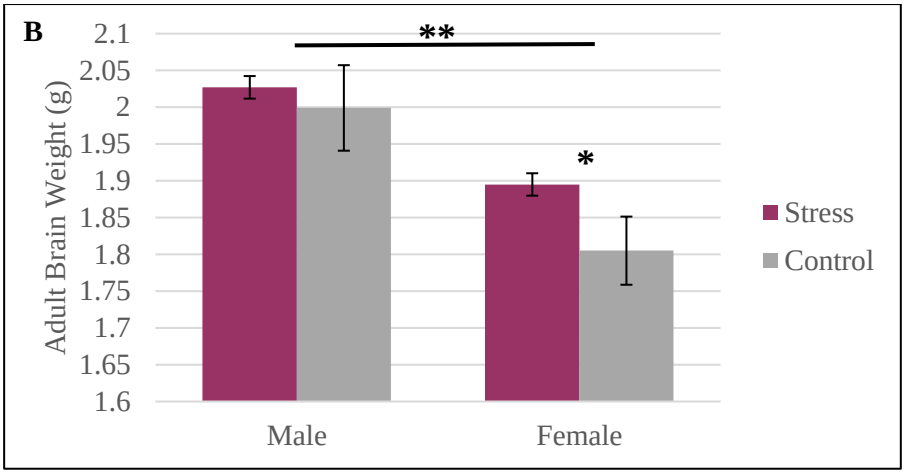

Figure 4.4b: Adult Brain Weight; MPS-females had significantly larger brains than control-females. Error bars = SE. * = $p < 0.05$. ** = $p < 0.01$.

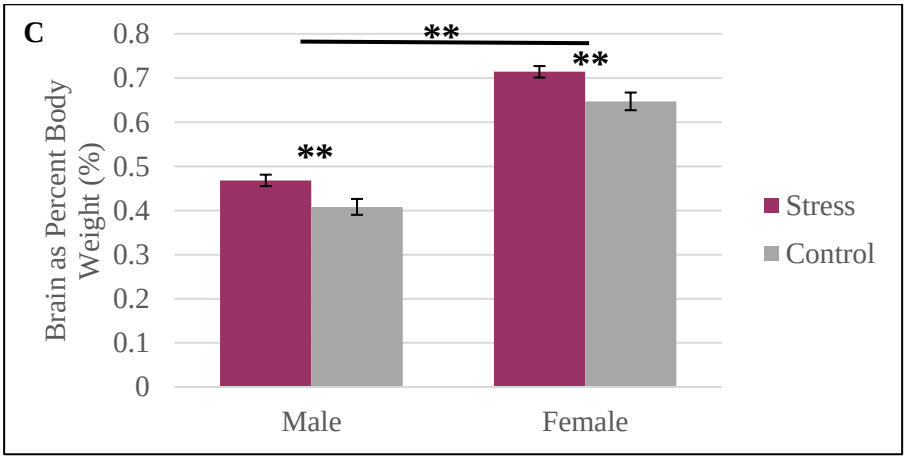

Figure 4.4c: Adult brain as percent of body weight; there were significant main effects of MPS and sex. Error bars = SE. ** = $p < 0.01$.

4.2.2. Cortical Thickness

4.2.2.1. P21. There was a main effect of MPS on cortical thickness in AID and LO cortices in P21 offspring ($F(1,33) = 4.401$, $p = 0.04$ for AID; $F(1,33) = 24.485$, $p < 0.001$ for LO). In both areas, MPS-offspring showed a reduction in thickness relative to control-offspring (Figure 4.5a). There were no significant differences in any other area examined (Table 4.1; Figure 4.5a-b).

Table 4.1: Summary of cortical thickness changes in P21 offspring. N may vary due to quality of staining. ↓ = significant decrease in MPS-offspring relative to control-offspring. --- = no significant change. * = $p < 0.05$. ** = $p < 0.01$.

		Condition		Sex		Interaction
	N	Change	*p*	Change	*p*	*p*
Fr1	33	---	0.194	---	0.325	0.157
AID	34	↓	0.044 *	---	0.864	0.759
LO	34	↓	<0.001 **	---	0.351	0.126
IL	34	---	0.701	---	0.353	0.737
PL	33	---	0.852	---	0.976	0.174
Medial	38	---	0.861	---	0.811	0.069
Central	38	---	0.124	---	0.276	0.169
Lateral	38	---	0.891	---	0.891	0.031 *

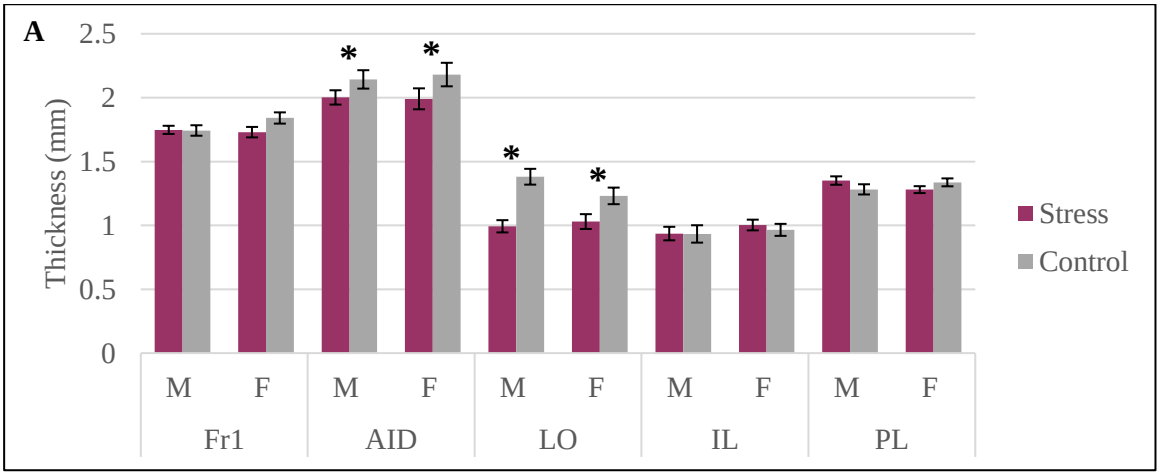

Figure 4.5a: P21 Cortical Thickness in anterior plane; thickness was reduced in agranular insular dorsal (AID) cortex and lateral orbital (LO) cortex in male and female MPS-offspring relative to control-offspring. Error bars = SE. * = $p < 0.05$.

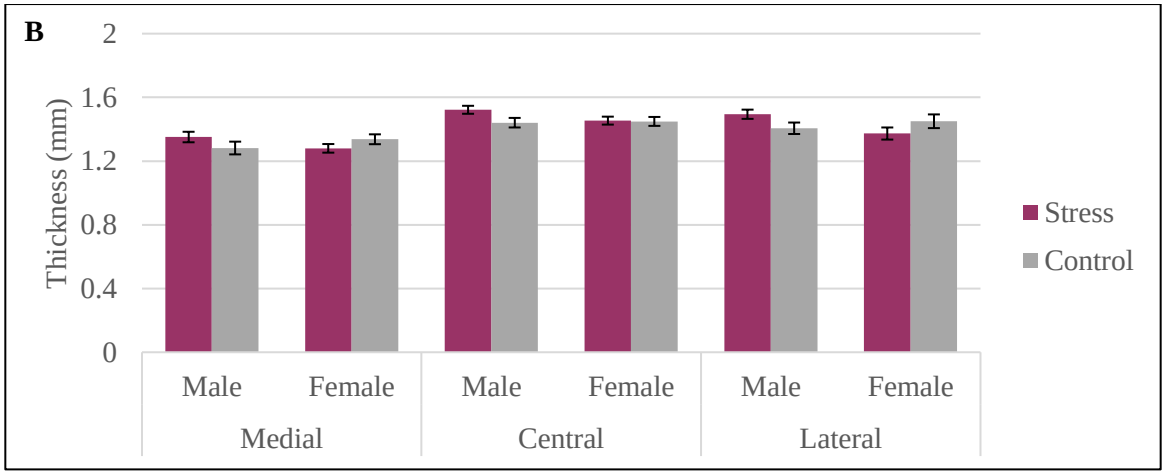

Figure 4.5b: P21 Cortical Thickness in posterior planes; thickness was unaffected by MPS at all three locations in the posterior five planes. Error bars = SE.

4.2.2.2. Adulthood. MPS affected cortical thickness in adulthood in a region- and sex-specific manner. There was a main effect of MPS in IL ($F(1,40) = 5.442$, $p = 0.03$) and PL ($F(1,40) = 6.881$, $p = 0.01$). Further analysis revealed that MPS-males showed a reduction in cortical thickness compared to control-males ($F(1,19) = 8.382$, $p = 0.01$ and $F(1,19) = 4.782$, $p = 0.04$ respectively). Female offspring of MPS- and control-dams were identical with respect to these regions ($F(1,20) = 0.077$, $p = 0.78$ for IL and $F(1,20) = 4.782$, $p = 0.15$ for PL; Figure 4.6). No other region examined was affected by MPS in either male or female offspring (Table 4.2).

Table 4.2: Summary of MPS-induced changes in cortical thickness in adult offspring. N may vary due to quality of staining. ↓ = significant decrease in MPS-offspring relative to control-offspring. --- = no significant change. * = $p < 0.05$.

		Condition		Sex		Interaction
	N	Change	*p*	Change	*p*	*p*
Fr1	41	---	0.883	---	0.121	0.1
AID	42	---	0.326	---	0.8	0.26
LO	42	---	0.736	---	0.98	0.98
IL	41	↓	0.025 *	---	0.375	0.058
PL	41	↓	0.013 *	---	0.232	0.612
Medial	42	---	0.424	---	0.215	0.383
Central	42	---	0.319	---	0.524	0.386
Lateral	42	---	0.069	---	0.774	0.791

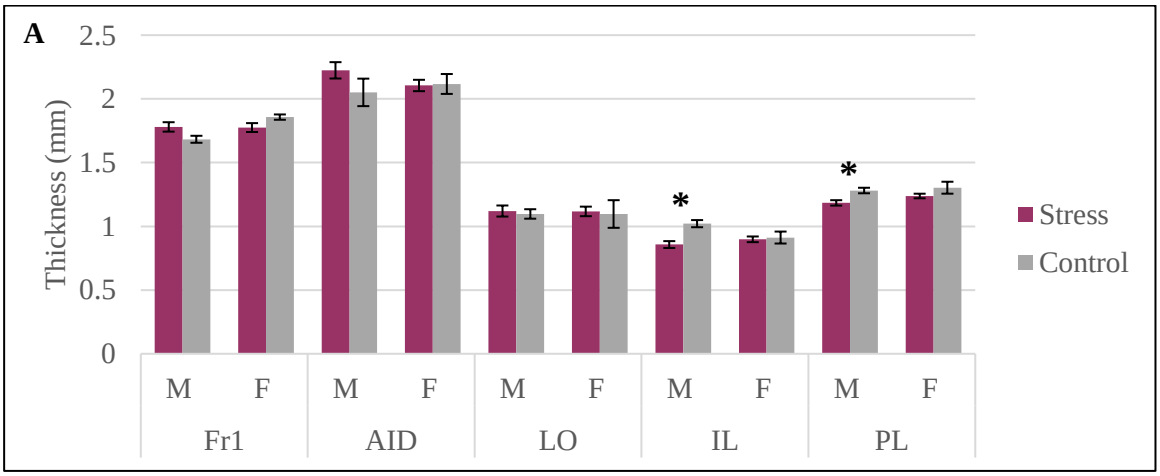

Figure 4.6a: Adult Cortical Thickness in anterior plane; thickness was reduced in infralimbic (IL) and prelimbic (PL) cortices in MPS-males compared to control-males; female offspring were unaffected. Error bars = SE. * = $p < 0.05$.

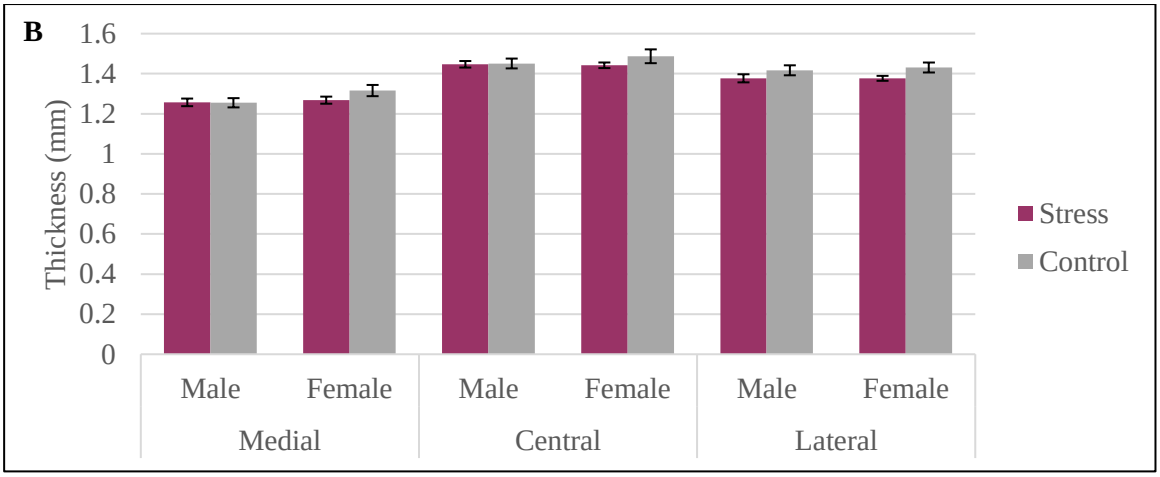

Figure 4.6b: Adult Cortical Thickness in posterior planes; thickness was unaffected by MPS in the five posterior planes at all locations. Error bars = SE.

4.2.3. Thalamic Area

4.2.3.1. P21. Thalamic area was not affected by MPS in male or female offspring. Neither the anterior ($F(1,9) = 1.158$, $p = 0.31$ for male; $F(1,8) = 0.738$, $p = 0.42$ for female) or the posterior ($F(1,9) = 0.012$, $p = 0.92$ for male; $F(1,8) = 0.040$, $p = 0.85$ for female) thalamus demonstrated a significant difference (Figure 4.7).

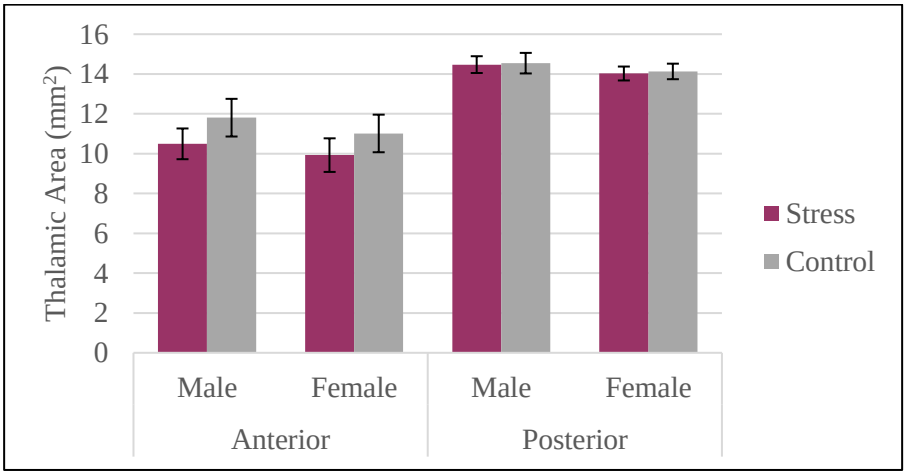

Figure 4.7: P21 Thalamic Area. There were no significant effects in either anterior or posterior thalamus in male or female offspring. Error bars = SE.

4.2.3.2. Adulthood. The size of both the anterior and posterior thalamus was unaffected by MPS in adult offspring (Anterior $F(1, 20) = 0.016$, $p = 0.90$; Posterior $F(1,20) = 1.249$, $p = 0.28$). Furthermore, male and female offspring did not differ (Anterior $F(1,20) = 0.931$, $p = 0.35$; Posterior $F(1,20) = 2.848$, $p = 0.11$; Figure 4.8).

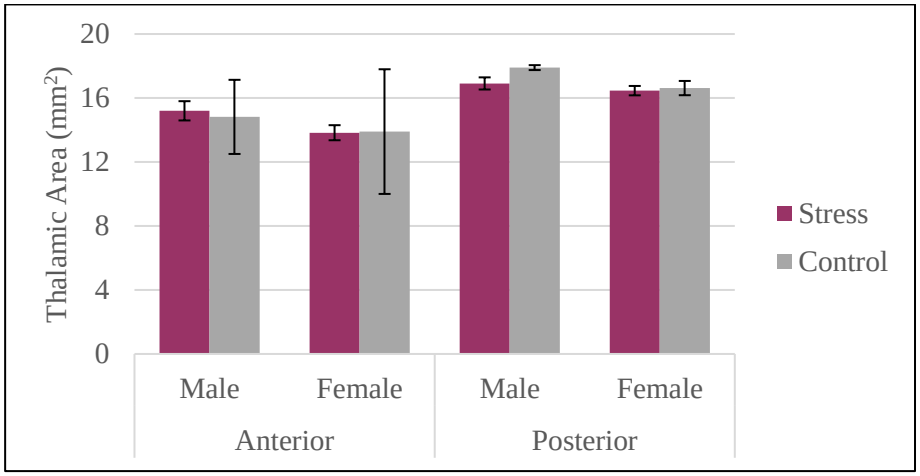

Figure 4.8: Adult Thalamic Area. There were no significant effects of MPS on the anterior or posterior thalamus. Error bars = SE.

4.3. Discussion

4.3.1. MPS Stunts Physical Growth of Offspring

MPS-offspring were smaller than control-offspring on P21 and in adulthood. Decreased weight at birth (Van den Hove et al., 2006) and on P21 (Mychasiuk et al., 2011) is observed following PS. Fetal pups of maternal rats exposed to CUS prior to pregnancy also show decreased body weight relative to controls (Huang et al., 2012). In humans, stress before and during gestation is associated with low birth weight and small for gestational age infants (Witt et al., 2014). Mueller and Bale (2006) found no differences in adult body weight in the offspring of mice exposed to PS during different stages of gestation.

Brain weight was largely unaffected by MPS; only adult female-MPS offspring showed a difference relative to controls, and their brain weight was increased. Preconception maternal CUS did not impact brain weight in fetal offspring (Huang et al., 2012). Severe PS decreases brain weight in male P21 offspring, but it is increased in female P21 offspring (Mychasiuk et al., 2011). PPS increases brain weight in adult male and female offspring (Harker, 2015).

4.3.2. MPS Reduces Cortical Thickness in a Region-Dependent Manner

Cortical thickness was reduced in MPS-offspring in a small subset of the areas examined. At both ages, there were no differences observed posteriorly from the caudate. On P21, LO and AID cortices were thinner in MPS-offspring relative to controls; in adulthood, IL and PL cortices were thinner due to MPS. Reduced cortical thickness is frequently associated with stress (e.g. Geuze et al., 2008), as well as other neuropsychological disorders such as depression (e.g. Sandman et al., 2015) and schizophrenia (e.g. Chiappelli et al., 2017). Furthermore, thinning is often restricted to the PFC (Geuze et al., 2008; Sandman et al., 2015). To contrast these findings, Kang and colleagues report increased cortical thickness in the frontal and temporal cortices of habitual meditators, a practice intended to alleviate stress (Kang et al., 2013). Prefrontal areas also show stress-induced changes in neuron morphology and connectivity (Kolb et al., 2012; Kolb and Gibb, 2015), the direction of change depending on the timing and intensity of the stress and the age of the animal at time of sacrifice. Bock et al. (2016) observed changes in neuron architecture in IL and PL cortices in the adult offspring of maternal preconception CUS dams, the two areas in which thickness was reduced in adult

male offspring. Interestingly, many of the findings of these authors were also restricted to male offspring.

Reduced cortical thickness in the PFC is associated with behavioral impairments, such as deficits in working memory, which were observed in the present study (Chapter 3). PL cortex of the medial PFC (mPFC) is especially important, in which thickness was reduced in adult MPS-offspring (Devilbiss, Jenison, & Berridge, 2012). The mPFC also includes anterior cingulate and IL cortices, of which the latter was also thinner in MPS-offspring (McEwen & Morrison, 2013).

No changes were observed in thalamic area. The thalamus sends dense projections to the cortex, including visual, auditory, motor, somatosensory, retrosplenial, cingulate, and prefrontal areas (Zhang et al., 2010). The PFC shares many connections with the medial-dorsal (MD) thalamus (Bolkan et al., 2017). Given that the PFC was the only area that showed changes in cortical thickness in the current study, it is likely that restricting measurements of the thalamus to area MD would yield significant results.

4.3.3. The Effects of MPS on Brain are Sexually-Dimorphic

Brain development was more greatly affected by MPS in male offspring than in female offspring. On both P21 and during adulthood, male offspring of preconception stressed-dams were significantly lighter than males born to control-dams, whereas females were unaffected at both ages. Furthermore, males showed decreased cortical thickness at both ages, whereas females only displayed cortical thinning on P21. In general, male offspring show more stress-typical effects on brain development than female offspring, although the mechanism is currently unclear.

4.4. Conclusion

MPS impacts brain development is a similarly manner as PS; offspring are lighter show reduction in cortical thickness with emphasis on PFC. As with behavior, a sex difference is observed in that males are more greatly affected. Furthermore, MPS-changes are observed throughout the lifespan.

Chapter 5

General Discussion

The previous two chapters discussed the effects of MPS on the development of offspring behavior and brain throughout the lifespan. The final chapter will discuss the efficacy of the elevated platform stress paradigm, and provide concluding comments regarding how MPS compares to PS and PPS, and the prevailing sex difference observed in stress research. Limitations and suggestions for further research will follow.

5.1. Elevated Platform Stress

The current research used a predictable chronic stress paradigm, which could be contrasted with the chronic unpredictable stress paradigm that is used by many of the studies conducted on preconception stress. Evidence for the efficacy of the elevated platform stressor comes from a collection of studies completed in our lab over the last several years on both preconception and prenatal stress (Mychasiuk et al., 2013; Harker et al., 2015; Mychasiuk et al., 2011; Muhammad et al., 2012). Rats subjected to elevated platform stress show overt signs of distress such as weight loss, frequent defecation/urination while on the platform, and barbering of the fur on the forelimbs. In the current study, dams showed all of these signs.

5.2. Prenatal vs. Preconception Stress

PS and MPS impact offspring in similar ways; both increase anxiety, impair memory and motor skills, and result in smaller offspring with thinner PFC. The most striking difference between maternal stress before and during pregnancy is the

disproportionate manner each sex is affected. Following PS, both male and females display changes in neuron architecture (Mychasiuk, Gibb, & Kolb, 2011; Mychasiuk, Gibb, & Kolb, 2012), although specific changes may vary due to the moderating effects of sex hormones (McEwan & Morrison, 2013). Females are much more likely to show increased anxiety- and depressive-like symptoms that are central to the findings of the impacts of stress (Weinstock, 2007). However, MPS appears to disproportionately affect male offspring in terms of both brain and behavior. Male-offspring demonstrated stress-typical changes in many of the measures included, whereas females were largely unaffected with a couple of exceptions (P36 Elevated Plus Maze, P21 Cortical Thickness, Adult Brain Weight). The mechanism behind this disparity is currently unknown, but is likely moderated by COR levels and sex hormones during development.

5.3. Maternal vs. Paternal Preconception Stress

This research and the research on PPS (Mychasiuk et al., 2013; Harker et al., 2015; Harker, 2015) utilized the same methods to assess how preconception stress experienced by either parent influences offspring development. The strain of rat, stress paradigm, and offspring test battery were all mirrored across studies, allowing us to make direct comparison between experiences. The effects of MPS and PPS on offspring behavior and brain will be considered in turn.

5.3.1. Comparison of the Effects of MPS and PPS on Behavior

PPS has a more substantial impact on the early development of offspring behavior than MPS. PPS-offspring show deficits in sensorimotor development on P9 as assessed by Negative Geotaxis (Mychasiuk et al., 2013); MPS-offspring showed no impairments in

this behavior. Anxiety-like behaviors were altered by both MPS and PPS. PPS-offspring, however, were less active overall than controls in adolescence, which was not observed with MPS. After PPS, both male and female offspring were impaired in skilled reaching (Harker, 2015), whereas just male offspring showed reaching impairments as a result of MPS.

5.3.2. Comparison of the Effects of MPS and PPS on Brain

The brains of female offspring are more greatly affected by PPS than the brains of male offspring. Females show a greater number of changes in neuron morphology following PPS than males (Harker et al., 2015). Furthermore, methylation in the frontal cortex is reduced in PPS-female, but not in PPS-males (Mychasiuk et al., 2013). Although the present study did not include morphological measures, Bock and colleagues (2016) found that following maternal preconception CUS, only males were affected with respect to neuron architecture. PPS did not induce changes in offspring body weight on P21 (Mychasiuk et al, 2013) or during adulthood (Harker, 2015), whereas MPS-males were lighter at both ages. Neither PPS or MPS affected brain weight in P21 offspring (Mychasiuk et al., 2013). In adulthood, MPS-females had larger brains than control-females; both male and female PPP-offspring had larger brain relative to controls (Harker, 2015).

5.4. Sex Differences

As discussed in the previous two chapters, the effects of MPS on the development of offspring behavior and brain are highly sexually-dimorphic. Males offspring are more greatly affected in term of both behavior and brain. Changes in brain and behavior usually

accompany each other, so it is unsurprising that both were similarly changed. Males showed increased anxiety, impaired memory, and deficits in motor control due to MPS, but females mostly did not. This could be due to increased levels of COR in male offspring, which was not assessed in the current study but has been found in other studies on MPS (Huang et al., 2012; Huang et al., 2013). Testosterone also potentially plays an important role and should be assessed in future studies.

5.5. Limitations and Future Directions

This research provided evidence that a consistent stressor prior to pregnancy induces changes in offspring that are comparable to stress at other times throughout development. However, a few questions remain unanswered. Firstly, this study did not measure COR levels in dams or offspring, as do many studies examining stress. Dam testing was intentionally omitted to avoid an additional stressor. Offspring COR analysis would allow us to correlate behavioral changes with changes in physiological stress reactivity. Furthermore, if a sex difference was found in COR levels, it would provide additional support for the finding that male offspring are disproportionately affected by MPS. Therefore, future studies should collect biological samples for COR assays.

Secondly, no epigenetic measures were included in this study, as in the PPS study. Considering epigenetic modification is a possible mechanism of MPS, this is a shortcoming of this study. The present study was exploratory and aimed to identify observable changes in offspring and how MPS compares with PPS and PS. Further studies will incorporate epigenetic analyses, such as DNA methylation and assessment of gene expression using Western Blot. If epigenetic changes are present in the offspring, this could provide evidence that the maternal experience is transmitted via stress-induced

epigenetic modifications in the germline that influence development. Of course, there are other potential mechanisms that should be considered and explored, such as chronic stress-induced changes in maternal physiology that could have impacted offspring, despite being no overlap between stress and gestation.

Finally, this thesis reports on minimal changes in offspring brain, including only brain weight, cortical thickness, and thalamic measurements. Currently in progress are estimates of neuron density and analyses of Golgi-Cox stained tissue including dendritic length and complexity and spine density.

5.6. Conclusion

The present research demonstrated that chronic predictable maternal stress immediately prior to conception impacts that development of offspring brain and behavioral development throughout the lifespan in a sexually-dimorphic manner. Major findings include increased anxiety-like behavior, impaired fine motor control and working memory, decreased body weight, and reduced cortical thickness in select areas. Male-offspring show MPS-induced impairments in all areas, whereas female offspring appear resistant to MPS. Hypotheses were generally supported with the exception of the direction of the sex difference. Further studies should include assessments of COR, testosterone levels, and epigenetic changes to attempt to explain this disparity between the sexes.

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