PRECONCEPTION PATERNAL STRESS: IMPACT ON OFFSPRING EPIGENOME, BRAIN, AND BEHAVIOR THROUGHOUT THE LIFESPAN

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DEDICATION

… Fathers Matter!

ABSTRACT

A rich literature has been amassed demonstrating the impact of early experience on the structure and function of the developing brain. Adverse maternal experience during the perinatal period has been implicated as a major risk factor for the development of psychopathologies later in life. Little information is available regarding the impact of experience during the preconception period. As spermatogenesis is a continuous process, experiences that have the ability to alter epigenetic regulation in fathers may actually change developmental trajectories of offspring. The aim of this thesis was to examine the lifelong impact of preconception paternal stress on the epigenome, brain morphology, and behavior of both male and female developing rat offspring. Profound and enduring changes in DNA methylation patterns, neuroanatomical measures, and behavioral outcomes of paternally stressed offspring were observed. Results from this study clearly demonstrate that preconception paternal stress during spermatogenesis can influence offspring epigenome, brain, and behavior.

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LIST OF ABBREVIATIONS

ACd	Anterior Cingulate Cortex
AID	Orbitofrontal Cortex
ANOVA	Analysis of Variance
CA1	Hippocampus
Cg3	Cingulate Cortex
HPA	Hypothalamic-Pituitary-Adrenal
HPC	Hippocampus
mPFC	Medial Prefrontal Cortex
NAc	Nucleus Accumbens
OFC	Orbitofrontal Cortex
Par1	Parietal Cortex
PFC	Prefrontal Cortex
PL	Prelimbic Cortex
IL	Infralimbic Cortex
PPS	Preconception Paternal Stress

CHAPTER 1

General Introduction

"*Pure logical thinking cannot yield us any knowledge of the empirical world; all knowledge of reality starts from experience and ends in it.*"

Albert Einstein (Ideas and Opinions, p.271)

Neurodevelopment is not a simple unfolding of a genetic blueprint, rather it is a continuous process that involves an intricate synthesis of genetic and experiential factors that shape developing brain architecture and, subsequently, behavior (Kolb, Mychasiuk & Gibb, 2014). A common theoretical framework that governs developmental research is the assertion that early life experiences allow offspring to form characteristics that will enhance adaptability to the environment (Sullivan et al., 2006). A rich literature has been amassed demonstrating that early life events alter the structure and function of the developing brain. Early experience, whether deemed favorable or aversive, has been found to induce epigenetic modifications and produce changes in brain architecture and behavior (Bock et al., 2015; Harker, et al., 2015; Kolb, 1995; Mychasiuk et al., 2013a; Susser et al., 1998; Weinstock, 2008).

Gibb, Gonzalez & Kolb (2014) found that a favorable prenatal experience (complex housing) influenced and somewhat reversed anatomical changes and behavioral deficits resulting from a perinatal cortical injury. In contrast, research suggests that exposure to adverse environmental perturbations (e.g., stress) during early life are

strongly associated with neurodevelopmental disorders later in life (Bale, 2014; Bock et al., 2015; Li, Gonzalez & Zhang, 2012; Turecki & Meaney, 2014). Abdolmaledy (2014) explains that studies using genome-wide association scans (GWAS) have failed to identify major gene(s) linked to psychiatric diseases, leaving the scientific community to reconsider the impact of environmental factors on brain development and function. When considering the nature versus nurture debate, growing research suggests that the milieu of the prenatal environment/nurture may be more crucial than genetics/nature in the development of psychiatric diseases later in life (Abdolmaledy, 2014). There is considerable evidence that suggests that stressful experiences during early development can have a lifelong impact on offspring brain and behavior (Bale, 2014; Bock et al., 2015; Harker et al., 2015; Hehar & Mychasiuk, 2015; Kolb et al., 2012a; Kolb & Gibb, 2015; Li, Gonzalez, & Zhang, 2012; Mychasiuk et al., 2011a, 2011b, 2011d; Mychasiuk et al., 2012; Nemati et al., 2013; Rodgers et al., 2013; Turecki & Meaney, 2014).

As it is believed that mothers exert the primary influence on offspring neurodevelopment, the preponderance of research examines the effect of maternal contribution on offspring during pre and postnatal developmental periods. Only recently has research attempted to examine the indirect role of preconception *paternal* experience on offspring brain development and behavior. The purpose of this thesis is to investigate the effects of preconception paternal stress on the epigenome, neurodevelopment, and behavioral outcomes of paternally stressed offspring. Through the years we have come to appreciate that this programming during early development occurs, in some measure, through epigenetic mechanisms influenced by environmental experiences during fetal development (Kim, Bale, & Epperson, 2015).

1.1. Epigenetics

The term epigenetics refers to a modification in gene activity, without a change in the primary DNA sequence, causing a change in phenotype not genotype. It is the way in which biology and experience work together to enhance adaptation to the environment and increase chances of survival. Only by considering the theories of both Charles Darwin and Jean-Baptiste Lamarck can we attempt to appreciate epigenetics. Darwin's theories of natural selection (*survival of the fittest*, or *decent with modification*) have rightfully withstood the test of time, and are the cornerstone of our views on biological evolution. However, Darwin's theories fail to account for phenotypic variation dependent on selective pressure, proposing that variation originates from random mutations only. Lamarck's theory of use and disuse, on the other hand, suggests that an organism can alter the phenotype of offspring by passing on characteristics acquired throughout the lifespan (*inheritance of acquired traits* or *soft inheritance*), while neglected characteristics are eventually lost (Rando & Verstrepen, 2007). Through the course of natural selection and random mutation, a genetic trait may take many generations to appear in a given population. Alternatively, the epigenome can react to signals from the environment and through epigenetic inheritance, pass along parental experiences to future generations, permitting perpetual adaptation to dynamic and fluctuating environmental conditions.

1.1.1. The Epigenome

Deoxyribonucleic acid (DNA) contains sequences of genes that provide the blueprint for development and regulation of the body, but this is only part of the story.

DNA is wound around proteins called “histones” much like thread around a spool. Chemical “tags” or “marks” cover both the DNA and the histones. This additional structure, known as the epigenome, shapes to the genome and provides a second set of instructions for the body. Genes can be expressed (turned on) or inhibited (turned off) by these epigenetic chemical tags, without changes to DNA. *Figure 1.4* illustrates three primary mechanisms used to regulate the transmission of the epigenetic message to either express or inhibit the specific gene (Kolb & Whishaw, 2014).

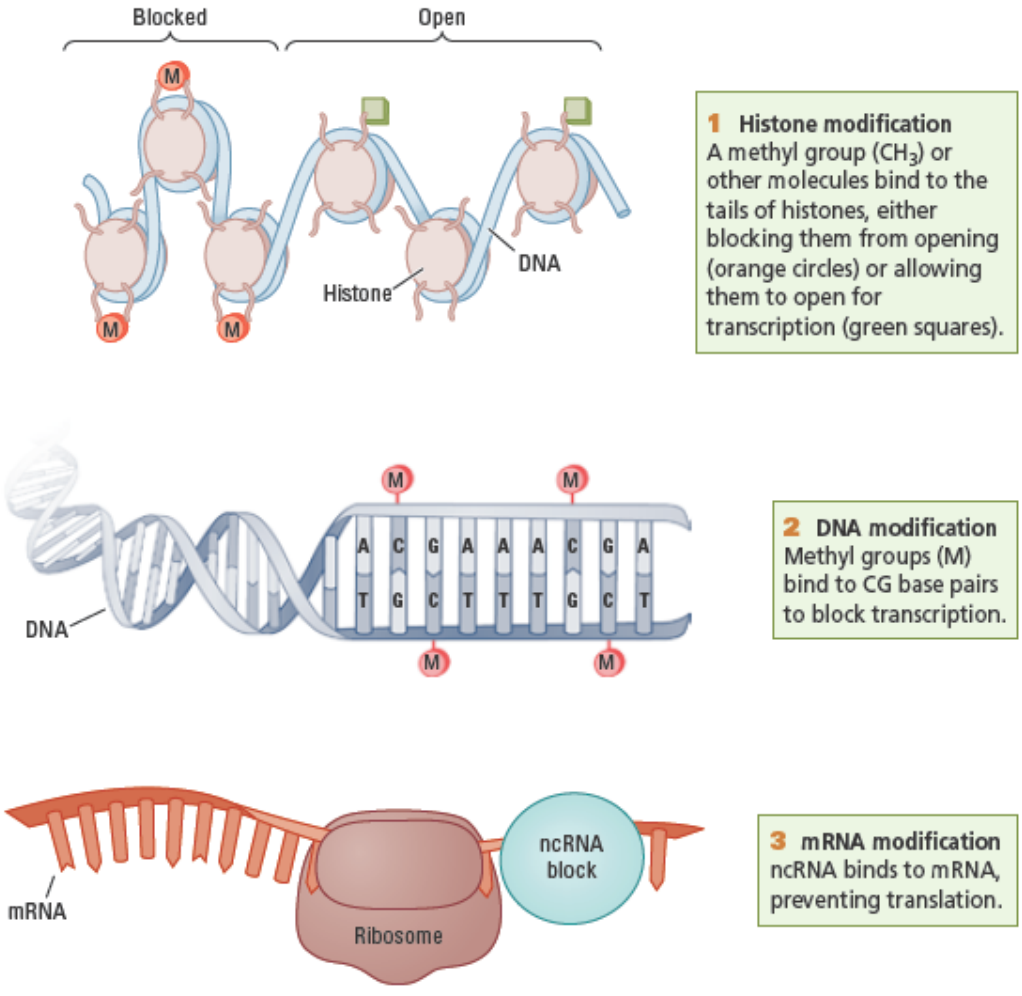


Figure 1.1. Epigenetic Mechanisms
(Adapted from: *Brain & Behaviour, Kolb & Whishaw, 2014*)

Mechanism 1: *Histone Modification.* The binding of epigenetic factors such as a methyl group (CH_3) or another molecule to histone "tails" altering the extent to which DNA is wrapped around histones and the availability of specific genes in the DNA to be expressed or inhibited (Kolb & Whishaw, 2014).

Mechanism 2: *DNA Modification.* One of more methyl groups can bind or "tag" the cytosine residue of a CpG island, blocking transcription of DNA to mRNA, thereby silencing the gene (Champagne, 2010; Kolb & Whishaw, 2014).

Mechanism 3: *mRNA Modification.* Noncoding RNAs (ncRNA) regulate the expression of genes at many levels and dominate transcriptional output during development, permitting genes to be either expressed or inhibited (Mattick, et al., 2009). Translation of mRNA can be inhibited through the binding of ncRNA to mRNA (Kolb & Whishaw, 2014).

To recap, epigenetic factors are cellular processes that are passed on to future generations through genetic "tags" or "marks" on DNA. These chemical tags interact with genetic materials that tell the genes what to do, where to go, and when to be expressed or inhibited. As the primary location of interactions between genes and the environment, the epigenome is important to the survival of the organism. The epigenome can be altered by exposure to environmental experiences (favorable/aversive) that can either generate or remove one or more of these blocks, thereby permitting regulation of gene expression by the environment. One example of an environmental experience that is

known to impact the regulation of gene expression through epigenetic mechanisms is "stress".

1.2. Stress

Hans Selye (1907-1982) is considered to be one of the founding fathers of stress research and recent studies on the effects of stress on the body can be traced back to his original work. Selye proposed the concepts of "eustress" and "distress" and introduced the phenomenon of the "General Adaption Syndrome" (GAS), later renamed the "stress response". *Eustress* is positive stress perceived as a pleasant form of stress caused by desirable stimuli. This type of stress can motivate an organism and enhance performance, for example, working hard to accomplish a goal or performing a presentation. *Distress* is perceived as a threat to quality of life and/or homeostasis. The threat is perceived to exceed an organism's capability. Prolonged exposure can have a negative effect on homeostasis. An example of distress is loss of employment or death of a family member (Szabo, Tache & Somogyi, 2012). Selye is credited with discovering the biologic stress response in 1956, and subsequently introduced his GAS theory, described below as a series of 3 stages. See *Figure 1.1*.

Selye found that rats that had been stressed in a variety of ways exhibited specific and reliable psychological and physical responses. He observed atrophy of the immune system, enlarged adrenal glands and gastrointestinal ulcers as a result of the adverse stressing conditions. Selye discovered that at appropriate levels, stress was adaptive and physiological processes worked to keep the organism in balance. However, if exaggerated for a period of time, these adaptive processes could damage the organism

much like the effect of illness. Following Selyes' launching of the biological stress concept, nearly a century of research on the effects of stress have led researchers to the awareness that stress plays an important role in the maintenance of an organism's health and homeostatic condition.

Table 1.1. General Adaptation Syndrome (GAS) - *Hans Seyle (1956) (Szabo, Tache & Somogyi, 2012)*

Stage	Description
Alarm!	In response to a stressor, the body prepares for immediate physical action. The sympathetic nervous system is activated, and stress hormones (cortisol, epinephrine, norepinephrine) are released in greater amounts.
Resistance	If the stressor continues, the body maintains a moderate level of physiological arousal. The organism has increased its ability to withstand the original stressor, which brought about the alarm reaction.
Exhaustion	With added stressors or depletion of the ability to continue resisting, the body enters a stage in which a variety of illnesses or even death may occur.

Stress in an unavoidable condition of life. An organism is designed to react and adapt to an environment in an effort to survive. A delicate balance of opposing forces, or equilibrium must be maintained. Maintenance of equilibrium is referred to as homeostasis. This physiological state of balance is vulnerable to diverse perturbations by internal or external events or perceptions. The term "stress" is used to describe an organism's method of reacting to an intrinsic or extrinsic challenge to homeostasis, whether actual or perceived. Once homeostasis has been threatened, a complex sequence of behavioral and physiological processes responds to reestablish desired equilibrium, known as an "adaptive stress response" or simply "stress response" (Kirschbaum & Hellhammer, 1994).

1.2.1. Hypothalamic-Pituitary-Adrenal (HPA) Axis

The cascade of events induced by the stress response involves both the central and peripheral nervous systems. Beginning with a response by the brain to stimulation received from its inputs, the paraventricular nucleus (PVN) in the hypothalamus produces two peptide hormones, corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). CRH is carried to its target, the anterior pituitary to which it binds, stimulating secretion of its own messenger, corticotropin. AVP prompts vasoconstriction and reabsorption of water by the kidneys, increasing blood pressure. Together, these two hormones stimulate the organism's pituitary gland thereby activating a system of feedback mechanisms involved in the Hypothalamic-Pituitary-Adrenal (HPA) axis, the primary biochemical pathway underlying the brain's response to stress (Adinoff et al., 1998; Maniam, Antoniadis, & Morris, 2014; Randall, 2011). See *Figure 1.2.*

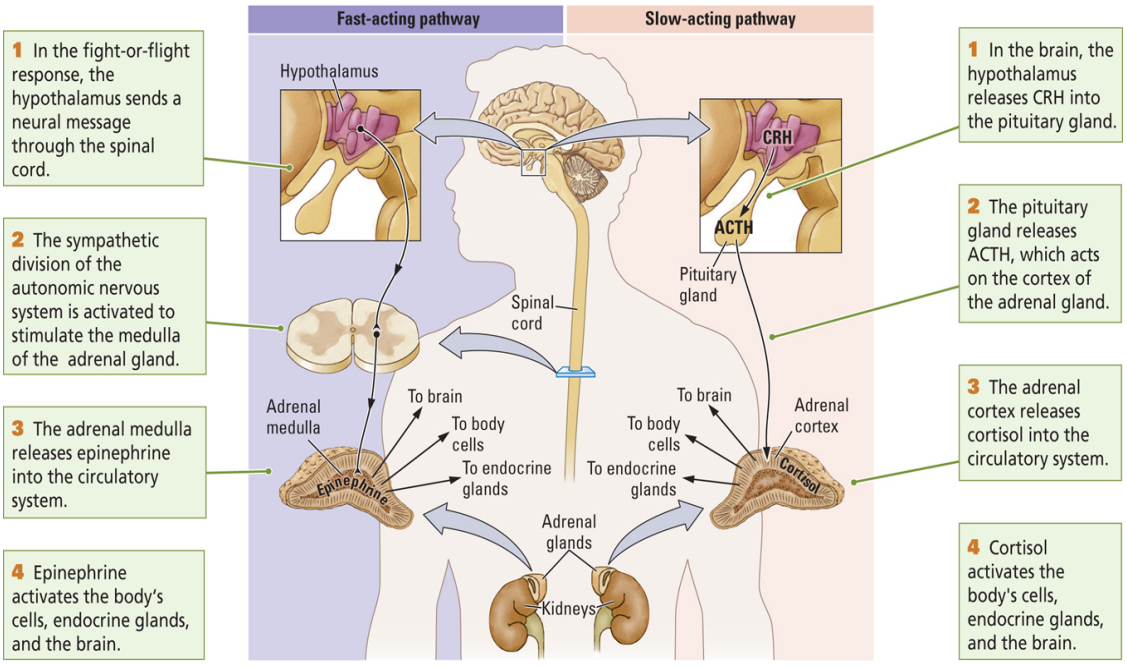


Figure 1.2. Activating a Stress Response
(Adapted from: *Brain & Behaviour, Kolb & Whishaw, 2014*)

Kolb and Whishaw (2014) describe two separate signaling pathways to the adrenal gland that control an organism's stress response. The fast-acting pathway sends a neural message through the spinal cord that primes an organism for "fight or flight" and stimulates the medulla of the adrenal gland to produce epinephrine (adrenaline) through activation of the sympathetic division of the autonomic nervous system. At the same time there is inhibition of the parasympathetic division, prompting the organism to "rest and digest". During the slow-acting response, the release of CRH from the hypothalamus activates the release of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH makes its way to the outer layer (cortex) of the adrenal gland via the bloodstream stimulating secretion of the steroid cortisol, a glucocorticoid (GC) hormone. Cortisol then travels through the circulatory system exerting widespread effects on the organism's cells, endocrine glands, and brain.

The main purpose of cortisol during the stress response is to divert cellular processes from long-term metabolic processes and toward systems required for immediate survival, including inhibiting bodily systems that are not essential to the stress response. The brain uses cortisol in an attempt to restore homeostasis by redistributing resources (energy/glucose) to critical organs (Kolb & Whishaw, 2014). Cortisol is the major GC hormone in humans, whereas corticosterone is the major GC hormone in rats.

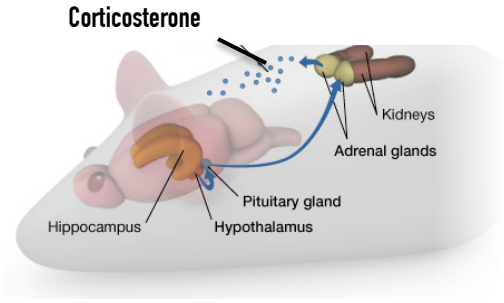


Figure 1.3. HPA Axis in Rodent Model *(University of Utah Health Sciences)*

The HPA axis is comprised of a negative feedback system that inhibits its own activation. This occurs once enough cortisol has been secreted to reestablish equilibrium and the immediate threat is no longer present, or perceived to be present. As cortisol rises to a certain level, binding to the hypothalamus and pituitary gland, feedback is sent to suppress further production of CRH and ACTH, essentially shutting down the HPA stress response (Adinoff et al., 1998). However, if there is chronic activation of the HPA axis and excess cortisol remains elevated for an extended period of time, a desired state of homeostasis cannot be achieved. This can lead to inhibition of growth hormone, muscle wasting and fatigue, gastrointestinal issues, decreased inflammatory response, and suppression of the immune (defense) system (Kolb & Whishaw, 2014). Programming and modification of the HPA axis commences *in utero*, during which time the maternal environment has the ability to impact offspring HPA axis function and behavior (Kapoor et al., 2006).

1.2.2. Fetal Glucocorticoid Exposure

It is understandable that a parent organism would have the ability to alter the HPA axis of offspring in order to better prepare the offspring for subsequent environmental conditions. For example, if conditions were highly hostile, increased vigilance would be a necessity for survival, requiring an alteration to the stress response (HPA axis) to allow for adaptation to the stressful environment. However, alterations transmitted to fetal HPA axis do not always correspond with environmental demands post birth. Prenatal stress can lead to permanent alterations in offspring HPA axis that are maladaptive and negatively impact neurodevelopment and behavioural outcomes of offspring (Kapoor et al., 2006).

Increased levels of glucocorticoids during gestation are a critical component for fetal growth and development, and are a contributing factor to immediate survival following birth. Acting as nuclear transcription factors, glucocorticoids bind to their respective receptors to control gene expression, proliferation, differentiation, apoptosis and survival. Maternal stress has been found to trigger an over-release of cortisol and disrupt barriers such as llß-Hydroxysteroid dehydrogenase (llß-HSD2) found in the placenta, normally available to protect the fetus from excess glucocorticoids. Overexposure of maternal cortisol (corticosterone in rodents) during stressful conditions can alter fetal HPA axis structure and function, leading to permanent reprogramming and perturbations in offspring developmental trajectories (Li, Gonzalez, & Zhang, 2012).

1.2.3. Maternal (Gestational) Stress

An extensive literature has been amassed examining the impact of maternal stress on fetal development. Prenatal stress has been shown to alter the epigenome, brain, and behavior of prenatally stressed rats (Bale, 2014; Bale et al., 2010; Bock et al., 2015; Bock et al., 2014a, 2014b; Bock & Braun, 2011; Brunson et al., 2005; Carrion & Wong, 2012; Das et al., 2015; Garrett & Wellman, 2009; Glover & Hill, 2012; Kapoor et al., 2006; Kim, Bale, & Epperson, 2015; Li, Gonzalez, & Zhang, 2012; McEwen & Morrison, 2013; Muhammad & Kolb, 2011a; Mychasiuk, Gibb, and Kolb, 2012; Mychasiuk, Gibb, & Kolb, 2011a, 2011b, 2011c; Mychasiuk, et al, 2011d; Paris & Frye, 2011; Rodgers & Bale, 2015; Shansky et al., 2009; Sunanda et al.,1995; Turecki & Meaney, 2014).

Recently, research examining the impact of stress *in utero* has become an area of considerable investigation and assessment. Compelling data suggest that the nature and

the intensity of the stressor mitigates the degree to which stress influences the maternal HPA axis, placenta, and immune system functioning, and is considered to be significantly involved in early fetal programming, leading to possible neurodevelopmental perturbations later in life (Kim, Bale, & Epperson, 2015; Mychasiuk et al., 2011d). Research to date has primarily focused on the prenatal/perinatal developmental period. There is, however, extremely limited investigation examining the outcome of environmental factors during the preconception period, prior to fetal development. I know of only one study exploring preconception maternal stress in a rat model (Bock et al., 2014a).

1.3. Preconception Stress

Bock et al., (2014a) showed complex changes in brain architecture in the mPFC, but not in the OFC in offspring whose mothers had been stressed during the preconception period. Offspring of mothers mated two weeks following the stressing period exhibited increases in pyramidal dendritic length, complexity, and spine density in the anterior cingulate (ACd) and prelimbic/infralimbic (PL/IL) regions in response to the preconception stress (PCS). The findings were regionally, temporally, and sex specific. This begs the question, how can preconception stress impact subsequent offspring? Bock et al., (2014) postulate that the observed structural changes in brain morphology resulted from (re)programming of future gene activity in the oocyte mediated by stress-induced epigenetic mechanisms. Transmission of the effects of PCS to offspring occurred via epigenetic changes in the germline that adjust neuronal and synaptic developmental processes in the oocyte. Although there are questions as to how these epigenetic changes

or “marks” are conserved during the processes of fertilization and fetal brain development, it is feasible that this transmission is possible as there abides a direct connection between mother and offspring. However, what would happen if the preconception stress came from the father? Is this possible, and how would transmission occur? Remarkably, or perhaps predictably, there is a possible avenue that would allow transmission of paternal experience to offspring - spermatogenesis.

1.4. Preconception Paternal Stress (PPS) and Spermatogenesis

Mychasiuk et al. (2013a) explain that owing to the continuous nature of spermatogenesis, experiences that change DNA methylation patterns in sperm before fertilization have the potential to alter epigenetic programming of future offspring. DNA methylation is used by spermatozoa and is uniquely regulated to play an important role in the development of future gametes and embryos (Jenkins and Carrell, 2011). Robust paternal epigenetic contribution to embryogenesis requires that the DNA in spermatozoa contain layers of regulatory elements, including methyl groups that drive gene activation or silencing upon contact with the egg. Although critical to normal development, this abundance of regulatory control also leaves the DNA susceptible to damage from outside agents (Jenkins and Carrell, 2011). Damaging outside agents are commonly believed to be chemical toxins or drugs of abuse, however, prior research has demonstrated that stress impairs spermatogenesis in adult rats (Potemina, 2008), and alters methylation patterns in the germline of F2 male mice (Franklin et al., 2010). Epigenetic mechanisms that would allow for transmission of paternal experience have very recently come to the forefront of investigation.

1.4.1. Possible Epigenetic Mechanisms of Paternal Transmission

New research is focusing on the examination of spermatogenesis and the epigenetic mechanisms that are involved in this dynamic process. Epigenetic marks in sperm have been proposed as a possible mechanism through which paternal experience can impact offspring neurodevelopment. Working with mice, Carone et al. (2010) found that paternal environmental conditions, in this case paternal diet, acted upon epigenetic information carriers in sperm, thereby influencing lipid and proliferation-related gene expression in offspring. Epigenetic reprogramming of the paternal germline in rats was observed by Vassoler et al., (2013) in males voluntarily ingesting cocaine, producing significant effects in male offspring resistance to cocaine reinforcement, and mPFC gene expression.

Research suggests that distinct epigenetic mechanisms and retained histone proteins - protamine (1% in rats, 10% in humans) and their modifications found in the testes make them an epigenetically advantaged site (Godman et al., 2009; Rogers & Bale, 2014). Of all infertility cases, 30-50% are related to paternal infertility, suggesting that spermatogenesis is a dynamic process that is prone to frequent error. Epigenetic patterns are ingrained and preserved during the critical period of germ cell development. It is postulated that exclusive epigenetic controls are engendered during stage and testis-specific gene expression (diploid spermatogonia progress to haploid spermatozoa), the transition of histone-protamine, and mitotic and meiotic division.

Rodgers et al., (2013) explain that genetic responses to environmental insults may occur during spermatogenesis through spermatozoa RNA populations, methylation patterns, and histone modifications, previously depicted (*Figure 1.1*). The impact of these

insults are believed to alter the offspring phenotype due to the dysregulation of the stress HPA axis.

These important findings provide possible mechanisms permitting paternal contribution to offspring. The aforementioned research attests to the possibility that the effects of *paternal* environmental perturbations could be transmitted to offspring, via epigenetic mechanisms. As stated earlier, although there is an abundance of research investigating maternal effects on offspring, there is a lack of studies examining the effect of preconception paternal experience and the consequences that these experiences have on offspring brain and behavior. This leads to the objective of this Thesis.

1.5. Objective of Thesis

The purpose of this thesis was to investigate the effects of preconception paternal stress on the neurodevelopment, behavioural outcomes, and epigenome in a rat model of paternally stressed offspring. The intent was to follow offspring throughout the lifespan to fully examine any enduring effects of PPS on brain development and behavior.

1.5.1. Modeling Stress in Animals

In order to better understand the mechanisms involved in long-term effects of preconception paternal stress and considering the obvious obstacles inherent to human research in this particular field, animal models of stress have been developed. An animal model provides an important opportunity to examine anatomical, morphological, epigenetic and behavioural analyses throughout the lifespan of the animal.

Wong et al. (2007) developed a stressing paradigm that has been shown to induce significant chronic stress in rats, supported by analysis of corticosterone levels and observation of behavioral effects (consistent urination and/or defecation during the stress procedure). We chose to follow this stressing protocol (described below) in order to forego unnecessary stress related to blood collection for corticosterone analysis, extensively examined previously by Wong and associates.

1.5.1.1. Stressing Paradigm. Paternal stress was administered for 27 consecutive days, immediately prior to the mating session. The paternal stress consisted of placing the male rat (n = 6) on an elevated Plexiglas® platform (1 m tall, 21 × 21 cm), in a brightly lit room twice daily for 30 min (Wong et al., 2007). Control males were removed from home cages twice daily for 30 min and placed in a cage in the same breeding room as their home cage. They were not submitted to the stressing paradigm.

1.5.2. Lesion Model as a Tool

One of the major concerns prior to the commencement of this research project was the ability to observe subtle changes in behavior of paternally stressed offspring through the employment of crude behavior tasks developed to evaluate brain lesions. Research has shown that an injured brain appears to be particularly sensitive to perinatal experiences, such as stress. Kolb and colleagues, through many years of research, have determined that the impact of cortical injury on brain and behaviour varies drastically with age at time of injury. An injury during postnatal day 1-5 is found to have devastating and enduring consequences on both brain and behavior. Conversely, lesions performed on postnatal days 10-12 allow for remarkable spontaneous recovery of brain and

behaviour. Recovery from a postnatal day 7 (P7) lesion is found somewhere on the continuum in between the two, allowing for partial recovery, but not complete (Kolb & Gibb, 2007 ; Kolb et al., 2011). With this in mind I decided to use a mPFC lesion on P7. A lesion at this developmental age would allow for analysis of potential changes in levels of recovery whether positive or negative. A subset of the offspring were subsequently given a P7 mPFC lesion in order to observe any subtle changes in brain and/or behavior induced by preconception paternal stress. See *Figure 1.4.*

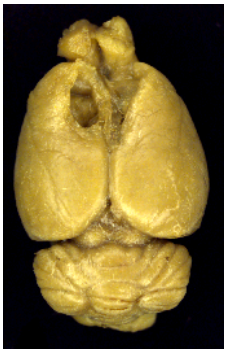
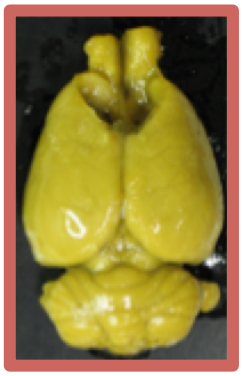
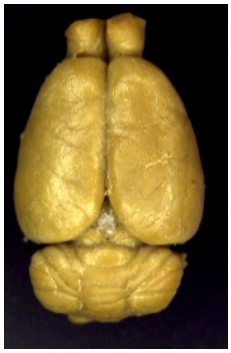
Postnatal Day 3 Lesion (P3)	**Postnatal Day 7 Lesion (P7)**	**Postnatal Day 10 Lesion (P10)**
		
Devastating impact on recovery of tissue and/or function	✔	**Remarkable spontaneous recovery of tissue and/or function**
Advantage of P3 Lesion	**Some recovery of tissue and/or function, but not complete**	**Advantage of P10 Lesion**
Ability to observe a subtle effect of stress on recovery of function	**Advantage of P7 Lesion**	Ability to observe a subtle effect of stress on loss of function
Disadvantage of P3 Lesion	Ability to observe a subtle effect of stress on loss of function	**Disadvantage of P10 Lesion**
Unable to observe a subtle effect of stress on loss of function	Ability to observe a subtle effect of stress on recovery of function	Likely ceiling effect

Figure 1.4. Lesion Model as a Tool *(Kolb et al., 2011, Kolb & Gibb, 2006)*

1.6 Theory

This research is guided by the fundamental theory that *early life experiences alter the structure and function of the developing brain, resulting in changes to epigenome and behavior.*

1.7 Hypothesis

I hypothesize that preconception *paternal* experience would impact neurodevelopment and behavioral outcomes of developing offspring.

1.7.1. Predictions

1. I predict that if I stressed male rats during the preconception period (PPS), I would observe changes in offspring epigenome, brain architecture, and behavior.

2. I predict that the impact of preconception paternal stress (PPS) on offspring brain and behavior would be observed throughout the lifespan.

3. I predict that the effects of PPS would be increased in offspring with neonatal prefrontal cortex injuries.

1.8 Organization of Thesis

The present thesis includes three experiments examining DNA global methylation, behavior throughout the lifespan, and brain morphology performed at two separate time points. Experiments are presented in 3 individual manuscripts, two of which are published, the third has been submitted for publication.

Experiment 1: *"Paternal Stress Prior to Conception Alters DNA Methylation and Behavior of Developing Rat Offspring"*

- Global Methylation Levels
- Early Behavior

Experiment 2: "*Preconception Paternal Stress in Rats Alters Dendritic Morphology and Connectivity in the Brain of Developing Male and Female Offspring"*

- P21 Brain Morphology – 3 measures in 5 brain areas

Experiment 3: *"Preconception Paternal Stress in Rats with Neonatal Prefrontal Cortex Lesion"*

- P110 Brain Morphology – 3 measures in 4 brain areas
- Juvenile/Adult Behavior in both sham and lesion animals

Chapter 2

Paternal Stress Prior to Conception Alters DNA Methylation and Behavior of Developing Rat Offspring[1]

2.1 ABSTRACT

Although there has been an abundance of research focused on offspring outcomes associated with maternal experiences, there has been limited examination of the relationship between paternal experiences and offspring brain development. As spermatogenesis is a continuous process, experiences that have the ability to alter epigenetic regulation in fathers may actually change developmental trajectories of offspring. The purpose of this study was to examine the effects of paternal stress prior to conception on behavior and the epigenome of both male and female developing rat offspring. Male Long-Evans rats were stressed for 27 consecutive days and then mated with control female rats. Early behavior was tested in offspring using the negative geotaxis task and the open field. At P21 offspring were sacrificed and global DNA methylation levels in the hippocampus and frontal cortex were analyzed. Paternal stress prior to conception altered behavior of all offspring on the negative geotaxis task, delaying acquisition of the task. In addition, male offspring demonstrated a reduction in stress reactivity in the open field paradigm spending more time than expected in the center of the open field. Paternal stress also altered DNA methylation patterns in offspring at P21. Global methylation was reduced in the frontal cortex of female offspring, but increased in the hippocampus of both male and female offspring.

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[1]Mychasiuk, R., Harker, A., Ilnytskyy, S., Kolb, B., & Gibb, R. (2015). Paternal Stress Prior to Conception Alters DNA Methylation and Behavior of Developing Rat Offspring. *Published in Neuroscience, 241, 100-105. doi:10.1016/j.neuroscience.2013.03.025*

The results from this study clearly demonstrate that paternal stress during spermatogenesis can influence offspring behavior and DNA methylation patterns, and these effects occur in a sex-dependent manner. Development takes place in the center of a complex interaction between maternal, paternal, and environmental influences, which combine to produce the various phenotypes and individual differences that we perceive.

Key words: epigenetics, frontal cortex, hippocampus, fathers.

2.2. INTRODUCTION

Although a relatively new field of investigation, researchers are beginning to examine indirect environmental influences capable of altering neurodevelopmental outcomes. As we have extended the macro-environment of the child to include fathers, paternal influences, as they pertain to maternal behavior/depression and co-parenting expectations (Field et al., 2006; Mashoodh et al., 2012; Outscharoff et al., 2006; Paulson and Bazemore, 2010; Seidel et al., 2011), have come under scrutiny. Despite this expansion however, the underlying theory has not really changed; mothers are still considered the primary influencer of child neurodevelopment, with fathers influencing offspring only through modification of maternal characteristics. Hence, there has been limited examination of experiences whereby fathers directly influence the neurodevelopment of offspring. Despite emerging evidence that paternal age, nutrition, and drug use, may place offspring at risk of psychopathologies (Kaat et al., 2007; Curley et al., 2010), very few studies have been designed to tease out the mechanisms responsible for these associations. When examining the role of the father from a genetic

perspective, the majority of this research has focused on possible paternal contributions to fetal alcohol syndrome (Abel, 1993, 2004; Randall et al., 1982) or genetic imprinting, with very limited analysis of epigenetic reprogramming of the sperm prior to conception (Franklin et al., 2010; Miller et al., 2010; Mychasiuk et al., 2012b).

Owing to the continuous nature of spermatogenesis, experiences that change DNA methylation patterns in sperm before fertilization have the potential to alter epigenetic programming of future offspring. DNA methylation is used by spermatozoa and is uniquely regulated to play an important role in the development of future gametes and embryos (Jenkins and Carrell, 2011). Robust paternal epigenetic contribution to embryogenesis requires that the DNA in spermatozoa contain layers of regulatory elements, including methyl groups that drive gene activation or silencing upon contact with the egg. Although critical to normal development, this abundance of regulatory control also leaves the DNA susceptible to damage from outside agents (Jenkins and Carrell, 2011). Damaging outside agents are commonly believed to be chemical toxins or drugs of abuse, however, prior research has demonstrated that stress impairs spermatogenesis in adult rats (Potemina, 2008), and alters methylation patterns in the germline of F2 male mice (Franklin et al., 2010).

The purpose of this study was to examine the effects of paternal stress prior to copulation on the epigenome and behavior of developing rat offspring. There is overwhelming literature outlining the acute and longterm effects of maternal stress on offspring development (e.g., Champagne and Meaney, 2006; Kapoor et al., 2006; Mychasiuk et al., 2011b, 2011d; Muhammad et al., 2012) but little examination of how paternal stress influences the same outcomes. In an effort to begin investigation of the

direct link between paternal experience and offspring sequel, we stressed male rats prior to conception and examined behavior and methylation patterns in the brain of young offspring. Early behavioral testing included the open field paradigm to measure exploratory behavior and the negative geotaxis paradigm to examine sensorimotor development. The frontal cortex and hippocampus were the two brain areas chosen for DNA methylation analysis. The frontal cortex receives input from all other cortical regions and is intricately involved in processes such as executive function, socio-emotional regulation, and psychopathologies, whereas the hippocampus is highly susceptible to the effects of stress and is imperative for learning and memory. As we were interested in detecting effects in the developing brain and wanted to avoid confounding interactions from pubertal hormones, DNA methylation analysis was conducted on brain tissue derived from offspring on postnatal day 21 (P21).

2.3. EXPERIMENTAL PROCEDURES

2.3.1. Subjects and Paternal Stress Procedure

All experiments were carried out in accordance with the Canadian Council of Animal Care and were approved by the University of Lethbridge Animal Care Committee. All of the animals in this experiment were maintained on a 12:12-h light:dark cycle in a temperature controlled (21 °C) breeding room and were given access to food and water *ad libitum*. All rats were bred in the facility for multiple generations. Ten female Long-Evans rats were mated with 10 different male Long-Evans rats (four control and six paternal stress). All mating pairs were successful. Paternal stress was administered for 27 consecutive days, immediately prior to the mating session. The

paternal stress consisted of placing the male rat (n = 6) on an elevated Plexiglas® platform (1 m tall, 21 × 21 cm) twice daily for 30 min (Wong et al., 2007). Control males were removed from their home cages twice daily for 30 min, but were not submitted to the stressing paradigm. The mating session was the only exposure female dams had with the stressed male rats. Following mating, female dams were pair-housed in shoe-box cages with another female dam. Each pair consisted of two females in the same experimental condition (ex. paternal stress–paternal stress vs. control–control). Once the pups were born, each of the mothers was housed individually with their litters.

2.3.2. Behavioral Protocols

2.3.2.1. Negative Geotaxis. Pups were tested on the on the negative geotaxis task at P9 and P10. Pups are individually placed facing downwards on a Plexiglas® board set to a 40° angle. The pups are filmed for 60 s each day. If the pup slid off of the board, they were placed back on the board facing downwards. Pups were scored for the amount of time they spent in an upwards direction by a research associate blinded to the experimental parameters. A pup was considered to be in the upward position when its head crossed the horizontal plane.

2.3.2.2. Open Field. Pups were tested on the open field task from P10–P13 and on P15. Pups are individually placed in the centre of a transparent Plexiglas® box (16 × 20 × 20 cm). The base of the open field box was divided into 130 squares measuring roughly 2 × 2 cm. Pups are filmed for 60 s on each testing day and scored for exploratory behaviour by calculating the total number of novel squares their front paws

enter. The box was cleaned with Virkon in between the filming of each pup. In addition to exploratory behaviour, we also conducted a gross estimate of anxiety by measuring the percentage of time animals spent in the centre of the open field and compared to the outer edges of the open field. A research associate who was blind to the experimental parameters scored each video.

2.3.3. Methylation Procedure

On P21, twenty pups who had previously undergone the negative geotaxis task and the open field testing (10 male:10 female – one from each dam) were subject to isofluorane inhalation, weighed and quickly decapitated. The brains were removed from the skull and weighed. The frontal cortex and hippocampi of each pup was removed, immediately flash frozen on dry ice, and stored at −80 °C. DNA was extracted from the tissue using the AllPrep DNA/RNA mini kit according to manufacturer recommendations (Qiagen; Valencia, California).

To determine the extent of global methylation a well-established radiolabeled [3H]-dCTP HpaII/MspI-based cytosine extension assay was utilized (Pogribny et al., 1999). This assay measures the proportion of CpG islands that have lost methyl groups on both strands of the DNA. To briefly summarize, 1 μg of genomic DNA is digested with the methylation-sensitive restriction endonuclease, HpaII (New England Biolabs; Beverly, MA) while a second 1 μg of genomic DNA is digested with the methylation insensitive endonuclease MspI (New England Biolabs; Beverly, MA). A third 1 μg of genomic DNA is left undigested to serve as the background control. The single nucleotide extension reaction is performed in a 25 μl reaction mixture containing 1 μg

DNA, 1× polymerase chain reaction (PCR) buffer II, 1.0 mM $MgCl_2$, 0.25 U AmpliTaq DNA polymerase, and 1 μl of [3H]-dCTP that is incubated at 56 °C for 1 h. Samples are then applied to DE-81 ion-exchange filter paper and washed three times with 0.5 M Na-phosphate buffer (pH 7.0). The filter paper is then dried and processed for scintillation counting. Two technical repeats of each experiment are conducted to ensure data consistency and reliability. The absolute percentage of double-stranded unmethylated CpG sites can then be calculated by relating the data from the HpaII and MspI digests (Pogribny et al., 1999).

2.3.4. Statistical Analysis

All statistical analysis was carried out using SPSS 20.0 for Mac. For behavioural tests, litter analysis was conducted to ensure results could not be attributed to a single litter and single parent. One-way analysis of variances (ANOVAs) with litter as the factor were run for each behavioural test and the main effect of litter was not discovered. For the global methylation analysis, a single male and female were randomly selected from each litter to ensure litter effects did not confound the data. For all parameters, two-way ANOVAs with paternal stress (PS) and sex as factors were run to compare the offspring in the paternal stress group to control offspring. Significant results in all graphical illustrations represent significant differences between offspring in the paternal stress group and offspring in the control group.

2.4. RESULTS

2.4.1. Parental and Litter Characteristics

The elevated platform stress paradigm has been demonstrated in the past to induce a significant stress response in rats that does not habituate over time (Wong et al., 2007). The male rats in this study (~P180 at the time of stressing) also displayed overt signs of substantial stress such as significant hair loss and attenuated weight gain. All male rats were sexually naïve. Sexual behaviors were observed for the first 2.5 h to ensure mating did occur, and although stressed males were considerably more aggressive towards females than the control males, there was no effect of paternal stress on the duration of pregnancy or ratio of male to female offspring. Weight gain during pregnancy did not differ between dams mated with paternally stressed males and dams mated with control males. Stressed males did not have difficulty conceiving and were equally as likely to mate with the female they were partnered with.

Ten dams (six mated with stressed males and four mated with control males) gave birth to a total of 131pups (78 Paternal Stress (PS):54 Control). Average litter size for dams of the paternal stress pups was 13.0 ± 1.6 whereas control dams had litters of 13.25 ± 1.9 pups. Average weight gain for dams during pregnancy was 126 g ± 19.8. Seventy-four of the pups were randomly selected from the 10 dams (44 PS:30 Control) for use in this study and the remainder were used in another study not described here. Of the 74 pups, 10 males and 10 females were sacrificed for global DNA methylation (one male and one female from each litter). Selection of pups was carried out to reduce possible litter effects. For DNA methylation a single male and female was selected from

each litter. For behavioral testing, an attempt was made to select equal numbers of male and female offspring from each litter, with approximately six or seven pups from each litter selected, the remaining pups were used in another study.

2.4.2. Brain and Body Weight.

Brain weight was computed as a percentage of body weight. Paternal stress prior to conception did not alter brain weight in offspring and there was not a significant difference between male and female offspring. A two-way ANOVA with paternal stress (PS) and sex as factors did not exhibit any main effects or a significant interaction. PS, $F(1, 73) = 0.483$, $p = 0.49$; sex, $F(1, 73) = 0.002$, $p = 0.96$; interaction, $F(1, 73) = 0.001$, $p = 0.97$.

2.4.3. Behavioral Outcomes

2.4.2.1. Negative Geotaxis. When examined on P9, offspring in the paternally stressed group spent significantly less time in the upwards direction than offspring in the control group. The repeated measures ANOVA with PS and sex as factors and day as variable failed to exhibit a main effect of PS, $F(1, 72) = 0.41$, $p = 0.44$, or sex, $F(1, 72) = 0.04$, $p = 0.85$. However, post hoc *t*-tests demonstrated a significant difference between PS and control offspring on P9, $t = -2.17$, $p = 0.03$, but not on P10, $t = -1.48$, $p = 0.14$. See Fig. 1. When calculating percentage of time spent in the upwards direction, scores on the negative geotaxis task ranged from 24.9 to 98.91. (Figure 2.1.)

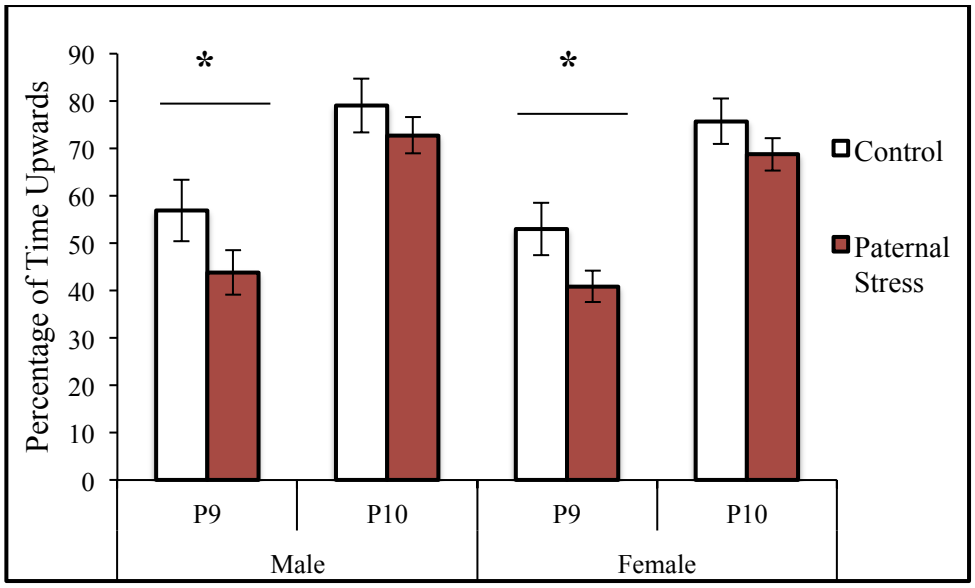

Figure 2.1. Negative Geotaxis
Average percentage of the total time male and female offspring spent in the upward direction on the negative geotaxis task. $^{*}p < 0.05$.

2.4.2.2. Open Field. Pups were tested in the open field for 5 days (P10–P13 & P15). Although there were no significant differences between offspring in the paternal stress group and the control group for novel squares entered on any of the days, following a repeated measures ANOVA, on the total number of novel squares entered, offspring in the paternal stress group did spend a greater percentage of their time in the middle of the open field when compared to control offspring. This was especially true for male offspring. A two-way ANOVA for percentage of time spent in the middle of the open field demonstrated a main effect of PS, $F(1, 73) = 5.92$, $p = 0.01$, and a trend towards significance for sex, $F(1, 73) = 3.69$, $p = 0.06$. The interaction was not significant. See Fig. 2.2. As described above, when examining the daily progression of novel squares entered, the repeated measures ANOVA with day and PS as factors failed to demonstrate significant effects for PS or sex, $F(3.11, 70)= 0.95$, $p = 0.42$; $F(3.11, 70) = 2.24$, $p = 0.08$,

respectively. The interaction was also not significant, $F(3.11, 70) = 1.18$, $p = 0.32$. (Figure 2.2./2.3.).

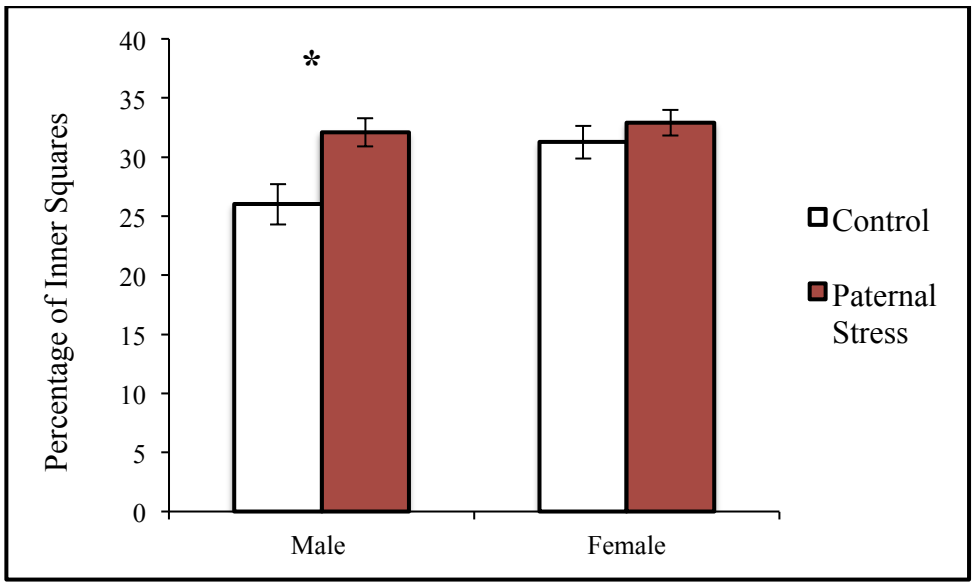

Figure 2.2. Open Field – Inner Squares
Average percentage of inner novel squares entered (total number of inner squares/total number of novel squares) by male and female offspring over the five testing days, for male offspring – $^{*}p < 0.01$.

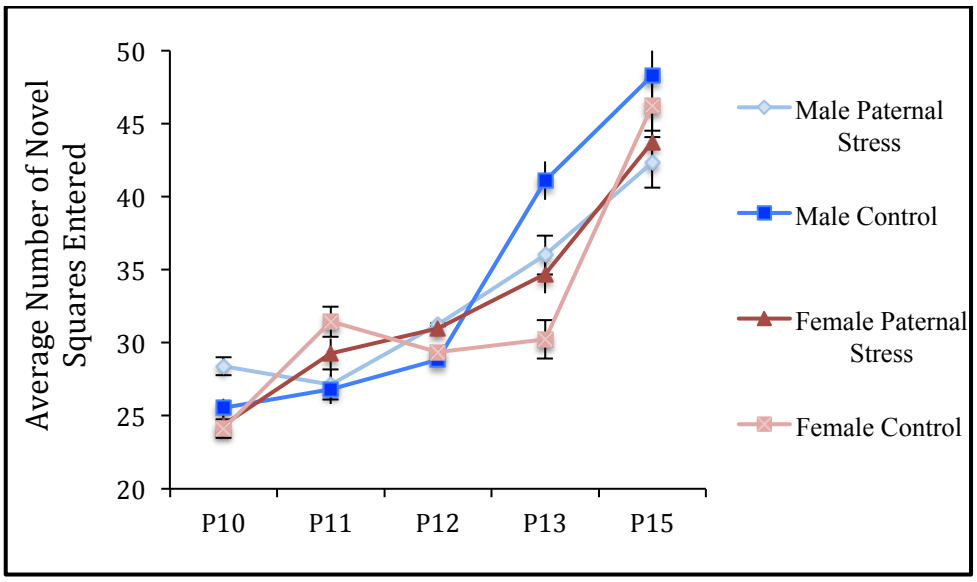

Figure 2.3. Open Field – Novel Squares
There were no significant differences in the average number of novel squares entered by male and female offspring over the five testing days.

2.4.4. Global DNA Methylation

2.4.4.1. Frontal Cortex. Global DNA methylation was significantly decreased in female, but not male offspring in the paternal stress group. A two-way ANOVA with PS and sex as factors demonstrated a significant main effect of PS, $F(1, 19) = 4.951$, $p = 0.04$, but not of sex, $F(1, 19) = 0.000$, $p = 0.99$. The interaction was significant, $F(1, 19) = 5.881$, $p = 0.03$, further emphasizing the significant decrease in methylation present in female offspring but not male offspring. In the frontal cortex, methylation levels ranged from 75.14 to 80.00, SD = 1.14. (Figure 2.4.).

2.4.4.2. Hippocampus. A significant increase in global DNA methylation was found in both male and female offspring in the paternal stress group when compared to control offspring. The two-way ANOVA demonstrated a main effect of PS, $F(1, 19) = 9.132$, $p < 0.01$, but not of sex, $F(1, 19) = 0.147$, $p = 0.71$. The interaction was also not significant, $F(1, 19) = 1.810$, $p = 0.20$. In the hippocampus, methylation levels ranged from 72.87 to 80.29, SD = 2.11. (Figure 2.4.).

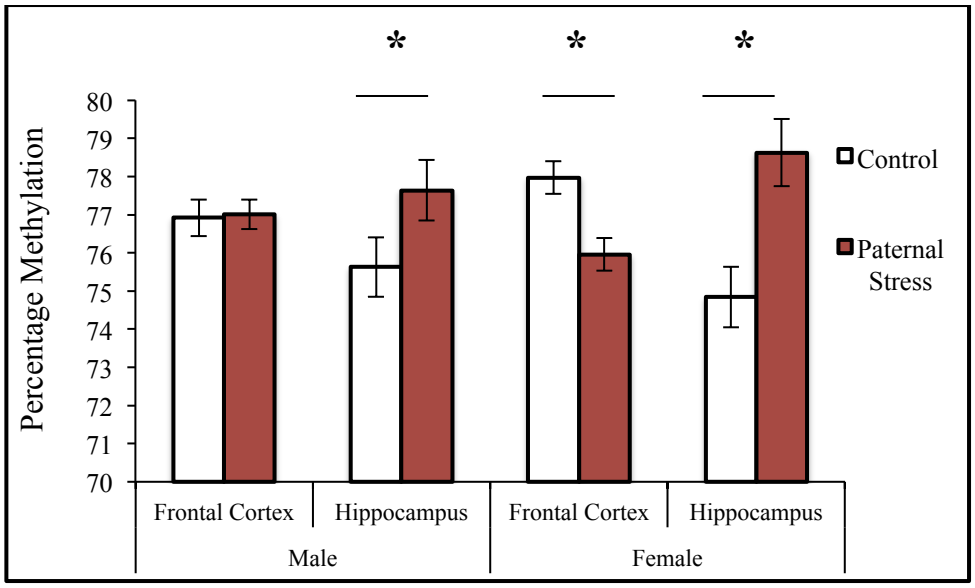

Figure 2.4. Methylation Percentage
Average percentage of global DNA methylation in the frontal cortex and hippocampus of male and female offspring at the time of sacrifice (P21), $^{*}p < 0.05$; $^{**}p < 0.001$.

2.5. DISCUSSION

Although there is an abundance of literature examining the effects of prenatal maternal stress, to our knowledge this is the first study designed to examine the effects of chronic paternal stress immediately prior to conception. As evolutionary success is not based upon longevity, but rather an individual's ability to reach reproductive age, very different processes and objectives would be expected to drive epigenetic adaptation in the face of adversity for males and females (Meaney et al., 2007 and Glover and Hill, 2012). The results clearly demonstrate that paternal stress during spermatogenesis does influence offspring behaviour and DNA methylation patterns in a sex-dependent manner.

2.5.1. Effects of Paternal Stress on Brain and Body Weight

Paternal stress prior to conception did not affect brain weight or body weight of offspring at P21. As this was an exploratory study and comparison literature is lacking, it is difficult to corroborate or oppose these findings. Interestingly however, postnatal paternal deprivation also failed to alter offspring brain weight (Helmeke et al., 2009), but paternal alcohol consumption (Abel, 2004) and paternal enrichment (Mychasiuk et al., 2012b) prior to conception both decreased offspring birth weights and organ weight at weaning. Despite the plethora of research, the prenatal maternal stress literature is conflicting; some studies report no change in offspring brain weights (Van Den Hove et al., 2006) and other studies have found reductions in offspring brain weight (Mychasiuk et al., 2011b).

2.5.2. Effects of Paternal Stress on Behavioral Development

Prenatal maternal stress has been shown to induce differential behavioral outcomes for male and female offspring, whereby females exhibit greater activation of the stress response and males demonstrate learning and memory deficits (Glover and Hill, 2012). In this study we have also demonstrated that paternal stress prior to conception alters behavioral development of male and female offspring. Although the skills and underlying neural circuitry required to complete the negative geotaxis task have not been fully appreciated, the paradigm has been reliably used as a measure of sensorimotor development (Alberts et al., 2004 and Patin et al., 2004). Offspring of fathers who experienced chronic stress were significantly impaired at the negative geotaxis task when tested on P9, but had recovered by P10 and were indistinguishable from control

offspring. This behavioral response is quite different from the response of offspring exposed to prenatal maternal stress. Prenatally stressed offspring were indistinguishable from controls on P9 but failed to demonstrate any learning/improvement that was typical of control offspring and therefore exhibited deficits on P10 (Mychasiuk et al., 2011d). The prenatally stressed offspring appeared to be impaired at learning the task, whereas pre-conceptually stressed offspring (paternal stress) could learn the task but begin somewhat delayed. Paternal stress may have slowed maturation of the developing brain.

Although paternal stress prior to conception did not alter exploratory behavior in preweanling offspring, it did significantly increase the amount of time male offspring spent in the middle of the open field. This may indicate that PS male offspring have reduced anxiety or deficits in behavioral control. This finding is consistent with research on mice that found stressful paternal events reduced stress sensitivity in male offspring, whereby their mean latency time to enter the center of the open field was much lower than control mice (Franklin et al., 2010). Similar to the study by Franklin et al. (2010), our results are not due to increased locomotor activity. Conversely, other paternal experiences do affect early locomotor behavior; paternal enrichment prior to conception increases early exploratory behavior in male offspring (Mychasiuk et al., 2012d), whereas paternal alcohol consumption prior to conception can also induce hyperactivity in rat offspring but effects appear to be strain-specific (Abel, 2004).

2.5.3. Paternal Stress Alters DNA Methylation Patterns in Brain

Although the term epigenetics has been redefined numerous times in the last decade, it is now generally used to refer to changes in gene expression that take place

without modification to the DNA sequence (Jirtle and Skinner, 2007). As DNA methylation is commonly used to repress gene expression and maintain genome stability (Bird, 2007) it can be used as a reliable measurement of epigenetic change. In response to paternal stress prior to conception, global DNA methylation was increased in the hippocampus of both male and female offspring. Low levels of chronic prenatal maternal stress also increased global DNA methylation in the hippocampus (Mychasiuk et al., 2011b), in contrast to parental enrichment (maternal – prenatally or paternal – pre-conception) which decreased global methylation in the same brain region (Mychasiuk et al., 2012b). Global DNA methylation patterns were only affected in the frontal cortex of female offspring, where methylation was significantly reduced. The frontal cortex may be more sensitive to demethylation in response to perinatal experiences, as high levels of prenatal maternal stress, prenatal maternal enrichment, and paternal enrichment prior to conception, all reduced global DNA methylation in the frontal cortex as well (Mychasiuk et al., 2011b and Mychasiuk et al., 2012b). Existing literature demonstrates that paternal exposure to substrates such as radioactive materials and carcinogenic drugs have the ability to affect offspring development by creating mutations in the actual DNA sequence (Curley et al., 2010 and Miller et al., 2010).

This study is important because it adds to the growing literature that demonstrates the powerful influence paternally induced epigenetic changes have on offspring brain development. Although the findings are interesting, it is important to note that without further investigation into the particular genes that have undergone methylation changes in response to paternal stress, it is difficult to speculate on the precise implications of an increase or decrease in methylation. While a decrease in methylation is generally

associated with the activation of gene expression, we are ignorant of the genes and specific pathways affected. As evidence is beginning to illustrate that paternal experiences can alter the epigenome of offspring, researchers need to be cognizant of the factors contributing to brain development that do not reside in the normal sphere of influence.

2.5.4. Conclusion

There is a complex interaction between maternal, paternal, and environmental influences that combine to produce the offspring phenotype that is observed. Although we have not established a concrete mechanism whereby chronic paternal stress alters the epigenome of developing offspring, we hypothesize that chronic stress modifies epigenetic expression of genes in maturing spermatozoa which in turn impacts gene expression and development in the embryo. We acknowledge that we are unable to differentiate between methylation of gene promoter regions and transposable elements with our global methylation protocol. However, basic science research has demonstrated that methylation status of transposable elements is also associated with gene activity (Rakyan et al., 2002). Future studies will strive to develop a mechanistic model to explain how paternal stress prior to conception actually modifies gene expression in the brain of developing offspring. In addition, these studies will select and investigate candidate genes and examine effects on offspring in the long-term

CHAPTER 3

Preconception Paternal Stress in Rats Alters Dendritic Morphology and Connectivity in the Brain of Developing Male and Female Offspring[2]

3.1. ABSTRACT

The goal of this research was to examine the effect of preconception paternal stress (PPS) on the subsequent neurodevelopment and behavior of male and female offspring. Prenatal (gestational) stress has been shown to alter brain morphology in the developing brain, and is presumed to be a factor in the development of some adult psychopathologies. Our hypothesis was that paternal stress in the preconception period could impact brain development in the offspring, leading to behavioral abnormalities later in life. The purpose of this study was to examine the effect of preconception paternal stress on developing male and female offspring brain morphology in 5 brain areas; medial prefrontal cortex (mPFC), orbitofrontal cortex (OFC), parietal cortex (Par 1), hippocampus (CA1) and nucleus accumbens (NAc). Alterations in dendritic measures and spine density were observed in each brain area examined in paternal stress offspring. Our two main findings reveal; 1) PPS alters brain morphology and organization and these effects are different than the effects of stress observed at other ages; and, 2) the observed dendritic changes were sexually dimorphic. This study provides direct evidence that PPS modifies brain architecture in developing offspring, including dendritic length, cell

[2] Harker, A., Raza, S., Williamson, K., Kolb, B., & Gibb, R. (2015). Preconception Paternal Stress in Rats Alters Dendritic Morphology and Connectivity in the brain of Developing Male and Female Offspring. *Published in Neuroscience, 303, 200-210. doi:10.1016/j.neuroscience.2015.06.058*

complexity, and spine density. Alterations observed may contribute to the later development of psychopathologies and maladaptive behaviors in the offspring.

Key Words: *paternal, development, prefrontal, nucleus accumbens, hippocampus, Golgi*

3.2. INTRODUCTION

A rich literature has been amassed demonstrating the impact of early life events on the structure and function of the developing brain. Research has shown that the effect of maternal stress on offspring development has been linked to an increased risk of psychopathologies, including attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), major depressive disorder (MDD), schizophrenia, and drug addiction (Arnsten, 2011; Bale et al., 2010; Beversdorf et al., 2005; Class et al., 2014; Dietz et al., 2011; Franklin et al., 2010; Khashan et al., 2008; Kolb et al., 2012a; Pelham et al., 2007). Although there is a well-established relationship between maternal stress and mental health, very little is known regarding the effect of preconception paternal stress (PPS) on the developing brain of offspring. In a recent study, we found that PPS had a significant effect on early behavior and stress reactivity in offspring (Mychasiuk et al., 2013a). PPS also altered global DNA methylation levels in postnatal day 21 (P21) offspring. Dietz et al., (2011) found that offspring of fathers that had been exposed to chronic social defeat stress exhibited anxiety-like and depressive behaviors, as well as decreased levels of endothelial growth factor and increased levels of corticosterone. However, these effects were absent when researchers used in vitro fertilization (IVF), suggesting limited epigenetic transmission. Epigenetic reprogramming of the germ cells

mediated by hypothalamic-pituitary-adrenal (HPA) axis dysregulation was found in offspring of chronically stressed fathers (Rodgers et al., 2013). Braun and Champagne (2014) review substantial literature that examines possible influences of fathers on offspring development. These researchers suggest three possible routes by which transmission may occur; direct paternal care, epigenetic transmission, and interactions between mother and infant, influenced by fathers.

The present study investigated brain morphology in postnatal day 21 (P21) offspring of fathers stressed prior to mating. Analyses of spine density, dendritic length, and cell complexity were examined in the following five brain areas, medial prefrontal cortex (mPFC), orbitofrontal cortex (OFC), parietal cortex (Par1), hippocampus (CA1), and nucleus accumbens (NAc). These brain regions were chosen because we (and others) have previously found them to be especially sensitive to stress in adult rats (Cg3, AID), gestational stress (Cg3, AID, CA1), maternal separation (Cg3, AID, NAcc), and bystander stress (Cg3, AID, CA1) (e.g., Liston et al., 2006; Muhammad & Kolb, 2011a; 2011b; Mychasiuk et al., 2011a; 2011b). We included an additional region, Par1, because it is extremely sensitive to early experience such as prenatal and infant tactile stimulation (e.g., Kolb & Gibb, 2010), but were unaware of any evidence showing it to be stress sensitive. Indeed, Shors, Chua, & Falduto (2001) found no effect of stress on parietal cortex in adult male and female rats.

Numerous studies within our lab have examined the effects of gestational stress on these specific brain areas (Mychasiuk et al., 2011a, 2011b; Muhammad et al., 2012; Mychasiuk et al., 2012a). Recently, we found that PPS significantly influenced early behavior, and had a significant effect on DNA global methylation levels in the prefrontal

cortex (PFC) (Mychasiuk et al., 2013a). We aim to further our understanding of the effect of paternal stress in the preconception period and the impact of this type of stress on these previously examined brain areas.

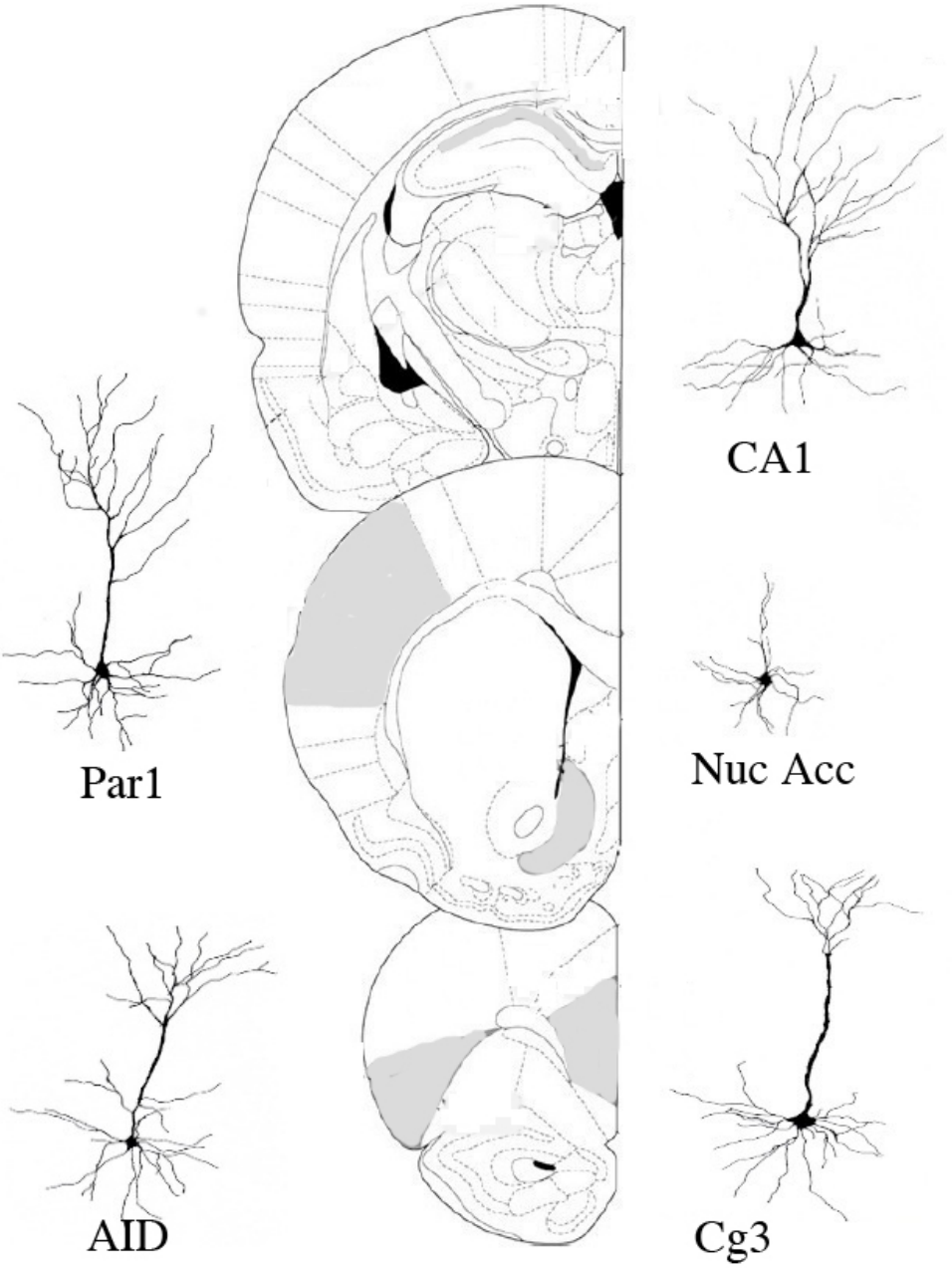

Figure 3.1. Brain regions (shaded areas) and example neurons analyzed. Adapted from Paxinos and Watson (1997).

3.3. EXPERIMENTAL PROCEDURES

3.3.1. 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. Ten female Long-Evans rats were mated with 10 male Long-Evans rats (six paternal stress and four control). All pairs successfully mated resulting in 131 pups (78 Paternal Stress (PS): 54 Control (C)). Animals were given access to food and water *ad libitum* and were maintained on a 12 h light/dark schedule (lights on from 07:30 to 19:30 h) in a temperature controlled (21º) breeding room.

3.3.2. Paternal Stress

Paternal stress was administered a total of 27 consecutive days prior to the mating session. Stressing consisted of placing the male rat (n=6) on an elevated Plexiglas® platform (1 m tall, 21 x 21 cm) in a brightly lit room for 30 min. (Wong et al., 2007). Following the stressing procedure, rats were transported back to their home cages. Stressing sessions occurred at 9:00 A.M. and 3:00 P.M. During stressing sessions, control males (n=4) were removed from their home cages for the 30 minutes, but did not participate in the stressing paradigm. Following the 27 days of stressing, paternal stress and control males were immediately mated with females. This was the only exposure that female dams had with the stressed male rats. Subsequent to mating, female dams were housed in shoebox cages with another female in the same experimental condition (eg. control–control vs. paternal stress–paternal stress). Female dams were separated and housed individually prior to birth of pups, following a weight gain ≥ 100g. Female dams

remained individually housed following the birth of their litter. Weight gain during pregnancy did not differ between female dams mated with paternal stress males or paternal control males. Average litter size for dams of the paternal control pups was 13.25 ± 1.9 whereas dams mated with paternal stress males had litters of 13.0 ± 1.6. We chose 1-2 male and female pups from each litter to reduce the possibility of litter effects. There were no other differences in litter characteristics to report. The remaining pups were used in parallel experiments.

3.3.2.1. Stressing paradigm. Wong et al. (2007) developed a stressing paradigm that has been shown to induce significant chronic stress in rats, supported by analysis of corticosterone levels and observation of behavioral effects (consistent urination and/or defecation during the stress procedure). We chose to follow this stressing protocol in order to forego unnecessary stress related to blood collection for corticosterone analysis, extensively examined previously by Wong and associates.

3.3.3. Anatomy

3.3.3.1 Perfusion and staining. Histological processing was performed on postnatal day 21. Animals were administered an overdose of sodium pentobarbital solution (i.p.) and perfused with 0.9% saline solution intracardially. The brains were extracted from the skull, brain weight was recorded and brains were preserved in bottles containing Golgi-Cox solution for 14 days in the dark. Following this period, brains were transferred to 30% sucrose solution for a minimum of 3 days. A Vibratome was used to section the brains at a thickness of 200 μm, and sections were mounted on gelatin-coated

slides. Brain sections were then processed for Golgi-Cox staining according to the procedures outlined by Gibb and Kolb (1998).

3.3.3.2. Dendritic analyses. Relevant cells in five brain regions (see Figure 1) were identified at low power (100X), and individual pyramidal cells from layer III were traced using camera Lucida (at 250X) in areas Cg 3 (medial prefrontal cortex), AID (orbitofrontal cortex), Par1 (parietal cortex), CA1 (hippocampus), as defined by Zilles (1985). The same manner was used to identify and draw medium spiny neurons in the shell of the nucleus accumbens. A total of 10 cells, five from each hemisphere, were drawn from each brain region of every animal. The mean of the five cells per hemisphere was used as the unit of analysis. Both apical and basilar dendritic branching were drawn for Cg3 and Par1. Basilar (no apical) dendritic branching was drawn for AID and CA1 because the plane of sectioning truncated many apical fields in AID and there was excessive blood vessel artifact obscuring much of the apical fields in CA1.

To meet the criteria for analysis, the dendritic tree of the cell had to be intact, well impregnated with stain and not obscured by blood vessels, astrocytes, or stain precipitations. The cell's dendritic arbor was quantified using two methods. First, dendritic complexity was estimated using branch order (based on the total number of branch bifurcations) (Coleman & Reisen, 1968). Second, a Sholl analysis was used to estimate dendritic length (a transparent grid of concentric rings, equivalent to 25 μm apart was placed over dendritic drawing and the number of ring intersections were counted) (Sholl, 1956).

3.3.3.3. Spine density. Apical and basilar dendrites were drawn from Cg3 and Par1 and basilar dendrites were drawn from AID and CA1 at 1000X from 10 neurons (5/hemisphere) per region. For Cg3 and Par1 pyramidal neurons one third-order terminal tip from both the basilar and apical dendritic trees was identified, and the total number of visible spines along the length of the dendritic segment (at least 40 μm long) was counted. For pyramidal neurons in AID and CA1, spines were counted on one third-order tip from only basilar dendrites. For medium spiny neurons, spines were counted on one terminal tip (third order or up) per neuron. Density was expressed as spines per 10 μm after the exact length of the dendrite portion drawn had been calculated. Care was taken to ensure that the dendritic traced segments met the criteria of being well impregnated with stain and not obscured by neighboring dendrites or blood vessels. A researcher blind to treatment conditions performed cell selection and tracing.

3.3.4. Statistical Analysis

Statistical analyses were performed using SPSS 21 for Mac. The anatomical data for each of the five brain areas were analyzed using three-way ANOVA's with treatment (preconception paternal stress (PPS) and control), sex and hemisphere as factors. The data points were the mean of 5 cells for dendritic measures or distal dendrite segments for spines per area per hemisphere per animal. However, hemisphere failed to show significant main effects or interactions with other factors and was therefore eliminated as a factor. Consequently, we report two-way ANOVA's with PPS and sex as factors. The following are the results of the two way ANOVA's on 10 cells per area, as hemisphere was no longer investigated. Pairwise comparisons followed all significant interactions

between factors. Statistical significance was considered $p < 0.05$. All illustrations portray comparisons between paternally stressed offspring and control offspring.

3.4. RESULTS

We observed the same behavioral responses including consistent urination and/or defecation of the animals while on the elevated platform as reported by Wong et al. (2007). Further, we observed attenuated weight gain of all stressed participants [F (1,9) = 104.4, $p > 0.001$] (See Figures 3.2A/B), as well as excessive barbering of hair on forearms, suggesting a severe anxiety response. The anatomical results showed significant effects of PPS on dendritic morphology in all regions measured; the effects overall being greater in females (see Figs. 3.1-7.1 and Table 3.1). We consider each area in turn.

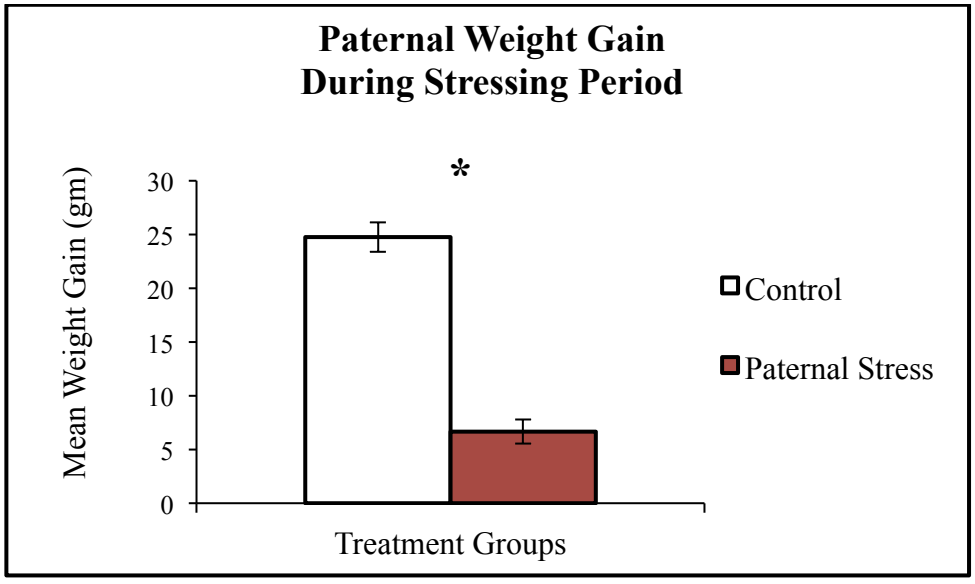

Figure 3.2 (A) Paternal weight gain during 27-day stressing period reveals attenuated weight gain for paternal stress males. Paternal stress males gained significantly less weight than did paternal control males. (*$p < 0.050$)

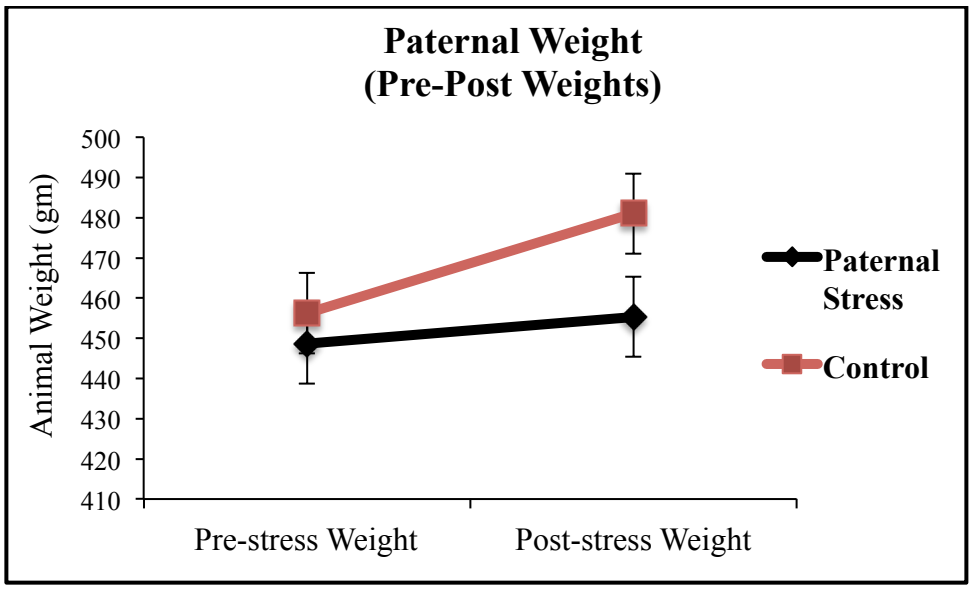

Figure 3.2.(B) Pre-stress weight and post-stress weight of paternal stress and paternal control males reveals attenuated weight gain for paternal stress males.

3.4.1. Brief Summary of Anatomical Results

3.4.1.1. Orbital frontal cortex (AID). PPS significantly reduced dendritic branching and length in the AID region of the OFC in both sexes, and significantly reduced dendritic spines in males. (See Figures 3.3.A/B, Table 3.1.).

3.4.1.2. Medial Prefrontal Cortex (Cg3). PPS significantly increased apical dendritic branching in Cg3 in both sexes and significantly decreased apical and basilar dendritic spine density in females. (See Figures 3.3.A/B, Table 3.1.).

3.4.1.3. Parietal Cortex (Par1). In Par1, PPS significantly increased basilar dendritic length in males. As well, PPS significantly decreased apical dendritic spines in

females, and significantly decreased basilar dendritic spines in both sexes. (See Figures 3.3.A/B, Table 3.1.).

3.4.1.4. Hippocampus (CA1). PPS significantly decreased dendritic length in CA1 in females. (See Figures 3.3A/B, Table 3.1).

3.4.1.5. Nucleus Accumbens (NAc). In NAc, PPS significantly increased dendritic branching and length in females. (See Figures 3.3A/B, Table 3.1).

3.4.2. Dendritic Branching

3.4.2.1. AID branching. A two-way ANOVA (Treatment x Sex) of dendritic branch order revealed a main effect of treatment [F (1,41) = 8.50, $p < 0.001$], no main effect of sex [F (1,41) = 1.78, $p = 0.191$], nor an interaction between the two [F (1,41) = 0.01, $p = 0.918$]. PPS significantly decreased dendritic branch complexity in AID regardless of sex (See Figs. 3.3A/B, Table 3.1).

3.4.2.2. Cg3 branching. A two-way ANOVA (Treatment x Sex) of dendritic branch order revealed a main effect of treatment in apical branching [F (1,41) = 12.61, $p = 0.001$], no main effect of sex [F (1,41) = 0.35, $p = 0.556$], nor an interaction between the two [F (1,41) = 0.15, $p = 0.698$]. Basilar branching in Cg3 revealed no main effect of treatment [F (1,41) = 3.04, $p = 0.089$], or of sex [F (1,41) = 0.12, $p = 0.726$], nor an interaction between the two [F (1,41) = 0.92, $p = 0.343$]. PPS increased apical dendritic branching in Cg3 regardless of sex (See Figs. 3.3A/B, Table 3.1).

3.4.2.3. Par1 branching. A two-way ANOVA (Treatment x Sex) of apical dendritic branching did not reveal a main effect of treatment [F (1,41) = 0.19, p = 0.668], or of sex [F = 1.68, p = 0.202], nor an interaction between the two [F (1,41) = 1.68, p = 0.202]. Basilar dendritic branching in Par1 did not reveal a main effect of treatment [F (1,41) = 0.25, p = 0.619] but there was a main effect of sex [F (1,41) = 4.33, p = 0.044], and no interaction between the two [F (1,41) = 0.05, p = 0.833]. Females had significantly higher dendritic branch complexity (See Figs. 3.3A/B, Table 3.1).

3.4.2.4. CA1 branching. A two-way ANOVA (Treatment x Sex) of dendritic branch order revealed no main effect of treatment [F (1,41) = 0.40, p = 0.529], or of sex [F (1.41) = 0.16, p > 0.692], nor an interaction between the two [F (1,41) = 0.52, p = 0.475] (See Figs. 3.3A/B, Table 3.1).

3.4.2.5. NAc branching. A two-way ANOVA (Treatment x Sex) of dendritic branch order revealed no main effect of treatment [F (1,41) = 0,67, p = 0.419], a main effect of sex [F (1,41) = 4.36, p = 0.044], and a significant interaction between the two [F (1,41) = 4.28, p = 0.045]. A pairwise analysis of Treatment by Sex revealed that PPS females had a significant increase in dendritic branching (p = 0.042), whereas PPS males had decreased branching (See Figs. 3.3A/B, Table 3.1).

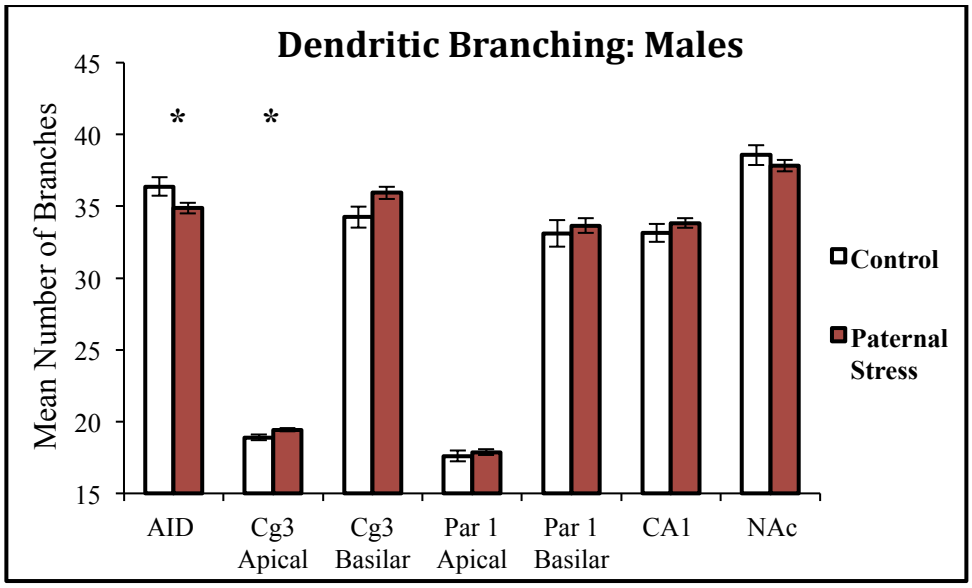

Figure 3.3. (A) Average dendritic branching of neurons in male rat offspring. Five brain areas have been used to compare Paternal Stress (treatment) and Control (no treatment) on postnatal day 21. (* $p < 0.050$)

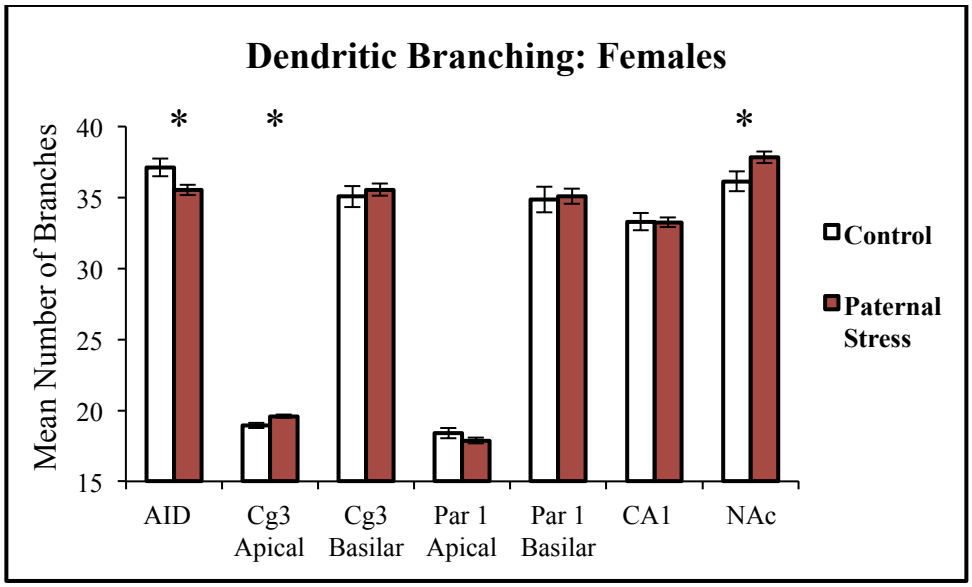

Figure 3.3. (B) Average dendritic branching of neurons in female rat offspring. Five brain areas have been used to compare Paternal Stress (treatment) and Control (no treatment) on postnatal day 21. (* $p < 0.050$)

3.4.3. Dendritic Length

3.4.3.1. AID length. A two-way ANOVA (Treatment x Sex) of dendritic length revealed a main effect of treatment [F (1,41) = 14.170, p = 0.001], no main effect of sex [F = 1.36, p = 0.252], nor an interaction between the two [F = 3.32, p > 0.076]. PPS significantly reduced dendritic length in AID in both sexes (Figures 3.4. A/B, Table 3.1).

3.4.3.2. Cg3 length. A two-way ANOVA (Treatment x Sex) of apical dendritic length did not reveal a main effect of treatment [F (1,41) = 0.71, p = 0.406], nor of sex [F (1,41) = 0.65, p = 0.426], nor an interaction between the two [F (1,41) = 0.01, p > 0.05]. Basilar dendritic length did not reveal a main effect of treatment [F (1,41) = 1.17, p = 0.286] but there was a main effect of sex [F (1,41) = 7.69, p = 0.009], and no interaction between the two [F (1,41) = 0.08, p = 0.780]. Basilar dendritic length was significantly longer in males (p = 0.008) (See Figs. 3.4A/B, Table 3.1).

3.4.3.3. Par1 length. A two-way ANOVA (Treatment x Sex) of apical dendritic length revealed no main effect of treatment [F (1,41) < 0.01, p = 0.971]. There was a main effect of sex [F (1,41) = 4.17, p = 0.048], but no interaction between the two [F (1,41) = 1.84, p = 0.182]. Females had significantly longer dendrites. Basilar dendritic length revealed no main effect of treatment [F (1,41) = 2.44, p = 0.126], but there was a main effect of sex [F (1,41) = 12.66, p = 0.001] and a significant interaction between the two [F (1,41) = 6.36, p = 0.016]. A pairwise analysis of Treatment by Sex revealed that PPS significantly increased basilar dendritic length in males (P = 0.001). However, females had significantly longer dendrites overall. (Figures 3.4. A/B, Table 3.1).

3.4.3.4. CA1 length. A two-way ANOVA (Treatment x Sex) of dendritic length revealed a main effect of treatment [F (1,41) = 4.136, p = 0.049], no main effect of sex [F (1,41) = 0.01, p = 0.950], nor an interaction between the two [F (1,41) = 1.18, p = 0.284]. Although PPS slightly decreased dendritic length in males, in females dendritic length was significantly decreased. (Figures 3.4. A/B, Table 3.1).

3.4.3.5. NAc length. A two-way ANOVA (Treatment x Sex) of dendritic length did not reveal a main effect of treatment [F (1,41) = 1.16, p > 0.288], but there was a main effect of sex [F (1,41) = 14.365, p = 0.001], and no significant interaction between the two [F (1,41) = 3.04, p = 0.089], Overall, females have significantly shorter dendrites. (Figures 3.4. A/B, Table 3.1).

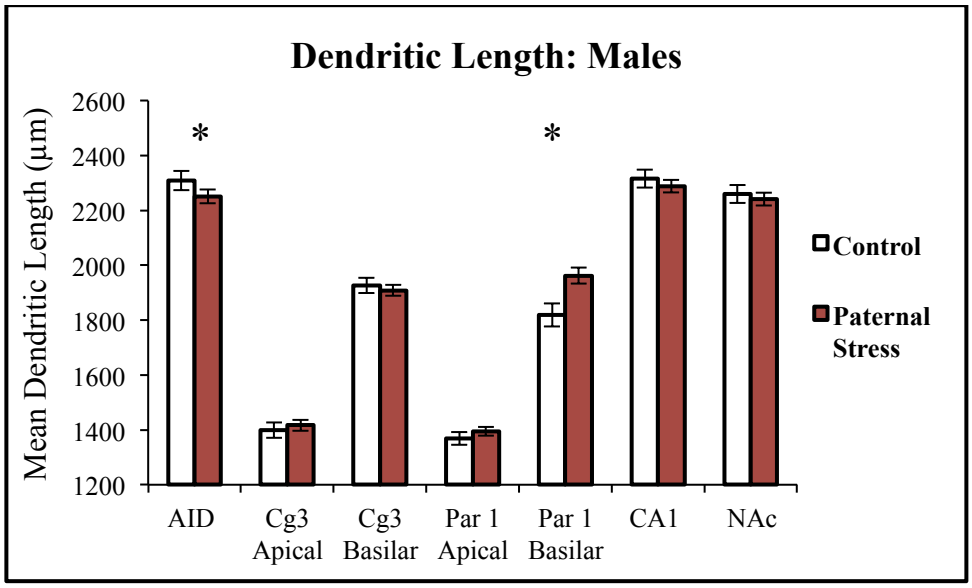

Figure 3.4. (A) Average length of dendrites in male rat offspring. Five brain areas have been used to compare Paternal Stress (treatment) and Control (no treatment) on postnatal day 21. (* p < 0.050)

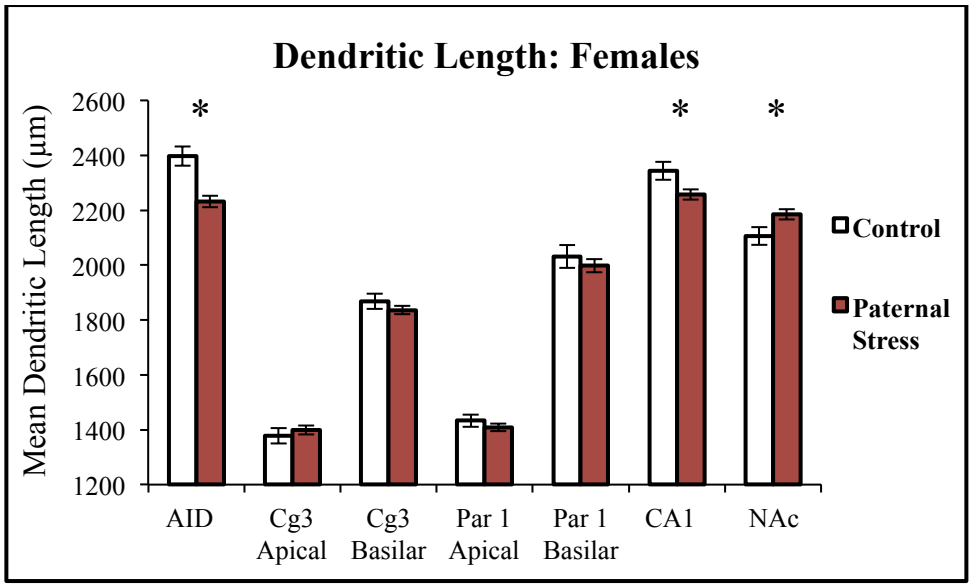

Figure 3.4. (B) Average length of dendrites in female rat offspring. Five brain areas have been used to compare Paternal Stress (treatment) and Control (no treatment) on postnatal day 21. (* $p < 0.050$)

3.4.4. Spine Density

3.4.4.1. AID spine density. A two-way ANOVA (Treatment x Sex) of dendritic spines revealed a marginal main effect of treatment [F (1,41) = 3.77, p = 0.060], no main effect of sex [F (1,41) = 1.26, p = 0.270], nor an interaction between the two [F (1,41) = 1.54, p = 0.222]. PPS tended to reduce dendritic spine density in AID. (Figures 5A/B, 6A/B, 7, Table 1).

3.4.4.2. Cg3 spine density. A two-way ANOVA (Treatment x Sex) of apical dendritic spines revealed a main effect of treatment [F (1,41) = 19.04, $p < 0.001$], a main effect of sex [F (1,41) = 5.08, p = 0.030], and an interaction between the two [F (1,41) = 8.49, p = 0.006]. A pairwise analysis of Treatment by Sex revealed a significant decrease in spine density in PPS females (p = 0.004). Basilar dendritic spines revealed a main effect of treatment [F (1,41) = 11.29, p = 0.002], and of sex [F (1,41) = 5.096, p = 0.030],

and an interaction between the two [F (1.41) = 6.96, p = 0.012]. A pairwise analysis of Treatment by Sex revealed a significant decrease in spine density in PPS females (p < 0.001). Thus, PPS significantly reduced apical and basilar dendritic spines in females. (Figures 5A/B, 6A/B, 7, Table 1).

3.4.4.3. Par1 spine density. A two-way ANOVA (Treatment x Sex) of apical dendritic spines revealed a main effect of treatment [F (1,41) = 15.004, p < 0.001], no main effect of sex [F (1,41) = 2.77, p = 0.105], and a significant interaction between the two [F (1,41) = 5.89, p = 0.020]. A pairwise analysis of Treatment by Sex revealed that PPS significantly reduced apical dendritic spines in females (p < 0.001). Basilar dendritic spines revealed a main effect of treatment [F (1,41) = 18.13, p < 0.001], no main effect of sex [F = 0.13, p = 0.724], nor a significant interaction between the two [F (1,41) = 0.44, p = 0.511], revealing a decrease in spine density regardless of sex. (Figures 5A/B, 6A/B, 7, Table 1).

3.4.4.4. CA1 spine density. A two-way ANOVA (Treatment x Sex) of dendritic spines revealed no main effect of treatment [F (1,41) = 1.43, p = 0.239]. There was a main effect of sex [F (1,41) = 4.41, p = 0.042], but no interaction of Treatment by Sex [F (1,41) = 0.30, p = 0.588] Females had significantly higher spine density in CA1. (Figures 5A/B, 6A/B, 7, Table 1).

3.4.4.5. NAc spine density. A two-way ANOVA (Treatment x Sex) of dendritic spines revealed no main effect of treatment [F (1,41) = 0.04, p = 0.844], or of sex [F (1,41) = 2.33, p = 0.135], nor an interaction between the two [F (1,41) = 2.35, p = 0.134].

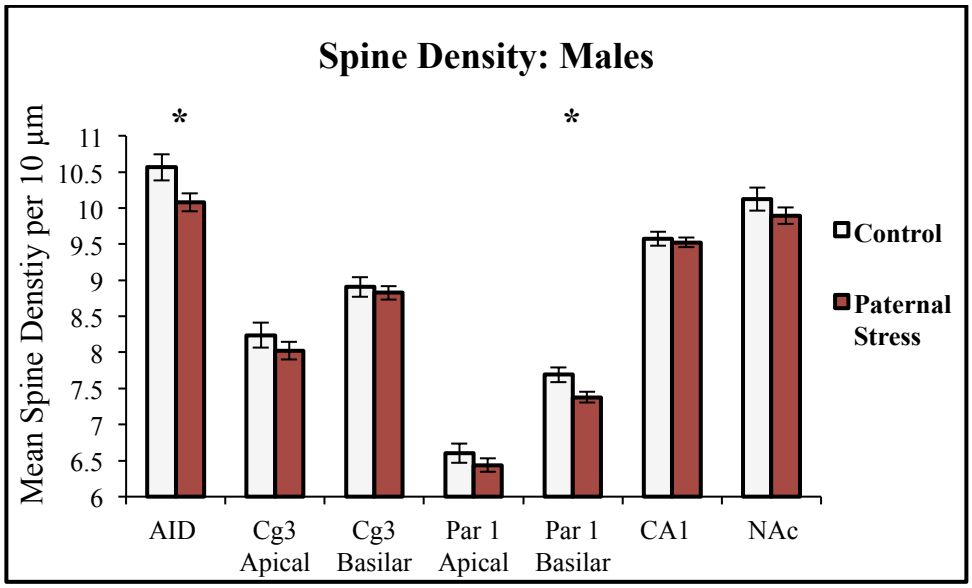

Figure 3.5. (A) Average spine density in male rat offspring. Five brain areas have been used to compare Paternal Stress and Control on postnatal day 21. (* $p < 0.050$)

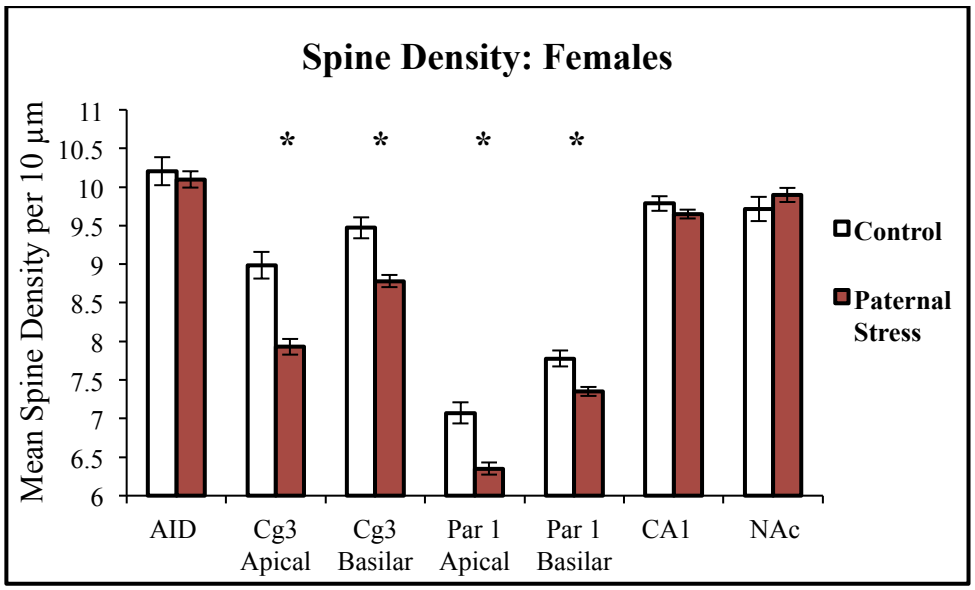

Figure 3.5. (B) Average spine density in female rat offspring. Five brain areas have been used to compare Paternal Stress and Control on postnatal day 21. (* $p < 0.050$)

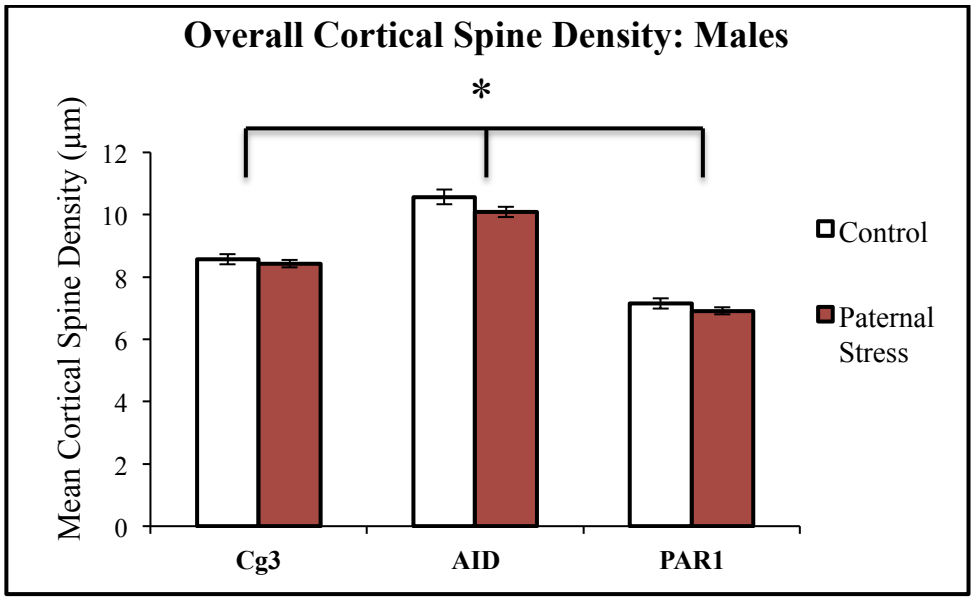

Figure 3.6. (A) Overall cortical spine density in male rat offspring. Three cortical (Cg3, AID, PAR 1) areas have been used to compare Paternal Stress (treatment) and Control (no treatment) on postnatal day 21. (* $p < 0.050$)

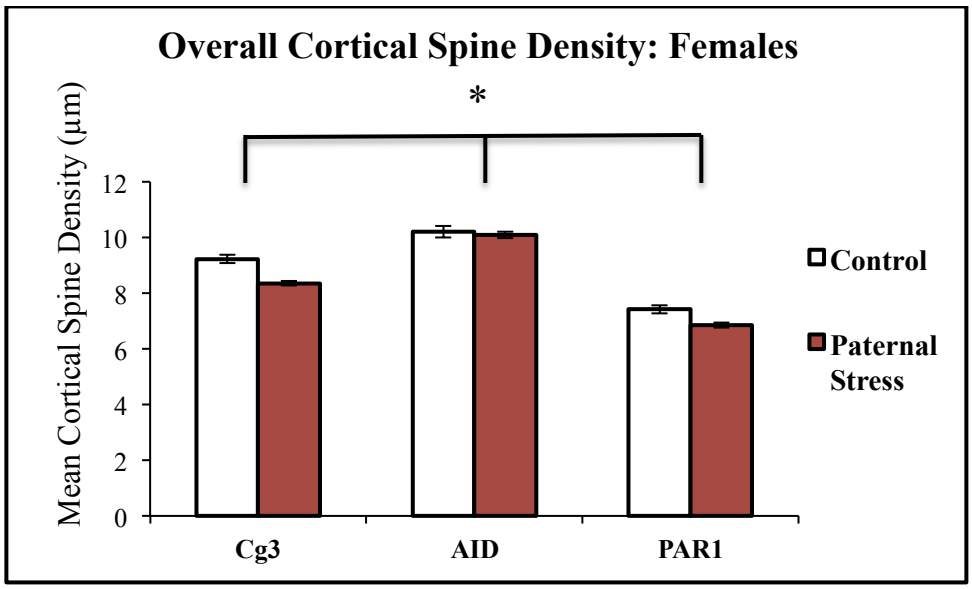

Figure 3.6. (B) Overall cortical spine density in female rat offspring. Three cortical (Cg3, AID, PAR 1) areas have been used to compare Paternal Stress (treatment) and Control (no treatment) on postnatal day 21. (* $p < 0.050$)

Table 3.1. Summary of two-way ANOVA evaluations of the effects of paternal stress by sex for 21 independent parameters measured in this study

Parameter	Male	Female
AID basilar dendritic branching	↓	↓
AID basilar dendritic length	↓	↓
AID basilar spine density	**(↓)**	**(↓)**
Cg3 apical dendritic branching	↑	↑
Cg3 apical dendritic length	**N**	**N**
Cg3 apical spine density	**N**	↓
Cg3 basilar dendritic branching	**N**	**N**
Cg3 basilar dendritic length	**N**	**N**
Cg3 basilar spine density	**N**	↓
PAR 1 apical dendritic branching	**N**	**N**
PAR 1 apical dendritic length	**N**	**N**
PAR 1 apical spine density	**N**	↓
PAR 1 basilar dendritic branching	**N**	**N**
PAR 1 basilar dendritic length	↑	**N**
PAR 1 basilar spine density	↓	↓
CA1 basilar dendritic branching	**N**	**N**
CA1 basilar dendritic length	**N**	↓
CA1 basilar spine density	**N**	**N**
NAc dendritic branching	**N**	↑
NAc dendritic length	**N**	↑
NAc spine density	**N**	**N**
% Significant	**29%**	**48%**

↑ Significant increase ($P < 0.050$); ↓ Significant decrease ($P < 0.050$); **(↓)** Near significant decrease ($P = 0.060$). **N**, Nonsignificant main effect (all comparisons were made between control offspring and paternal stress offspring).
% Significance = total number of increases and/or decreases per sex, divided by total number of measures. Cell complexity, dendritic length and spine density analyses were performed on Golgi-Cox stained brains.

3.5. DISCUSSION

This study examined the effects of prenatal paternal stress (PPS) on dendritic structure and spine density in five brain regions in the offspring. We report two major findings here: 1) PPS alters dendritic organization in the offspring's brains and these effects differ from the effects of stress at other ages; and, 2) the dendritic changes were sexually-dimorphic. We consider each in turn. Before proceeding with the discussion we would like to emphasize that changes in dendritic and spine measures are really proxies for changes in neural networks that bind the various regions together – and especially the prefrontal, nucleus accumbens and hippocampal regions. Because these networks function to produce behavior we can therefore anticipate that network reorganization will be related to changes in behavior, but at present little is known about what behaviors might be affected and what changes might occur.

3.5.1. The Effects of Stress Vary With Age

Stress in adult rats alters the morphology of dendritic arbor, spine, and synapse number in many brain regions, including both the medial and orbital prefrontal cortex and the hippocampus. The prefrontal regions show opposite effects with *decreased* spine density in mPFC compared to *increased* density in the OFC (see review by McEwen & Morrison, 2013). Although the loss in spines in mPFC is dramatic (about 30%), the effect is reversed with time in young adult, but not mature, animals (Goldwater et al., 2009). It is not known what happens over this time frame in OFC. The CA3 pyramidal neurons of the hippocampus of adult male rats show dendritic atrophy following prolonged stress but females do not (e.g., Galea et al., 1997; Margarinos & McEwen,

1995; Watanabe, Gould, & McEwen, 1992). Surprisingly, whereas there is dendritic atrophy, there is an increase in spine density on CA3 neurons (Sunanda, Rao, & Raju, 1995) (but see below for complications).

In contrast to the effects of adult stress on prefrontal neurons, Mychasiuk, Gibb, & Kolb (2011c; 2012a) found that when the brains were examined at P21, gestational stress *increased* spine density in both regions as well as the hippocampus, although it *decreased* dendritic length in OFC, *increased* it in CA1, and induced no changes in mPFC. When Muhammad & Kolb (2011a) looked at the brains of adult rats who had experienced gestational stress, they found a different pattern of changes: there was *decreased* spine density in mPFC and OFC, a result also seen in adult degus stressed gestationally (Murmu et al., 2006). Neither Muhammad & Kolb nor Murmu et al. examined CA1 but Muhammad & Kolb found *increased* spine density in NAc. Finally, Muhammad & Kolb (2011c) reported that adult rats who experienced infant maternal separation showed *increased* spine density in both mPFC and OFC.

The current study examined the effects of preconception paternal stress in the brains of weanling rats and found a general *decrease* in spine density across all regions measured (mPFC, OFC, CA1, NAc, Par1). We are unaware of any other studies that examine the effect of PPS on the neuronal morphology of P21 offspring, although Helmeke et al. (2009), showed that *postnatal* paternal separation *reduced* spine density in prefrontal cortex of degus at both P21 and in adulthood. There is, however, one study looking at the effect of preconception stress in females. Bock et al. (2014a) gave virgin female rats unpredictable stress for a week and then paired the females with males two weeks later. Analysis of the offspring's brains in adulthood showed that dendritic

complexity and spine density were increased in male but not female Cg3 area and the effects were significantly larger in the left hemisphere. Analysis of prelimbic/infralimbic cortex showed a similar increase in dendritic complexity but the effects were not sex nor hemisphere specific. There were no effects of the experience on OFC. In a parallel study they gave females the same stress but paired the females with males with no poststress delay. There were no effects of the preconception stress on the PFC neurons in this paradigm.

There are two important points to raise here. First, the transgenerational effect of preconception stress on females is very different from the effect of PPS reported here. Second, the Bock et al. results show a temporal effect with no stress effects when mating occurred at one time point but not at another. In the current study the paternal stress lasted longer than in the Bock et al. study (27 days versus 7 days), the males were paired with the females immediately after the stress ended, and the animals were older (65 days versus over 90 days). Thus, it is not clear if the differences in the results were related to sex of the stressed animals, the duration of the stress, or the age at perfusion.

Taken together, all the studies discussed here demonstrate that stress alters the morphology of cerebral neurons but the timing of the stress (preconception to adult) and the time at which the brain is examined result in differing plastic changes in neuronal circuits and that these alterations often evolve over protracted intervals. The effects of stress are correlated with changes in cognitive function as well as emotional regulation and other self-regulatory behaviors, the details varying again with age at stress and age at study (e.g., Bock et al. 2014b; Ferdman et al., 2007; McEwen and Gianaros, 2011; McEwen & Morrison, 2013; Muhammad & Kolb, 2011a; 2011c).

A fundamental question that arises is why does stress have different effects at different ages. One reason is that the stress is acting on very different brain substrates at different ages, likely producing different epigenetic changes. For example, gestational stress is acting on a brain undergoing rapid neurogenesis whereas neonatal stress is acting on neurons that are migrating, differentiating, and undergoing synaptogenesis. Stress in adolescence would be acting on neurons that are pruning and forming mature neuronal networks. Stress in adulthood would be acting on neuronal networks that are relatively stable. Furthermore, we can anticipate that the differing epigenetic effects at different ages will influence how the brain responds to stressors and other experiences later in life. The idea that there will be differing epigenetic effects is conjecture at this point but we do know that adult stress produces striking epigenetic changes.

Kolb et al. (2014) showed using both global methylation and a gene chip array analysis that although both the mPFC, OFC, and HPC showed areal-specific changes following two weeks of adult stress (and two weeks recovery) similar to what was used in the current study, the epigenetic effects were strikingly areal specific. In particular, both PFC regions showed an *increase* in global methylation whereas the HPC showed a *decrease*. Furthermore, the gene chip array showed that although about 20 genes changed (mostly increased gene expression) in each region, there was almost no overlap among genes showing significant expression changes in the three regions. Mychasiuk et al. (2013a) looked at the effects of PPS on global methylation in PFC (both regions combined) and HPC and also found decreased methylation in the PFC but increased methylation in the HPC – just the opposite of what was seen in adults.

It is difficult to conclude whether the synaptic changes seen in the current study

and in other stress studies are necessarily adverse rather than adaptive in some way. In fact, they may be both. We have found in a study of adult littermates of the current animals that PPS led to impairments in skilled motor behavior but enhanced the acquisition of a spatial navigation task (Harker et al., 2015).

There is a general consensus that perinatal adversity is a significant risk factor for later behavioral and psychiatric disorders (Bock et al., 2014b). It is less clear, however, whether preconception trauma has a similar effect. Bock et al. (2014a) proposed that proliferative changes (increased dendritic complexity and/or spine density) may reflect adaptive effects promoting resilience to later stress whereas retarded growth (or enhanced pruning) of dendrites and spines may reflect neuropathologic adaptation. It would be instructive to investigate the effects of adult stress in the offspring of both paternal and maternal preconception stressed animals.

3.5.2 The Effects of Stress are Sexually Dimorphic

Although it has not been studied systematically across PFC regions and HPC, there are consistent sex differences in the effects of stress throughout the lifetime. Garrett and Wellman (2009) showed that whereas males show a general shortening of dendrites in mPFC, females do not. The effect was estradiol dependent. Shansky et al. (2009; 2010) showed, however, that the details of the sex difference are circuit specific. They showed that in male rats mPFC neurons projecting to the basolateral amygdala (BLA) did not show the dendritic retraction whereas those projecting elsewhere did. In contrast, females showed an expansion of dendrites of the BLA-projecting neurons as long as the animals had circulating estrogen. Ovariectomized animals showed no change. mPFC

neurons projecting elsewhere failed to show any dendritic changes after chronic stress whether or not females had circulating estrogen. In contrast to the effects on dendritic length, spine density was increased in both regions by stress in estrogen-intact animals (Shansky et al., 2010).

As noted above, the effects of stress is sexually dimorphic in HPC as males but not females show dendritic atrophy of CA3 pyramidal dendrites (Galea et al., 1997). Similarly, Shors et al., (2001) showed that whereas males showed an *increase* in spine density in CA1, females showed a more complex effect: there was an *increase* in spines if the females were perfused in diestrus but a *decrease* if perfused in proestrous. One difference between the Shors et al. study and most other studies is that the animals were perfused 24h after the end of the stress. We are unaware of studies examining chronic sex differences in the effects of stress on hippocampal spine density. The epigenetic effects of adult stress are also sexually dimorphic. As noted earlier, mPFC, OFC, and HPC show changes in gene expression that are areal-specific, but in addition, there is virtually no overlap in the specific genes in the two sexes (Kolb et al., 2014).

Less is known about the sex differences in response to early life or preconception stress but one general result is that although generally similar effects are observed in both sexes, there are larger and/or more changes in females. In the current study, for example, 48% of the measures changed in females compared to 29% in males (see Table 1). Parallel findings were seen in gestational stress too, with 72% change in females versus 56% in males (Mychasiuk et al., 2012a) when examined at weaning, although the sex difference had disappeared in adulthood (Muhammad & Kolb, 2011a).

3.5.3. Possible Mechanisms

It is known that the maternal environment during the prenatal period has a direct influence on the neurodevelopment of offspring, but less is understood regarding possible mechanisms involved in paternally mediated effects on offspring brain and behavior. Epigenetic changes in sperm have been proposed as a possible mechanism through which paternal experience can impact offspring neurodevelopment. Carone et al. (2010) found that paternal environmental conditions, in this case paternal diet, acted upon epigenetic information in sperm, thereby influencing lipid and proliferation-related gene expression in offspring. Epigenetic reprogramming of the paternal germline was observed by Vassoler et al. (2013) in males voluntarily ingesting cocaine, producing significant effects in male offspring's resistance to cocaine reinforcement, and mPFC gene expression.

New research is focusing on the examination of spermatogenesis and the epigenetic mechanisms that are involved in this dynamic process. Godmann et al. (2009) suggest that distinct epigenetic mechanisms and protein players found in the testes make them an epigenetically advantaged site. Mychasiuk et al. (2013a) showed that PPS alters DNA methylation in PFC and hippocampus in the offspring, which would be consistent with an epigenetic mechanism. Unfortunately, they did not measure gene expression directly so that remains to be done.

If the effect of PPS is via an epigenetic mechanism, there are several issues to consider (see also Bock et al., 2014b). For example, how is the epigenetic change in sperm reflected in brain development and how do changes in gene expression influence the development and maturation of neural networks? Similarly, why are the neuronal

changes regionally different? Finally, why are the effects of maternal and paternal preconception stress so different?

3.5.4. Conclusion

It is generally believed that stressed-induced insults on fetal neurodevelopment can initiate or influence human mental illness. It is likely that the alterations to dendritic morphology and spine density, induced by PPS, result in important functional changes that generate consequences for behavior. Perturbations to the structural plasticity of relevant brain areas induced by preconception stress exposure have been shown to lead to a dysregulation in hypothalamic-pituitary-adrenal (HPA) axis reactivity, long associated with neuropsychiatric disease, affective disorders and autism. According to the Mental Health Commission of Canada, one in five persons (more than 6.7 million people in Canada) are living with mental health issues and similar results are reported in other countries as well. Mental health problems are currently experienced by 21.4% of the working population, potentially affecting productivity, work performance and home life (Statistics Canada, 2014). While it is clear that fathers play an important role in offspring neurodevelopment, it is necessary to delineate underlying mechanisms involved in the paternal transmission of stress to offspring. Further research examining the impact of environmental influences on the processes involved in epigenetic patterning of the germline is necessary if we are to fully understand the paternal contribution to brain and behavioral development in the offspring.

Chapter 4

Preconception Paternal Stress in Rats with Neonatal Prefrontal Cortex Lesion[3]

4.1 ABSTRACT

Whereas environmental challenges during gestation have been repeatedly shown to alter offspring brain architecture and behavior, research examining the impact of paternal preconception experience on offspring outcome is limited. The goal of this study was to examine the effects of preconception paternal stress (PPS) on the offspring, half of whom also received a lesion of the medial prefrontal cortex (mPFC) on postnatal day 7. We hypothesized that PPS would impact cerebral plasticity in the offspring. Based on previous work in our lab, we expected to observe reduced behavioral and anatomical recovery in the brain injured animals, as well altered behavior and cortical morphology in the sham animals. Several behavioral assays were performed on offspring between days P33 and P101. Following behavioral testing, the brains were harvested and dendritic morphology was examined on cortical pyramidal cells in orbital frontal cortex (OFC), parietal cortex (PAR1), and the CA1 area of the hippocampus of all animals, as well as the mPFC of sham animals. As anticipated, behavior was altered on both the activity box assay and elevated plus maze and performance decreased in the Whishaw reaching task. Unexpectedly, PPS appeared to somewhat improve paternal stress female sham behavior on the Morris water task. Neuroanatomical measures revealed a decrease in spine density in both mPFC and OFC, and PAR1, but not in CA1. Thus, PPS impacted both behavior and neuronal morphology of offspring. These effects may have an epigenetic basis given

[3] Harker, A., Carroll, C., Raza, S., Kolb, B., & Gibb, R. (2015). Preconception Paternal Stress in Rats with Neonatal Prefrontal Cortex Lesion *Submitted to Journal of Neuroscience (August, 2015).*

that in a parallel study of littermates of the current animals we found extensive epigenetic changes at P21.

4.2. INTRODUCTION

There is growing evidence that both maternal and paternal preconception experience can influence the development of brain and behavior in the offspring (Bale, 2014; Rodgers & Bale, 2015). For example, Bock et al. (2015) stressed female rats daily for one week before pairing them with males two weeks later. Analysis of the offspring's brains in adulthood found that dendritic complexity and spine density in medial prefrontal cortex (mPFC) were increased in males but not in females. We recently showed that twice-daily stressing of males for 27 consecutive days, followed by pairing with females, decreased spine density in mPFC and orbital frontal cortex (OFC) in both sexes of offspring when the animals' brains were harvested on postnatal day 21 (P21) (Harker et al., 2015). In an earlier paper based on littermates of animals reported in the current study, Mychasiuk et al. (2013) found the same paternal stress experience altered DNA methylation patterns in offspring at postnatal P21: global methylation was reduced in the frontal cortex of female offspring but increased in the hippocampus of both female and male offspring. These findings point to an epigenetic mechanism for preconception experiences (see Bale, 2015).

One difficulty with studying the effects of preconception experiences on behavior of the rat offspring is that most behavioral tests that might be used have been devised to examine the effect of brain injury in rats, and thus are not very sensitive to more subtle perturbations. One way to solve this problem is to examine how the offspring's brains

respond to an additional perturbation, such as a neonatal cerebral injury. We have shown that the behavioral and morphological outcomes of perinatal cortical injury in rats is exquisitely age dependent: damage to the mPFC in the first few days of life leads to a miserable behavioral outcome and general atrophy of dendritic fields of pyramidal cells across the cortex; similar damage at 10 days of age allows virtual total functional recovery associated with hypertrophy of dendritic fields; and, injury around day seven falls in between, the extent of recovery varying with the extent of cortical injury (e.g., Kolb & Gibb, rev). We therefore decided to repeat the preconception paternal stress studies and to add a P7 mPFC lesion (or sham control). Given the intermediate level of recovery with a lesion at this age, we predicted that we would be able to either interfere with recovery, or less likely, improve it. Behaviors were studied at several ages beginning at P33 and ending at P101. Based upon our earlier studies, we anticipated that the adult P7 lesion rats would not be impaired (or for females marginally impaired) at the Morris water task but significantly impaired at a skilled reaching task (Kolb, Stewart & Sutherland, 1997). Following the behavioral testing, the brains were harvested and stained using a modified Golgi-Cox technique (Gibb & Kolb, 1998) and the dendritic organization and spine density of cortical pyramidal cells in OFC, parietal cortex (PAR1), and CA1 of all animals and the mPFC of sham animals were examined.

4.3. MATERIALS AND METHOD

4.3.1. Subjects

Ten female Long-Evans rats were mated with 10 male Long-Evans rats (6 paternal preconception stress (PPS); 4 control). All pairs successfully mated resulting in

132 pups (78 paternal stress, 54 control). Animals were given access to food and water *ad libitum* and were maintained on a 12 h light/dark schedule (lights on from 07:30 to 19:30 h) in a temperature controlled (21º) breeding room. 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. Data from a subset of the offspring of these pairings were already reported by Harker et al. (2015) in which a Golgi analysis was done on offspring killed on P21. Using a split-litter design, 1 or 2 animals assigned to each group were chosen from different litters. When behavioral testing began there were 10 sham (5M, 5F), 11 PPS (5M, 6F), 12 Lesion (7M, 5F), and 12 Lesion + PPS (8M, 4F). Data for sham littermates of these animals were previously reported for both Golgi (Harker et al., 2015) and epigenetic analyses (Mychasiuk et al., 2013).

4.3.2. Paternal Stress

PPS was administered using the same procedures as in our previous studies (e.g., Harker et al., 2015). Paternal stress was administered a total of 27 consecutive days prior to the mating session. Stressing consisted of placing the male rat (n=6) on an elevated Plexiglas® platform (1 m tall, 21 x 21 cm) in a brightly lit room for 30 min. (Wong et al., 2007). Following the stressing procedure, rats were transported back to their home cages. Stressing sessions occurred at 9:00 A.M. and 3:00 P.M. During stressing sessions, control males (n=4) were removed from their home cages (placed in new cage in same room as home cage) for the 30 minutes, but did not participate in the stressing paradigm. Following the 27 days of stressing, paternal stress and control males were immediately mated with females. This was the only exposure that female dams had with the stressed

male rats. Subsequent to mating, female dams were housed in shoebox cages with another female in the same experimental condition (ex. control–control vs. paternal stress–paternal stress). Female dams were separated and housed individually prior to the birth of their pups, following a weight gain ≥ 100g or 2 days prior to their expected delivery date. Female dams remained individually housed following the birth of their litter. The offspring were weaned at P21and housed in same-sex groups of 5 or 6 throughout behavioral testing.

Although we did not measure corticosterone levels in the current study, previous analysis by Wong et al. (2007) using the same procedure found significant increases in corticosterone after the stressing. As in the Wong et al. study, we also observed consistent urination and defecation by the stressed animals while on the platform. In addition, we observed attenuated weight gain in all stressed males relative to the controls [F (1,9) = 104.4, $p > 0.001$], as well as excessive barbering of hair on forearms, suggesting a severe anxiety response.

4.3.3. Surgery

On P7, offspring rats were anaesthetized by cooling them in a Thermatron cooling chamber until their rectal body temperatures were in the range of 18-20^{o}C. For the mPFC lesion rats, the frontal bone was removed by cutting it with iris scissors, and mPFC decortication was achieved by gentle aspiration. The intent was to remove the medial subfields of the prefrontal cortex including the Zilles' (1985) presumptive regions Cg3 and PL. Access to these regions in such a small brain also made it likely that part of Cg1 and Fr1 was also injured. The animals were sutured with 5-0 Vicryl as soon the lesion

was achieved. The normal control group animals were anaesthetized in the same manner, the skin was incised and sutured. All the animals were warmed in cupped hands until the rats were moving. They were then placed in a shoebox cage sitting on a heating pad with the temperature controlled at 21˚C.

4.3.4. Behavior

The rats were given 4 behavioral tests: 1) open field activity at P33 and P61; 2) elevated plus maze (EPM) at P35 & P63; 3) Whishaw tray reaching task at P78; 4) Morris water task at P101.

4.3.4.1. Open field activity, P33/61. The exploratory activity was evaluated using Accuscan activity monitoring Plexiglas® boxes (L 42 cm, W 42 cm, H 30 cm). The activity was recorded as the number of sensors beam breaks in a 10 min period. The number of horizontal beam breaks were recorded on a computer with a VersaMax program and converted to a spreadsheet using VersaDat software. Recorded on postnatal days 33 and 61.

4.3.4.2. Elevated plus maze, P35/P63. The EPM was an elevated '+' shape maze with two closed and two open arms. The rats were placed in the center of the EPM facing a closed arm and were allowed to explore for 5 min. The behavior was videotaped and the time spent in closed arms and number of entries in each open and closed arm were scored and analyzed to assess anxiety-like and exploratory behaviors, respectively. Recorded on postnatal days 35 and 63.

4.3.4.3. Whishaw tray reaching task, P78. Prior to training the rats were food restricted and maintained at 90% of their normal weight, adjusting for continued growth. The testing apparatus was a cage that measured 30.5 cm high by 20.5 cm wide by 28 cm long. The sides and the back of the cage were made of clear Plexiglas. Thin metal bars formed the front of the cage, and the bottom was made of wire mesh. A food tray ran the length of the cage front with about .5 cm between the tray and the cage. In order to obtain food the rats had to reach through the bars and grasp chicken feed pellets, bringing them back through the bars for consumption. Individual rats were habituated to the cages for one hour per day. Once habituated, the rats were tested and allowed to retrieve and consume pellets for 30 min/day. Once each animal was consistently reaching and obtaining food (14 days habituation) they were individually videotaped on the following day for 5 min. A reaching attempt was scored each time the forepaw was extended through the bars and an attempt was made to grasp food. Reaches were scored as successful or not and the percentage of successes over attempts was calculated for analysis.

4.3.4.4. Morris water task, P101. Animals were tested using the standard place task version of the Morris water task as described by Morris (1984). The circular pool measured 1.5m across and 45 cm high and was located in a room with a number of visible distal visual cues located on the walls. The pool was filled with ≤23 ◦C water and made opaque with ~250 ml of nontoxic liquid Tempera paint. A square platform (12 cm x 12 cm) was placed in the pool about 2 cm beneath the water surface. Each animal was given four trials per day (one trial block) in which they were started pseudo-randomly

from four different locations (east, west, south, and north) and tested for 6 consecutive days. The rats were placed into the water facing the wall at one of the four compass points each day and allowed a maximum of 60 s to search for the platform. The rats were removed from the pool, dried off, and placed in a holding cage for about 5 min before beginning a new trial. Following the last trial on day 6 the rats were given a probe trial in which they placed into the pool but no platform was present. Their behavior was observed for 30 sec before they were removed from the pool. The swim distance and latency to find the hidden platform, and swim speed were recorded for each trial.

4.3.4.5. Statistical analyses. For all measures, three-way ANOVAs with stress, lesion, and sex were performed.

4.3.5. Anatomy

The anatomical analysis was done as in our previous papers (e.g., Harker et al., 2015). At the completion of the reaching task the animals were administered an overdose of sodium pentobarbital solution (i.p.) and perfused with 0.9% saline solution intracardially. The brains were extracted, weighed, and placed in Golgi-Cox solution for 14 days. The brains were then transferred into 30% sucrose for at least 3 days before being sliced on a Vibratome at 200 μm. Slices were transferred to gelatin-coated slides for staining as outlined by Gibb and Kolb (1998).

Neurons selected for analysis were derived from the Cg3 (layer III) region of the anterior cingulate cortex of the medial PFC, the dorsal agranular insular cortex (AID, layer III) of the orbital frontal cortex (OFC), and the PAR I region (layer III) of the

parietal cortex, as described by Zilles (1985), and CA1 of the hippocampus. Individual neurons from the Golgi-Cox stained brains were traced at 250X using a camera Lucida (12.5 magnification) mounted on a microscope. A total of 10 cells (5 per hemisphere) were traced from each brain region for each individual brain. The mean of the cells from each hemisphere comprised the data points for statistical analysis. Neuronal investigation included: Sholl analysis, a measure of total dendritic length, derived from the number of dendritic branches that intersect concentric circles spaced 25μm from the center of the cell body; dendritic branch order, an estimation of dendritic complexity, which is based upon the number of branch bifurcations; and spine density, which is calculated as the number of spine protrusions on a distal dendrite traced at 1000X. The exact length of the segment is calculated and spine density is expressed as the number of spines per 10μm. A research technician blinded to all experimental conditions drew the cells for this analysis.

Statistical analyses were performed using SPSS 21 for Mac. The anatomical data for each of the five brain areas were analyzed using three-way ANOVA's with treatment (preconception paternal stress (PPS) and control), lesion and hemisphere as factors. The data points were the mean of 5 cells per area per hemisphere. However, hemisphere failed to show significant main effects or interactions with other factors and was therefore eliminated as a factor.

4.3.5.1. Statistical analyses. For all measures except those in Cg3, three-way ANOVAs with stress, lesion, and sex were performed on dendritic branches, dendritic length, and spine density for each region drawn. For Cg3 only sham animals were drawn (the P7 lesions removed this region) so only two-way ANOVAs with stress and sex as

factors were performed. For all measures there was an initial additional comparison of hemisphere but as they were not significant the data were collapsed across hemispheres.

4.4. RESULTS

4.4.1. Behavior

Rats with P7 mPFC lesions performed similarly on the Water Task and Whishaw reaching as in our earlier studies (e.g., Kolb et al., 1997). Stress produced impairments on the skilled reaching task and a lesion effect was observed in PPS females. Interestingly, stress did not interact with the lesion, but rather affected lesion and control animals similarly. We had not previously tested rats with P7 mPFC lesions on measures of either activity or the EPM. There was a transient lesion effect at P35 but not P63 in the current study. There were no lesion effects on elevated plus maze. The activity results contrast with the effects of adult mPFC lesions which show increased activity, the effect being larger in females (Kolb, 1974).

4.4.1.1. Open field activity, P33. There were significant effects of stress (males less active, females unaffected), lesion (increased activity), and sex (females more active) (see Figure 4.1A.). A three way ANOVA revealed main effects of stress ($F(1,38)=12.63$, $p<.001$), lesion ($F(1,38)=5.88$, $p=.02$), and sex ($F(1,38)=17.66$, $p<.001$). There were also significant stress X lesion ($F(1,38)=4.49$, $p=.04$), and stress X sex ($F(1,38)=4.1$, $p<.05$) interactions. These interactions reflected the absence of a stress effect in the lesion animals and an absence of stress effects in females.

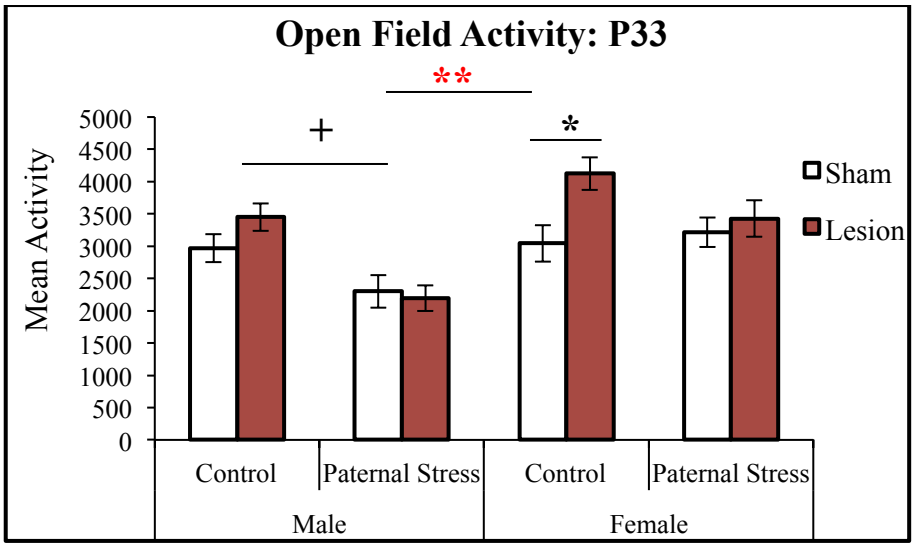

Figure 4.1A. Mean activity measure at P33. * $p < 0.05$ Sham/Lesion; + $p < 0.05$ Control/Paternal Stress; ** $p < 0.05$ Male/Female

4.4.1.2. Open field activity, P61. The lesion and stress effects had reversed by P61 but there was still a large sex effect (females more active) (see Figure 4.1B.). A three way ANOVA found a significant effect of sex ($F(1,38)=23.86$, $p<.0001$) but not stress ($F(1,38)=1.03$, $p=.32$) or lesion ($F(1,38)=0.53$, $p=.47$). No interactions were significant (p's>.05).

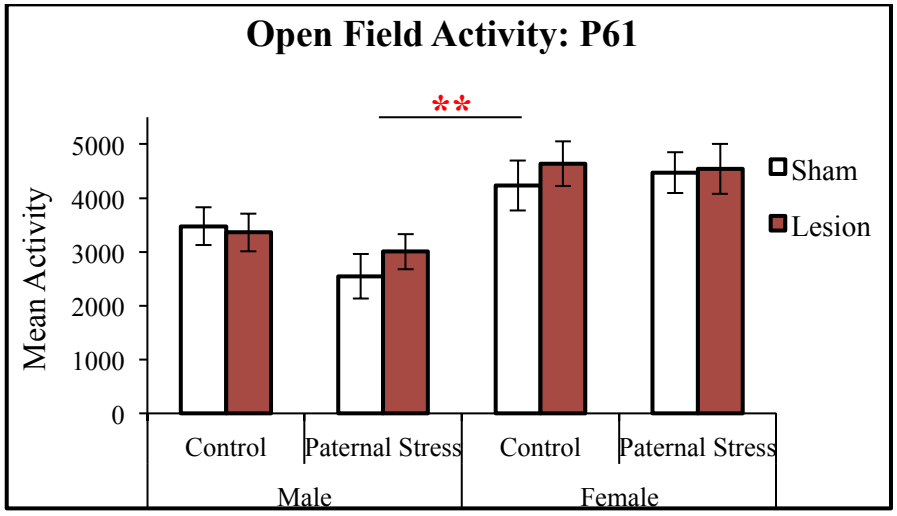

Figure 4.1B. Mean activity measure at P61. ** $p < 0.05$ Male/Female

4.4.1.3. Elevated plus maze, P35. Animals with paternal stress showed a decrease in time spent in the closed arms, the effect being larger in females, reflecting reduced anxiety in the stressed groups (see Figure 4.2A.). A three way ANOVA on time in the closed arms showed a main effect of stress, ($F(1,38)=4.83$, $p=.03$) but not sex ($F(1,38)=0.010$ $p=.92$) or lesion ($F(1,38)=2.14$, $p=.15$). There were no significant interactions (p's>.05).

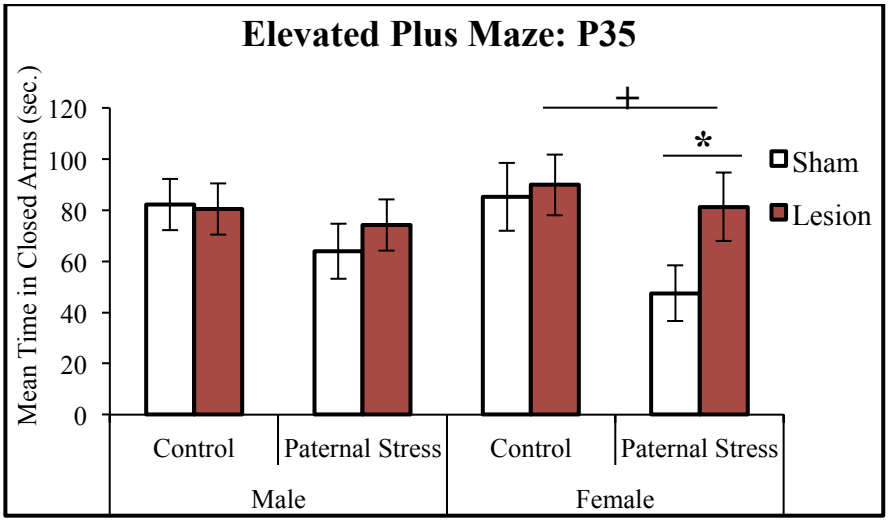

Figure 4.2A. Mean time in the closed arm of the Elevated Plus Maze at P35.
* $p < 0.05$ Sham/Lesion; + $p < 0.05$ Control/Paternal Stress

4.4.1.4. Elevated plus maze, P63. Opposite effects were seen on the second EPM test compared to the first. Stressed animals spent more time in the closed arms (see Figure 4.2B.). A three way ANOVA on time in the closed arms found a main effect of stress, ($F(1,38)=6.99$, $p=.01$) and sex ($F(1,38)=5.18$, $p<.03$) but not lesion ($F(1,38)=0.18$, $p=.68$). There were no significant interactions (p's>.05).

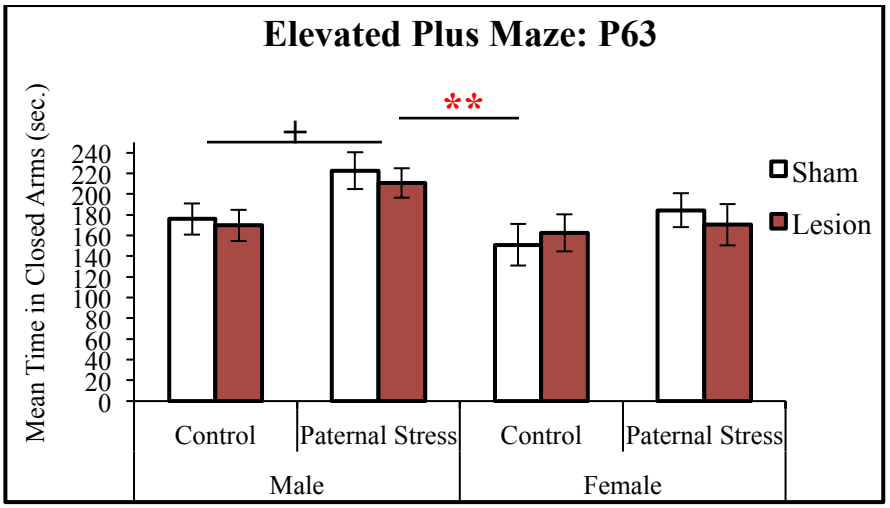

Figure 4.2B. Mean time in the closed arm of the Elevated Plus Maze at P63.
+ $p < 0.05$ Control/Paternal Stress; ** $p < 0.05$ Male/Female

4.4.1.5. Whishaw tray reaching task, P78. Sham rats learn to reach quickly and typically reach asymptote of about 60-80% accuracy. Rats with mPFC lesions are generally impaired at reaching, seldom exceeding 60% accuracy. PPS reduced accuracy in both control and lesion groups by about 10% (see Figure 4.3.). A three-way ANOVA found a main effect of stress ($F(1,38)=13.65$, $p<.001$) and lesion ($F(1,38)=13.93$, $p<.001$) but not sex ($F(1,38)=0.007$, $p=.93$). There were no significant interactions (p's>0.05).

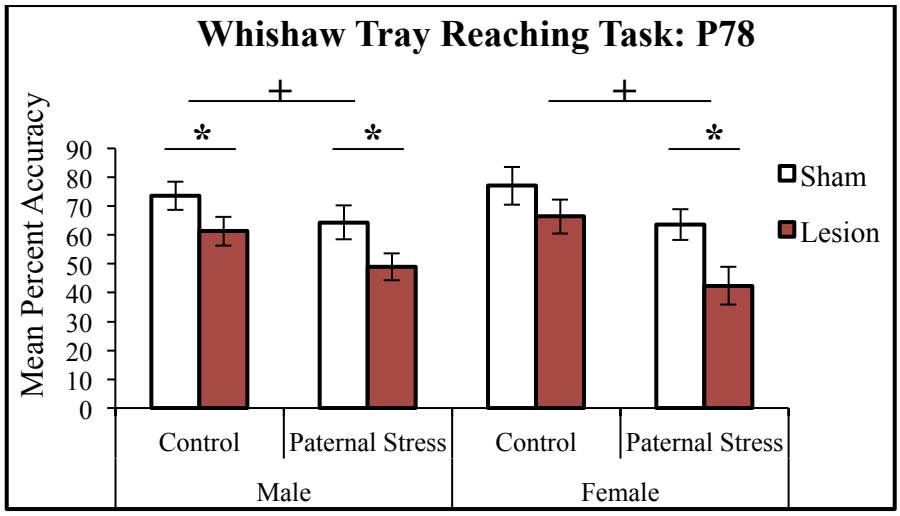

Figure 4.3. Mean percent reaching acuracy at P78.
* $p < 0.05$ Sham/Lesion; + $p < 0.05$ Control/Paternal Stress;

4.4.1.6. Morris water task, P101. As in our previous studies, both control and P7 mPFC lesion rats learned the task quickly and males had decreased latency compared to females. A lesion effect was observed in paternal stress females, with decreased latency in PPS shams (see Figure 4.4.). A three way ANOVA of latency in the Morris water task found a significant effect of sex ($F(1,38)=15.50$, $p<.001$) and lesion ($F(1,38)=5.56$, $p=.024$) but not stress ($F(1,38)=0.31$, $p<.578$). There were no significant interactions (p's>.05). For the probe trial all groups spent 20-25 sec (out of 30) in the previously correct quadrant. There were not significant effects (p's>.05).

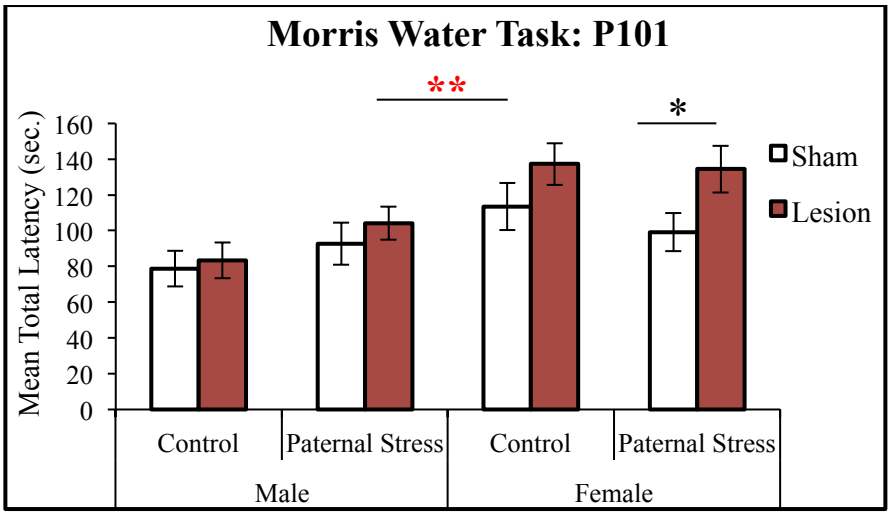

Figure 4.4. Mean total latency in Morris Water Task at P78.
* $p < 0.05$ Sham/Lesion; ** $p < 0.05$ Male/Female

4.4.2. ANATOMY

4.4.3. General morphology.

The mPFC lesions included the medial prefrontal regions (Zilles Cg3, FR2, and IL) as well as variable amounts of adjacent motor cortex (Fr2) as shown in Figure 4.5. The lesions of the PPS and non-stressed rats were similar. There was little evidence of regeneration of mPFC in the lesion rats, which is a variable finding at this age (see Kolb et al., 1998; Kolb, Petrie & Cioe, 1996). Neither stress nor lesion affected body weight but both factors affected brain weight, but in opposite ways: Lesions reduced brain weight and stress increased brain weight.

Figure 4.5. Drawings through serial sections of a representative medial frontal lesion. The midline frontal cortex is largely destroyed, and there is gliosis (marked by dots) both in cortex and striatum.
Abbreviations: Zilles' parietal

4.4.3.1. Effects of PPS on body and brain weight at P110

A 3-way ANOVA on the body weight (lesion x stress x sex) found no significant effects of lesion ($F(1,37)=2.88$, $p=.10$), or stress ($F(1,37)=0.32$, $p=.57$), there was a main effect of sex, reflecting the much heavier male body weight ($F(1,37)=543.5$, $p<0.0001$) (see Fig. 4.6).

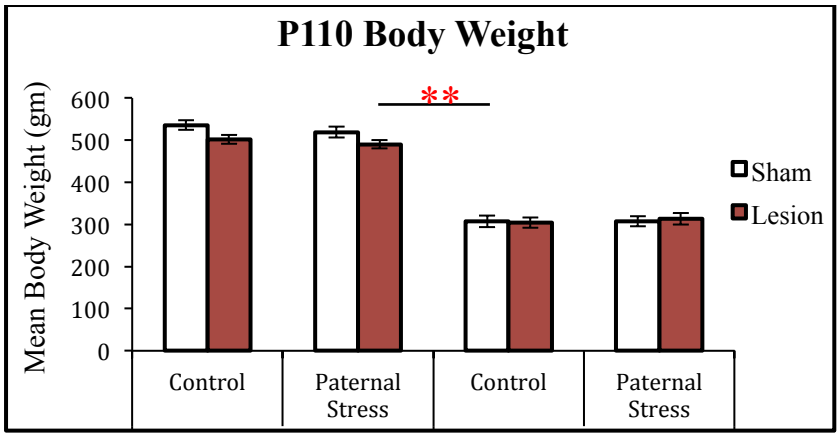

Figure 4.6A. Mean brain weight at P110. ** $p < 0.05$ Male/Female

A 3-way ANOVA on the brain weight (lesion x stress x sex) showed significant effects of stress ($F(1,37)=5.53$, $p=.02$), lesion ($F(1,37)=27.98$, p,.0001), and sex ($F(1,37)=45.0$, $p<.0001$). There were no significant interactions (p's>.05) (See Fig. 4.6B).

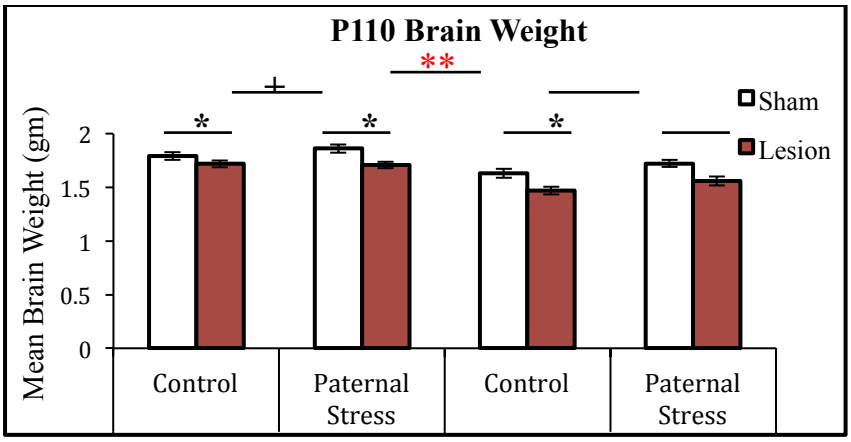

Figure 4.6B. Mean brain weight at P110. * $p < 0.05$ Sham/Lesion; + $p < 0.05$ Control/Paternal Stress; ** $p < 0.05$ Male/Female

4.4.3.2. Cg3 dendritic morphology in sham animals. Although the P7 lesion rats did not have area Cg3 to draw, we analyzed this region in the sham rats to determine if there was an effect of stress. There was an effect of stress, although it differed in different measures (see Figs. 4.7A,B,C).

Two-way ANOVA on the apical branches showed a main effect of stress (1,41)=6.92, p=.013) and sex (F(1,41)=4.76, p=.036) but no interaction (F(1,41)=0.31, p=.58). Stress decreased dendritic branches and males had more branches than females. We have seen this sex difference before (Kolb & Stewart, 1991).

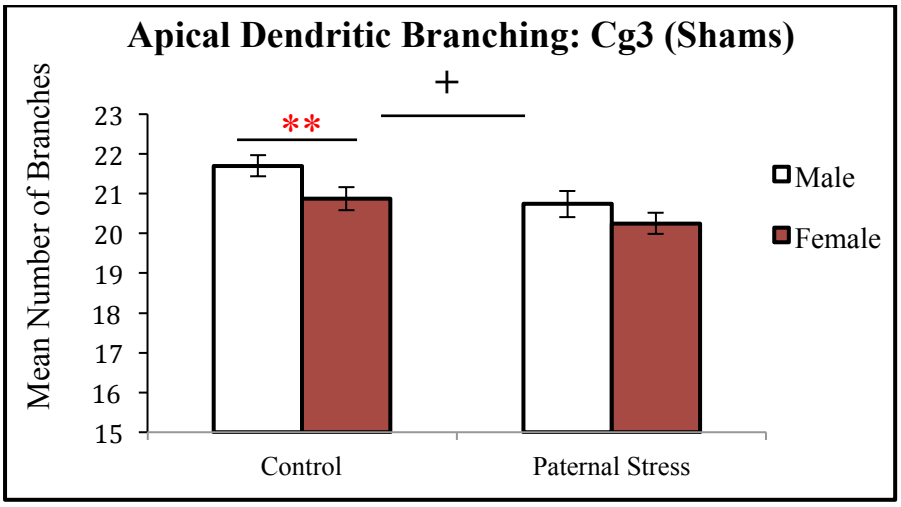

Figure 4.7A. Mean number of Cg3 apical dendritic branches at P110. + p < 0.05 Control/Paternal Stress; ** p < 0.05 Male/Female.

Two-way ANOVA on the apical length showed a main effect of stress (F(1,41)=11.70, p=.002), but not sex (F(1,41)=1.01, p=.33) nor an interaction (F(1,41)=3.33, p=.07). Stress increased dendritic length.

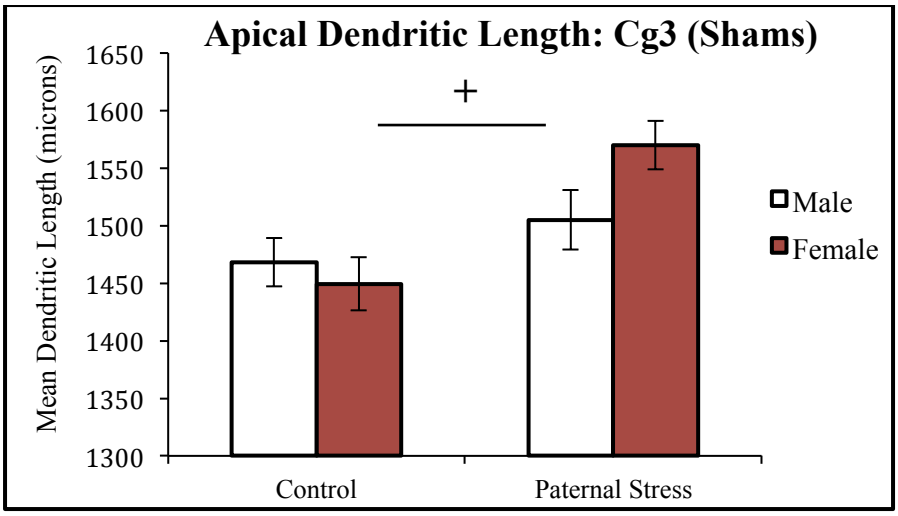

Figure 4.7B. Mean Cg3 apical dendritic length at P110.
+ $p < 0.05$ Control/Paternal Stress

Two-way ANOVA on apical spine density showed no effect of stress ($F(1,41)=2.54$, $p=.120$) sex ($F(1,41)=0.92$, $p=.34$) nor an interaction ($F(1,41)=0.79$, $p=.38$).

Two-way ANOVA on the basilar branches showed no effect of stress ($F(1,41)=0.06$, $p=.82$), sex ($F(1,41)=0.003$, $p=.96$), nor an interaction ($F(1,41)=0.34$, $p=.57$). The means were virtually identical across the groups.

In contrast, ANOVA on the basilar length showed an effect of stress ($F(1,41)=8.64$, $p=.006$) but not of sex ($F(1,41)=.30$, $p=.59$) nor an interaction ($F(1,41)=1.10$, $p=.30$). Stress increased dendritic length.

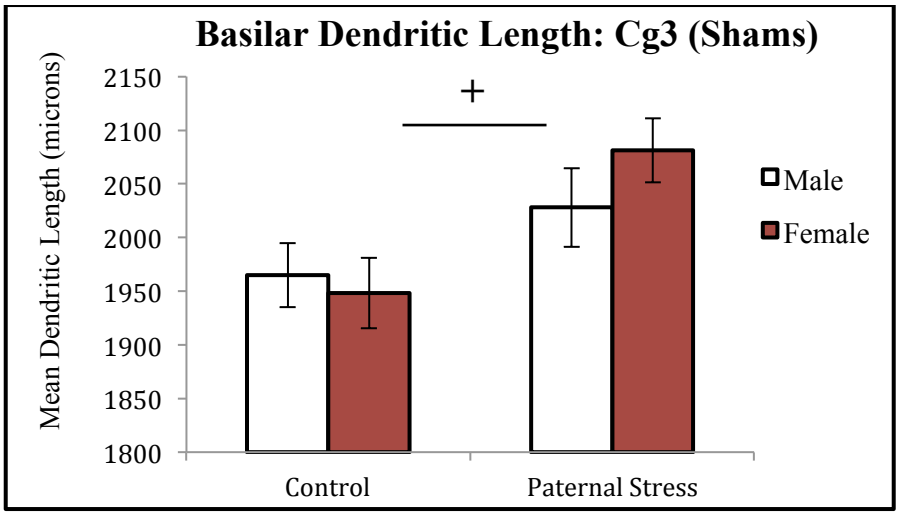

Figure 4.7C. Mean Cg3 basilar dendritic length at P110.
+ $p < 0.05$ Control/Paternal Stress

Two-way ANOVA on basilar spine density found no main effect of stress ($F(1,41)=.24$, $p=.63$) sex ($F(1,41)=0.24$, $p=.63$) nor an interaction ($F(1,41)=0.11$, $p=.74$).

4.4.3.3. AID dendritic morphology. Only the basilar fields were drawn owing to the significant truncating of the apical fields because of the angle of sectioning. The significant effects were an increase in dendritic length and a decrease in spine density in the stress groups (see Figs. 4.8A,B,C).

Three-way ANOVA on the basilar branches found no main effect of stress ($F(1,82)=1.82$, $p=.18$), lesion ($F(1,82)=0.26$, $p=.61$) or sex ($F(1,82)=0.02$, $p=89$. The three-way interaction was significant ($F(1,82)=5.68$,$p=.02$). This complex interaction resulted from small sex differences that varied across the groups. The one interesting sex difference was in the sham controls where the females had more branches than the males. As in the sex difference in Cg3, we have reported this before (Kolb & Stewart, 1991).

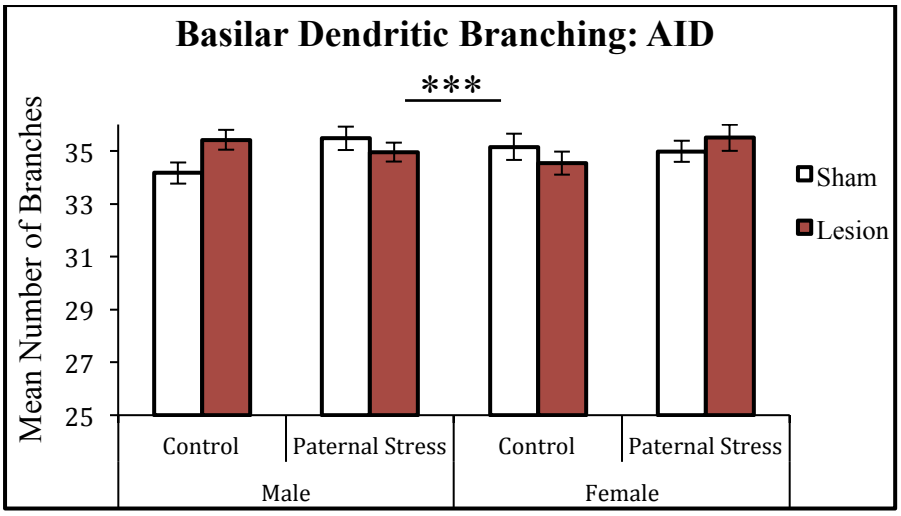

Figure 4.8A. Mean number of AID basilar dendritic branches at P110.
*** $p < 0.05$ Three way interaction (stress-lesion-sex)

Three-way ANOVA on the basilar length revealed a main effect of stress ($F(1,82)=31.62$, $p<.0001$) but not of lesion ($F(1,82)=0.13$, $p=.72$), nor sex ($F(1,82)=1.2$, $p=.28$). There were no significant interactions (p's>.05). The effect of stress was to increase dendritic length as it did in Cg3.

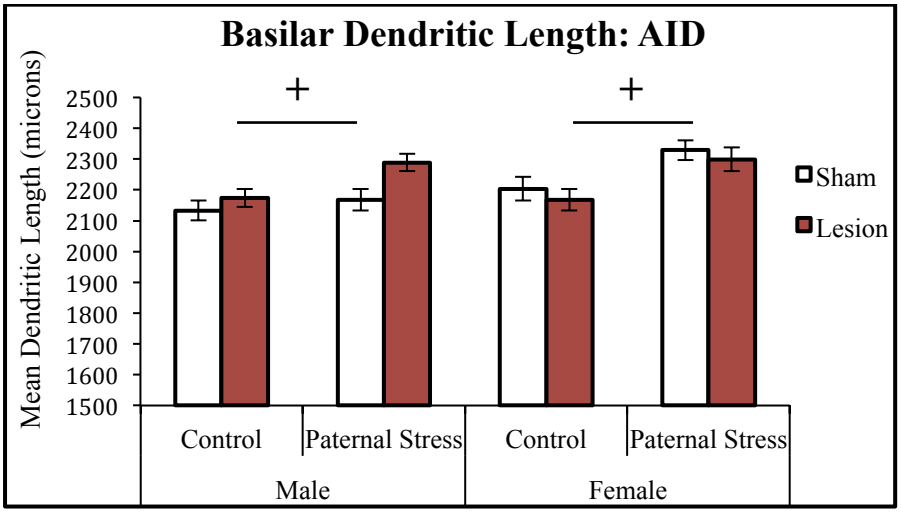

Figure 4.8B. Mean AID basilar dendritic length at P110.
+ $p < 0.05$ Control/Paternal Stress

Three-way ANOVA on the basilar spine density revealed a main effect of stress ($F(1,82)=10.35$, $p=.002$) and sex ($F(1,82)=4.74$, $p=.03$), but not of lesion ($F(1,82)=0.02$, $p=.89$). There was a significant interaction of stress and sex ($F(1,82)=4.09$), $p<.05$). The interaction shows that the spine density was higher in non-stressed males (sham and lesion) than non-stressed females but was virtually identical in the stressed groups.

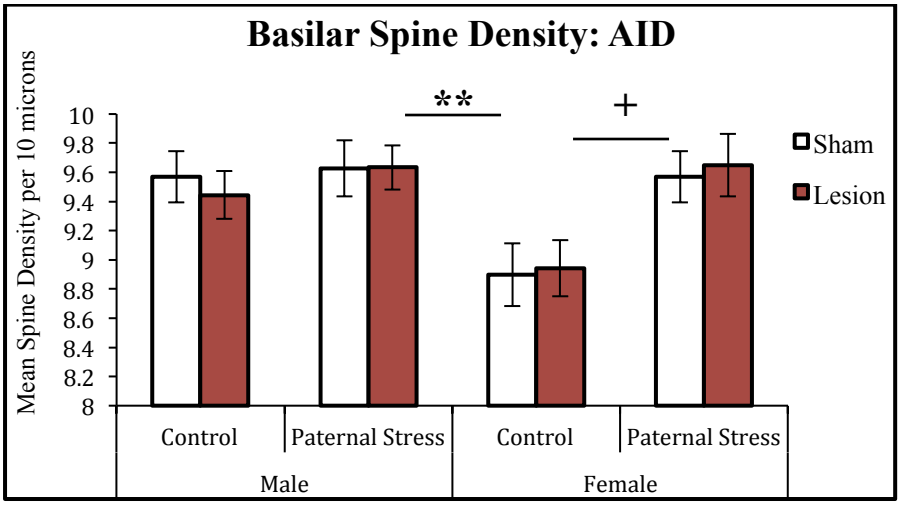

Figure 4.8C. Mean AID spine density at P110.
+ $p < 0.05$ Control/Paternal Stress
** $p < 0.05$ Two-way interaction (stress-sex)

4.4.3.4. PAR1 dendritic morphology. Stress reduced both dendritic branching and length in the basilar field but had no significant effect on the apical field. There was no effect of stress or lesion (see Figs. 4.9A,B).

Three-way ANOVA on the apical branching found no effects of stress ($F(1,82)=1.23$, $p=.27$), lesion ($F(1,82)=0.003$, $p=.96$), sex ($F(1,82)=0.09$, $p=.77$). There also were no significant interactions (p's>.05).

Similarly, Three-way ANOVA on the apical length showed no effects of stress ($F(1,82)=0.41$, $p=.53$), lesion ($F(1,82)=0.02$, $p=.96$), sex ($F(1,82)=0.06$, $p=.81$). There also were no significant interactions (p's$>.05$).

Three-way ANOVA on the apical spine density found no effects of stress ($F(1,82)=0.64$, $p=.43$), lesion ($F(1,82)=0.98$, $p=.33$), sex ($F(1,82)=0.40$, $p=.53$). There also were no significant interactions (p's$>.05$).

Three-way ANOVA on the basilar branching found a large effect of stress ($F(1,82)=9.5$, $p=.003$), but no effect of lesion ($F(1,82)=0.72$, $p=.40$), or sex ($F(1,82)=0.001$, $p=.98$). There was one significant interaction, the stress x lesion x sex ($F(1,82)=3.94$, $p=.05$). This interaction is clearly complex, reflecting a decrease in branching in the stressed groups with the effect being larger in female shams and larger in male lesion animals.

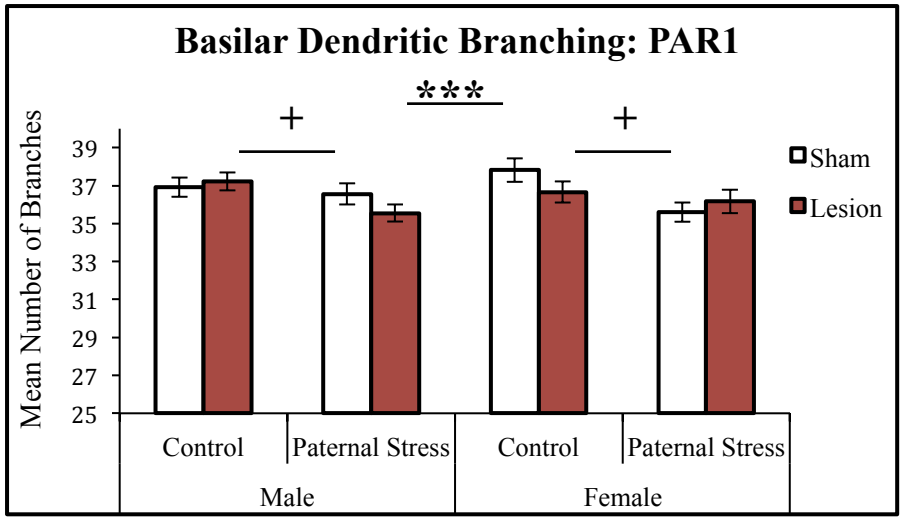

Figure 4.9A. Mean number of PAR1 basilar dendritic branches at P110.
+ $p < 0.05$ Control/Paternal Stress
*** $p < 0.05$ Three way interaction (stress-lesion-sex)

Three-way ANOVA on the basilar length found an effect of stress ($F(1,82)=4.09$, $p<.05$), but no effect of lesion ($F(1,82)=2.5$, $p=.11$), or sex ($F(1,82)=1.22$, $p=.27$). There also were no significant interactions (p's>.05).

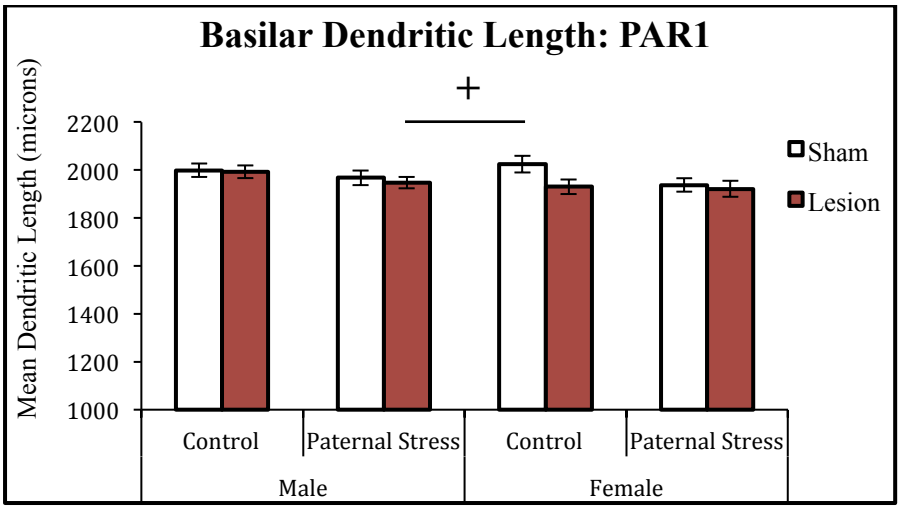

Figure 4.9B. Mean AID basilar dendritic length at P110.
+ p < 0.05 Control/Paternal Stress

Three-way ANOVA on the basilar spine density found no effects of stress ($F(1,82)=3.59$, $p=.06$), lesion ($F(1,82)=0.06$, $p=.80$), or sex ($F(1,82)=3.1$, $p=.08$). There was, however, a significant Stress x Sex interaction, which reflected an increase in spine density in stressed females but not males.

4.4.3.5. CA1 dendritic morphology. Stress increased dendritic branching and length in males but only length in females. There was a sex difference in branching (F<M) as has been reported by Juraska (1990) (see Figs. 4.10A,B).

Three-way ANOVA on the basilar branching showed a main effect of sex ($F(1,82)=5.19$, $p<.03$) but not of lesion ($F(1,82)=0.32$, $p=.57$), or stress ($F(1,82)=3.21$,

p=.077). There was also an sex x stress interaction (F(1,82)=4.73, p<.03). No other interactions were significant (p's>.05).

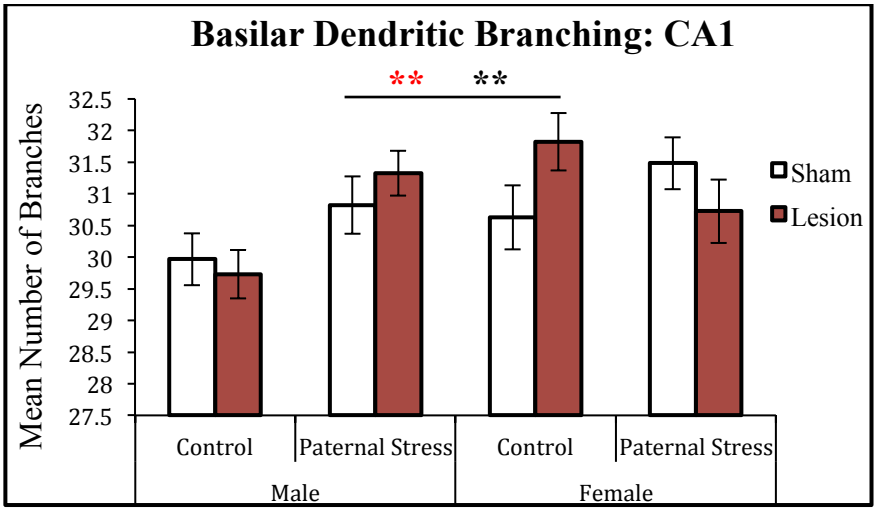

Figure 4.10A. Mean number of CA1 basilar dendritic branches at P110.
** p < 0.05 Male/Female; ** p < 0.05 Two-way interaction (stress-sex)

Three-way ANOVA on the basilar length revealed a main effect of stress (F(1,82)=4.66, p=.03) but not of lesion (F(1,82)=1.03, p=.31), or sex (F(1,82)=1.97, p=.16). There were no significant interactions (p's>.05). Stress increased dendritic length in every group.

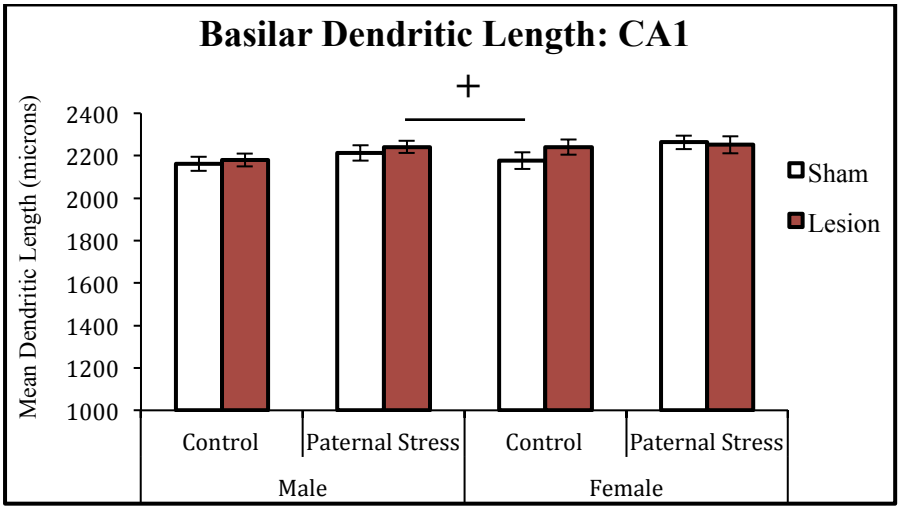

Figure 4.10B. Mean CA1 basilar dendritic length at P110.
+ p < 0.05 Control/Paternal Stress

Three-way ANOVA on the basilar spine density found no effects of stress ($F(1,82)=0.02$, $p=.89$), lesion ($F(1,82)=0.06$, $p=.80$), or sex ($F(1,82)=3.1$, $p=.08$). There also were no significant interactions (p's$>.05$).

4.5 DISCUSSION

There were 3 major findings of this study: 1) Preconception paternal stress had significant effects on brain and behavior of adult offspring; 2) P7 mPFC lesions affected adult behavior but there was little interaction with the effects of preconception stress; and, 3) the effects of paternal stress were sexually dimorphic. We consider each finding in turn (for a summary see Figure 4.11).

	P21 Brains		P110 Brains		
Measure	Stress	Sex	Stress	Sex	Lesion
Activity, P33	NA	NA	X	X	X
Activity, P61	NA	NA	--	X	--
EPM, P33	NA	NA	X	--	--
EPM, P61	NA	NA	X	--	X
Reaching, P100	NA	NA	X	--	X
MWT, P100	NA	NA	--	--	--
Brain weight	↓	X	↑	X	↓
CG3 (Shams) Branch apical	↑	--	↓	X	NA
CG3 (Shams) Length apical	--	--	↑	--	NA
CG3 (Shams) spines apical	↓	X	--	--	NA
CG3 (Shams) Branch basilar	--	--	--	--	NA
CG3 (Shams) Length basilar	--	--	↑	--	NA
CG3 (Shams) spines basilar	↓	--	--	--	NA
AID Branch basilar	↓	--	--	X	--
AID Length basilar	↓	--	↑	--	--
AID spines basilar	↓	--	↑	X	--
Par1 Branch apical	--	--	--	--	--
Par1 Length apical	--	--	--	--	--
Par1 Spines apical	↓	--	--	--	--
Par1 Branch basilar	--	--	↓	--	--
Par1 Length basilar	↑	X	↓	--	--
Par1 Spines basilar	↓	--	--	--	--
CA1 Branch basilar	--	--	--	X	--
CA1 Length basilar	↓	X	↑	--	--
CA1 Spines basilar	--	--	--	--	--
Proportion changed	10/19	4/19	13/25	7/25	1/19

Figure 4.11. Summary of the effects of stress and lesions on the brains for animals killed at P21(from Harker et al., 2015) or P110 (current study). X=Significant main effect; -- =No significant effect; ↑=significant increase; ↓=significant decrease

4.5.1. Preconception Paternal Stress.

As anticipated, PPS altered behavior on activity and elevated plus maze and worsened it on reaching behavior. On Morris Water Task we did not observe a significant effect on latency in male or female offspring, however, PPS female sham offspring latency times were remarkably similar to those of PPS male sham offspring, suggesting enhanced cognitive performance in female sham offspring. We had anticipated that female shams would display longer latency times, as observed in control animals, but this did not occur. There is prior evidence that adult stress negatively influences skilled reaching (Metz, Jadavji & Smith (2005), and alters activity and elevated plus maze (e.g., Das et al., 2015).

A comparison of the anatomical findings from animals killed at P21 vs P110 reveal a number of interesting changes. Overall AID showed the most distinct changes over the lifespan (all effect of stress measures sampled changed). This may reflect the nature of the inputs to AID and its function. There are reciprocal connections with amygdala and the amygdala's influence on behavior may be tempered by development of other PFC areas as the animal matures. In addition, the developmental trajectories are not the same for AID and CG3. According to van Eden et al. (1990) there is an enormous increase in volume of AID at P21 and in CG3 the volume decreases at this age. The different effects observed are thus not surprising as these two areas are developing at different rates and in different manners. Generally speaking changes observed in AID were opposite to those seen in CG3 and that held true at both P21 and P110. This likely reflects the distinctive nature of these two PFC areas. Both CG3 and PAR1 showed intermediate levels of change (approximately 60% of the anatomical areas sampled

changed). The CA1 field in the HPC showed the most resistance to change (only 50% of features sampled showed changes in adulthood that varied from P21 to P110). This may reflect CA1 area involvement in context dependent memory. It may be important to preserve the connectivity in this area to provide stability for context dependent memory. It should be noted that the animals in the P110 group were behaviorally tested several times over their lifespan. It is likely that this testing had an impact on the anatomical measures observed. Overall, more changes were observed in female offspring than in male offspring. (see Fig. 4.12).

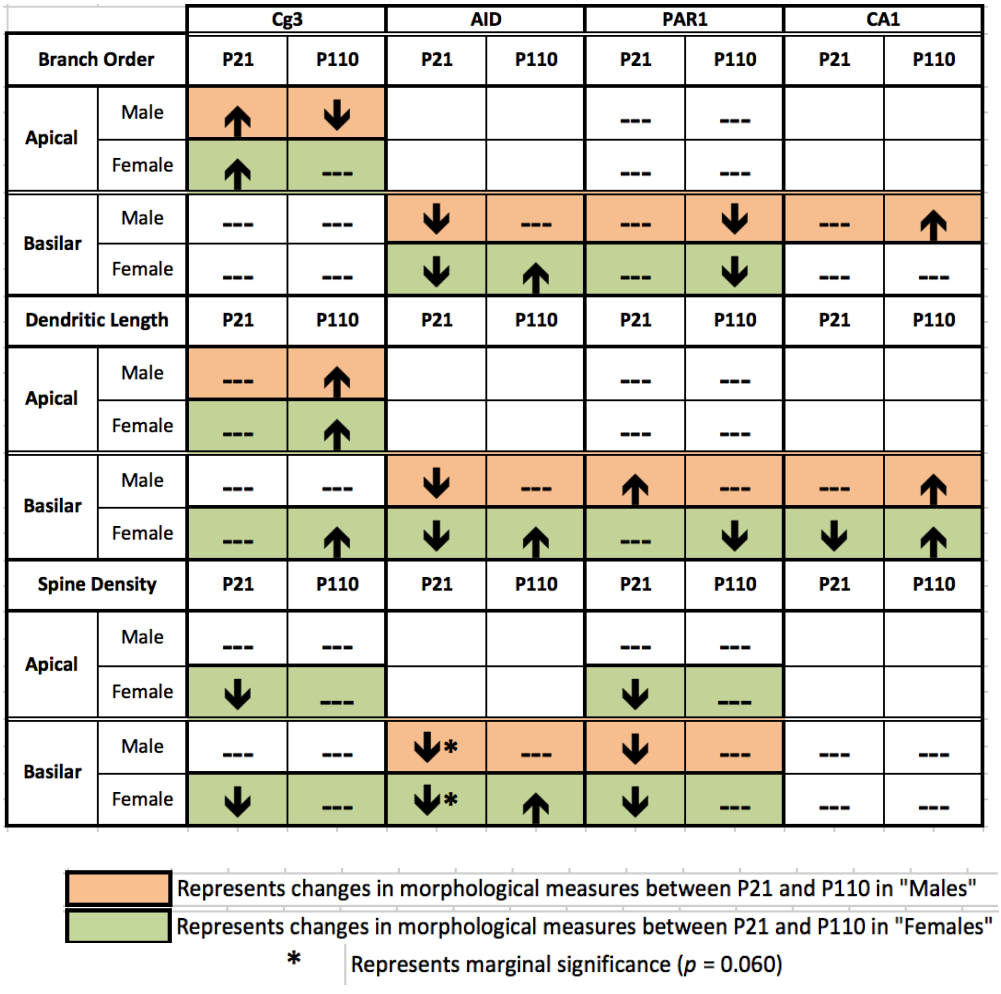

Figure 4.12. Summary of anatomical findings comparing two separate time points, P21 (from Harker et al., 2015) and P110 (current study).

Stress in adult rats alters the morphology of neurons in both medial and orbital prefrontal cortex and the hippocampus. For example, spine density is decreased in mPFC, but increased in OFC and hippocampus; (see review by McEwen & Morrison, 2013; Sunanda, Rao, & Faju, 1995). In the current study PPS did not change spine density in the mPFC or the hippocampus, however, it increased spine density in the OFC. In a study examining the littermates of the animals in the current study, spine density was reduced in both mPFC and OFC when the brains were harvested at P21 (Harker et al., 2015) (see Fig. 4.12). This was correlated with a reduction in global methylation in mPFC and an increase global methylation in hippocampus (Mychasiuk et al., 2013a). These spine density results parallel the findings of Muhammad & Kolb (2011a) who showed that gestational stress also reduces spine density in both mPFC and OFC. In contrast, Bock et al. (2014a) showed that preconception stress of female rats had the opposite effect in mPFC of adult offspring but they did not measure OFC or hippocampus.

It is clear that preconception, gestational, and neonatal stress all influence spine density in the adult offspring but the effects vary with age and which parent was stressed. The aforementioned studies also examined dendritic branching and length and although the details of the changes are slightly different than the spine results, as they were in the current study, the general point is the same: these preconception stressors modify the organization of cerebral cortex in adulthood. It is likely that all of these transgenerational effects of stress are mediated by (re)programming of later gene activity in the oocyte (in dams) and spermatocytes (in males).

There is a significant literature looking at the effects of postnatal stressors on the brain morphology in children (see review by Carrion & Wong, 2012) but we are unaware of any human studies examining the effects of PPS on this measure. Overall, the research demonstrates that children with severe early postnatal stress show significant decreases in volume and activity in both the prefrontal cortex and hippocampus, a result consistent with the rodent studies discussed above. Human MRI studies examining children whose father experienced severe stress prior to their conception might be revealing.

Although there is an extensive literature examining the effects of gestational and neonatal stress on neuronal morphology, there is surprisingly little research on the behavioral sequelae of such stress in laboratory animals. There is evidence, however of increased anxiety, reduced play behavior, abnormal social interaction, and cognitive impairments from various forms of early stress including both gestational stress and maternal separation (e.g., Muhammad & Kolb, 2011a, 2011b; Ferdman et al., 2007; Paris & Frye, 2011). In addition, Brunson et al. (2005) showed that early life stress is related to cognitive decline in middle-aged, but not young adult, rats. They found memory disturbance in hippocampal-related tasks as well as disturbances in hippocampal long-term depression and dendritic atrophy. There is also considerable literature linking early adversity to adolescent or adult mental health problems in humans (e.g., Bock & Braun, 2011). Apart from the literature cited, we are unaware, of other studies looking at PPS on the behavior of either laboratory animals or humans and know of no other studies on the effects of PPS on brain anatomy.

4.5.2. P7 mPFC lesions and stress.

The effects of neonatal injury to the prefrontal cortex vary exquisitely with age (see review by Kolb & Gibb, 2006). In particular, damage to the mPFC in the first 5 postnatal days leads to a very poor behavioral outcome on tests of both cognitive and motor behaviors and this is correlated with a small brain, thin cortex, and atrophied pyramidal neurons across the cortex (e.g., Kolb, 1987; Kolb, Gibb & van der Kooy, 1994). In contrast, similar damage at 7-10 days of age allows remarkable functional recovery, which is correlated with extensive compensatory changes in the cortex (e.g., de Brabander, van Eden, & de Bruin, 1991a; 1991b; Klein, Koch & Schwabe, 2008; Kolb, 1987; Kolb et al., 1998; Kolb & Whishaw, 1981; Schnider & Koch, 2005; Schwabe et al., 2004). In many of these studies there is extensive filling-in the lesion cavity with smaller lesions, which is associated with lesion-induced neurogenesis, especially with lesions around P10 (e.g., Klein et al., 2008; Kolb, Petrie & Cioe, 1996; Kolb et al., 1998). The behavioral data in the current study are consistent with earlier studies. There was no filling-in of the lesion cavity in the current study, likely because we made larger lesions, which invaded the subventricular zone. The goal was to increase the likelihood that the effects of another perturbation (i.e., PPS) might interfere with the functional and anatomical sequelae of the lesions.

One surprising effect in the current study was the complete absence of effects of the lesion on spine density, especially in parietal cortex, in either the control or stressed animals. Although most of our previous studies looking at morphological changes after early lesions were done in animals with lesions from P1-P5 or P10, in which there are extensive changes in parietal neurons, in two studies of the effects of P7 mPFC lesions on

recovery we found increased spine density in males in parietal cortex (Kolb & Stewart, 1995; Kolb, Stewart & Sutherland, 1997). There is a key difference in the earlier and current studies. The animals in this study were studied behaviorally during development, both as neonates (Mychasiuk et al., 2013) and as juveniles (this study) whereas the earlier animals were not. (Although the Mychasiuk et al. paper described only the behavior of the non-lesion animals, the littermate lesion animals were also tested.) It is difficult to know how such experiential differences might influence cortical development and why there might be differential effects in lesion and sham animals. We (BK and RG) also have unpublished observations that early behavioral testing (P25-P30) of animals with P10 hemidecortications reduced the lesion-induced increases in spine density in parietal cortex relative to untested animals when the brains were examined in adulthood, so our suspicion is that the early behavioral testing may alter cerebral organization. Indeed, there is a lot of evidence that behavioral training in adults can have very large effects on cortical and subcortical neurons (e.g., Muhammad et al., 2013).

We have shown previously that prenatal experiences, including maternal complex housing, Fibroblast Growth Factor-2, and bromodeoxyuridine, can influence recovery from neonatal mPFC lesions (e.g., Comeau, Hastings & Kolb, 2007; Gibb, Gonzalez, & Kolb, 2014; Kolb, Pedersen & Gibb, 2012b). We know of no evidence that preconception experiences might interact with neonatal brain injury in the offspring but it was our expectation that PPS might interfere with recovery from such injury. In contrast to our prediction, PPS had no effect on functional recovery from the lesions. This is especially surprising given that the PPS had such extensive effects on cerebral neurons. It is possible that the stress-lesion interaction might show up later in life, however. As

noted earlier, Brunson et al. (2005) found that early stress caused deterioration of memory and dendritic atrophy in the hippocampus of middle-aged but not young adult rats.

Finally, we have shown elsewhere that rats with P10 mPFC lesions show behavioral deficits at P22-25 but not at P55-58 (Kolb & Gibb, 1993). There was a similar result here as the lesion rats were hyperactive when studied at P33 but not at P61, suggesting that they had grown out of their deficits. We must note, however, that the rats did not show anxious behavior in the elevated plus maze at P33 but they did at P61, suggesting that they also grew into deficits, much as is seen in hamsters and rhesus monkeys with neonatal prefrontal lesions (Goldman, 1974; Kolb & Whishaw 1985. It is likely that animals grow out of deficits as brain regions mature and take over functions whereas animals grow into deficits as functions are released to later maturing regions that are dysfunctional.

4.5.3. The effects of stress were sexually dimorphic in prefrontal cortex pyramidal cells.

There are sexually dimorphic effects of stress on mPFC neurons in rats stressed as adults. Garrett and Wellman (2009) showed that whereas males show a general shortening of dendrites, females do not and this effect was estradiol dependent. This sex effect is circuit specific, however, as Shansky et al. (2009, 2010) found that in male rats mPFC neurons projecting the basolateral amygdala (BLA) did not show dendritic retraction whereas those projecting elsewhere did. In contrast, females showed an

expansion of dendrites of the BLA-projecting neurons in intact, but not ovariectomized, females.

There are also sex-related differences in the effects of gestational stress. Mychasiuk et al. (2011d) measured dendritic length, spine density, and neuron number in mPFC and OFC in P21 animals who had experienced gestational stress. When neuron number, dendritic length, and spine density were combined to estimate synapse number there was a clear stress-related sex difference: males showed an increase in mPFC and a decrease in OFC whereas females showed the opposite. Muhammad and Kolb (2011a) did a less thorough analysis but also found sex differences in the effect of gestational stress in mPFC in adult offspring.

There are few studies looking at the effects of preconception stress on the dendritic morphology of the offspring. Bock et al. (2014a) found that maternal preconception stress only affected mPFC neurons in males (longer basilar dendrites). Harker et al. (2015) examined the brain of P21 animals with PPS (the littermates of the current animals) (See Fig. 4.12) and found that there was a decrease in spine density in mPFC in females, but not males whereas the stress-related effects were similar in both sexes in OFC. In the current study there were no sex differences in mPFC but females and not males had increased spine density in OFC.

The only clear conclusion from the stress-related sexual dimorphism studies examining stressors at different developmental ages is that there are sex differences but they appear to vary in complex ways depending on the age. It is important to note that the stress is acting on prefrontal regions that are sexually dimorphic in animals that are not stressed, and these differences are hormone related (Kolb & Stewart, 1991; current

data). It is possible that preconception stressors might epigenetically influence the effects of gonadal hormones on prefrontal neurons later in life. An epigenetic analysis of littermates of the current animals found a decrease in global methylation in the frontal cortex at P21 of female, but not male, offspring (Mychasiuk et al., 2013).

4.5.4. Conclusion

We have identified both behavioral and neuroanatomical effects of preconception paternal stress on the adult offspring. Although we have no direct evidence of epigenetic changes in the adult offspring of the stressed males, epigenetic changes are a feasible explanation given that they were present in littermates at P21. Although we failed to demonstrate an interaction between preconception stress and the effects of neonatal mPFC injury, we believe it is likely that the effects of PPS will interact with other postnatal experiences that induce brain plasticity such as the effects of psychoactive drugs or complex housing. This remains to be shown.

Chapter 5

General Discussion

The studies included in this thesis contribute essential documentation to support the growing literature examining the impact of preconception paternal experience on offspring neurodevelopment and behavioral outcomes. The cardinal finding is that preconception paternal stress (PPS) altered offspring global methylation levels, brain morphology, and behavioral outcomes, as hypothesized, also confirming the first prediction, that we would observe changes in epigenome, brain architecture and behavior of offspring of fathers stressed in the preconception period. However, we had not anticipated how profound and enduring the effects on offspring epigenome, brain, and behavior would be. That the effects were life long supports the second prediction, that we would observe changes to brain and behavior throughout the lifespan; this underscores the importance of preconception paternal experience on offspring development.

Surprisingly, preconception paternal stress did not exacerbate behavioral impairments or brain morphology in animals with neonatal medial prefrontal cortex injuries, contrary to our third prediction, that the effects of PPS would be increased in offspring with neonatal prefrontal cortex injuries. Rather, the lesion animals showed a similar degree of behavioral change as did the controls and the extent of the lesion was unaffected by paternal experiences. This finding was entirely unanticipated and suggests that preconception experience is fundamentally and mechanistically different than prenatal experiences. Overall, this research supports my hypothesis that preconception

paternal experience would impact neurodevelopment and behavioral outcomes of developing offspring.

5.1 Effect of PPS on Dendritic Morphology and Spine Density

Complex changes in brain morphology were observed in all brain regions examined. The effects varied with brain region, age at time of perfusion, and sex. Animals with PPS examined at P21 were found to have a decrease in spine density in the mPFC in females, but not males, whereas the stress-related effects were similar in both sexes in OFC. When littermates were examined at P110, there were no sex differences observed in the mPFC, however, females had increased spine density in OFC and males showed no change. In the Bock et al. (2014) study of preconception *maternal* stress, researchers observed dendritic and synaptic changes that were dependent on brain region, timing of stress exposure, sex, and the type of dendrite. Spine density, dendritic length and complexity all increased in the mPFC, but no changes were observed on the OFC. Clearly, the sex of the parent that experiences stress may be a factor in determining how stress will affect the developing brain.

Overall, the OFC of PPS animals showed the most distinct changes over the lifespan (all measures sampled changed). This may reflect the nature of the inputs to OFC and its function. There are reciprocal connections with amygdala and the amygdala's influence on behavior may be tempered by development of other PFC areas as the animal matures. In addition, the developmental trajectories are not the same for OFC and mPFC. According to van Eden et al. (1990) there is an enormous increase in volume of OFC at P21 and in mPFC the volume decreases at this age. The different effects observed are

thus not surprising as these two areas are developing at different rates and in different manners. Generally speaking, changes observed in OFC were opposite to those seen in mPFC and that held true at both P21 and P110. This likely reflects the distinctive nature of these two PFC areas. Both the mPFC and PAR1 showed intermediate levels of change (approximately 60% of the anatomical areas sampled changed). The CA1 field in the hippocampus showed the most resistance to change (only 50% of features sampled showed changes in adulthood that varied from P21 to P110)(see Figure 4.12). This may reflect CA1 area involvement in context dependent memory. It may be important to preserve the connectivity in this area to provide stability for context dependent memory. It should be noted that the animals in the P110 group were behaviorally tested several times over their lifespan. It is likely that this testing had an impact on the anatomical measures observed. Overall the changes were equally distributed between males and females but the observed changes were more dramatic in females. What the impact of this is on future generations remains to be seen and is not in the scope of the present thesis.

5.2 Effect of PPS on Global Methylation Levels

The epigenome is essentially a second set of genetic instructions that can inhibit genes rendering them unreadable by wrapping them tightly in chromatin, or by relaxing the chromatin allowing genes to be read and expressed. Unlike the fixed DNA code, the epigenome is flexible and can be influenced by environmental experiences, such as toxins, diet, stress, etc. Epigenetic “tags” or “marks” react to signals from the environment providing an adaptive response (inhibition/expression) to rapidly changing environmental conditions. One method frequently used to maintain genome stability and

repress gene expression is DNA methylation (Bird, 2007). Therefore, global DNA methylation can be used as a reliable means of assessing changes occurring in the epigenome. PPS altered DNA methylation patterns in offspring at P21. Global methylation was reduced in the frontal cortex of female offspring, but increased in the hippocampus of both male and female offspring. Mychasiuk et al. (2011a) found increased levels of global methylation in the hippocampus of offspring where their mothers had chronically experienced mild stress, similar to the findings in this study. However, studies of both maternal and preconception paternal enrichment showed decreased global methylation in the hippocampus (Mychasiuk et al., 2012b). In this study females, but not males, showed reductions in the frontal cortex. The frontal cortex may be more sensitive to demethylation in response to perinatal experiences, as high levels of prenatal maternal stress, prenatal maternal enrichment, and paternal enrichment prior to conception, all reduced global DNA methylation in the frontal cortex. Unfortunately, global methylation levels were not examined on littermates at P110, making a comparison between these two different developmental time periods impossible at this time.

5.3 Effect of PPS on Behavior

Early behavioral testing showed that offspring of fathers who experienced chronic stress were significantly impaired/delayed at the negative geotaxis task when tested on P9, but had recovered by P10 and were indistinguishable from control offspring. This was opposite the observed effect of prenatally stressed offspring who were indistinguishable from controls on P9 but failed to demonstrate any

learning/improvement that was typical of control offspring and therefore exhibited deficits on P10 (Mychasiuk, 2011b). It appears that the prenatally stressed offspring were learning impaired, whereas the PPS were developmentally slow. This may suggest that PPS slows brain maturation. Whereas the open field test did not show significant effects in exploratory activity for PPS offspring, observations revealed that pre-weanling male offspring spent significantly more time in the center of the open field. There are two schools of thought here; first, this behavior may indicate that PPS males have less anxiety, or second, that PPS males demonstrated deficits in behavior control. The fact that a prey animal is spending a significant amount of time in the center of a field appears to be maladaptive and an example of a deficit in behavior control.

PPS had an intriguing and unexpected impact on the behavior of juvenile and adult offspring. Other studies on adult stress (Metz, Jadavji, & Smith, 2005) have demonstrated a negative impact on skilled reaching; precisely the findings in this PPS study. As well, Das et al. (2015) demonstrated alterations to activity and elevated plus maze assessment, which was similar to our findings in PPS offspring. However, we found that PPS female sham offspring actually had improved performance on the Morris water task, a measure of enhanced cognitive performance. It is interesting that PPS decreased latency in female shams, bringing them very close to male sham measures of latency. Further research in this area would be helpful in order to fully understand this observation.

5.4 Transmission of Paternal Experience

Braun and Champagne (2014) suggest that in mammals there are several avenues by which paternal effects can be transmitted to offspring. First, fathers, like mothers, can overtly influence offspring through direct care, observed in biparental species (prairie voles, degus, humans, etc.). However, rats are a non-monogamous species that do not invest in care of offspring subsequent to birth. Fathers did not have contact with offspring or their mate following conception. Therefore, this route of transmission of paternal effect is not valid.

Second, fathers may exert an indirect influence on offspring through direct contact with the mother, impacting mother-infant interactions, and generating paternally-induced maternal effects. In this study, contact between mother and father was limited to the mating period only; contact was restricted between partners prior to conception and following conception. Although this route of transmission is unlikely due to the limited contact between father and mother, it is still a possibility. Studies have shown that a female's reproductive investment is determined by the observed phenotype or perceived quality of her mate (Bluhm & Gowaty, 2004; Cunningham & Russell, 2000). Braun and Champagne explain that through the use of *in vitro* fertilization it is possible to examine the paternal influence on offspring absent any possible paternal effect on maternal investment. In this case female mice were implanted with sperm from fathers whom had undergone chronic social defeat stress. There was no contact between father and mother, or father and offspring. The paternal effect of chronic social defeat stress revealed that some phenotypes (i.e. depressive-like behavior) were observed in offspring, while others

(i.e. anxiety-like behavior) were not, suggesting that there is transmission of paternal effect to offspring, however it is incomplete.

Third, fathers can transmit effect of environmental perturbations to offspring through epigenetic mechanisms. The process of PPS transmission to offspring, although unknown at this point, is suspected to be a combination of routes two and three involving a dynamic interplay between maternal investment and paternal transmission of epigenetic marks on germ cells, with more emphasis on the transmission of paternal experience inherited by offspring, through the germline.

5.5 Limitations and Future Directions

One of the limitations of this study was the lack of collection and analysis of global methylation levels on P110 littermates. It would have been an asset to examine the effects of global methylation at two separate time points in the lifespan of the PPS offspring. Brain morphological analyses at P21 and P110 provided valuable insight into the differences in synaptic connectivity and dendritic length and complexity between the two time points. Much can be learned from observations at different developmental time periods.

Although expensive, it would have been valuable to examine possible transgenerational effects of PPS on offspring. Owing to the fact that there were significant changes in all brain areas examined, global methylation levels and behavioral assays, it would be interesting to observe possible transmission of the effect of PPS to the next generation.

Another limitation was that the examination of possible resilient tendencies was not conducted. It is possible that PPS provides an epigenetic inheritance that, while deficits in learning and motor ability may be present, resilience in stressful situations may occur. It would have been advantageous to examine PPS offspring in behavioral tasks, such as the foot shock task, in order to assess possible resilience in PPS offspring during extreme stress. Rodgers and Bale (2014) suggest that epigenetic marks in sperm produce a lasting effect on offspring stress reactivity that programs either disease susceptibility or resilience. Perhaps PPS provided an advantage to offspring that was not observed through the use of the developmental tasks we chose. It would also be beneficial to examine possible paternal experiences such as complex housing or tactile stimulation on both PPS dads and PPS offspring in order to assess possible strategies to mitigate or remediate the effects of PPS on brain structure and behavioral outcomes.

In the future, it would be important to administer several different paternal experiences prior to mating. Having examined the adverse effects of preconception paternal stress, it would be intriguing to investigate the effect of a positive environmental condition, such as complex housing. Would the effect of a positive experience trigger a response similar in strength, but in the opposite direction, to what was observed in PPS offspring?

Finally, we know that maternal alcohol use or pharmacological use of SSRIs during gestation can have significant and possibly devastating effects on offspring brain and/or behavior. It would be pertinent to the investigation of paternal experience to examine the effects of both these teratogens on the brain and behavior of affected offspring. Research in this area would be especially useful if the same experimental

procedures were maintained as in the paternal stress experiment. Exploring these additional paternal experiences would further our knowledge in this area, and provide the impetus for much needed education regarding the influences of paternal contribution, of which would ultimately lead to healthier outcomes for future generations of offspring.

5.6 Conclusion

Research detailed in this thesis provides evidence that preconception paternal stress can produce dramatic and lasting changes in brain architecture and function. The mechanism by which paternal experience directs the epigenetic reprogramming is still unclear. However, I hypothesize that the effect of preconception paternal stress is disseminated to offspring through epigenetic marks in the germline, thereby causing a (re)programming of offspring stress response and reactivity. This (re)programming may lead to disease or resilience in offspring, dependent on environmental conditions to which offspring are exposed (Rodgers & Bale, 2014).

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