INTENSITY MATTERS: EFFECTS OF PRENATAL STRESS ON THE DEVELOPING BRAIN

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ABSTRACT

This thesis examines the behavioral, structural, cellular, and epigenetic changes observed in offspring exposed to different prenatal stressors. A number of questions were answered in this thesis that contribute to a basic understanding of the mechanisms by which early experiences alter long-term outcomes. These include: 1) What epigenetic modifications are associated with prenatal stress? 2) What are the structural and cellular changes in the brains of offspring that correspond to prenatal stress exposure? 3) How do these epigenetic and structural changes manifest as behavioral changes? And 4) What are the consequences of varying the level of prenatal stress?

The key findings were that not all prenatal stress is the same. Variations to the intensity and nature of the stress dramatically alter offspring outcomes. Second, prenatal stress produces changes at many levels and these changes can be functionally related. Expression changes were identified in genes involved in altering dendritic morphology, which in turn modifies behaviour. For the first time, a comprehensive examination of brain plasticity occurred following prenatal stress. Additionally, this thesis demonstrated that brain changes related to prenatal stress are age-dependent and sex-dependent. The effects of prenatal stress on the pre-weaning brain are dramatically different than those observed in adulthood. Also, the sex of the offspring significantly influences neuroanatomical and epigenetic modifications. This finding is of critical importance because a majority of prenatal stress research is conducted on male offspring only. Taken together these discoveries emphasize that perturbations to development during the prenatal period produce persistent changes in the structure and functioning of the brain that will influence all subsequent experiences.

DEDICATION

This thesis is dedicated to societies greatest natural resource – the children

ACKNOWLEDGEMENTS

I would first like to thank my supervisors, R.Gibb and B. Kolb for their unwavering support over the past 3 years; their commitment to learning and promotion of excellence provided an amazing environment for development of my new found love for research. I would also like to thank my committee member O. Kovalchuk for her help with all of the genetic methodology and C. Rankin for making the journey as my external. I owe a great debt of gratitude to the entire Kolb Lab for their expertise and assistance over the years. I would also like to thank K. Benzies for her patience and assistance. Finally, I would like to thank my family and friends for their continuous support and encouragement, especially my husband Aaron, my dad, my mom, and my sister.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
Adcy1	Adenylyl cyclase 1
AID	Used to represent the orbital frontal cortex
Alg-2	Apoptosis linked gene 2
Alix	Alg-2 interacting protein X
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxozole-propionate
Apo E	Apolipoprotein E
AVP	Arginine vasopressin
BS	Bystander stress
Cg3	Used to represent the medial prefrontal cortex
CNS	Central nervous system
CRF	Corticotropin releasing factor
Dbn1	Drebrin
DNA	Deoxyribonucleic acid
ERα	Estrogen receptor alpha
ERK	Extracellular regulated kinase
ES	Extreme stress
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FGF-2	Fibroblast growth factor 2
GC	Glucocorticoid
Gria2	Glutamate receptor 2

HPA	Hypothalamic-pituitary-adrenal
Hsp27	Heat shock protein 27
IGF2	Insulin-like growth factor 2
Igf2r	Insulin-like growth factor 2 receptor
IP	Intra-peritoneal
Lrp8	Low density lipoprotein receptor-related protein 8
LTD	Long term depression
LTP	Long term potentiation
M6P	Manose-6-phosphate
MAPK	Mitogen-associated protein kinase
mPFC	Medial prefrontal cortex
MS	Mild stress
NOV	Nephroblastoma overexpressed gene
NS	No stress
ncRNA	Non-coding RNA
PCD	Programmed cell death
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PVN	Paraventricular nucleus
RNA	Ribonucleic acid
Safb1	Scaffold attachment factor B1
Sema7A	Semaphorin 7A
Shisa4	Shisa homolog

USV	Ultrasonic vocalization
Wnt	Wingless Type
11β-HSD-2	11β-hydroxysteroid dehydrogenase type 2

Chapter 1: Introduction

An organism's ability to survive is dependent upon its capacity to adapt to its environment. For mammals this adaptive response begins prenatally, as the fetus adjusts to the intrauterine environment, with the assumption that the prenatal and postnatal environments are similar (Talge, Neal, Glover, & Early Stress Translational Research and Prevention Science Network, 2007). A fundamental characteristic of the nervous system, and more specifically the brain, is it's propensity to change in response to experience (Kolb, Gibb, & Robinson, 2003). The prenatal brain is exceeding susceptible to environmental influence, as this is a sensitive period for brain development (Fagiolini, Jensen, & Champagne, 2009). In response to environmental stimuli the brain changes at the genetic, cellular and structural level. These early changes are often stable and result in long-term patterning that may be maintained through out the life span (Meaney, Szyf, & Seckl, 2007). The purpose of this thesis was to investigate the effects of varying prenatal stress paradigms on offspring brain, behaviour, and epigenome.

Stress

An organism's existence is based upon maintenance of their homeostatic equilibrium, which is constantly challenged by stressors. The brain is responsible for interpreting these stressors as threatening or non-threatening and then initiating the appropriate physiological and behavioural responses (McEwen, 2007). A stressor is any force that disturbs or threatens homeostasis and activates the stress response. There are 2 main types of stressors: 1) Real Threats – represent genuine challenges to homeostasis, and 2) Predicted Threats – are anticipated challenges to homeostasis. The response to

these stressors is generally the same and includes mobilization of a variety of endocrine, metabolic, immune and neural defenses (Meaney, et al., 1985). Once the stressor is eliminated the stress response is terminated and the organism returns to homeostasis.

The hypothalamic-pituitary-adrenal (HPA) axis is the primary biological mechanism underlying the brain's response to stress. See Figure 1.1. When the brain perceives a stressful stimulus the paraventricular nucleus (PVN) in the hypothalamus releases corticotropin releasing factor (CRF) and arginine vasopressin (AVP). CRF and AVP stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which in turn, activates the release of glucocorticoids (GC) and mineralocorticoids from the adrenal glands. The GCs circulate in the bloodstream and enhance catabolic processes by increasing availability of energy substrates. The HPA axis is regulated by negative feedback inhibition; when levels of GCs rise above a certain point, GCs inhibit further release of ACTH and CRF, shutting down the response (Kofman, 2002). The major GC in rodents is corticosterone, whereas the major GC in humans is cortisol.

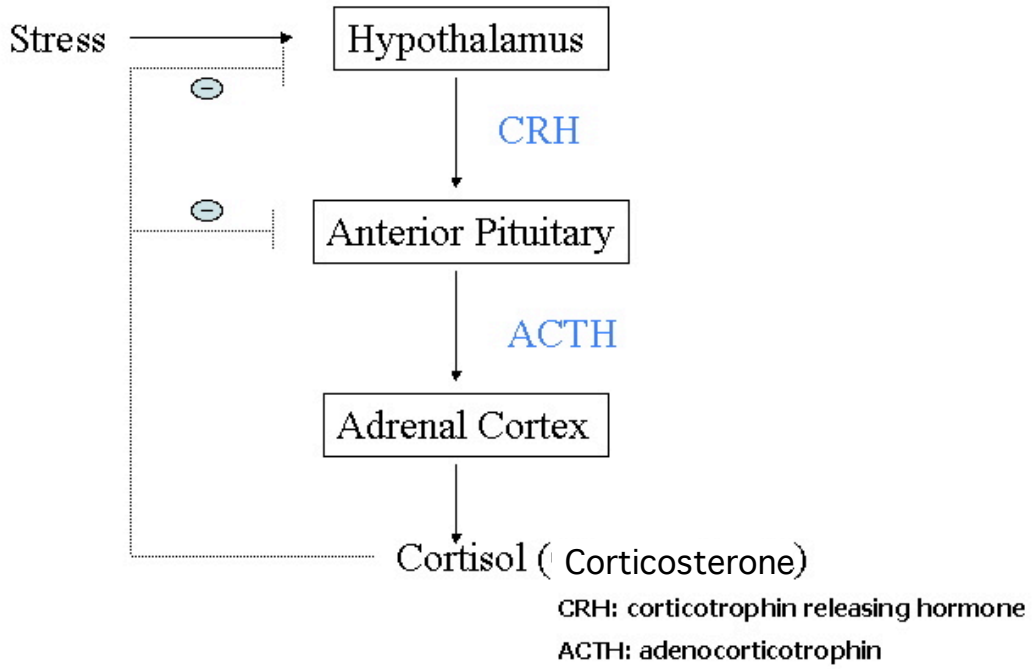

Figure 1.1: *Simplified representation of the HPA axis*

Activation of the HPA axis temporarily inhibits the immune response and processes involving cellular growth and reproduction, to ensure the animal can use all available resources to eliminate the stressor (Kofman, 2002). This response is beneficial and advantageous for circumstances involving acute stress that can be mastered. Chronic hyper-activation of the HPA axis, as a result of prolonged, recurrent, dangerous stress, produces a maladaptive state of homeostasis (Talge, et al., 2007). Prolonged stress and chronic release of glucocorticoids is associated with tissue damage, altered metabolism and immunosuppression (Meaney, et al., 1985). When the stress response is activated during pregnancy the effects on the HPA axis are not limited to the mother.

The HPA axis is a target for environmental influence and therefore, a mediator between maternal experience and offspring development (Meaney, et al., 2007). Lipophilic steroids like GCs released from the mother's adrenal glands can easily cross

the placental barrier and act upon the developing fetus (White, Mune, & Agarwal, 1997). Glucocorticoid receptors are found in most fetal tissue by gestational day 13 (Matthews, 2000) and GCs are important for normal brain growth and maturation (Meyer, 1983). Nevertheless, there is strong evidence for fetal sensitivity to elevated levels of GCs and a protective mechanism has evolved to shelter the developing offspring from over exposure. 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2) is an enzyme that catalyzes the conversion of active glucocorticoids to inert 11-keto forms. Placental expression of 11β-HSD-2 allows only 10-20% of maternal GCs, under normal conditions, to cross the placenta intact and act upon the developing brain (White, et al., 1997). However, compensatory mechanisms can only protect the fetus from maternal GCs to a certain threshold. Given that maternal GC levels are higher than fetal levels, abnormal maternal GC secretion as a result of chronic stress, may profoundly affect fetal GC exposure and brain development (Meaney, et al., 2007).

The rapid period of cellular proliferation and differentiation that occurs during fetal development provides a critical window during which gestational exposure to GCs may lead to long-term disruption of cortical plasticity (Champagne, 2010). GC receptors are found throughout the brain and optimal brain development is dependant upon GCs to initiate terminal maturation, remodel axons and dendrites, and affect cell survival (Meyer, 1983). Too much GC exposure, however, impairs brain development and functioning. Increased GCs during pregnancy are associated with reduced birth weight (Meaney, et al., 2007), a reduction in cell proliferation in the dentate gyrus (Lemaire, Koehl, Le Moal, & Abrous, 2000), and adult health disorders such as hypertension and type 2 diabetes (Seckl, 2004). Evidence suggests that the maternal GCs must be acting upon the

developing brain and altering gene expression to help the fetus adapt to its environment (Meaney, et al., 2007). Increased circulation of glucocorticoids activate epigenetic mechanisms producing sustained effects at the genetic level which form the basis for changes in brain function (Darnaudery & Maccari, 2008).

Epigenetics

"*…from so simple a beginning, endless forms most beautiful and most wonderful, have been and are being evolved." Charles Darwin.*

The theory of evolution is based upon the concept of 'natural selection' (survival of the fittest, or descent with modification): the idea that heritable variation exists within populations and this variability alters an organism's ability to survive and reproduce. Essentially, individuals that are most adapted to their environments are more likely to survive and pass along their DNA. These genetic adaptations occur as a result of mutations or changes to the DNA, and have diversified organisms over millions of years to a multitude of niche-specific species. What happens however, if an environment changes? Is there a mechanism by which individuals can adapt to their environments and then carry this forward? Contrary to Charles Darwin who believed adaptation took thousands of years, Jean-Baptiste Lamark proposed the concept of *soft evolution*. Lamarkian evolution is based upon the theory of use and disuse; essentially that individual efforts acquired during a lifetime were passed on to offspring, while those that were neglected exhibited diminished and eventually a loss of functional capacity in future generations. Until very recently, a mechanism by which environment could influence the stable propagation of use and disuse was inconceivable.

The term epigenetics refers to variations in gene expression rather than gene sequence (Fagiolini, et al., 2009). Epigenetics seem to allow an organism to respond instantly to its environment and provides stable propagation of a gene activity state from one generation of cells to the next (Jaenisch & Bird, 2003). This variation in gene expression is tightly regulated and vital for adaptation and survival of the organism (Champagne, 2010). While adaptations based on changes to the DNA sequence may take multiple generations to come into fruition, epigenetic alterations respond to the environment in the short term. In essence, epigenetics is the genome's method by which to learn from experience (Jaenisch & Bird, 2003). Consequently, epigenetic changes may provide an immediate adaptive response for the organism but may also generate risk later in life (Meaney, et al., 2007; Talge, et al., 2007). Success in the context of evolutionary adaptation is based upon reproductive fitness, not longevity or health (Meaney, et al., 2007). Inferring from this, epigenetic modifications may be an underlying source of the outcomes seen in developing offspring as a result of prenatal stress.

Gluckman and Hanson (2005) hypothesized that the epigenetic modifications experienced by the fetus in response to the intrauterine environment prepared the fetus for the particular environment it is likely to be exposed to later in life. These early life responses, also known as fetal programming, occur rapidly in a single generation, to increase the offspring chances of survival (Talge, et al., 2007). The fetal basis of adult disease assumes that perinatal experiences influence cellular plasticity (Dolinoy & Jirtle, 2008). Vulnerability and risk for disease occur when there is a discrepancy between the intrauterine environment and the postnatal environment (Desai, Gayle, Babu, & Ross,

2005). Epigenetic modifications are produced with ease during the sensitive gestational period and are then generally propagated in a stable and persistent fashion.

There are 3 main mechanisms of epigenetic regulation used to transmit these genetic adaptations: RNA regulation, post-translational modification to histone proteins, and most important to this thesis DNA methylation. See Figure 1.2.

RNA Regulation. Many aspects of chromatin structure regulation have been demonstrated to be directed by RNA. Non-coding RNAs (ncRNAs) dominate the transcriptional output of mammals and regulate many levels of gene expression during development (Mattick, Amaral, Dinger, Mercer, & Mehler, 2009). RNA-binding co-regulators are recruited to transcriptional units by gene-specific ncRNAs to modulate chromatin structure (Mattick, et al., 2009). ncRNAs may recruit transcriptional repressors or transcriptional activators functioning in both gene activation and gene silencing. Additionally, microRNAs are post-transcriptional regulators that bind to the complementary sequence of mRNA in the 3' untranslated region (UTR) usually resulting in gene silencing (Mattick, et al., 2009).

Post-translational Modification to Histone Proteins. Post-translational modification to histone proteins includes processes such as, methylation, acetylation, and ubiquitination. Acetylation is the most commonly studied post-translational modification. Acetylation is associated with increased transcriptional activity whereas histone deacetylation is linked to transcriptional repression (Fagiolini, et al., 2009). The acetylation state is controlled by histone deacetylases (HDACs) and histone acetyltransferases (HATs) (Fagiolini, et al., 2009).

DNA Methylation. DNA methylation is the primary contributor to the stable maintenance of particular gene expression states (Jaenisch & Bird, 2003). Most epigenetically induced changes in mammalian gene expression are mediated by DNA methylation coupled with chemical modification of histone tails (Kim, Samaranayake, & Pradham, 2009). Gene silencing via DNA methylation occurs when a methyl group binds to the cytosine residue of a CpG island (Champagne, 2010). This addition attracts other methyl-binding proteins to the site; when these proteins are bound to the gene's promoter region, RNA polymerase cannot bind and the gene cannot be transcribed (Champagne, 2010). This process does not alter the DNA sequence. It merely limits access to the gene's promoter region. DNA methyltransferases, methylbinding proteins, and histone deacetylases are critical factors for the establishment and maintenance of DNA methylation (Das, Hampton, & Jirtle, 2009). Switching between expression and silence is unlikely to occur by chance due to the presence of self-reinforcing mechanisms (Bird, 2002). DNA methylation is tightly regulated; epigenomes are highly organized and surprisingly non-random with respect to methylation patterns (Borrelli, Nestler, Allis, & Sassone-Corsi, 2008). There are few methylation differences from neuron to neuron, or even neuron to neuron in two unrelated individuals.

The DNA sequence is only a foundation for understanding how the genetic program is read. A second layer of interpretation is required to fully appreciate the possibilities arising from the original sequence. An individual's "epigenome" is modifiable by nature, making it vulnerable, but adaptable to environmental influences (Das, et al., 2009).

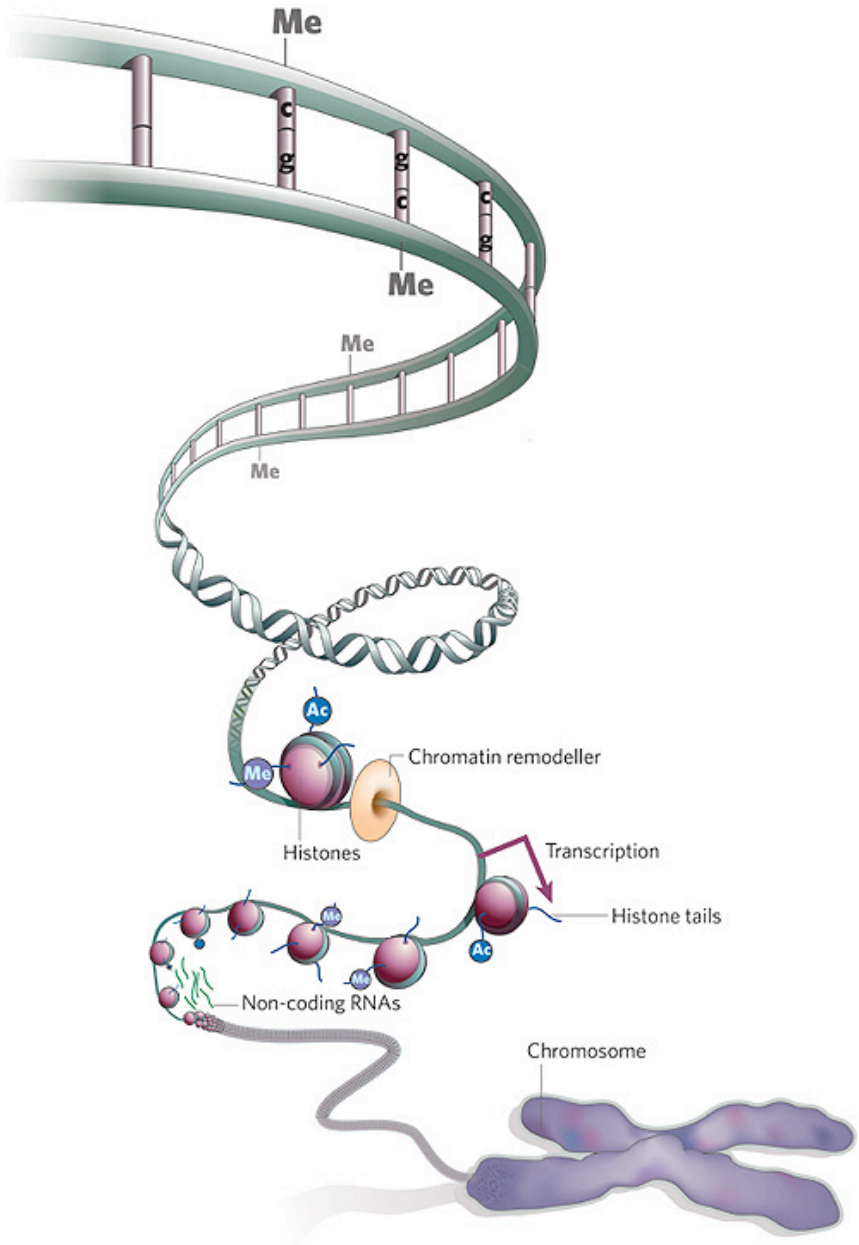

Figure 1.2: *Schematic representation of epigenetic mechanisms employed by the cell. Reproduced from the National Institute of General Medical Sciences (2010). Permission granted by NIGMS to use image for educational, news media, or research purposes (http://images.nigms.nih.gov)*

Bystander Stress

The majority, if not all, literature related to prenatal stress focuses on direct stress to mothers. It is becoming apparent that the realm of maternal experiences capable of altering the intrauterine environment extends much further than this. The social

environment that a female is exposed to during pregnancy has the potential to influence development of the offspring. In an effort to advance understanding of these interactions, the relationships between pregnant dams and their cage-mates were examined. For the purpose of this thesis; *bystander stress* (BS) refers to indirect stress experienced by mothers who are cohabiting with a stressed individual.

Brain Areas of Interest

The hippocampus and prefrontal cortex were chosen for study because they are critical to processes such as executive functioning, cortical plasticity, and learning and memory. Disruption of normal functioning to either of these brain areas as a result of prenatal stress would have significant long lasting effects on development. The hippocampus plays a chief role in the formation of new memories, spatial navigation and learning (Kolb & Whishaw, 2008). Additionally, this region is critical to brain plasticity and is a site of adult neurogenesis. Because the hippocampus is constantly regenerating brain cells it is the perfect candidate for epigenetic studies. Furthermore, because the hippocampus contains high levels of glucocorticoid receptors, it is extremely vulnerable to toxic stress (Watanabe, Gould, & McEwen, 1992).

The frontal cortex is vital for the control of our behavior with respect to time and place. It houses our main executive functions which include: planning, weighing costs and benefits, choosing socially appropriate responses, recognizing future consequences and ignoring extraneous stimuli (Kolb & Whishaw, 2008). This area of the brain is rich with dopamine cells, which allows it to be a significant contributor to the reward circuit. Two areas of particular interest in the frontal cortex are the medial prefrontal cortex

[(mPFC), for purpose of this thesis, Cg3 as determined by Zilles, 1985] and the orbitofrontal cortex (OFC) for purpose of this thesis, AID as determined by Zilles, 1985. The mPFC has traditionally been implicated in attentional processes, behavioural flexibility and working memory (Heidbreder & Groenewegen, 2003). It has more recently been divided into 2 functionally distinct regions - the dorsal mPFC and the ventral mPFC. The dorsal mPFC is particularly involved in temporal shifting of behavioural sequences while the ventral mPFC is specifically responsible for shifting strategies related to spatial cues and integrating environmental cues for the guidance of behaviour (Heidbreder & Groenewegen, 2003). The OFC is believed to be important for response inhibition, control of mood and drive, and is engaged in reward and decision making mechanisms (Cavada & Schultz, 2000).

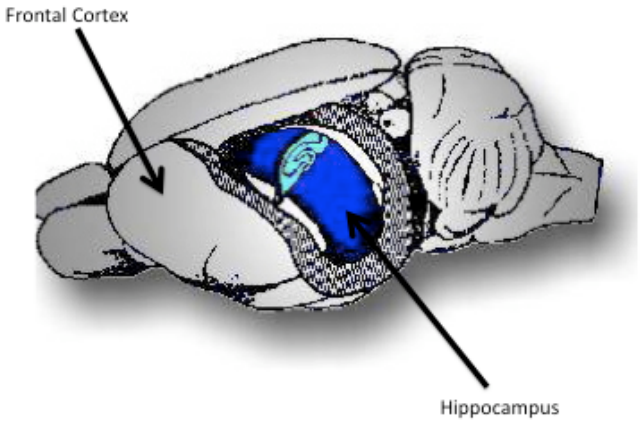

Figure 1.3: *Visual depiction of the rat brain. Reproduced from the O'Keefe and Burgess Research Group (2010)*

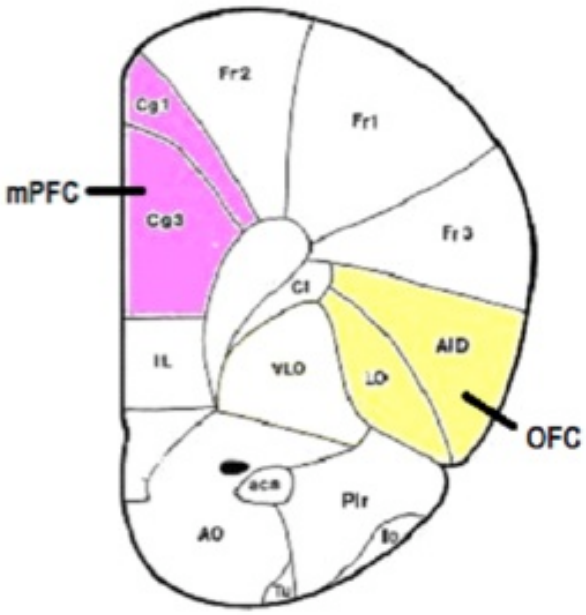

Figure 1.4. *Illustration of coronal section of sub areas of rat prefrontal cortex modified from Zilles 1985*

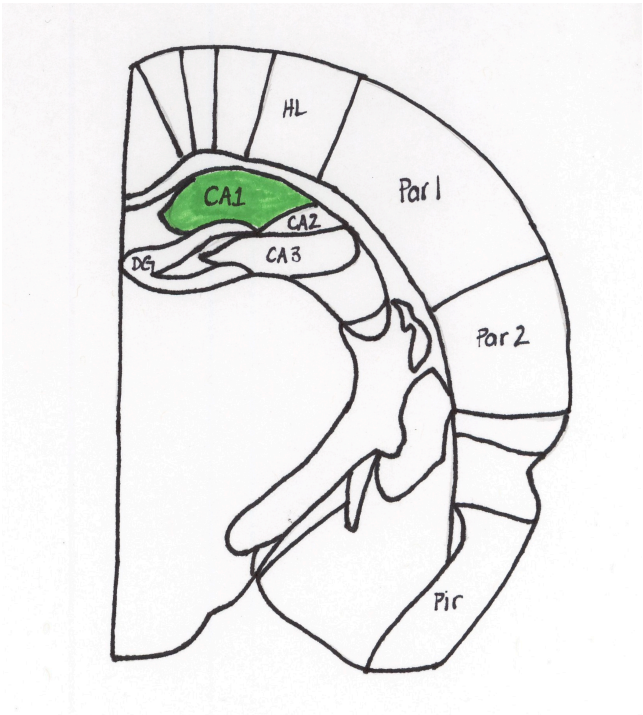

Figure 1.5. *Illustration of coronal section of sub areas of rat cortex modified from Zilles (1985)*

The Experiments

The general purpose of this thesis was to determine how variations in prenatal stress affect the developing brain. Based on current knowledge, it was hypothesized that different levels of prenatal stress would alter offspring brain development in a dose-dependant manner. It was predicted that a linear relationship would exist between the intensity of prenatal stress and the degree of offspring developmental impairment. To generate a comprehensive understanding of this phenomenon, examination of the brain occurred at multiple levels and with a variety of techniques. Chapter 3 of this thesis addresses the behavioural aspect of these experiments. As behaviour is the functional output of the brain, behavioural testing of dams and offspring was used to identify relationships between the experimental manipulations and changes in brain functioning. Based upon the theory that changes in behaviour result from changes in the brain, Chapter 4 concentrates on anatomical analysis. This aspect of the thesis was designed to look for changes in cell numbers as well as examine structural changes in neurons. Finally, the intention of Chapter 5 was to cultivate an understanding of how genetic plasticity is related to experience dependent brain changes. This chapter explores the relationship between epigenetic modification and various prenatal stress paradigms in 2 distinct brain regions.

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Chapter 2: Methods

This section will describe the research methods used for all experiments addressed in this thesis. All measurements were based upon the same experimental paradigm; for simplicity it will only be presented once. Figure 1 is a schematic demonstration of the type of data collected for pups in each experimental group.

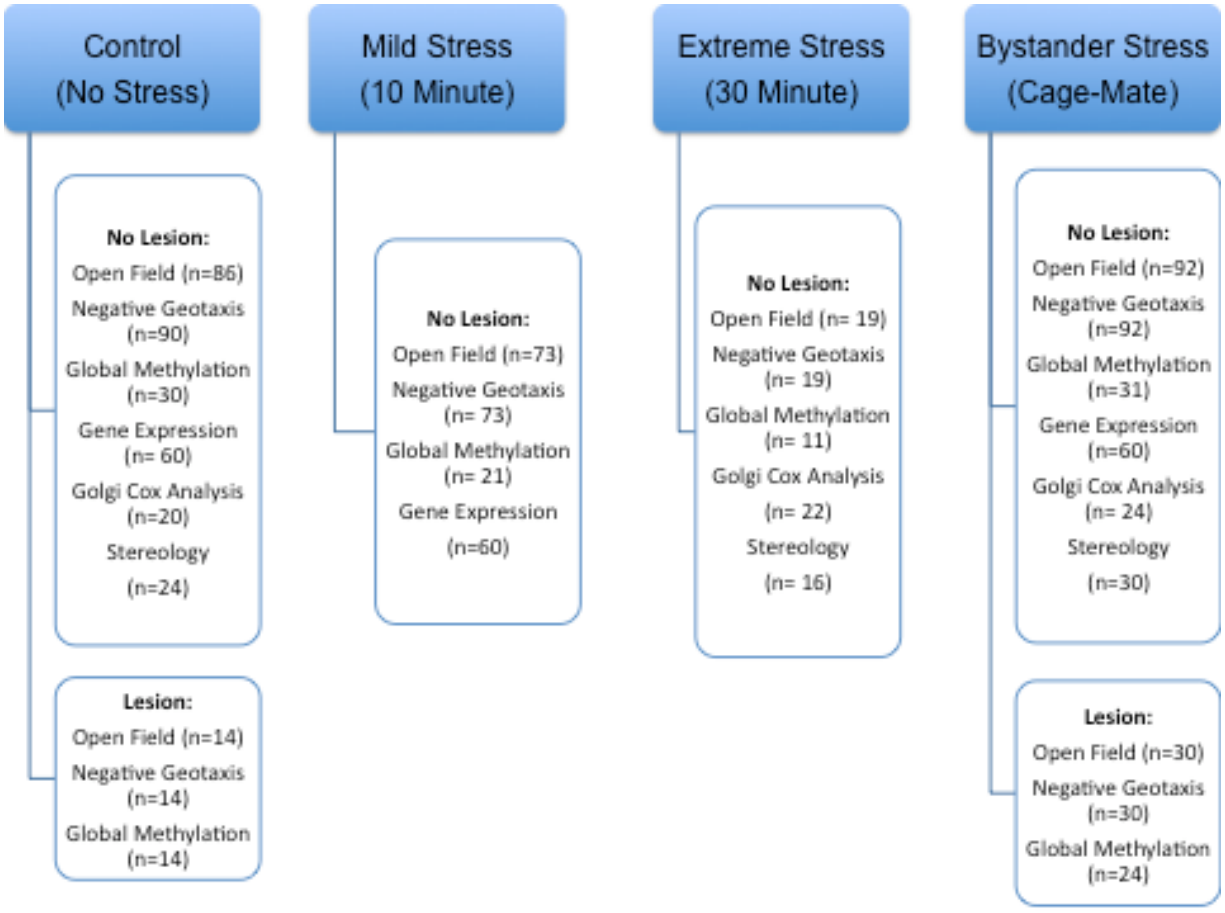

Figure 2.1. *Schematic illustration of experimental groups and analyses conducted.*

Subjects and Stressing Procedures

All experiments were carried out in accordance with the Canadian Council of Animal Care and approved by the University of Lethbridge Animal Care Committee. All animals were maintained on 12:12 hour light:dark cycle in a temperature controlled

breeding room (21°C) and were given access to food and water *ad libitum.* Female Long-Evans rats were housed in pairs in shoe-box cages. One male Long-Evans rat was introduced into each of the cages for a single 24-hour period; this ensured accurate establishment of conception date. The prenatal stress treatment was performed daily on gestational days 12-16 (G12-G16). All female dams were weighed daily; preconception to birth. Once the pups were born, each of the mothers with their litters, were housed separately.

Mild Prenatal Stress (MS): The pregnant dams (n=5) were placed on an elevated Plexiglas® platform (1m tall, 21 x 21 cm) and exposed to bright light for 10 minutes twice a day (Wong, et al., 2007). Stressing occurred at 9:00 am and 3:00 pm.

Extreme Prenatal Stress (ES): The pregnant dams (n=4) were placed on an elevated Plexiglas® platform (1m tall, 21 x 21 cm) and exposed to bright light for 30 minutes twice a day (Wong, et al., 2007). Stressing occurred at 9:00 am and 3:00 pm.

Bystander Stress (BS): The female cage-mates of the pregnant dams (n=9) were placed on an elevated Plexiglas® platform (1m tall, 21 x 21 cm) and exposed to bright light for 30 min twice a day (Wong, et al., 2007). Stressing occurred at 9:00 am and 3:00 pm.

Control (NS): Control dams (n=8) were left in their cages undisturbed.

Ultrasonic Vocalization Recording

Ultrasonic vocalizations (USVs) from some of the female rats were recorded and analyzed using Noldus UltraVox 2.0 equipment and software. USVs were recorded during pregnancy following the stressing procedure. The recording chamber consisted of

a transparent Plexiglas® box containing 3 chambers (see figure 2). An USV microphone was fixed from above into each of the two outer chambers. The outer chambers measured 25 x 60 x 36 cm. The middle chamber was left empty and acted as a buffer, preventing cross recording of USVs from the opposing chamber. The middle chamber measured 15 x 60 x 36 cm. The female rats could see, hear, and smell each other however, direct contact was not possible. The female rats that were housed together were individually placed into the two outer chambers of the box. On gestational day 9 (G9) female rats were habituated to the recording box for 2, 10-minute sessions. On G10 and G11 baseline recordings of USVs were taken for 10 minutes at 9:00 am and 3:00 pm. Immediately following the stressing procedure, on days G12-G16, the stressed rat and her *bystander* cage-mate were placed in the recording chamber and USVs were recorded for 10 minutes. USVs for non-stressed control females were also recorded in the same manner on G12-G16.

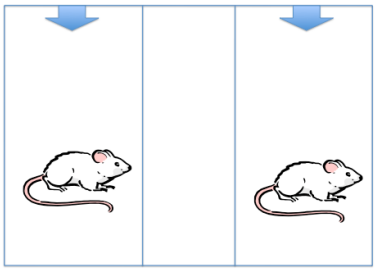

Figure 2.2. *Diagram of USV recording chamber*
**Blue arrows indicate USV microphones*

Lesion Procedure

On postnatal day 8, pups were cooled at -3°C in a Thermotron cooling chamber and anesthetized via cryoanesthesia when their body temperature reach approximately 20˚C (Kolb & Cioe, 2001). Some of the control offspring and some of the bystander

stress offspring received bilateral aspiration removals of the medial prefrontal cortex (mPFC) while another portion of the offspring received sham injuries. Sham animals received incisions and sutures, but no aspiration. Following surgery, all pups were warmed and returned to their mothers. Pups were frequently checked to ensure recovery was without incident.

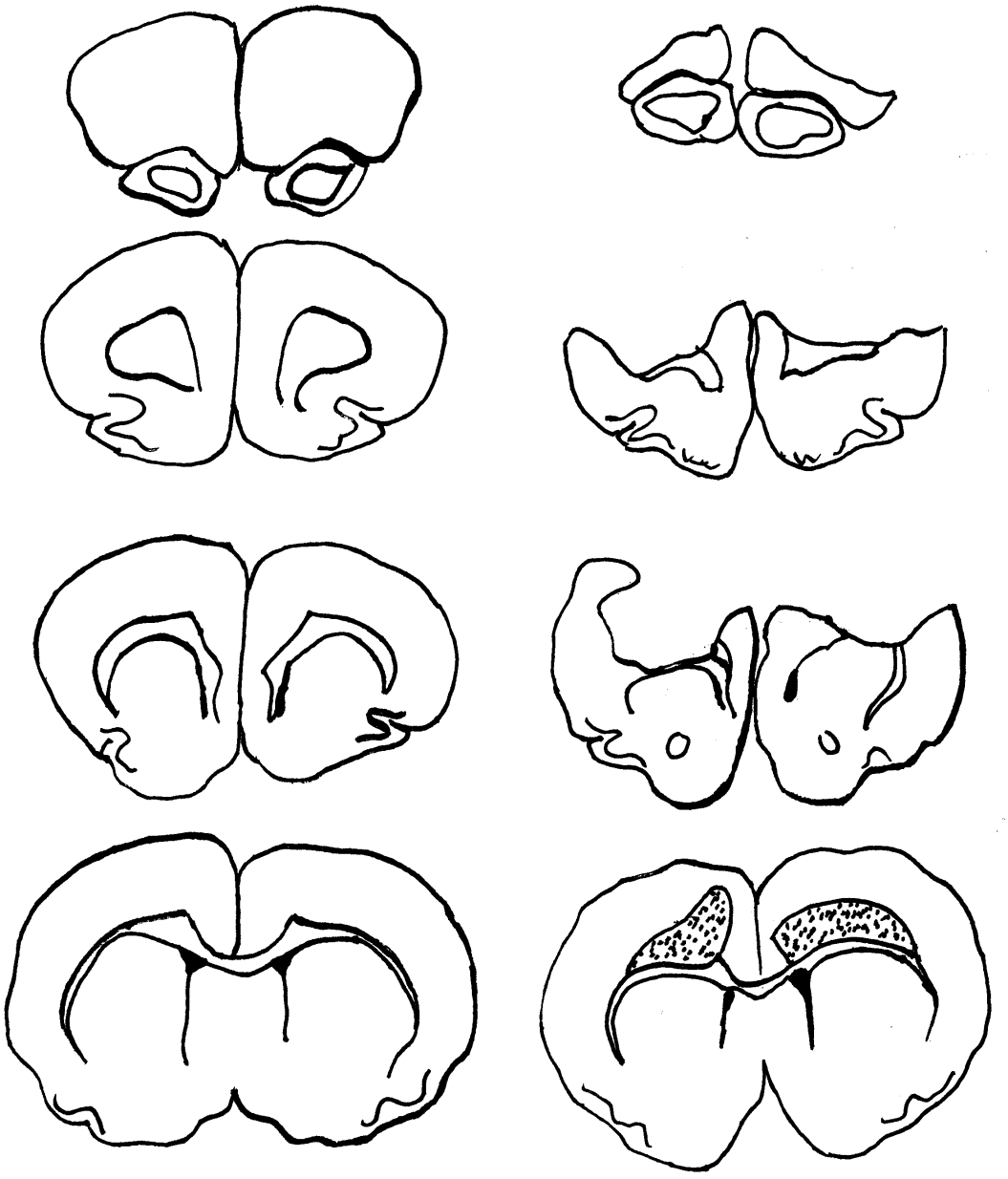

Figure 2.3. *Illustrative representation of normal brain at P23 (left) and brain at P23 with day 7 mPFC lesion (right).*

Behavioural Methods

Negative Geotaxis- Pups were tested on postnatal day 9 (P9) and 10. Pups were individually placed facing downward on a Plexiglas® board set to a 40° angle and filmed for 60 seconds. If the pup slid off the board, they were replaced in the downward position. Pups were scored for the amount of time they spent facing in an upward direction. A pup was considered to be in an upward position when its head crossed the horizontal plane.

Open Field- Pups were tested on P10-P13 and P15. Pups were individually placed in the centre of a transparent Plexiglas box measuring 16 x 20 x 30 cm. The base of the box was divided into roughly 130 squares measuring 2 cm x 2 cm. Pups were filmed for 60 seconds and scored for the total number of novel squares their front paws entered. Two analysts scored each video and the average score for each pup was used.

Sacrifice and Preparation

Cresyl Violet - At approximately 21 days of age, pups were given an overdose intra-peritoneal (I.P.) injection of sodium pentobarbital. They were then weighed and perfused with 0.9% saline solution followed by equal volume of 4% paraformaldehyde (PFA). The brains were then removed from the skull (cutting at the optic nerves ahead of the optic chiasm and removing the olfactory bulbs), weighed and preserved in 4% PFA for 1-2 days. Following preservation the brains are transferred to a solution of 30% sucrose in 4% PFA and stored until they were ready to be sectioned.

Golgi-Cox - At approximately 21 days of age, pups were given an overdose I.P. injection of sodium pentobarbital. They were then weighed, and perfused intracardially

with 0.9% saline solution. The brains were removed from the skull (cutting the optic nerves ahead of the optic chiasm and removing the olfactory bulbs), then weighed and preserved in Golgi-Cox solution for 14 days in the dark. After 14 days the brains were transferred to a 30% sucrose solution for a minimum of 3 days before they were sectioned.

Epigenetics - At approximately 21 days of age, pups were anesthetized with isoflurane, weighed and quickly decapitated. The brains were then removed from the skull (cutting the optic nerves ahead of the optic chiasm and removing the olfactory bulbs), and weighed. The frontal cortex and dorsal hippocampi of each pup was removed, immersed in RNAlater RNA Stabilization Reagent (Qiagen, Valencia), immediately flash frozen on dry ice and stored at -80°C.

Neuroanatomical Techniques

Stereology - Brains were removed from the sucrose/PFA solution, and 60 μm sections through the frontal cortex were cut with a cryostat. Each section was mounted on glass slides and stained with cresyl violet (1% cresyl violet acetate in distilled water). Following staining sections were dehydrated, cleared and cover-slipped with permount.

Neurons and glia were counted from 6 sections of Cg3, AID, and CA1 in the right and left hemispheres using a Zeiss Axio Imager M1. Stereo Investigator 9.03 was used to quantify cell numbers based on the Optical Fractionator method. Software parameters were set as: Counting frame- 40μm x 40μm; Grid size- 225μm x 225μm; Optical dissector height- 15μm; Section evaluation interval- 1; Section periodicity- 4; and remained constant throughout counting. Selection of neurons and glia for counting was

based on the following rules: 1) If the cell at any point touched the exclusion line, crossed the exclusion line or was outside the counting frame, it was not counted; 2) A cell was counted if it was inside the counting frame, touched an inclusion line from either inside or outside the counting frame, or crossed the inclusion line; 3) If a cell touched or crossed both the inclusion line and exclusion line, it was not counted; and 4) The cell had to be in focus within the virtual space to be counted. Cell counts were performed with a 100X objective.

Golgi-Cox - Brains were removed from the sucrose solution and cut at 200 μm with a vibratome. Sections were mounted onto gelatin coated glass slides and stained according to the procedures described by Gibb and Kolb (Gibb & Kolb, 1998). Pyramidal cells from region three of the anterior cingulate cortex (Cg3), the dorsal agranular insular cortex (AID), and CA1 of the hippocampus (Ziles, 1985) were chosen for analysis. Individual neurons were traced from Golgi-Cox stained brain sections using a camera lucida mounted on a microscope. A total of 10 cells, 5 from each hemisphere were traced at 250X from each brain region. Neuronal investigation included; basilar dendritic branch order (the number of branch bifurcations, an estimate of dendritic complexity), basilar Sholl analysis (the number of dendritic branches that intersect concentric circles spaced 25 μm apart, an estimate of dendritic length), as well as, spine density (the number of spine protrusions on a 10 μm segment of basilar dendrite at 1000X).

Epigenetic Techniques

Global Methylation - The brain tissue was removed from the -80°C freezer and permitted to thaw at room temperature. The tissue was transferred to a homogenization

buffer (buffer RTL) and immediately homogenized with a digital sonifier until the solution was clear. Genomic DNA and total RNA was extracted from each sample using the Qiagen Allprep DNA/RNA Mini Kit according to the manufacture's recommendations (Qiagen, 2005). A spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific) was used to determine the quality and quantity of DNA and RNA extracted from each sample. The total RNA was stored at -20°C until needed for gene expression analysis.

The genomic DNA was used for global methylation testing as previously described (Pogribny, Yi, & James, 1999). Briefly, the DNA is divided into 3 solutions. The first aliquot contains the DNA plus the restriction endonuclease *Hpa*II, which is sensitive to unmethylated CCGG sites. The second aliquot contains the DNA plus the restriction endonuclease *Msp*I, which is sensitive to CCGG in a methylation independent manner and leaves a 5'- guanine overhang. The third aliquot contains only DNA and serves as a control. The solutions are left to incubate for 16-18 hours at 37°C and are then prepared for polymerase chain reaction (PCR). The PCR master mix contains a radioactive dCTP, which will bind to the 5'- guanine overhangs during the extension cycle. Following PCR, samples are suspended on Watman Ion Exchange filter paper and the radioactive incorporation is measured using a liquid scintillation counter. The percent methylation is determined by comparing the amount of radioactive incorporation in the *Hpa*II aliquots to the radioactive incorporation in the *Msp*I aliquots. This can be demonstrated using the following equation:

$$1 - \{ (\text{HpaII} - \text{control}) / (\text{MspI} - \text{control}) \} \times 100$$

Gene Expression Analysis - Total RNA was extracted from brain tissue and quantified as mentioned above. Owing to financial limitations RNA samples were pooled for gene expression microarray analysis. Three samples were prepared for each experimental group (ie: experimental group = bystander stress-frontal cortex-males), for a total of 36 samples. Each sample contained RNA from 5 rats in that particular group (total number of rats sampled = 180). Effort was made to choose high quality RNA from rats of different litters. Hence, samples would contain RNA from 5 different rats, all of which had different mothers. The optimal starting concentration for the Affymetrix gene chip Rat Gene 1.0 ST is 100 ng/μl in a 100μl aliquot. An algorithm was generated in Microsoft Excel 2008, to ensure equal quantities of RNA were used from each of the 5 rats to generate the pooled sample. This would ensure equal representation in the pooled sample during gene expression analysis. Once pooled samples were generated from the 5 individual samples, they were quantified with nano-drop, and shipped on dry ice over-night to Genome Quebec in Montreal.

Gene expression analysis was carried out at McGill University using the Affymetrix GeneChip Rat Gene 1.0 ST. This microchip provides gene level analysis based on multiple probes of different exons across the whole gene rather than just the 3'end. Hence, the microchip contains 722,254 probes, generated from ~25 oligonucleotide sequences, which represent 27,342 well-annotated genes within the rat genome (Affymetrix, 2007). The McGill University and Genome Quebec Innovation Centre completed platform analysis of the 36 samples and provided data in the form of CEL files. These files contained information regarding gene expression levels for the 36 samples at all 27,342 genes.

FlexArray, a statistical data analysis software program designed specifically for use with gene expression microarray data (Blazejczyk, Miron, & Nadon, 2007); along with R, the open-source language for statistical computing program, were used to analyze the abundant amount of data obtained from the GeneChip platform. Statistical tests were only run on normalized data. The computational programs were able to run two-way ANOVAs to examine the effect of stress and sex on gene expression level changes. As gene expression microarray analysis generates an exuberant number of tests, the chances of encountering a type I error greatly increase. In order to account for this, a *false discovery rate* algorithm (Benjamini & Hochberg, 1995) was run by FlexArray in combination with R. The *false discovery rate* increases the probability that the observed gene expression changes are a result of the experimental manipulations, rather than chance.

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Chapter 3: Behavioural Changes

Introduction

Why study behaviour? Behaviour is the principal function of the brain and changes in the brain are often manifest as changes in behaviour (Whishaw & Kolb, 2005). Through meticulous observation and manipulation of behavioural tasks it is possible to learn a great deal about normal and abnormal brain functioning. In many cases, subtle changes in behaviour, detected only as a result of carefully designed tests, reflect dramatic changes in the brain.

Why study laboratory rats? Rats are behavioural generalists, as are humans. They have proved to be adaptable and successful in virtually all environmental conditions (Whishaw & Kolb, 2005). Additionally, the behaviour of the rat is extremely complex and consequently so is the cortical organization. Most structures of the rat brain are remarkably qualitatively similar to that of humans. Through study of the relationship between rat behaviour and brain it is possible to make accurate inferences about human functioning without the moral constraints of human testing.

The following chapter will address the behavioural aspect of this thesis. Dam and offspring behaviours were observed and analyzed in attempt to determine if various prenatal stress paradigms were capable of altering behaviour and brain.

Timeline

The time period for prenatal stress and postnatal testing were chosen because of their applicability to specific brain development milestones. Gestational days 12-16 (G12-G16) were chosen for stressing because this is a period of major neurogenesis and gliogenesis in all brain regions. See figure 3.1. Furthermore, glucocorticoid receptors have been found in most regions of the brain by gestational day 13 allowing maternal

stress hormones to influence brain development from this time period and on (Meaney, et al., 1985). Surgery was performed to administer mPFC lesions on P8 because this time period is associated with moderate recovery from brain injury. Moderate recovery permits investigation of the relationship between the prenatal experience and the lesion. Pups receiving lesions earlier (~P3) show little or no recovery, making it difficult to examine additive effects that might worsen the outcome, as the offspring are already terribly impaired. Likewise, pups receiving lesions later (~P10) demonstrate full recovery with modest impairment, making it equally as difficult to observe measurable effects that might enhance recovery. Animals with lesions on P8 could be expected to show additive effects leading to impairments or enhanced recovery. Lesions were only performed on NS and BS pups due to scheduling conflicts with the surgeon. Furthermore, early analysis did not reveal the hypothesized additive effect of stress and mPFC lesion so the procedure was abandoned. Postnatal days 9-15 were chosen for behavioural analysis because this time period is correlated with infancy in humans, a developmental stage characterized by rapid learning, synaptogenesis and brain plasticity. For future use of remedial efforts, it was important to determine if behavioural deficits related to prenatal stress could be identified early. The open field and negative geotaxis tasks were chosen based on their identified ability to discriminate between treatment and control groups (unpublished data from the Gibb lab)

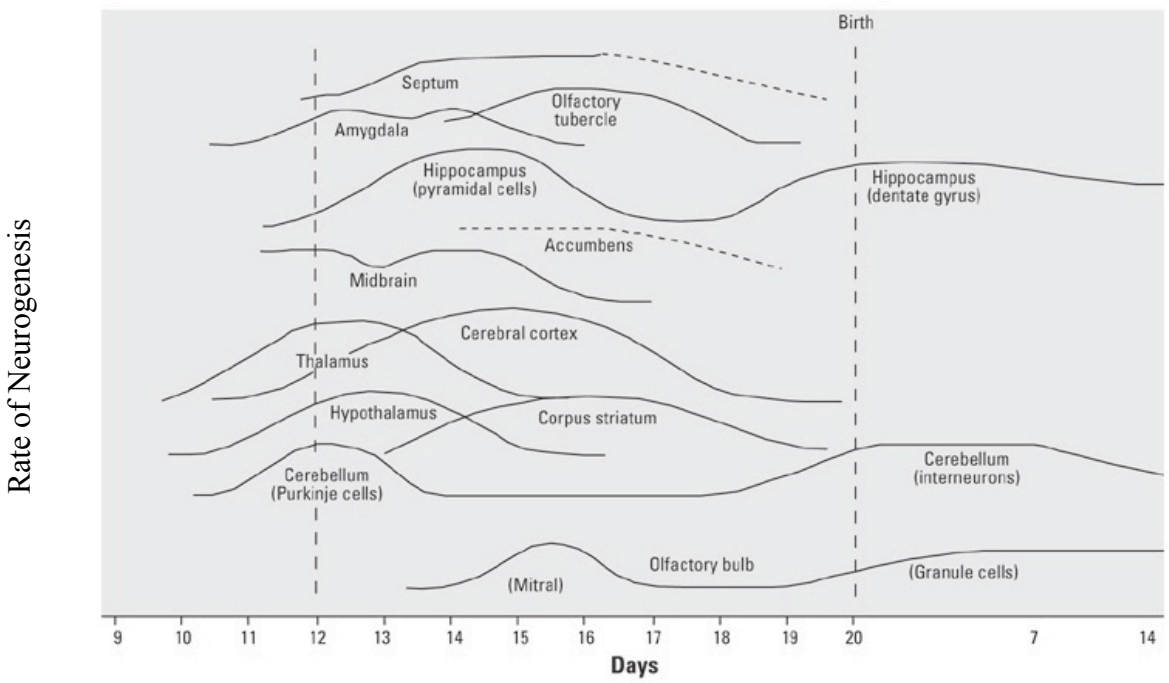

Figure 3.1. *Developmental time line of gliogenesis in major brain areas of the rat. Figure reproduced from Rodier (1977)*

Ultrasonic Vocalizations

Adult rats are known to produce 2 distinctively separate USVs: a low frequency, 22 kHz call and a high frequency, 50 kHz call. Research has demonstrated that low frequency USVs are emitted under distressing situations and generally represent negative affective states (Knuston, Burgdorf, & Panksepp, 2002). Low frequency calls are often produced when rats are placed in fearful situations or when rats are forced into subordinate positions by dominant rats (Sokoloff & Blumberg, 2005). Conversely, high frequency USVs are assumed to represent positive affective states associated with reward or joy (Knuston, et al., 2002). They are often produced in anticipation of play or during reproductive behaviour (Knutson, Burgdorf, & Panksepp, 1998). By recording dam USVs immediately after the stress procedure it is possible to make inferences about their affective states.

Negative Geotaxis

Although the processes involved in the negative geotaxis task are not fully understood it continues to be used as a milestone for early sensorimotor development in rats. Negative geotaxis is generally considered a motor task (Alberts, Motz, & Schank, 2004; Patin, Lordi, & Caston, 2004) and can be learned implicitly over time. It is hypothesized that negative geotaxis depends on 1) vestibular and proprioceptive detection, 2) processing of the vestibular and proprioceptive inputs by the central nervous system and 3) motor competence to change orientation (Alberts, et al., 2004). Independent of the processes required to complete the task, stark differences can be demonstrated in offspring abilities and these differences can be used to discriminate between experimental treatments.

Open Field

The open field test has been utilized to assess behavioural responses such as locomotor activity (Ivinskis, 1970), exploration (Patin, et al., 2004), hyperactivity (Gentsch, Lichtsteiner, Frischknecht, Feer, & Siegfried, 1988), and anxiety or fear (Perrot-Sinal & Petersen, 1997). Rats tend to avoid bright, novel, open spaces and the open field box simulates these environmental conditions. By monitoring the extent of their activity and the places they travel in the box valuable insight related to the effects of prenatal stress on offspring behaviour can be ascertained. For the purpose of this thesis, the open field was used as a test of locomotor activity and exploratory behaviour.

Results

Dams and Litters

Dams were weighed daily from preconception until the day they gave birth. Dams receiving extreme stress (ES) gained less weight through their pregnancy when compared to no stress (NS) dams. Conversely, dams in the mild stress (MS) group gained more weight through pregnancy than their NS counterparts. There was no significant difference in weight gain for bystander stress (BS) and NS dams (See Figure 3.2). A one-way ANOVA for dam weight gain with stress level as a factor revealed main effect of stress, $F(1, 25) = 3.636$, $p = .029$.

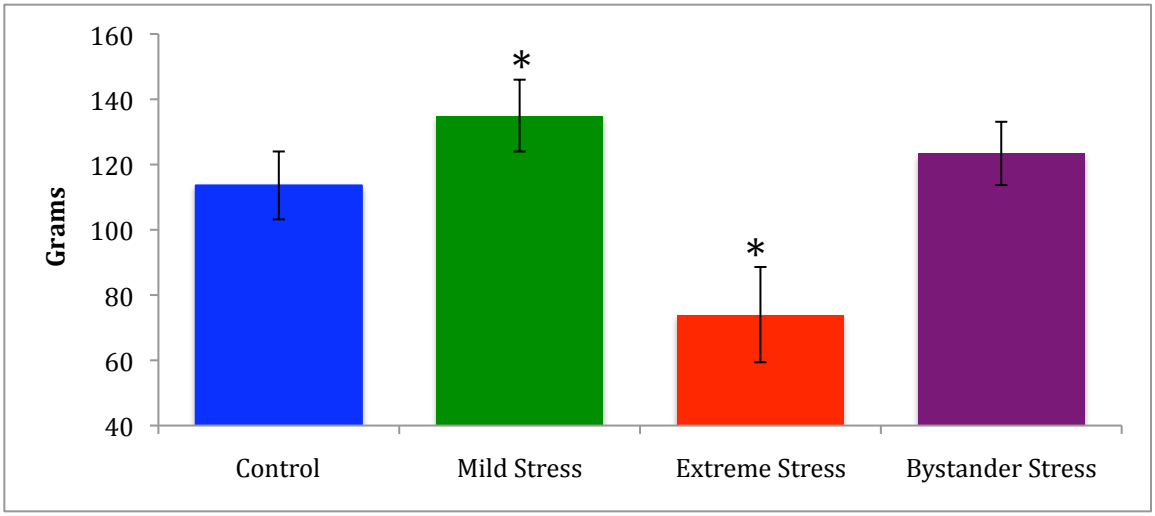

Figure 3.2. *Average weight gain by pregnant mothers from G0-G21. (*p < .05)*

Stress did not result in significant differences in dam litter sizes, $F(1, 25) = 2.258$, $p = .110$, nor did it have an effect on the ratio of male to female offspring, $F(1, 25) = .848$, $p = .483$. There were a total of 335 (169 male, 166 female) pups born to 26 different females (8 NS, 5 MS, 4 ES, and 9 BS). Seven pups died before they could be sacrificed; this was random and unrelated to any particular stress protocol. This resulted in the sacrifice of 328 pups (166 male, 162 female).

Variations in prenatal stress affected offspring body weights at P21. Male and female offspring born to MS mothers demonstrated a significant decrease in body weight while female offspring of ES mothers exhibited a substantial increase in body weight when compared to no stress offspring. BS did not have a significant effect on body weight for male or female offspring (See Figure 3.3 for male offspring, Figure 3.4 for female offspring). A two-way ANOVA with sex and stress as factors revealed a main effect of stress, $F(3, 291) = 14.997$, $p < .0001$, but not of sex, $F(1, 291) = 2.999$, $p = .084$. The interaction was not significant.

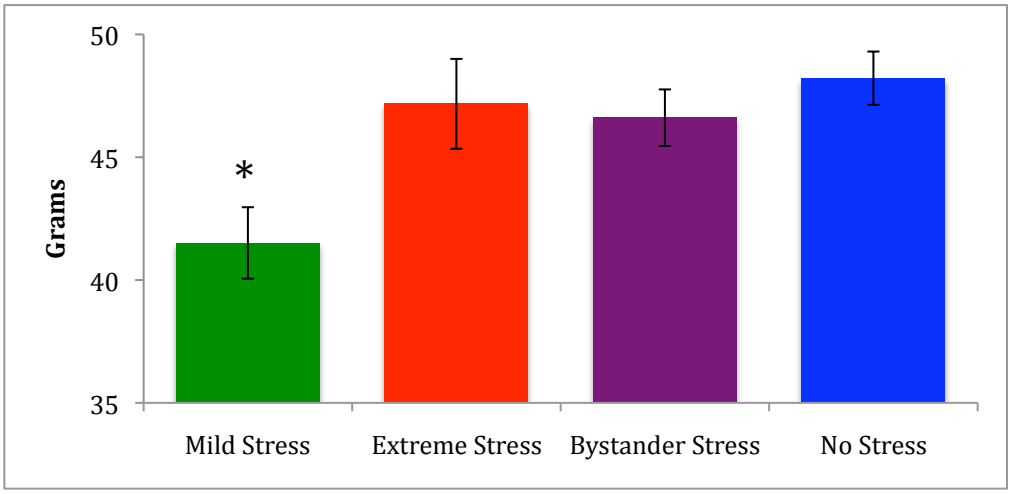

Figure 3.3. *Mean body weight of male offspring at the time of sacrifice (P21) (*p < .001)*

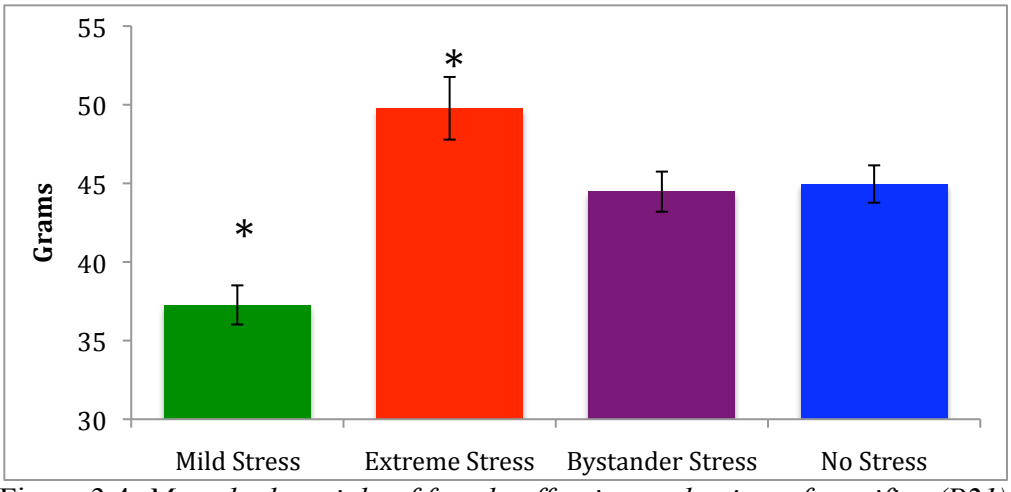

Figure 3.4. *Mean body weight of female offspring at the time of sacrifice (P21) (*p < .001)*

Body Weight – Lesion

Analysis of body weight for the subset of pups in the lesion study produced an interesting finding. Prenatal stress and sex still produced significant differences in body weight, but the mPFC lesion had no effect on body weight. A three way ANOVA with sex, stress and lesion as factors revealed a main effect of sex, $F(1, 155) = 4.070$, $p = .045$, and stress, $F(1, 155) = 6.551$, $p = .011$, but not of lesion, $F(1, 155) = .240$, $p = .625$. None of the interactions were significant (p's > .050).

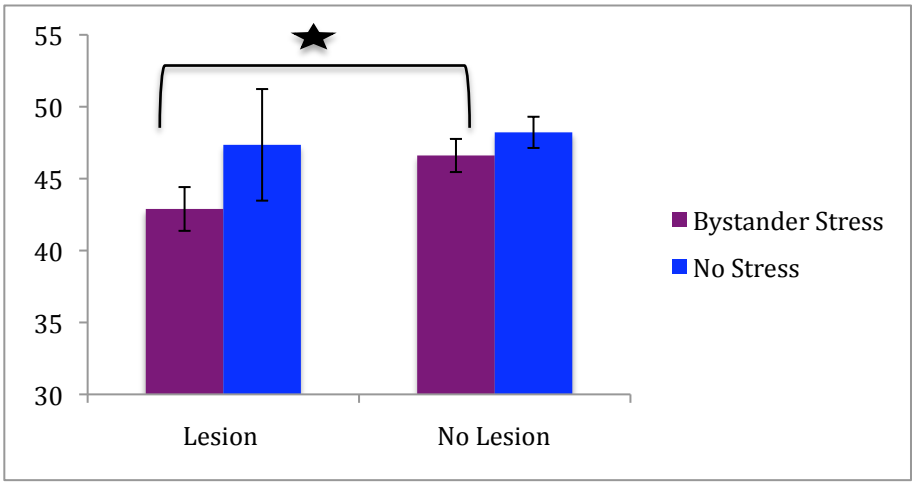

Figure 3.5. *Mean body weight of male offspring with lesions and prenatal bystander stress at time of sacrifice (P21)(*p < .05)*

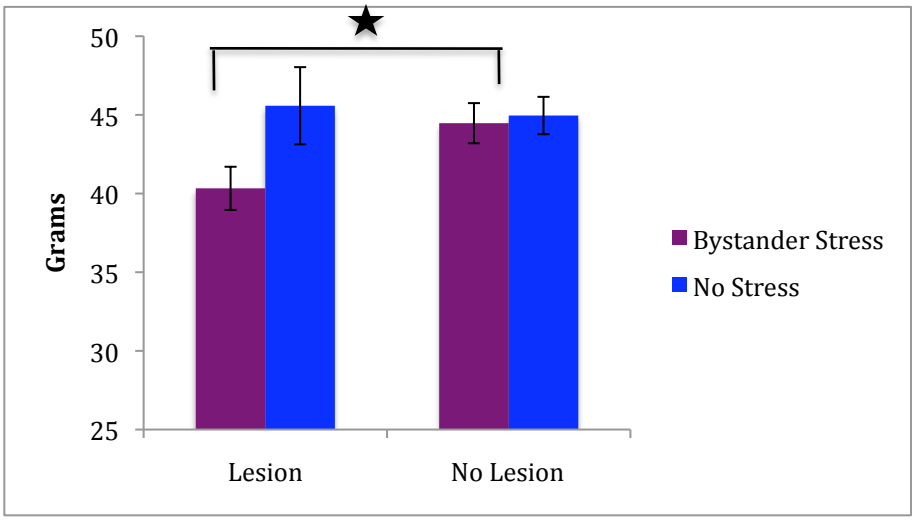

Figure 3.6. *Mean body weight of female offspring with lesions and prenatal bystander stress at time of sacrifice (P21)(*p < .05)*

Ultrasonic Vocalization

There were no significant differences in high frequency or low frequency calls between any of the females before the stressing procedures commenced (data not shown). However, following the BS protocol, the stressed females exhibited a significant increase in the number of low frequency calls (See Figure 3.7) and the unstressed *bystanders* demonstrated an increase in high frequency calls (See Figure 3.8). A univariate ANOVA of the low frequency calls demonstrates a main effect of stress level, $F(2, 9) = 4.601$, $p = .042$. A univariate ANOVA of the high frequency calls exhibits a trend towards a main effect of stress, $F(2, 9) = 3.302$, $p = .084$, however post hoc analysis of the relationship between the bystander and stressed females reveals a significant difference $p = .032$.

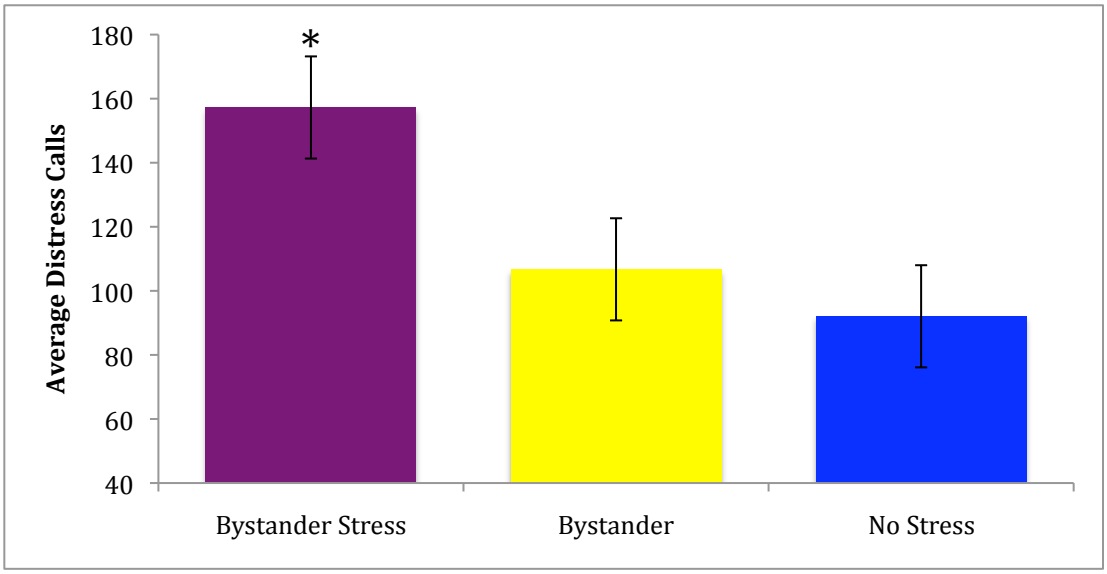

Figure 3.7. *Average number of distress calls recorded in the 10 minute period following the stress protocol (*p < .05)*

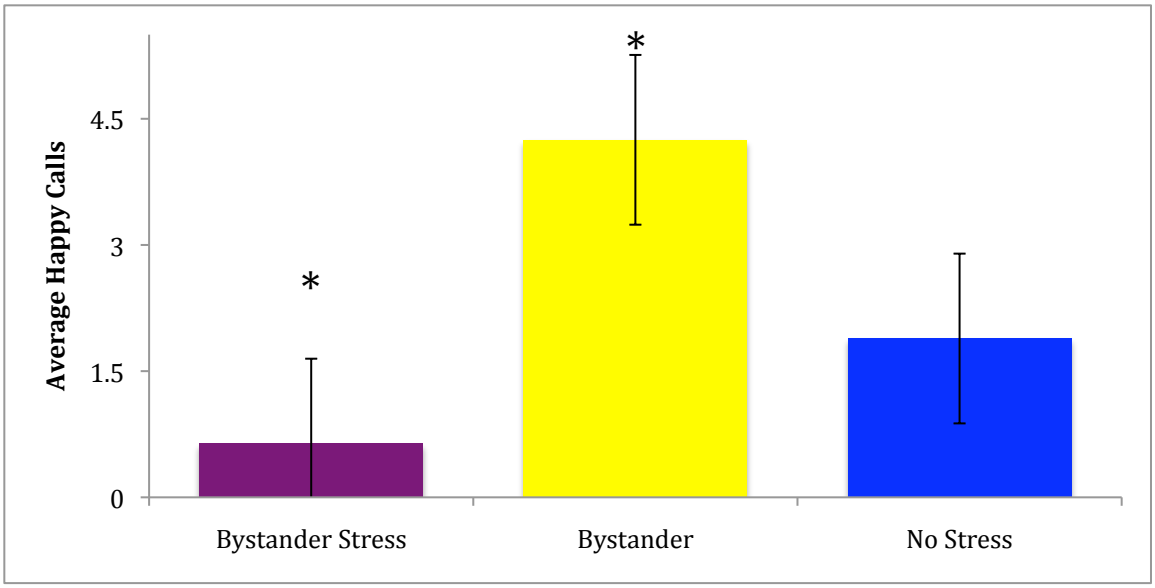

Figure 3.8. *Average number of happy calls recorded in the 10 minute period following the stress protocol (*p < .05)*

Negative Geotaxis

Analysis of the negative geotaxis behaviour demonstrated that male and female offspring born to ES mothers were not different from NS offspring on P9, however they failed to show the same learning as NS offspring on P10. Conversely, offspring born to MS and BS mothers demonstrated the same learning curve as the NS offspring but due to their significant initial deficits they were slightly behind NS offspring on P10 (Figure 3.9 & 3.10). A two way ANOVA with stress and sex as factors illustrated a main effect of stress, $F(3, 243) = 15.564$, $p < .000$, but not of sex, $F(1, 243) = .148$, $p = .701$, for P9. The interaction was not significant. For P10, a two way ANOVA with sex and stress as factors also demonstrated a main effect of stress $F(3, 243) = 11.584$, $p < .000$, but not of sex, $F(1, 243) = .094$, $p = .759$, The interaction was also not significant on P10.

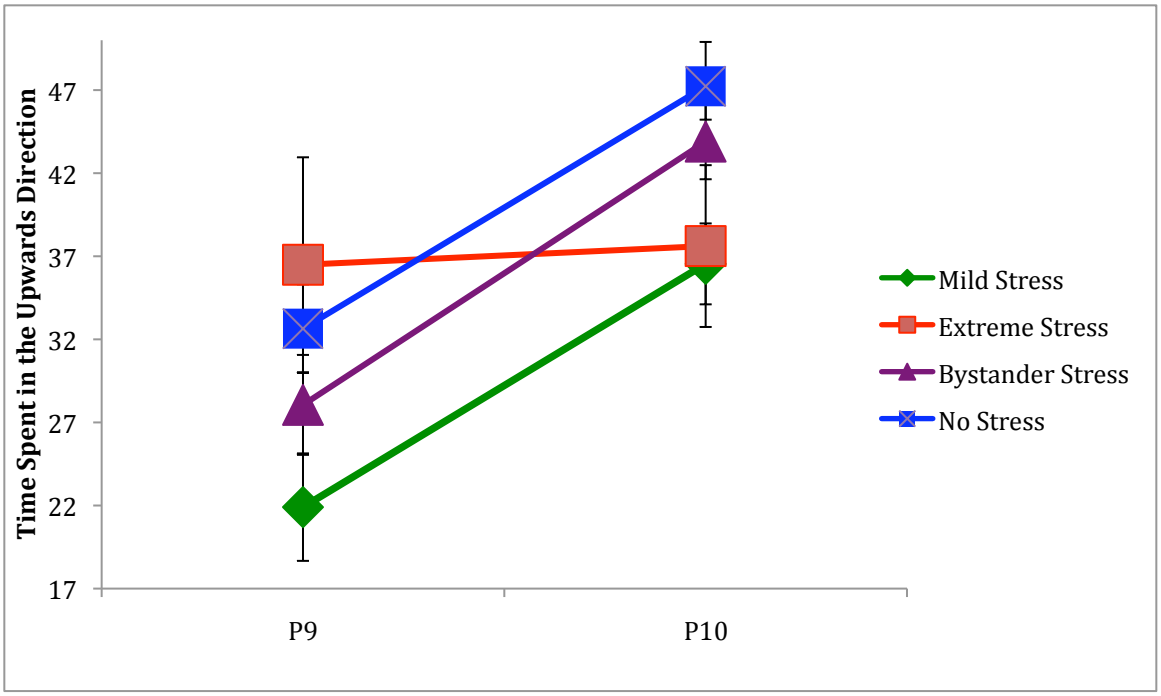

Figure 3.9. *Negative geotaxis scores for male offspring on P9 and P10*

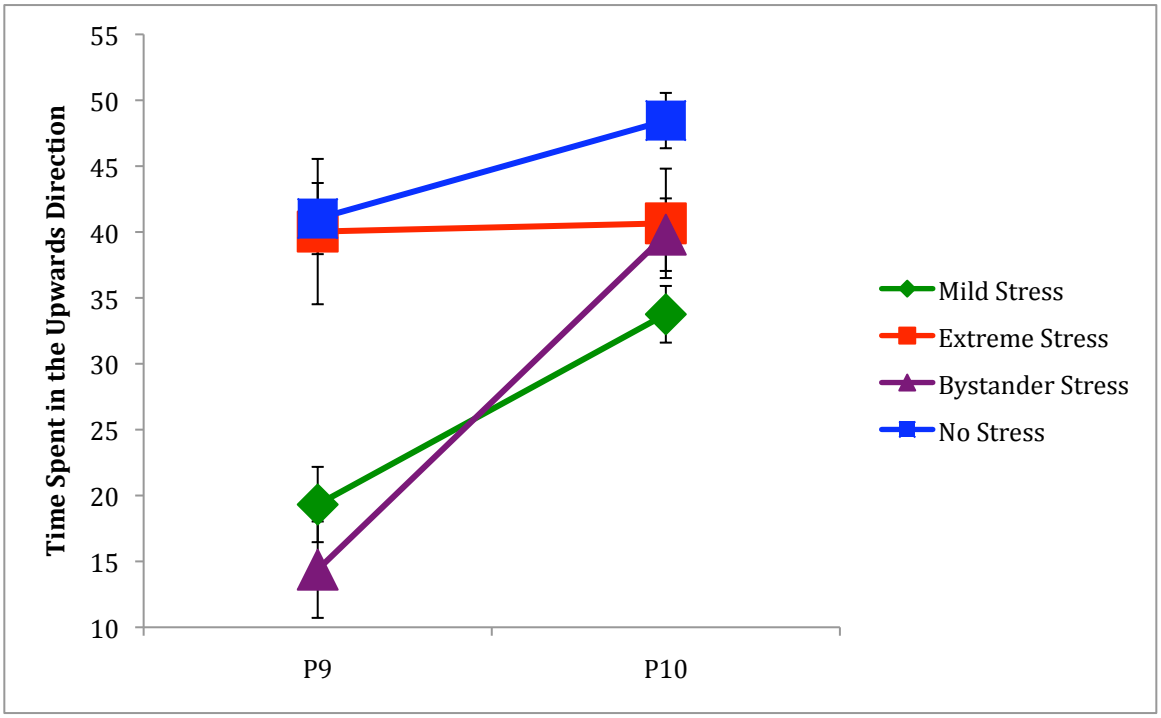

Figure 3.10. *Negative geotaxis scores for female offspring on P9 and P10*

Negative Geotaxis – Lesion

The mPFC lesion did not have an effect on the offspring's ability to complete the negative geotaxis task. BS offspring demonstrated the same learning curve as NS offspring but were significantly impaired at the task. There was not a cumulative effect of BS and mPFC lesion (Figure 3.11 & 3.12). A three way ANOVA with sex, stress and lesion as factors revealed a main effect of stress, P9: $F(1, 188) = 9.417$, $p < .000$, P10: $F(1, 188) = 11.584$, $p < .000$, but not of sex or lesion. None of the interactions were significant for P9 or P10.

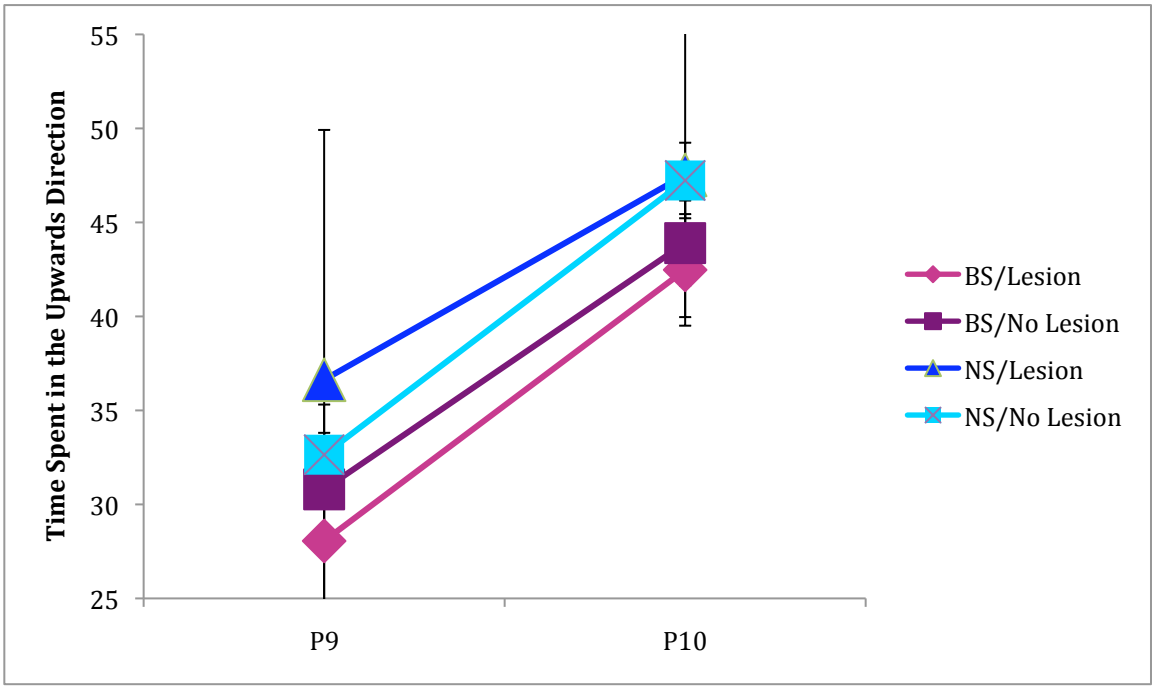

Figure 3.11. *Negative geotaxis scores for male offspring with lesions and prenatal bystander stress on P9 and P10*

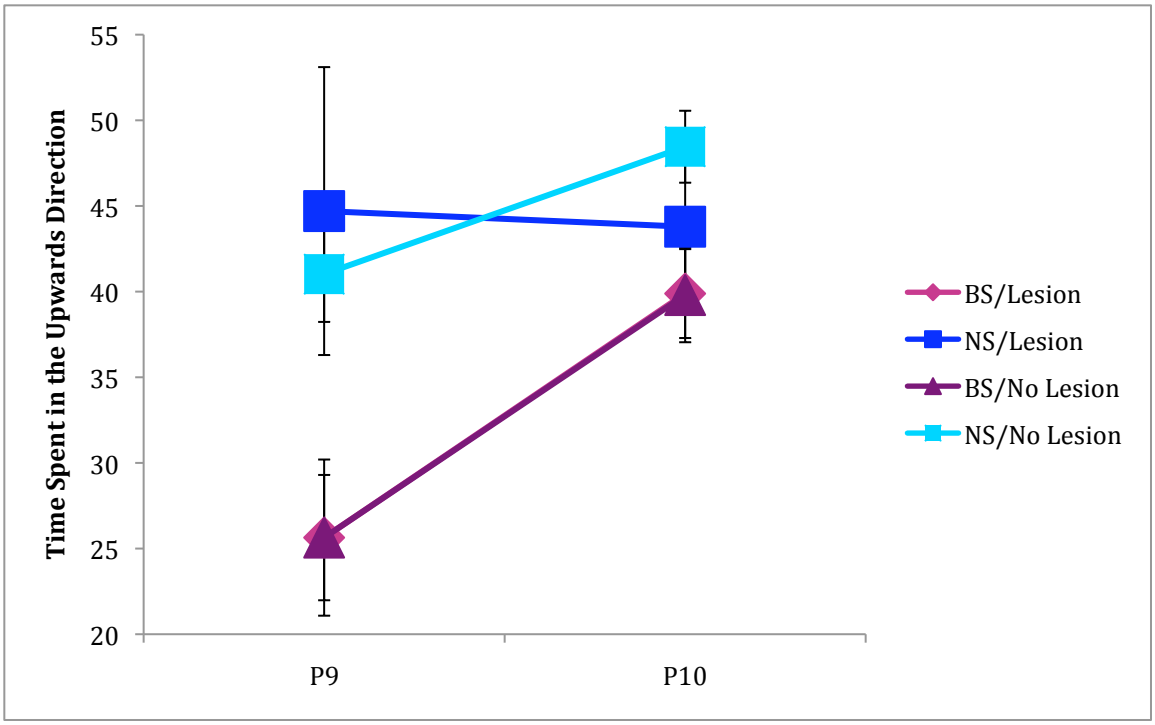

Figure 3.12. *Negative geotaxis scores for female offspring with lesions and prenatal bystander stress on P9 and P10*

Open Field

Male offspring exposed to prenatal MS exhibited a reduction in the number of novel fields entered when compared to NS offspring. Conversely, males born to ES mothers exhibited an increase in the number of novel fields entered over the 5 days of testing. Female offspring, however, demonstrated a decrease in the number of novel fields entered when their mothers experienced either BS or MS. A two way repeated measures ANOVA with sex and stress as factors for the five days of testing revealed a main effect of sex, $F(1, 193) = 13.929$, $p < .001$, and stress, $F(3, 193) = 12.646$, $p < .001$. The interaction was also significant, $F(1, 193) = 3.748$, $p = .012$.

Open Field – Lesion

When total number of novel fields entered is summed across all 5 days, the activity level of animals is not affected by lesion (See Figure 3.17 & 3.18) . A three-way

ANOVA with lesion, sex, and stress as factors showed a main effect of stress, $F(1, 191) = 23.835$, $p < .001$, but not of lesion, $F(1, 191) = 1.485$, $p = .226$, or sex, $F(1, 191) = .280$, $p = .598$. None of the interactions were significant (p's < .05).

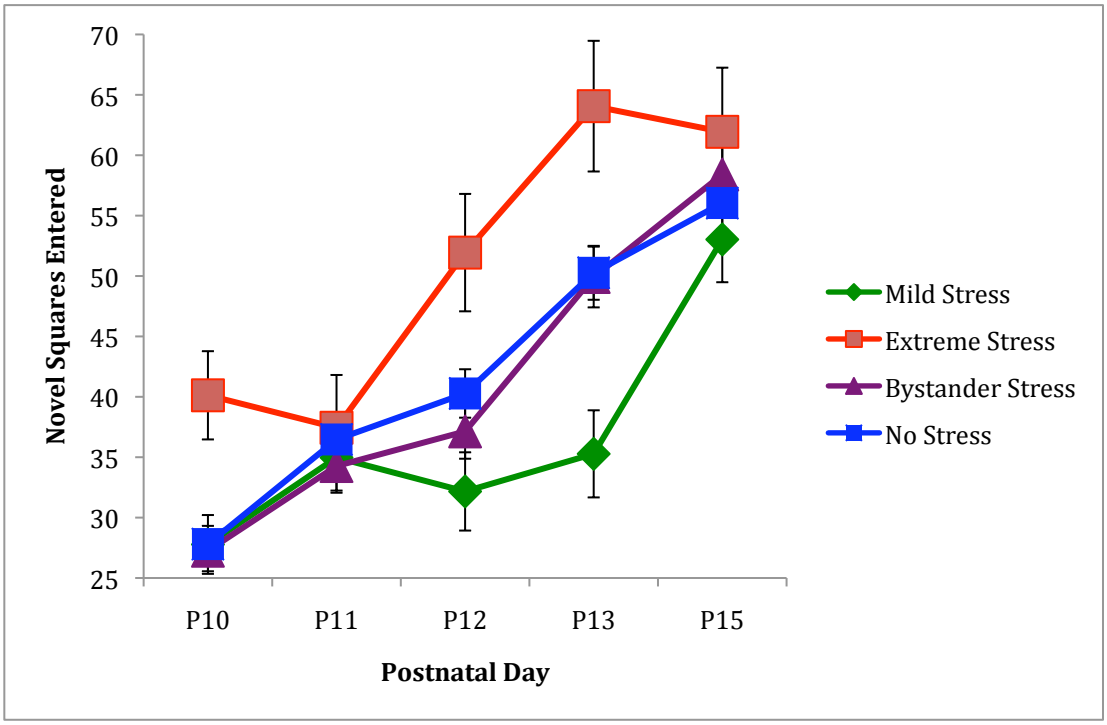

Figure 3.13. *Average number of novel fields entered by male offspring on P10-P13 and P15*

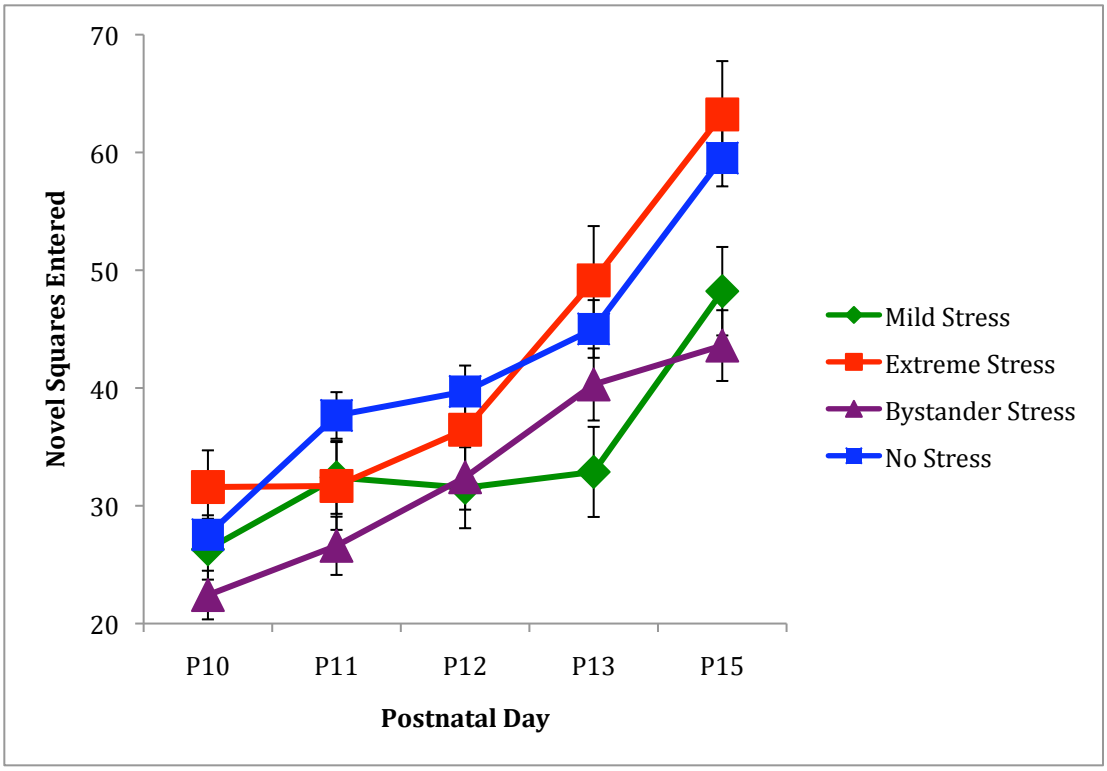

Figure 3.14. *Average number of novel fields entered by female offspring on P10-P13 and P15*

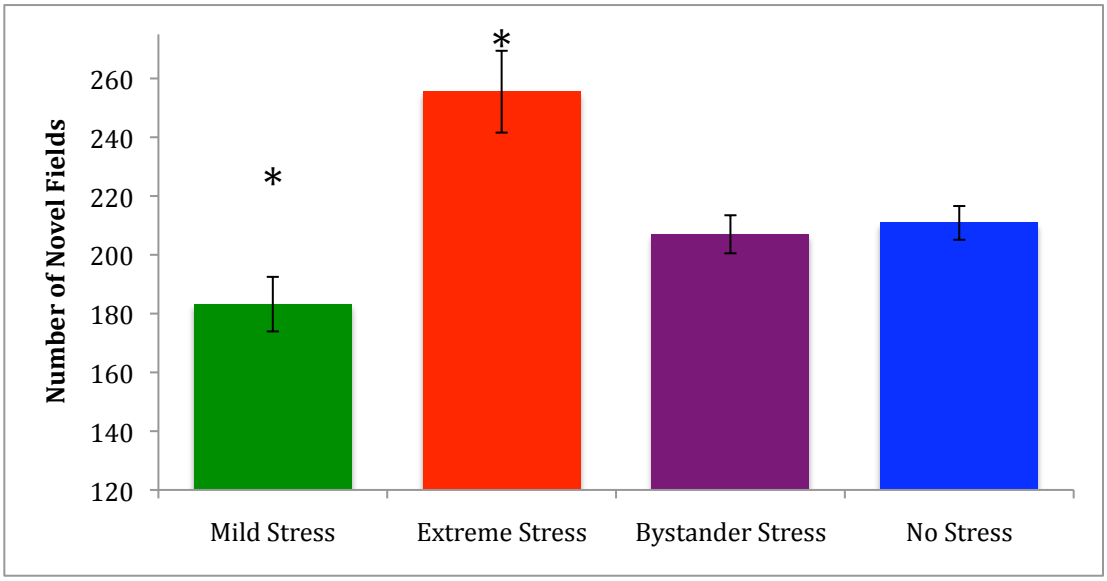

Figure 3.15. *Average number of novel fields entered for male offspring over the 5 testing days (*p < .001)*

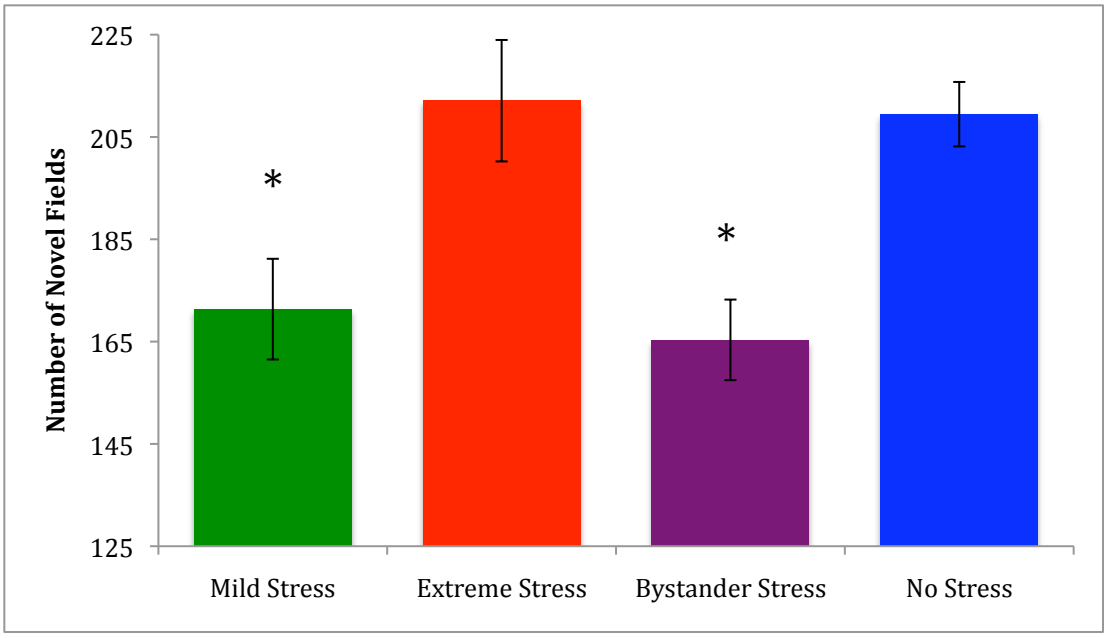

Figure 3.16. *Average number of novel fields entered for female offspring over the 5 testing days (*p < .001)*

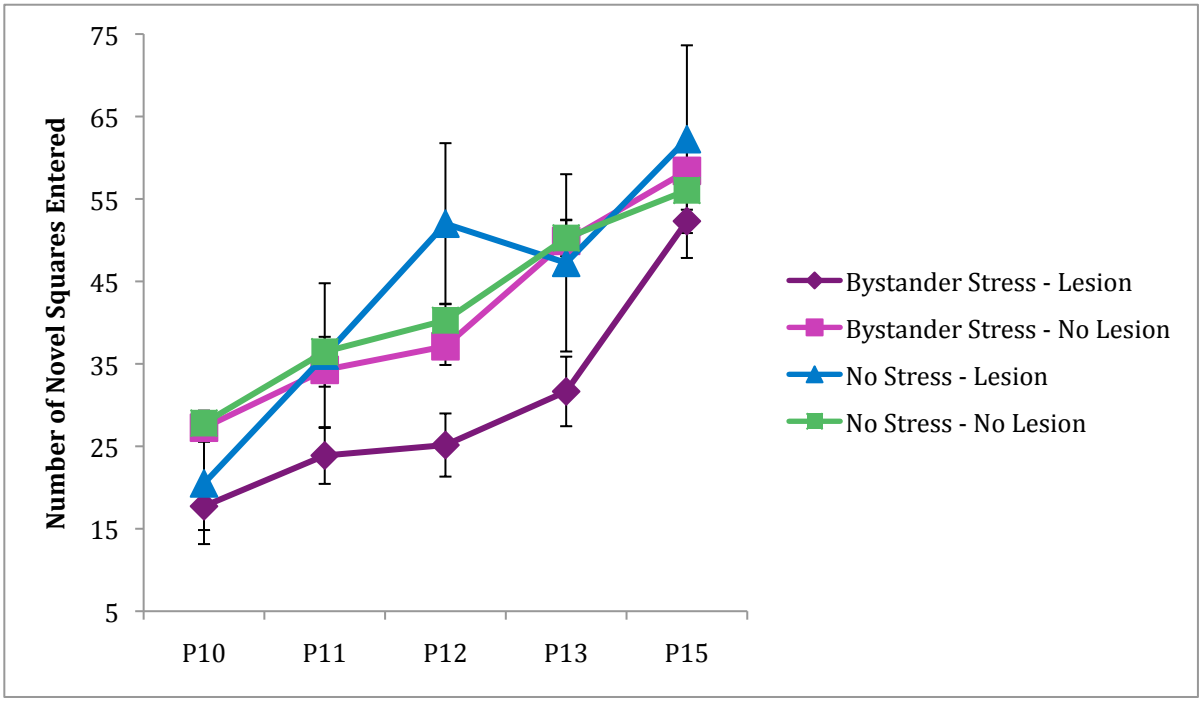

Figure 3.17. *Average number of novel squares entered for male offspring with lesions*

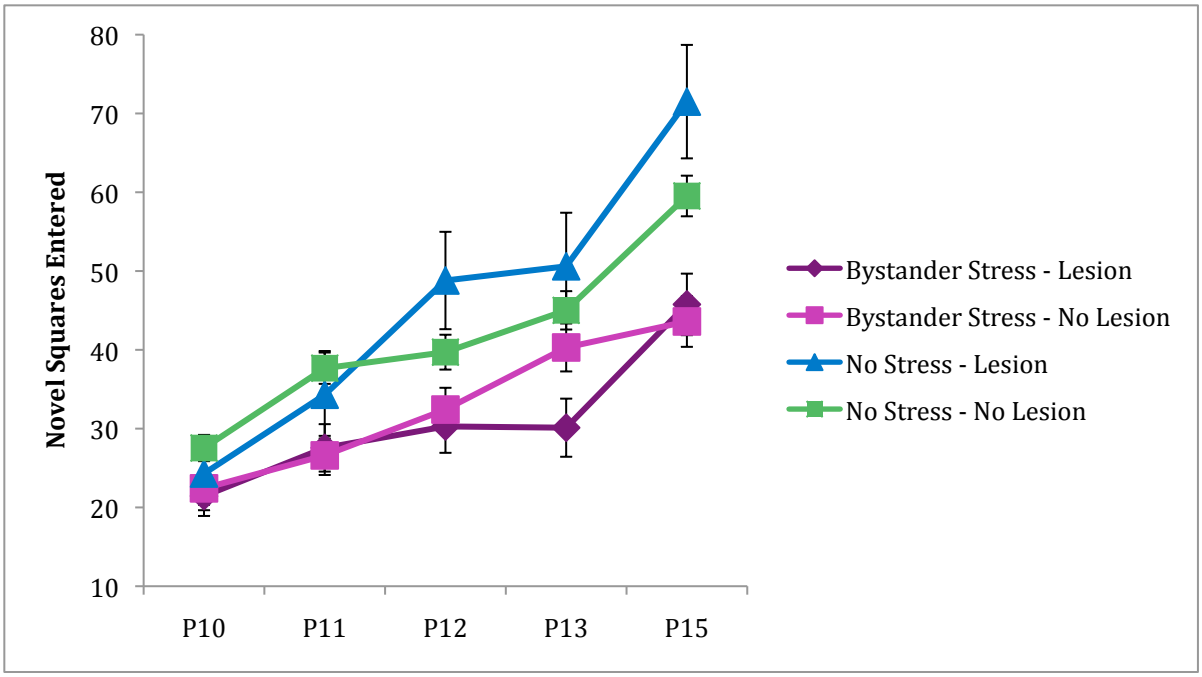

Figure 3.18. *Average number of novel squares entered for female offspring with lesions*

Discussion

This chapter clearly demonstrates that not all prenatal stress is equal; at least with regards to pre-weaning behaviour. Variations in the prenatal stress paradigm produced time-dependent, sex dependent, and stress-dependent behavioural changes in offspring. It is particularly fascinating that behavioural changes can be identified in offspring whose mothers were not even directly stressed (BS). These changes in offspring behaviour likely result from a combination of epigenetic changes and behavioural changes of the mother.

Although maternal behaviour was not monitored throughout pregnancy and weaning, inferences can be made from the ultrasonic vocalization recording and changes in weight gain. As there were no differences in ultrasonic vocalizations at baseline, the stressing procedure appears to have altered communication between cage-mates. It was

not surprising that the stressing procedure significantly increased the number of low frequency calls emitted by the stressed rats and this is consistent with other research (Blanchard, Blanchard, Agullana, & Weiss, 1991). What was surprising however, was the significant increase in high frequency calls produced by the bystander females (the dams living with the stressed rats). Because bystander females did not show elevated levels of high frequency calls at baseline, it can be interpreted that bystanders were attempting to soothe or relax their stressed cage-mate (Taylor, Klein, Lewis, & Gruenewald, 2000). As there were no changes in vocalizations of the no stress controls upon return to their partners following a 30-minute separation without stress, this increase in high frequency calls is unlikely due to the excitement of merely returning of an absent cage-mate. Communication was not the only maternal characteristic modified by the stress protocols, weight gain during pregnancy was also changed.

Given that two female rats were housed in a single cage it was impossible to determine if changes in weight gain were the result of modifications to eating habits or changes in metabolism. However, there is a strong inverse correlation between dam weight gain and offspring weight at sacrifice. Dams experiencing MS exhibited a significant increase in weight gain, while their offspring (both male and female) were substantially lighter than their NS compliments at P21. Conversely, dams in the ES group demonstrated a dramatic reduction in weight gain through out pregnancy and their female offspring exhibited increased weight at time of sacrifice. Finally, BS dam weight gain was not different from controls nor was weight gain in their offspring.

Offspring behaviour was evaluated with 2 assessments over 6 days. The negative geotaxis task was evaluated on two days (P9-P10) and provided fascinating results. As

the task involves righting of the body to orient to the upward position it can be inferred that the rat pup can learn to do this more efficiently from trial 1 to trial 2. Both male and female pups born to BS mothers and MS mothers were able to learn the task with the same efficiency of NS pups. However, there was such a deficit in their initial ability that they were unable to reach the performance of NS pups at trial 1 or trial 2. There is evidence that prenatal stress slows the maturation of the nervous system in the developing offspring (Meek, Burda, & Paster, 2000; Patin, et al., 2004). The initial deficit seen in pups born to BS and MS mothers may merely be the result of delayed maturation. Conversely, all pups born to ES mothers performed equally as well as NS pups on trial 1, but failed to show any learning on trail 2. Due to this inability to learn the task, ES pups appeared to have no deficit on trial 1 but were significantly impaired at trial 2. In summary, BS and MS pups were able to learn the task but started so far behind they were unable to catch up, while ES pups showed no deficit in initial ability but were unable to improve. The results of this task demonstrate that there can be variation in the behavioural impairment on a single task and the use of sensitive well-designed tests are necessary for effective behavioural analysis of rat pups.

The second behavioural task, open field, was evaluated on 5 days (P10-P13 & P15). The open field task assesses the balance between a rat's innate curiosity and their fear of bright open spaces. An increase in activity can be inferred to demonstrate hyper-activity related to hyper-reactivity and increased sensitivity to the environment (Gentsch, et al., 1988; Pijlman, Wolterink, & Van Ree, 2003), whereas a decrease in activity is generally believed to represent increased anxiety (Perrot-Sinal & Petersen, 1997) or a loss of motivation to explore (Patin, et al., 2004). Locomotor activity of male offspring

was only affected by direct stress (MS or ES) to the mother; BS had no effect. Male pups born to ES mothers exhibited an increase in activity. The high degree of prenatal stress may have increased the sensitivity and emotionality of these pups to a novel environment. This notion is supported by the observation that the hyperactive response is most pronounced on the first day of open field testing and is somewhat tempered by the last day. Conversely, male pups born to MS mothers showed a reduction in activity likely related to lowered exploratory motivation (Patin, et al., 2004). With the exception of testing day 1, locomotor activity was not affected for females born to ES mothers. Female offspring in this group likely exhibit the same heightened sensitivity to novel environments as their male siblings but extinguish this reaction by day 2. However, females born to MS and BS mothers both showed a reduction in locomotor activity. These 2 prenatal stress paradigms decreased exploratory behaviour and may have lowered offspring emotionality (Ivinskis, 1970). Research carried out in other laboratories have identified reductions in spontaneous locomotion (Patin, et al., 2004), whereas others have found increases in activity for offspring exposed to prenatal stress (Gue, et al., 2004; Vallee, Mayo, Le Moal, Simon, & Maccari, 1997). Pijlman and colleagues (2003), have also found opposing results in locomotor activity for rats exposed to physical versus emotional stress. It is clear from the research conducted for this dissertation that discrepancies in the actual stress experienced by the mother may account for the variations in open field behaviour of the offspring.

Lesion Study

It was hypothesized that there would be an additive effect for offspring of having a mPFC lesion and prenatal BS. This was true for some aspects of offspring behavioural

development but not for others. An additive effect was seen in the reduction of offspring body weight for both males and females with prenatal BS and a mPFC lesion. Offspring with a mPFC lesion and BS showed a significant reduction in body weight when compared to offspring with only a lesion, only BS, or neither. The lesion had no effect on offspring performance on the negative geotaxis task regardless if they were exposed to BS or not. Offspring with lesions were not different from shams on this task. The lesion did however, have an effect on offspring locomotor activity. For female offspring of BS dams, receiving a mPFC lesion was confounding; the lesion reduced the already decreased locomotor activity of BS offspring. This is contradictory to the finding that female offspring who received a mPFC lesion without prenatal stress exhibited an increase in locomotor activity. A lesion alone had no effect on locomotor activity of male offspring. However, mPFC lesion in combination with prenatal BS, lowered emotionality and the motivation to explore, as inferred by the reduction of locomotor activity of male offspring (Ivinskis, 1970; Patin, et al., 2004). This was an even greater reduction in activity than that observed in animals exposed to BS alone. As the lesions were localized to the mPFC and the offspring without prenatal BS did not show a reduction in activity following lesion, the hypoactivity is unlikely the result of damage to motor regions of the brain. It is more probable that the lesion in combination with the prenatal stress is influencing the motivational and stress systems.

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Chapter 4: Neuroanatomy

Introduction

If the brain is to change in response to experience, changes will have to occur at the cellular level. Neural modifications associated with experience include: changes in brain size and weight, variations in dendritic branching and length, alterations in spine density, and changes in neuron, glia, or synapse numbers (Kolb & Whishaw, 1998). The neuroanatomical aspect of this thesis was designed to investigate the consequences of various prenatal stress paradigms on offspring brain development. In addition to quantifying changes in cell numbers, it was also important to know how the morphology of cells had changed. Therefore, stereological techniques were used in conjunction with Golgi-Cox methods in attempt to generate a 'bigger picture' of brain change.

Stereology

Stereology is an interdisciplinary field that is primarily concerned with the three-dimensional interpretation of planar sections. In essence, higher dimensional information is obtained from lower dimensional samples (The Stereology Group, 1999-2010). Stereology is a method that exploits random, systematic sampling to generate unbiased quantitative data (West, Slomianka, & Gundersen, 1991). The most commonly used stereological techniques in neuroscience form a group designated as "Design-Based". These methods do not assume the brain is homogenous and sampling schemes are defined independent of the size, shape, spatial orientation and spatial distribution of the cells under investigation (West, 2002). Furthermore, design-based methods assume the cells are fixed and the probes that investigate the objects are random (West, 2002). The design-based method used for stereological analysis in this thesis was the optical fractionator.

The optical fractionator method uses systematic random samples of unbiased virtual counting spaces in directions X, Y, and Z, to estimate the total number of cells from the number of cells counted (The Stereology Group, 1999-2010). This method is much more efficient than older methods; what previously involved a week's worth of work, can now be accomplished in an hour (Gundersen, 1992). Furthermore, the optical fractionator method permits easier implementation of stereological analysis because it does not need a density estimation, and it is immune to tissue shrinkage associated with histological staining (West, et al., 1991). Similarly, it is easier to estimate the coefficient of error (CE – the quality of the quantitative estimate obtained from a design-based method) with this technique (West, et al., 1991).

Golgi-Cox

The Golgi-Cox stain is a valuable modification of a technique for studying the morphology of neurons and glia that was developed by Camillo Golgi in 1873. The Golgi-Cox technique allows for complete visualization of individual neurons (Pasternak & Woolsey, 1975). While it does not afford information about neurotransmitters or the chemical characteristics of the neuron, it does provide extensive information about dendritic and axonal arborization. What may have been considered a drawback to this technique, the random selectivity of neuronal impregnation, is actually a major advantage. As only 1-5% of neurons are stained in their entirety, it is easy to observe the structure of a single neuron on a relatively clear background (Pasternak & Woolsey, 1975). This however, is not the only benefit gained by employing the Golgi-Cox technique. As it is often impossible to predict the extent of brain change resulting from any given treatment, Golgi-Cox stains cells throughout the entire brain allowing the

researcher the ability to go back and investigate areas or cell types originally omitted (Kolb & Whishaw, 1998). In summary, Golgi-Cox impregnation is a reliable, cost-effective procedure that provides a means to correlate behavioural outcome with brain anatomy.

Results

Brain Weight

Male offspring born to MS and ES mothers demonstrated a decrease in brain weight when compared to controls. Female offspring of MS mothers also exhibited a decrease in brain weight but female offspring born to ES mothers had heavier brains than controls. Brain weights of both male and female offspring born to BS dams were not different than controls (See Figure 4.1 & 4.2). A two-way ANOVA with prenatal stress and sex as factors showed a main effect of stress, $F(3, 291) = 6.759$, $p < .001$ and sex, $F(1, 291) = 24.006$, $p < .001$. The Stress level by Sex interaction was not significant, $F(3, 291) = 1.958$, $p = .121$.

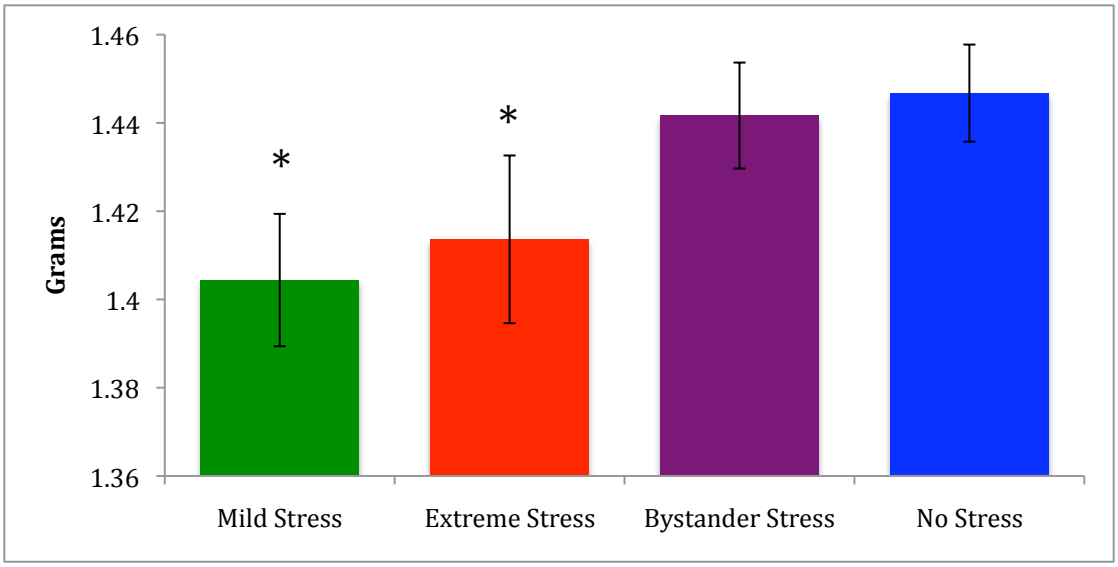

Figure 4.1. *Mean brain weights of male offspring at time of sacrifice (P21)(*p < .001)*

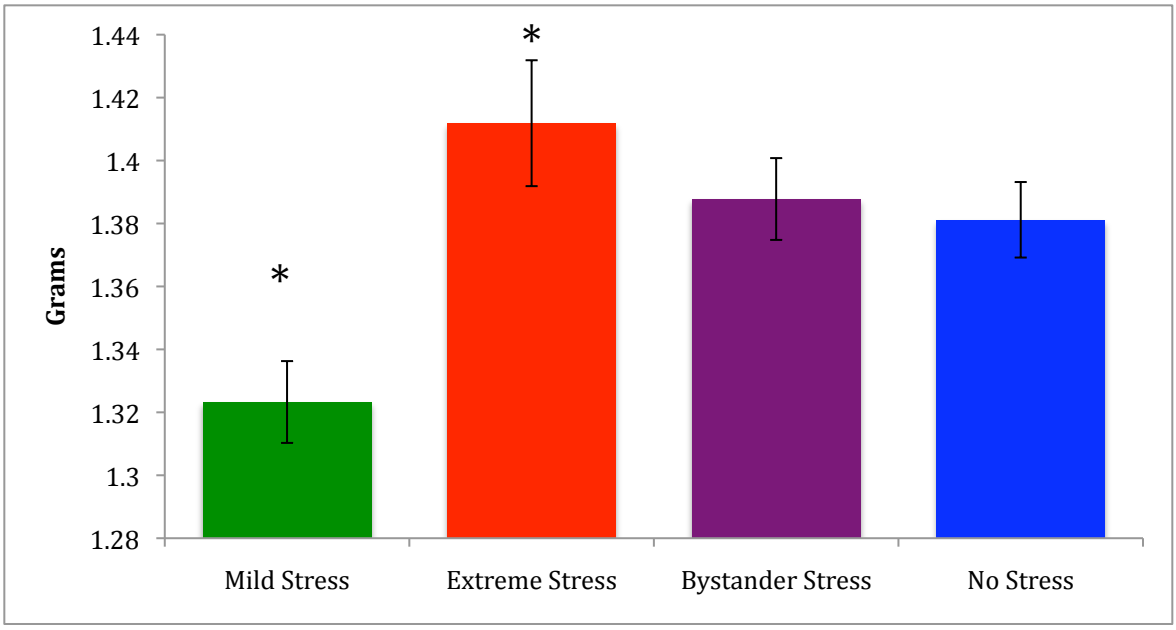

Figure 4.2. *Mean brain weight of female offspring at sacrifice (P21)(*p < .001)*

Brain Weight – Lesion

A subset of BS and NS offspring were given mPFC lesions on P8. As expected, male and female offspring that received P8 lesions had reduced brain weights when compared to offspring with sham surgeries or no lesion. There was not a combined effect on brain weight for either sex, if the offspring experienced BS and then received a mPFC lesion later in life (See Figure 4.3 & 4.4). A three way ANOVA with stress level, sex, and lesion as factors showed a main effect of sex, $F(1, 156) = 13.076$, $p < .001$, and lesion, $F(1, 156) = 44.227$, $p < .001$, but not of stress level, $F(1, 156) = 2.342$, $p = .128$. None of the interactions were significant (p's >.05).

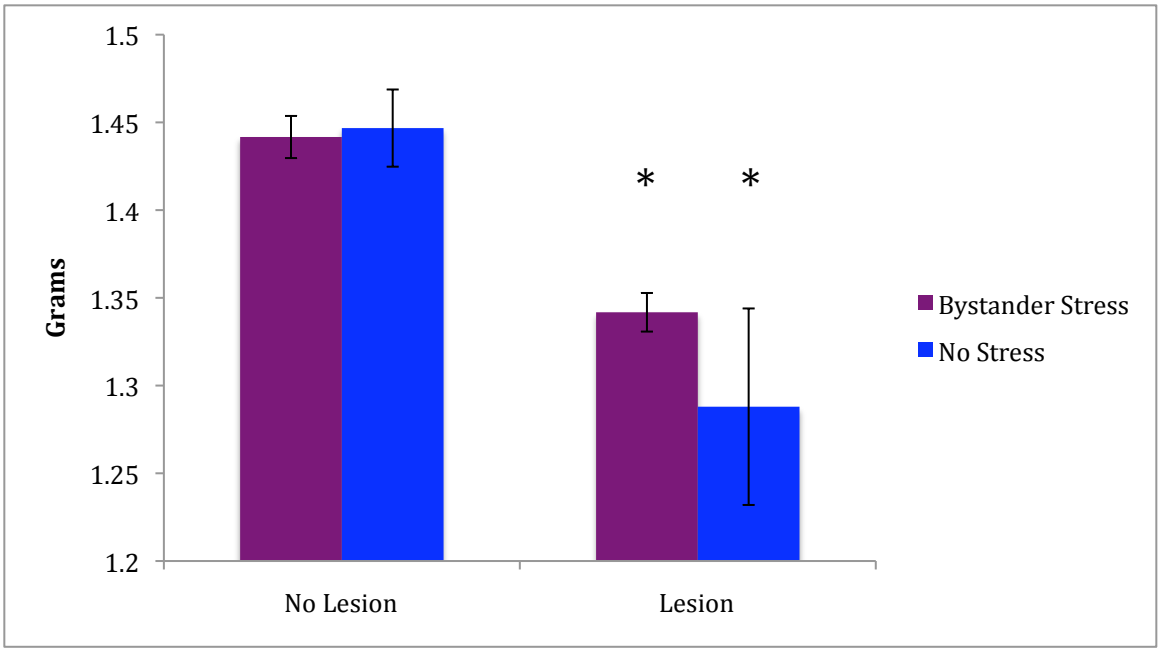

Figure 4.3. *Mean brain weights of male offspring with lesions at time of sacrifice (P21) (*p < .001)*

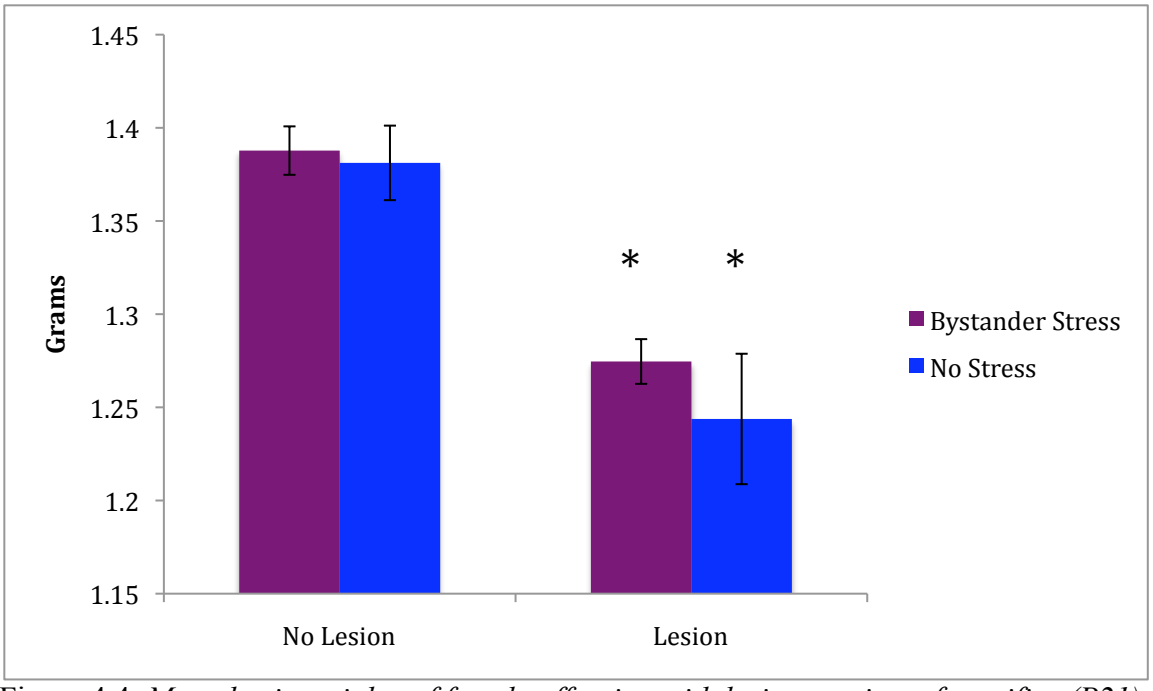

Figure 4.4. *Mean brain weights of female offspring with lesions at time of sacrifice (P21) (*p < .001)*

Stereology

Cg3 - Stereology

Male offspring who experienced prenatal BS showed a reduction in the number of neurons in Cg3 but no change in the number of glial cells in this brain area. In contrast, females born to BS dams exhibited an increase in the number of neurons and glia in Cg3. Both male and female offspring born to ES dams demonstrated a reduction in glial cell number in Cg3 but no change in neuronal cell count (See Figure 4.5 & 4.6). Two-way ANOVAs were run with stress level and sex as factors for neuron, glia and total cell numbers. When analyzing the neuronal population there were no effects of stress, $F(2, 39) = .271$, $p = .765$ or sex, $F(1, 39) = .658$, $p = .423$, but the interaction was significant, $F(2, 39) = 6.109$, $p = .005$. There was a main effect of stress $F(2, 39) = 8.317$, $p < .001$ and sex, $F(1, 39) = 6.968$, $p = .007$ on glial numbers: but the interaction was not significant. Analysis of total cell numbers (neurons + glia) in Cg3 revealed a main effect of stress, $F(2, 39) = 4.132$, $p = .025$, and sex, $F(1, 39) = 3.959$, $p = .050$, and a significant interaction, $F(2, 39) = 5.010$, $p = .012$.

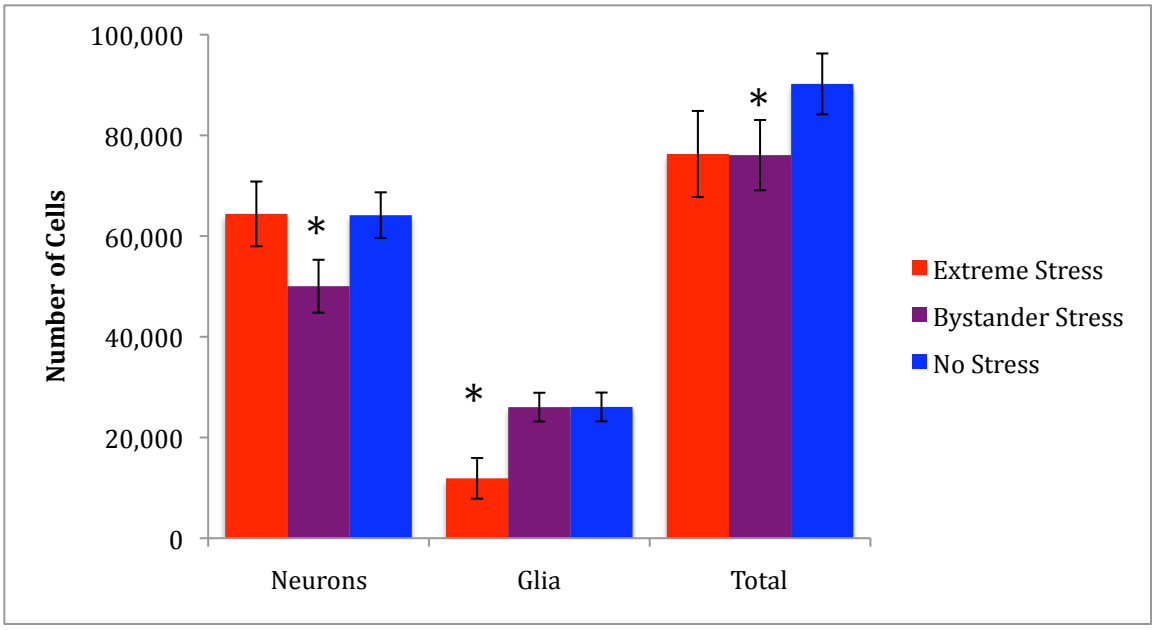

Figure 4.5. *Number of cells in Cg3 for male offspring at P21 (*p < .001)*

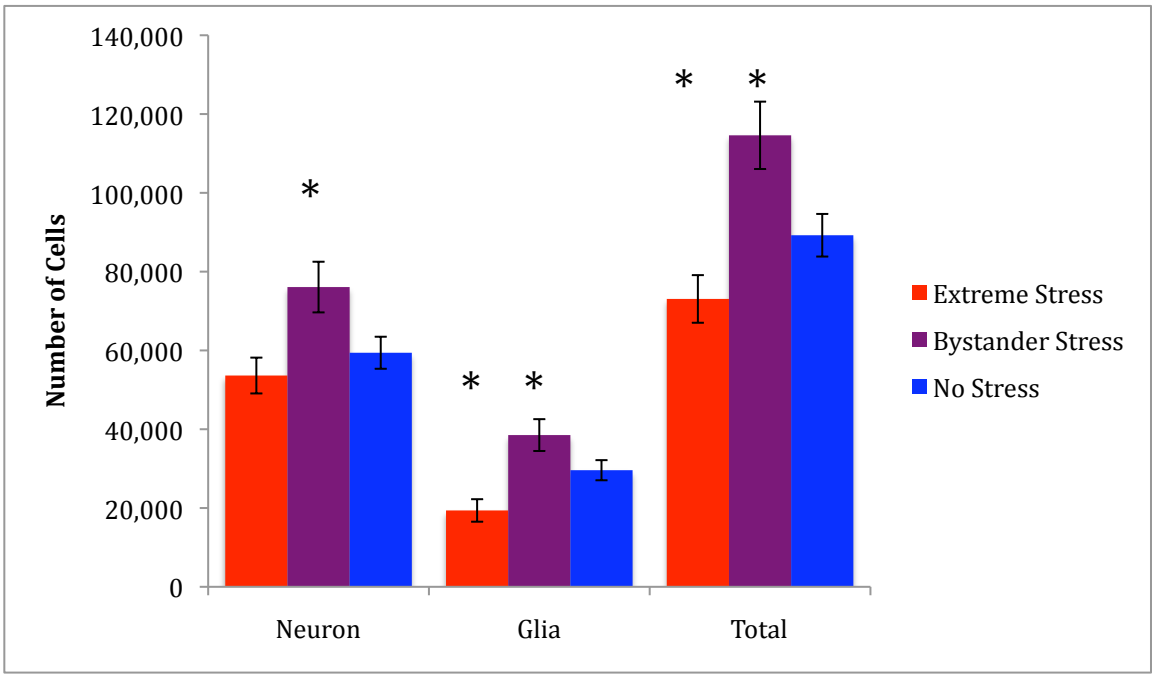

Figure 4.6. *Number of cells in Cg3 for female offspring at P21 (*p < .001)*

AID-Stereology

Although offspring born to BS mothers exhibited significant differences in glia and neuronal cell quantity in Cg3 no differences were found for these offspring in AID.

AID appeared to develop normally in both male and female offspring of BS mothers. Conversely, males born to ES mothers demonstrated significant reductions in glia and neurons in AID, whereas females of ES mothers exhibited a dramatic increase in neuron quantities (See Figure 4.7 & 4.8). Two-way ANOVAs were run with stress level and sex as factors for neuron, glia and total cell numbers. Neuronal population analysis revealed a main effect of sex $F(1, 61) = 5.790$, $p = .019$, but not of stress $F(2, 61) = .011$, $p = .989$, and the Sex by Stress interaction was significant, $F(2, 61) = 5.798$, $p = .005$. Analysis of glia cell numbers failed to produce any significant results (stress; $F(2, 61) = 1.218$, $p = .304$, sex; $F(1, 61) = .984$, $p = .326$, Sex by Stress interaction; $F(2, 61) = .478$, $p = .623$). As observed with the neuronal population, total cell number was affected by sex, $F(1, 61) = 6.235$, $p = .015$, but not stress, $F(2, 61) = .145$, $p = .865$. However, a significant interaction of Sex and Stress was observed, $F(2, 61) = 5.518$, $p = .006$.

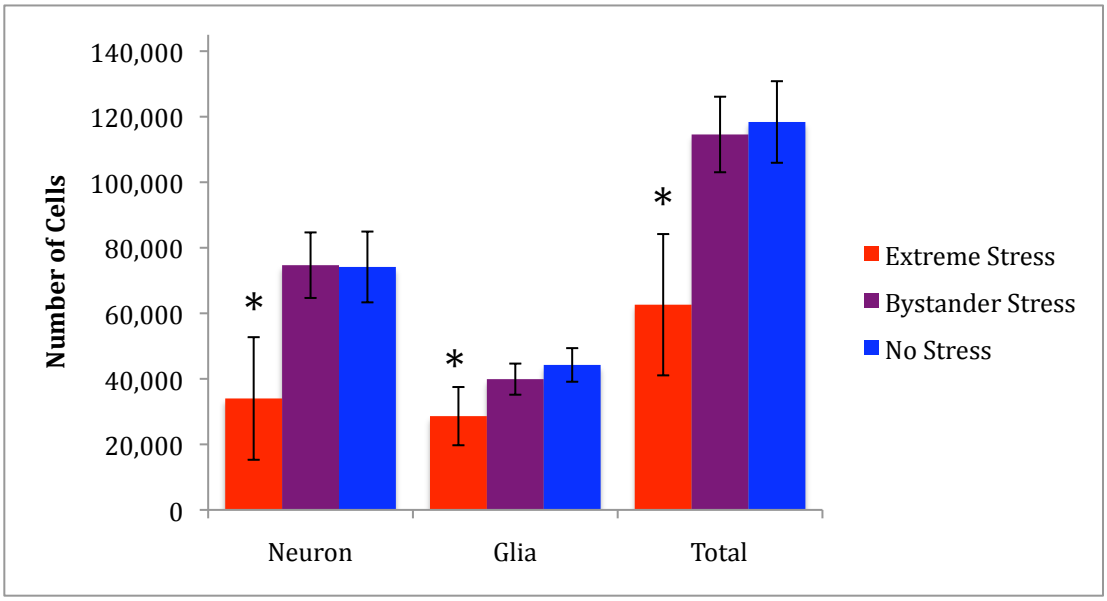

Figure 4.7. *Number of cells in AID for male offspring at P21 (*p < .05)*

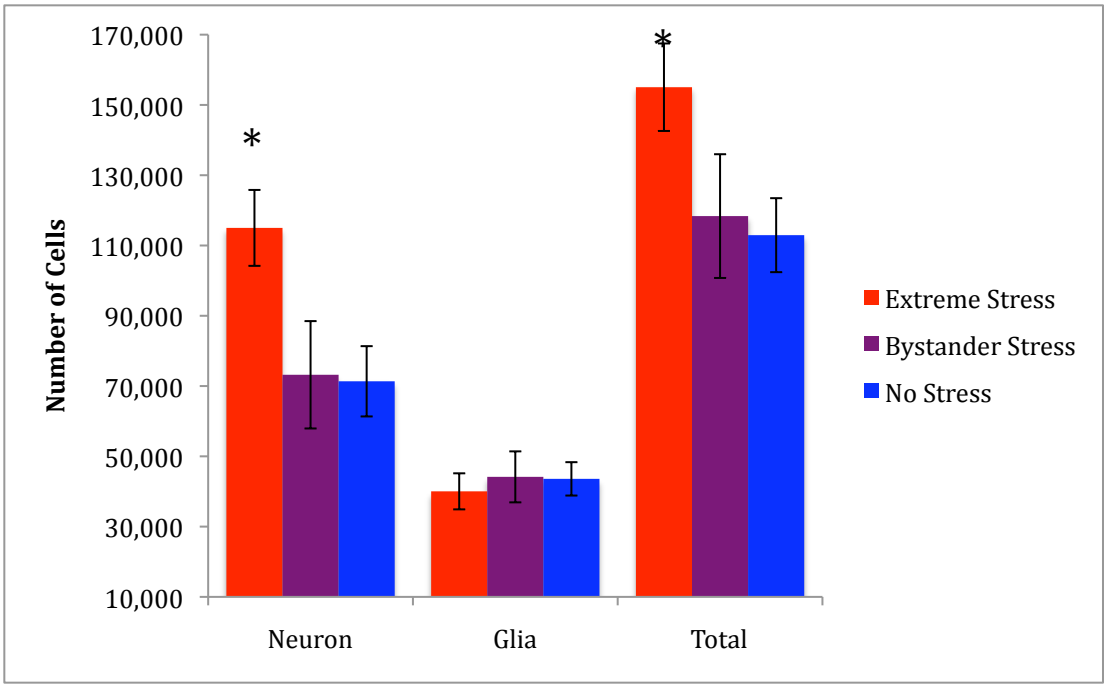

Figure 4.8. *Number of cells in AID for female offspring at P21 (*p < .05)*

HPC-Stereology

Offspring neuronal and glial cell numbers in CA1 of the hippocampus were significantly impacted by exposure to prenatal stress. Both male and female offspring of ES mothers demonstrated a reduction in neuron and glia cell numbers, whereas only female offspring of BS mothers exhibited a reduction in both of these cell types (See Figure 4.9 & 4.10). Two-way ANOVAs were run with stress level and sex as factors for neuron, glia, and total cell numbers. Neuronal population analysis revealed a main effect of stress $F(2, 22) = 6.337$, $p = .009$, but not of sex, $F(1, 22) = 2.037$, $p = .172$. The interaction was not significant, $F(2, 61) = .180$, $p = .837$. Analysis of glia cell numbers demonstrated a significant effect of stress, $F(2, 22) = 6.397$, $p = .008$, but not of sex; $F(1, 22) = 3.014$, $p = .101$. The interaction was not significant, $F(2, 22) = .481$, $p = .626$. As observed with the neuronal population, total cell number exhibited a main effect of stress,

$F(2, 22) = 6.650$, $p = .007$, but not sex, $F(1, 22) = 2.538$, $p = .130$. Again the interaction was not significant, $F(2, 22) = .289$, $p = .753$.

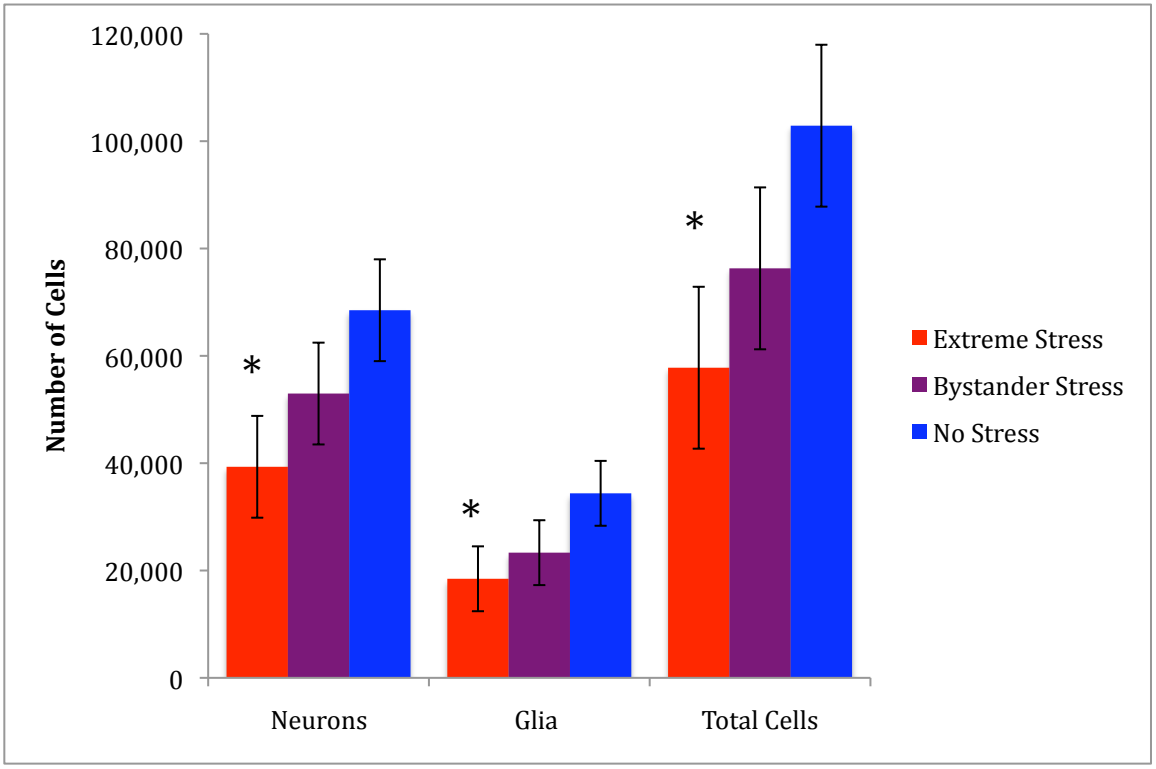

Figure 4.9. *Number of cells in CA1 of the hippocampus for male offspring at P21 (*p < .01)*

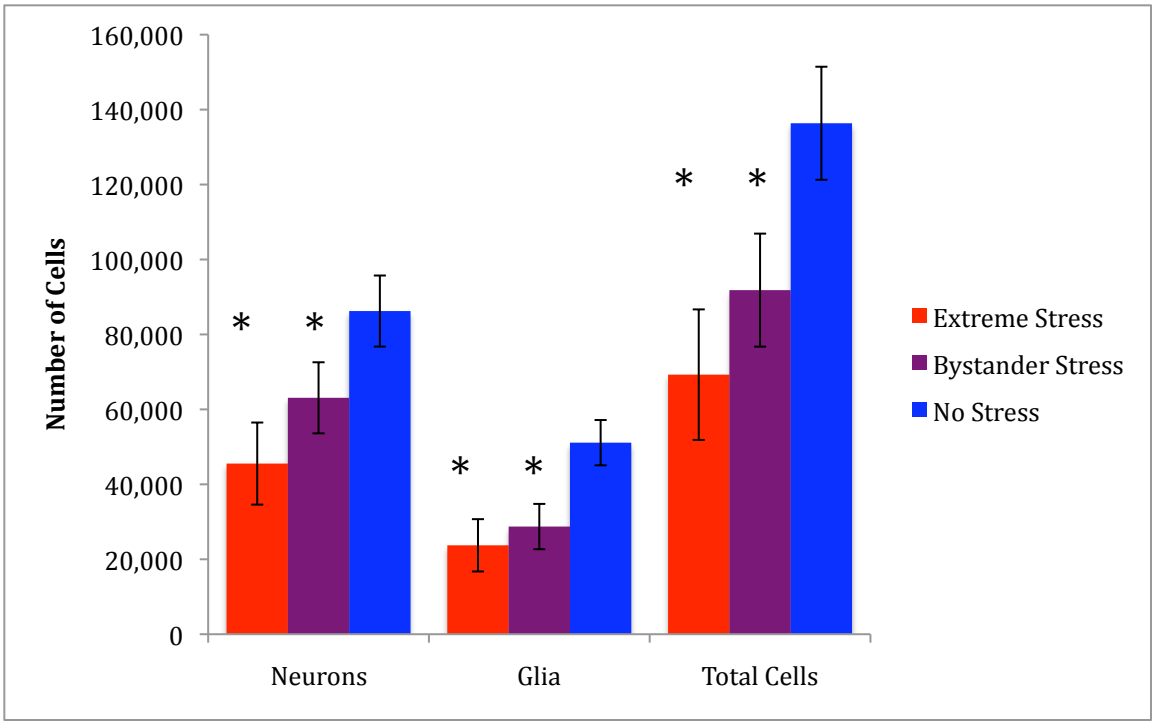

Figure 4.10. *Number of cells in CA1 of the hippocampus for female offspring at P21 (*p < .01)*

Dendritic Morphology

In summary, both BS and ES reduced the basilar dendritic field in neurons of the orbital frontal cortex (AID) for all offspring. Extreme stress had a sex-dependent effect on layer III neurons of the Cg3. Males demonstrated an increase in apical dendritic length in both hemispheres and an increase in basilar length and bifurcations in the left hemisphere. Females born to ES mothers, however, exhibited no change in apical or basilar dendritic length or branch order. All offspring born to BS mothers exhibited an increase in apical and basilar dendritic length as well as, an increase in bifurcations in the left hemisphere. Interestingly, analysis of dendritic morphology in CA1 of the hippocampus revealed a stress-dependent effect. Both male and female offspring of ES mothers exhibited an increase in basilar dendritic length, whereas offspring of BS mothers demonstrated a significant decrease in basilar dendritic length.

Cg3 (Layer III)

Apical Field – Sholl analysis revealed that prenatal BS produced an increase in apical dendritic length irrespective of sex, whereas, prenatal ES only produced an increase in apical dendritic length for male offspring. Prenatal stress had no effect on apical branch order (See Figure 4.11 & 4.12). A two-way ANOVA of apical dendritic length with prenatal stress and sex as factors showed a main effect of stress, $F(2, 65) = 3.219$, $p = .047$, but not of sex, $F(1, 65) = .479$, $p = .492$. The interaction was not significant, $F(2, 65) = 1.969$, $p = .149$. A two-way ANOVA of apical branch order with prenatal stress and sex as factors revealed no significant effects. Sex, $F(1, 65) = .380$, $p = .540$; stress, $F(2, 65) = .058$, $p = .944$; interaction, $F(2, 65) = .338$, $p = .714$.

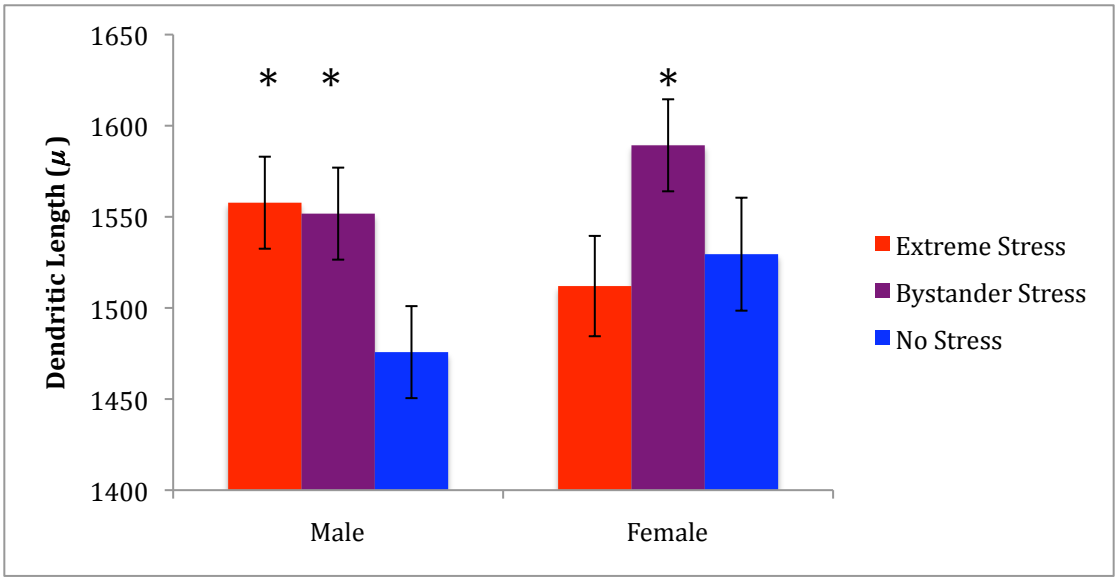

Figure 4.11. *Apical dendritic length of neurons in Cg3 (layer III) for offspring sacrificed on P21 (*p < .05)*

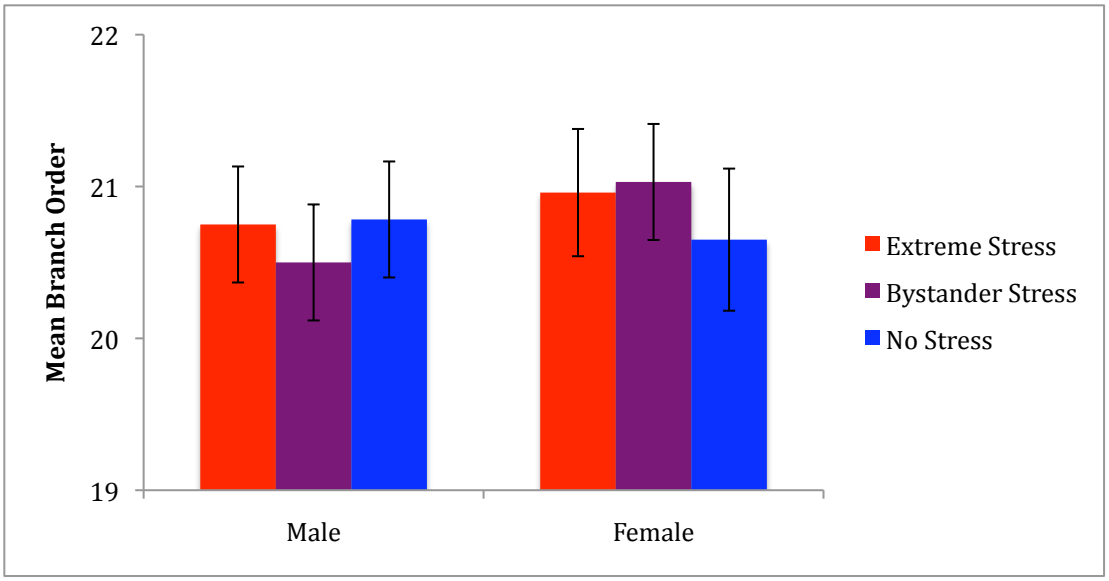

Figure 4.12. *Apical branch order of neurons in Cg3 (layer III) for offspring sacrificed on P21*

Basilar Field – Analysis of basilar dendritic morphology revealed a hemisphere dependent effect. Prenatal ES did not alter basilar dendritic length or branch order for female offspring but did increase basilar dendritic length and branch order in the left hemisphere of male offspring. Similarly, prenatal BS produced an increase in basilar dendritic length and branch order in the left hemisphere of male offspring. However, basilar dendritic length was increased in the right hemisphere, and branch order in the left hemisphere, of females born to BS mothers. There were no hemispheric effects on basilar spine density; all offspring demonstrated a significant increase in spine density when compared to non-stressed offspring (See Figure 4.13 - 4.17). A three-way ANOVA for basilar dendritic length with prenatal stress, sex and hemisphere as factors revealed a main effect of hemisphere, $F(1, 65) = 20.386$, $p < .001$, sex, $F(1, 65) = 9.058$, $p = .004$, and stress, $F(2, 65) = 5.622$, $p = .006$. None of the interactions were significant. A three-way ANOVA for basilar branch order with prenatal stress, sex, and hemisphere as factors revealed a main effect of hemisphere, $F(1, 65) = 15.267$, $p < .001$, and prenatal stress,

$F(2, 65) = 4.116$, $p = .022$, but not of sex, $F(1, 65) = 1.110$, $p = .297$. None of the interactions were significant. A two-way ANOVA for basilar spine density with prenatal stress and sex as factors demonstrated a main effect of stress, $F(2, 65) = 118.717$, $p < .001$, but not of sex, $F(1, 65) = 2.212$, $p = .412$. The interaction was not significant, $F(2, 65) = .077$, $p = .926$.

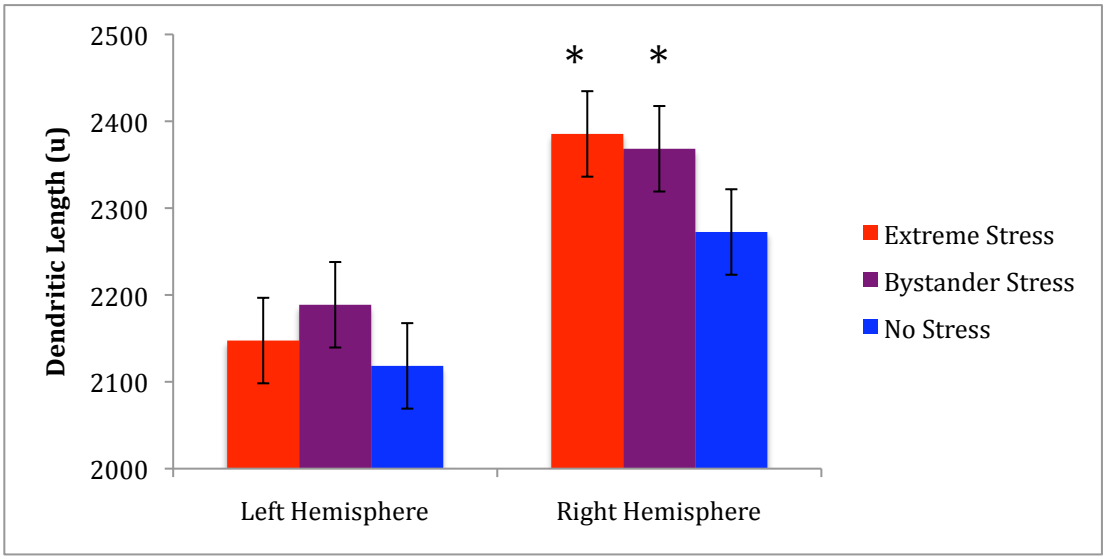

Figure 4.13. *Basilar dendritic length of neurons in Cg3 (layer III) for male offspring sacrificed on P21 (*p < .01)*

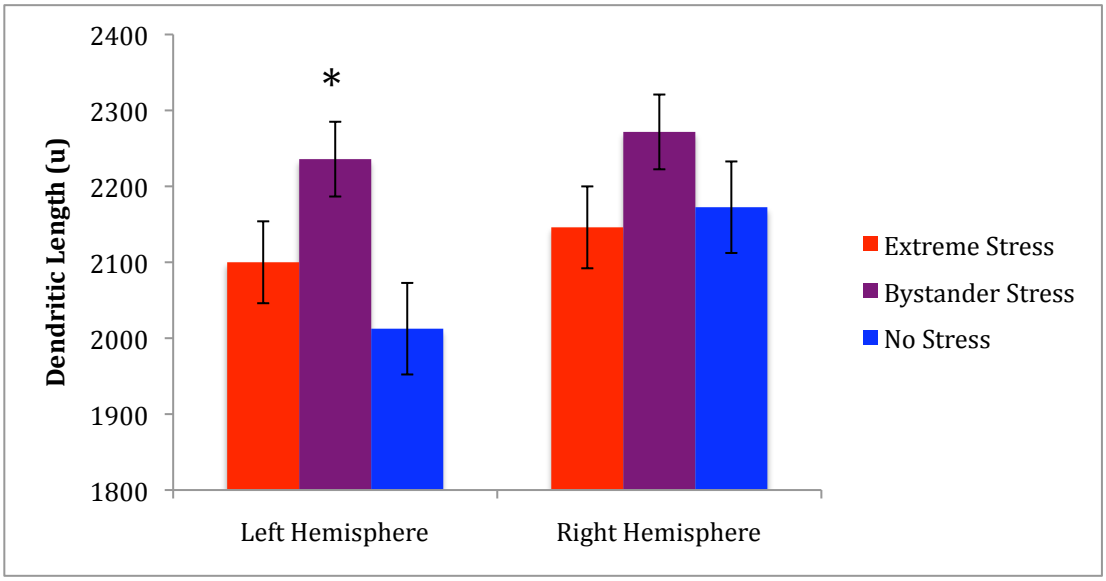

Figure 4.14. *Basilar dendritic length of neurons in Cg3 (layer III) for female offspring sacrificed on P21 (*p < .01)*

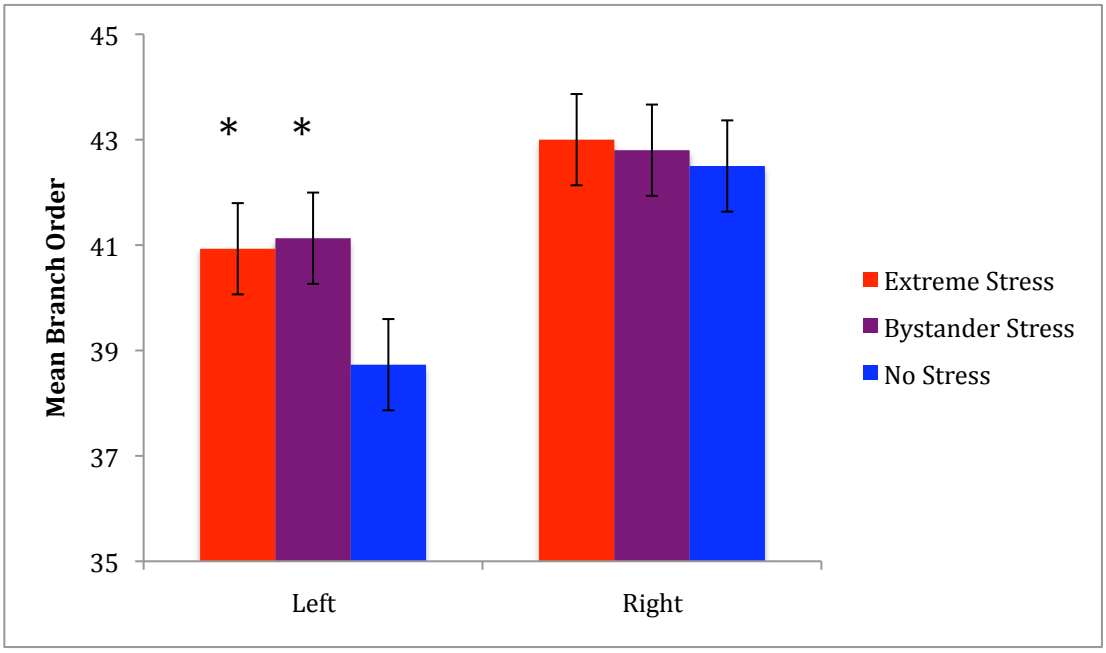

Figure 4.15. *Basilar branch order of neurons in Cg3 (layer III) for male offspring sacrificed on P21 (*p < .05)*

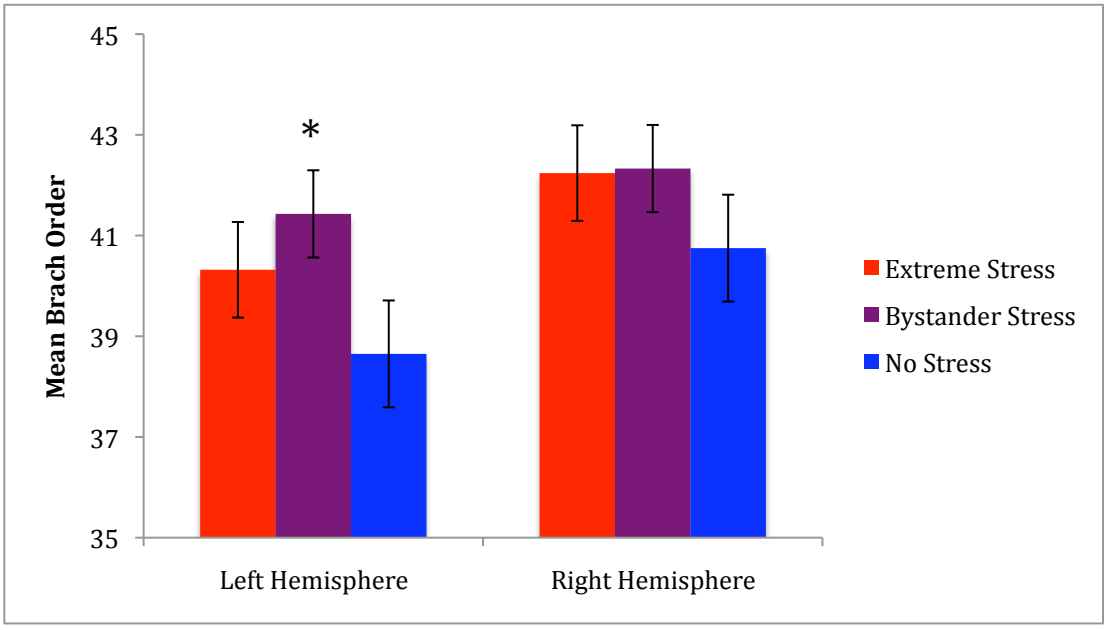

Figure 4.16. *Basilar branch order of neurons in Cg3 (layer III) for female offspring sacrificed on P21 (*p < .05)*

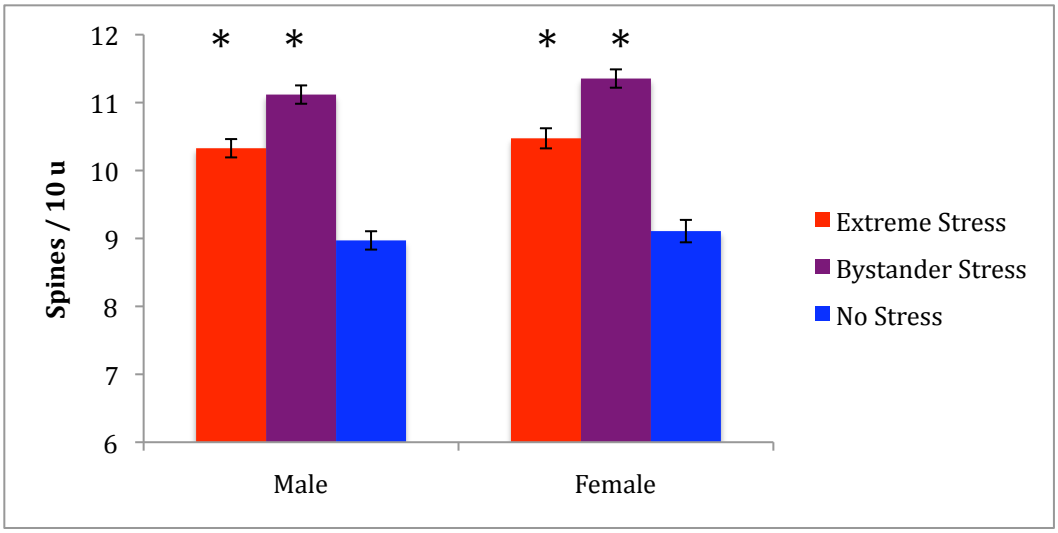

Figure 4.17. *Basilar spine density for neurons in Cg3 (layer III) of offspring sacrificed on P21 (*p < .001)*

AID

Sholl analysis of basilar dendrites in the orbital frontal cortex revealed a decrease in dendritic length for male and female offspring of both prenatal stress groups. Basilar branch order was decreased in female offspring of ES mothers only. Conversely, all offspring except females of ES mothers demonstrated an increase in basilar spine density (See Figure 4.18 – 4.20). A two-way ANOVA of basilar dendritic length with prenatal stress and sex as factors demonstrated a main effect of stress, $F(2, 65) = 23.598$, $p < .001$, but not of sex, $F(1, 65) = .267$, $p = .607$. The interaction was not significant, $F(2, 65) = 1.077$, $p = .347$. A two-way ANOVA of basilar branch order with prenatal stress and sex as factors showed a main effect of stress, $F(2, 65) = 3.360$, $p = .041$, but not of sex, $F(1, 65) = .032$, $p = .860$. The interaction was not significant, $F(2, 65) = .364$, $p = .697$. A two-way ANOVA of basilar spine density with prenatal stress and sex as factors revealed a main effect of stress, $F(1, 65) = 46.406$, $p < .001$, but not of sex, $F(2, 65) = .670$, $p = .087$. The interaction was not significant, $F(2, 65) = 2.539$, $p = .087$.

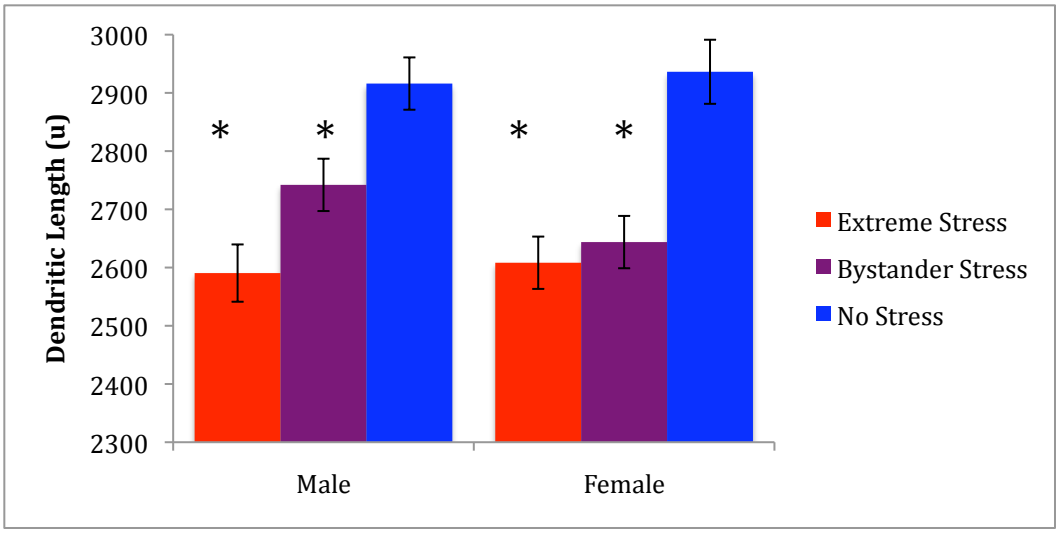

Figure 4.18. *Basilar dendritic length of neurons in AID for offspring sacrificed on P21 (*p < .001)*

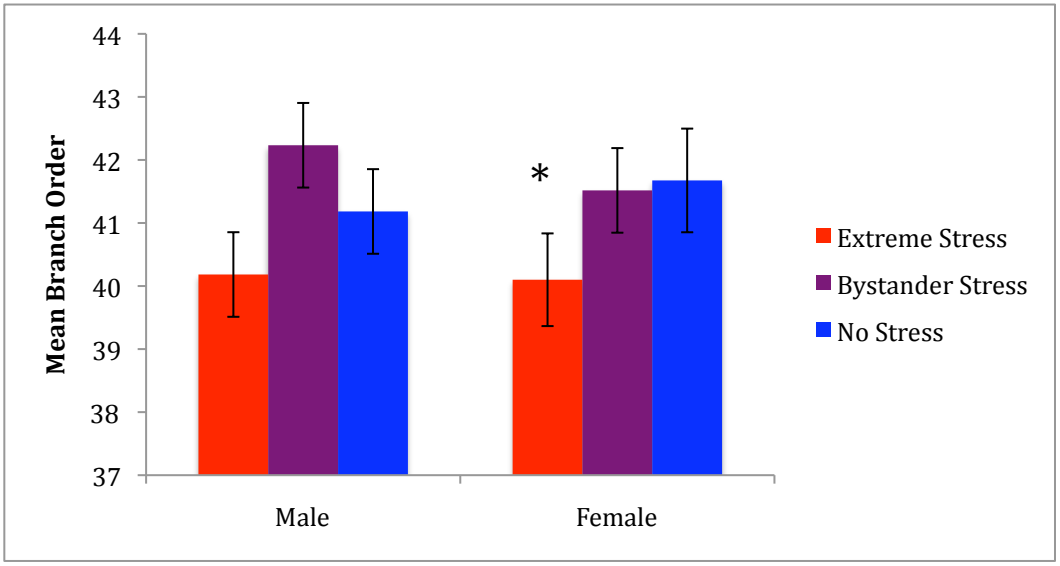

Figure 4.19. *Basilar branch order of neurons in AID for offspring sacrificed on P21 (*p < .05)*

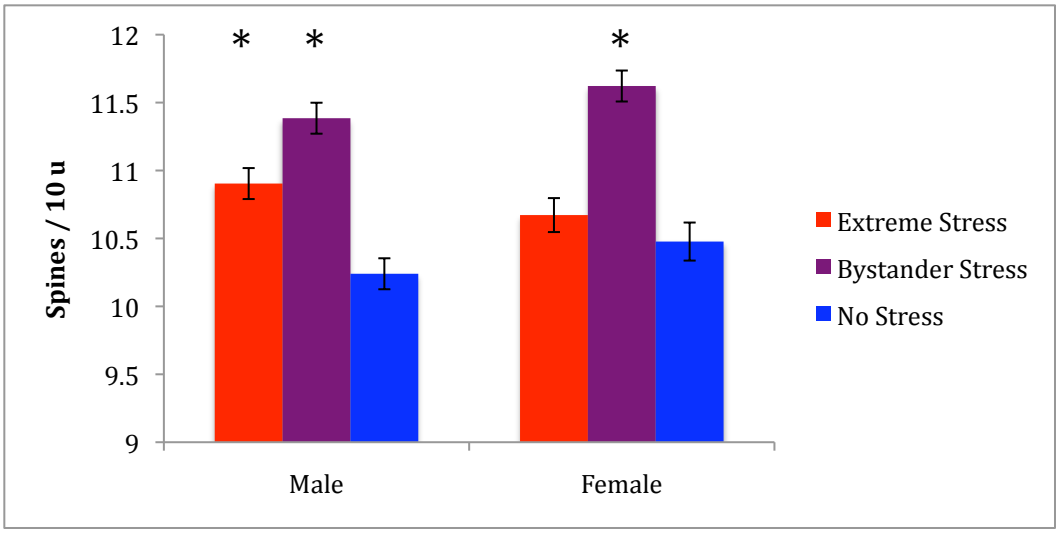

Figure 4.20. *Basilar spine density of neurons in AID for offspring sacrificed on P21 (*p < .001)*

CA1

Dendritic morphology analysis of the neurons in CA1 of the hippocampus revealed a stress dependent effect. Both male and female offspring of ES mothers demonstrated an increase in basilar dendritic length and basilar branch order (See Figure 4.21). Conversely, both male and female offspring of BS mothers exhibited a decrease in basilar dendritic length. Male offspring of BS mothers also showed a decrease in basilar branch order (See Figure 4.22). Both male and female offspring of BS or ES mothers, demonstrated an increase in spine density (See Figure 4.23). A two-way ANOVA of basilar dendritic length with prenatal stress and sex as factors revealed a main effect of stress, $F(2, 65) = 55.857$, $p < .001$, and sex, $F(1, 65) = 23.29$, $p < .001$. The interaction was not significant, $F(2, 65) = .593$, $p = .556$. A two-way ANOVA of basilar branch order with prenatal stress and sex as factors demonstrated a main effect of stress, $F(2, 65) = 23.651$, $p < .001$, and sex, $F(1, 65) = 21.008$, $p < .001$. The interaction was not significant, $F(2, 65) = 2.752$, $p = .072$. A two-way ANOVA of basilar spine density with

prenatal stress and sex as factors revealed a main effect of stress, $F(2, 65) = 132.603$, $p < .001$, but not of sex, $F(1, 65) = 1.151$, $p = .288$. The interaction was not significant, $F(2, 65) = 1.359$, $p = .264$.

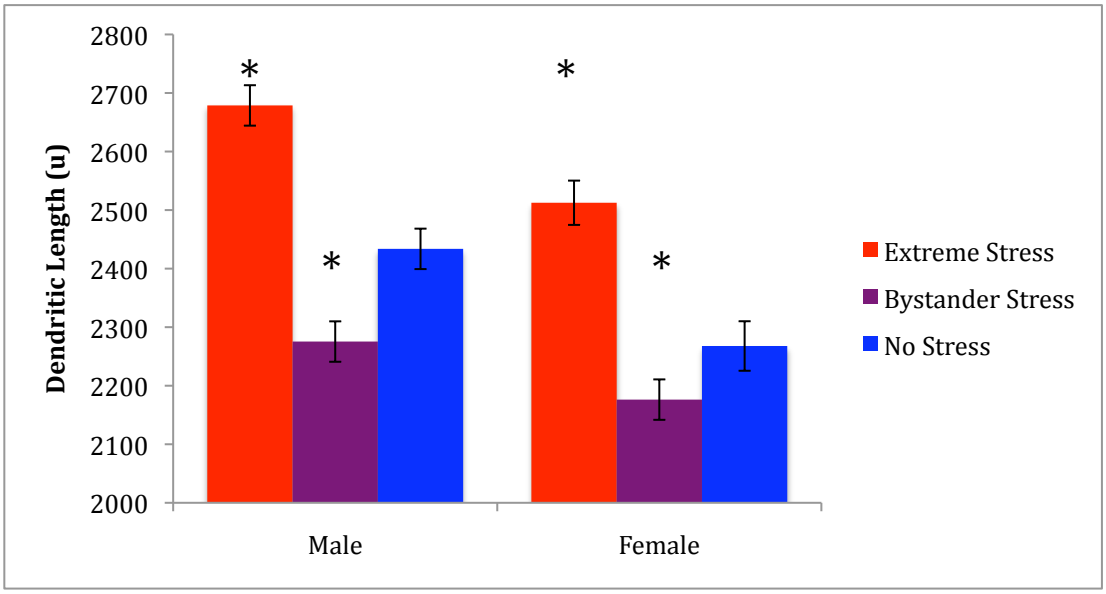

Figure 4.21. *Basilar dendritic length of neurons in CA1 of the hippocampus for offspring sacrificed on P21 (*p < .001)*

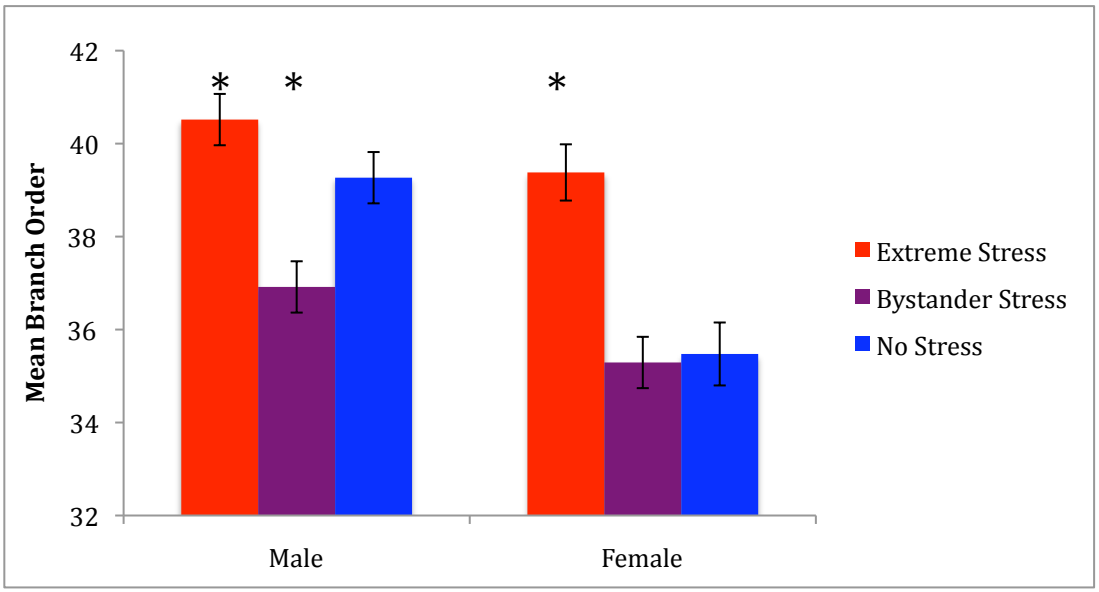

Figure 4.22. *Basilar branch order of neurons in CA1 of the hippocampus for offspring sacrificed on P21 (*p < .001)*

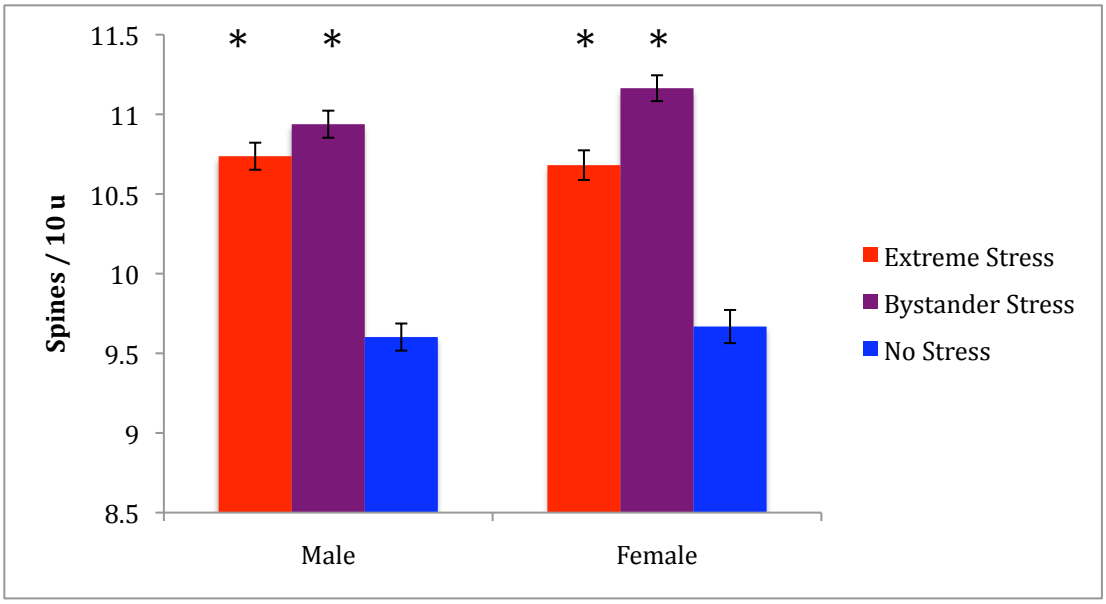

Figure 4.23. *Basilar spine density of neurons in CA1 of the hippocampus for offspring sacrificed on P21 (*p < .001)*

Estimation of Synapse Number

Utilizing data regarding dendritic length, spine density and neuronal count, an estimation of synapse number was generated for each experimental group. In Cg3, male offspring born to ES mothers exhibited an increase in synapse number compared to BS and NS offspring. Conversely, female offspring born to BS mothers demonstrated an increase in synapse number when compared to ES and NS offspring. In AID, both male and female offspring born to ES mothers demonstrated changes in synapse number when compared to NS offspring. Male offspring exhibited a decrease in synapse number whereas female offspring exhibited an increase. Offspring born to BS mothers demonstrated no change in synapse number when compared to NS offspring. In CA1 of the hippocampus, all offspring born to ES and BS mothers, demonstrated a decrease in synapse numbers when compared to offspring born to NS mothers (See Figure 4.24 & 4.25).

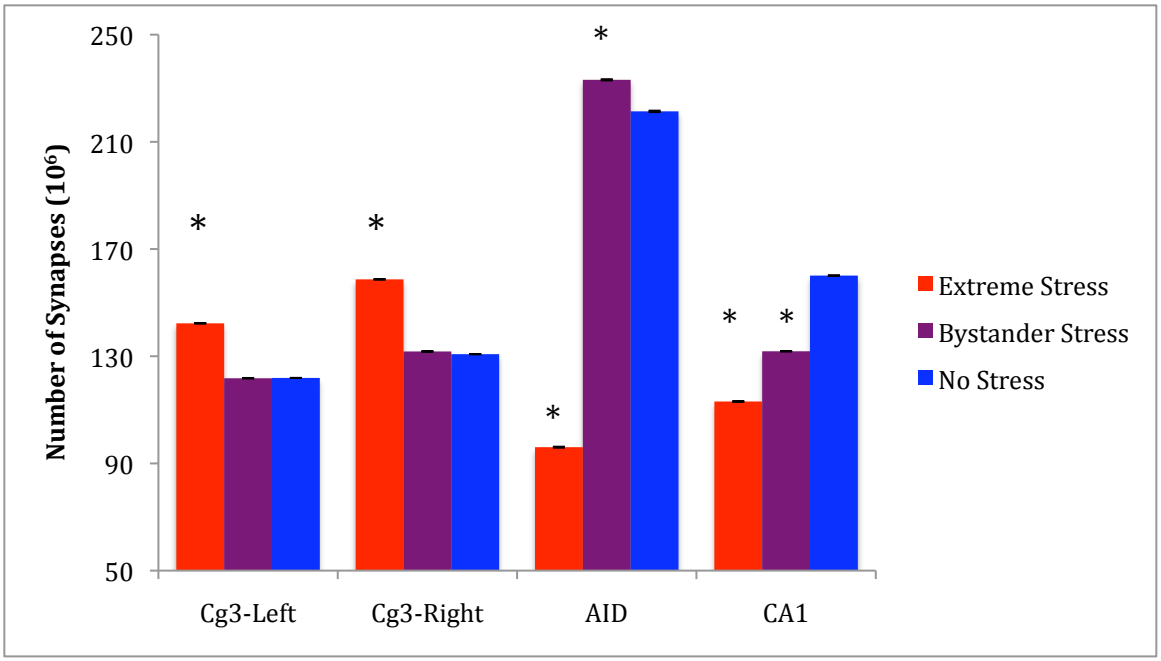

Figure 4.24. *Estimation of the number of synapses in 3 brain regions for male offspring at P21*

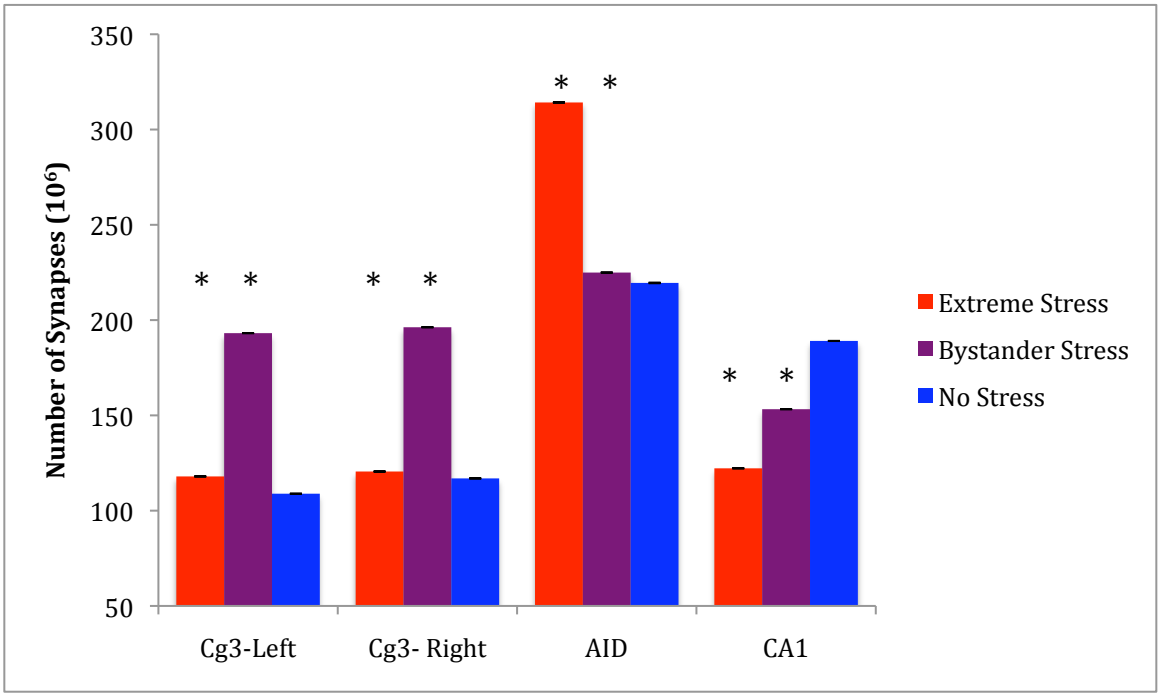

Figure 4.25. *Estimation of the number of synapses in 3 brain regions for female offspring at P21*

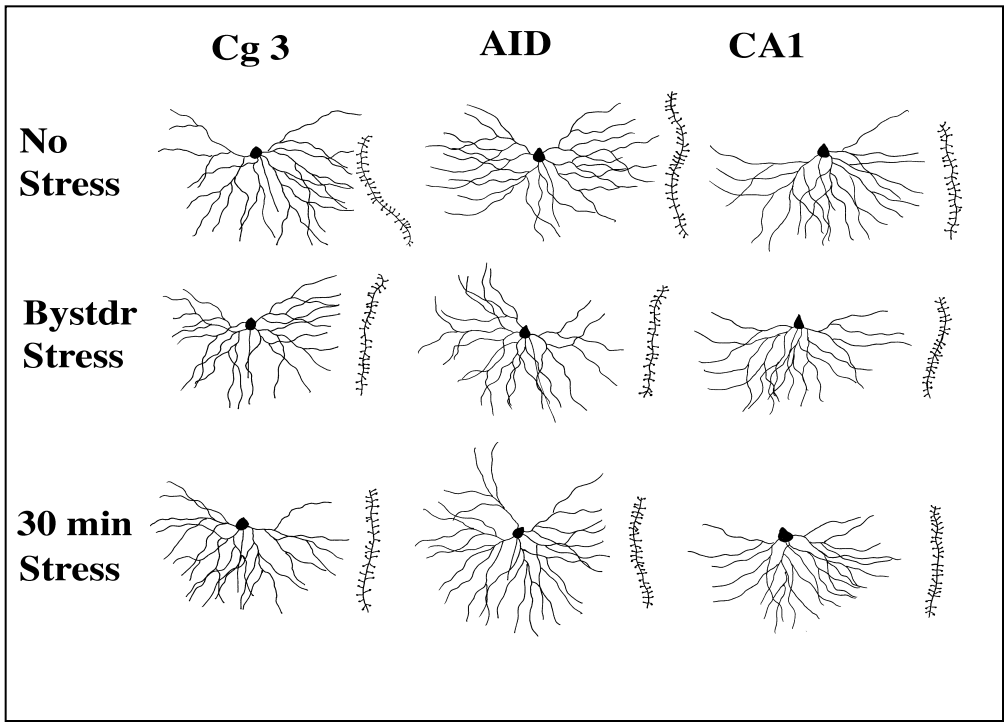

Figure 4.26. *Illustrative summary of neurons and basilar spines from male offspring at P21*

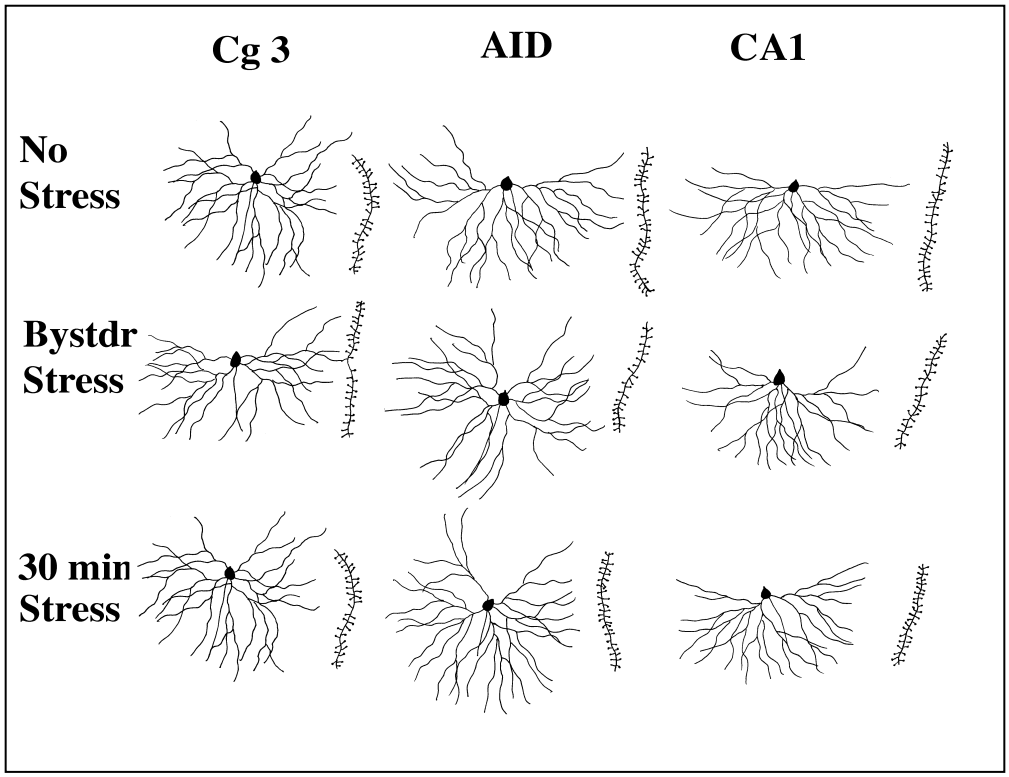

Figure 4.27. *Illustrative summary of neurons and basilar spines of female offspring at P21*

Table 4.1. *Summary of neuroanatomical findings for prenatally stressed male offspring compared to no stress male offspring*

	AID	**Cg3**	**HPC**
Neurons			
Bystander Stress	No Change	↓	No Change
Extreme Stress	↓	No Change	↓
Glia			
Bystander Stress	No Change	No Change	No Change
Extreme Stress	↓	↓	↓
Total Cells			
Bystander Stress	No Change	↓	No Change
Extreme Stress	↓	↓	↓
Dendritic Length			
Bystander Stress	↓	↑ (L)	↓
Extreme Stress	↓	↑ (L)	↑
Branch Order			
Bystander Stress	No Change	↑ (L)	↓
Extreme Stress	No Change	↑ (L)	↑
Spine Density			
Bystander Stress	↑	↑	↑
Extreme Stress	↑	↑	↑
Synapse Number			
Bystander Stress	No Change	No Change	↓
Extreme Stress	↓	↑	↓

Table 4.2. *Summary of neuroanatomical findings for prenatally stressed female offspring compared to no stress offspring*

	AID	Cg3	HPC
Neurons			
Bystander Stress	No Change	↑	↓
Extreme Stress	↑	No Change	↓
Glia			
Bystander Stress	No Change	↑	↓
Extreme Stress	No Change	↓	↓
Total Cells			
Bystander Stress	No Change	↑	↓
Extreme Stress	↑	↓	↓
Dendritic Length			
Bystander Stress	↓	↑ (R)	↓
Extreme Stress	↓	No Change	↑
Branch Order			
Bystander Stress	No Change	↑ (L)	No Change
Extreme Stress	↓	No Change	↑
Spine Density			
Bystander Stress	↑	↑	↑
Extreme Stress	No Change	↑	↑
Synapse Number			
Bystander Stress	No Change	↑	↓
Extreme Stress	↑	No Change	↓

Discussion

The findings from these studies provide compelling evidence for dose-dependent effects of prenatal stress on the neuroanatomical development of rat offspring. Furthermore, the profound morphological changes identified in these experiments are specific to brain region and offspring sex. It is clear that the developing brain employs numerous adaptive mechanisms in attempt to compensate for the abnormal maturation environment. It is therefore necessary to investigate cell architecture at multiple levels, as

examination of individual areas or specific morphologies may overlook valuable information. Understanding brain plasticity as it relates to prenatal stress is more complex than combining the functioning of single cells; the whole is not always just the sum of the parts. However, to fully comprehend plasticity in this respect, there must first be an understanding of how and where specific cells are changing.

Stereology

Neurogenesis and/or apoptosis in the prefrontal cortex and hippocampus appears to be influenced by offspring sex and the intensity of prenatal stress. Female offspring show increases in neuronal cell numbers in Cg3 and AID as a result of BS and ES respectively. Conversely, male offspring exposed to prenatal stress exhibit reductions in neuronal populations in the prefrontal cortex (BS-Cg3, ES-AID). These opposing alterations to neuron populations as a result of prenatal stress seen in male and female offspring could be related to differential rates of cortical brain maturation (Kolb & Whishaw, 2008), variations in estrogen receptor localization (Yokosuka, Okamura, & Hayashi, 1997), or discrepancies in epigenetic susceptibility (McCarthy, et al., 2009), all of which are easily influenced by maternal glucocorticoids. Although neuronal populations in the hippocampus were unaffected by offspring sex, they were dramatically reduced as a result of the prenatal stress. Both male and female offspring of ES and BS mothers exhibited significantly diminished neuron numbers in CA1. This is consistent with research carried out by Lemaire and colleagues (2000) who found an inhibition of neurogenesis in the hippocampus related to prenatal stress. Furthermore, it has long been known that perinatal glucocorticoid levels influence cell proliferation (Meyer, 1983) and increased GC levels are often toxic to the hippocampus (Radley & Morrison, 2005)

Dendritic Morphology

Analysis of dendritic morphology generated an abundance of valuable information regarding brain plasticity and prenatal stress. Most significantly, it is clear that prenatal stress impacts the prefrontal cortex differently than postnatal stress. In these experiments, offspring born to ES or BS mothers had neurons with no change or increased dendritic length and branch order in Cg3, whereas rats exposed to postnatal stress show a reduction in dendritic length and branch order in the same area (Brown, Henning, & Wellman, 2005; Radley, et al., 2005; Radley, et al., 2004). Additionally, offspring in these experiments born to prenatally stressed mothers exhibited increases in spine density in Cg3, whereas postnatally stressed rats often demonstrate spine density reductions in the same brain area (Radley, et al., 2008). Similarly, these prenatally stressed rats exhibited decreases in spine density in AID, even though others have found increases in AID spine density (Murmu, et al., 2006). In conjunction with this, asymmetries in the frontal cortex also vary with offspring age. Consistent with these results, Van Eden et al., (1984), found left-right asymmetries in Cg3 of the developing brain between P10-P18 that had disappeared by P24. They also noted that asymmetries in AID were not present until P30-P60. This is likely the reason why the prenatally stressed offspring sacrificed at P21 do not exhibit left-right morphological asymmetry in AID but they do in Cg3. Finally, in line with data from this experiment, Van Eden demonstrated that anatomical asymmetry is far more pronounced in males than females (Van Eden, et al., 1984).

The effects of prenatal stress on hippocampal anatomy appear to be intensity dependent. Prenatal bystander stress and postnatal stress produce similar morphological

changes in the hippocampus. Similar to rats enduring restraint stress, offspring born to BS mothers, exhibit decreased dendritic branching and decreased dendritic length (McKittrick, et al., 2000; Watanabe, et al., 1992). In contrast to this, both male and female offspring born to ES mothers exhibited an increase in dendritic branch order and dendritic length in CA1 of the hippocampus. It has been postulated that shrinkage of dendritic trees in the HPC serves as an adaptive mechanism; to reduce input of the over-active stress response on hippocampal neurons (McKittrick, et al., 2000). It is therefore puzzling that offspring of ES mothers do not show the same modifications. Even more perplexing is the finding that all prenatally stressed offspring exhibited an increase in spine density in HPC. This is consistent with results from other laboratories on postnatal stress (Sunada & Raju, 1995), but is contrary to expectation that in an effort to adapt, stress produces a reduction in spine density in the hippocampus (Silva-Gomez, Rojas, Juarez, & Flores, 2003).

Synapse Formation

The synapse is the site of neuronal communication. It is the means by which information is relayed from one neuron to the next in order to generate complex networks (Kolb & Whishaw, 2008). By combining the data collected regarding dendritic length, spine density, and neuronal number, an estimation of synapse quantity was generated. An estimation of synaptic quantity will denote whether or not the morphological modifications and changes in cell numbers amount to actual changes in neuronal communication. Due to the extremely large number of synapses that exist in any given brain area, there is very little data regarding changes in synapse number. While new

research has investigated changes in synapse morphology as a result of chronic stress (Donohue, et al., 2006), the findings in this dissertation are the first to examine alterations in synapse numbers related to prenatal stress.

Evidence from this dissertation demonstrates that synaptic plasticity in the hippocampus is affected most profoundly by prenatal stress. Both male and female offspring of BS and ES mothers exhibited decreases in synapse number in CA1 of the hippocampus. This decrease in synapse number occurred concomitantly with all offspring displaying increases in spine density. Contrary to expectation, increased spine density in the hippocampus was correlated with a decrease in overall synapse number. Such a marked reduction in synapse number would have likely lead to deficits in hippocampal dependent tasks such as Morris Water Maze (Morris, Garrud, Rawlins, & O'Keefe, 1982) had these animals been tested.

Synapse number in the frontal cortex was most affected in male offspring of ES mothers. These males demonstrated increases in synapse number in Cg3 and decreases in synapse number in AID. Despite significant changes in neuronal numbers, dendritic length, spine density, and branch order, prenatal stress produced no alteration in synapse number in male offspring of BS mothers. Frontal cortex of female offspring differentially responded to the two prenatal stress paradigms. Females of ES mothers showed no change in synapse number in Cg3, but increases in synapses in AID. Conversely, female offspring of BS mothers exhibited increases in Cg3, but not AID. These dramatic alterations to neuronal communication in the frontal cortex would have significantly influenced offspring response to all future experiences (Radley, et al., 2004).

In summary, prenatal stress produces sexually dimorphic, stress-dependent effects that can be measured with different techniques. The results indicate that anatomical modifications occur not only in cell number but also with respect to cell morphology. Furthermore, these results demonstrate that merely utilizing one method of analysis does not provide a complete picture of the anatomical changes that are possible in response to prenatal stress. It is recommended that future studies also use multiple measures rather than relying on a single, possibly misleading one.

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Chapter 5: Epigenetics

Introduction

As demonstrated in chapters 3 and 4, prenatal stress is capable of producing behavioural and anatomical brain changes in the developing offspring. The mechanisms behind these changes are currently unknown but likely involve modifications to gene expression. Every cell in an individual's body contains the exact same DNA sequence. If cells are to distinguish themselves and maintain various properties associated with their function there must be tissue and cell specific differences in the genes being expressed. It has become apparent that experiences are capable of turning genes on and off, thereby altering gene expression patterns and consequently changing the brain and behaviour. The epigenetic portion of this thesis was designed to investigate the modifications in gene expression associated with brain changes in the developing offspring of prenatally stressed rats. Global methylation analyses, along with a gene expression microarray, were utilized to generate a comprehensive picture of the genetic changes occurring in response to these early experiences.

Global Methylation

One of the epigenetic mechanisms regulating gene expression is DNA methylation. This highly stable form of transcriptional repression involves the covalent addition of a methyl group to the 5'-carbon of cytosine residues in CpG islands (Champagne, 2010; Fujiwara & Ito, 2002). Methylated CpG islands are included in the promoter regions of most human genes and are randomly distributed through out the genome (Pogribny, Yi, & James, 1999). The cytosine extension assay utilizes the selectivity of methylation-sensitive enzymes (*Msp*I - cuts at all CpG islands, methylated

or unmethylated, *Hpa*II – only cuts at CpG islands that are not methylated) to digest the DNA leaving 5' guanine overhangs. A single nucleotide primer extension procedure with [^{3}H]dCTP follows the enzymatic cleavage. The extent of [^{3}H]dCTP incorporation opposite to the 5' guanine is directly proportionate to the number of unmethylated CpG islands in the genome of interest (Pogribny, et al., 1999). A notable limitation to this procedure however, is the lack of cleavage specificity to the CpG islands of genes. Approximately 50% of the mammalian genome is comprised of transposable elements which also contain CpG islands (Thornburg, Gotea, & Makalowski, 2006). Transposable elements are repetitive sequences of DNA capable of moving in the genome from one chromosomal location to another (McDonald, 1995). Although, transposable elements are thought to be maintained in a heavily methylated state (Rakyan, Blewitt, Druker, Preis, & Whitelaw, 2002), it is clear that their methylation levels can vary (Das, Hampton, & Jirtle, 2009).

Gene Expression MicroArray

The "chip-based" microarray is a powerful and cost effective tool for the rapid quantification of gene expression for thousands of genes in parallel (Kerr & Churchill, 2001). The use of robotics for gene expression profiling enables high-speed, high-capacity output from a single RNA sample (Schena, 2005). A DNA microarray is based upon the principle of hybridization and the expectation that two DNA strands with complimentary nucleic acid sequences will bind specifically. The microchip contains "arrays" of immobilized DNA oligonucleotides representing specific coding regions from all annotated genes (Affymetrix, 2007). Purified mRNA from the tissue of interest is

reverse-transcribed into cDNA and labeled with fluorescent dyes (Kerr & Churchill, 2001). The newly generated cDNA is hybridized to the microchip and stringently washed to remove any non-complimentary strands (Kerr & Churchill, 2001). The remaining cDNA probes are scanned with a laser and the fluorescence intensity provides an accurate measure of the expression level of each gene (Schena, 2005).

Results

Global Methylation – Frontal Cortex

Methylation in the frontal cortex of males was affected by all prenatal stress paradigms. An increase in methylation was noted for males born to MS and BS dams, whereas a decrease in methylation was found for males born to ES mothers. Only prenatal BS and ES affected methylation patterns in the frontal cortex of female offspring. ES resulted in a significant decrease in methylation whereas BS increased methylation (See Figure 5.1 & 5.2). A two-way ANOVA with sex and stress as factors revealed a main effect of sex, $F(1, 74) = 7.065$, $p = .010$, and stress, $F(3, 74) = 36.684$, $p < .001$. The interaction was not significant. The sex effect reflected the generally higher levels of methylation in all female groups.

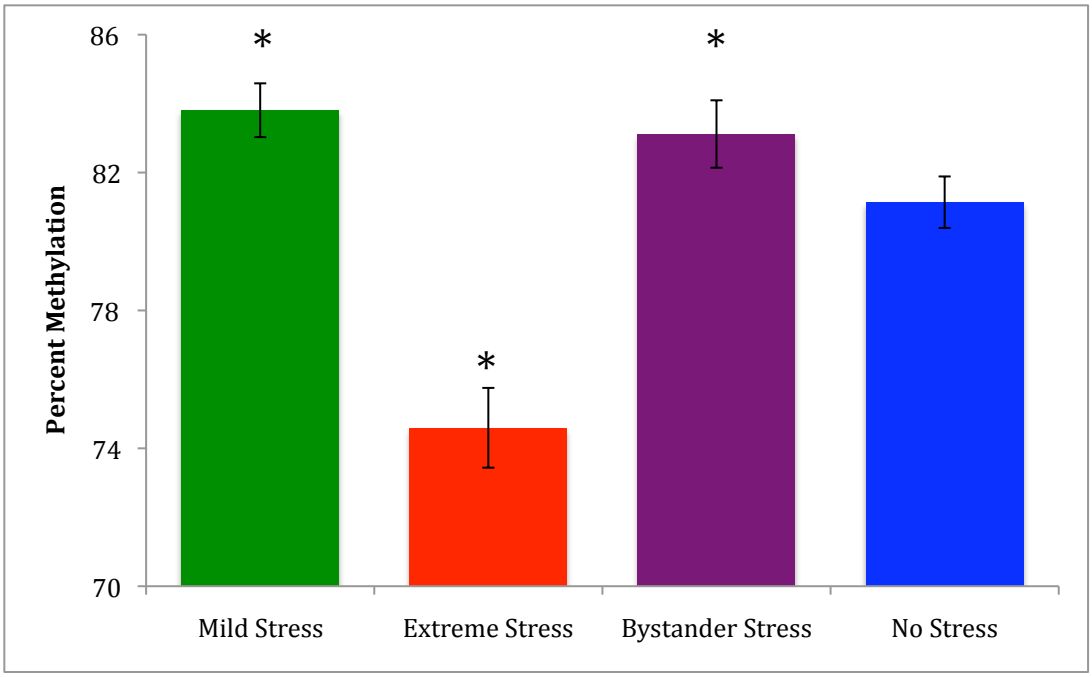

Figure 5.1: *Global methylation in frontal cortex of male offspring sacrificed at P21 (* p < .001)*

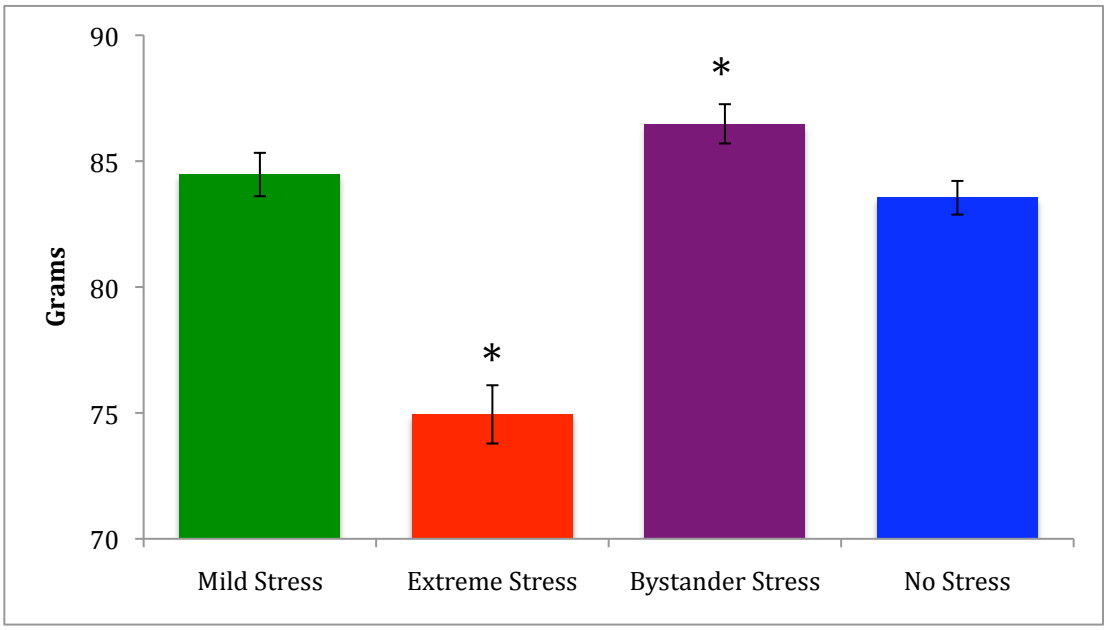

Figure 5.2: *Global methylation in frontal cortex of female offspring sacrificed at P21 (*p < .001)*

Global Methylation – Frontal Cortex, Lesion Study

For both male and female offspring, BS increased methylation in the frontal cortex adjacent to the lesion, whereas mPFC lesion decreased methylation levels.

However, the combination of mPFC lesion and BS appears to produce methylation levels indistinguishable from NS-no lesion offspring (See Figure 5.3 & 5.4). A three-way ANOVA with sex, stress and lesion as factors revealed a main effect of sex, $F(1, 74) = 19.970$, $p < .001$, stress, $F(2, 74) = 19.116$, $p < .001$, and lesion, $F(1, 74) = 12.796$, $p = .001$. None of the interactions were significant.

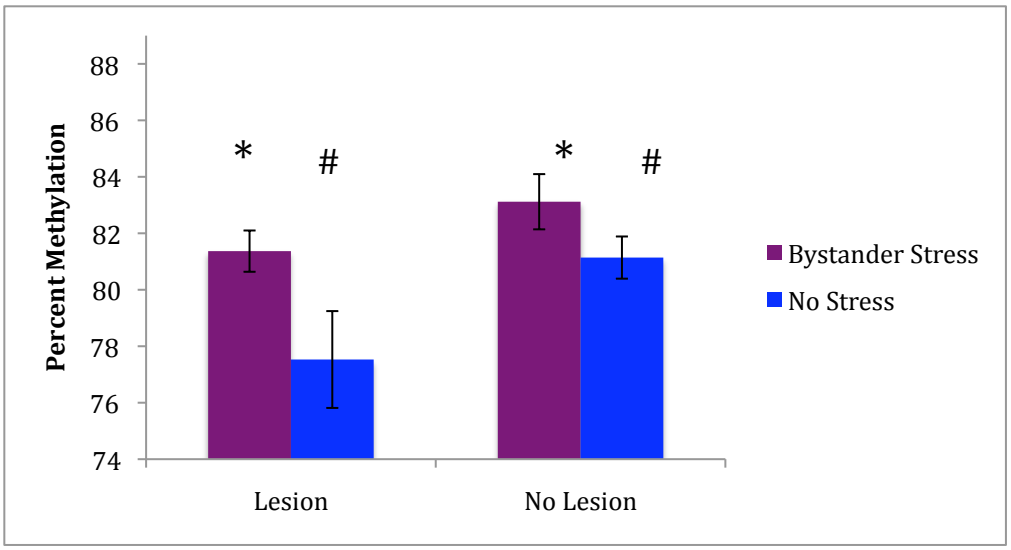

Figure 5.3: *Methylation levels in frontal cortex of male offspring sacrificed at P21 with lesions and prenatal bystander stress (*p < .001 – stress compared to no stress) (#p < .001 – lesion compared to no lesion)*

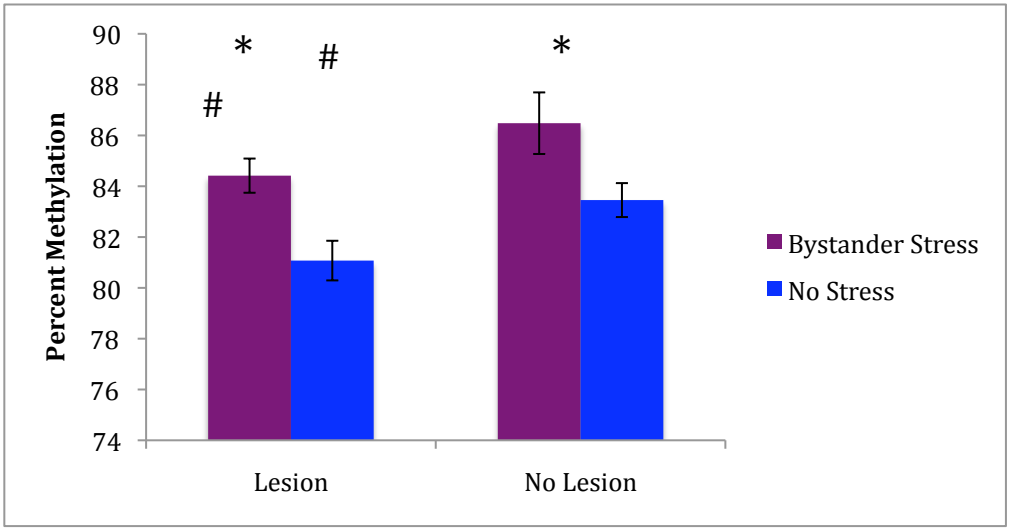

Figure 5.4: *Methylation levels in frontal cortex of female offspring sacrificed at P21 with lesions and prenatal bystander stress (* p < .001 – stress compared to no stress) (#p < .001 – lesion compared to no lesion)*

Global Methylation – Hippocampus

Global methylation in the hippocampus was affected similarly by all stress paradigms for both male and female offspring. BS and MS produced increases in global methylation in the hippocampus whereas ES produced a decrease in methylation (See Figure 5.5 & 5.6). A two-way ANOVA with sex and stress as factors demonstrated a main effect of stress, $F(3, 69) = 97.467$, $p < .001$, but not of sex, $F(1, 69) = .771$, $p = .383$. Similarly, the interaction was not significant.

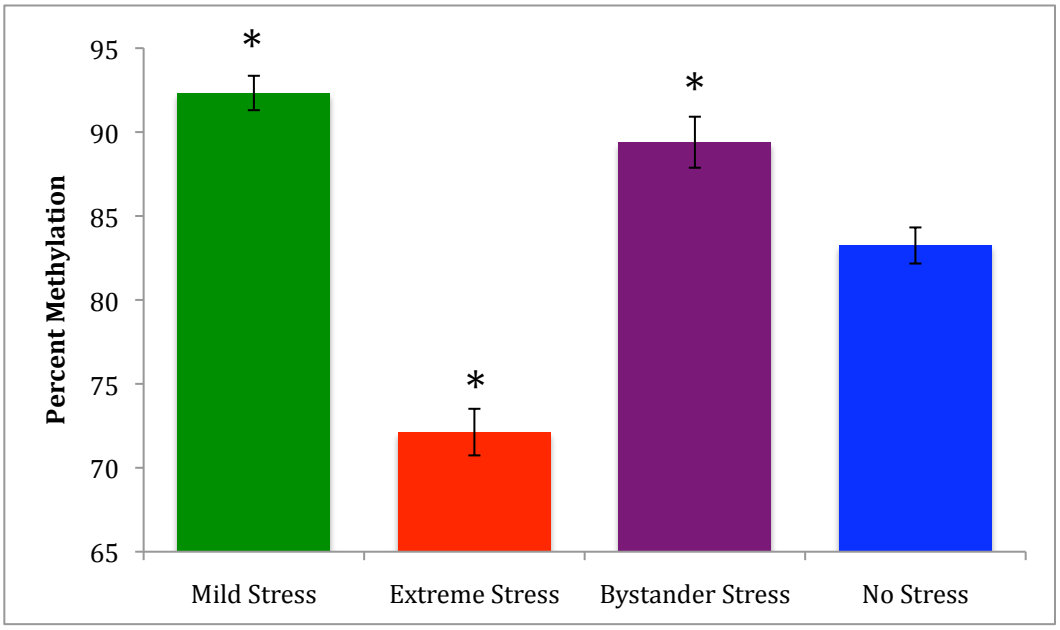

Figure 5.5: *Methylation levels in the hippocampus of male offspring sacrificed at P21 (*p < .001)*

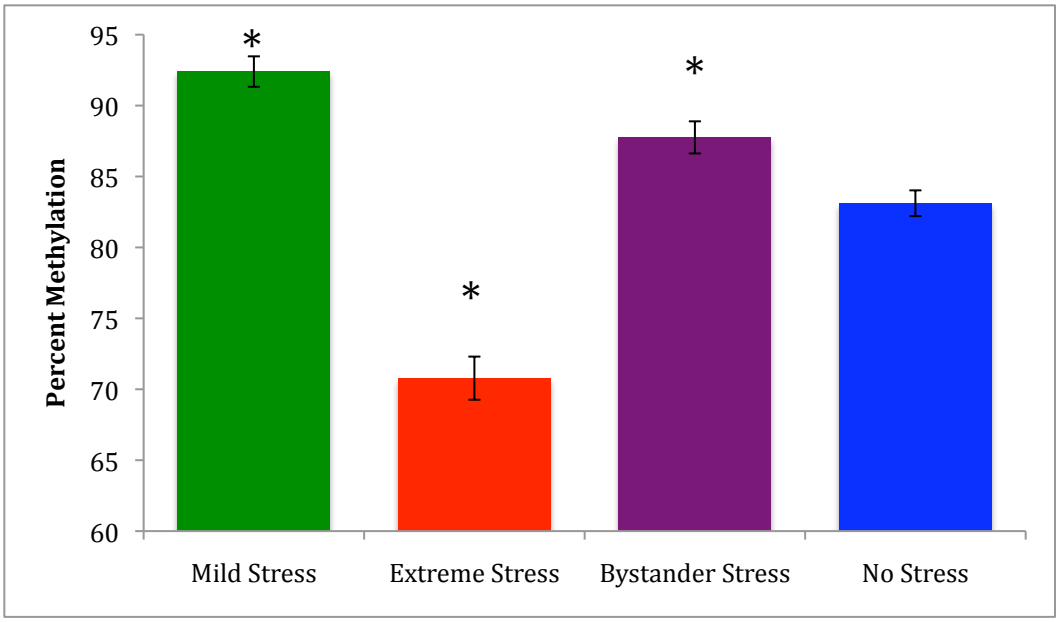

Figure 5.6: *Methylation levels in the hippocampus of female offspring sacrificed at P21 (*p < .001)*

Global Methylation – Hippocampus, Lesion Study

The mPFC lesion had no effect on methylation patterns in the hippocampus for either offspring. The only effect seen was an increase in methylation resulting from the BS paradigm (See Figure 5.7 & 5.8). A three-way ANOVA with sex, stress and lesion as factors revealed a main effect of stress, $F(1, 60) = 16.452$, $p < .001$, but not of sex, $F(1, 60) = .629$, $p = .431$, or lesion, $F(1, 60) = .949$, $p = .334$. None of the interactions were significant.

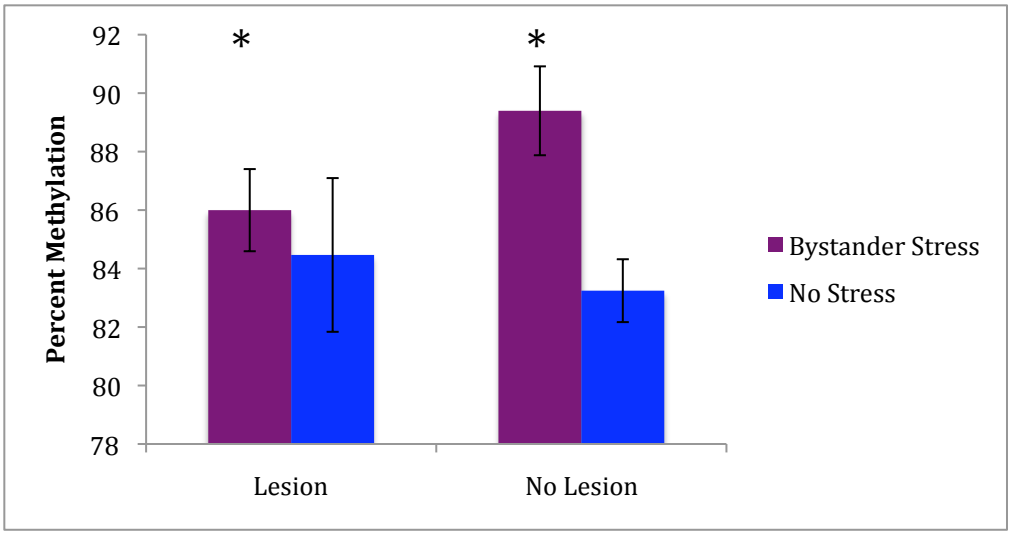

Figure 5.7: *Methylation levels in the hippocampus of male offspring sacrificed at P21 with lesions (*p < .001)*

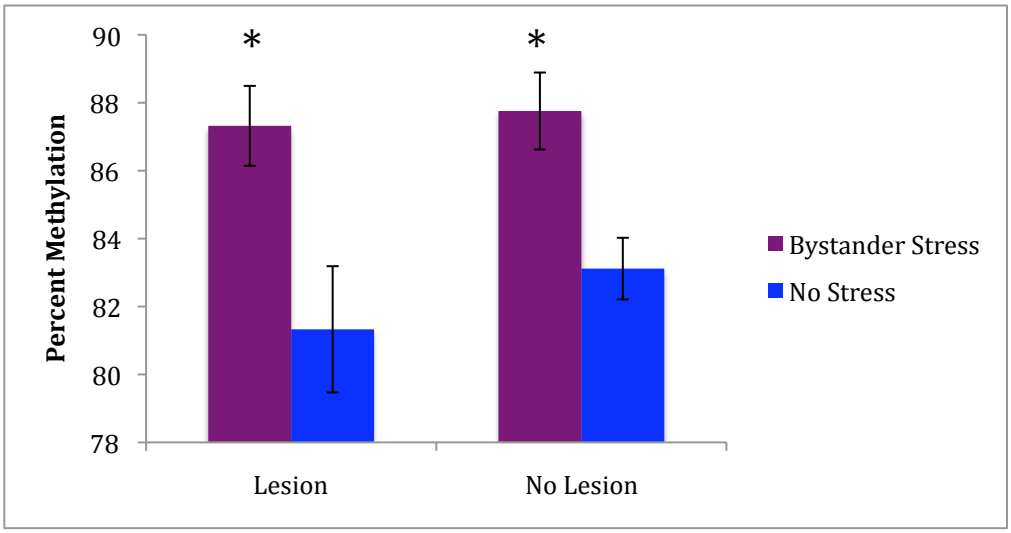

Figure 5.8: *Methylation levels in the hippocampus of female offspring sacrificed at P21 with lesions (*p < .001)*

Gene Expression – Mild Stress

In accordance with the false discovery rate, FlexArray completed a two-way ANOVA for expression level, with sex and stress as factors. This revealed a main effect of stress ($p < .00001$) for 236 genes. For a summary of these genes see Appendix 1. Of the 236 genes, 114 had changes in expression level in the frontal cortex and 128 exhibited expression level changes in the hippocampus. (Six of the genes were altered in frontal

cortex and hippocampus, 114 + 128 = 242.) Forty-seven of the genes with altered expression levels in frontal cortex were up-regulated whereas, 67 were down-regulated. In the hippocampus, 63 of the genes were up-regulated and 65 were down-regulated. Genes were further classified into 1 of 12 functional categories, see figure 5.9.

A significant Stress by Sex interaction ($p < .00001$) was also revealed for 44 genes as a result of MS. For a summary of these genes see Appendix 2. Thirty of the genes demonstrating an expression change dependent upon the Sex by Stress interaction were localized to the hippocampus and 14 to the frontal cortex. For functional classification of these genes see figure 5.10.

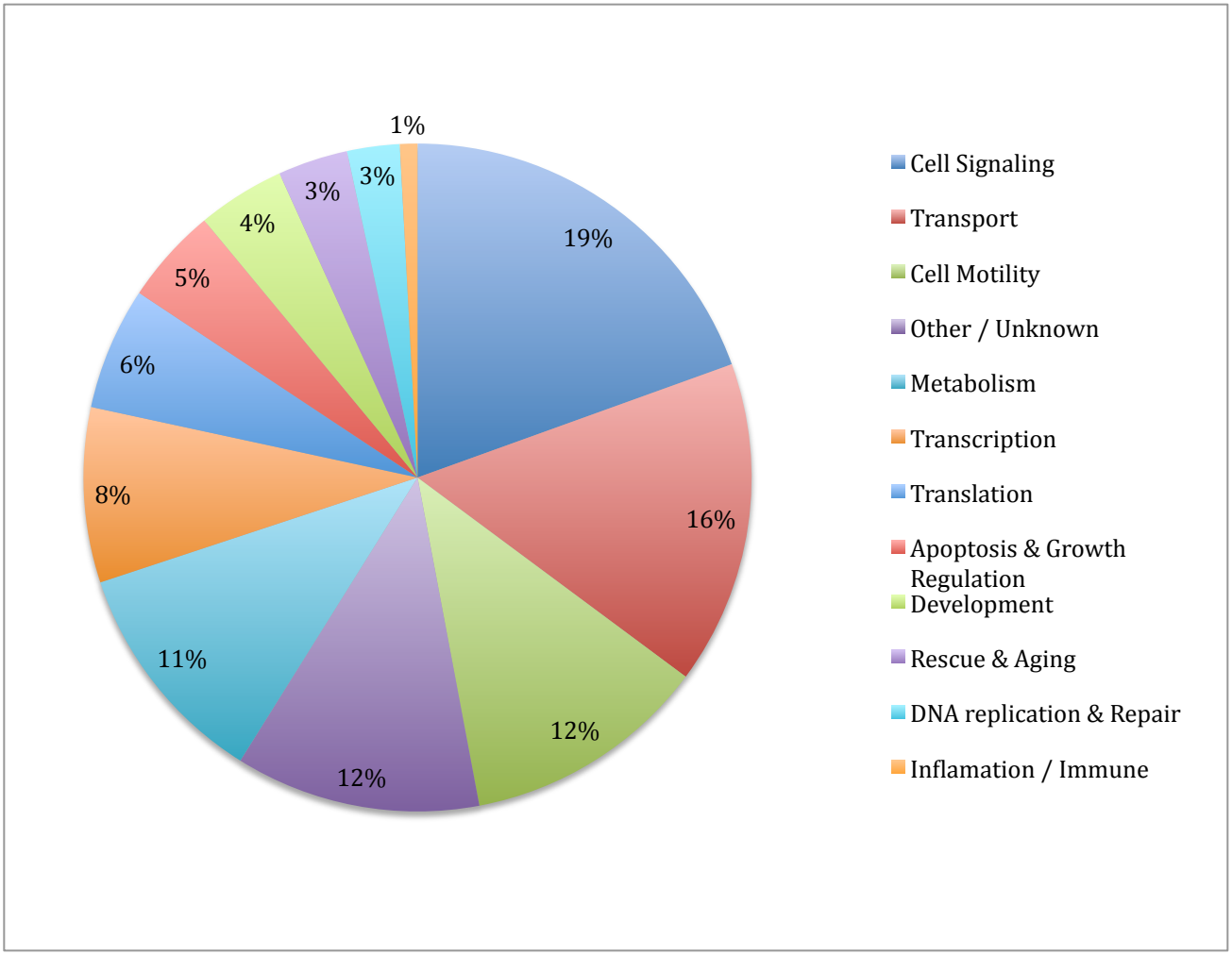

Figure 5.9: *Functional classification of genes altered by MS.*

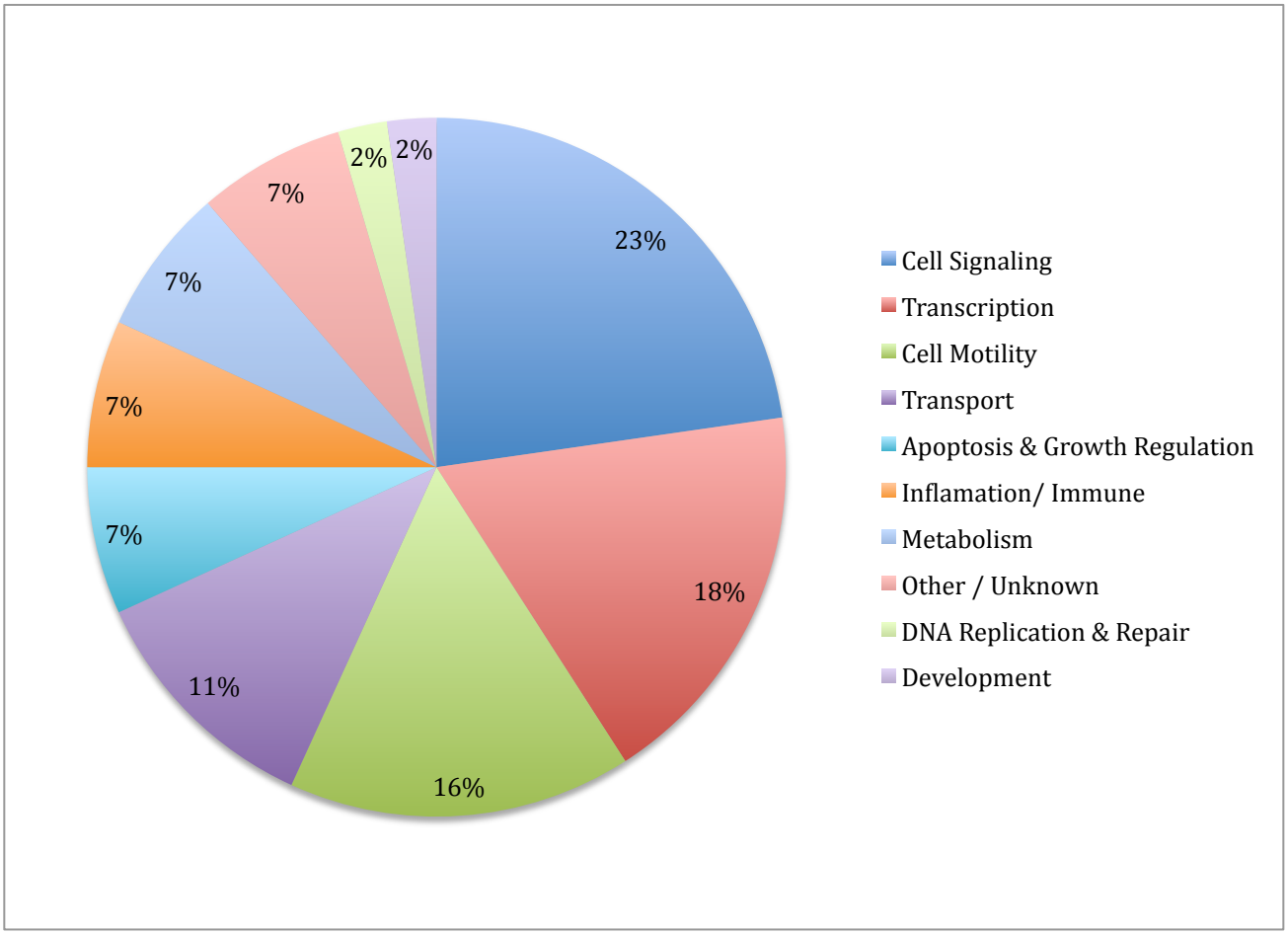

Figure 5.10: *Functional classification of genes demonstrating a significant interaction effect with sex and stress for expression level following MS*

Gene Expression – Bystander Stress

In accordance with the false discovery rate, FlexArray completed a two-way ANOVA for expression level, with sex and stress as factors. This revealed a main effect of stress ($p < .00001$) for 74 genes. For a summary of these genes see Appendix 3. Of the 74 genes, 32 had changes in expression level in the frontal cortex and 42 exhibited expression level changes in the hippocampus. Twenty-one of the genes with altered expression levels in frontal cortex were up-regulated whereas 11 were down-regulated. In

the hippocampus, 13 of the genes were up-regulated and 29 were down-regulated. Genes were further classified into 1 of 12 functional categories, see figure 5.11.

A significant Stress by Sex interaction ($p < .00001$) was also revealed for 53 genes as a result of BS. For a summary of these genes see Appendix 4. Thirty of the genes demonstrating an expression change dependent upon the sex by stress interaction were localized to the frontal cortex and 23 to the hippocampus. For functional classification of these genes see figure 5.12.

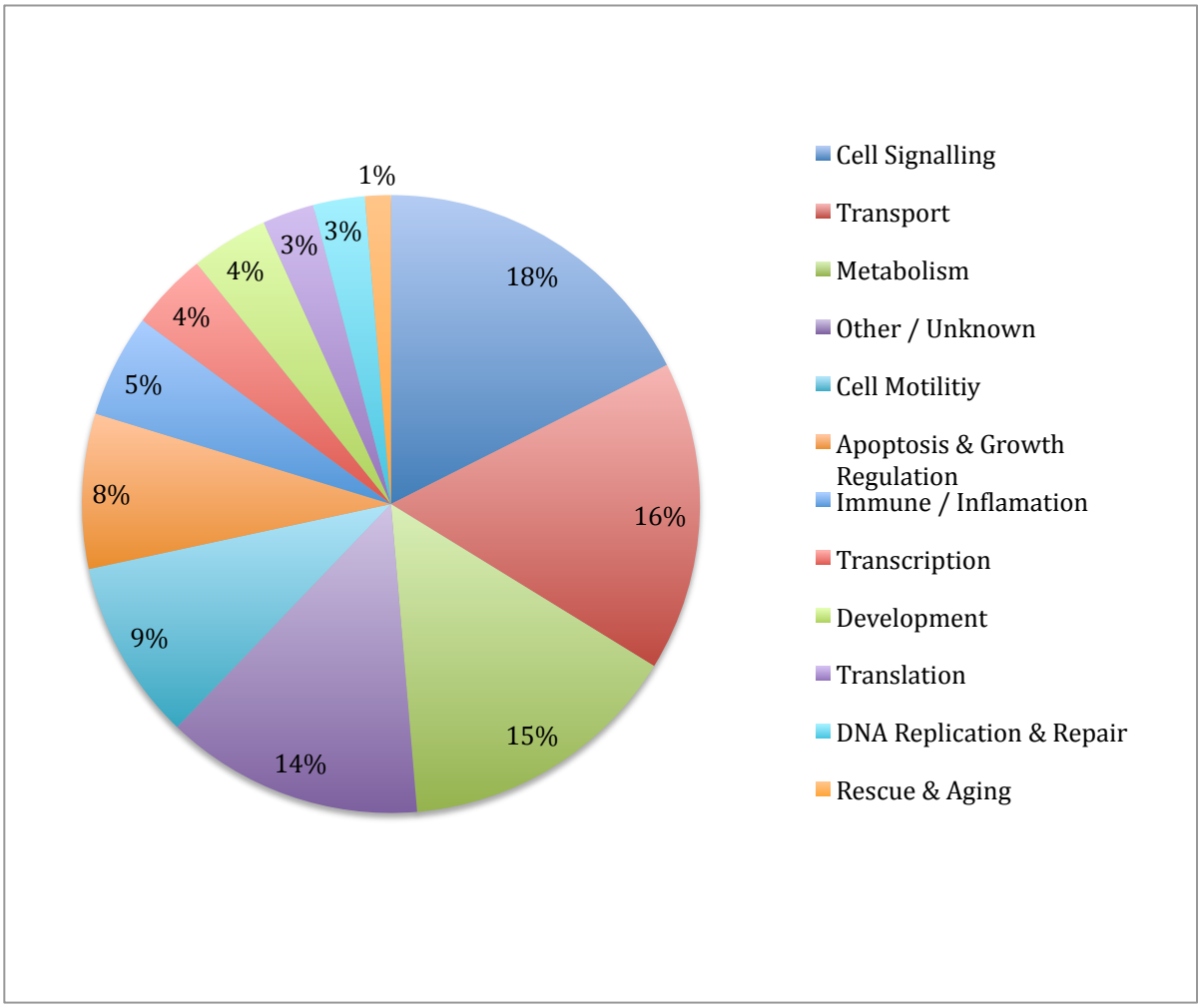

Figure 5.11: *Functional classification of genes altered by BS*

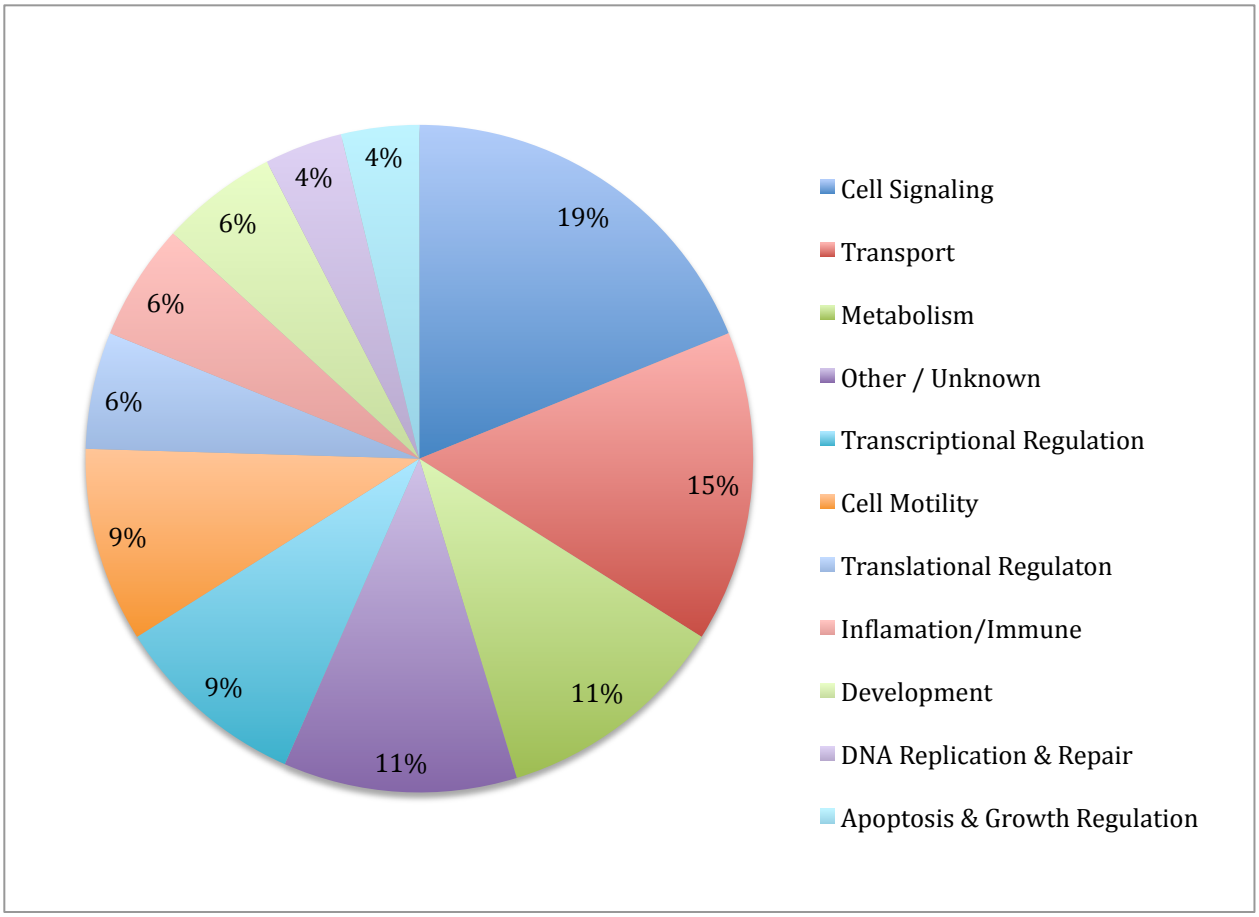

Figure 5.12: *Functional classification of genes demonstrating a significant interaction effect with sex and stress for expression level following BS*

Particular Genes of Interest

Although a perfect world would permit description of all genes demonstrating significant expression level changes as a result of the prenatal stress manipulations, it is not realistic. In an effort to generate a thesis that is readable and informative, 10 genes with particular importance to brain plasticity have been selected for further depiction. While all genes exhibiting significant changes can be found tabulated in Appendices 1-4, this section will highlight some of the exciting information that was obtained from the microarray study.

Alg-2 (Apoptosis-linked-gene 2)

Alg-2 is a 28 kDa cytosolic Ca^{2+} binding protein that forms dimers and belongs to the penta-EF (PEF) family (Sadoul, 2006; Vito, Lacana, & D'Adamio, 1996). The protein coded by Alg-2 gene is required for T-cell receptor, Fas and glucocorticoid –induced apoptosis (Missotten, Nichols, Rieger, & Sadoul, 1999; Sadoul, 2006; Vito, et al., 1996). Alg-2 is the first Ca^{2+} binding protein demonstrated to be directly involved in programmed cell death (PCD). It is hypothesized that Alg-2 acts as a Ca^{2+} sensor; as Ca^{2+} levels rise, Alg-2 binds Ca^{2+} resulting in a conformational change in Alg-2 that allows it to bind to other proteins (Krebs & Klemenz, 2000). Research has shown that Alg-2 forms a complex with Alg-2 interacting protein X (Alix) in response to abnormal Ca^{2+} levels, to regulate caspase-activation and the endolysosomal system necessary for the active destruction of cells (Sadoul, 2006). Upregulation of Alg-2 or Alix has been correlated with neuronal cell death. Conversely, cells expressing reduced levels of Alg-2 have been demonstrated to be resistant to T-cell receptor, steroid and staurosporine –induced apoptosis (Vito, et al., 1996).

Homeostasis is achieved through a delicate balance between cell proliferation, differentiation and apoptosis. PCD is an extremely important mechanism for the removal of unnecessary, damaged or aged cells during development (Krebs & Klemenz, 2000). Alterations to the genes regulating this balance may have long-term consequences for healthy maturation of the brain. The experiments carried for this thesis demonstrated a significant down regulation of Alg-2 in the frontal cortex of offspring born to prenatally stressed mothers (See figure 5.13). As research indicated, down-regulation of Alg-2 allows neurons to be resistant to glucocorticoid/steroid induced cell death (Vito, et al.,

1996). This may have served a compensatory purpose in utero, as the prenatally stressed offspring were likely exposed to higher levels of maternal stress hormones. However, in the absence of stress, abnormal resistance to apoptosis postnatally may actually pose as a risk for the development of autoimmune disorders or cancer (Krebs & Klemenz, 2000).

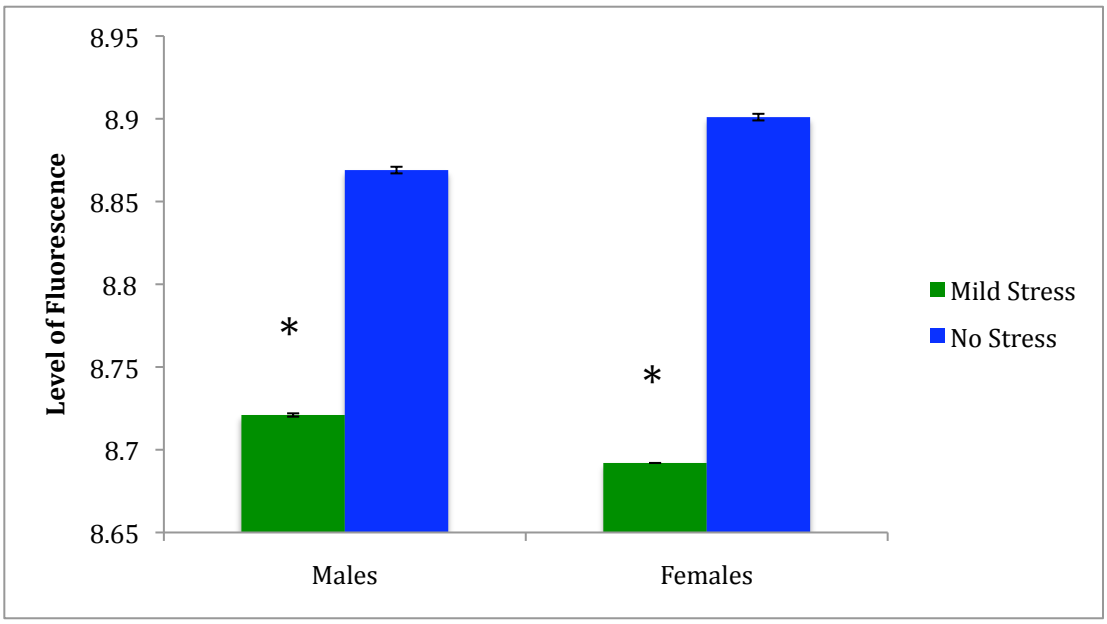

Figure 5.13: *Alg-2 expression level in the frontal cortex of offspring sacrificed at P21 following MS (*p < .00001)*

Adcy1 (Adenylyl cyclase 1)

Adcy1 is a membrane-bound, neurospecific enzyme that catalyzes the formation of cAMP which in turn directly stimulates neurite outgrowth (Inan & Crair, 2007). Adcy1 is commonly expressed in areas of the brain associated with neuroplasticty, such as the hippocampus and cerebral cortex (Abdel-Majid, et al., 1998). cAMP and Adcy1 are positive regulators of synaptic plasticity and are required for hippocampus-dependent memory (Wang, Ferguson, Pineda, Cundiff, & Storm, 2004), long-term potentiation (LTP) (Wang, et al., 2004; Wu, et al., 1995), and the association of conditioned and unconditioned stimuli (Abdel-Majid, et al., 1998). Adcy1 is vital for neuroplasticity because it integrates multiple signals to produce optimal cAMP transduction in neurons

(Wu, et al., 1995). Research has demonstrated that over-expression of Adcy1 increases LTP, increases memory for object recognition, and slows extinction of contextual memories (Wang, et al., 2004). Conversely, a disruption in Adcy1 perturbed LTP in the hippocampus (Wu, et al., 1995) and altered performance on the Morris water task (Wu, et al., 1995), while complete elimination of Adcy1 prevented barrel formation in the somatosensory cortex of mice (Abdel-Majid, et al., 1998).

The current experiments identified a significant reduction in Adcy1 protein expression in the hippocampus of offspring born to prenatally stressed mothers (See Figure 5.14). Research has clearly illustrated that modifications to Adcy1 expression have profound implications for the organism. Although there were identified behavioural differences in these offspring prior to weaning, they were not tested on the Morris water task so results cannot be compared with regards to this test. Because MS female *Adcy1* levels appear to approximate male control levels, it would be predicted however, that females may actually demonstrate an improvement on this task.

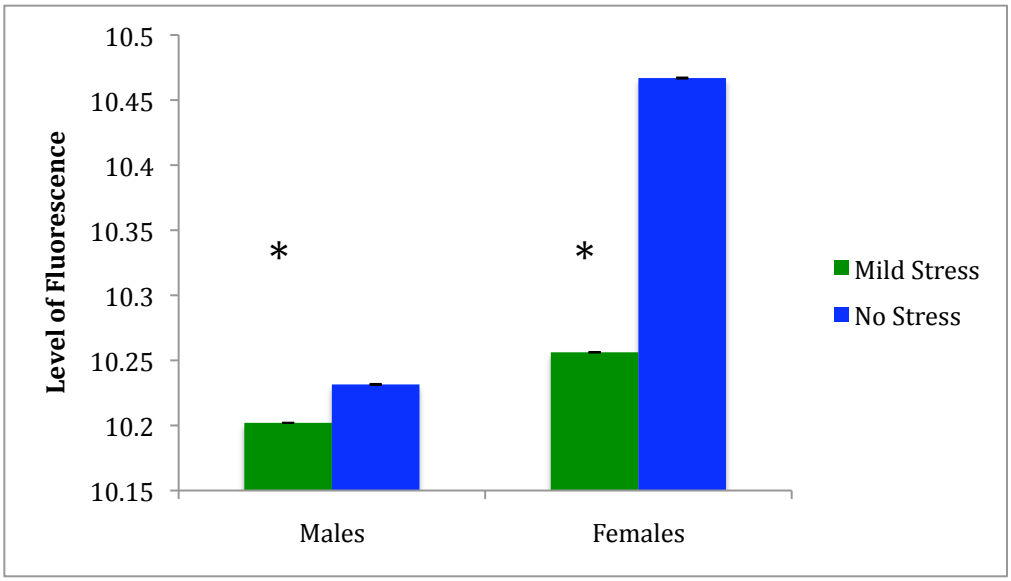

Figure 5.14: *Adcy1 expression level in the hippocampus of offspring sacrificed at P21 following MS (*p < .00001)*

Gria2 (Glutamate receptor 2 – Ionotropic AMPA, aka: GluR2)

Gria2 is a subunit of the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) specific glutamate receptor. In the mammalian CNS, AMPA-type glutamate receptors mediate the vast majority of excitatory synaptic transmission and play a key role in LTP and LTD (Isaac, Ashby, & McBain, 2007). AMPA receptors are composed of combinations of the 4 Gria subunits: Gria 1-4. Neurons and glia generally have AMPA receptors composed of 4 Gria2 subunits (Isaac, et al., 2007). Gria2 is a critical subunit in determining AMPA receptor function. This subunit establishes many of the biophysical properties of the receptor including: kinetics, channel conductance, ligand binding and most importantly Ca^{2+} permeability (Isaac, et al., 2007). AMPA receptors with Gria2 subunits are impermeable to Ca^{2+}, while AMPA receptors lacking Gria2, like those of GABAergic interneurons, are Ca^{2+} permeable (Isaac, et al., 2007). Normal Gria receptor subunit expression has been found to be important for extinction learning in fear expression paradigms (Gourley, Kedves, Olausson, & Taylor, 2009).

Research has demonstrated a relationship between neurological disorders such as pain and cerebral ischemia, and disruption in Gria2 function (Isaac, et al., 2007). Similarly, studies in humans have identified significant reductions in Gria2 AMPA receptors in the brains of schizophrenics when compared to age matched controls (Vawter, et al., 2002). Furthermore, clozapine, a potent atypical antipsychotic, has been shown to upregulate AMPA receptor density and these changes were hypothesized to mediate the drugs antipsychotic effects (Vawter, et al., 2002). Offspring exposed to prenatal stress in these experiments exhibited significant down regulation of Gria2 subunit expression (See Figure 5.15). This gene expression change would have perturbed

glutamatergic excitatory synaptic transmission in the frontal cortex. While it is unlikely that this epigenetic change would have caused the offspring to develop schizophrenia, it may have increased the predisposition to develop diseases like this.

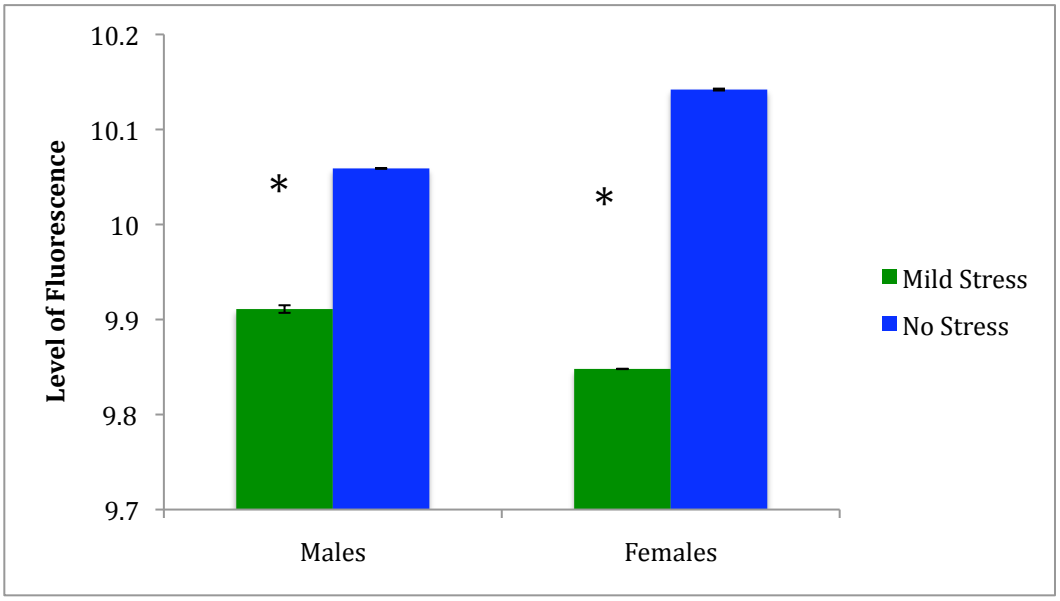

Figure 5.15: *Gria2 expression level in the frontal cortex of offspring sacrificed at P21 following MS (*p < .00001)*

NOV (Nephroblastoma overexpressed gene, aka: CCN3)

Nov is a small, secreted, cystein-rich protein of the CCN regulatory family. It is a immediate-early gene that is thought to play a multitasking matrical role in cell growth regulation, migration, differentiation and organogenesis (Dreau, et al., 2010; Sin, Bechberger, Rushlow, & Naus, 2008). By interacting with Notch, Nov regulates cell growth and differentiation (McCallum & Irvine, 2009; Sin, et al., 2009). Notch is a negative regulator of neural progenitor cell proliferation and differentiation (McCallum & Irvine, 2009; Van De Bor & Giangrande, 2001) and will repress neurogenesis or gliogenesis once activated by Nov. Overexpression of Nov leads to growth arrest in several tumor cell lines (Dreau, et al., 2010; Sin, et al., 2008) whereas down-regulation

increased growth of malignant gliomas (Sin, et al., 2008). Nov is also involved in cell motility. Through interaction with integrin receptors, Nov mediates cell-adhesion and can alter cell morphology (Sin, et al., 2009).

The perinatal period is a critical window for brain growth and development. Healthy brain maturation depends on a delicate balance between cell proliferation and pruning. Alterations to this balance are likely to have long-lasting effects on functioning and plasticity. Nov expression was found to be up-regulated in the frontal cortex of both male and female offspring exposed to prenatal MS (See Figure 5.16). By interacting with Notch, the increase in Nov would repress neurogenesis and gliogenesis; two very important processes for the developing brain. However, this increase in Nov expression may function to the benefit of the offspring later in life, as it is a known tumor suppressor for gliomas.

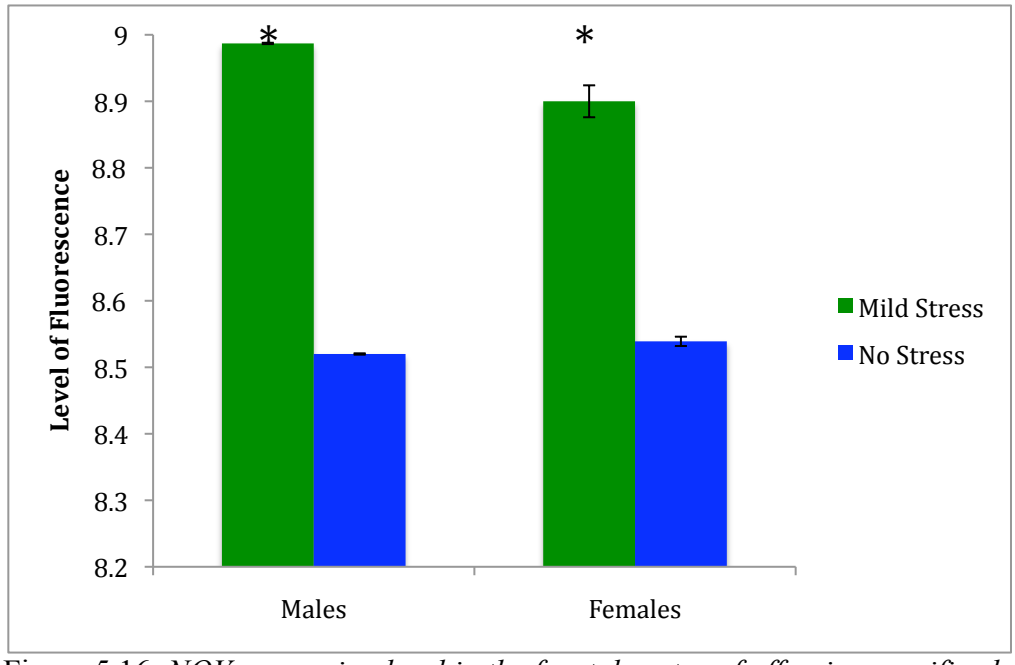

Figure 5.16: *NOV expression level in the frontal cortex of offspring sacrificed on P21 following MS (*p < .00001)*

Dbn1 (Drebrin)

Dbn1 encodes a cytoplasmic actin binding protein thought to play a role in neuronal morphogenesis. In the developing brain, Dbn1 is first expressed at the time of outgrowth and maturation, in cell bodies, growing axons and dendrites (Shirao, 1995; Shirao, Kojima, & Obata, 1992). In the adult brain, Dbn1 is characteristically localized at dendrites, which are postsynaptic terminals of excitatory synapses (Hatanpaa, Isaacs, Shirao, Brady, & Rapoport, 1999; Shirao, 1995). Dbn1 plays a critical role in communication between presynapses and postsynapses by regulating the neurite processes and wiring of the brain (Shim & Lubec, 2002). Dbn1 accumulates in the spines of neurons and modulates dendritic morphology by regulating actin polymerization (Hatanpaa, et al., 1999). Inoculation of non-neuronal cells with Dbn1 induces the formation of highly branched neurite-like cell processes while suppression of Dbn1 abolishes a neuron's ability to extend neurite processes (Furushima, et al., 2007).

Dendrite formation is the most characteristic feature of structural plasticity in mature neuronal synapses (Shim & Lubec, 2002). Furthermore, expression patterns of Dbn1 are associated with defined windows of cortical plasticity (Shirao, et al., 1992). For example, in the cat visual cortex, expression of Dbn1 is most extensive during the sensitive period of ocular dominance (Shirao, et al., 1992). The research conducted for this thesis identified a decrease in Dbn1 expression in the frontal cortex of both male and female offspring born to MS mothers (See Figure 5.17). Decreased Dbn1 levels might reflect a loss of spines that could seriously diminish a neuron's ability to modulate synaptic input. The decrease in Dbn1 may be reducing synaptic plasticity during an extremely important sensitive period for brain maturation.

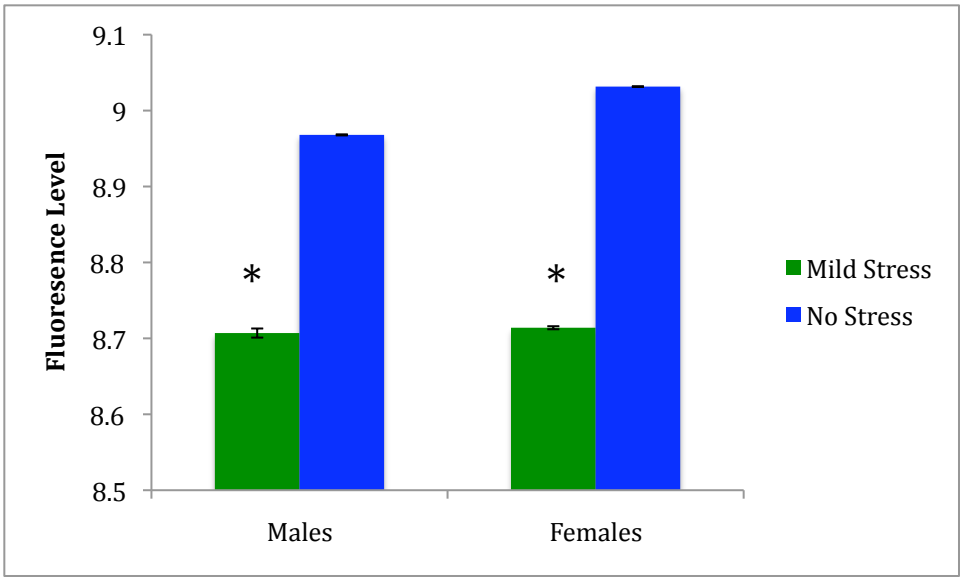

Figure 5.17: *Dbn1 expression level in the frontal cortex of offspring sacrificed at P21 following MS (*p < .00001)*

Shisa4 (Shisa homolog)

The Shisa4 gene encodes a single-pass type I membrane protein that is an antagonist to both Wnt and FGF signaling (Furushima, et al., 2007; Yamamoto, Nagano, Takehara, Hibi, & Aizawa, 2005). Shisa4 prevents Wnt and FGF signaling by suppressing the maturation and trafficking of the cell surface expression of Frizzled and the FGF receptor (Furushima, et al., 2007). (Wnt is the ligand for the frizzled receptor while FGF binds FGF receptors 1-4.)

Adult hippocampal progenitor cells express receptors and signaling pathways for Wnt proteins and Wnt signals have been demonstrated to regulate adult hippocampal neurogenesis (Lie, et al., 2005). Overexpression of Wnt is sufficient to increase neurogenesis while blocking Wnt expression reduces neurogenesis (Lie, et al., 2005). Similarly, FGF is also involved in hippocampal neurogenesis. FGF-2 specifically has been regarded as one of the most potent growth factors in the CNS (Yoshimura, et al.,

2003). Overexpression of FGF-2 has been found to reduce neuronal death and up-regulate neurogenesis in the dentate gyrus (Yoshimura, et al., 2003).

From this it can be inferred that, an increase in Shisa4 expression would result in a reduction of Wnt and FGF signaling, consequently reducing hippocampal neurogenesis. On the other hand, a decrease in Shisa4 expression would up regulate Wnt and FGF signaling, promoting neurogenesis in the hippocampus. A seemingly small sex-dependent alteration in expression of a single gene is modifying multiple signal transduction pathways producing a spectrum of changes. A significant Stress x Sex interaction was identified for Shisa4 expression levels in the hippocampus of offspring exposed to prentatal stress (See Figure 5.18). Males experienced a reduction in Shisa4 expression and females exhibited an increase in Shisa4 gene expression following prenatal stress. This sex-dependent epigenetic change impacts two distinctive and essential signaling pathways in the hippocampus and may help explain sex differences in behaviour.

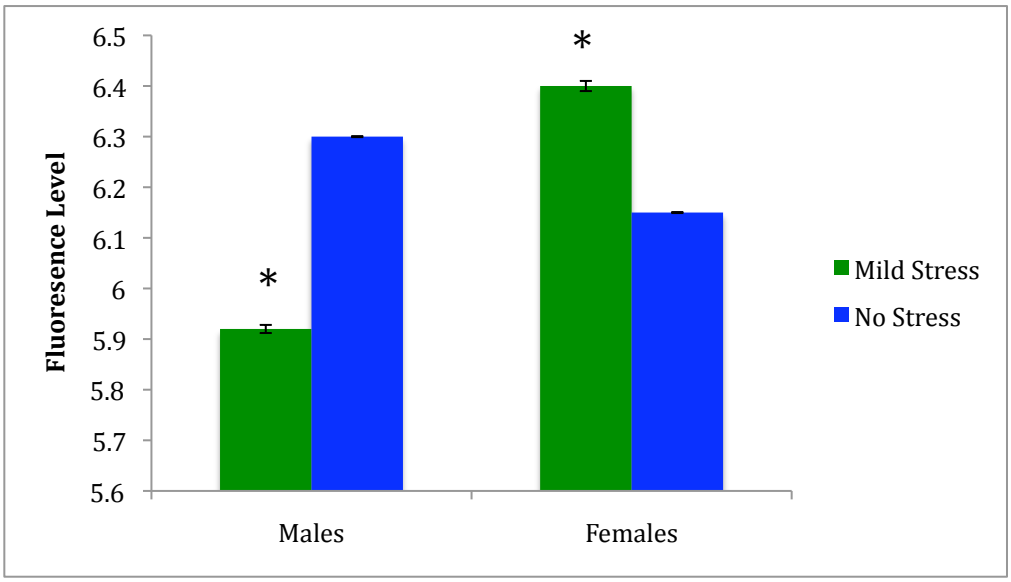

Figure 5.18: *Shisa4 expression level in the hippocampus of offspring sacrificed at P21 following MS (*p < .00001)*

Sema7A (Semaphorin 7A)

Semaphorins are a large family of secreted and membrane-associated proteins which are instrumental in formation of the neural network (Pasterkamp, Kolk, Hellemons, & Kolodkin, 2007; Suzuki, et al., 2007). Semaphorins are axon guidance factors that help growing axons find appropriate targets, influence axonal and dendritic growth, and assist axons in synapse formation (Pasterkamp, et al., 2007). Sema7A is a glycosylphosphatidylinositol-anchored semaphorin that is widely expressed in the adult CNS and promotes axon outgrowth through interaction with β1-integrin receptors (Suzuki, et al., 2007). Sema7A has chemotropic effects and is a potent growth promoting factor for embryonic axons and postnatal dendrites (Pasterkamp, et al., 2007). Early in life, Sema7A is involved in neural crest migration and/or differentiation, but later on promotes branching and enhances neurite outgrowth of cortical, olfactory and sensory neurons (Pasterkamp, et al., 2007). It is also important for activation of mitogen-associated protein kinase (MAPK) and phosphorylation of focal adhesion kinase (FAK) and extracellular regulated kinase (ERK) (Suzuki, et al., 2007). Pasterkamp (2007) has speculated that Sema7A contributes to the formation and maintenance of efferent and afferent dopaminergic midbrain connections.

It is clear that Sema7A plays a critical role in brain development. Dendritic branching and synaptic formation are important for network formation and learning and memory. Sema7A gene expression was up-regulated in the frontal cortex of both male and female offspring born to mothers in the prenatal bystander stress group (See Figure 5.19). The effects of this up-regulation could be beneficial or detrimental. While one would assume an increase in dendritic branching and axon outgrowth would confer an

advantage for learning, memory and pattern consolidation this is not always the case (Kolb, Gorny, Li, Samaha, & Robinson, 2003).

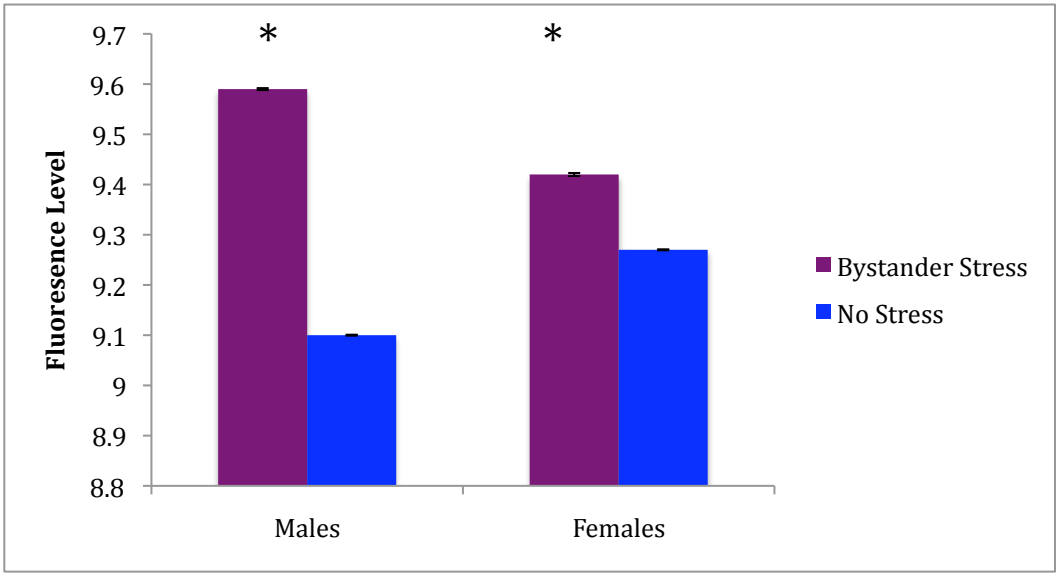

Figure 5.19: *Sema7a expression level in the frontal cortex of offspring sacrificed at P21 following BS (*p < .00001)*

Lrp8 (Low density lipoprotein receptor – related protein 8, aka: apolipoprotein e receptor)

Lrp8 encodes a 34 kDa apolipoprotein e receptor that is involved in cellular recognition and internalization of lipoproteins (Ma, et al., 2002). The Lrp8 receptor is multifunctional. It can mediate endocytosis and signal transduction (Andersen, Benhayon, Curran, & Willnow, 2003). Known ligands of the Lrp8 receptor include Apo E and Reelin. Apo E is a major cholesterol and triglyceride carrying lipoprotein. Lipoproteins are important for specialized neuronal membranes like myelin (Kim, et al., 1996). Most lipids in the CNS, are synthesized by astrocytes in the CNS and neurons are thought to take them up via Lrp8 receptor mediated endocytosis (Kim, et al., 1996). It has also been hypothesized that Apo E interacts with neuronal Lrp8 receptors to affect synaptic plasticity (Clatworthy, et al., 1999). Reelin also binds the Lrp8 receptor and is

important for neuronal positioning, axonal branching and layer formation in the developing brain (Andersen, et al., 2003). Reelin is secreted by cells in the outermost layer of the developing cortex and orchestrates the migration of neurons (Beffert, et al., 2006). Lrp8$^{-/-}$ mice show a disruption in cortical layering and disturbed neuronal patterning in the hippocampus (Ma, et al., 2002).

Through differential ligand binding Lrp8 is important for myelin formation, neuronal migration, axonal branching, and synaptic plasticity; all of which are vital for healthy brain development. Modifications to the expression level of Lrp8 would likely have long-term effects on offspring brain functioning. A sex-dependent change in expression of Lrp8 was found for offspring born to BS mothers (See Figure 5.20). While final expression levels for male and female offspring were equivalent, males exhibited an up-regulation and females exhibited a down-regulation of Lrp8 in the frontal cortex. The epigenetic change appears to have neutralized the sex-difference seen in NS rats and could be altering sex-differences in cortical patterning and myelination.

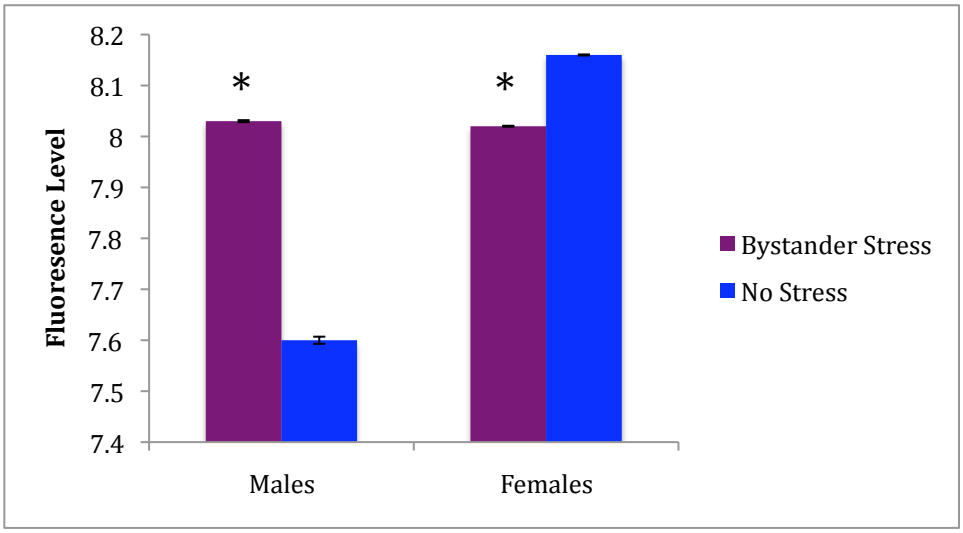

Figure 5.20: *Lrp8 expression level in the frontal cortex of offspring sacrificed at P21 following BS (*p < .00001)*

Safb1 (Scaffold attachment factor B1)

Safb1 is a large nuclear protein with multiple functional domains (Oesterreich, 2003). It has been implicated it in a wide variety of cellular processes, such as RNA splicing, transcriptional regulation, signal transduction, and the cellular response to stress (Lee, Colley, Norman, Biamonti, & Uney, 2007; Sergeant, et al., 2007). Two processes of particular interest to this research are; Safb1's ability to repress the heat shock protein 27 (hsp27) promoter and estrogen receptor α (ERα) – mediated transcription. Hsp27 is involved in the apoptotic signaling pathway; an increase in Hsp27 inhibits the pro-caspase 9 pathway and enhances activation of the NF-$_K$B pathway that controls cell growth, inflammation, and the cellular stress response (Oesterreich, 2003). ERα is a steroid receptor which dimerizes upon ligand binding and activates transcription of target genes important for cellular proliferation and differentiation (Oesterreich, 2003).

A decrease in Safb1 gene expression was found in the hippocampus for both male and female offspring exposed to MS (See Figure 5.21). Given that Safb1 represses the hsp27 promoter, a decrease in Safb1 would permit increased expression of hsp27 and consequently reduce apoptosis and activate the cellular stress response. Similarly, because Safb1 also represses ERα - mediated transcriptional activation, a decrease in Safb1 would allow for an increase in transcription of ERα target genes. Decreased activation of apoptotic pathways in conjunction with an increase in cell proliferation and differentiation could lead to uncontrollable cell growth in the hippocampus of these offspring.

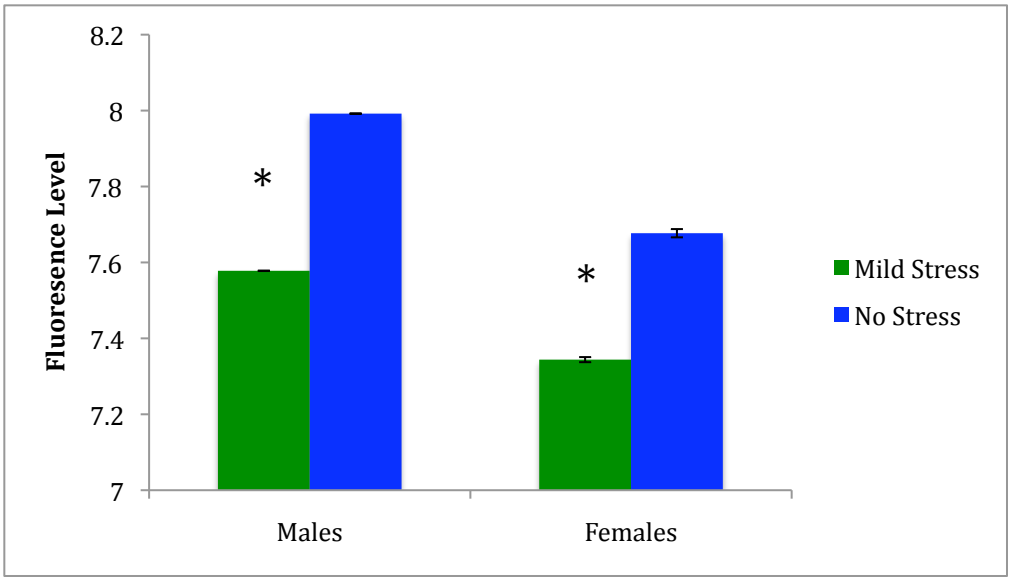

Figure 5.21: *Safb1 expression level in the hippocampus of offspring sacrificed on P21 following MS (*p < .00001)*

Igf2r (Insulin-like growth factor 2 receptor)

The Igf2r gene encodes a transmembrane receptor molecule for both insulin-like growth factor II (IGF2) and manose-6-phosphate (M6P). The receptor is involved in protein trafficking, transmembrane signaling, fetal organogenesis, tumor suppression and T-cell mediated immunity (Hawkes & Kar, 2003; Kornfeld, 1992; Wylie, et al., 2003). Despite imprinted expression in most tissues, Igf2r is bi-allelically expressed in the brain (Yamasaki, et al., 2005). The maternal allele is expressed throughout the body and in the glia and fibroblasts of the brain, while the paternal allele is expressed in neurons (Yamasaki, et al., 2005). Igf2r levels reach their peak between gestational days 16-20 and then decline thereafter. This is accompanied by the same pattern of IGF2 expression, implicating IGF2 and Igf2r as important players for fetal growth and development (Kornfeld, 1992). In support of this, an Igf2r knockout results in fetal overgrowth and lethality (Wylie, et al., 2003).

The main function of the Igf2r is to regulate the extracellular bioavailablity of IGF2 (Hawkes & Kar, 2003; Kornfeld, 1992; Wylie, et al., 2003). Igf2r binds and internalizes IGF2 at the cell surface resulting in degradation of IGF2 at the lysosome (Wylie, et al., 2003). High levels of IGF2 stimulate proliferation and differentiation of neuronal and glial cells early in development (McKelvie, Rosen, Kinney, & Villa-Komaroff, 1992). In the postnatal period, increased IGF2 is involved in the potentiation of endogenous acetylcholine release from the hippocampus (Hawkes & Kar, 2003). Igf2r expression is highest in pyramidal cells, the polymorphic layers of the hippocampus and the granule cell layer of the dentate gyrus – regions of the brain important for information processing, memory formation, and plasticity (Wylie, et al., 2003). It is hypothesized that Ifg2r contributes to the development of these brain areas and has been identified as the 1st putative "IQ gene" (Wylie, et al., 2003).

Igf2r is particularly important to this dissertation, as it was the only gene in which a main effect was demonstrated for all 4 analyses. This gene appears to be easily modifiable and particularly susceptible to the effects of prenatal stress. A main effect of stress was found in the frontal cortex of both male and female offspring of MS mothers in which there was a reduction in Igf2r expression (See Figure 5.22).

A Stress by Sex interaction was seen in male and female offspring of MS mothers in the hippocampus in which males exhibited an increase in expression and females exhibited a decrease. A main effect of stress was also found in both males and females born to BS mothers in whom there was reduction in Igf2r expression in the hippocampus (See Figure 5.23). The Stress by Sex interaction also demonstrated a significant alteration in Igf2r expression for BS offspring in the hippocampus. These changes to Igf2r

expression occurred during a critical window for fetal development and will likely have long-term effects. Modifications to Igf2r levels affect the concentration of circulating IGF2 consequently altering neuronal proliferation and differentiation, memory consolidation, and plasticity.

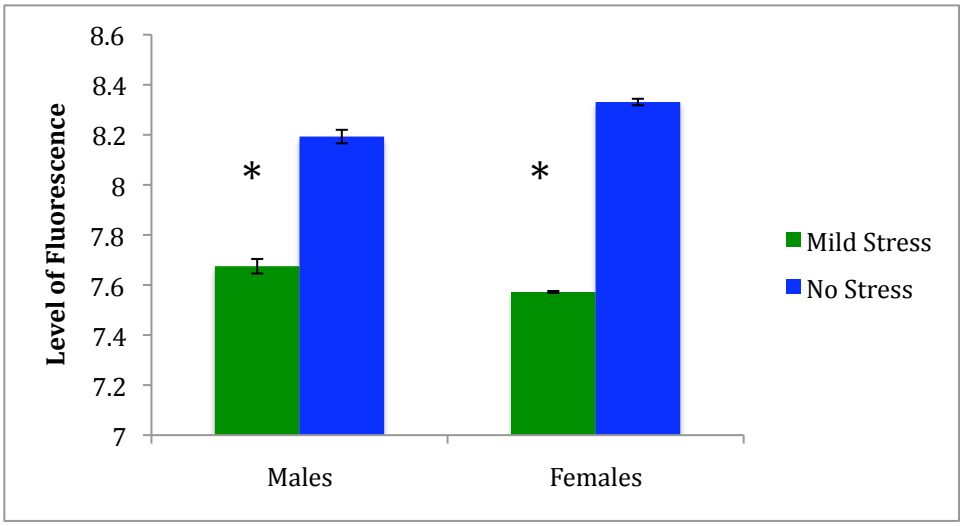

Figure 5.22: *Igf2r expression level in the frontal cortex of offspring sacrificed at P21 following MS (*p < .00001)*

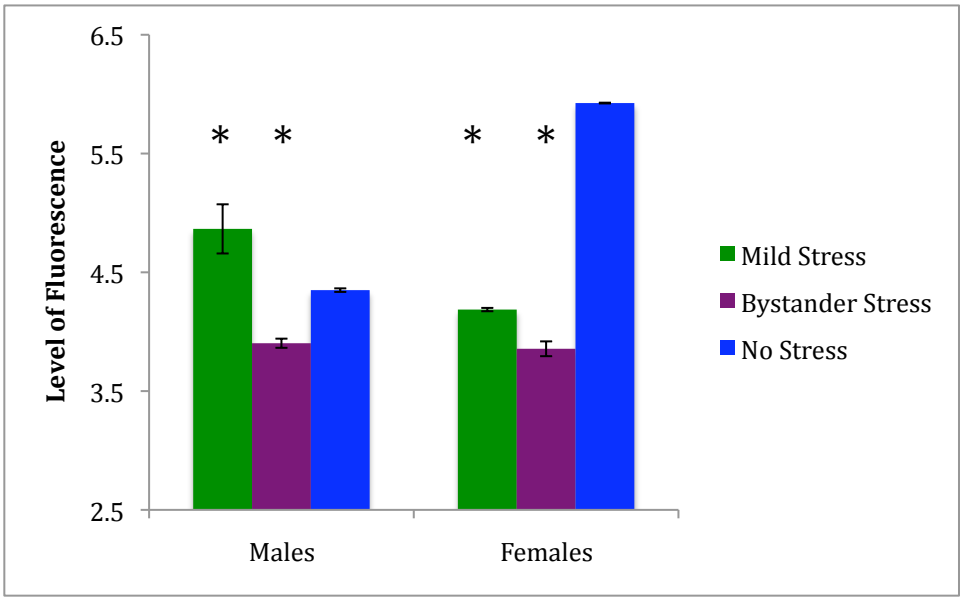

Figure 5.23: *Igf2r expression level in the hippocampus of offspring sacrificed at P21 following prenatal stress (*p < .00001)*

Discussion

The genetic aspect of this thesis clearly demonstrates that modifications to the intrauterine environment have widespread effects on the epigenome of the developing offspring. Recent research supports the notion that epigenetic mechanisms, which apply lasting control over gene expression without changing the actual genetic sequence, are mediating stable changes in brain function (Darnaudery & Maccari, 2008). Consistent with the previous 2 chapters, and supporting the theory that modifications to gene expression are mediating changes in brain, variations in the prenatal stress paradigm produced dramatically different results.

In contrast to expectation, prenatal stress did not always produce an increase in global methylation. Whereas offspring born to MS and BS dams exhibited significant increases in global methylation in both the frontal cortex and hippocampus, offspring born to ES mothers experienced significant global methylation reductions in these brain areas. It is likely that these epigenetic changes are the consequence of the maternal stress response. The HPA axis is a well-studied mediator of the relationship between maternal experiences and fetal development (Matthews, 2002; McCormick, Smythe, Sharma, & Meaney, 1995; Meaney, et al., 1985). Lipophilic steroids like glucocorticoids easily cross the placenta (White, Mune, & Agarwal, 1997) and glucocorticoid receptors are expressed in most fetal tissues early in embryonic development (Meaney, et al., 1985). While the placenta and the fetus have developed mechanisms to protect the fetus from maternal cortisol this can only compensate to a certain threshold (White, et al., 1997). This evidence suggests that small increases in maternal-stress hormone-release can silence genes by increasing global methylation, whereas a much greater increase in maternal

stress hormone release activates genes by decreasing global methylation. This bidirectional change would seem to imply an advantageous adaptation for one of the groups; however, both hypo- and hyper-methylation of the genome have been associated with health risks and adverse outcomes (Jaenisch & Bird, 2003). Additionally, as previously mentioned, it is possible that the methylation changes identified in this research occurred in transposable elements as well as in gene promoter regions. Consistent with gene expression, hypomethylation of transposable elements is associated with increased activity whereas, hypermethylation is linked to inactivation (Rakyan, et al., 2002). Therefore, reductions to global methylation, as seen in ES offspring, may be increasing activity of transposable elements in addition to raising gene expression. As the methylation of transposable elements has been deemed a defense mechanism to prevent their replication (Das, et al., 2009), this would likely result in negative outcomes for the offspring.

For both male and female offspring, mPFC lesion was correlated with a decrease in global methylation of the frontal cortex, but no change in the hippocampus. It can be hypothesized that in response to the mPFC lesion, methylation was decreased to activate genes in an effort to compensate and repair the brain. Similarly, offspring exposed to BS also demonstrated a decrease in frontal cortex methylation following mPFC lesions but no change in the hippocampus. Interestingly, the combination of BS (an increase in global methylation) and mPFC lesion (a decrease in global methylation) produced a methylation pattern that was not different from NS-no lesion offspring. As anticipated, the mPFC lesion had no effect on hippocampal methylation pattern as the damage to the brain was quite localized.

The gene expression microarray provided results equally fascinating. While MS and BS offspring showed similar changes in global methylation the actual genes undergoing modification were surprisingly different. Of the 316 genes changed as a result of either prenatal stress paradigm only 4 (*Dock9, Gja5, Igf2r, Slc9a6)* or 1.3% were common to both groups. Classification of the main function of all of the genes as they pertain to brain also revealed interesting findings. Offspring genes altered in response to MS exposure were heavily involved in cell signaling, transport, and cell motility. Similarly, sex-dependent gene changes in offspring of MS dams were mainly responsible for cell signaling, transcription, and cell motility. Considering the majority of genes altered in response to BS were implicated in cell signaling, transport, and metabolism it is remarkable that the overlap between BS and MS gene-expression change is minimal.

In addition to the intriguing gene expression changes identified, there were notable absences in genes hypothesized to demonstrate expression changes in response to prenatal stress. Most significantly, due to prior experimentation in this laboratory (Kolb & Gibb, 2007), alterations to levels of *FGF* and *FGF-2* expression were anticipated. Although expression of *FGF-2* was altered at $p < .0001$, the change was not seen once the false discovery algorithm was employed. Similarly, in accordance with research in other laboratories (Meaney, et al., 1996; Sapolsky, 2004) changes in GC receptor concentrations were expected. Again, this dissertation found alterations in GC receptor concentrations before the false discovery algorithm, but not after. The failure to reproduce these results exemplifies an important concern with gene expression analysis. It is difficult to attribute meaningfulness to genome wide changes at the genetic level. Two genes may have similar *p* values but one has a fold change twice that of the other

arbitrarily making it more important. In order to appropriately define meaningfulness, a weight related to the functioning of each gene and its significance with respect to the system being studied, along with the *p* value and fold changes, would need to be compared. However, current knowledge of gene function and system specific efficacy is limited preventing a true comparison of meaningfulness.

Despite this limitation, there is strong evidence demonstrating that variations to prenatal stressors have highly variable effects on the epigenome. Both prenatal stress paradigms (BS & MS) are primarily influencing cell-signaling pathways but the vehicles of change are completely different. As illustrated in the *particular genes of interest* portion of this chapter small changes to a signaling pathway can result in significantly diverse outcomes. Furthermore, cell signaling, motility, transcription, and metabolism are vital processes for the maintenance of brain plasticity. These findings undoubtedly illustrate that both MS and BS are influencing offspring brain development and long-term plasticity.

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Chapter 6: General Discussion and Conclusion

This thesis contributes to the understanding of the interaction between fetus and mother; specifically how prenatal stress impacts the developing brain. The detrimental effects of prenatal stress have been documented extensively in laboratory animals (Gue, et al., 2004; Lemaire, et al., 2000; Meaney, et al., 2007; Mueller & Bale, 2008) and humans (Huizink, Robles de Medina, Mulder, Visser, & Buitelaar, 2003; Ruiz & Avant, 2005). For a review see Lupien, McEwen, Gunnar, & Heim (2009). However, until now, there has been limited research regarding offspring outcomes as they pertain to variations in intensity of prenatal stress. Furthermore, this dissertation is unique insofar as it examines the development of the offspring brain at multiple interdependent levels.

The main goals of these studies were to build an understanding of how variations in dose and experience of prenatal stress alter brain plasticity at the genetic, cellular, morphological, and behavioural level. Genes or environment alone are insufficient to predict most developmental outcomes; this thesis examines the synergistic interaction between brain and experience and how they interact to produce differential outcomes. The results of these experiments produced a number of novel findings that can be used to advance our understanding of this critical developmental window. The findings are summarized below.

1. Not all prenatal stress is the same.

One of the most interesting findings from this dissertation is that variations to the intensity and nature of prenatal stress alter the developmental sequelae of offspring. Even more fascinatingly, the prenatal stress does not have to be experienced by the mother directly to affect offspring development.

Bystander stress, or stress to the dam's cage-mate, changes the uterine environment and in turn modifies offspring maturation. Research has identified different outcomes related to physical versus emotional stress (Pijlman, et al., 2003; Ramsey & Van Ree, 1993), but the effects of emotional stress as they relate to prenatal environments have not been studied previously. Through analysis of ultrasonic vocalizations, it is clear that by stressing one of the two cage-mates their existing relationship is altered and in turn stresses both animals.

The evidence from this dissertation demonstrates that prenatal stress always has an effect on the developing brain, but the effect varies both quantitatively and qualitatively depending on the nature of the stressor. Although the changes that occurred were dose-specific, offspring from all 3 prenatal stress paradigms exhibited modifications to brain. Remarkably, higher doses did not always produce the most dramatic effects. This is evidence that not all prenatal stress is the same and implies that not all prenatal stress is bad - just different. These findings also indicate that there needs to be a reexamination of previous work done regarding prenatal stress and offspring outcomes. Owing to variations in lab protocols and stress paradigms it may be impossible to generalize results. If the outcomes are not the same, universal remedial efforts will be ineffective.

2. Prenatal Stress & Interdependent Changes

Prenatal stress produces interdependent changes in the brain evident at the genetic, cellular, structural, and behavioural level. Changes in behaviour are often correlated with changes in brain. Structural changes occur in response to stimuli

and must be related to changes at the genetic level. In attempt to avoid the chicken vs egg argument this thesis is based upon the following assumptions: 1) elevated glucocorticoid levels resulting from maternal stress change the developmental environment of neurons (White, et al., 1997); 2) this hormonal change produces adaptive epigenetic modifications (Talge, et al., 2007); and, 3) the epigenetic modifications result in changes to neuron number, structure, morphology, and communication. These structural changes in turn alter offspring behaviour and response to internal and external stimuli (Radley, et al., 2004).

This thesis identified significant genetic modifications in the frontal cortex and hippocampus of offspring born to prenatally stressed mothers. A majority of these epigenetic changes (over 50%) occurred in genes involved in cell signaling, transcription, cell motility, and cell metabolism; all elements necessary for substantial changes in neuronal morphology and synaptic plasticity. Additional experiments revealed considerable alterations to dendritic morphology, cell number, and synapse number in the same two brain areas. The anatomical changes outlined in chapter 4 could easily be attributed to the genetic modifications described in chapter 5 based upon knowledge of the genes' functionality. For brief description of gene functionality see Appendices 1-4. Just as anatomical changes cannot arise without changes in protein synthesis, alterations in behaviour cannot exist without modifications to neuronal organization. Although it cannot be guaranteed that the behavioural changes identified in the prenatally stressed offspring were directly related to the anatomical changes noted, prenatal stress did result in significant alterations to

normal behavioural patterns. The identified behavioural changes may be related to changes in brain areas that were not examined; the early behavioural test battery utilized in this dissertation was not specific enough to relate specific brain regions to specific behavioural deficits.

Despite the fact that there is no mechanism by which to completely verify the relationship between the prenatal stress and the genetic, cellular, structural, and behavioural changes in offspring, this dissertation provides compelling evidence for a causal pathway. Data obtained from these experiments have clearly illustrated that manipulating only the prenatal environment results in substantial changes in the brain at multiple synergistic levels.

3. Prenatal Stress & Sex Differences

Variations in the prenatal stress paradigms produced measurable sex-dependent differences in brain. These sex differences were evident at all investigated levels. Normal sex differences in the brain are generated by exposure to steroid hormones during a sensitive prenatal period, which alter subsequent hormonal and non-hormonal stimuli through out the lifespan (McCarthy, et al., 2009). Recent evidence suggests there are sex-differences in both the capacity to transmit traits as well as to inherit them (McCarthy, et al., 2009). There is an abundance of literature demonstrating sex-dependent outcomes related to prenatal stress (Bowman, et al., 2004; Champagne & Curley, 2005; Kaiser, Kruijver, Swaab, & Sachser, 2003) and the research for this dissertation further supports these claims. Consistent with other research (Bowman, et al., 2004; Kaiser, et al.,

2003), the current studies demonstrated that prenatally stressed male offspring were sometimes partially feminized whereas female offspring were sometimes partially masculinized. These were not all-or-none changes but tended to be selective to specific measures

In addition to this, findings revealed many other sexually dimorphic brain changes. Data collected in chapter 3 revealed that, behaviorally, male offspring were affected most by direct stress to the mother and often showed little behavioural disruption when born to BS mothers. On the contrary, female offspring generally exhibited behavioural alterations in a dose-dependent manner. Anatomically, male offspring of BS mothers showed the least amount of disruption to normal neuronal organization, whereas females born to these mothers exhibited the most. Male neuroanatomy changed dramatically in all three brain regions studied as a result of prenatal ES but only in one brain area in the female counterparts. Finally, although there were no sex-dependent changes in global methylation in the frontal cortex or hippocampus, gene expression analysis did identify 44 genes altered in a sex-dependent manner in relation to prenatal MS and 53 genes altered as a result of prenatal BS. Research has demonstrated that epigenetics contribute to both the establishment and maintenance of sex differences in the brain (McCarthy, et al., 2009). The sex-dependent epigenetic modifications resulting from prenatal stress exposure (as identified in this dissertation) will continue to propagate sex-differences in response to subsequent experiences. These changes will contribute to the consistently observed sex-differences in long-term health outcomes.

4. Critical Periods and Subsequent Experiences

Experiences, including prenatal stress, can impact the structure and circuitry of the brain, altering subsequent responses to a variety of situations (Radley, et al., 2004). There is strong evidence for stress hyporesponsiveness during gestation suggesting that the fetus has developed mechanisms to prevent over exposure to glucocorticoids during this sensitive period (Douglas, 2005). During this sensitive period, the HPA axis has been touted as both a target and a mediator between early life events and adult health outcomes (Meaney, et al., 2007). In theory, the adaptations made by the fetus prenatally are done to prepare for postnatal life under the expectation that the intrauterine environment is representative of the postnatal environment (Talge, et al., 2007). Through out an organism's lifespan there is a continuous reciprocal relationship between environmental stimuli and epigenetic response. Protein synthesis is in a constant flux, resulting in a brain that demonstrates remarkable plasticity. However, the prenatal period is a critical time for epigenetic processes, many of which are vital for development and differentiation (Jaenisch & Bird, 2003). The methylation patterns created at this time are highly stable and resistant to change. Hence, the prenatal experiences can set the brain on a developmental trajectory that is difficult to reprogram and follows the individual throughout life.

Just as early drug exposure alters the capacity of the brain to change in response to later experiences (Halliwell, Comeau, Gibb, Frost, & Kolb, 2009), it is possible that prenatal stress is having a similar effect. It has been hypothesized that there is a limit to brain plasticity. Therefore, if brain changes are made to

compensate for prenatal stress, resources may not be available later in life. There is evidence that stressful experiences accelerate brain aging (McEwen, 2007) and the loss of plasticity seen in adulthood may be related to epigenetic changes at sites of synaptic transmission (Fagiolini, et al., 2009; Siegmund, et al., 2007). This dissertation has identified numerous dramatic changes in the brains of prenatally stressed offspring and it is likely that these changes will influence the ability of their brains to respond to later experiences. It is possible that some forms of prenatal stress reduce offspring 'potential'. Offspring of ES mothers demonstrated an inability to learn the negative geotaxis task and this was early in development, namely at P10. The interaction between genes and environment is an extremely delicate and perpetual relationship. As a result it is difficult to predict long-term outcomes without controlling for pre-existing genetic composition and all subsequent experiences. However, evidence from this thesis clearly illustrates that prenatal stress drastically alters normal development of the brain, and therefore, must be influencing the impact of future experiences.

Limitations and Future Directions

There are numerous, exciting experiments that could be conducted to build upon the data collected for this dissertation. Some of these investigations could be used to remedy the limitations of the study and others for sheer interest. One of the most significant limitations to this study was the age in which the offspring were sacrificed. Future studies could use the same stressing protocols but keep the offspring until adulthood. In adulthood, offspring could be tested on numerous behavioural tasks to determine long-term differences in behaviour. The anatomical and epigenetic analysis

could also be repeated at this time point to determine if the results found at P21 are persistent. Subsequent to this, it would be interesting to breed these prenatally stressed rats and determine if the epigenetic changes identified in the first generation are propagated to their offspring. Owing to the fact that there were significant methylation changes and DNA methylation is one of the most stable forms of epigenetic regulation, intergeneration transmission would be predicted. An interesting discovery from finding, however, would be identification of the specific genes, or cluster of genes affected.

Another limitation to this dissertation was the level of analysis available for the gene expression data. The amount of data obtained from this analysis is remarkable, however, without the correct software and technology a majority of it went unused. Furthermore, current knowledge of gene function is quite limited. Many identified genes had no known function or the function was understood in tissues other than the brain. However, because this data will retain its validity, when genes are discovered in the future or new knowledge comes to light, genes can be cross-referenced for interesting findings. Additionally, it would have been interesting to determine gene expression changes for specific learning or memory pathways rather than single genes. In order to do this, generation of novel parametric pathway analysis algorithms would have been required, which was beyond the scope of this dissertation. Again, if these algorithms are generated in the future data from this study could easily be re-analyzed.

Finally, in the future it would be beneficial to determine if early postnatal experiences could remediate the brain changes related to varying prenatal stress paradigms. Owing to the different changes that arise from variations to the paradigm, it is unlikely, but possible, that a single therapeutic intervention would have universal effects.

It therefore, may require individually tailored efforts to remediate the variety of changes that stem from the different prenatal experiences. Nonetheless, searching for these remedial means are necessary to ensure the healthy development of future generations of children. Tactile stimulation or environmental enrichment hold the most promise as effective intervention strategies and would be an excellent starting point.

Conclusion

The dichotomous conception of genes and environments as separate contributors to development fails to consider the ways in which they cooperate to produce individual differences (Harper, 2005). This dichotomy also fails to appreciate the role the developing individual has as an active participant in it's own surroundings. Epigenetic regulation of gene expression is particularly important in the early stages of brain growth and organization (Champagne, 2010). It is at this time point that changes in environment have the best chance of altering genetic regulation. Evidence from this dissertation illustrates that even small perturbations during the prenatal period produce persistent changes in the structure and functioning of the brain.

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APPENDIX 1:

Summary of Genes Altered by Mild Stress

Legend

Chr – Rat Chromosome Number
H.H. – Chromosome Number For Human Homolog
FC – Frontal Cortex
HPC – Hippocampus
Purple Text – Change Occurred in Both Brain Areas
Blue Text – Change Varied Based on Offspring Sex

Gene	Gene Name	Chr	H.H.	FC	HPC	Description
Aatk	*Apoptosis-associated tyrosine kinase*	10	Chr 17	-	↓	Is essential for neuronal differentiation, and promotes neurite extension in developing neurons [1].
Abi2	*Abl-interactor 2*	9	Chr 2	-	↑	Involved in cell migration, dendritic spine formation, spine density, & short and long term memory [2].
Abr	*Active BCR-related gene*	10	Chr 17	-	↑	Has a GTP exchange factor with GAP activity towards members of the Rac/Rho family [3]
Aco1	*Aconitase 1, soluble*	5	Chr 9	↓	-	Involved in regulation of iron metabolism proteins. Aco1 binds to iron responsive elements [4]
Acoxl	*Acyl-Coenzyme A oxidase-like*	3	Chr 2	-	↑	Function Unknown.
Actn4	*Actinin alpha 4*	1	Chr 19	↓	↓	Actin-binding proteins important in organization of the cytoskeleton and cell adhesion found in neuroblastoma cell variants [5].
Adam9	*ADAM metallopeptidase domain 9 (meltrin gamma)*	16	Chr 8	↓		Mediates cell adhesion and cleaves the insulin B-chain. Overexpression of ADAM9 results in metastatic foci in the brain [6].
Adam23	*ADAM metallopeptidase domain 23*	9	Chr 2	-	↑	Mediates cell adhesion. Lethal mutant with homozygous deletion. ADAM23 deficiency results in tremor and severe ataxia [7]
Adcy1	*Adenylate cyclase 1 (brain)*	14	Chr 7	-	↓	Catalyzes the formation of cyclic AMP. Adcy1 activity is regulated by neurotransmitter and hormone receptors. May play a role in memory acquisition and learning [8]
Adora1	*Adenosine A1*	13	Chr 1	↓	-	G-protein-coupled receptor

	receptor					involved in sleep and wakefulness [9].
Agphd1	*Aminoglycoside phosphotransferase domain containing 1*	8	Chr 15	-	↓	Function Unknown.
Akna	*AT-hook transcription factor*	5	Chr 9	-	↓	A protein that binds the A/T promoters of CD40 and CD40 ligand and regulates their expression [10].
Alg2	*Asparagine-linked glycosylation 2 homolog (S. cerevisiae, alpha-1,3-mannosyltransferase)*	5	Chr 9	↓	-	A Ca2+ binding protein required for T-cell receptor, Fas, and glucocorticoid-induced cell death [11].
Ampd2	*Adenosine monophosphate deaminase 2 (isoform L)*	2	Chr 1	↓	-	A highly regulated enzyme involved in purine nucleotide catabolism and interconversion [12].
Ankrd17	*Ankyrin repeat domain 17*	14	Chr 4	↑	-	Earliest specific in situ marker of hepatic differentiation.
Ankrd33b	*Ankyrin repeat domain 33b (similar to RIKEN)*	2	Chr 5	-	↓	Function Unknown.
Ank3	*Ankyrin 3, epithelial*	20	Chr 10	-	↑	A modular protein, plays a critical role in clustering voltage gated Na2+ channels in nodes of Ranvier [13].
Anp32e	*Acidic (leucine-rich) nuclear phosphoprotein 32 familiy member E*	2	Chr 1	-	↓	Involved in the signal transduction pathway that directs differentiation of certain neurons [14].
Arhgef2	*Rho/rac guanine nucleotide exchange factor (GEF) 2*	2	Chr 1	-	↑	Plays a role in activation of Rac and/or Rho and in bringing activated GTPase to microtubules [15].
Arf1	*ADP-ribosylation factor 1*	1	Chr 1	-	↑	Subset of the regulatory GTP-binding proteins

						[16].
Armc5	*Armadillo repeat containing 5*	1	Chr 16	↓	-	Function Unknown.
Arnt	*Aryl hydrocarbon receptor nuclear translocator*	2	Chr 1	-	↑	A DNA binding protein that forms a heterodimer with Ahr in response to environmental pollutants [17].
Atp5d	*ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit*	7	Chr 19	↓	-	Subunit of mitochondrial ATP synthase.
Atp5j2	*ATP synthase, H+ transporting mitochondrial F0 complex, subunit F2*	12	Chr 7	↑	-	Subunit of mitochrondrial ATP synthase. Part of the complex F(0) domain. Minor subunit located with subunit a in the cell membrane.
Atp6v0c	*ATPase, H+ transporting, lysosomal 16kDa, V0 subunit C*	10	Chr 16	↓	-	Encodes a component of vacuolar ATPase.
Bai2	*Brain specific angiogenesis inhibitor 2*	5	Chr 1	-	↓	A seven span transmembrane protein thought to belong to the secretin receptor superfamily [18]
Bhlhe41	*Basic helix-loop-helix domain containing class B3*	4	Chr 12	-	↑	Inhibits transcription of genes involved in mammalian circadian rhythms [19].
Bicd2	*Bicaudal D homolog 2 (Drosophila)*	17	Chr 9	-	↓	Developmental protein involved in dynein-mediated microtubule motility [20].
Bin1	*Bridging integrator 1*	18	Chr 2	-	↓	May act as a tumor suppressor and inhibit malignant cell transformation by binding to Myc [21].
Cacnb2	*Calcium channel, voltage-dependent, beta 2 subunit*	17	Chr 10	-	↑	Subunit of a voltage-dependent calcium channel found in neurons and important for polarizing cells.

Cadm3	*Cell adhesion molecule 3*	13	Chr 1	↑	-	Localizes at the contact sites among axons, their terminals, and glia cell processes that cooperatively form synapses, axon bundles and myelinated axons [22].
Cadps	*Ca++ dependent secretion activator*	15	Chr 3	↓	-	A calcium binding protein essential for late-stage exocytosis of neurotransmitters from synaptic terminals [23].
Ccdc39	*Coiled-coil domain containing 39*	2	Chr 3	↑	-	Function Unknown.
Cherp	*Calcium homeostasis endoplasmic reticulum protein*	16	Chr 19	-	↑	Protein has an important function in cell Ca2+ homeostasis, growth and proliferation [24].
Chmp7	*CHMP family, member 7*	15	Chr 8	↓	↓	An escort related protein involved in the endosomal sorting pathway [25].
Chp	*Calcium binding protein, p22*	3	Chr 15	↓	-	A phosphoprotein that may be an important signal controlling mitogenic regulation of NHE1 [26].
Cip98	*CASK-interacting protein*	5	Chr 16	↓	-	Localized to the dendritic processes of neurons and interacts with CASK [27].
Cited2	*Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2*	1	Chr 6	-	↑	Acts as a dose dependent coactivator of peroxisome proliferator-activated receptor transcription and both proteins participate in signaling cascades [28].
Clasp2	*Cytoplasmic linker associated protein 2*	8	Chr 3	↑	-	A microtubule-stabilizing protein that mediates the interaction between microtubule ends and the cell cortex [29].
Clec4a1	*C-type lectin domain family 4, member a1*	4	Chr 12	-	↑	Modulates cell-cell and cell-matrix interactions and may also modulate cell adhesion [30].
Clptm1l	*CLPTM1-like*	1	Chr 5	↓	-	Enhances cisplatin-mediated apoptosis.

Cltc	*Clathrin, heavy chain (Hc)*	10	Chr 17	↓	-	Major protein of coats of coated pits and vesicles.
Cnksr2	*Connector enhancer of kinase suprressor of Ras 2*	X	Chr X	-	↓	Regulates Ras signaling pathways and may integrate signals between MAPK and Ral pathways [31].
Cobll1	*Cobl-like 1*	3	Chr 2	-	↓	Developmental gene that interacts with the neurulation gene to facilitate midbrain neural tube closure [32].
Col5a1	*Collagen, type V, alpha 1*	3	Chr 9	-	↓	Produces a component of procollagen.
Coro1c	*Coronin, actin binding protein 1C*	12	Chr 12	↓	-	Involved in myelin formation [33].
Cpsf2	*Cleavage and polyadenylation specific factor 2*	6	Chr 14	↓	-	Plays a key role in pre-mRNA 3'end formation recognizing specific sequences.
Cpsf7	*Cleavage and polyadenylation specifc factor 7*	1	Chr 11	-	↑	Plays a key role in pre-mRNA formation; binds to cleavage and polyadenylation sites.
Cr1l	*Complement component (3b/4b) receptor 1-like*	13	Chr 1	↓	-	A single-pass type I membrane glycoprotein receptor of complement activation.
Crtc2	*CREB regulated transcription coactivator 2*	2	Chr 1	-	↓	A CREB coactivator that is triggered in response to camp signaling [34].
Cttnbp2	*Cortactin binding protein 2*	4	Chr 7	-	↑	Brain specific protein; plays role in the actin cytoskeleton
Cul2	*Cullin 2*	17	Chr 10	↑	-	Ubiquitinates and degrades hypoxia-inducible factor [35].
Dbn1	*Drebrin 1*	17	Chr 5	↓	-	Protein located in dendritic spines which plays a role in synaptic plasticity with actin filaments [36].
Ddx58	*DEAD (Asp-Glu-Ala-Asp) box polypeptide 58*	5	Chr 9	↓	-	Involved in innate immune defense against viruses and inhibits signaling of Ras/mitogen kinases [37].

Dennd4b	*DENN/MADD domain containing 4b*	2	Chr ?	↓	-	Function Unknown.
Derl1	*Der1-like domain family, member 1*	7	Chr 8	-	↓	A protein essential for the degradation of misfolded ER proteins [38].
Dgkz	*Diacylglycerol kinase zeta*	3	Chr 11	-	↑	Enzyme that metabolizes DAG and regulates Ras activation [39].
Dhcr24	*24-dehydrocholesterol reductase*	5	Chr 1	-	↓	Acts as an antiapoptotic factor in neurons through an oxidative stress-specific resistence [40].
Dld	*Dihydrolipoamide dehydrogenase*	6	Chr 7	↑	-	A component of the glycine cleavage system.
Dmxl2	*Dmx-like 2*	8	Chr 15	↑	↑	Regulates Ca2+ dependent exocytosis of neurotransmitters [41].
Dnajc13	*DnaJ (Hsp40) homolog, subfamily C, member 13*	8	Chr 3	-	↑	A receptor involved in regulating trafficking of the EGF receptor [42].
Dock9	*Dedicator of cytokinesis 9*	15	Chr 13	-	↑	Small GTPase responsible for total dendrite length and the number of branch points [43].
Dpys12	*Dihydropyrimidinase-like 2*	15	Chr 8	↓	-	Plays a role in axon guidance, neuronal growth cone collapse and cell migration [44].
Dync1i2	*Dynein cytoplasmic 1 intermediate chain 2*	3	Chr 2	-	↑	Intermediate chain of dynein complex which help dynein bind to dynactin.
Eaf2	*ELL associated factor 2*	11	Chr 3	↑	-	An RNA polymerase II elongation factor with a transcriptional activation domain [45].
Eif2b2	*Eukaryotic translation initiation factor 2B subunit 2*	6	Chr 14	↓	-	Mediates the regeneration of active eIF2-GTP complexes which is critical for initiation of mRNA translation [46].
Eif4a1	*Eukaryotic translation initiation factor*	6	Chr 17	↑	-	A translation initiation factor with RNA helicase abilities [47].

	4A1					
Eltd1	*EGF, lactrophilin and seven transmembrane domain containing*	2	Chr 1	-	↑	A seven-transmembrane receptor observed in cytoplasmic vesicles [48]
Eno2	*Enolase 2, gamma neuronal*	4	Chr 12	↓	-	Has neurotrophic and neuroprotective properties on a broad spectrum of CNS neurons
Epha8	*Eph receptor A8*	5	Chr 1	-	↓	Regulates cell adhesion and migration while inducing neurite outgrowth [49].
Epn2	*Epsin 2*	10	Chr 17	-	M↓, F↑	Proteins involved in membrane dynamics and clathrin mediated endocytosis [50].
Epsti1	*Epithelial stromal interaction 1 (breast)*	15	Chr 13	↑	-	A novel gene whose expression is induced by epithelial-stromal interaction and involved in invasion [51].
Fam5b	*Family with sequence similarity 5, member B*	13	Chr 1	-	↓	Function Unknown.
Fam5c	*Family with sequence similarity 5, member C*	13	Chr 1	↓	-	Function Unknown.
Fbrsl1	*Fibrosin-like 1*	12	Chr 12	↓	-	Function Unknown.
Fbxw11	*F-box and WD repeat domain containing 11*	10	Chr 5	-	↑	Substrate recognition component of a E3 ubiquitin protein ligase complex; involved in Wnt signaling.
Fchsd2	*FCH and double SH3 domains 2*	1	Chr 11	↑	-	Function Unknown.
Fkbp3	*FK506 binding protein 3*	6	Chr 14	↑	-	Member of the immunophilin protein family, which plays a role in immunoregulation.
Fkbp4	*FK506 binding protein 4*	4	Chr 2	-	↓	Member of the immunophilin protein family, thought to form

						steroid hormone receptors.
Fxr2	*Fragile X mental retardation, autosomal homolog 2*	10	Chr 17	-	↓	A cytoplasmic RNA-binding protein that interacts with the 60S ribosomal subunit; found in differentiated neurons [52].
Fzr1	*Fizzy/cell division cycle 20 related 1 (Drosophila)*	7	Chr 19	↓	-	Regulates ubiquitin ligase activity of the anaphase promoting complex/cyclosome.
Garnl1	*GTPase activating Rap/RanGAP domain-like 1*	6	Chr 14	-	↑	A transcriptional regulator of E12 which is important for brain formation, neuronal differentiation and maintenance [53].
Gatad1	*GATA zinc finger domain containing 1*	4	Chr 7	M ↓ F↑	-	Likely plays a role in transcription regulation but function is unknown.
Gfpt1	*Glutamine fructose-6-phosphate transaminase 1*	4	Chr 2	↓	-	A rate-limiting enzyme in the hexosamine biosynthetic pathway [54].
Gfra2	*GDNF family receptor alpha 2*	15	Chr 8	-	↑	Receptor for glia cell line-derived neurotrophic factor [55].
Gja5	*Gap junction protein, alpha 5*	2	Chr 1	↑	-	The connexons by which materials of low molecular weight diffuse from one cell to a neighboring cell; enables synchronized rapid responses [56].
Gpr162	*G protein-coupled receptor 162*	4	Chr 12	-	↓	Orphan receptor, function unknown.
Gria2	*Glutamate receptor, ionotropic, AMPA 2*	2	Chr 4	↓	-	Main excitatory receptor in the brain; binds glutamate, AMPA, NMDA, & kainite [57].
Gtf3c1	*General transcription factor III C1*	1	Chr 16	-	↓	A multisubunit transcription factor for RNA polymerase III. It initiates transcription of tRNA and 5s rRNA [58].
Gusb	*Glucuronidase, beta*	12	Chr 7	-	↓	An enzyme required for the degradation of glycosaminoglycans [59].

Gyg1	*Glycogenin 1*	2	Chr 3	-	↑	Self-glucosylates via an inter-subunit mechanism to form an oligosaccharide primer that serves as a substrate for glycogen synthesis.
Hbp1	*HMG-box transcription factor 1*	6	Chr 7	-	↑	A transcriptional repressor that participates in RAS- and p38 MAPK-induced premature senescence [60].
Hcfc1r1	*Host cell factor C1 regulator 1 (XPO1-dependent)*	10	Chr 16	-	↓	Regulates host cell factor C1 by modulating subcellular localization.
Hdac3	*Histone deacetylase 3*	18	Chr 5	-	↓	Deacetylases nucleosomal histones which results in DNA condensation and a repression of transcription [61].
Herc4	*Hect domain and RLD 4*	20	Chr 10	-	↑	A ubiquitin ligase involved in cell signaling [62].
Hexb	*Hexosaminidase B*	2	Chr 5	-	↑	Provides instructions for making two related enzymes.
Hiat1	*Hippocampus abundant gene transcript*	2	Chr 1	↑	-	Function Unknown.
H13	*Histonecompatability 13*	3	Chr 20	-	↓	Catalyzes intramembrane proteolysis of some signal peptides after they have been cleaved from a preprotein.
Hmcn1	*Hemicentin1*	13	Chr 1	-	↓	An extracellular matrix protein which facilitates mechanosensory neuron anchorage to the epidermis [63].
Hnrnpa3	*Heterogeneous nuclear ribonucleoprotein A3*	8	Chr 2	-	↓	A nuclear transcription factor that may interact with the regulatory region of some Hox genes [64].
Hprt1	*Hypoxanthine phosphoribosyltransferase 1*	X	Chr X	↑	-	A purine salvage enzyme that catalyzes the conversion of hypoxanthine and guanine

						to their respective mononucleotides [65].
Icmt	*Isoprenylcysteine carboxyl methyltransferase*	5	Chr 1	-	↓	Catalyzes the posttranslational modification of isoprenylcysteine [66].
Igf2r	*Insulin-like growth factor 2 receptor*	1	Chr 6	↓	-	A multifunctional receptor involved in fetal development, tumor suppression and cognitive ability [67].
Ipo13	*Importin 13*	5	Chr 1	-	M↑ F↓	Functions in nuclear protein import as a nuclear transport receptor.
Iqsec3	*IQ motif and Sec7 domain 3*	4	Chr 12	-	↑	A member of the guanine nucleotide exchange factor with activity toward ARF1 [68].
Kcnab2	*Potassium voltage-gated channel, shaker-related subfamily, beta member 2*	5	Chr 1	-	↑	A K+ channel β subunit though to play a critical role in K+ channel inactivation [69].
Kcnc2	*Potassium voltage gated channel, Shaw-related subfamily, member 2*	7	Chr 12	-	↓	A voltage gated K+ channel found in lymphocytes [70].
Khsrp	*K-H-type splicing regulatory protein*	?	Chr 19	↓	-	Promotes rapid mRNA decay by recruiting degredation machinery to ARE-containing mRNAs [71].
Kifap3	*Kinesin-associated protein 3*	13	Chr 1	↑	-	A microtubule-based ATPase motor for organelle transport [72].
Klhl18	*Kelch-like 18 (drosophila)*	8	Chr 3	↓	-	Function Unknown.
Lrp1	*Low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)*	7	Chr 12	↓	-	Catabolizes numerous ligands including proteinases, proteinase inhibitor complexes and lipoproteins. May also modulate cell signaling events [73].
Lrp1b	*Low density*	3	Chr 2	-	↑	A multifunctional cell

	lipoprotein-related protein 1B (deleted in tumors)					surface receptor for tissue-type plasminogen & receptor-associate protein [74].
Lrp11	*Low density lipoprotein receptor-related protein 11*	1	Chr 6	↑	-	Function Unknown.
Lrrc16a	*Leucine rich repeat containing 16a*	17	Chr 6	↓	-	Adapter protein that binds capping protein at the end of filaments. Decreasing this protein lowers F-actin and slows migration [75].
Lrrc57	*Leucine rich repeat containing 57*	3	Chr 15	-	↑	Function Unknown.
Ly6h	*Lymphocyte antigen 6 complex, locus H*	7	Chr 8	-	↓	Involved in the development and homeostasis of hematopoietic cells in the CNS and immune system [76].
Macf1	*Microtubule-actin crosslinking factor 1*	5	Chr 1	-	↓	A protein that can associate with both actin microfilaments and microtubules [77].
Map4k3	*Mitogen-activated protein kinase kinase kinase kinase 3*	6	Chr 2	-	↑	A pro-apoptotic kinase that acts in response to environmental stress [78].
March6	*Membrane-associated ring finger (C3HC4) 6*	2	Chr 5	-	↑	A novel ER-resident ubiquitin ligase, also is an ER degradation substrate for itself [79].
Mfsd6	*Major facilitator superfamily domain containing 6*	9	Chr 2	-	↑	Function Unknown.
Mical2	*Microtubule associated monoxygenase, calponin and LIM domain containing 2*	1	Chr 11	↓	-	Member of a conserved family of secreted and transmembrane proteins utilized as neuronal receptors to signal repulsive axon guidance [80].
Mll5	*Myeloid/lymphoid*	4	Chr 7	↓	-	A trithorax gene. The

	or mixed-lineage leukemia 5 (trithorax homolog, Drosophila)					protein forms complexes that may play an important role in chromatin remodeling and cellular growth suppression [81].
Morc2	*MORC family CW-type zinc finger 2*	14	Chr 22	-	↓	Highly expressed in smooth muscle, function unknown.
Morf412	*Mortality factor 4 like 2*	X	Chr X	↑	-	Has a transcription factor-like domain which induces senescence in immortal cells [82].
Msto1	*Misato homolog 1 (Drosophila)*	2	Chr 1	↓	-	Involved in the regulation of mitochondrial distribution and morphology.
Mthfd1l	*Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like*	1	Chr 6	↓	-	A functional cytoplasmic folate metabolizing enzyme that catalyzes the interconversion of tetrahydrofolatem 10-formyl-THF and 5-10-methylene-THF [83].
Mtr	*5-methyltetrahydrofolate-homocysteine methyltransferase*	17	Chr 1	↓	-	An enzyme that catalyzes the transfer of a methyl groups to generat H_4folate and methionine [84].
Mysm1	*Myb-like, SWIRM and MPN domains 1*	5	Chr 1	↑	-	A chromatin-associated protein that binds DNA and regulates transcription [85].
Ncln	*Nicalin homolog (zebrafish)*	7	Chr 19	-	↓	An antagonist to a key vertabrate development gene family: Nodals [86].
Ndufa6	*NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6 (B14)*	7	Chr 22	↑	-	Accessory subunit to the NADH dehydrogenase complex of the mitochondria [87].
Nf1	*Neurofibromin 1*	10	Chr 17	-	↑	A RAS GTPase activating protein [88]
Nipsnap3A	*Nipsnap homolog 3A*	5	Chr 9	-	↑	Thought to play a role in vesicle transport.
Nov	*Nephroblastoma*	7	Chr 8	↑	-	Member of the connective

	overexpressed gene					tissue growth factor family, acts as a growth factor & is an immediate early gene [89].
Npepps	*Aminopeptidase puromycin sensitive*	10	Chr 17	↑	-	Contributes to the degradation of enkephalins [90].
Nrxn3	*Neurexin 3*	6	Chr 14	-	↓	Neuronal proteins that function as cell adhesion molecules during synaptogenesis and intercellular signaling [91].
Nsmce2	*Non-SMC element 2 homolog (MMS21, S. cerevisiae)*	7	Chr 8	↑	-	Prevents DNA damage-induced apoptosis by facilitating DNA repair [92].
Ntan1	*N-terminal asparagine amidase*	10	Chr 16	↓	-	Responsible for side chain deamination of N-terminal asparagine and glutamine residues [93].
Nudt3	*Nudix (nucleoside diphosphate linked moiety X)-type motif 3*	20	Chr 6	↓	-	A nudix-type hydrolase that acts on Ap6A and [PP]InsP5 [94]
Oc90	*Otoconin 90*	7	Chr 8	-	↑	Involved in sensing orientation relative to gravity [95]
Olr49	*Olfactory receptor 49*	1	Chr 11	↑	-	Members of the olfactory receptor family, which is the largest in the genome. They are members of the G-protein coupled receptors.
Olr1160	*Olfactory receptor 1160*	8	-	-	↑	
Osbpl6	*Oxysterol binding protein-like 6*	2	Chr 2	-	↑	A cytosolic protein that is implicated in the regulation of cellular cholesterol metabolism [96].
Otub1	*OUT domain, ubiquitin aldehyde binding 1*	1	Chr 11	-	↑	A negative regulator of transcription mediated by ERalpha [97].
Oxr1	*Oxidation resistence 1*	7	Chr 8	-	↑	Functions to protect cells from oxidative damage [98].

Oxsr1	*Oxidative-stress responsive 1*	8	Chr 3	↓	-	Involved in protecting cells from osmotic stress [99].
Parp6	*Poly (ADP-ribose) polymerase family, member 6*	8	Chr 15	-	↑	An enzyme whose catalytic activity is stimulated by DNA strand breaks and contributes to the survival of injured proliferating cells [100].
Pclo	*Piccolo (presynaptic cytomatrix protein)*	4	Chr 7	-	↑	A structural component of the presynaptic cytomatrix which anchors vesicles to presynaptic plasmalemma [101]
Pcnx	*Pecanex homolog (Drosophila)*	6	Chr 14	↓	-	Function Unknown.
Pde1b	*Phosphodiesterase 1B, Ca2+ calmodulin dependent*	7	Chr 12	↑	-	A calmodulin-stimulated 3,5-cyclic nucleotide phospho-diesterase [102].
Pdxdc1	*Pyridoxal-dependent decarboxylase domain containing 1*	10	Chr 16	↓	-	Function Unknown.
Pfkp	*Phosphofructokinase, platelet*	17	Chr 10	↓	↓	A key enzyme responsible for the regulation of glycolosis in the brain [103].
Pfkm	*Phosphofructokinase, muscle*	7	Chr 12	↓		An enzyme that is involved the the phosphorlyation and metabolism of fructose-6-phosphate [104].
Pkm2	*Pyruvate kinase, muscle*	16	Chr 15	-	↓	Kinase specifically expressed in proliferating cells [105].
Plekha7	*Pleckstrin homology domain containing, family A, member 7*	1	Chr 11	↓	-	Forms a complex which is responsible for regulating the zonula adheren integrity [106].
Pop1	*Processing of precursor 1, ribonucleas P/MRP subunit*	7	Chr 8	-	↓	Component of ribonuclease P, a protein complex that generates mature tRNA molecules

	(S. cervisiae)					by cleaving their 5' ends. Also a component of MRP.
Ppil4	*Peptidylprolyl isomerase (cyclophilin)-like 4*	1	Chr 4	↑	↑	Has an RNA recognition motif and plays an important role in protein folding [107].
Prmt5	*Protein arginine methyltransferase 5*	15	Chr 14	↑	-	Regulates cell growth and proliferation through transcriptional activation or repression [108].
Psme4	*Proteasome (prosome, macropain) activator subunit 4*	14	Chr 2	↑	-	Activates proteasomal cleavage of peptides in an energy-independent manner. May be involved in DNA repair.
Qk	*Quaking*	1	Chr 6	-	M↓ F-	Gene product is necessary in embryogenesis and myelination, has signal transduction properties [109].
Qsox2	*Quiescin Q6 sulfhydryl oxidase 2*	3	Chr 9	↑	-	Plays a major role in regulating the sensitization of neuroblastoma cells for IFN-δ-induced apoptosis [110].
Rab26	*RAB26, member RAS oncogene family*	10	Chr 16	-	↓	Member of the RAB protein family; an important regulator of vesicular fusion and trafficking [111].
Rac1	*Ras-related C3 botulinum toxin substrate 1*	12	Chr 7	-	↓	Essential components of signal transduction pathways linking growth factors to the organization of polymerized actin [112].
Rbm5	*RNA binding motif protein 5*	8	Chr 3	↓	-	Enhances the formation of proapoptotic Casp 2L [113].
Rcn2	*Reticulocalbin 2, EF-hand calcium binding domain*	8	Chr 15	↑	-	A Ca2+ binding protein which shows ubiquitous expression patterns [114].
Rhbdd2	*Rhomboid domain containing 2*	12	Chr 7	-	↓	Function Unknown. Over-expressed in cancer [115].
Rnpep	*Arginyl*	13	Chr 1	↓	-	A Zn2+ dependent

	aminopeptidase (aminopeptidase B)					exopeptidase involved in processing in the secretatory pathway or the plasma membrane [116].
Rpe	*Ribulose-5-phosphate-3-epimerase*	9	Chr 2	↑	-	Unknown in Mammals.
Rplp2	*Ribosomal protein, large P2*	1	Chr 11	↑	-	A ribosomal phosphoprotein that is a component of the 60S subunit.
Rpl3l	*Ribosomal protein L3-like*	13	Chr 6	↑	-	Shares similarities to ribosomal protein L3; found in skeletal muscle.
Rpsa	*Ribosomal protein SA*	8	Chr 3	-	M↑ F-	A high affinity, non-integrin family, lamin receptor. Also acts as a receptor for viruses and bacteria [117]
Rps15a	*Ribosomal protein S15a*	1	Chr 16	↑	-	Component of the 40S ribosomal subunit.
Rps21	*Ribosomal protein S21*	3	Chr 20	↑	-	Component of the 40S ribosomal subunit.
Rps27	*Ribosomal protein S27*	2	Chr 1	↑	-	Covalently links the 40S subunit to the eIF3 complex.
RT1-CE13	*RT1 class I, locus CE13*	20	-	-	↓	Function Unknown.
Rtn3	*Reticulon 3*	1	Chr 11	-	↓	Plays an important role in the development of axons through the transport of liquids and proteins [118]
Ryr2	*Ryanodine receptor 2, cardiac*	17	Chr 1	↓	-	Receptor that transfers Ca2+ within cells.
Sacm1l	*SAC1 suppressor of actin mutations 1-like (yeast)*	8	Chr 3	-	↑	A PIP phophatase important for signal transduction events [119].
Safb	*Scaffold attachment factor B*	9	Chr 19	-	↓	Can function as an estrogen receptor coreceptor [120].
Scg5	*Secretogranin V*	3	Chr 15	↑	-	Plays a role in regulating pituitary hormone secretion [121].
Sdk2	*Sidekick homolog 2 (chicken)*	10	Chr 17	-	↓	Protein that guides axonal terminals to specific

						synapses in developing neurons.
Selk	*Selenoprotein K*	8	Chr 3	-	↑	A novel antioxidant related to the regulation of the cellular redox balance [122].
Serpinb6a	*Serine (or cysteine) peptidase inhibitor, clade B, member 6a*	17	Chr 6	-	↑	Major transport protein for glucocorticoids and progestins in the blood.
Sim2	*Single-minded homolog 2 (Drosophila)*	11	Chr 21	-	↑	A transcription factor that may be a master gene of CNS development in cooperation with Arnt [123].
Slc3a2	*Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2*	1	Chr 11	↓	-	An integrin association protein that contributes to integrin-dependent cell spreading, cell migration and cell survival [124].
Slc6a6	*Solute carrier family 6 (neurotransporter , taurine), member 6*	4	Chr 3	↓	-	A transport protein required for the uptake of taurine; transports taurine and beta-alanine. Can use GABA as a substrate [125].
Slc9a6	*Solute carrier family 9 (sodium/hydrogen exchanger), member 6*	X	Chr X	-	↓	A monovalent Na2+ selective sodium / hydrogen exchanger. Mutations have been correlated to Angelman syndrome [126].
Slc24a2	*Solute carrier family 24 (sodium/potassiu m/calcium exchanger), member 2*	5	Chr 9	-	↑	Na/Ca/K exchanger; only mechanism for extruding Ca2+ that enters ROS via the light sensitive and cGMP-gated channels.
Slc25a11	*Solute carrier family 25 (mitochondrial carrier, oxoglutarate*	10	Chr 17	↓	-	An inner-membrane protein of mitochondria that plays a role in several metabolic processes such as nitrate metabolism

	carrier) member 11					[127].
Slc30a10	*Solute carrier family 30, member 10*	13	Chr 1	-	↓	Transporter involved in zinc homeostasis [128].
Slco1b2	*Solute carrier organic anion transporter*	4	-	↓	-	A transport protein at the BBB that mediates uptake of a variety of organic anions including steroids [129].
Sf4	*Splicing factor 4*	16	Chr 19	↓	-	Splices mRNA precursors [130].
Sf1	*Splicing factor 1*	1	Chr 11	↑	-	A negative transcription regulator of many genes [131].
Smc1a	*Structural maintenance of chromosomes 1A*	X	Chr X	-	↓	Plays an important role in sister chromatid cohesion, chromosome condensation and DNA recombination and repair [132].
Snx1	*Sorting nexin 1*	8	Chr 15	-	↓	Plays a role in sorting EGFR to the lysosome [133].
Spna2	*Spectrin alpha 1*	3	Chr 1	-	↓	A protein involved in maintaining neuronal membrane integrity; acts as an early neuronal defense mechanism [134].
Spock2	*Sparc/osteonectin , cwcv and kazal-like domains proteoglycan 2*	20	Chr 10	-	↓	A Ca2+ binding proteoglycan with the potential to participate in diverse steps of neurogenesis [135].
Stac3	*SH3 and cysteine rich domain 3*	7	Chr 12	-	↑	Function Unknown.
Strn3	*Striatin, calmodulin binding protein 3*	6	Chr 14	-	↑	An intracellular protein that is a signaling or scaffold protein localized to the soma and dendrites [136].
Strn4	*Striatin, calmodulin binding protein 4*	1	Chr 19	↓	-	An intracellular protein that is a signaling or scaffold protein localized to the soma and dendrites [136].
Stxbp51	*Syntaxin binding*	11	Chr 3	-	↓	Has a potential role in

	protein 5-like					vesicle trafficking and exocytosis.
Surf4	*Surfeit gene 4 (predicted)*	3	Chr 9	↓	-	A cargo receptor essential for maintaining the architecture of the ERGIC [137].
Syne1	*Spectrin repeat containing, nuclear envelope 1*	1	Chr 6	↑	-	Participates in migration of myonuclei in myotubes and/or their anchoring at the postsynaptic apparatus [138].
Tacc1	*Transforming, acidic coiled-coil containing protein 1*	16	Chr 8	↓	-	A protein located at the centrosome which interacts with microtubules and is required for mitosis [139].
Tbl3	*Transducin (beta)-like 3*	10	Chr 16	↓	-	Roles in many functions including signal transduction.
Tceb1	*Similar to transcription elongation factor B (SIII), polypeptide 1*	16	Chr 8	↑	-	Protein elongin C; a subunit of the transcription factor B complex.
Tfpi	*Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)*	3	Chr 2	↑	-	Prevents participation of Tissue Factor in the coagulation process. Found in increased levels in gioblastoma and intercerbral metastases [140].
Thoc2	*THO complex 2*	X	Chr X	-	↓	A heterotetrameric complex involved in transcription and export of poly (A) RNA and mRNA [141].
Tmem55b	*Transmembrane protein 55B*	15	Chr 14	↑	-	An enzyme responsible for the degradation of EGF receptors [142].
Tmem71	*Transmembrane protein 71*	7	Chr 8	↓	-	Function Unknown.
Tmsb4x	*Thymosin, beta 4, X chromosome*	10	Chr X	-	↑	Plays a role in regulation of actin polymerization. Also in cell proliferation, migration and differentiation.

Tmtc4	*Transmembrane and tetratricopeptide repeat containing4*	15	Chr 13	-	↓	Function Unknown.
Tnfrsf9	*Tumor necrosis factor receptor superfamily, member 9*	5	Chr 1	-	↑	Initiates a signaling cascade leading to activation of nuclear factor kappaB [143].
Traf7	*Tumor necrosis factor receptor-associated factor 7*	10	Chr 16	↓	-	Involved in MEKK3 signaling and induces caspase-dependent apoptosis [144].
Tuba4a	*Tubulin, alpha 4A*	9	Chr 2	-	↑	Major constituent of microtubules.
Tyw1	*tRNA-yw synthesizing protein 1 homolog (S. cerevisiae)*	12	Chr 7	-	↓	Supports reading frame maintenance by stabilizing codon-anticodon interactions [145].
Ubap1	*Ubiquitin-associated protein 1*	5	Chr 9	↓	-	A ubiquitin associate protein; is a risk factor for frontotemportal lobe degeneration [146].
Ube2cbp	*Ubiquitin-conjugating enzyme E2C binding protein*	8	Chr 6	-	↓	A ubiquitin-conjugating enzyme for the anaphase-promoting complex/cyclosome [147].
Ube2d3	*Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)*	2	Chr 4	-	↑	A ubiquitin conjugating enzyme with many different functions [148].
Ube2g1	*Ubiquitin-conjugating enzyme E2G 1 (UBC7 homolog, C. elegans)*	10	Chr 17	-	↑	Protein required for normal and substrate-induced ubiquitination and proteolysis [149].
Ubl5	*Ubiquitin-like 5*	8	Chr 19	↑	-	A ubiquitin-like protein modifier important for modulation of protein function [150].
Unc5a	*Unc-5 homolog A (C. elegans)*	17	Chr 5	↓	-	Receptor for netrin-1 which when unbound induces apoptosis [151].
Uqcrc2	*Ubiquinol*	1	Chr 16	↑	-	A component of the

	cytochrome c reductase core protein 2					ubiquinol-cytochrome c reductase complex; part of the mitochondrial respiratory chain.
Urb1	*URB1 ribosome biogenesis 1 homolog (S. cerevisiae)*	11	Chr 21	-	↓	Function unknown in mammals.
Usp22	*Ubiquitin specific peptidase 22*	10	Chr 17	↓	-	A subunit of the SAGA transcriptional cofactor complex recruited to genes like Myc [152].
Usp48	*Ubiquitin specific peptidase 48*	5	Chr 1	-	↓	A ubiquitin protease with cell signaling capabilities [153].
Vapa	*VAMP (vesicle-associated membrane protein)-associated protein A*	9	Chr 18	-	↓	Plays a role in vesicle fusion in both neuronal and non-neuronal cells [154].
Vsnl1	*Visinin-like 1*	6	Chr 2	↑	-	A member of the neuronal Ca2+ sensor protein family that modulate Ca2+ dependent cell signaling [155].
Wbp11	*WW domain binding protein 11*	4	Chr 12	-	↑	A novel pre-mRNA splicing factor and interactor of protein phosphatase-1 [156].
Wdfy2	*WD repeat and FYVE domain containing 2*	15	Chr 13	-	↓	Plays a role in endocytosis as the first stage of endocytic processing of internalized cargo [157].
Wdr6	*WD repeat domain*	8	Chr 3	↓	-	A WD repeat protein that is implicated in the cell growth inhibitory pathway of LKB1 [158].
Wrnip1	*Werner helicase interacting protein 1*	17	Chr 6	-	↓	A replication factor protein involved in normal aging of cells; disruption of gene results in premature aging [159].
Wwp1	*WW domain containing E3 ubiquitin protein*	5	Chr 8	-	↑	A ubiquitin ligase that has been shown to regulate the Notch receptor [160].

	ligase 1					
Xpr1	*Xenotropic and polytropic retrovirus receptor 1*	13	Chr 1	-	↑	A retrovirus receptor, may have G protein-coupled signal transduction properties [161].
Zcchc7	*Zinc finger, CCHC domain containing 7*	5	Chr 9	↓	-	Likely involved in transcriptional regulation but function is currently unknown.
Zinki	*Arc mRNA-binding zinc finger*	12	-	↑	↑	Suppresses Arc expression.
Znf512	*Zinc finger protein 512*	6	Chr 2	-	↓	Thought to be involved in regulation of transcription.

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APPENDIX 2:

Summary of Genes Demonstrating a Sex by Stress Interaction via Mild Stress

Legend

Chr – Rat Chromosome Number
H.H. – Chromosome Number for Human Homolog
FC/HPC – Frontal Cortex or Hippocampus
Fem – Female
Red Text – Expression Change in Same Direction for Both Sexes

Gene	Gene Name	Chr	H.H.	FR/ HPC	Male	Fem	Description
Abra	*Actin-binding Rho activating protein*	7	Chr 8	HPC	↓	↑	Specifically enhances Rho-dependent transcription and links changes in actin dynamics to gene transcription [1].
Abt1	*Activator of basal transcription 1*	3	Chr 6	HPC	↓	↑	A TATA binding protein that can function as a basal transcription activator of class II promoters [2].
Acadvl	*Acyl-coenzyme A dehydrogenase very long chain*	10	Chr 17	HPC	↓	↑	A straight-chain acyl-CoA dehydrogenase enzyme which catalyzes the initial step of fatty acid beta oxidation [3].
Actb	*Actin, beta*	12	Chr 7	FR	↑	↓	Globular protein involved in cell motility, structure and integrity. Is a major constituent of the contractile apparatus.
Ccar1	*Cell division cycle and apoptosis regulator 1*	20	Chr 10	FR	↑	↓	A coactivator protein which regulates expression of key proliferation-inducing genes through transcriptional regulation [4].
Centd3	*ArfGAP with RhoGAP domain, ankyrin repeat and PH domain*	18	Chr 5	FR	↑	↓	A binding protein important for rearrangements in the cell cytoskeleton and cell shape [5].
Col6a3	*Collagen, type VI, alpha 3*	9	Chr 2	HPC	↑	↓	The alpha 3 chain of collagen VI [6].
Cstf1	*Cleavage stimulation factor, 3' pre-RNA, subunit 1*	3	Chr 20	HPC	↑	↓	Factor responsible for polyadenylation of mammalian pre-mRNAs [7].
Cubn	*Cubilin (intrinsic*	17	Chr 10	HPC	↓	↑	An endocytic receptor for apolipoprotein and

	factor-cobalamin receptor)						transferrin involved in transferrin catabolism [8].
Dkk1	*Dickkopf homolog 1 (Xenopus laevis)*	1	Chr 10	HPC	↓	↑	A secreted protein that co-elutes with neuregulin and inhibits Wnt signaling [9]
Ebf1	*Early B-cell factor 1*	10	Chr 5	HPC	↑	↓	A DNA-binding protein that participates in regulation of signal transduction of tissue specific component genes [10].
Epn2	*Epsin 2*	10	Chr 17	HPC	↓	↑	A protein implicated in membrane dynamics at the cell surface that is involved in clathrin mediated endocytosis [11]
Ermap	*Erythroblast membrane-associated protein*	5	Chr 1	HPC	↓	↑	A transmembrane immunoglobulin protein that localizes to the cell surface [12].
Exo4	*Exocyst complex component 4*	4	Chr 7	FR	↑	↓	Component of the exocyst complex involved in docking of exocystic vesicles with fusion sites in plasma membranes.
Fam92a 1	*Family with sequence similarity 92, member A1*	5	Chr 8	FR	↓	↑	Encodes a tumor-related gene with oncogenic potential [13].
Gata1	*GATA binding protein 1*	X	Chr X	FR	↓	↑	A erythropoietic transcription factor important for the survival aspect of erythroid cells [14].
Hipk1	*Homeodomain interacting protein kinase 1*	2	Chr 1	HPC	↓	↑	A transcriptional regulator important in transducing growth-regulatory signals [15].
Iars	*Isoleucyl-tRNA synthetase*	17	Chr 9	HPC	↑	↓	Isoleucyl-tRNA synthetase protein which may be the target of antibodies in

							autoimmune diseases [16].
Igf2r	*Insulin-like growth factor 2 receptor*	1	Chr 6	HPC	↑	↓	A multi-functional receptor with binding sites for IGF2 and retinoic acid. Involved in development of cognitive ability [17].
Ipo13	*Importin 13*	5	Chr 1	HPC	↑	↓	An import/export receptor on the nuclear envelope. Imports the RBM8 protein and exports eIF1A [18]
Jmjd8	*Jumonji domain containing 8*	10	Chr 16	HPC	↓	↑	Function Unknown
Muc1	*Mucin 1, cell surface associated*	2	Chr 1	HPC	↑	↓	Prevents the degradation of estrogen receptor alpha by binding to the DNA binding domain and stabilizing it. Also stimulates estrogen receptor alpha transcription [19].
Myh3	*Myosin, heavy chain 3, skeletal muscle, embryonic*	10	Chr 17	HPC	↓	↑	A contractile protein involved in muscle contraction.
Ncam1	*Neural cell adhesion molecule 1*	8	Chr 11	HPC	↓	↑	A protein known to play important roles in cell migration, neurite growth, axonal guidance and synaptic plasticity [20].
Pcdh24	*Protocadherin 24*	17	Chr 5	FR	↑	↓	A protein that directly binds PDZ and is a molecular switch for contact inhibition of epithelial cells [21].
Pde1a	*Phosphodiesterase 1A, calmodulin-dependent*	3	Chr 2	FR	↑	↓	A Ca2+ calmodulin-stimulated phosphodiesterase that is important for VSMC growth and survival

							[22].
Pde4b	*Phophodiesterase 4B, camp specific*	5	Chr 1	HPC	↑	↓	Protein that metabolically inactivates the second messenager camp to regulate intracellular signaling in neurons [23].
Plce1	*Phospholipase C, epsilon 1*	1	Chr 10	HPC	↑	↓	Catalyzes the hydrolysis of polyphosphoinositides to generate second messengers which leads to a cascade of intracellular responses that result in cell growth and differentiation [24].
Plekhb 2	*Pleckstrin homology domain containing, family B (evectins) member 2*	9	Chr 2	FR	↑	↓	Function Unknown
Poln	*Polymerase (DNA directed) nu*	14	Chr 4	FR	↑	↓	A nuclear DNA polymerase with helicase abilities involved in DNA repair.
Ptch2	*Patched homolog 2*	5	Chr 1	HPC	↑	↓	A transmembrane protein that may act as a repressor of sonic hedgehog (SHH) and as a target of hedgehog signaling [25].
QK	*Quaking*	1	Chr 6	HPC	↓	↑	Encodes a protein with signal transduction properties which is necessary for myelination [26].
Rac2	*Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP*	7	Chr 22	HPC	↓	↑	A Rho-family GTPase that regulates the actin cytoskeleton and superoxide anion production in

	binding protein Rac2)						neutrophils [27].
Rapgef 6	*Rap guanine nucleotide exchange factor (GEF)*	10	Chr 5	HPC	↑	↓	A guanine nucleotide exchange factor for Rap1 and Rap2 downstream of M-Ras [28].
Rpsa	*Ribosomal protein SA*	8	Chr 3	HPC	↑	-	An extracellular glycoprotein receptor for laminin. Plays a role in cell adhesion.
Rufy3	*RUN and FYVE domain containing 3*	14	Chr 4	HPC	↑	↓	A single axon related protein which accumulates in the growth cone of axons and ensures the robustness of neuronal polarity [29].
Shisa4	*Shisa homolog 4 (Xenopus laevis)*	13	Chr 1	HPC	↓	↑	Is an antagonist to Wnt and Fgf signaling [30].
Si	*Sucrase-isomaltase (alpha-glucosidase)*	2	Chr 3	HPC	↑	↓	Plays an important role in the final stage of carbohydrate digestion
Snip	*SNAP25-interacting protein*	10	Chr 17	FR	↑	↓	A tyrosine phosphorylated molecule involved in integrin and EGF dependent signaling for controlling actin cytoskeleton organization [31].
Spock2	*Sparc/osteonectin, cwcv and kazal-like domains proteoglycan 2*	20	Chr 10	HPC	↓	↓	A Ca2+ binding proteoglycan required for neurogenesis [32].
Ssbp4	*Single stranded DNA binding protein 4*	16	Chr 19	FR	↑	↓	A sequence specific single stranded DNA binding protein with tumor suppressor capabilities [33].
Taok3	*TAO kinase 3*	12	Chr 12	HPC	↓	↑	A dendrite specific protein kinase that activates the

							ERK1/ERK2 and JNK/SAPK pathways [34].
Tdrd5	*Tudor domain containing 5*	13	Chr 1	FR	↓	↑	Function Unknown
Vps16	*Vacuolar protein sorting 16 homolog (S.cerevisiae)*	3	Chr 20	FR	↑	↓	Subunit of the vacuole/prevacuole-associated complex which directs multiple reactions during the docking and fusion of vesicles with the vacuole [35].

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APPENDIX 3:

Summary of Genes Altered via Bystander Stress

Legend

Chr – Rat Chromosome Number
H.H. – Chromosome Number for Human Homolog
FC – Frontal Cortex
HPC – Hippocampus

Gene	Gene Name	Chr	H.H.	FC	HPC	Description
Abcb1a	*ATP-binding cassette, Subfamily B, Member 1A*	4	Chr 7	↓	-	A energy- dependent efflux pump responsible for decreased drug accumulation in multi-drug resistant cells [1].
Abcd4	*ATP-binding cassette, Subfamily D, Member 4*	6	Chr14	-	↑	Encodes for a half-size peroxisomal transporter that has to dimerize to become functional [2].
Abr	*Active BCR-related gene*	10	Chr17	↑	-	A GTPase-activating protein. Interacts with members of the Rho family to regulate & coordinate cellular signaling [3]
Adh5	*Alcohol dehydrogenase 5 (class-3)*	2	Chr4	↑		Is a zinc-containing dimeric enzyme responsible for the oxidation of long-chain alcohols and omega-hydroxyfatty acids [4].
Ano4	*Anoctamin 4*	7	Chr12	-	↓	A calcium activated chloride channel [5].
Asip	*Agouti signaling protein*	3	Chr20	↑	-	Inhibits melanocortin action by binding to melacortin receptors [6].
Atp2b1	*ATPase, Ca++ transporting, plasma membrane 1*	7	Chr12	-	↓	Ion transport ATPase responsible for removing bivalent calcium ions against the concentration gradient. Plays a critical role in intracellular calcium homeostasis [7].
Atp5sl	*ATP5S-like*	1	Chr19	↑		Function Unknown
Blk	*B lymphoid tryrosine kinase*	15	Chr8	↑	-	Induces apoptosis through activation of the cytochrome c-Apaf-1-caspase-9 pathway [8].
Btg3	*B-cell translocation gene 3*	11	Chr21	↑		Is a target of p53 and inhibits the activity of E2F1 transcription factor. May act to suppress tumorigenesis [9].
Bwk1	*Leukemia-related gene*	3	Chr20	-	↑	Function Unknown
C1qb	*Complement component 1, q*	5	Chr1	↑	-	C1q associates with the proenzymes C1r and C1s to

	subcomponent, beta polypeptide					yield C1, the first component of the serum complement system.
Cadps	*Ca++dependent secretion activator*	15	Chr3	-	↑	A Ca2+ binding protein essential for late-stage exocytosis of neurotransmitters from synaptic terminals [10]
Cetn2	*Centrin 2*	X	ChrX	↑		Is a calcium binding protein that localizes to the centrioles and centrosomes, is essential for centrosome duplication [11].
Clasp1	*Cytoplasmic linker associated protein 1*	13	Chr2	-	↓	Microtubule stabilizing protein that mediate interactions between distal microtubule ends and the cell cortex [12]
Cohh1	*Cohen syndrome homolog 1*	7		-	↓	This gene encodes a transmembrane protein involved in vesicle mediated transport.
Col17a1	*Collagen, type XVII, alpha 1*	1	Chr10	↑	-	Is an immediate-early response gene for transforming growth factor β (TGF-β) /SMAD signaling pathway [13].
Coro1c	*Coronin, actin binding protein 1C*	12	Chr12	↓	-	Protein involved in myelin formation [14]
Cstf3	*Cleavage stimulation factor, 3' pre-RNA, subunit 3*	3	Chr11	-	↓	A heterotrimeric protein necessary for the first step of polyadenylation of mRNA precursors [15].
Cul2	*Cullin 2*	17	Chr10	↑	-	A component of the VHL tumor suppressor complex that ubiquinates and degrades elements important for vasculogenesis [16]
Dnah17	*Dynein, axonemal, heavy chain 17*	10	Chr17	-	↑	A heavy chain microtubule associated motor protein associated with axonemal dyein.
Dock 1	*Dedicator of cytokinesis 1*	1	Chr10	-	↓	A guanine exchange factor that has a role in cell surface extension of engulfing cell

						around a dying cell during apoptosis.
Dock 9	*Dedicator of cytokinesis 9*	15	Chr13	-	↑	A guanine exchange factor important for dendritic length and branch number [17]
Elf3k	*Eukaryotic translation initiation factor 3, subunit k*	1	Chr19	↑	-	A multisubunit complex that binds to the 40S ribosomal subunit to initiate translation [18].
Eml4	*Echinoderm microtubule associated protein like 4*	6	Chr2	-	↓	A novel microtubule-associated protein that is necessary for correct microtubule formation [19].
Ern2	*Endoplasmic reticulum to nucleus signaling 2*	1	Chr16	-	↓	Stress response protein that attenuates transcription of ER proteins [20].
Ext1	*Exostoses (multiple) 1*	7	Chr8	↑	-	Encodes a glycosyltransferase involved in heparan sulfate biosynthesis [21].
Fer1l5	*Fer-1like 5 (C. elegans)*	9	Chr2	-	↑	Function Unknown
Flt3	*Fms-related tyrosine kinase 3*	12	Chr13	↓	-	Encodes a receptor-type tyrosine kinase [22].
Fndc3b	*Fibronectin type III domain containing 3B*	2	Chr3	↑	-	A positive regulator of adipogenesis [23].
Gja5	*Gap junction protein, alpha 5*	2	Chr1	↑	-	A connexon that allows materials of low molecular weight to diffuse from 1 cell to a neighboring cell. Enables synchronized rapid responses [24].
Gramd1b	*GRAM domain containing 1B*	8	Chr11	-	↑	Function Unknown.
Grik2	*Glutamate receptor, ionotropic, kainite 2*	20	Chr6	-	↓	Predominant excitatory neurotransmitter receptor in the mammalian brain [25].
Hras	*Harvey rat sarcoma virus oncogene*	1	Chr11	-	↑	An oncogene that has been shown to stimulate and regulate the expression of VEGF, which is necessary for tumor vascularization

						[26].
Ibtk	*Inhibitor of Bruton agammaglobulinemia tyrosine kinase*	8	Chr6	-	↓	Inhibits Btk, which is required for B cell development [27].
Idh3g	*Isocitrate dehydrogenase 3 (NAD), gamma*	X	ChrX	-	↑	In the presence of divalent metal ions, functions to catalyze the oxidative decarboxylation of isocitrate [28].
Igf2r	*Insulin-like growth factor 2 receptor*	1	Chr6	-	↓	A multifunctional receptor with binding sites for IGF2 and retinoic acid. Involved in development of cognitive function [29].
Jarid1a	*Jumonji, AT rich interactive domain 1A*	4	Chr12	-	↓	A ubiquitously expressed protein that binds other proteins to control cell proliferation.
Kcnb2	*Potassium voltage gated channel, Shab-related subfamily, member 2*	5	Chr8	-	↓	A K+ channel β subunit which enhances the expression of CDRK [30].
Loxl4	*Lysyl oxidase-like 4*	1	Chr10	↑	-	Is a negative feedback regulator of TGF-beta1 (TGF-β1 is a multifunctional cytokine involved in regulation of cell proliferation and differentiation) [31].
Lrp1b	*Low density lipoprotein-related protein 1B (deleted in tumors)*	3	Chr2	-	↓	Is a low density lipoprotein multifunctional cell surface receptor. Also a candidate tumor suppressor [32].
Mapkapk 2	*Mitogen-activated protein kinase-activated protein kinase 2*	13	Chr1	↑	-	Is a member of the DNA damage checkpoint kinase family; initiates cell cycle arrest in response to DNA damage [33].
Mettl9	*Methyltransferase like 9*	1	Chr16	↑	-	Function unknown, but hypothesized to be a methyltransferase.
Mfsd7	*Major facilitator*	14	Chr4	↓	-	Function Uknown.

	superfamily domain containing 7					
Mical1	*Microtubule associated monoxygenase, calponin and LIM domain containing 1*	20	Chr6	-	↓	A cytoskeletal regulator that connects NEDD9 to intermediate filaments.
Mmd2	*Monocyte to macrophage differentiation-associated 2*	12	Chr7	↓	-	A 7-transmembrade pass motif functional receptor [34].
Myl6	*Myosin, light chain 6, alkali, smooth muscle and non-muscle*	7	Chr12	↑	-	A myosin alkali light chain that is expressed in smooth muscle and non-muscle tissues which is important for cell motility.
Ndor1	*NADPH dependent diflavin oxidoreductase 1*	3	Chr9	-	↓	A NADPH-dependent flavin reductase that may play a role in carcinogenicity and cell death associated with one-electron reductions [35]
Palmd	*Palmdelphin*	2	Chr1	↑	-	A protein kinase A anchor protein involved in regulating intracellular signaling and membrane-cytoskeleton interactions [36].
Pgbd5	*PiggyBac transposable element derived 5*	19	Chr1	-	↑	A transposase, function unknown.
Pgm3	*Phosphoglucomutase 3*	8	Chr6	-	↓	A catalytic enzyme in the hexosamine biosynthesis pathway [37].
Rab43	*Member RAS oncogene family*	4	Chr3	-	↑	A Ras-related small GTPase of particular relevance to membrane trafficking [38].
Rhot1	*Ras homolog gene family, member T1*	10	Chr17	-	↓	A Rho-like GTPase important for mitochondrial homeostasis and apoptosis [39].
Samd8	*Sterlie alpha motif domain containing 8*	15	Chr10	↑	-	Function Unknown.
Scd	*Stearoyl-*	1	Chr10	-	↓	An iron containing enzyme

	Coenzyme A desaturase					that catalyzes the rate limiting step in the synthesis of unsaturated fatty acids [40].
Sel1	*Suppressor of lin-12*	6	Chr14	-	↓	Seems to serve as an adapter protein for the assembly of macromolecular complexes and is activated in response to cellular stress [41].
Sema7a	*Sema domain, immunoglobulin domain (Ig) and GPI membrane anchor (semaphorin) 7A*	8	Chr15	↑	-	The protein that carries the JMH blood group antigen, is involved in immune response, and plays an important role in axon growth and guidance [42].
Sin3a	*SIN3 homolog A, transcription regulator (yeast)*	8	Chr15	-	↓	Member of the type II nuclear hormone receptor subfamily [43].
Slc7a2	*Solute carrier family 7 (cationic amino acid transporter, y+ system) member 2*	16	Chr8	↓	-	An enzyme that regulates inducible nitric oxide synthase [44].
Slc9a6	*Solute carrier family 9 (sodim/hydrogen exchanger) member 6*	X	ChrX	-	↓	A monovalent sodium-selective sodium/hydrogen exchanger that exchanges Na+ for K+ across the membrane to maintain Ca2+ homeostasis [45]
Slc37a3	*Solute carrier family 37 (glycerol-3-phosphate transporter) member 3*	4	Chr7	-	↓	A transmembrane sugar transporter [46].
Spopl	*Speckle-type POZ protein-like*	3	Chr2	↓	-	A negative regulator of the Hedgehog signaling pathway [47].
St3gal1	*ST3 beta-galactoside alpha-2,3-sialytransferase*	7	Chr8	↓	-	A sialyltransferase [48].
Stil	*SCL/Tal 1 interrupting locus*	5	Chr1	-	↓	A cystolic protein that may play an important role in cellular growth and proliferation [49].

Strbp	*Spermatid perinuclear RNA binding protein*	3	Chr9	-	↓	A microtubule-associated RNA-binding protein [50].
Taf1	*TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated 1*	X	ChrX	-	↓	A general transcription factor that initiates preinitiation complex assembly through interaction with the TATA box [51].
Tmem40	*Transmembrane protein 40*	4	Chr3	-	↓	Function Unknown.
Tmem41b	*Transmembrane protein 41b*	1	Chr11	↓	-	Function Unknown.
Tmem131	*Transmembrane protein 131*	9	Chr2	-	↓	A transmembrane protein that may play a role in the immune response to viral infection.
Tmem132d	*Transmembrane protein 132d*	12	Chr12	-	↓	May serve as a cell surface marker for oligodendrocyte differentiation [52].
Ttc231	*Tetratricopeptide repeat domain 23-like*	2	Chr5	↓	-	Function Unknown.
Usp4	*Ubiquitin specific peptidase 4 (proto-oncogene)*	16	Chr3	-	↑	Encodes a nuclear ubiquitin protease whose overexpression leads to oncogenic transformation [53].
Zfat	*Zinc finger and AT hook domain containing*	7	Chr8	-	↓	Functions as a critical transcription regulator in B and T lymphocytes [54].
Zswim5	*Zinc finger, SWIM-type containing 5*	5	Chr1	-	↑	Transcriptional regulation.

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APPENDIX 4:

Summary of Genes Demonstrating a Sex by Stress Interaction via Bystander Stress

Legend

Chr – Rat Chromosome Number
H.H. Chromosome Number for Human Homolog
FR/HPC – Frontal Cortex or Hippocampus
Fem – Female
Red Text – Expression Change in Same Direction for Both Sexes

Gene	Gene Name	Chr	H.H.	FR/ HPC	Male	Fem	Description
Abi2	*Abl interactor 2*	9	Chr2	HPC	↑	↓	An interactor protein with dual roles as a regulator and an effector of c-Abl. May also function as a tumor suppressor [1].
Arhgap10	*Rho GTPase activating protein 10*	19	Chr4	HPC	↑	↓	A GTPase activating protein with specificity to RhoA and Cdc42Hs [2].
Cdc7	*Cell division cycle 7 (S. cerevisiae)*	14	Chr	HPC	↓	↑	Seems to phosphorylate critical substrates that regulate G1/S phase transition and DNA replication [3].
Cdh2	*Cadherin 2 (neuronal)*	18	Chr18	FR	↑	↓	A neuronal adhesion molecule involved in migratory and differentiational behavior of granule cells [4].
Cdig2	*Cadmium-inducible*	18	Chr18	FR	↑	↓	Protein that mediates intracellular cholesterol trafficking.
Centd3	*Centaurin, delta 3, pseudo*	18	?	FR	↑	↓	Pseudogene.
Clgn	*Calmegin*	19	Chr4	FR	↓	↑	A ubiquitous ER chaperone protein that retains incompletely folded or misfolded proteins from entering the cell [5].
Cmtm5	*CKLF-like MARVEL transmembrane domain containing 5*	15	Chr14	HPC	↑	↓	Chemokine-like factor that induces apoptosis through the caspase-dependent pathway (susceptible to epigenetic inactivation) [6].
Col2a1	*Collagen, type II, alpha I*	7	Chr12	HPC	↑	↓	Alpha-1 chain of type II collagen, which is essential for embryonic development of the

							skeleton.
Col6a3	*Collagen, type VI, alpha 3*	9	Chr2	HPC	↑	↓	Alpha chain of type VI collagen, which acts as a cell binding protein found in most connective tissues.
Crhr2	*Corticotropin releasing hormone receptor 2*	4	Chr7	HPC	↓	↑	Receptor for corticotrophin releasing factor whose activity is mediated by G proteins which activate adenylyl cyclase [7].
Fam132b	*Family with sequence similarity 132, member B*	9	Chr2	HPC	↓	↑	Function Unknown
Fgfbp3	*Fibroblast growth factor binding protein 3*	1	Chr10	HPC	↑	↓	A protein that is involved in angiogenesis by mobilizing endogenous FGF's from their extracellular matrix storage, also prevents FGF2 from binding heparin [8].
Fkbp9	*FK506 binding protein 9, 63kDa*	4	Chr7	FR	↓	↑	Are peptidly-prolyl cis/trans isomerase PPlases that may be intimately involved in organogenesis [9].
Gcn1l1	*GCN1 general control of amino-acid synthesis 1-like 1 (yeast)*	12	Chr12	FR	↑	↓	Encodes a protein with elongation factor related functions that upregulates translation [10].
Ghrh	*Growth hormone releasing hormone*	3	Chr20	FR	↑	↓	A hormone released from neurosecretory nerve terminals that travels to the pituitary to stimulate release of growth hormone. Defects in this gene cause dwarfism, while hypersecretion causes gigantism [11].

Gldc	*Glycine dehydrogenase (decarboxylating)*	1	Chr9	HPC	↑	↓	Encodes an enzyme responsible for glycine cleavage. Glycine is a neurotransmitter and an amino acid [12].
Gnao1	*Guanine nucleotide binding protein, alpha O (G protein)*	19	Chr16	FR	↑	↓	The alpha subunit of a major heterotrimeric G protein of the brain and neuroendocrine cells [13].
Igf2r	*Insulin-like growth factor 2 receptor*	1	Chr6	HPC	↓	↓	A multi-functional receptor with binding sites for IGF2 and retinoic acid. Involved in development of cognitive ability [14]
Irx2	*Iroquois homeobox 2*	17	Chr5	HPC	↓	↑	A member of the Iro and Irx class of homeobox genes. Gene plays a major role in pattern formation of the brain [15].
Klc2	*Kinesin light chain 2*	1	Chr11	HPC	↓	↑	A microtubule associated force protein that generates ATP [16].
Lilrb3	*Leukocyte immunoglobulin-like receptor, subfamily B*	1	Chr19	FR	↑	↓	May act as a receptor for class I MHC antigens on dendritic cells and natural killer cells [17].
Lrp1b	*Low density lipoprotein-related protein 1B (deleted in tumors)*	3	Chr2	HPC	↓	↑	Potential cell surface proteins that bind and internalize ligands in the process of receptor mediated endocytosis [18].
Lrp8	*Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor*	5	Chr1	FR	↑	↓	Cell surface receptor for Reelin and Apolipoprotein E that elicits intracellular signal transduction and is important for cell positioning [19].
Map3K10	*Mitogen*	1	Chr19	FR	↓	↑	A protein kinase that

	activated protein kinase kinase kinase 10						activates the JUN N-terminal pathway playing a role in cell growth regulation [20].
Mga	*MAX gene associated*	3	Chr15	FR	↑	↓	Transcriptional regulator (repressor or activator) of MYC-MAX genes involved in regulation of cell proliferation [21].
Mtmr1	*Myotubularin related protein 1*	18	ChrX	FR	↑	↓	Lipid phosphatase that acts on phosphatidylinositol 3-phosphate and phosphatidylinositol (3,5)-biphosphate [22].
Nap5	*Similar to Nck-associated protein 5*	13	Chr2	FR	↑	↓	Function Unknown.
Ndufa9	*NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9*	4	Chr12	FR	↑	↓	Accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase which transfers electrons from NADH to the respiratory chain [23].
Neb	*Nebulin*	3	Chr4	HPC	↓	↑	Binds and stabilizes F-actin, determines thin filament length and regulates actomyosin interaction [24].
Nell2	*NEL-like 2 (chicken)*	7	Chr12	FR	↑	↓	Is a glycoprotein hypothesized to play a role in neural cell growth and differentiation (specifically motor and sensory neurons), and has oncogenic properties [25].
Neurl4	*G protein pathway suppressor 2-like*	10	Chr17	HPC	↓	↑	Function Unknown.
Parc	*p53-associated*	9	Chr6	FR	↓	↑	A cytoplasmic anchor

	parkin-like cytoplasmic protein						protein in p53-associated protein complexes and is a critical regulator of p53 function [26]
Pcdh24	*Protocadherin 24*	17	Chr5	FR	↑	↓	A Ca2+ dependent cell-cell adhesion molecule that plays a role in contact inhibition at the surface of epithelial cells.
Pcnx	*Pecanex homolog (Drosophila)*	6	Chr14	HPC	↑	↓	Comes from a family of maternal-effect neurogenic genes.
Ppp2r5c	*Protein phosphatase 2, regulatory subunit B' gamma isoform*	6	Chr14	HPC	↓	↑	A major intracellular protein phosphatase that regulates multiple aspects of cell growth and metabolism [27].
Prdm2	*PR domain containing 2, with ZNF domain*	5	Chr1	FR	↑	↓	A transcription factor that plays an important role in cell growth and differentiation in the brain [28].
Ptpn22	*Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)*	2	Chr1	HPC	↑	↓	A regulatory gene for T cell and B cell activity associated with autoimmune disease through elaboration of autoantibody production [29].
Reep6	*Receptor accessory protein 6*	7	Chr19	FR	↑	↓	May enhance cell surface expression of odorant receptors by translocating the ORs to the plasma membrane [30].
Rfpl4a	*Ret finger protein-like 4*	1	Chr19	HPC	↓	↑	An E3 ubiquitin-protein ligase that mediates protein degredation [31].
Sema7a	*Sema domain, immunoglobulin domain (Ig) and*	8	Chr15	FR	↑	↓	A cell surface protein that plays an important role in the

	GPI membrane anchor, (semaphoring)						nervous system and in modulating immune function
Smad1	*SMAD family member 1*	19	Chr4	HPC	↑	↓	Transcriptional modulator. Functions downstream of TGF-β receptors, provides a link between receptor serine/threonine kinases and the nucleus [32].
Srm	*Spermidine synthase*	5	Chr1	FR	↑	↓	Enzyme involved in polyamine metabolism that is regulated by TGF-β [33].
St3gal1	*ST3 beta-galactoside alpha-2,3-sialyltransferase 1*	7	Chr8	FR	↓	↓	A golgi membrane-bound type II glycoprotein that catalyses the transfer of sialic acid residues to glycolipids [34].
Strc	*Sterocilin*	3	Chr15	FR	↑	↓	A superhelical lectin that binds to carbohydrate moieties of extracellular glycoproteins [35].
Tnrc4	*Trinucleotide repeat containing 4*	2	Chr1	FR	↑	↓	Encodes a member of the CELF family of RNA binding proteins selectively expressed in the brain and acting on *tau* splicing [36].
Trrap	*Transformation/transcription domain-associated protein*	12	Chr7	HPC	↑	↓	Is a component of several different histone acetyltransferase complexes ie: An epigenetic transcription activator [37].
Tubgcp2	*Tubulin, gamma complex associated protein*	1	Chr10	FR	↑	↓	Subunit of the gamma-tubulin complex which is necessary for nucleation of microtubule polymerization [38].

Ubl4	*Ubiquitin-like 4*	X	ChrX	FR	↑	↓	A housekeeping gene that plays a specific role in cellular processes through protein-protein recognition [39].
Vps4b	*Vacuolar protein sorting 4 homolog B (S.cerevisiae)*	13	Chr18	FR	↑	↓	A member of the AAA protein family and is a key player in intracellular protein trafficking [40].
Wdr64	*WD repeat domain 64*	13	Chr1	FR	↑	↓	Function Unknown
Zfp39	*Zinc finger protein 39*	10	?	FR	↑	↓	Transcriptional regulation.
Zfp94	*Zing finger protein 94*	1	?	HPC	↓	↑	Transcriptional regulation.

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