INVESTIGATING THE EFFECTS OF ANCESTRAL STRESS, ENVIRONMENTAL ENRICHMENT AND THE CONCEPT OF ALLOSTATIC LOAD

J. KEIKO McCREARY
Bachelor of Arts and Science, University of Lethbridge, 2010
Master of Science, University of Lethbridge, 2012

A Thesis
Submitted to the School of Graduate Studies
Of the University of Lethbridge
In Partial Fulfilment of the
Requirements for the Degree

DOCTOR OF PHILOSOPHY

Department of Neuroscience
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

© J. Keiko McCreary, 2016

INVESTIGATING THE EFFECTS OF ANCESTRAL STRESS, ENVIRONMENTAL ENRICHMENT AND THE CONCEPT OF ALLOSTATIC LOAD

J. KEIKO MCCREARY

Date of Defence: February 23, 2016

Dr. G. Metz Supervisor	Professor	Ph.D.
Dr. A. Luczak Thesis Examination Committee Member	Associate Professor	Ph.D.
Dr. A. Hontela Thesis Examination Committee Member	Professor	Ph.D.
Dr. C. Currie Internal Examiner	Assistant Professor	Ph.D.
Dr. B. Kerr External Examiner, University of Alberta, Edmonton, Alberta	Professor	Ph.D.
Dr. A. Iwaniuk Chair, Thesis Examination Committee	Associate Professor	Ph.D.

Dedicated to my Mom and Dad.

"We all carry, inside us, people who came before us."
- Liam Callanan, The Cloud Atlas

INVESTIGATING THE EFFECTS OF ANCESTRAL STRESS, ENVIRONMENTAL ENRICHMENT AND THE CONCEPT OF ALLOSTATIC LOAD

ABSTRACT

The stress that our ancestors experienced resonates with us in their stories, and memoires, but does it affect our health? In this thesis, the impact of ancestral stress on offspring was investigated. We hypothesized that ancestral stress has a significant impact on the development of the stress response, neuromorphology, and behaviour. Moreover, as stress is multi-systematic in its effects, this study examined the use of a new stratification tool termed the cumulative animal allostatic load index (CAALM). In addition, we tested the influence of postnatal enriched environment (EE) on ancestral stress-induced deficits. Lastly, we investigated a "two-hit hypothesis" of maternal stress and inflammation on offspring development. Overall, results indicate that ancestral stress is transferred across generations and increases allostatic load, and EE reverses detrimental effects. Moreover, inflammation exacerbates the effect of stress. Altogether, our results shed light on the influence of maternal and ancestral health on offspring development and the benefit of EE.

ACKNOWLEDGEMENTS

There are so many people that without, this thesis would not have been possible. I hope that I get the chance to thank each of you personally, but for now, this acknowledgments section will have to do. To all of you, thank you for your guidance, support, patience, kindness and encouragement.

First, I would like to thank my supervisor Dr. Gerlinde Metz. Thank you for your guidance, and most of all, thank you for working so hard to ensure your students success. You are truly amazing and that cannot be said enough.

To my committee; Dr. Luczak, Dr. Hontela, Dr. Kerr, Dr. Currie and Dr. Iwaniuk; thank you for your advice, guidance, and support. To all my fellow lab members; especially Sorina, Erin, Ashlee, Nasrin, Jamshid, Zac and Melinda, your helpfulness, patience and friendship have been invaluable both academically and personally; for this I am truly grateful. Dr. Cross, thank you for allowing me to pursue my interest in MRI and its applications, and for your support along the way. To Naomi, Amanda and the animal care staff, thank you for all the vital work you do.

To my friends who heard constantly "sorry I'm busy writing-can we re-schedule?", thank you for your patience and loyalty (and for forcing me to eat and breathe at times). To Patrick-what would I have done without you? Thank you for your insight and knowledge. You are the best team-mate. Thank you for your support, patience and love. I am forever and always grateful.

Lastly, to my family; thank you for your unwavering support. Mom, Dad and Kris, thank you for constantly teaching me that hard work and kindness does pay off. Grandma and Grandpa, thank you for being my source of inspiration and motivation.

Love you most.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

11 β-HSD2	11β-hydroxysteroid dehydrogenase type 2
ABC	avidin-biotin-peroxidase complex
AI	Allostatic load index
AL	allostatic load
ANOVA	analysis of variance
BDA	biotinylated dextran amine
BDNF	brain-derived neurotrophic factor
C	cervical
CAALM	Cumulative Animal Allostatic Load Measure
CNS	central nervous system
CORT	corticosterone
CSF	cerebrospinal fluid
CST	corticospinal tract
DAB	3'3-diaminobenzidine
DG	dentate gyrus
EE	enriched environment
EPM	elevated plus maze
F	filial
FA	fractional anisotropy
G	gestational day
GAD	generalized anxiety disorder
GC	glucocorticoids
GR	glucocorticoid receptors
HDL	high density lipoprotein
HNS	horse normal serum
HPA	hypothalamic-pituitary-adrenal
HPC	hippocampus
IL	Interleukin
IL-1β	interleukin-1 beta
LFL	left forelimb
LG	licking and grooming
LPS	lipopolysaccharides
MC	motor cortex
MGV	mean gray value
miRNA	microRNA
MPS	multigenerational prenatal stress
MRI	magnetic resonance imaging
MWT	Morris water task
NT-3	neurotrophin-3
OF	open field
P	postnatal day
PBS	phosphate buffer solution
PFA	paraformaldehyde
PFC	prefrontal cortex
Poly(I:C)	polyinosinic-polycytidylic acid

PS	prenatal stress
RHL	right hind limb
ROI	region of interest
SAM	sympathetic-adrenal-medullary axis
SD	standard deviation
SEM	standard error of the mean
SES	socioeconomic status
T	thoracic
T_2	transverse relaxation times
TBS	tris-buffered saline
TE	echo time
TPS	transgenerational prenatal stress
TR	repetition time

Chapter 1: Introduction

*Section 1.3 and 1.5 were adapted from the manuscript in submission entitled "**Environmental Enrichment as an Intervention for Adverse Health Outcomes: Postnatal Therapy for Prenatal Stress**", which is included in appendix B.*

1.1 General Introduction

From early on, we are dependent on our mothers for vital nourishment, protection and overall care. Based on recent research, we are becoming more aware that what a mother consumes and what experiences she is exposed to can affect the developing child *in utero* with consequences that potentially last into old age. For example, it is common knowledge that consuming alcohol during pregnancy can lead to the development of Fetal Alcohol Spectrum disorder, and that smoking causes atypical brain development and altered temperament in offspring.

From a vast array of studies conducted in animals and humans, we have recognized that the stress a mother endures during pregnancy can also affect the offspring. The term stress can be used to describe any physical or psychological challenge that threatens, or has the potential to threaten the natural regulatory capacity of an organism (Koolhaas et al., 2011). To a pregnant mother, stress can present itself in a multitude of ways including financial burdens, psychosocial stress, emotional stress, and physical stress or trauma. With the surmounting and ever increasing amount of research being conducted, it is apparent that the prenatal stress (PS) caused by maternal adverse experience may be an extremely influential factor in development; however, it may be overlooked because its causes and consequences are so variable.

The mechanisms of transfer and the effects of PS on the brain and behaviour in the offspring (first generation) are well documented and have been studied for the past 50+ years. However, less is known about the mechanism by which exposure to PS affects development and pathologies in the subsequent generations (second filial (F2), F3 and beyond). Or in other words, the effects of stress that our grandmothers endured and how they have been passed on to our generation are still being investigated.

The overall goals of this thesis are to investigate the heritable effects of ancestral prenatal stress on the F3 generation. Moreover, because the effects of PS are frequently associated with adverse health outcomes, a therapy known to mitigate the consequences of PS, enriched environment (EE) will be investigated for efficacy in ancestrally stressed offspring. Furthermore, because the effects of PS may not be as concrete as those seen from prenatal alcohol or nicotine exposure, a new method of quantifying the cumulative and often antagonistic effects of stress is introduced as a new translational tool to predict adverse health outcomes. Lastly, the cumulative and synergistic burden of psychological maternal stress, in combination with another stressor, such as inflammation, will be examined. Accomplishing these goals may prove critically important to the discovery of biomarkers that allow prediction and prevention of major complex diseases, and may lead to advances in personalized medicine.To elucidate the background of this thesis, the following will introduce the concepts of prenatal programming by maternal and ancestral stress, environmental enrichment and allostatic load.

1.2 Perinatal Programming by Stress

To date, there have been four main mechanisms of programming of the fetal and newborn stress response documented in the literature. These include; 1) prenatal programming due to alterations to the fetal milieu, or the transfer of maternal stress hormones or alterations of fetal hormones, 2) postnatal programming due to altered maternal behaviour, 3) epigenetic inheritance and 4) paternal influence in the transfer of stress. As this thesis focuses on maternal stress, the first three mechanisms will be discussed below and are illustrated in Figure 1.1.

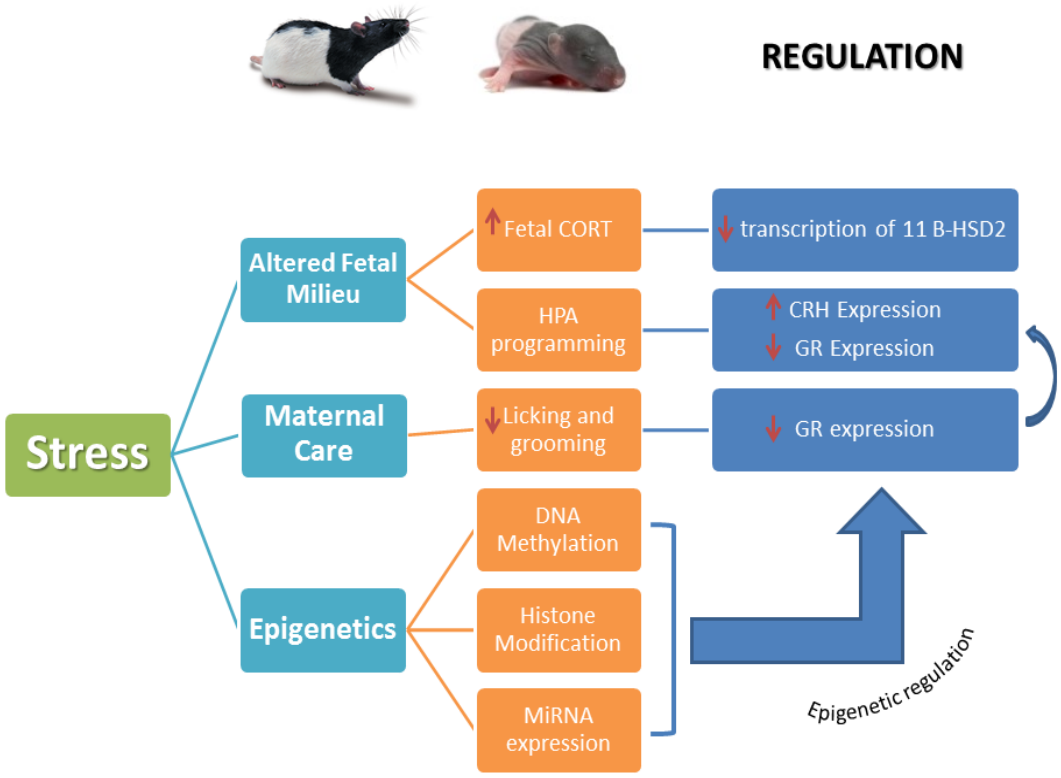

Figure 1.1. The transfer of stress from mother to pup and the regulation of the changes seen in pups. Illustration represents Stress is transferred through an altered fetal milieu, differences or absences in maternal care, and through epigenetic regulation. Epigenetic regulation, through DNA methylation, histone modification and miRNA expression, cause the changes in gene expression which help to regulate the phenotype in the pups (i.e., increased corticosteroid levels, programming of HPA axis, that are also affected by maternal care and altered fetal milieu).

1.2.1 Alterations to the Fetal Milieu

The experience of stress by the pregnant mother activates the hypothalamic-pituitary-adrenal (HPA) axis to initiate the release of stress hormones, such as cortisol in humans or corticosterone in rats, which may enter the fetal circulation (Migeon et al., 1956). In homeostatic conditions, the placenta moderates the fetal exposure to maternal circulating levels of glucocorticoids (GCs) (Cottrell and Seckl, 2009). Thus, GC levels are significantly lower in fetus than in the mother. This is due to the regulatory effects of

the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (11 β-HSD2), which breaks down GCs and provides a protective barrier for the fetus. Consequently, this barrier allows only 10-20% of maternal GCs to reach the fetus (Glover et al., 2009). However, when the homeostatic conditions are challenged through maternal stress, excessive GCs levels may saturate this protective barrier and gain access across the placenta (Benediktsson et al., 1997). In addition to the saturation of the protective barrier which decreases its effectiveness, experimental data has also shown that placental 11 β-HSD2 activity in prenatally stressed offspring is also reduced (Harris and Seckl, 2011).

If basal levels of 11 β-HSD2 in placenta are altered, the fetus will be exposed to excess corticosteroids. When comparing levels of cortisol in paired maternal and fetal plasma samples, researchers have shown that fetal concentrations of cortisol are linearly related to maternal concentrations (Gitau et al., 1998, Gitau et al., 2001b) indicating a transfer of HPA programming (Bosch et al., 2007, Glover et al., 2009). Thus, chronic stress may cause prolonged elevation in plasma cortisol in both the maternal and fetal circulation (Takahashi, 1998).

1.2.2 Alterations in Maternal Behaviour

Once the offspring is born, the external environment becomes the primary stressor, with the ability to cause alterations in the offspring which can be transferred to multiple generations. At this point, the offspring is most dependent on maternal care, and any alterations in this variable will impact the development of the offspring. It is well established that maternal stress and the quality of maternal care (Meaney, 2001, Champagne et al., 2003a, Champagne et al., 2003c, Smit-Rigter et al., 2009, Champagne, 2011, Ward et al., 2013) influence offspring development, behaviour and stress responses

(Champagne and Meaney, 2006, Galea and Brummelte, 2008). These consequences can last to adulthood and therefore have been shown to be programmed transgenerationally, impacting multiple generations (Ward et al., 2013). The extent of maternal care is influenced by stressors endured during pregnancy as well as stressors which may present postnatally. In animals, maternal care refers to feeding, licking and grooming (LG), nesting and overall availability to the offspring. In humans, maternal behaviour refers to the overall maternal mood, care and availability of resources given to the offspring.

The quality and frequency of maternal care is closely associated with maternal stress and mood. Approximately 20-30% of women experience peripartum mood disorders, ranging from mild to more severe postpartum depression within the first six weeks postpartum (Ehlert et al., 1990, Halbreich and Karkun, 2006). Multiple animal studies have demonstrated that stress during pregnancy affects maternal mood and therefore maternal behaviour postpartum (Ehlert et al., 1990, Meaney, 2001, Champagne et al., 2003a, Smit-Rigter et al., 2009). Animal models of maternal behaviour most often study LG behaviours (licking and grooming of the pups), and arched back feeding behaviours (active feeding). LG occurs synchronously while the dam nurses the offspring with arched back behaviour. Dams who show low licking and grooming behaviours tend to have a more passive feeding posture (laying on side or on top of pups during nursing).

Importantly, LG forms the basis for tactile stimulation for the offspring, which is vital for the development of offspring physiology and central nervous system (Champagne and Meaney, 2006). In prenatally stressed dams, LG behaviours are shown less frequently (Champagne and Meaney, 2006). The lack of tactile stimulation causes drastic alterations to the pup brain, including a decrease in glucocorticoid receptors (GRs)

in the hippocampus (HPC) and alterations to oxytocin receptors (Champagne et al., 2001) compared to rats who receive high LG behaviour.

Adult offspring born to mothers that exhibited low LG behaviours, show increased behavioural and endocrine responses to stress by comparison with the offspring of high LG mothers (Caldji et al., 1998). This indicates that alterations in maternal licking and grooming are associated with modifications in offspring stress response (Champagne and Meaney, 2007, Ho and Burggren, 2010). Hence, changes in adult offspring behaviour will then be transferred to the next generation, indicating a generational impact due to maternal behaviour. Moreover, the concept of LG behavioural studies in animals was recently studied in the human population through a self-report study, where maternal stroking over the first weeks of life modified physiological and behavioral outcomes in infancy (Sharp et al., 2012). Although it is clearly demonstrated that maternal behaviour itself causes alterations in the offspring's behavioural traits and their stress responses, it is proposed that these changes are determined and guided by epigenetic mechanisms (Francis et al., 1999).

1.2.3. Epigenetics

Epigenetics is the study of heritable changes in gene expression that occur without alteration of the DNA sequence (Babenko et al., 2012). Such changes can be brought on by prenatal experiences, during postnatal development and aging, or be passed on from the parent to offspring (Meaney and Szyf, 2005, Franklin et al., 2010). Epigenetic processes readily respond to environmental conditions and so allow rapid modifications to an adverse environment, such as stress (Zucchi. et al., 2010). Transfer of stress through epigenetic regulation can occur through multiple events such as DNA cytosine

methylation, histone modifications, and microRNA (miRNA) regulation, with the latter being most relevant to this thesis. MiRNA regulation occurs by binding to the 3' end of gene transcripts (Campbell, 2005). Depending on various factors, the miRNA-protein complex then either degrades the target messenger RNA (mRNA) or blocks its translation (Campbell, 2005). miRNA does not require perfect complementarity to their targets, and therefore, each miRNA can potentially regulate thousands of different mRNAs (Babenko et al., 2012). Furthermore, miRNA detection is valuable as they may be used as biomarkers to predict disease, and may be used for future therapeutic purposes.

The majority of the mechanisms of stress transfer from parent to offspring have an influence on the offspring's epigenome. It is apparent that the mechanisms through which stress contributes across generations will likely involve a complex interaction between the genes and the environment (Meaney, 2010, Dunn et al., 2011c). For example, the transfer of multigenerational stress may induce epigenome alterations through an interaction between the altered fetal milieu, maternal behaviour and resulting epigenetic programming of the embryo (Meaney, 2010, Dunn et al., 2011c).

1.3 Behavioural, Morphological and Molecular Changes Associated With PS

The programming by prenatal stress causes a multitude of detrimental effects in the offspring, including dysregulation of the HPA axis and stress response, altered neuromorphology, and subsequent changes in behaviour. Specifically, molecular markers of synaptic plasticity, neuronal development, growth and early programming in the HPC and cortex are downregulated due to PS (Koo et al., 2003, Lui et al., 2011b, Peng et al., 2011, Li et al., 2012, Zhang et al., 2012a). Markers of synaptic plasticity altered due to PS include neural cell adhesion molecule, synaptophysin, N-Methyl-D-aspartate receptor,

B1-integrin and Tissue plasminogen activator. Each molecule plays a pivotal role in synaptic efficacy, growth, neuronal development, regeneration, and plasticity (Nakamura et al., 1999, Koo et al., 2003). Markers of neurogenesis and neuronal growth altered due to PS include 5-Bromodeoxyuridine (BrdU), BDNF and Growth-associated protein 43 (GAP-43). These molecules act as mitotic markers during development (Kolb et al., 1999), regulate dendritic and axonal morphology (Levine et al., 1995, Thoenen, 1995, Schinder and Poo, 2000) and act as a marker for structural and functional changes in neuronal populations (Chao and McEwen, 1994, Gauthier-Campbell et al., 2004). These molecular changes due to PS lead to overall morphological changes which include decreased dendritic area and perimeter, reduced spine density, and diminished number of granular cells in the HPC and prefrontal cortex (PFC) (Mychasiuk et al., 2012).

The molecular and morphological changes associated with prenatal stress manifest in a plethora of behavioural abnormalities and are thought to contribute to multiple psychopathologies. PS has an anxiogenic effect with increased emotionality (Laviola et al., 2004, Pascual et al., 2015, Zubedat et al., 2015), along with reduced age-typical rough and tumble play (Laviola et al., 2004). Likewise, PS increases addictive behaviour (Yang et al., 2006) and causes impaired cognition (Koo et al., 2003, Yang et al., 2007, Lui et al., 2011b, Li et al., 2012, Zhang et al., 2012a, c) and attention (Zubedat et al., 2015).

In motor-learning tasks, PS animals show superior performance, indicating that PS may also lead to adaptive mechanisms thought to help in overall survival (Ulupinar et al., 2015, Zubedat et al., 2015). This indicates that not all consequences of stress are negative and may be beneficial and adaptive to an adverse environment; however the chronic exposure of stress prenatally seems to cause mostly detrimental consequences. Although these deficits have been thoroughly studied in the F1 generation, the effect of stress

across multiple generations is still in its infancy, and it is unknown whether stress across multiple generations will lead to similar outcomes.

1.4 The Next Generations: Transgenerational and Multigenerational Stress

Previous research from our laboratory has shown that the consequences of prenatal stress cause a wide range of metabolic, cardiovascular and behavioural disorders that may last into adulthood, and may be passed on to future generations (Zucchi. et al., 2013, Yao et al., 2014). Central models of generational stress include transgenerational and multigenerational models, and it is important to differentiate between the two.

Transgenerational inheritance of stress has become a popular topic, and is defined as stress transmitted through the germ line in the absence of direct exposure (Skinner et al., 2011). This indicates an involvement in germ line transmission between generations without direct exposure to the environmental factor (Skinner, 2008). Therefore, in studies involving gestational exposure to stress in the female lineage, the F3 or great-grand offspring must be investigated as the F2 and F1 generations were directly exposed (Skinner, 2008, Zucchi et al., 2013). The F3 generation is therefore necessary to investigate true transgenerational inheritance (Figure 1.2). There is limited evidence of the true epigenetic transfer of stress signatures transgenerationally that propagate to the F3 and even the F4 generation. A previous study investigated maternal separation and restraint stress in F0 mice and found that stress across generations caused social anxiety and altered cognition in adult F2 and F3 offspring (Franklin et al., 2010). Moreover, the defects were associated with impaired serotonergic signaling and serotonin levels. Using gestational restraint and swim stress, Yao and colleagues (2014) more recently showed that prenatal stress is transferred across generations and results in a higher risk of preterm

birth in the F2 generation and low offspring body weight and altered developmental trajectories in the F3 generation. Our laboratory has also confirmed similar effects lasting to the F4 generation (Ambeskovic et al., 2015).

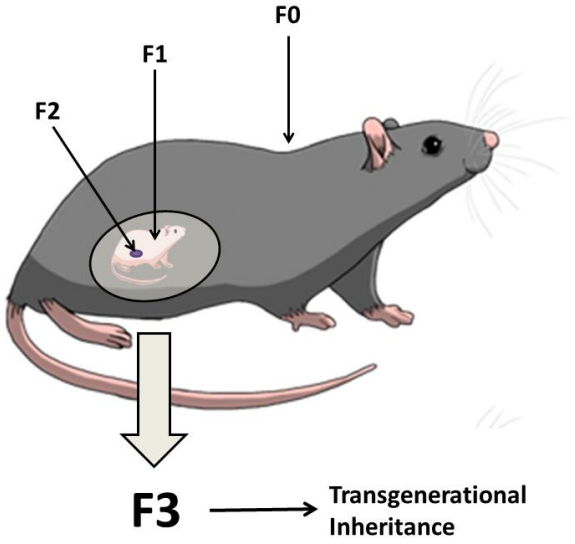

Figure 1.2. Illustration of the prenatal transgenerational inheritance in the maternal lineage. This diagram shows that stress in the F0 dam directly affects the female F1 offspring and its germline (F2). Therefore, the F3 generation is the first generation that would show true transgenerational inheritance in the absence of direct stress.

In comparison to transgenerational stress, multigenerational stress affects every consecutive generation through direct transfer. Multigenerational stress is defined here as an exposure that directly influences multiple generations, i.e. exposure to the pregnant female in each generation. By investigating the cumulative effect of multigenerational stress by directly stressing pregnant dams in each generation one can investigate the effect of cumulative recurring stress. This model is novel and is currently only used by our laboratory in order to mimic generations of individuals living in continuously stressful conditions.

Our data show that the effects of multigenerational stress differ from those of transgenerational stress, and also differ across sex and lifespan (Ambeskovic, 2013, Erickson et al., 2014, McCreary et al., 2015). Specifically, multigenerational stress (restraint and swim stress) results in motor activity profiles in males that are normal during the peak sexual reproductive age, but differ during development and aging, the most vulnerable periods in life (Erickson et al., 2014). From an experiment conducted during my Master's thesis, it was shown that each additional generation of PS incrementally elevates HPA axis activation, anxiety-like and aversive behaviours in adult female offspring (McCreary et al.,2015). Moreover, these changes are accompanied by reduced neural density and altered gene expression related to the regulation of neuronal maturation, arborization and synaptic plasticity (McCreary et al., 2015). Overall, current studies emphasize that recurrent stress across generations may cumulatively increase stress vulnerability and the risk of adverse health effects through perinatal programming.

1.4.1 Significance of Animal Models of Ancestral Stress

The implications in examining models of ancestral stress are plentiful, however mechanisms are still poorly understood as research in this area is in the early stages. Research which investigates ancestral stress will help to understand evolutionary origins of adaptive and maladaptive responses to stress, and the clinical and etiological significance of ancestral stress in disease due to poverty, famine or natural disaster. Complex disease etiology in particular is still poorly understood; understanding the mechanisms of transgenerational programming of complex diseases offers the potential for ground-breaking strategies of disease prediction and treatment.

Evolutionary theory predicts that constant stress over successive generations can shape those who survive (Nacci et al., 2002). Theoretically, adapted genotypes may become predominant in populations subject to multigenerational stress (Nacci et al., 2002). For example, one team of researchers hypothesized that long-term, multigenerational stress would produce populations that were adapted and/or resilient to toxic conditions through shifts in life history and/or physiological response traits (Nacci et al., 2002). They found that a species of fish multigenerationally exposed to environmental contaminants produced a toxin-adapted population, although the mechanisms were not discussed (Nacci et al., 2002). However, they also found that while adapted populations could persist and cope with the toxic environmental stressor, long-term consequences may include increased vulnerabilities. The latter may include increased sensitivity to newly introduced contaminants, as well as disturbances in ecosystem function (Nacci et al., 2002).

In terms of clinical or etiological significance, the investigation of generational stress may help to understand disease manifestations later in life, and prevalence of disease which arises without genetic determinism. A plethora of external environmental factors, such as stress, can alter epigenetic components that are passed on transgenerationally and therefore heighten or lessen stress resilience, and subsequently the risk of disease (Babenko et al., 2012, Zeybel et al., 2012, Zucchi. et al., 2013, Skinner, 2014).

Generational transfer of stress through epigenetic mechanisms may provide vital insights to many psychiatric and neurological disorders such as bipolar disorder, schizophrenia, autism, depression, early onset Alzheimer's, and inflammatory conditions (Gardiner et al., 2012, Matrisciano et al., 2012, Zucchi et al., 2014, Metz et al., 2015d), all

of which seem to be affected in part by PS. Other epidemics, such as obesity, diabetes and cardiovascular disease (Matthews and Phillips, 2012) may also be linked to ancestral stress. In areas of poverty or famine, where populations are chronically or repeatedly exposed to stress, models of multigenerational stress may help explain the profound impact on health and disease across generations and early prediction of disease risk.

1.5 Enriched Environment

Based on the evidence that consequences of PS can propagate across multiple generations, treatments to prevent or reverse the detrimental effects of PS are of significant interest. Environmental enrichment is one treatment which has shown promise in reversing the effects of PS. Enriched environment (EE; used interchangeable with environmental enrichment) is a non-invasive treatment that produces robust changes in neuronal morphology and behaviour. EE was first studied in the 1940's by Donald O. Hebb when he brought rats to his home and had his children play with them, essentially "enriching" their environment. He noted that rats reared as pets in his house performed better on memory tasks compared to rats reared in standard conditions (Hebb, 1947). Further study by Hebb's team found that laboratory dogs that were treated as pets are superior in problem-solving than those reared in simple or deprived environments (Clarke et al., 1951). In addition, the authors suggested that social behaviour and motivation appeared increased in the enriched dogs (Clarke et al., 1951).

Since Hebb's first experiments there has been extensive research about how EE affects the brain and behaviour. EE provides animals with enhanced social, motor, cognitive and sensory stimulation (Kolb and Metz, 2003). The combination of these factors is thought to produce many anatomical, molecular, and behavioural alterations in

animals. In general, neuroanatomical studies have revealed increased cortical weight, cortical thickness and more complex dendritic organization in EE rats (Rosenzweig et al., 1962, Bennett et al., 1964, Jung and Herms, 2014). Molecular studies have shown that EE raises expression of brain-derived neurotrophic factor (BDNF), which is involved in plasticity in the HPC and cortex (Falkenberg et al., 1992).

EE has been used in an attempt to reverse many neurological disorders, such as stroke and Parkinson's disease (Jadavji et al., 2006, Knieling et al., 2009), but studies addressing PS are somewhat limited. EE after PS is unique due to its attempt to reverse deficits that were primarily induced *in utero*. From the literature review conducted (see Appendix B), it was concluded that EE is able to reverse behavioural deficits, along with morphological and molecular deficits. However, lacking are studies which investigate the usefulness of enrichment in animals that were generationally stressed along with studies investigating epigenetic mechanisms of EE mediation.

1.6 Allostasis and Allostatic Load

Stress is unlike any other pathological condition, as it triggers non-specific and non-linear responses that are unique to each individual (Selye, 1984). Moreover, the response to stress impacts multiple physiological systems and leads to variable and complex symptomology. The range of symptoms and deficits may be due to individual stress response, along with the multiple systems trying to cope with the stress. In order to better understand the mechanisms of stress-associated outcomes, the concept of allostatic load and allostasis has been introduced (Sterling and Eyer, 1989). "Allostasis" is defined as the ability to achieve stability through change (Sterling and Eyer, 1989) as opposed to the more common term homeostasis, or the body's ability to maintain equilibrium (Cannon,

1932). Stress causes the body to react via allostasis by triggering multiple and often opposing physiological systems including the immune system, metabolic system and the neuroendocrine system.

The concept of allostatic load (AL) was created based on the concept of allostasis in order to help understand the health outcomes of the cumulative stress and the resulting mediators of the stress effect. AL was first defined by McEwen and Stellar in 1993 as the cost of chronic exposure to fluctuating or heightened neural or neuroendocrine response resulting from repeated or lasting environmental challenge (McEwen and Stellar, 1993). It is the cumulative burden of stress, which is expressed in the physiological dysregulation across numerous systems that are involved in regulating and coping with stress. The benefit of the biological concept of AL is that it incorporates several elements of stress pathophysiology in one comprehensive model (Nugent et al., 2015). The AL model includes many multi-systemic interactions including primary and secondary mediators of stress, and has been shown to have the predictive capacity to detect individuals at high risk of tertiary outcomes (Juster et al., 2010).

To aid in quantifying the cumulative effects of stress, Seeman and colleagues in 1997 proposed operationalizing AL through the use of an allostatic load index (AI (Seeman et al., 1997)). AI is measured as the sum of dysregulated physiological biomarkers (Seeman et al., 1997) that in turn reflect a multi-systemic view of the physiological toll that is placed on the body to maintain allostasis. Studies have shown the usefulness and effectiveness of AI and have shown that it better predicts future health risks, especially high risk groups, than any biomarker individually (Seeman et al., 2001, Karlamangla et al., 2002). Essentially, the AI is a comprehensive model that better links

often contradictory effects while providing insights into how individuals differ in their vulnerability or risk to develop diseases or tertiary outcomes (McEwen, 2000a).

Although the concepts of allostasis and AL have been considered useful for animal studies of stress and disease (Tannenbaum et al., 2002, Korte and De Boer, 2003, Van der Meer et al., 2004, Korte et al., 2005), there is currently no cumulative measure of AL, or an AI available for animal models. An animal AI offers a unique potential to advance knowledge translation of biomarker research in personalized medicine approaches.

1.7 Summary: Outline and Objectives

This thesis is composed of three experimental chapters and one conceptual application using experimental data presented in previous chapters. The present research is designed to aim at furthering the understanding of how maternal stress affects offspring directly and across multiple generations, and how EE can aid in reversing the effects of stress. Moreover, this thesis aims to introduce a novel method of measuring the effect of ancestral stress and how it could predict vulnerability or resilience to stress.

The first portion of this thesis will investigate the effects of transgenerational and multigenerational stress and the therapeutic efficacy of EE. The overall hypothesis is that both transgenerational and multigenerational stress will lead to an altered stress response as well as atypical behaviour. Moreover, we propose that ancestral stress will lead to altered neuronal morphology along with abnormal corticospinal tract fiber density. We propose that EE will mitigate the neuromorophological, neuroendocrine and behavioural consequences of ancestral stress.

The second portion will introduce a new method of measuring chronic stress known as AL. We hypothesize that the cumulative burden of ancestral stress will generate a

higher quantitative allostatic score compared to control animals; additionally enrichment will lead to decreased scores. Lastly, because the majority of psychopathologies are not linked to one individual origin and AL can be aggravated by more than one insult, the last experiment tested the hypothesis that stress combined with inflammation will cause more severe detrimental effects than stress alone. Thus, this experiment tested the concept of a two-hit hypothesis in which two stressors may synergistically enhance the risk of disease. Outlined below are the experiments by chapter.

- **Chapter 2**: The first experiment outlines the effects of transgenerational and multigenerational stress on physiology and affective behaviour, and the reversal by EE;
- **Chapter 3**: The second experiment outlines the effects of EE and ancestral stress on pyramidal neuronal morphology, corticospinal tract density using Biotinylated Dextran Amine, and motor skills outcome;
- **Chapter 4**: Aims to describe a new method of measuring ancestral stress using the concept of allostatic load;
- **Chapter 5**: The third experiment investigates the synergistic effects of two prenatal stressors, stress and inflammation, on neuromorphological changes using *in vivo* magnetic resonance imaging;
- **Chapter 6:** Summarizes the experimental findings and significance of this study.

Chapter 2

Experiment 1: Enriched Environment as an Effective Therapy to Mitigate Transgenerationally Programmed Stress Sensitivity

Chapter 2 has been submitted in its entirety.

2.1 Abstract

Prenatal stress (PS) can program stress hyper-responsiveness and is associated with impaired neurodevelopment and psychopathologies in later life. Here we report that PS generates a transgenerationally heritable physiological footprint and elevated stress sensitivity which can be mitigated by beneficial experiences in later life.

Dams of the parental F0 generation experienced late pregnancy psychosocial stress. Their pregnant daughters (F1) and grand-daughters (F2) were either stressed (multigenerational PS) or remained unstressed (transgenerational PS). Their male adolescent F3 offspring was exposed to either standard or enriched housing conditions. Endocrine and behavioural stress responses in F3 rats were determined in adulthood.

A family history of stress downregulated glucocorticoid receptor (GR) and prefrontal cortex neuronal density along with precocious development of anxiety-like behaviours in both multigenerational and transgenerational stress lineages. Deep sequencing of prefrontal cortex microRNA (miRNA) profiles in stressed lineages revealed altered regulation of pathways associated with brain-derived neurotrophic factor and signatures of schizophrenia, depression and neuroplasticity. Exposure to EE restored GR levels and reduced hypothalamic-pituitary-adrenal (HPA) axis activity across all groups. EE mitigated stress-induced anxiety-like behaviours in stressed lineages and normalized miRNA expression profiles.

Ancestral stress programs elevated stress sensitivity, affective behaviour and neuroplasticity through programming of the HPA-axis and epigenetic modifications involving miRNAs. Environmental enrichment serves as an effective intervention for stress programming. The identification of miRNAs that mediate the actions of EE may allow the development of new predictive biomarkers of disease and therapeutic targets.

2.2 Introduction

The prevalence of mental health and substance use disorders has increased by 37.6% since 1990 (Whiteford et al., 2013). Dysregulation of the stress response represents a major risk factor for these disorders. Through programming of the stress response, prenatal stress has been shown to increase the risk of developmental disorders and psychopathologies in later life (Gitau et al., 2001a, Lupien et al., 2009). The main mechanism of developmental programming by prenatal stress involves altered hypothalamic-pituitary-adrenal (HPA) axis function and elevated stress responsiveness in the F1 generation (Harris and Seckl, 2011, Moisiadis and Matthews, 2014). More recently, early life stress was shown to not only influence the F1 offspring, but also future filial generations. Transgenerational programming by stress (F2-F3 generations) was shown to alter affective state and sensorimotor behaviour (Erickson et al., 2014), endocrine functions (Babb et al., 2014) and heritable changes in DNA methylation status and microRNA (miRNA) expression (Gapp et al., 2014, Yao et al., 2014). The understanding that stress is transferred transgenerationally to filial generations and is mainly uncontrollable, highlights the need to identify interventions that can reverse adverse effects of programming by ancestral stress.

Beneficial environmental and lifestyle changes are powerful, ecologically valid strategies to mitigate adverse programming by stress. For example, exposing rodents to an enriched environment (EE) with rich social and sensorimotor stimulation decreased anxiety-like behaviours in an elevated plus maze (Pena et al., 2006, Galani et al., 2007, Pena et al., 2009, Leshem and Schulkin, 2012), improved sensorimotor skills (Jadavji et al., 2006, Jadavji and Metz, 2009) and led to larger cell proliferation and neuronal density (van Praag et al., 2000) along with increased expression of brain-derived neurotrophic

factor (BDNF) (Koo et al., 2003, Cao et al., 2014a). In prenatally stressed F1 offspring, EE decreases anxiety-like and fear behaviours (Leshem and Schulkin, 2012), improves social behaviour (Morley-Fletcher et al., 2003), restores dendritic and synaptic morphology (Peng et al., 2011, Pascual et al., 2015) and rescues density of glucocorticoid receptors (Li et al., 2012). Here we aim to determine if EE represents an effective therapy for the endocrine and behavioural consequences of epigenetically inherited manifestations of transgenerational stress.

The present study was designed to (1) characterize transgenerational and multigenerational phenotypes and microRNA of stress response and affective behaviour and (2) to determine if environmental intervention by EE can mitigate the transgenerationally programmed phenotype of stress. To determine the impact of EE on transgenerational versus cumulative ancestral stress, the design used (1) a lineage of transgenerational prenatal stress in which the parental generation but not the F1-F3 generations experienced stress, and (2) a lineage of multigenerational prenatal stress, in which each the parental and the offspring generations experienced gestational stress (Zucchi et al., 2012, Ward et al., 2013, Erickson et al., 2014, Yao et al., 2014). The F3 generation is of particular relevance because it is the first generation in the maternal lineage that is not directly exposed to prenatal stress and therefore changes can be considered truly programmed through epigenetic inheritance (Skinner, 2008). The focus on miRNA signatures in this study has relevance for the discovery of heritable predictive biomarkers of altered stress response. We hypothesized that ancestral stress alters HPA axis activity and affective behaviour in relation to specific miRNA signatures of stress, which then can be reversed by EE.

2.3 Materials and Methods

2.3.1 Experimental Design

The study involved forty-eight adult male Long-Evans hooded rats, bred and housed at the Canadian Centre for Behavioural Neuroscience. Rats were F3 offspring born to one of the following three maternal lineages: non-stress controls (n = 16), transgenerational prenatal stress (*TPS*; n = 16), and multigenerational prenatal stress (*MPS*; n = 16). TPS rats were the F3 generation of a filial line in which only the F0 dams were stressed during gestation. MPS rats were the F3 generation of a filial line in which dams from each consecutive generation (F0, F1, F2) were gestationally stressed. Each F3 group was gathered from 4 different litters (4 control, 4 TPS, 4 MPS), and animals in each litter were then split into standard or enriched housing conditions.

At weaning, rats derived from the three lineages were assigned to either housing in standard cages, or housing in an enriched environment (EE). Thus, the following groups were tested: non-stress controls in standard (*Control*; n = 8) and EE (*Control-EE*; n = 8) housing conditions, TPS in standard (*TPS*; n = 8) and EE (*TPS-EE*; n = 8) housing, and MPS in standard (*MPS*; n = 8) and EE (*MPS-EE*; n = 8) housing. Figure 2.1 illustrates the experimental design of the present study.

The animals were housed under a 12 h light/dark cycle with lights on at 7:30 AM. The room temperature was maintained at 20^{o}C with relative humidity at 30%. Body weight was regularly recorded as the rats aged. During adolescence (postnatal day (P) 21 to P60), rats were assessed in the open field and elevated plus maze tasks. At 100 days old, corticosterone (CORT) measurements were taken and at 180 days old, animals were euthanized for tissue collection. All procedures were performed in accordance with the

guidelines of the Canadian Council on Animal Care and approved by the University of Lethbridge Animal Welfare Committee.

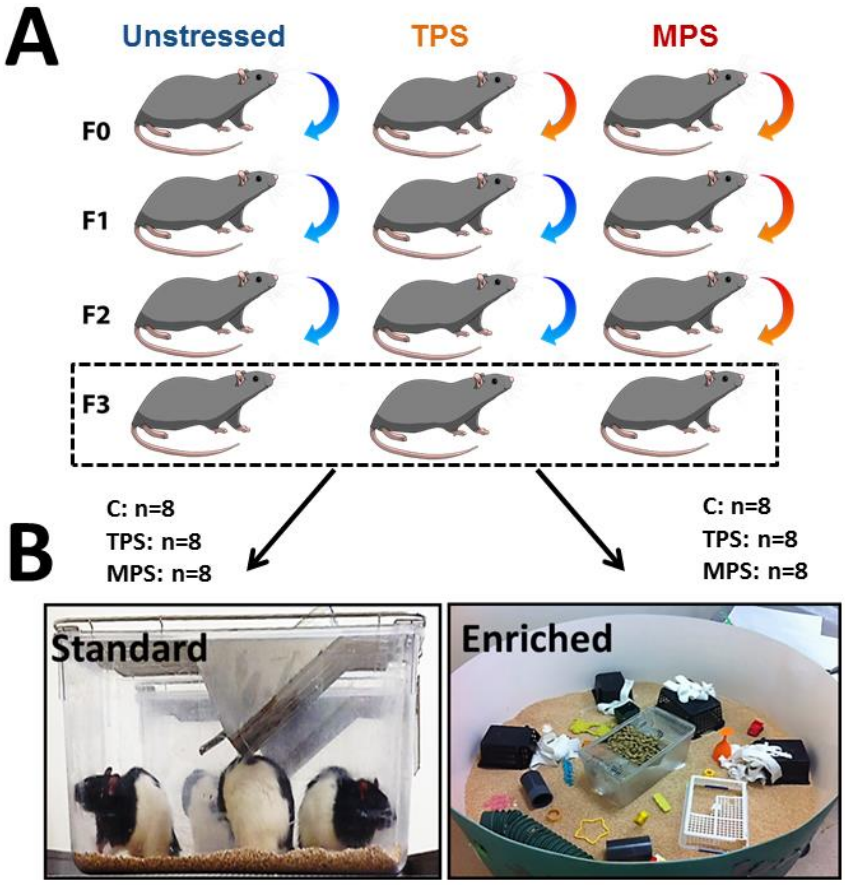

Figure 2.1: Animal model of transgenerational and multigenerational stress. The animal model included a non-stress control lineage, a transgenerational stress lineage in which gestational stress only occurred in F0 dams, and a multigenerational stress lineage in which F0-F1-F2 dams were stressed during pregnancy. Males from four F3 litters per group were tested. Four male rats per litter were used; 2 animals from each litter were placed in a standard environment and 2 rats from each litter were placed in an enriched environment at the age of 35 days.

2.3.2 Prenatal Stress

Pregnant dams were stressed by social isolation, which results in mild psychosocial stress (Hawkley et al., 2012). Each dam was housed alone and did not experience any direct contact with conspecifics from P90 until the weaning of her offspring. Control rats were housed in pairs until gestational day 21.

2.3.3. Standard and Enriched Housing Conditions

On P21, animals were weaned and assigned to two housing conditions. In the standard housing condition rats were housed in non-sibling pairs in Macrolon shoebox cages. From P21 to P35, the EE rats were housed in groups of four living in large Macrolon housing units. At P35, each group of four EE rats was moved to large circular housing units. In addition to the increased social interactions and space, the EE was equipped with multiple shelters and toys that were exchanged weekly. In addition to standard rat chow, EE rats were regularly provided with novel types of foods, including raw pasta, non-sweetened breakfast cereal, and seeds. In total, three identically arranged EE were used in the study, each housing all of the rats from a single stress treatment group.

2.3.4 Behavioural Analyses

2.3.4.1 Open Field Task

The open field task represents a rather aversive environment that allows the quantification of motor activity, anxiety-like behaviours, and exploration of an open arena (Smith et al., 2008, Jadavji et al., 2011). In this study, the open field task was conducted using the VersaMax Legacy Open Field system (Omnitech Electronics, Inc., Dartmouth,

NS, Canada; Figure 2.5A), which measured an animal's activity for a period of 10 minutes using an array of infrared sensors connected to a computer. Behavioural measures included total time spent moving during the test interval (*Movement Time*), the amount of time spent within the margins of the open field (*Margin Time*), and time spent rearing (*Vertical Time*).

2.3.4.2 Elevated Plus Maze

The elevated plus maze (Figure 2.5B) allows the assessment of anxiety-like behaviours in a novel, aversive environment (Lister, 1987). In this study, the elevated plus maze consisted of an opaque black Plexiglas maze suspended 50 cm above the ground. Two arms (50 cm x 10 cm) were enclosed by Plexiglas walls 40 cm high ("*closed arms*") and two identically sized arms were without walls ("*open arms*"). Individual rats were placed in the center of the maze and allowed to explore freely for a period of five minutes. Behavioural measures included *total time* spent in the closed arms, open arms and at the end of open arms; *number of entries* into closed arms, open arms, and end of open arms; and *latency to enter* the closed arms as well as number of *risk assessment* behaviours (i.e. stretch-extend postures).

2.3.5 Corticosterone Assay

Blood samples (0.6 ml) were collected from the lateral tail vein using a 23 gauge butterfly needle coated in heparin under 4% isoflurane anesthesia. Blood was transferred to centrifuge tubes and plasma was obtained by centrifugation at 5,000 rpm for ten minutes at 4°C. The samples were stored at –80°C. Plasma CORT concentrations were determined by radioimmunoassay, ran in duplicates, using commercial kits (ELISA, Abcam Inc., ON, Canada).

2.3.6 Tissue Collection

At the age of 180 days rats were euthanized with an overdose of Euthanosol® (Merck, QC, Canada) and perfused transcardially with phosphate buffer solution (PBS ; approximately 200 ml) followed by a transcardial injection of approximately 200 ml of 4% Paraformaldehyde (PFA; Sigma-Aldrich, MO, USA). Brains were extracted, stored in brain bottles containing 4% PFA and refrigerated for 24 h and then transferred to sucrose solution for at least three days.

2.3.7 Histology

2.3.7.1 Immunohistochemistry

Brains were cut in coronal sections with a microtome at a thickness of 40 µm and 12 series interval. Sections were stored in 0.01 M PBS with a 1:1000 concentration of sodium azide. The sections were washed in PBS (0.01 M, pH 7.6) for 3×10 min and pre-treated with 3% hydrogen peroxide for 10 min to reduce endogenous peroxidase activity. Sections were then rinsed in 0.01 M PBS for 3x10 min prior to blocking with 10% horse normal serum (HNS) (catalog no. s-2000; Vector Laboratories, CA, USA) with 0.01 M PBS for 1 h. Next the sections were incubated in the GR antibody (3D5; mouse monoclonal antibody, catalog no. sc-56851; Vector Labs) for 24 h at room temperature on a belly dancer (Stovall Life Science Inc,, USA). GR (3D5) was diluted in 10% HNS/PBS solution (1:500) with 2% Triton X-100. After incubation, the sections were washed 4x8 min in 0.01 M PBS and applied by biotinylated horse anti-mouse IgG (H+L) (1:200) (catalog no. BA-2001; Vector Labs) for 24 h at room temperature. The sections were then washed again in 0.01 M PBS (4x8 min) and subsequently incubated with avidin-biotin-peroxidase complex (ABC; Vectastain® Elite ABC Kits, catalog no. PK-6100; Vector

Labs) for 45 min at room temperature. Following 3 washes for 10 min each in 0.01M PBS, the sections were developed using 3'3-diaminobenzidine (DAB; DAB substrate Kit, catalog no. SK-4100; Vector Labs) for 30 sec. The sections were rinsed immediately for 4x8 min after staining, and then mounted on slides and air-dried for overnight. After dehydration in ethanol and HemoDe, the sections were coverslipped for microscopic observation.

2.3.7.2 Unbiased Stereology

All stereological data were blindly collected by a single investigator using Stereo Investigator® (MicroBrightField Inc., 2013, Version 10). The density of glucocorticoid receptors (GRs) was calculated by dividing the cell numbers obtained with the optical fractionator by the volume of each interested region as calculated by Cavalieri's principle (Garcia-Amado and Prensa, 2012). Briefly, an optical fractionator probe was used to estimate the populations of hippocampal GRs, including areas of CA1/2, dentate gyrus (DG) and CA3. Systematically, randomly positioned grids (150 µm x 150 µm) containing counting frames (80 µm x 80 µm) were superimposed on the areas of investigation. The counting frame contains a red exclusion line and a green inclusion line so that traversing along the Z-axis generates an optical or 3D dissector probe that counts the cells. A 120 µm x 120 µm grid was randomly placed on each section containing each hippocampal areas and all crosses in which the upper right quadrant was overlaying the structure were counted in order to determine an unbiased volume estimate. The Gundersen coefficient of error for cell number and volume estimates was ranging from 0.02-0.04 and 0.01-0.03, respectively (Gundersen et al., 1999).

2.3.7.3 Neural Density Analysis - Cytoarchitectonics

Every third series of sections was mounted and stained with cresyl violet to detect Nissl bodies. The slides were captured using a motorized Zeiss AxioImager M1 microscope (Zeiss, Jena, Germany) at 1X magnification. The quantitative cytoarchitectonic analyses in cresyl violet-stained sections corresponding to a region of interest (ROI) measuring 0.766 mm^2 slices at Bregma level 3.70 (caudal prefrontal cortex; PFC) were performed with Image J V1.36 (http://rsb.info.nih.gov/ij/download.html). The "absolute grey level index" was ascertained as the measured parameter (Zilles et al., 1980). A step tablet was used (http://rsb.info.nih.gov/ij/download.html) to calibrate the optical density in the 8-bit images.

2.3.7.4 Prefrontal Cortical Thickness

PFC thickness was measured on cresyl stained sections at Bregma level 3.70 mm using ImageJ software (NIH). Thickness coordinates were compared to Paxinos and Watson rat atlas (Paxinos, 1998) to determine location of measurement. The vertical (dorsoventral) distance was measured parallel to the midsagittal line, using the rhinal fissure endpoint to center the placement of the line. The horizontal (mediolateral) distance was measured perpendicular to the midsagittal line, crossing midway through the dorsoventral line (Spivey et al., 2009).

2.3.8 microRNA Deep Sequencing

Deep sequencing of miRNA expression was performed with fresh PFC tissue using Illumina GAIIx genomic platform (Illumina, CA,USA). Briefly, base calling and demultiplexing was completed using CASAVA 1.8.1 software pipeline with default settings. Short read quality was examined using FastQC software. Adapters were trimmed

using cutadapt software (http://code.google.com/p/cutadapt/) with options specified to search for adapters anywhere in the read sequence and retain only sequences over 15 nucleotides in length. Quality trimming was performed with Sanger quality score cutoff of 30. Over 92% were retained after trimming in each of the libraries. FastQC quality check was performed after trimming. MiRNA detection and counting was performed using standalone MicroRazerS 41 version 1.0 (Emde et al., 2010). Potential targets of selected miRNAs of interest were predicted using the 3'UTR available for Rat rn5 (UCSC) genome. Target prediction was based on miRanda v.3.3a (Computational Biology Center of Memorial Sloan-Kettering Cancer Center, NY, USA), an algorithm for finding genomic targets for microRNAs, with default options. Gene set enrichment analysis was performed using GOstats package v.2.34 (Bioconductor, MD, USA) as a way to identify significantly over-represented gene ontology categories and KEGG pathways.

2.3.9 Statistical Analysis

Statistical computations were based on Statview software version 5.0 (SAS Institute, NC, USA). All behavioural measures were analyzed on a per-variable basis using a mixed-design analysis of variance (ANOVA). The between-group independent variables included stress treatment (non-stress controls, TPS, MPS) and housing (standard and EE). The within-group variable was time. Post-hoc comparisons were performed using Tukey HSD for between-enrichment group differences in groups, and independent sample t-tests for between-group differences for enrichment. Additionally, non-parametric correlation was conducted to determine the relationship between the dependent variables. A *p*-value of less than 0.05 was chosen as the significance level.

For miRNA analysis, raw count data underwent normalization and regularized *log* transformation using statistical routines implemented in the DESeq2 bioconductor package (Love et al., 2014) as described in the DESeq2 user manual. Pairwise comparisons between experimental groups were performed using DESeq2 with default settings applied to normalization and statistical testing. Small RNAs with false discovery rate adjusted p-values <0.1 were considered differentially expressed.

2.4 Results

2.4.1 Ancestral stress alters HPA axis activity and is reversed by EE

2.4.1.1 GR Receptor Density

A summary of the total hippocampal GR density in all areas is shown in Figure 2.2B and measurements of all areas are shown in Figure 2.3. GR measurements included total markers and GR density of the DG, CA1-2, and CA3. In the CA1-2 areas of the hippocampus, there was a significant main effect of stress causing a decrease in total GR markers ($F(2,34)=15.871$, $p<0.0001$) and density ($F(2,34)=10.96$, $p<0.001$) and a significant effect of enriched environment causing an increase in total GR markers ($F(1,34)=75.842$, $p<0.0001$) and GR density ($F(1,34)=36.11$, $p<0.0001$). In the CA3 area of the hippocampus, there was a significant increase in total GR markers ($F(2,34)=8.423$, $p=0.001$) due to stress and a significant decrease of total markers ($F(1,34)=92.016$, $p<0.0001$) and GR density ($F(1,34)=92.016$, $p<0.0001$) due to enrichment.

2.4.1.2 Corticosterone

Overall, animals that were ancestrally stressed had higher baseline CORT levels, although these results were not significant. However, enrichment significantly decreased the level of basal circulating CORT ($F(1,42)=16.16$, $p<0.001$) across all groups (Figure 2.2C). Thus, CORT levels in enriched groups were at least on average 80% lower than in standard housing groups.

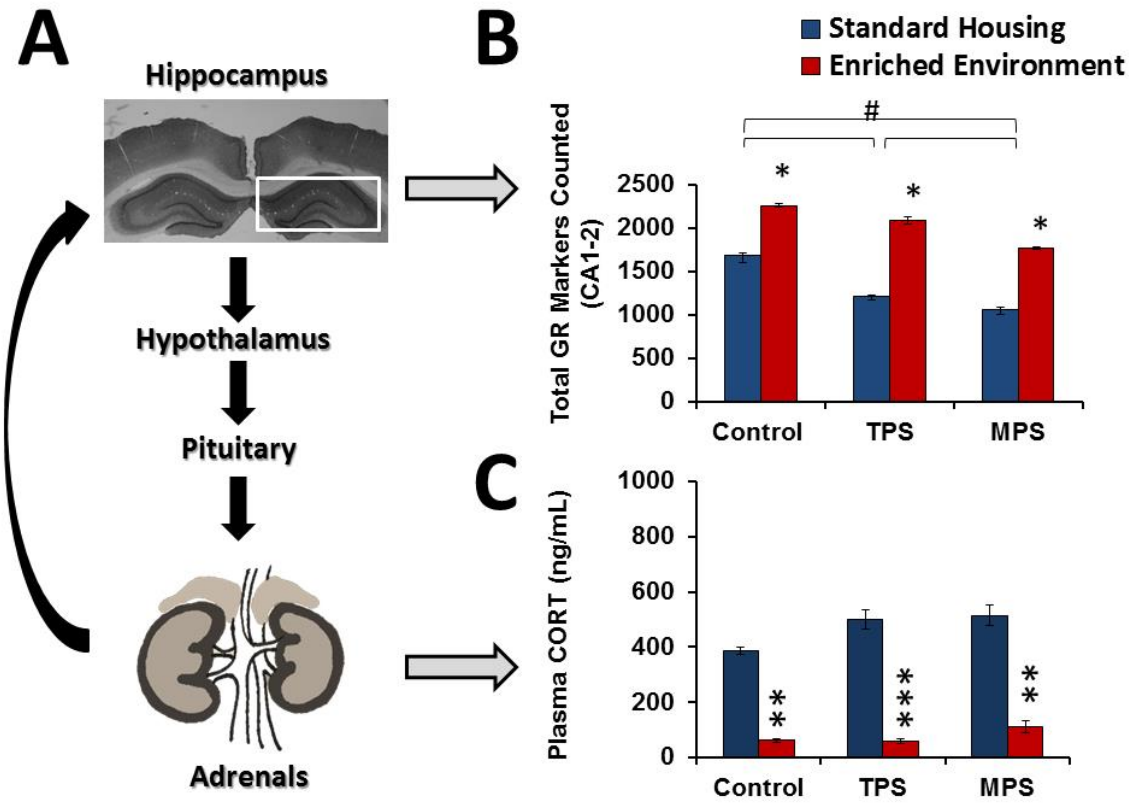

Figure 2.2: Enrichment therapy improves dysregulated hypothalamic-pituitary-adrenal axis activity. A) Illustration of critical components of the HPA axis assessed in this study. (B) Both transgenerational (TPS) and multigenerational (MPS) stress reduced glucocorticoid receptor density in the hippocampus. Exposure to enriched environment increased glucocorticoid receptor density. (C) Circulating plasma corticosterone levels were reduced by enriched environment across all groups. Enrichment therapy thus improved HPA axis feedback regulation. Asterisks denote significances due to EE (*$p<0.05$, **$p<0.01$,***$p<0.001$); # denotes significant difference due to ancestral stress ($^{\#}p<0.05$).

2.4.2 Ancestral stress alters prefrontal cortex neuroanatomy and cell density

2.4.2.1 Mean Gray Value

A summary of stress and enrichment-associated changes in neural density in the PFC is shown in Figure 2.4C. The density of the ROI drawn on the coronal sections corresponding to the caudal PFC (Figure 2.4A, right) was significantly reduced in the TPS and MPS animals compared to controls ($p<0.001$). There was a significant main effect of stress on mean gray values ($F(2,106)=37.456$, $p<0.0001$) where stressed animals had lower values, but there was no effect of enrichment.

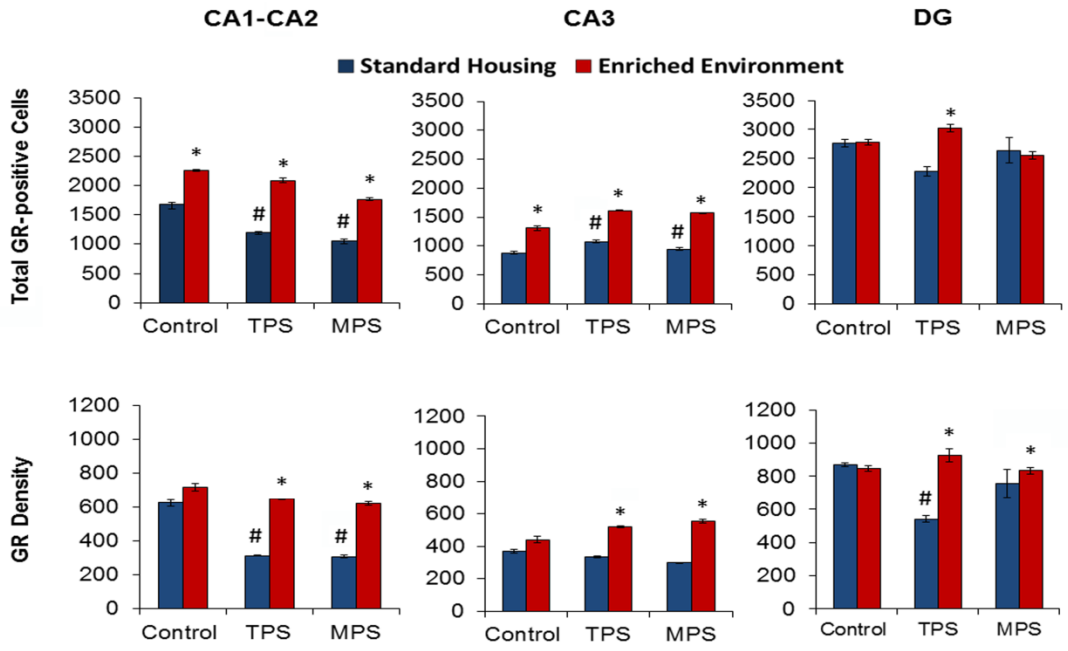

Figure 2.3: Enrichment therapy improves stress-programmed glucocorticoid receptor density. Stereological analysis of total GR-positive cells counted and GR density in hippocampal CA1-CA2, CA3 and dentate gyrus areas in control, transgenerationally (TPS) and multigenerationally (MPS) stressed rats revealed that ancestral stress reduced GR density, especially in the CA1-CA2 regions. In the CA3, TPS and MPS had significantly more GR markers compared to controls. Enrichment increased total GR markers counted and GR density in most cases. Asterisks denote significances due to EE (*$p<0.05$, **$p<0.01$), # denotes significant difference due to stress ($^{\#}p<0.05$).

2.4.2.2 Cortical Thickness

Figure 2.4B summarizes the stress- and enrichment-associated changes in medial-lateral cortical thickness in the PFC. There was a significant effect of stress on dorsoventral thickness ($F(2,103)=8.701$, $p<0.001$), indicating that stressed animals had diminished cortical thickness. There also was a significant effect of enrichment on dorsoventral thickness ($F(1,103)=6.113$, $p<0.05$). There was no significant effect of stress or enrichment on mediolateral thickness.

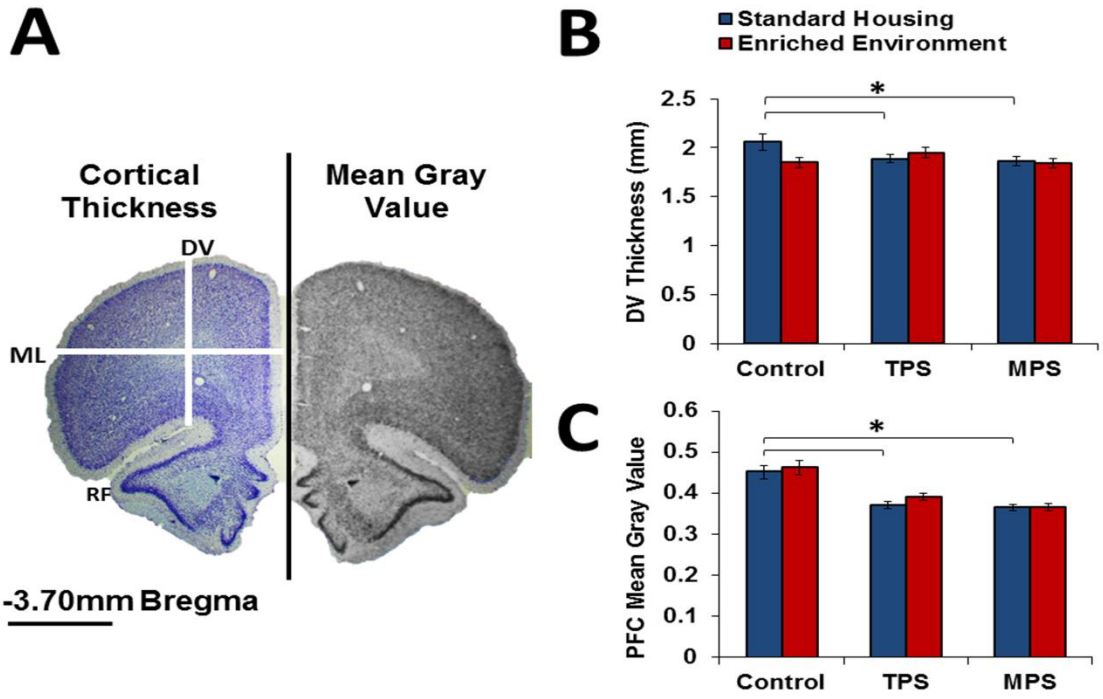

Figure 2.4: Ancestral stress reduced prefrontal cortex thickness. A) Image of a representative cresyl violet-stained coronal brain section corresponding to 3.70 mm relative to bregma illustrating the measurements of cortical thickness (DV: Dorsoventral, ML: Mediolateral, RF: Rhinal fissure, scale bar represents 2 mm). The right portion shows the gray scale 8-bit image used for mean gray value analysis. B) DV thickness and C) mean gray value were significantly decreased due to transgenerational and multigenerational stress, with no significant effect of enrichment therapy. PFC, prefrontal cortex. Asterisks denote significances (*$p<0.05$).

2.4.3 Ancestral stress leads to precocious hyperactivity and risk assessment behaviour which is mitigated by EE

2.4.3.1 Open Field

During development (P35 - P90), a statistically significant main effect of Enrichment was found in all variables, indicating that enriched housing rats displayed decreased Movement Time (Figure 2.5A; $F(1,42)=19.45$, $p<0.001$), Vertical Time ($F(1,42)=18.45$, $p<0.001$) and increased Margin Time ($F(1,42)=15.25$, $p<0.001$). The statistically significant effect of Age reflects an age-related increase in Movement Time ($F(2,84)=15.28$, $p<0.001$) and Vertical Time ($F(2,84)=48.22$, $p<0.001$), and an age-related decrease in Margin Time ($F(2,84)=48.06$, $p<0.001$). The statistically significant interaction between Age*Environment indicates that enriched housing rats showed a greater age-related increase in Movement Time ($F(2,84)=8.11$, $p<0.001$) and Vertical Time ($F(2,84)=9.70$, $p<0.001$), as well as a greater age-related decrease in Margin Time ($F(2,84)=10.68$, $p<0.001$). Additional post-hoc comparisons revealed that enriched unstressed and MPS rats showed decreased activity in all behavioural measurements at the age of 60 and 100 days.

2.4.3.2 Elevated Plus Maze

During development (P35-P90), the time spent in the open arms revealed a significant main effect of Age, with older rats spending more time in the open arms than younger rats ($F(1.49,62.58)=4.60$, $p<0.05$). Additionally, a significant main effect of Enrichment in the number of closed arm entries indicates that enriched housing rats made fewer entries into the closed arms than standard housing rats ($F(1,42)=7.12$, $p<0.05$). Further pairwise comparisons revealed that, at P35, enrichment resulted in an increase in time spent in open arms in MPS rats ($p<0.05$); at P60 enrichment resulted in an increase

in time spent in the open arms among TPS and MPS rats ($p<0.01$); and, at P100, enriched TPS and MPS rats spent more time in the open arms ($p<0.01$). Risk assessment behaviour also showed a significant main effect of age, (Figure 2.5B; $F(1,42)=19.182$, $p<0.0001$) with older rats having more risk assessment behaviours. Further pairwise comparisons indicate that stressed animals had more risk assessment behaviours at P60 ($p<0.05$) compared to non-stressed. Pairwise comparisons also revealed that enrichment in the TPS (P60) and MPS (P100) groups showed significantly reduced risk assessment behaviours compared to the standard housing rats ($p<0.05$).

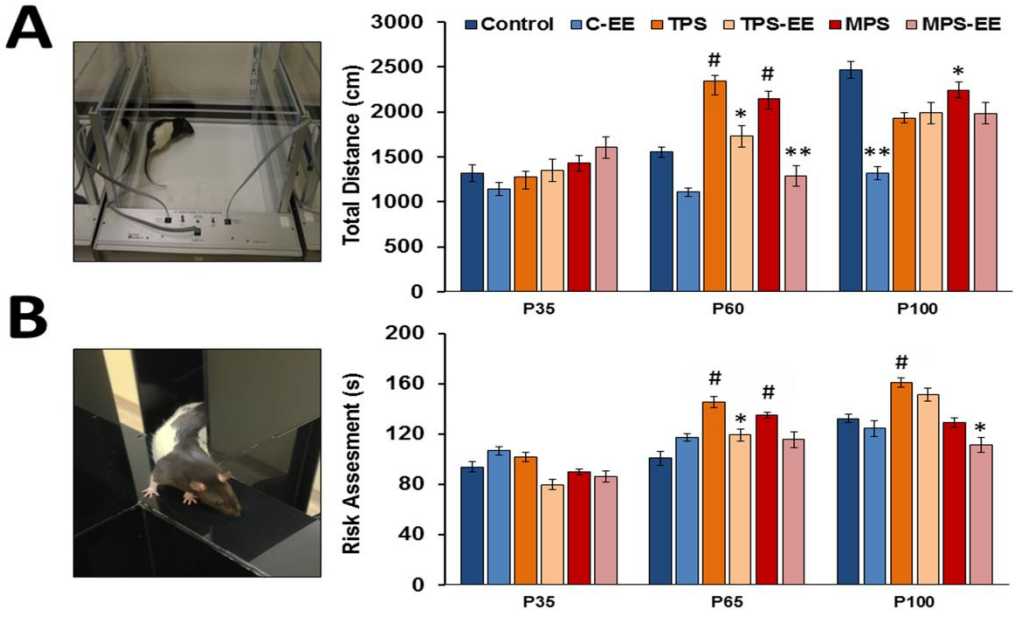

Figure 2.5: Enrichment therapy mitigates precocious anxiety-like behaviours induced by ancestral stress. A) Photograph of a rat in the automated open field and total distance travelled measured at P35, P60 and P100 showing an increase in movement time due to ancestral stress and a decrease due to enrichment. B) Photograph of a rat in stretch-extend posture in the elevated plus maze observed to measure the time spent risk assessing, showing an increase in the time spent risk assessing due to ancestral stress, and a decrease due to enrichment. Note that transgenerational and multigenerational stress generated precocious anxiety-like behavioural traits, and enrichment therapy normalized the developmental trajectory of these behaviours. Asterisks denote significances due to EE (*$p<0.05$, **$p<0.01$), # denotes significant difference due to stress ($^{\#}p<0.05$).

2.4.4 Ancestral stress-induced changes in miRNA expression are reversed by EE

Deep sequencing revealed that 59 miRNAs (TPS: 30, MPS: 29) were differentially expressed in response to ancestral stress, and 29 miRNAs were differentially expressed in response to enriched environment (TPS-EE: 7, MPS-EE: 22). After adjusting p-values using the Benjamini and Hochberg correction (Benjamini et al., 2001), miRNAs of interest that were significantly differently expressed included miR-182, miR-10a-5p and miR-124-3p (FDR $p<0.05$). Other miRNAs of interest that approached significance included miR-3553, miR-24-3p, miR-219a-5p, miR-411-5p (upregulated due to stress) and miR-3577 (downregulated due to stress).

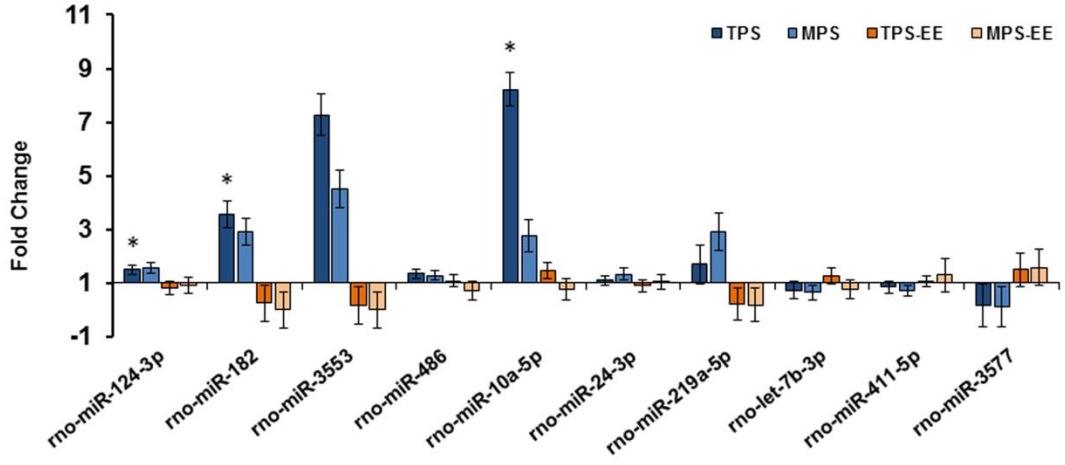

Figure 2.6: Trans- and multigenerational stress program miRNA expression profiles related to psychopathologies, which are reversed by enrichment therapy. Fold changes of the miRNAs of interest in TPS and MPS rats in reference to controls. Note that ancestral stress led to up- or down-regulation of miRNA expression, respectively, and enrichment returned these changes to normal levels. Asterisks denote significances (FDR adjusted; *$p<0.05$).

Importantly, exposure to an enriched environment reversed the effect of ancestral stress of all miRNA changes by either upregulating or downregulating their expression, respectively. Fold changes of the miRNAs of interest differentially expressed in the animals housed in the enriched environment compared to standard housing controls are summarized in Figure 2.6. Target prediction and hypergeometric test found pertinent biochemical pathways for miR-182 including neurotrophin signaling (Figure 2.7) and axon guidance (Figure S1). These data show that miR-182 directly regulates BDNF and neurotrophin-3 (NT-3) expression and is involved in axonal guidance mechanisms particularly involving netrin, semaphorin, and ephrin (Figure S1). The main small RNAs that were differentially regulated by EE and stress are summarized in Table S1.

2.5 Discussion

Ancestral exposure to a stressful environment contributes to altered stress response and raises the risk of neuropsychiatric disease. Our results show that experience of stress during pregnancy in the great-grandmother leads to higher stress sensitivity, i.e., by reduced negative feedback capacity of the HPA axis, and cortical atrophy in association with an altered response to novel and aversive environments. In particular, ancestral stress in the F3 generation led to precocious onset of motor hyperactivity and risk assessment behaviours in response to a novel environment. A similar phenotype resulted from exposure to multigenerational stress. Therapeutic exposure to an environmental enrichment drastically mitigated the stress sensitive phenotype induced by ancestral stress and improved HPA axis function across all groups. The results also provide evidence that transgenerational inheritance of altered stress response was linked to miRNAs,

particularly those involved in brain development and affective state. EE reversed the programming of miRNA signatures that was induced by ancestral stress.

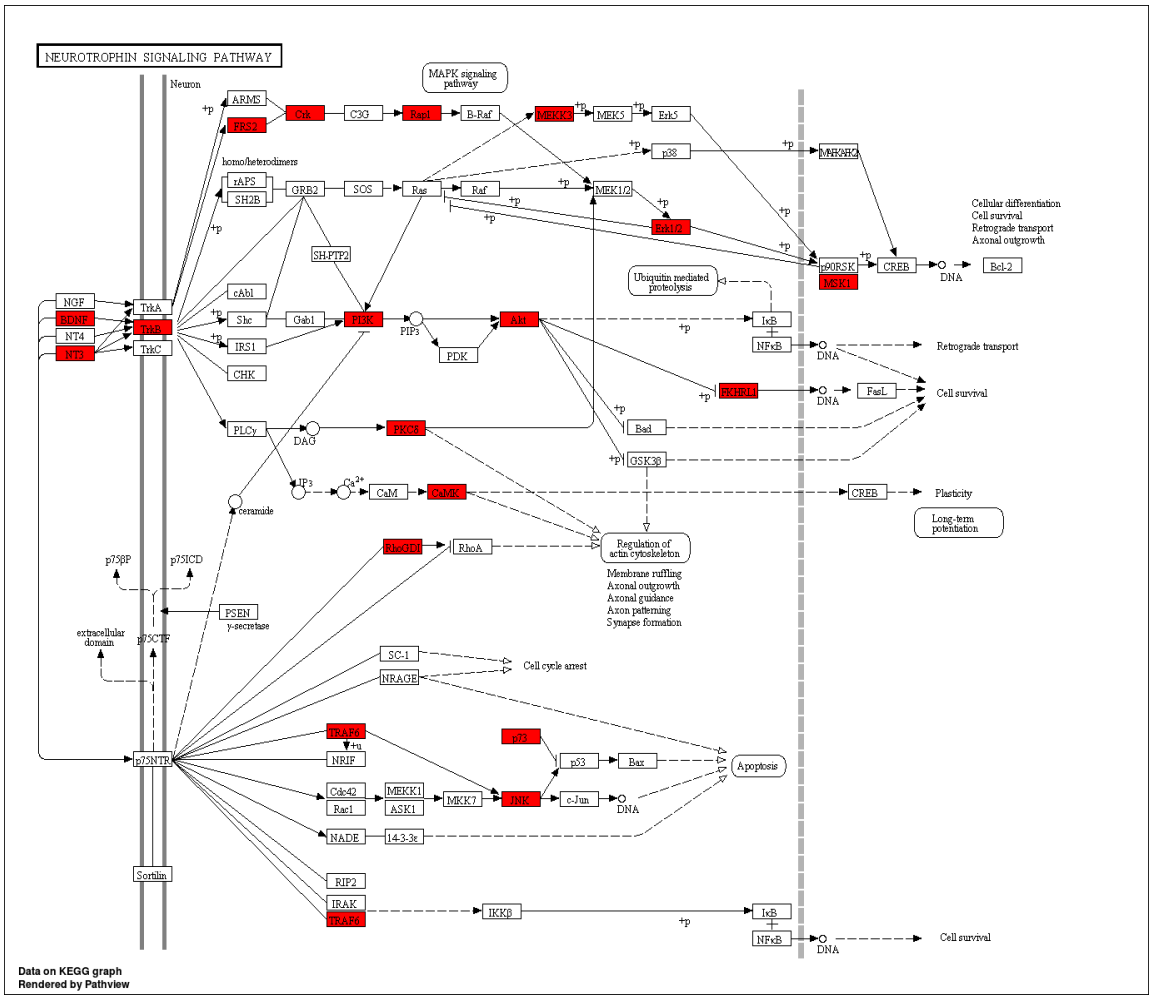

Figure 2.7: Neurotrophin signaling pathway as target for environmentally-regulated miR-182 expression. Predicted direct targets of mir-182 within major neurotrophin pathways are shown in red. The 3'UTRs of BDNF and NT-3 genes show up as high scoring targets in miR-182 binding prediction. The pathway suggests a mechanistic link between miRNA-mediated post-transcriptional regulation of BDNF and NT-3 and EE-associated behavioural and endocrine improvements.

Rats are highly social animals. Thus, the present study exposed pregnant rat dams to social isolation during pregnancy to induce psychological stress in a model of particular ecological validity for humans (Hawkley et al., 2012). The severity of the stress was

comparable to an established semi-random stress procedure used in our earlier transgenerational studies (Erickson et al., 2014, Zucchi et al., 2014).

Transgenerational programming by stress induce lasting modifications at multiple levels of HPA axis function. TPS and MPS both reduced the hippocampal density of GR, which resembles changes commonly seen in the prenatally stressed brain (Henry et al., 1994, Maccari et al., 1995, Harris and Seckl, 2011), specifically in the CA1-CA2 and DG which are known to have particularly high GR density (Van Eekelen et al., 1988). The resulting impairment in negative feedback regulation of the HPA axis poses a risk for greater stress sensitivity (McEwen et al., 1986). Thus, a decrease in GR density may be accompanied by increased CORT levels.

Negative feedback regulation is also mediated by the PFC, which was found to be of reduced thickness in TPS and MPS animals. Neuromorphology in the PFC is susceptible to prenatal stress (Mychasiuk et al., 2012), thus providing a mechanism by which ancestral stress can modulate higher-order executive functions, sensory perception and social reasoning (Arnsten, 2009). Moreover, studies investigating the relationship between PFC regulation of HPA axis function have concluded that the PFC is part of a regulatory circuitry involved in the stress response (Kern et al., 2008). Reduced cortical thickness and diminished neural density, as induced by TPS and MPS, may therefore indicate weakened inhibitory regulation of HPA axis activity (Diorio et al., 1993, Sullivan and Gratton, 2002) and affective state (Wagner et al., 2012). Effective inhibition of the HPA axis is essential for stress resilience and cognitive aspects of stress coping, and disturbances in PFC morphology may in part explain the present HPA dysfunction (Sullivan and Gratton, 2002). Since many neuropsychiatric diseases, such as anxiety and depressive disorders, share an association with both HPA axis dysregulation and PFC

abnormalities (for review see (Sullivan and Gratton, 2002)), current findings suggest that transgenerational programming by stress may present a causal mechanism for their clinical symptoms.

Programming of the developing brain and HPA axis by prenatal stress has consequences that extend into adulthood, which may result in changes in motor activity and affective behaviour (Holmes et al., 2006, Lupien et al., 2009, Harris and Seckl, 2011). In the present study, animals were assessed at the age of 30, 45, 60 and 90 days, thus covering the main stages of adolescent development. The longitudinal findings revealed that ancestral stress favors precocious onset of elevated motor activity and risk assessment behaviours in response to the novel and slightly aversive environments of open field and elevated plus maze. Both tests are standard assessment tools in the assessment of anxiety-like behaviour and response to novelty (Smith et al., 2008, Jadavji et al., 2011). The indication that ancestral stress (both TPS and MPS) enhances the vulnerability to hyperactive and anxiety-like behaviours agrees with findings made in prenatally stressed animals (Li et al., 2007, Metz, 2007, Field et al., 2008, Gatzke-Kopp, 2011). Our results also validate those of other studies that found precocious development of fear-related behaviours in rat pups after maternal stress (Zuluaga et al., 2014) and CORT exposure (Moriceau et al., 2004). A possible mechanism is that stress-induced hormonal imbalance prior to pubescence may accelerate testicular development, thus increasing the brain's exposure to androgens during development followed by decreased testosterone during adulthood (Pallares et al., 2013). The present findings concur with observations from the 'Global Burden of Disease Study' in 2010 which showed that behavioural disorders in boys become symptomatic at young ages (Whiteford et al., 2013).

The formation of stress-induced precocious anxiety-related behavioural traits in the present study was reversed by exposure to EE during adolescence. EE in ancestrally stressed rats reduced motor hyperactivity and anxiety-like behaviours along with lowering HPA axis activity across all groups. Reduced HPA axis activity may directly translate to reduced anxiety-like behaviour (Belz et al., 2003). The present findings strongly suggest that multiple levels of HPA axis activity, and therefore lifelong stress sensitivity, are programmed by ancestral stress. These consequences of adverse HPA axis programming by maternal stress can be mitigated by early beneficial experiences in the filial generations. Although the present study used EE exposure during adolescence, data suggest that EE at any time in life is beneficial to promote brain health and behaviour. In animals, EE is effective to promote skilled motor function (Jadavji et al., 2006, Jadavji and Metz, 2009, Knieling et al., 2009), learning and memory (Falkenberg et al., 1992, Gibb et al., 2014) and affective behaviours (Galani et al., 2007). In humans, enriched early childhood experiences have been linked to improved motor and cognitive function during old age (Metzler et al., 2013), risk of inattentive and hyperactive/impulsive behaviours (Forns et al., 2012) and lowered risk of anger and fear (Sharp et al., 2012) in later life. The latter findings suggest a role for HPA axis function in mediating beneficial effects of EE. In addition, EE was shown to decrease CORT production, further supporting the claim that EE reduces stress-associated anxiety-related behaviour (Galani et al., 2007). The present study is the first to suggest that EE is able to reduce the impact of adverse transgenerational experiences with drastic benefits for HPA axis function and affective state.

The mechanisms through which ancestral stress determines HPA axis function and forms new behavioural traits involves a complex interaction between genes and

environment (Meaney, 2010, Dunn et al., 2011a). A central component of transgenerational inheritance is the transmission of epigenetic marks across generations, including changes in DNA methylation (Meaney and Szyf, 2005, Franklin et al., 2010) and differential expression of miRNAs (Gapp et al., 2014, Yao et al., 2014). MiRNAs are post-transcriptional regulators of gene expression with particular implications as biomarkers of mental health (Metz et al., 2015a). Indeed, miRNAs that were differentially expressed in the PFC of stressed rats are recognized biomarkers of mental health, including miR-10a-5p for depression (Wan et al., 2015), miR-219a-5p for schizophrenia (Kocerha et al., 2009) and miR-182 for fear memory, depression and synaptic plasticity (Griggs et al., 2013). The present data on miR-182 suggest that an underpinning mechanism is the regulation of axonal pathfinding and synaptic maintenance during development and maturation through altered expression of guidance and neurotrophic molecules.

Recent findings have highlighted a central role of miR-182 in amygdala-dependent memory formation by targeting key actin-regulating proteins during structural plasticity (Griggs et al., 2013). The latter study linked miR-182 upregulation to disruption of long-term fear memory (Griggs et al., 2013). Importantly, miR-182 overexpression was associated with depression-like behaviours and decreased BDNF expression in the hippocampus of stressed rats, and miR-182 silencing led to anti-depressant-like effects (Li et al., 2015). As shown by this study and others, miR-182 and miR-10a-5p directly target BDNF expression (Li et al., 2013, Prins et al., 2014), thus proposing a key mechanism for stress to induce affective behaviour changes. Notably, EE was most effective in multigenerationally stressed animals, as MPS rats showed three times the number of miRNAs differentially expressed compared to TPS, which possibly indicates

that the chronic nature of MPS causes more miRNA activation in order to alleviate the stress effects. The miRNA downregulation by EE suggests that improved HPA axis regulation, through miRNA regulation, restores cortical and hippocampal BDNF levels to support neuronal maintenance and neuroplasticity during brain maturation. Indeed, EE has been shown to influence behaviour and brain plasticity mainly through the action of BDNF (Zhu et al., 2006, Cao et al., 2014a). The present findings suggest a mechanistic link between enriched environmental conditions, improved behavioural and brain development involving miRNA regulation of gene expression.

Further studies have also associated miR-182 with the regulation of NT-3 expression. In particular, enhanced neuroplasticity and improved learning and problem-solving ability in EE-exposed rats was linked to higher NT-3 mRNA levels in the cerebral cortex (Ickes et al., 2000, Hu et al., 2013). However, compared to a large body of research focusing on EE-induced changes in BDNF expression, the regulation of NT-3 received less attention. As shown in Figure 2.7, however, NT-3 and its potent effects on neuronal survival, differentiation, neurogenesis and synapse formation through interaction with the trkC and trkB receptors (Gomez-Palacio-Schjetnan and Escobar, 2013) is a likely candidate or EE-mediated neuroplasticity and behavioural change.

2.6 Conclusions

By investigating the role of ancestral stress in HPA axis regulation, the present study is the first to develop an intervention strategy to mitigate transgenerationally programmed stress sensitivity. We showed that an ancestral history of stress leads to precocious developmental trajectories of anxiety-like behaviour during adolescence along with elevated HPA axis activity via miRNA-regulated pathways. We show that EE serves

as an effective therapy not only for phenotypic changes of affective state and potential mental health risks, but also for re-programming pathogenic pathways leading to these conditions.

The present data highlight that investigation of ancestral stress is critical to the understanding of disease manifestations later in life, and prevalence of disease which arises without genetic determinism. Environmental interventions and enriched life style may provide an effective means by which the consequences of adverse ancestral experiences can be mitigated at any time in life (Zucchi et al., 2014). It is reasonable to assume that an enriched early childhood environment or life style improvements at an older age may benefit families at risk for psychopathological disorders such as attention deficit hyperactivity disorder, schizophrenia, anxiety, and depression. In addition, epigenetic regulation through miRNA pathways provides a possible biochemical basis for the large behavioral and morphological alterations generated by EE exposure during adolescence and in later life. The identification of miRNAs that mediate the actions of EE may allow the development of predictive biomarkers of disease and therapeutic targets.

Chapter 3

Experiment 2: The Effects of Ancestral Stress and Enrichment on Corticospinal Tract Density, Pyramidal Morphology and Motor Performance in Rats

3.1 Abstract

An adverse fetal environment *in utero* has been associated with long-term alterations in brain structure and function, and a higher risk of neurological disorders in later life. In this study we investigated the effect of ancestral stress on neuronal morphology, corticospinal tract (CST) density as well as motor behaviour. Our hypothesis was that ancestral stress would cause a decrease in axonal density in the CST along with impaired dendritic complexity, which may lead to compromised motor performance. Animals were trained and tested in a skilled walking task and were then injected with a neural tract tracer, biotinylated dextran amine (BDA), into the motor cortex for anterograde tracing of the CST. Brains and spinal cords were extracted for tissue processing two weeks after BDA injection. The results revealed an overall decrease in axonal density in the CST and dendritic complexity in the parietal cortex. Furthermore, ancestral stress was associated with significant deficits in skilled walking, as indicated by reduced foot placement accuracy and inter-limb coordination. Therapeutic intervention by exposure to environmental enrichment was able to reverse the major neuromorphological consequences associated with ancestral stress and restored skilled walking ability. Thus, ancestral stress represents a significant impact on motor system development and movement ability, while intervention by enriched environment can be applied as a rehabilitation strategy to reduce the uncontrollable impact of ancestral stress and promote fine motor control.

3.2 Introduction

Central nervous system (CNS) development is a dynamic and complex process that begins *in utero* and continues prominently throughout infancy, adolescence, and even adulthood. The pre- and postnatal environment represents a significant impact to modify brain development and maturation (Lenroot and Giedd, 2008). Notably, environmental factors can both positively and negatively impact both brain structure and function through altering neuronal plasticity throughout our life (Kolb et al., 1998). Consequently, a hostile condition *in utero*, such as prenatal stress (PS), has been associated with long-term alterations in neuronal structure and function and has been linked to susceptibility to neurological diseases in later life (Monk et al., 2012, Metz et al., 2015a). An ideal system to study the impact of stress is the motor system and associated pathways, since motor outputs are unambiguously testable and quantifiable (Metz et al., 2005, Jadavji et al., 2011). The motor system includes the primary motor cortex and its main efferent projection, the corticospinal tract (CST). The corticospinal tract is the major descending white matter pathway controlling voluntary movement and is critically involved in skilled reaching and skilled walking (Metz and Whishaw, 2002, Farr et al., 2006, Hurd et al., 2013). Little information is available however, that investigates the influence of stress on CST development and its function.

A few studies have found that an adverse environment *in utero* and in early life influences CST development and associated motor function. For example, preterm birth and low birth weight, both associated with PS (Vidal et al., 2014) and ancestral stress (Yao et al., 2014), may determine the developmental trajectory of the CST. Specifically, illness severity of the very preterm neonate along with pain-related stress exposure has been linked to slower microstructural development of the CST (Ranger et al., 2013,

Zwicker et al., 2013). Moreover, prenatal and postnatal malnutrition in the rat has been associated with neuronal loss, reduced brain weight, and a reduction of the conduction velocity along the corticospinal tract (Sima and Sourander, 1976, 1978, Quirk et al., 1995).

PS has been shown to modulate neuronal development in the frontal cortex (Shaw et al., 2008) and thus it may also potentially affect the motor cortex and subsequently alter motor control (Zucchi et al., 2014). In addition to the multitude of deficits accompanied with PS, there is emerging evidence that an adverse intrauterine environment or maternal stress during pregnancy impairs neuromotor behavioural development in rats and non-human primates (Spinillo et al., 1993, Patin et al., 2004, Grace et al., 2015). PS induced motor deficits include disabilities in fine motor skills (Ulupinar et al., 2015), reflexes (Patin et al., 2004) along with coordination and balance (Cao et al., 2014c).

It seems reasonable to expect that remote ancestral stress may influence the corticospinal tract based on the prominent effects on motor development that were reported for PS. Previous studies have found that ancestral stress is passed on from the maternal lineage to subsequent generations through programming of the hypothalamic-pituitary adrenal axis (HPA) and through epigenetic mechanisms (e.g. Chapter 2 and McCreary et al., 2015). Moreover, a recent transgenerational PS study (across four generations) found that PS compromised skilled movement in males but not females in both the skilled reaching and skilled walking task (Ambeskovic, 2013, Thesis). Defects in the CST and associated systems may be one mechanism to explain some of these impairments. Abnormalities of the CST have been linked to motor impairments (Thallmair et al., 1998), and gross and skilled movement impairments have been

associated with PS (Patin et al., 2004, Cao et al., 2014c). Thus, ancestral stress may, at least in part, alter motor control through influencing neuronal development and plasticity within the CST and motor cortex pathway.

Despite the fact that investigating the CST is one of the keys to appreciating the extent of the effects of PS, there has been no study of ancestral programming of motor function. Our previous experiment (Chapter 2) showed that transgenerational and multigenerational stress in the F3 generation causes a decrease in neuronal density and prefrontal cortical thickness. These data suggest that ancestral stress may also affect associated areas including the CST. Therefore, a history of ancestral stress may represent a powerful determinant of neuronal morphology, CST fiber density and ultimately motor performance. Moreover, we hypothesized that environmental enrichment, which has been shown to reverse neuromorphological deficits due to stress, will aid to reduce deficits caused be ancestral stress.

3.3 Materials and Methods

3.3.1 Animals and Experimental Design

For a description of animals and experimental design, see the *Experimental Design* section in Chapter 2.

3.3.2 Skilled Walking Task

3.3.2.1 Training and Testing

Two weeks prior to BDA injection, animals were pretrained and tested in the ladder rung walking task (Metz and Whishaw, 2002, 2009). The ladder rung walking task

assesses both fore- and hind limb coordination and limb placement (Metz and Whishaw, 2002). Rats were trained to cross a 1-m long horizontal ladder with metal rungs arranged at random distances, ranging from 1 to 5 cm. In each test session, the rung pattern was conserved for consistency. Rats were trained to cross the ladder and in each test session, rats were videotaped for further analysis.

3.3.2.2 Skilled Walking Task Scoring

The skilled walking task rating system used was developed by Metz and Whishaw (2002) for qualitative analysis. Briefly, the rating system was based on seven categories of scores from 0-6 for limb placement on the rungs: (0) Total Miss: The limb misses the rung, interrupting walking pattern (fall) (1) Deep Slip: The limb is placed on the rung, but then slips off, interrupting walking pattern (fall) (2) Slight Slip: The limb is placed on the rung but then slips off, walking is uninterrupted (slight fall). (3) Replacement: The limb is placed on one rung, but rapidly is lifted and placed on another rung. (4) Correction: The limb is aimed at one rung, but prior to placement, is placed onto a different rung; alternatively, the limb was placed on a rung and then readjusted on that rung. (5) Partial placement: The limb is placed on the rung, with the wrist/digits of the forelimb, or heel/toes of the hind limb. (6) Correct placement: The middle portion of the limb is placed onto the rung, in one accurate motion. Foot fault scores were averaged across three five trials, and time needed to cross the length of the ladder was measured. The number of errors was recorded by calculating the mean number of errors (Score of 0,1 or 2) per step, which was averaged over five trials.

3.3.2.3 Video recording

Skilled walking was recorded manually by using a Canon ZR50 MD camcorder set at a shutter speed of 1/500 s. During filming, additional light was supplied (Lowel-

light Mtg Inc, New York, USA). Frame-by-frame analysis and scoring by a blind investigator was performed using a Sony GV-D1000 NTSC miniDV player.

3.3.3 Anterograde Tract Tracing Using BDA

BDA is suitable for anterograde and retrograde neuronal pathway tracing. High molecular weight BDA (10 k) yields sensitive and detailed labeling of axons and terminals, while low molecular weight BDA (3 k) yields sensitive and detailed retrograde labeling of neuronal cell bodies (Reiner et al., 2000). BDA can be visualized at the light microscopy or electron microscopy level and it can be combined with various other pathway tracing or immunohistochemical methods (Reiner et al, 2000). An example of the pathway traced in this study is illustrated in Figure 3.1. In this study, BDA was injected in to the motor cortex, which travelled through the internal capsule and midbrain, decussated in the medulla and traced the CST in the spinal cord.

3.3.4 Surgical Procedure for BDA Anterograde CST Tracing

Two weeks prior to euthanization, rats underwent anterograde tract tracing of the CST with BDA (n =5 rats/group, total of 30 animals). Under isoflurane anesthesia, rats were positioned in a stereotaxic frame. Using a Hamilton syringe, 1 ml of BDA (10%, 10,000 MW; Invitrogen, Eugene, OR) was slowly (over 5 minutes) injected bilaterally into the forelimb areas of the motor cortex using the following coordinates based on Paxinos and Watson (1998): (1) 1 mm anterior and 2 mm lateral; (2) 1 mm anterior and -2 mm lateral (in reference to Bregma). Injections were made 1.5 mm from the surface of the cortex. The syringe remained in place for another 3 min following the injection. The

area of incision was sutured, and the animal was left to recover overnight on a heating pad and monitored until recovery.

Animals were euthanized as described below for tissue collection two weeks after the BDA injection. Previous protocols have indicated that the two-week interval between BDA labeling and animal euthanization is sufficient for anterograde labelling of the CST in cervical (C) and thoracic (T) regions (Veenman et al., 1992, Reiner et al., 2000).

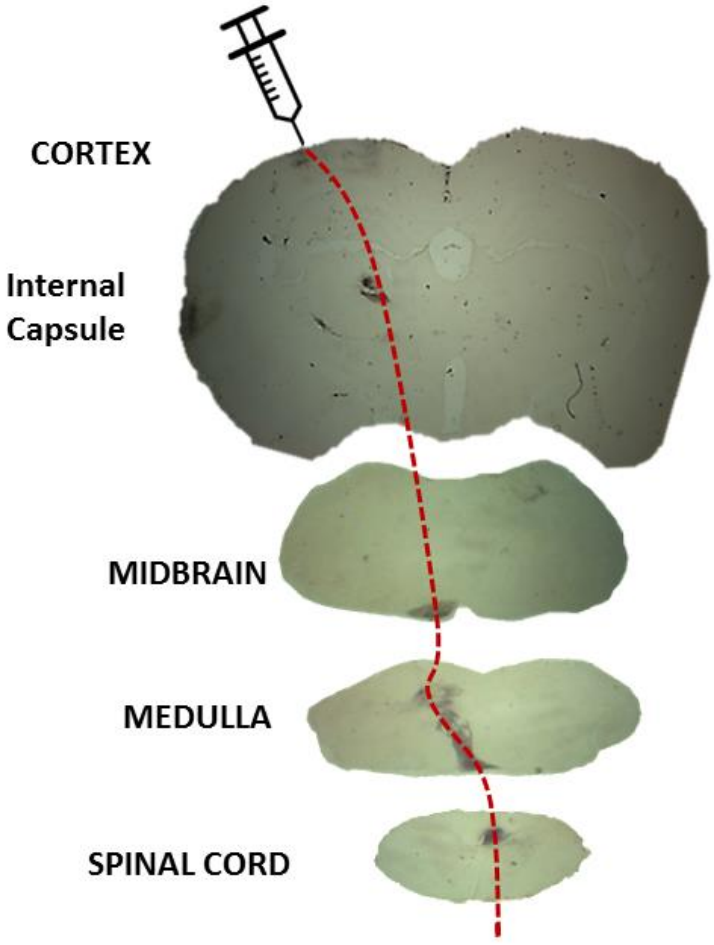

Figure 3.1. Illustration of anterograde pathway tracing (red) of the corticospinal tract using injection of BDA into the motor cortex.

3.3.5 Tissue Processing

Two weeks following the BDA injections rats were euthanized with an overdose of Euthansol® (Merck, QC, Canada) and perfused transcardially with phosphate buffer solution (PBS; approximately 200 ml) followed by a transcardial injection of approximately 200 ml of 4% Paraformaldehyde (PFA; Sigma-Aldrich, MO, USA). The spinal cords and brains were removed, post-fixed overnight in 4% PFA and then transferred to a 30% sucrose solution for 3 days. Brains were cut in coronal sections with a microtome at a thickness of 40 µm and 12 series interval. Sections were stored in 0.01 M PBS with a 1:1000 concentration of sodium azide. Every third series of sections was mounted and stained with cresyl violet to detect Nissl bodies. Every 4th series was processed with the ABC-DAB procedure as outlined below.

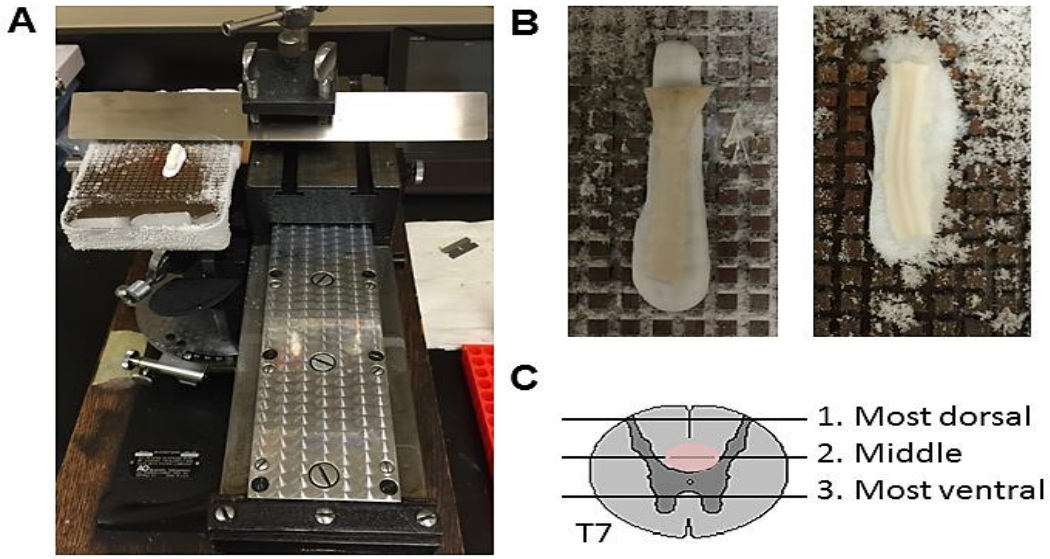

Figure 3.2. A) Microtome used to section the spinal cord horizontally, B) Setup of spinal cord segments unsectioned (left) and sectioned (right) and C) division of sections that were used for analysis (in pink: area of interest, dorsal corticospinal tract).

The spinal cord portion extending from cervical vertebrae C3 to thoracic vertebrae T7 (Figure 3.2B) was partially embedded in Tissue Tek (O.C.T Compound) and cut transversely on the microtome (Figure 3.2A). Transverse sections at 40 µm were chosen to illustrate fiber density along the CST. Slices were separated into three series, representing the most ventral, middle and dorsal portions of the spinal cord (Figure 3.2C). Analysis focused on the dorsal CST as the area of interest. The entire middle portion of the spinal cord was processed with ABC-DAB and 5 slices from both the most ventral and most dorsal portions were also processed. All sections that did not show any BDA visualization were stained with cresyl violet for general anatomy. The slides were captured using a motorized light microscope, Zeiss AxioImager M1 (Zeiss, Jena, Germany).

3.3.5.1 Immunohistochemistry

BDA staining was performed according to procedures previously published (Vavrek et al., 2006). Slides were stained using the free floating technique (Herzog and Brosamle, 1997). The slices were washed 3 times for 10 minutes in tris-buffered saline (TBS), pH 7.4, followed by two 45 min washes with TBS containing 0.5% Triton X-100. Afterwards, the slides were incubated overnight with an avidin–biotin–peroxidase complex (ABC; ABC Elite, Vector Laboratories, Burlingame, CA, USA) in TBS with 30% Triton according to the instructions of the manufacturer. The slices were then washed in TBS 3 times for 10 minutes. Subsequently, the 3.3'-Diaminobenzidine (DAB) reaction was performed using the Vector DAB kit (DAB substrate Kit, catalog no. SK-4100; Vector Labs). The reaction was monitored and stopped by extensive washing in TBS. The slides were dehydrated with alcohol and cleared with HemoDe and then coverslipped in Permount (Fischer Scientific Ltd. Ottawa, ON, Canada).

3.3.6 Histological Analysis

3.3.6.1 Integrated Density. Images were transformed to 8-bit images and adjusted for brightness and contrast (same threshold was applied across all images). The background was subtracted and the image was inverted and threshold was adjusted for optimal visualization of fibers. A square region of interest of the same area in each image was drawn. The integrated density (the average density over the area of interest) from three sections per animal was measured using ImageJ (V1.36, NIH), and an average integrated density was calculated.

3.3.6.2 Analysis of Pyramidal Neuronal Morphology

BDA stains the entire neuron in a similar fashion to Golgi-Cox staining as shown in Supplemental Figure 2 (Gelfo et al., 2009). Therefore, BDA labelled neurons are suitable for a Sholl-type analysis. Neurons were selected if their labeling was uniform and basal dendritic arborizations were intact and visible (Leggio et al., 2005). In parietal cortrex layer III, three neurons were selected from each of the groups. Thus, the present research sampled 18 cells (Table 3.1). Neurons from layer III were selected because previous research has shown that they are particularly responsive to experience, such as stress and enriched environment (Kolb et al., 1998).

For each neuron, the basal dendritic trees were examined using Sholl Analysis (Kolb et al., 1998). This procedure was based on virtually including the cell body in a set of concentric shells at a 10 μm interval. Each dendrite was manually traced (Figure 3.3A), and the image was imported into Image J for Sholl Analysis. Dendritic complexity was analyzed by counting the number of intersections at each 10 μm interval.

Table 3.1. Number of neurons per animal included in Sholl analysis.

Rat	Treatment	Number of Neurons
3	C	1
7	C	1
9	C	1
8	CEE	1
12	CEE	2
19	TPS	1
23	TPS	1
29	TPS	1
36	TPSEE	1
30	TPSEE	2
43	MPS	1
45	MPS	2
46	MPSEE	2
44	MPSEE	1

3.4 Results

3.4.1 Enriched Environment Promotes Pyramidal Dendritic Complexity

Sholl analysis was performed on three neurons per group from the parietal cortex layer III, and data are shown in Figure 3.3B. There was no significant effect of ancestral stress on the number of intersections counted in the Sholl analysis. However, transgenerational prenatal stress (TPS) caused a decrease in dendritic length and arborisation, which approached significance ($F(5,174)=1.922$, $p=0.0929$). There was no significant effect of enrichment on the control or TPS groups. However, a significant effect of enrichment was found in the multigenerational prenatal stress (MPS) group ($p<0.05$) where EE promoted dendritic complexity.

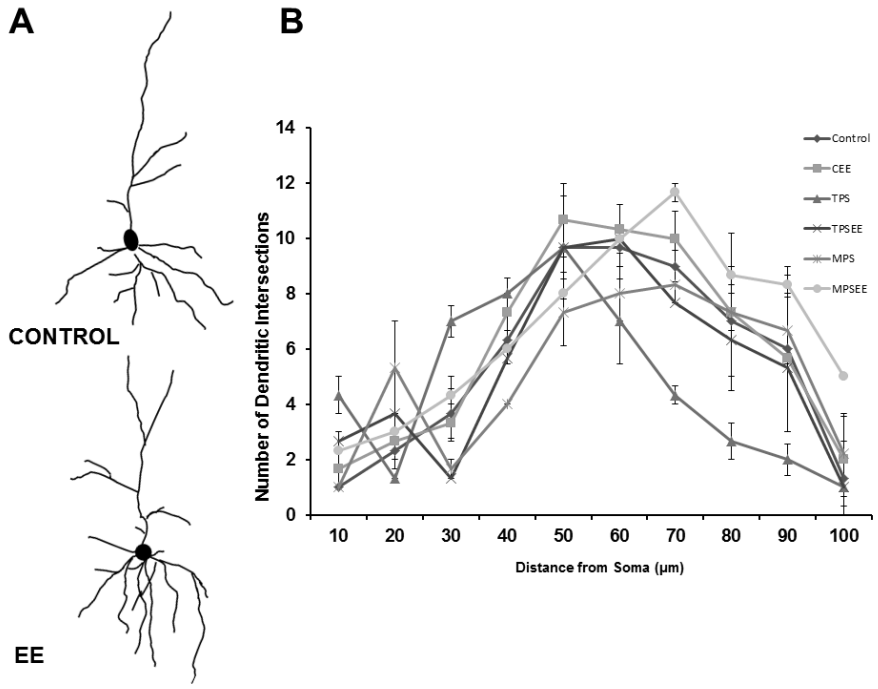

Figure 3.3. A) Illustrations of representative parietal cortex layer III pyramidal neurons from rats reared in standard (top) or enriched (bottom) conditions. B) Summary of the effects of TPS and MPS in standard and enriched conditions on the number of dendritic intersections measured from basal dendrites of rat parietal cortex layer III pyramidal neurons. All values represent the mean ± SEM.

3.4.2 Enichment Restores Reduced Corticospinal Tract Axonal Density Linked to Ancestral Stress

The spinal cord region from vertebrae C3 to T7 was sectioned horizontally and stained with cresyl violet for anatomical comparison, and with DAB for BDA visualization (Figure 3.4). Fiber density across all groups, measured by integrated density, is shown in Figure 3.5. There was a significant effect of treatment on integrated density ($F(5,37)=5.433$, $p<0.001$). Specifically, ancestral stress significantly decreased fiber density in both TPS ($p<0.001$) and MPS ($p<0.05$) groups. There was also a significant effect of enrichment in the MPS group ($p<0.05$), where enrichment increased fiber

density. Enrichment also increased fiber density in the TPS group although this result was not significant.

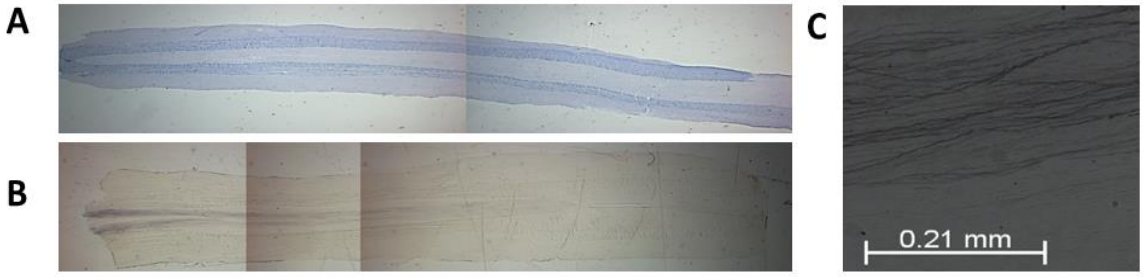

Figure 3.4. A) Cresyl violet stained section used for anatomical comparisons, B) ABC-DAB processed section of BDA tract tracing, and C) 20X magnification of corticospinal tract stained with BDA for integrated density measurement.

3.4.3 Ancestral Stress Modulates Skilled Walking Ability

There were no significant differences in the skilled walking overall score however there were significant differences in the number of errors made. Figure 3.6 shows the number of errors made on each limb in the ancestrally stressed and enriched animals. The error rates in skilled walking revealed an effect of stress ($F(1,42)=2.18$, $P<0.05$) as TPS and MPS rats made on average more left forelimb (LFL) errors (TPS, $p<0.05$). Furthermore, right hind limb (RHL) errors revealed a main effect of EE, ($F(1,42)=9.08$, $p<0.05$), where MPSEE animals showed significantly less errors than MPS rats ($p<0.05$). Enrichment did not show a significant effect on the TPS group.

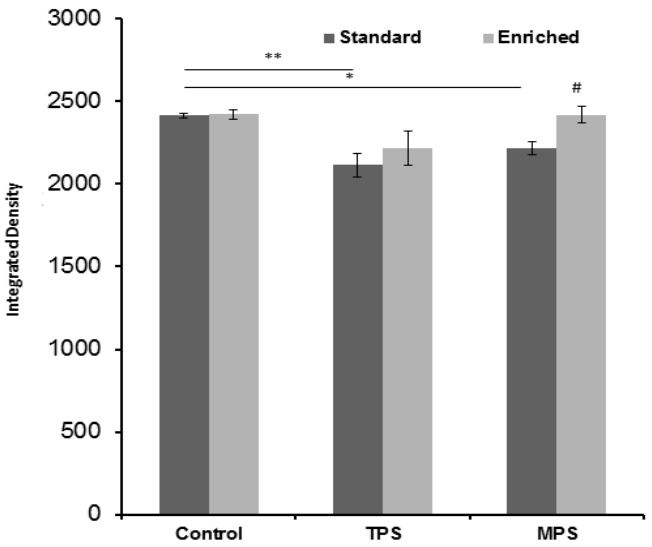

Figure 3.5. Mean integrated density across all treatment groups. Fiber density in TPS and MPS animals was significantly lower than density in controls. EE increased integrated density in MPS. Asterisks indicate significances: effect of stress * $p < 0.05$, ** $p < 0.01$, effect of EE #$p<0.05$. All values represent the mean ± SEM.

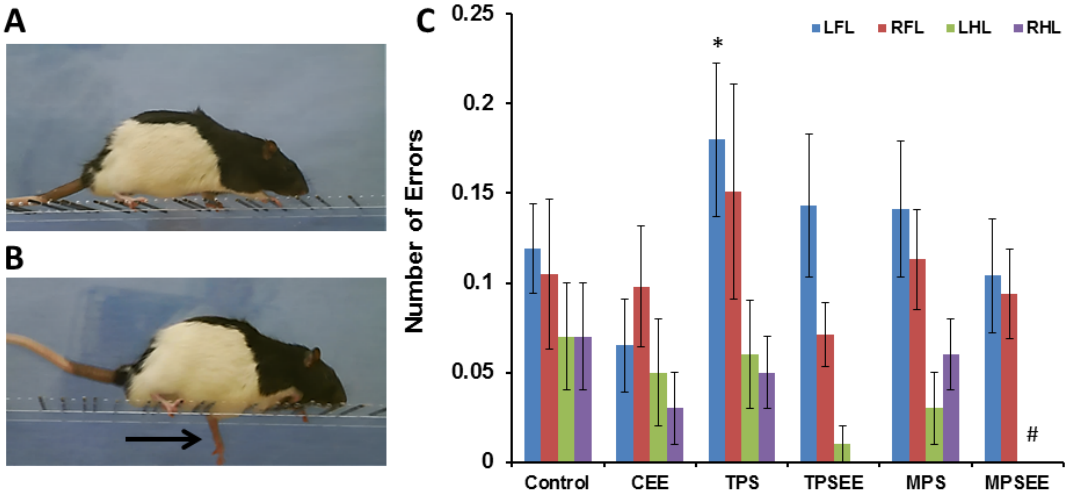

Figure 3.6. Skilled Walking Task. A) Image of a rat crossing the horizontal ladder with normal paw placements, B) animal crossing with an deep limb slip or error depicted by the arrow, C) mean number of errors in each paw measure from the number of errors divided by the number of steps. Asterisks indicate significances: effect of stress * $p < 0.05$, effect of EE #$p<0.05$. All values represent the mean ± SEM.

3.5 Discussion

The present study confirms the hypothesis that ancestral stress affects skilled motor function in association with altered neuromorphology of the CST tract and pyramidal neurons. There were three main findings from this study. First, ancestral stress led to motor deficits as reflected by an increased number of errors in a skilled walking task. This deficit was improved with enrichment therapy. Second, ancestral stress decreased dendritic length and neuronal complexity in the parietal cortex, and enrichment therapy reversed these effects in the MPS group. Lastly, and possibly most intriguing, ancestral stress significantly decreased fiber density in the thoracic region of the CST, which was ameliorated with enrichment therapy.

The present study was the first to investigate the influence of PS in ancestors on motor system function in the F3 generation. Earlier research has provided evidence of reduced motor skills and impaired balance in the F1 generation exposed to PS (Schneider and Coe, 1993, Patin et al., 2004). A recent study demonstrated that trans- and multigenerational PS (across four generations) compromised skilled movement in males but not females in both skilled reaching and skilled walking tasks (Ambeskovic, 2013, Thesis). More specifically, in this study MPS males made more limb placement errors in skilled walking and exhibited a lower success score in skilled reaching performance. In the present study, there were no differences in the overall time it took to cross the ladder, or in the changes in overall score. However, supporting the previous study, we did find an increase in the number of errors made. These motor deficits may indicate impaired inter-limb coordination and reduced placement accuracy, which are supported by recent human observations. For example, maternal stress during pregnancy results in lower motor development scores in offspring (Buitelaar et al., 2003, Huizink, 2012, Grace et al.,

2015). Specifically, Grace et al. (2015) most recently found that pregnancy stress during the last trimester resulted in lower motor development (or "clumsiness") at ages 10, 14, and 17 years. Mothers who experienced three or more stressful events during their pregnancy, such as marital problems, income problems, death of relative, had offspring with a lower motor competence than those who experienced between zero and two events (Grace et al., 2015). Another study by Buitelaar et al. (2003) reported that PS was predictive of lower motor development in infants at 8 months (Buitelaar et al., 2003). Moreover, one study also linked increased maternal cortisol levels to a decline in mental and motor developmental scales in infants (Huizink et al., 2003). Although the present study focused on males, findings showed that males exhibit more severe motor deficits than females (Hands et al., 2009, Ambeskovic, 2013) indicating that motor development may be a sexually dimorphic trait.

Disturbances of the developing cortex, which occur later in the ontogeny of the nervous system (last trimester), may be a key etiological factor for motor programming (Gramsbergen, 2003). The most rapid stage of cortical development mainly occurs during the second and third trimesters in humans, which corresponds to gestational day 11 to postnatal day 10 in the rat. This stage of neurodevelopment includes neurogenesis, cell migration, and dendritic growth, and therefore it represents a critical stage to establish CST axonal pathfinding and connectivity (Andersen, 2003, Gramsbergen, 2003). These developmental stages are characterized by maturation of fine motor control and posture, coordination and balance (Gramsbergen, 2003).

The present observations of impaired motor performance reflect disturbances in motor system development, including the motor cortex and CST. Changes may also arguably be found in subcortical systems which were not investigated at this time.

Dendritic morphology, i.e., branching, complexity and length, reflect the synaptic connectivity. More complex dendritic branching provides more space for synaptic connections which ultimately alters the functional capacity of the respective brain region (Muhammad et al., 2011).

The effects of PS on dendritic complexity have previously been studied showing that PS causes changes in dendritic arborisation depending on location as well as sex and age. For example, Muhammad et al. (2012) reported that PS caused a decrease in length in the basilar orbitofrontal pyramidal neurons in both males and females along with a decrease in spine density of the pyramidal neurons in the medial PFC (Muhammad et al., 2012). Moreover, Bustamante et al. (2013) found that PS reduced dendritic growth only in male mice which was accompanied by reduced branching of apical dendrites in layer II/III pyramidal neurons of the parietal cortex (Bustamante et al., 2013). Another study found that PS-induced reduction in spine density is evident at postnatal day 65 but not on day 35, while no alteration in dendritic length was observed (Martinez-Tellez et al., 2009).

The effect of ancestral stress on pyramidal morphology has also recently been studied in animals that were generationally stressed consistently for four generations. Multigenerational PS exposure decreased spine density in the parietal neurons and dendritic length was increased in both males and females (Ambeskovic, 2013). Conversely, our results indicate a decrease in dendritic length, as deduced from the number of intersections in the Sholl-like analysis. All in all, results from these previous studies indicate that experience-dependent neuromorphology varies with the type of experience, its duration, sex and age of an animal (Murmu et al., 2006, Mychasiuk et al., 2012). Differences in ancestral studies may be due to the type of stress used (restraint and

swim stressors in Ambeskovic (2013), versus social isolation), or the generational effects from the third to fourth generation.

Although motor deficits and dendritic complexity have previously been linked to PS across one and multiple generations, the novel finding from this study comes from the measurement of CST fibers in the spinal cord. Studies linking CST damage to changes *in utero* have been executed, however, these studies have only been related to nutritional restrictions or preterm birth (Spinillo et al., 1993, Quirk et al., 1995), which are often associated with PS. For example, infants who had restricted intrauterine growth have increased rates of long-term neurological and neuromotor problems that last into childhood (Spinillo et al., 1993). Further, prenatal and postnatal malnutrition in the rat leads to neuronal loss, reduced brain weight, and a reduction of the conduction velocity along the CST (Quirk et al., 1995). Malnutrition has also been associated with decreased diameter of corticospinal axons and reduced myelination of corticospinal fibers (Sima and Sourander, 1976, 1978). Moreover, illness severity of the very preterm neonate along with pain-related stress exposure has been found to contribute to slower microstructural development of the corticospinal tract (Ranger et al., 2013, Zwicker et al., 2013). Our results confirm the vulnerability of the CST *in utero*, as ancestral stress also reduced CST fiber density.

There are also many psychopathologies and related disorders associated with PS that have been linked to alterations in white matter tract density. These include anxiety disorders, depression and schizophrenia (Kim et al., 2005, Liao et al., 2014, Ublinskii et al., 2015). However, the majority of studies have been performed on areas related to emotional processing and associated behaviours including the cingulate gyrus and the white matter tracts of the limbic system. For example, in one study which compared

adolescents with generalized anxiety disorder (GAD) to healthy controls, individuals with GAD showed significantly reduced fractional anisotropy (FA; uniformity of water flow) as measured by diffusion tensor imaging in white matter tracts of the limbic system (Liao et al., 2014). In studies investigating post-traumatic stress disorder in trauma survivors, researchers have found significant changes in FA in various white matter tracts, predominantly tracts that are proximal to the cingulate gyrus (Kim et al., 2005, Schuff et al., 2011).

On the other hand, in patients with schizophrenia, a disorder which has been highly linked to PS (van Os and Selten, 1998, Holloway et al., 2013) and more recently ancestral stress (Yao et al., 2014, Babenko et al., 2015), there have been more studies investigating motor areas of the brain including the CST. For example, a decrease in FA has been found in the posterior limb of the internal capsule and an increase in diffusion coefficient in the motor cortex and associated areas of the CST (Ublinskii et al., 2015) Moreover, in early-onset schizophrenia, white matter maturation is delayed, especially in bilateral frontal lobes and the pyramidal tract (Gogtay et al., 2008, Douaud et al., 2009). Furthermore, multiple studies have linked a decrease in motor activity with the structural connectivity of the premotor cortex (Perez-Iglesias et al., 2010, Bracht et al., 2013, Kamiyama et al., 2015). Results from the present study show that ancestral stress alters CST density and causes changes in motor performance similar to the studies concerning associated psychopathologies. These insights confirm a link between ancestral stress and a possible increase in vulnerability to neurological deficits and psychopathologies.

A central aspect of the present study is the use of enrichment therapy to intervene adverse consequences of ancestral stress. Animals placed in an EE after weaning respond with improved neural morphology and restored motor skill. The effects of EE on dendritic

complexity have been vastly studied. EE enhances dendritic complexity and spine density in the hippocampus (Bindu et al., 2007) as well as in pyramidal neurons of the parietal cortex (Kolb and Gibb, 1991, Leggio et al., 2005, Mychasiuk et al., 2014), and in the somatosensory cortex (Johansson and Belichenko, 2002). The present results confirm these findings by showing that enrichment has an overall beneficial effect on dendritic complexity. However, studies on enrichment and motor deficits are less common. Our laboratory has shown that EE improves skilled reaching performance in healthy and lesion animals (Jadavji et al., 2006, Jadavji and Metz, 2009, Knieling et al., 2009). The impact of EE on adverse programming by PS has been studied twice, but with differing outcomes. One study showed a negative effect of EE on motor learning tasks and reaching performance (Ulupinar et al., 2015), and the other showed superior performance in motor tasks induced by EE (Zubedat et al., 2015). The present results in both trans- and multigenerational stress confirm the latter by demonstrating improved limb placement accuracy and fine motor control in treated animals. Lastly, the present study is the first to show that enrichment altered plasticity to alleviate some of the negative effects of PS on CST development and long-term connectivity. One other study was found which used enrichment to reverse detrimental effects in an animal model of cerebral palsy (Marques et al., 2014). This group found an average increase in the mean area of motoneurons and an increase in the expression of synaptophysin in the ventral horn of the spinal cord of the enriched animals compared to the standard housed animals modeled for cerebral palsy. Along with synaptophysin as a possible mechanism for axonal mitigation with EE, in Chapter 2, mir-182 which was found to control axonal guidance and neurotrophin signalling pathways was found to be differentially expressed in standard and EE animals. As shown, mir-182 controls proteins such as Ephrin, which have been found to be

important in the proper guidance of motor neuron axons in the spinal cord (Kao et al., 2012). Overall, this could indicate that the beneficial effects of EE, as described in Chapter 2 and in particular the influence of mitigating microRNAs, may influence pyramidal cell morphology, CST development, and skilled movement.

3.6 Conclusion

A growing body of evidence has linked motor disorders to altered prenatal neurodevelopment. In this study we show that ancestral stress causes alterations in pyramidal cell morphology of the parietal cortex along with changes in axonal density in the CST. These alterations manifested as poor motor control particularly in skilled movement ability as measured by the number of errors in a skilled walking task. The present findings in the motor system suggest similarities of neuromorphological changes to those seen in the limbic system in major psychopathologies. The adverse consequences of ancestral stress in motor system plasticity were mitigated by an early intervention therapy. The present findings provide evidence linking ancestral programming to motor system development and illustrates that both pre- and postnatal experience shapes motor skill and development.

Chapter 4:

Developing the Cumulative Animal Allostatic Load Measure (CAALM): Applying CAALM to Measure the Burden of Ancestral Stress and Mitigation by Environmental Intervention

4.1 Abstract

Stress causes the body to react via allostasis by triggering multiple physiological systems to try and maintain stability. If stress is chronic, it becomes more difficult for the body to maintain allostasis. One concept or model that has proven useful in understanding cumulative stress and the resulting mediators of the stress effect on the body is "allostatic load" (AL). In this study, the cumulative burden of stress in the ancestral stress model was measured using a new allostatic load index termed the cumulative animal allostatic load measure (CAALM) which was measured as the sum of dysregulated physiological biomarkers. Using the CAALM index, we assessed if ancestral stress lead to higher allostatic load, and if enrichment works to decrease allostatic load. Furthermore, we investigated if the CAALM index could predict future health deficits. It was found that CAALM is a valid method to predict the burden of chronic stress by measuring the ability to maintain allostasis. Moreover, this cumulative measure can be used to predict stress resiliency and vulnerability and the risk of neural deficits. Lastly, we showed that enrichment therapy can offset the adverse health outcomes linked to a high AL.

4.2 Introduction

The burden of ancestral stress is felt across all major physiological systems and influences many aspects of health. As shown in previous chapters, we know that generational stress from prenatal circumstances programs the HPA axis, alters brain morphology and anatomy, and that epigenetic mechanisms may be a key component to these changes. Importantly, our bodies are also equipped with the ability to adapt to the stressful environments, which is a crucial mechanism, but is also a pertinent risk to our health and well-being when exhausted. One term that has been helpful in defining these adaptive mechanisms is "allostasis", which is defined as the ability to achieve stability through change (Sterling and Eyer, 1989). Stress causes the body to react via allostasis by triggering the multiple and often opposing physiological systems including the immune system, metabolic system and the neuroendocrine system. As shown in previous chapters, these events, if consistent or chronic, may lead to adverse health events. It is often difficult to determine the ultimate cause of the adverse effects because stress is so dynamic in its cause and effect. This especially applies to ancestral stress, where changes occur not only in the offspring, but at each generation (Metz et al., 2015a). One concept or model that has proven useful in understanding cumulative stress and the resulting mediators of the stress effect on the body is "allostatic load".

Allostatic load (AL) was first defined by McEwen and Stellar in 1993 as the cost of chronic exposure to fluctuating or heightened neural or neuroendocrine response resulting from repeated or chronic environmental challenge (McEwen and Stellar, 1993). AL refers to the cumulative burden of stress, which is expressed in the physiological dysregulation across numerous systems that are involved in regulating and coping with stress. The

benefit of the biological concept of AL is that it incorporates several elements of stress pathophysiology in one comprehensive model (Nugent et al., 2015).

The AL model includes many multi-systemic interactions including primary and secondary mediators of stress, and has been shown to have the predictive capacity to detect individuals at high risk of tertiary outcomes (Juster et al., 2010). Primary mediators include stress hormones activated by the sympathetic-adrenal-medullary axis (SAM axis; epinephrine, norepinephrine), the hypothalamic-pituitary-adrenal axis (HPA axis; cortisol or corticosterone) and primary immune modulators or cytokines directly influenced by stress (e.g. interleukin (IL)-6). Secondary mediators that are a result of chronic or long-term stress responses include metabolic changes (e.g., glucose, cholesterol, fat deposition), cardiovascular alterations (e.g., blood pressure) and immune regulators (e.g., IL-1β, IL-2). Tertiary outcomes or comorbidities from elevated primary and secondary mediators include a decline in health and cognition, accelerated aging, diseases of the metabolic, cardiovascular and immune systems (cardiovascular disease, diabetes), and also death (Seeman et al., 2001, Leahy and Crews, 2012). Figure 4.1 depicts the primary and secondary mediators of stress and AL, and tertiary outcomes in this study.

The allostasis-adaptation, or allostasis-allostatic overload process is thought to include physiological mediators that are interconnected, reciprocal and non-linear in their effects [Figure 4.1; (Juster et al., 2010, McEwen and Gianaros, 2011)]. Non-linearity in this case would refer to the fact that changes in each biomarker could cause subsequent changes in any other biomarker in any direction. This often makes it difficult to analyze cumulative effects when only considering single biomarkers. An AI would therefore facilitate the detection of sub-clinical consequences of stress and increase the diagnostic and predictive sensitivity by using a fingerprint of biomarkers instead of a single marker.

Thus, Seeman and colleagues in 1997 proposed operationalizing AL through the use of an allostatic load index [AI (Seeman et al., 1997)].

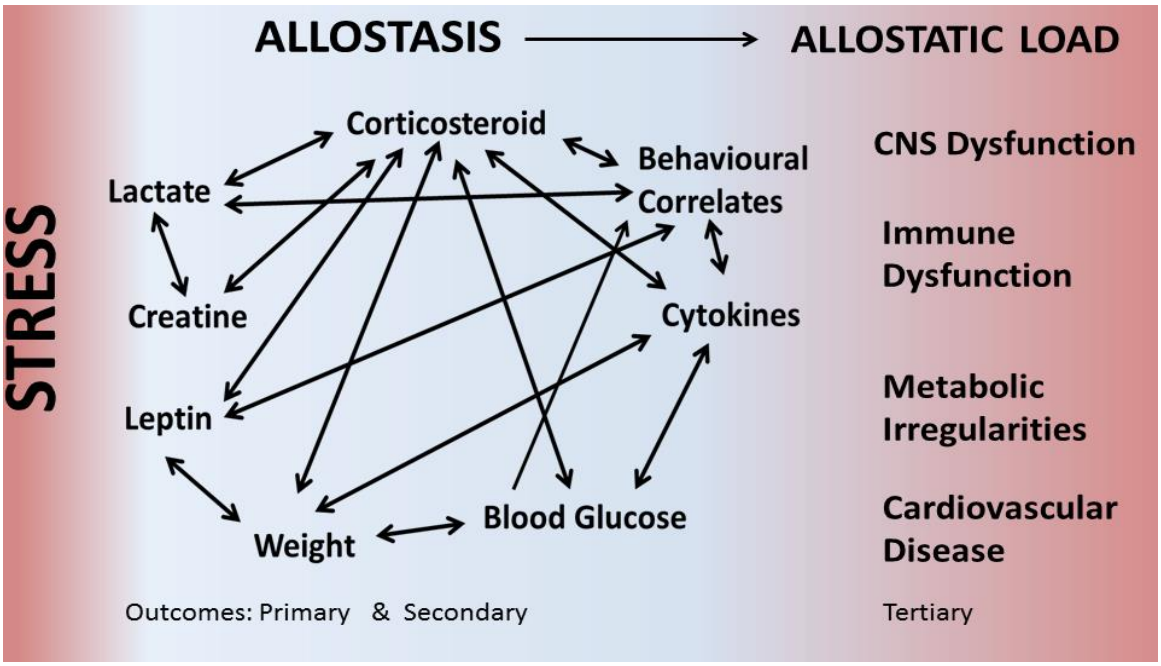

Figure 4.1. Non-linear representation of the effects of stress on multiple systems of the body, illustrating that primary and secondary outcomes lead to adverse tertiary outcomes due to allostatic overload. Illustrated here are the potential interactions of the biomarkers used within this study.

AI is measured as the sum of dysregulated physiological biomarkers (Seeman et al., 1997) that in turn reflect a multi-systemic view of the physiological toll that is placed on the body for allostasis. AI first quantified in 1997 when Seeman and colleagues used the multiple biomarkers to predict cognitive decline associated with aging using the MacArthur Studies of Successful Aging (Seeman et al., 1997, Seeman et al., 2001, Seeman et al., 2004a). The authors quantified AL through a series of ten biomarkers that are now commonly used in AI studies including; blood pressure, waist-hip ratio, serum high density lipoprotein, total cholesterol, glycosylated hemoglobin serum dihydroepiandrosterone sulfate, overnight urinary cortisol and overnight urinary

noradrenalin (or norepinephrine) and adrenalin (epinephrine). Together, these represent indices of cardiovascular activity, metabolism, HPA axis activity and sympathetic nervous system activity (McEwen, 2000a). In using the AI, Seeman and colleagues were able to predict associated high risk for cognitive decline.

The AI is now commonly used in clinical and research populations to visualize the extent of dysregulation caused by multiple systems. The AI was shown to be able to predict adverse health outcomes, diabetes, physical and cognitive decline, and elevated mortality risk, and has been applied to many populations (McEwen, 2000a, g, Seeman et al., 2001, Seeman et al., 2004a, Langelaan et al., 2007, Danese and McEwen, 2012, Bizik et al., 2013). Studies demonstrate the validity and effectiveness of AI and have shown that it better predicts future health risks, especially high risk groups, than any biomarker individually (Seeman et al., 2001, Karlamangla et al., 2002). Essentially, the AI is a comprehensive model that better links often contradictory effects while providing insights into how individuals differ in their vulnerability or risk to develop diseases or tertiary outcomes (McEwen, 2000a).

Although the concepts of AL and allostasis has been mentioned in animal studies of stress and disease (Tannenbaum et al., 2002, Korte and De Boer, 2003, Van der Meer et al., 2004, Korte et al., 2005), there is currently no measure of AL, or an AI that has been used in animal models. In stress research, an AI could be useful as a more comprehensive interpretation of the impact of stress that we hypothesize would be helpful in predicting disease risk, susceptibility and vulnerability. Furthermore, an AI would facilitate the translation of animal studies to humans. Therefore, in this study we created an AI using biomarkers commonly used in stress research. We called the new index the ‘Cumulative Animal Allostatic Load Measure’ or CAALM.

The objectives of this study were to:

1) Develop an allostatic load index for the use in animal models;

2) Determine if ancestrally stressed animals have a higher allostatic load;

3) Determine if environmental enrichment decreases allostatic load; and,

4) Determine if allostatic load predicts adverse health outcomes in terms of neuronal deficits.

The present chapter will describe how CAALM was created, and how it was used to measure the cumulative effect of ancestral stress.

4.3 Materials and Methods

4.3.1 Animals

See chapter 2 for complete animal information.

Data used for the CAALM index was collected from F3 offspring rats born to one of the following three maternal lineages: non-stress controls (n = 16), transgenerational prenatal stress (*TPS*; n = 16), and multigenerational prenatal stress (*MPS*; n = 16). TPS rats were the F3 generation of a filial line in which only the F0 dams were stressed during gestation. MPS rats were the F3 generation of a filial line in which dams from each consecutive generation (F0, F1, F2) were gestationally stressed. At weaning, rats derived from the three lineages were assigned to either housing in standard cages, or housing in an enriched environment (EE). Thus, the following groups were tested: non-stress controls in standard (*Control*; n = 8) and EE (*Control-EE*; n = 8) housing conditions, TPS

in standard (*TPS*; n = 8) and EE (*TPS-EE*; n = 8) housing, and MPS in standard (*MPS*; n = 8) and EE (*MPS-EE*; n = 8) housing.

4.3.2 Weight

Animals were weighed every other day between 7:30AM-9:30AM. Weight used for the CAALM index was collected at P120.

4.3.3 Corticosterone Assay

See chapter 2 for corticosterone assay protocol.

4.3.4 Blood Glucose

Blood glucose was measured between 8:00AM and 9:00AM using an Ascensia Breeze Blood Glucose Meter (Bayer, Toronto, ON, Canada) with glucose test strips.

4.3.5 Behaviour

See chapter 2 for elevated plus maze and open field descriptions.

4.3.5.1 Morris Water Task

The Morris water task (MWT) was conducted over the course of nine days using a pool filled with room temperature water. The water was made opaque by adding non-toxic white tempura paint and visual cues were placed on the walls for spatial orientation. A computer assisted tracking system (HVS Image Water 2020™) was used to track rat position and collect data obtained from an overhead video camera. Swim speed and latency were used as a biomarker for CAALM.

4.3.6 Nuclear Magnetic Resonance H^1

At 100 days old, 6.0mL blood samples were collected from the lateral tail vein while rats were anaesthetized using 4% isoflurane. Blood was transferred to centrifuge tubes and plasma was obtained by centrifugation at 5,000 rpm for ten minutes at 4°C. The samples were then stored at –80°C until further processing.

4.3.6.1 NMR Data Acquisition and Processing

NMR spectra were collected on a 700 MHz Bruker Avance III HD spectrometer. The 1-D NOESY gradient water suppression pulse sequence noesygpr1d was used (Bruker). Each sample was run for 512 scans to a total acquisition size of 256k. The spectra were zero filled to 512k, automatically phased and baseline corrected, and line-broadened by 0.3Hz. The processed spectra were then exported to MATLAB for statistical analysis. All peaks were referenced to formate (8.22δ) and a reference metabolite library was used. Concentrations were measured using the MestreNova 10.0.1 qNMR plugin, referenced to an internal standard.

4.3.7 Cytokine Multiplex Analysis

Cytokine biomarkers were quantified using a Discovery Assay® called the Rat Cytokine/Chemokine Array 27-plex (Eve Technologies Corp, Calgary, AB, Canada). The multiplex array was performed at Eve Technologies by using the Bio-Plex™ 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and a Milliplex Rat Cytokine/Chemokine kit (Millipore, St. Charles, MO, USA) according to their protocol. The 27-plex consisted of Eotaxin, EGF, Fractalkine, IL-1α, IL-1 β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-13, IL-17A, IL-18, IP-10, GRO/KC, IFN-γ, TNF-α, G-CSF, GM-CSF, MCP-1, Leptin, LIX, MIP-1α, MIP-2, RANTES, VEGF. The assay sensitivities of

these markers range from 0.3 – 30.7 pg/mL. Individual analyte values and other assay details are available on Eve Technologies' website or in the Milliplex protocol. Cytokines used in the CAALM score included IL-2, IL-6, IL-1β and Leptin.

4.3.8 Neural Density

See Chapter 2 for methods of brain extraction, preparation and mean gray value (MGV) measurement.

4.4 Developing the 'Cumulative Animal Allostatic Load Measure' (CAALM)

4.4.1 Biomarkers

An allostatic load index termed "CAALM" to differentiate for animal use, was developed using guidelines from the first operationalized study of AI (Seeman et al., 1997). The index was developed using 12 biomarkers commonly measured in relation to stress physiology and behaviour in animal studies. Biomarkers were chosen so the CAALM score would represent markers of the acute (primary mediators) and chronic (secondary outcomes) manifestations of stress involving multiple system levels (e.g. immune system, neuroendocrine, metabolic). A score based on multiple systems will create a composite measure with a better predictive capacity (Seplaki et al., 2005). The biomarkers that were selected and measured are summarized below.

4.4.2 Rationale for Biomarker and Description

4.4.2.1 Neuroendocrine

Corticosterone is the primary hormonal mediator of the stress response in rats. In response to acute and chronic stress, the brain activates many hormonal pathways including the hypothalamic-pituitary-adrenal axis (HPA). This response leads to the release of corticosteroid hormones from the adrenal glands, which then consequently feed-back on the brain by binding to glucocorticoid receptors (GR) and mineralocorticoid receptor, terminating the stress response. This response has been shown to be programmed by prenatal stress (Glover et al., 2010, Harris and Seckl, 2011). Moreover, the effects of cortisol and corticosteroids are felt all over the body with primary targets including metabolism, immunity and memory.

4.4.2.2 Markers of Affective State

The open field (OF) task allows the quantification of locomotor activity, affective or anxiety-like behaviours, and exploratory behaviour (Smith et al., 2008, Jadavji et al., 2011). The time spent in the margins of the open field was used as a marker for the CAALM index. The elevated plus maze (EPM) allows the quantification of motor activity and anxiety-like behaviours (Lister, 1987). A particularly robust measurement of anxiety-like behaviours in response to stress is risk assessment, which was chosen for the AI (Roy and Chapillon, 2004).

The Morris water task is most often used as a learning and memory task, but can also be used to assess hyperactivity by measuring swim speed or the latency to locate and escape to a hidden platform (Leggio et al., 2005). Both swim speed and latency were used as a marker for hyperactivity.

4.4.2.3 Immune Markers

Three cytokines were chosen as immune markers for the CAALM index. Cytokines are small glycoproteins that regulate the physiological functions of immunity and inflammatory responses (Khan, 2008). The cytokines included in the CAALM index were the pro-inflammatory interleukins (IL) IL-1β, IL-2, and IL-6. IL-1β and IL-2 have been suggested to be mediators of changes in immune and neuroendocrine functions during stress at both peripheral and central nervous system (CNS) levels (Tanebe et al., 2000). IL-1β is a potent mediator of stress-induced reactions especially in the neuroendocrine systems that control the secretion of pituitary hormones which lead to the secretion of glucocorticoids. The presence of IL-2 has also been demonstrated in the hypothalamus, the pituitary gland and in the locus coeruleus, all of which are involved in the control of the neuroendocrine axes (Tanebe et al., 2000). IL-6 is the cytokine most documented in AL studies. It has major effects on non-immunological tissues and is termed a pleiotropic or endocrine cytokine for this reason. IL-6 is one of the most important biomarkers of chronic or systemic low grade inflammation. Numerous studies have shown that IL-6 is crucial for interaction between the immune system and the CNS in inflammatory disease and plays a role in both acute and chronic stress responses (Segerstrom and Miller, 2004). Overall, the cytokine biomarkers chosen play a physiological role as neuroendocrine and immune modulators.

4.4.2.4 Metabolic Markers

4.4.2.4.1 Body weight, blood glucose and leptin

Body weight is recognized as an indicator of chronic stress and AL, and excess cortisol is found to be positively correlated with body weight (Kiess et al., 1995). Blood glucose functions as a major source of energy and higher plasma levels are positively

correlated to elevated cortisol levels and weight gain (Eigler et al., 1979). A marker of increased body weight is leptin, which is a fat-derived hormone that plays a pivotal role in the regulation of body weight and food intake (Friedman and Halaas, 1998). Moreover, leptin is also thought to be involved in brain development, with its connection to normal neuronal and glial maturation in the mouse nervous system (Ahima et al., 1999). Studies have shown that patients suffering from schizophrenia or major depression have normal body mass indices but reduced leptin levels. In order to determine pathologies associated with altered CAALM index it is useful to include both weight and leptin levels.

4.4.2.4.2 Lactate

Lactate serves as a marker of sympathetic nervous system activation (Hamann et al., 2001, Meyer et al., 2005), muscle activity and psychosocial stress (Kubera et al., 2012), as well as liver function (Phypers and Pierce, 2006). Plasma lactate is also an alternative energy substrate to glucose and may act as the preferred energy source for activated neurons within the CNS (Smith et al., 2003, Glenn et al., 2015). Thus, these studies indicate that lactate levels may represent an indicator of cerebral activity.

4.4.2.4.3 Creatine

The primary physiological function of creatine is to buffer energy concentrations in tissues with large and shifting energy demands, especially in muscles and the brain (Wyss and Schulze, 2002). In the brain, creatine serves its primary role as an energy shuttle and regulator of energy homeostasis (Brosnan and Brosnan, 2007, Mak et al., 2009). Deviations in creatine levels may indicate altered metabolic or mitochondrial function and energy demand (Allen, 2012).

4.4.3 Computing the Composite CAALM Score: Standardization and Measurement

In order to determine high risk measures, the sum of individual z-scores for each biomarker was calculated based on the samples distribution. Calculation of z-scores for standardization allows for each biomarker to have a weight which differs conditionally on its own deviation from the samples mean (Figure 4.2). Once the biomarkers were standardized, each individual marker was analyzed for high risk. The percentiles used for an indicator of high risk for each biomarker are listed in Table 4.1.

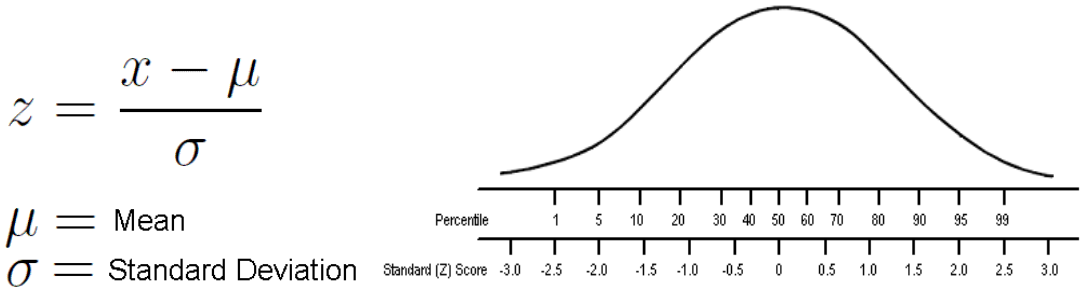

Figure 4.2: Formula for z-score calculation and illustration of percentile distribution at each score

The AI is based on a clinical assessment tool in which biomarkers in the highest or lowest 25% were deemed high risk (Seplaki et al., 2004). Accordingly, values falling within the high risk percentile (above 75th) were dichotomized as "1" and those within the standard ranges as "0". In some instances, lowest percentiles were given a score of "1" as scores too low presented as high risk. Faster swim speed in morris water maze, indicated by lower latency time, indicate higher anxiety and therefore higher risk. The CAALM score was then calculated by summing the number of biomarkers for which the animal fell into the high risk category, so that the overall sum value was between 0, indicating

low risk, and 12, indicating the maximum risk. It should be noted that cut-off points could be set to the 10th percentiles (below 10 and above 90) as they could produce an even stronger predictor of health outcome. However, the 25th and 75th percentiles were chosen to maximise the predictive power for early signs of tertiary health outcomes.

Table 4.1. Information on the biomarker's category, the 10th, 25th, 50th, 75th and 90th percentile along with the high risk cut-off levels (highlighted in pink).

			Percentile				
Category	**Marker Category**	**Biomarker**	**10th**	**25th**	**50th**	**75th**	**90th**
Primary	Neuroendocrine	Corticosterone (ug/mL)	19.004	86.108	182.885	498.75	639.45
Primary	Immune	IL-6 (ug/ml)	8.3	9.5	11.5	15	20.5
Secondary	Behavioural	Open Field (margin time)	497.9	539	552.2	574.55	589.86
Secondary	Behavioural	EPM (Risk assessment)	65	79	91	120.5	126.4
Secondary	Metabolic	Blood Glucose (mmol/L)	5.800	6.200	6.900	8.350	9.54
Secondary	Immune	IL-1β (ug/ml)	171.31	222.645	363.125	585.84	779.318
Secondary	Immune	IL-2 (ug/ml)	21.188	40.585	81.235	120.54	156.893
Secondary	Metabolic	Leptin (ug/ml)	5387.031	8008.255	10382.54	14871.96	26872.85
Secondary	Metabolic	Weight (g)	523.7	558	597	624	653.6
Secondary	Metabolic	Lactate (mmol)	1.336	1.583	1.868	2.147	2.347
Secondary	Metabolic	Creatine (mmol)	0.225	0.265	0.323	0.351	0.379
Secondary	Behavioural	MWT (Latency Time)	4.772	7.178	9.366	10.851	12.457

Elevated plus maze (EPM); interleukin(IL); morris water task (MWT)

4.4.4. Statistics

Statistical computations were based on Statview software version 5.0 (SAS Institute, NC, USA). Descriptive statistics are reported where results represent means ± standard deviations. Analysis of variance (ANOVA) was used to compare the mean levels of each biomarker across all groups, followed by Fisher's post-hoc tests or pairwise

student t-tests. The alpha level was set to 0.05 and significant p-values were designated with an asterisk or hashtag in all tables and figures.

Because CAALM is an ordinal scoring system and is not normally distributed, the non-parametric Kruskal-Wallis test was used to compare the distributions of CAALM scores across groups. Pairwise comparisons were performed by collapsing groups and applying separate Mann-Whitney U tests. In order to investigate whether CAALM levels, along with individual biomarkers, could predict changes in neuronal density we computed Simple regressions (R) were used to determine the relationship between biomarkers, CAALM and MGV.

4.5 Results

4.5.1 Descriptive Allostatic Load Index

The means and standard deviations of individual biomarkers are summarized in Table 4.1. Corticosterone levels showed a main effect of Enrichment (Chapter 2). Animals that were ancestrally stressed had higher baseline CORT levels, although these results were not significant. However, enrichment significantly decreased the level of basal circulating CORT ($F(1,42)=16.16$, $p<0.001$) across all groups . The EPM revealed that stressed animals had more risk assessment behaviours ($p<0.05$) compared to non-stressed. Further pairwise comparisons also revealed that enrichment in the TPS and MPS groups showed significantly reduced risk assessment behaviours compared to the standard housing rats ($p<0.05$). Blood glucose levels showed an effect of Enrichment in the control group ($p<0.05$), and an effect of TPS ($p<0.001$) and MPS ($p<0.0001$) compared to controls. Latency in the Morris water task revealed a significant increase in

swim speed in CEE ($p<0.05$) and TPS-EE ($p<0.05$) compared to their respective control groups (C, TPS) and an increase in swim speed in MPS compared non-stress controls ($p<0.05$). Furthermore, MPS elevated lactate and creatine levels in urine compared to controls ($p<0.05$).

The 10^{th}, 25^{th}, 50^{th}, 75^{th} and 90^{th} percentile distribution of each biomarker and the cut-offs used for dichotomizations are summarized in Table 4.2. The CAALM index was able to reveal effects of housing conditions on stress responses. Mean CAALM scores were highest in the MPS and TPS animals and were lowest in groups exposed to environmental enrichment. The summary of the average CAALM scores are shown in Figure 4.5A. CAALM scores were significantly different between treatment groups ($H=19.866$, $p=0.0013$). Specifically, MPS were significantly higher than control animals ($p<0.0001$), and TPS animals ($p=0.0003$) and enrichment had a significant effect on the MPS group ($p=0.0023$), while decreasing the score across all groups.

The individual weights of each biomarker above the high risk percentile in each group are illustrated in Figure 4.3. The dichotomized biomarkers showed significant differences in swim speed in the Morris water task ($H=12.796$, $p=0.0254$), IL-1β ($H=11.51$, $p=0.0422$), creatine ($H=18.18$, $p=0.0027$) and lactate ($H=17.2$, $p=0.0041$) levels. Figure 4.4 illustrates the score for each individual animal and biomarker in a heat map. Notably, all of the high risk animals (above a CAALM score of 5) have elevated IL-1β and creatine.

Table 4.2. Summary of descriptive statistics.

Biomarker	C		CEE		TPS		TPSEE		MPS		MPSEE	
	Mean	±	Mean	±	Mean	±	Mean	±	Mean	±	Mean	±
OF	552.3	29.25	560.52	15.00	551.32	45.82	525.73	54.99	557	27.03	523.17	42.35
EPM	93.875	31.12	106.5	24.51	101.62	28.47	79.75*	33.01	89.87	19.43	86.00 *	35.20
CORT	387.688	106.99	61.43*	56.96	501.05	287.47	58.68*	68.16	514.22	295.83	111.38*	174.65
BG	6.325	0.52	6*	0.78	5.3375#	0.32	5.48	0.59	6.58#	0.34	6.35	0.65
IL-1β	339.506	197.25	392.19	285.55	346.60	202.40	276.33	231.24	370.45	302.67	320.85	291.25
IL-2	106.006	104.04	82.23	73.03	128.92	157.93	81.68	46.99	114.93	85.04	64.14	39.38
IL-6	15.5	6.46	14.25	4.70	14.25	4.07	11.87	4.78	11	3.13	13.25	3.50
Leptin	3.15E4	2.57E3	1.82E4	1.37E3	2.10E4	3.28E3	1.57E4	1.78E3	1.17E4	4.78E3	9.84E3	2.50E3
Weight	574.75	56.12	556.87	59.80	591.12	27.32	586.75	50.94	598.75	47.88	579.25	90.42
Lactate	1.61	0.21	1.49	0.16	1.89	0.36	1.83	0.28	2.08#	0.11	1.97	0.68
Creatine	0.27	0.04	0.25	0.017	0.31	0.05	0.31	0.03	0.34#	0.02	0.33	0.11
MWT	10.85	2.25	7.16*	2.47	10.59	2.97	*7.94	2.29	8.30#	2.36	7.36	1.92

*denotes significant effect of EE ($p<0.05$), # denotes significant effect of stress ($p<0.05$)
Open field (OF); Elevated plus maze (EPM); corticosterone (CORT); Blood glucose (BG); interleukin(IL); morris water task (MWT)

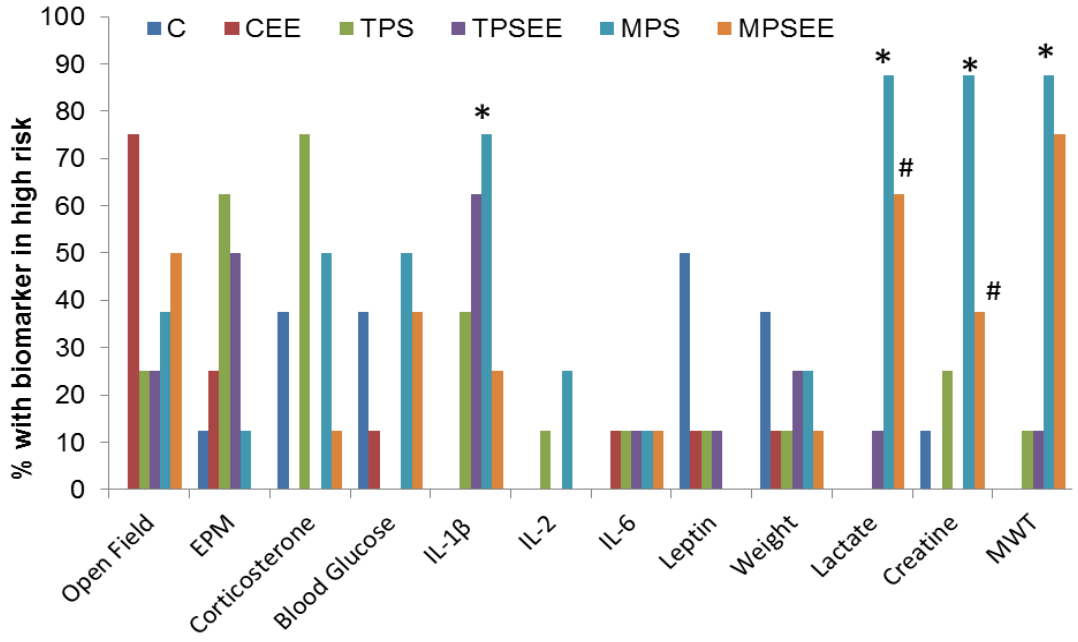

Figure 4.3. Individual components contributing to the allostatic load score. Percent of animals designated as high risk within each biomarker further divided by group. Values represent each biomarker above high risk that was counted and divided by total number of animals in each group. Absent groups indicate that no animals were within the high risk category. *denotes significant effect of stress (*$p<0.05$), # denotes significant effect of EE ($p<0.05$). Elevated plus maze (EPM), Morris water task (MWT)

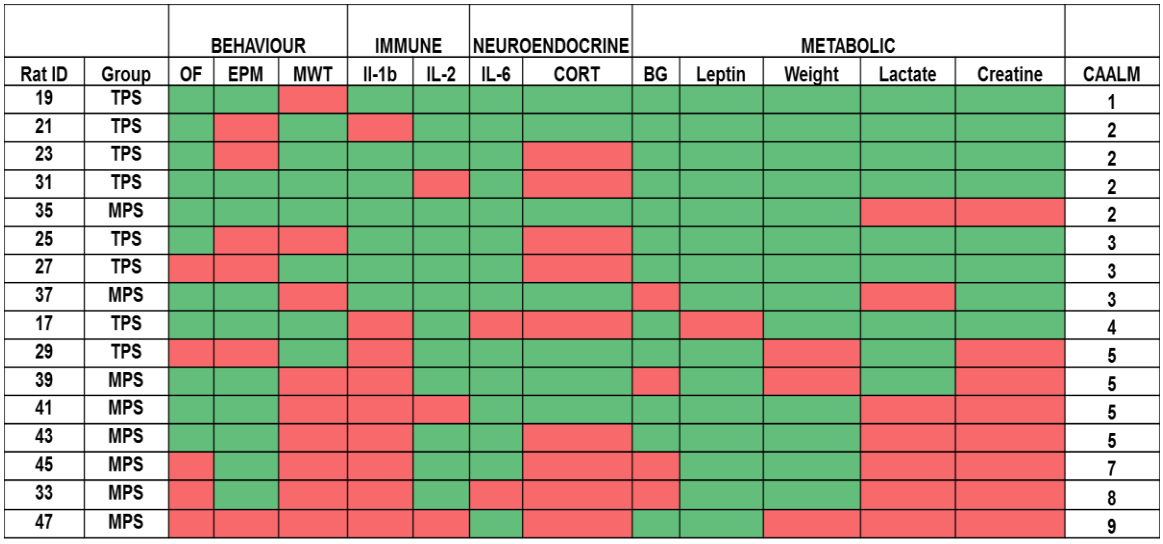

Figure 4.4 Heat map illustrating the individual CAALM score for all animals in the ancestrally stressed groups broken down in to the individual biomarkers. Green depicts a score of 0, red depicts a score of 1. CAALM score is organized according to lowest risk score to highest risk in the last column.

4.5.2 Predictive Value of the CAALM Index and Individual Biomarkers

Correlations for all individual biomarkers are shown in Supplemental Table S2 in Appendix A. Values that correlate to MGV include lactate ($R=-0.42$, $p<0.05$), creatine ($R=-0.41$, $p<0.05$) and the CAALM score ($R=-0.551$, $R^2=0.3036$, $p<0.05$; Figure 4.5B). In order to determine if CAALM still has predictive value without the influence of lactate and creatine, both were removed from the AI index to test the predictive value. CAALM scores without the influence of lactate and creatine were still significantly different between treatment groups ($H=13.110$, $p=0.0224$). CAALM still had the largest values in MPS and TPS animals, with enriched environment lowering CAALM (Figure 4.6A). Moreover, CAALM without these biomarkers was still correlated to MGV ($R=-0.490$, $R^2=0.2401$, $p<0.05$; Figure 4.6B). However, the significance factor was stronger when creatine and lactate were included in the index indicating that overall, using all biomarkers and a multisystem approach provides the highest predictive value.

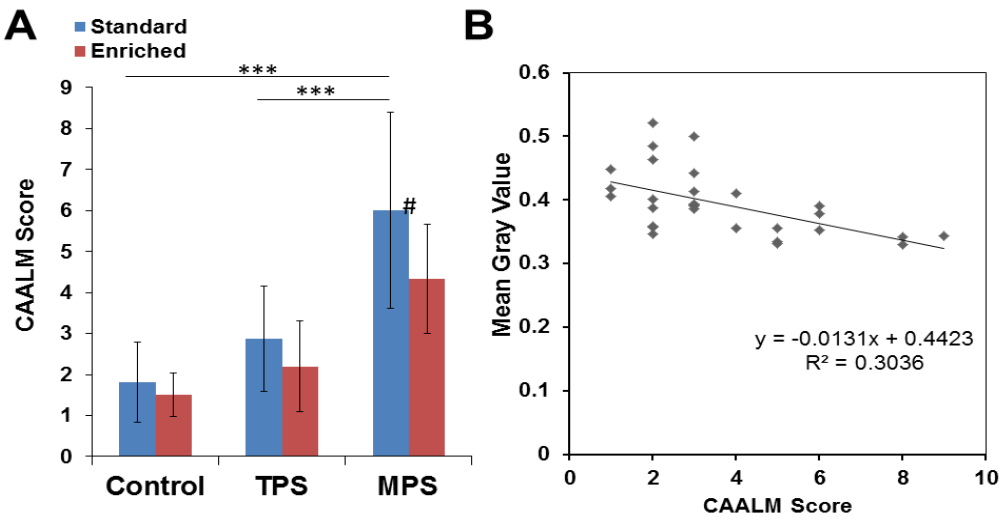

Figure 4.5. A) The average CAALM in each group. B) CAALM score and mean gray value associations. CAALM was significantly correlated with MGV. Values represent Mean +/- SD. *denotes a significant effect of stress (***p<0.001), # denotes significant effect of enriched environment (p<0.05).

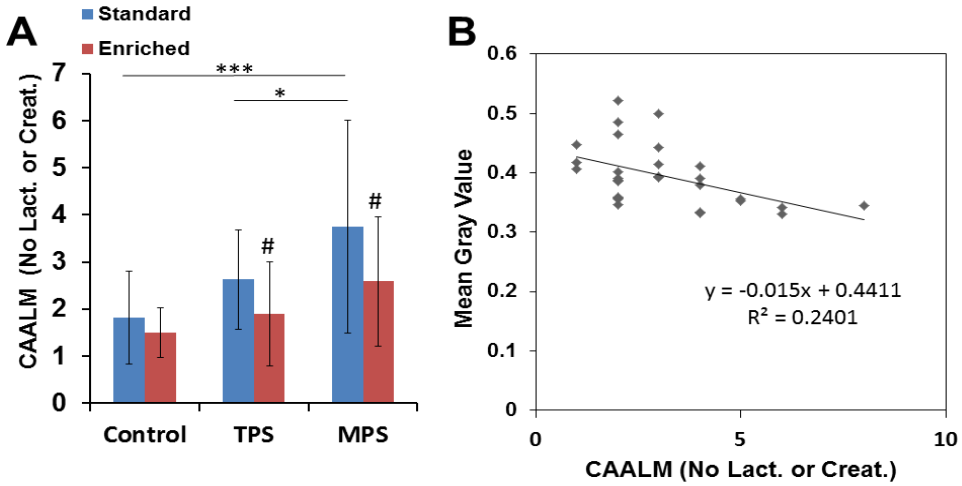

Figure 4.6. A) The average CAALM score minus lactate and creatine in each group. B) CAALM score minus lactate and creatine and mean gray value associations. CAALM minus lactate and creatine was significantly correlated with MGV. Values represent Mean +/- SD. *denotes significant effect of stress (*p<0.05, ***p<0.001), # denotes significant effect of enriched environment (p<0.05)

4.6 Discussion

The goal of this study was to create a comprehensive and cumulative model that measures non-linear effects of stress (McEwen, 2000a) and assesses the risk of chronic health impacts generated by trans- and multigenerational stress. Recent evidence suggest that transgenerational stress is a central risk factor in complex disease etiology (Zucchi. et al., 2013, Babenko et al., 2015, Metz et al., 2015d). However the burden of chronic stress induced by ancestral stress has not been systematically quantified. While indices for AL have become a valuable tool for predicting stress-related diseases (Juster et al., 2010), the lack of prospective human cohort data spanning at least three generations emphasizes the need for modeling ancestral stress in laboratory animals. Here we developed a novel allostatic load index for use in laboratory rodents termed "CAALM" based on guidelines by Seeman et al. (1997). The index was developed using 12 biomarkers commonly measured in relation to stress physiology and behaviour in rat and mouse studies. The findings show that most biomarkers when analyzed individually, did not predict high risk for neuronal deficits. However, when all biomarkers were standardized and dichotomized to dictate high risk, CAALM was able to predict neuronal deficits.

Validity of this new tool was confirmed by CAALM predicting elevated AL in ancestrally stressed rats, showing that remote ancestral stress raises the risk for low neuronal density. This indicates that the cumulative burden of stress is greater in ancestrally stressed animals, and the compensation and adaptation to stress has increased their allostatic load and therefore vulnerability to stress-induced disease (McEwen, 2000). By contrast, treatment with enriched environment, a powerful therapy that improves recovery in animal models of neurological disease (Knieling et al., 2009, Baldini et al.,

2013), reduces the cumulative burden by AL and improves neuronal density in ancestrally stressed rats particularly in the MPS group.

The CAALM index, like AI, was developed to measure the effect of cumulative stress based upon the notion of primary mediators leading to primary effects and then to secondary outcomes which lead to tertiary outcomes that characterize tangible diseases (McEwen and Seeman, 1999). In this study, the tertiary outcome measured was neuronal density as measured by MGV in the prefrontal cortex. The PFC is particularly important in higher-order executive functions, sensory perception and social reasoning (Kolb et al., 2012). Behavioural deficits and disorders characterized by alterations in neuronal density in the PFC include anxiety, attention deficit hyperactivity disorder (Brennan and Arnsten, 2008) and depression (Frodl et al., 2010), to name a few. Moreover, studies have investigated the relation between PFC function and HPA axis activation and concluded that the PFC is a part of the regulatory circuitry involved in the stress response (Kern et al., 2008). The decrease in MGV may therefore diminish the cortical negative feedback on HPA axis activity and contribute to the observed slight increase in CORT levels. In this study, greater allostatic load was significantly associated with a decrease in neural density, as measured by MGV.

The MPS group, with the highest allostatic load score, seemed to benefit most from enrichment. This group is thought to have larger cumulative effects due to stress, as direct stress is present in each generation (see Chapter 2 and McCreary et al., 2015), and therefore the benefit of enrichment may reach multiple physiological systems. Many studies have investigated the effect of enrichment on allostatic load and measurement by the AI index in the human population. An "enriched" environment in the human population could be interpreted as those with higher socioeconomic statuses (SES) and

higher education, however, this study more closely relates to social enrichment, or increased social support. Both higher SES and education have both been correlated to a lower AI score (Seeman et al., 2004d), and notably, social support has also shown specific protection against AL, decreasing AI score (Seeman et al., 2004a, Brooks et al., 2014). Findings of this study show that enrichment has the capacity to promote resiliency against the cumulative effects of AL, and may therefore represent a therapeutic and a preventative measure against some of the negative tertiary outcomes of AL.

4.6.1 Advantages of CAALM Over Individual Biomarkers of Ancestral Stress

Biomarkers that exhibited a significant effect of stress when comparing raw means included risk assessment behaviour, blood glucose, swim speed, lactate and creatine. Biomarkers that showed a significant effect of enrichment included risk assessment, corticosterone, blood glucose, swim speed, lactate and creatine. This indicates that ancestral stress does affect the metabolic system, as measured by blood glucose, lactate and creatine, as well as behaviour, specifically hyperactivity and anxiety as measured by MWT and EPM. Moreover, enrichment can reverse some of these detrimental effects (as shown in Chapter 2). However, the majority of these individual biomarkers were not able to predict changes in neuronal density, the tertiary outcome. The individual biomarkers that were individually significant at high risk while acting as predictive indicators for low neural density included lactate and creatine. Changes in metabolism, as measured by lactate and creatine, assist in illustrating the large and pertinent effects of cumulative stress on metabolism. Impaired cerebral energy metabolism, which is linked to alterations in neuronal plasticity, is among the leading hypotheses in the pathogenesis and etiology of some psychiatric disorders including mood disorders, major depression and bipolar

disorder (Stork and Renshaw, 2005, Yildiz-Yesiloglu and Ankerst, 2006, Koene et al., 2009, Wood et al., 2009, Kondo et al., 2011, Allen, 2012).

Other individual biomarkers that did not predict changes in neural density, but significantly contributed to the overall CAALM score included IL-1β, and swim speed in MWT. Notably, animals with ancestral stress had higher risk for elevated IL-1β and all animals with a CAALM score greater than 5 received an IL-1β score of 1. This score indicates increased inflammation and immune responses that may indicate compensatory mechanisms linked to an insufficient stress response by glucocorticoids, or an increased response due to higher metabolic rates (Johnson et al., 2012). Understanding interactions between the stress response and the immune system will shed light on the association between HPA axis dysfunction and psychopathologies such as depression and schizophrenia that are associated with altered immune status. Moreover, high risk for immune dysregulation due to stress may also shed light on vulnerability and severity of certain autoimmune and inflammatory diseases (Marques et al., 2009).

A recent question proposed by Juster et al. (2010) and Seeman et al. (2001) concerns the masking of the predictive value of individual AL components. By breaking the AI into neuroendocrine and metabolic biomarkers, previous studies found that the individual clusters did not overlap and may therefore individually contribute to health risks (Seeman et al., 2001, Juster et al., 2010). Clustering biomarkers provide biological signatures that are vital in predicting morbidity and mortality (Gruenewald et al., 2006). Previous results show mixed support for the inclusion of a comprehensive AI instead of subgroups of fewer biomarkers (Juster et al. 2010). The present study addressed this issue in two ways. First, it was determined that CAALM was better correlated to MGV than individual biomarkers. Second, when lactate and creatine were removed from CAALM,

the index was still significantly correlated to MGV. The overall focus of an AI should be on identifying levels of biomarkers that identify high risk for better prevention of tertiary outcomes.

4.6.2 Effectiveness of the CAALM Index

Stratification tools such as the AI and CAALM are effective discriminating tools to dissociate stress resiliency versus stress vulnerability. For example, a score of behavioural traits may provide a better characterization of an individual's vulnerability to prolonged stress and stress-induced depression (Castro et al., 2012). Accordingly, our data show that higher multivariable CAALM scores are associated with increased risk to lower neuronal density. Likewise, ancestral stress also caused epigenetic variations, such as differentially expressed micro RNAs (miRNAs), which may explain increased disease vulnerability (see Chapter 2). Interestingly, some measures of stress such as corticosterone, did not vary much between transgenerational and multigenerational stress experiences, yet the respective overall CAALM scores were higher in the multigenerational group. This observation indicates that ancestral stress through allostatic mechanisms, promotes adaptation to stress in some functions, while creating vulnerabilities in others.

The value of assessing multiple biomarkers, including primary and secondary mediators, may improve high risk detection as well as intervention strategies to promote health and wellbeing (McEwen, 2003). Multivariable tools, such as AI and CAALM increase the ability to identify individuals at higher or lower risk for developing complex diseases due to stress. AI scoring are critical tools to stratify individuals at risk. As a result, targeted prevention strategies and personalized medicine approaches that focus on high-risk individuals may be more effective than population-based strategies (Zulman et

al., 2008). By determining high risk groups, treatment efficiency could be better delineated, as shown by the usefulness of enrichment in the high-risk multigenerational stress group.

4.6.3 Limitations and Suggestions for Future Work on CAALM

The present study presents the first attempt to study the impact of cumulative stress and allostatic load in rats. Potential limitations of this approach pertain to the following. Firstly, improvements could be made to the CAALM score and chosen biomarkers. This was a retrospective study, where the biomarkers were chosen after data collection. Our measures of AL were designed to summarize dysregulation across multiple physiological systems. Biomarkers were selected based on theoretical grounds from those assessed in clinical populations and from previous literature, and also reflected constraints of the project itself. Therefore I suggest, for more accurate comparisons to the human AI index, to include more measurements that were not included in CAALM. These would include; body-mass index, lipoproteins, as well as blood pressure measurements. Secondly, an index should include epigenetic signatures, such as miRNA and DNA methylation marks. Although this study made reference to miRNA data, there was not sufficient epigenetic data collected from each animal to include as a biomarker or predictive outcome measure. Lastly, the addition of temporal data, sampled at multiple time points throughout development and aging, would add to the predictive power and capabilities of the CAALM index.

Future studies should continue to elaborate on the ability to represent cumulative effects of stress in animals using the CAALM index or a new comparable index. The CAALM or similar indices should be applied to more animal models of stress or trauma,

including aging populations to determine the longitudinal effects of stress. The overall objectives for future work should focus on illustrating the global or cumulative effects of stress on multiple biological systems, which may allow researchers to better predict risk for adverse outcomes compared to individual elements of stress pathophysiology.

4.7 Conclusions

Physiological systems are designed to adapt and respond to stressful environments in an effort to maintain allostasis. However, programming by ancestral stress may increase stress sensitivity and challenge allostasis to potentially provoke maladaptive responses with serious health consequences. For the first time we introduce an index for animals, CAALM, as a valid method to predict the burden of chronic stress by measuring the ability to maintain allostasis. We show that this cumulative measure could be used to predict stress resiliency and vulnerability and the risk of neural deficits. By contrast, we show that enrichment therapy can offset the adverse health outcomes linked to a high AL. As chronic stress, trauma and associated diseases create a rapidly growing economic burden to our society, refined detection strategies are of utmost importance for treatment and prevention. Future treatments using indices such as AI and CAALM could be adapted to include multiple levels of biological, psychological and social functions. Such optimized predictive and diagnostic strategies are critical to advance personalized medicine approaches to promote health in future generations.

Chapter 5

Experiment 4: The "Two-Hit" Hypothesis: Impaired Neurodevelopmental Outcome in an Animal Model of Maternal Stress and Inflammation as Measured by T_2-Relaxometry

5.1 Abstract

Growing evidence suggests that maternal infection and stress affect the developing foetal brain and lifetime mental health trajectories. One critical mechanism in the priming of brain development may be the transition of pro-inflammatory cytokines, particularly interleukin-1 beta (IL-1β), across the foetal-placental barrier. Other factors, such as stress, are known to trigger or synergistically aggravate existing inflammatory processes, thus exacerbating potential adverse effects on brain development. The purpose of this study was to investigate the effects of maternal stress and inflammation on offspring neurodevelopmental trajectories and mental health in later life. We used *in vivo* magnetic resonance imaging (MRI) to analyze maps of transverse relaxation times (T_2) to provide a quantitative measure of brain tissue changes. The findings show that exposure to IL-1β and stress during gestation is associated with reduced neuronal density in the prefrontal cortex, an area critically involved in higher cognitive functions. The reduced neuronal density may indicate disturbed neuronal pruning during brain development, which represents a prominent neuropathological finding in human neurodevelopmental disorders.

5.2 Introduction

Prenatal stress has been linked to a greater risk of psychopathologies in later life, such as schizophrenia, autism, anxiety and depression. A unifying concept that links prenatal stress to psychopathologies is a stress-associated increase in allostatic load (AL) in the direct offspring and in subsequent generations (McEwen, 2000a). Another emerging model in neuropsychology that may help explain susceptibility to psychopathologies and has been shown to increase AL is the "two-hit hypothesis" or the "multiple-hit" model. The "multiple-hit" theory was introduced by Nordling in 1953, and presents the concept that risk of incidence, such as cancer in the studies by Nordling, grows proportionally as the number of biologically relevant mutations or alterations increase (Nordling, 1953). This theory has recently become relevant in psychiatric diseases where multiple adverse events, that are biologically significant, accumulate during development and early life, and result in the development of schizophrenia-like diseases (Giovanoli et al., 2013, Meyer, 2013). There are multiple variations of the "two-hit" hypothesis and its connection to psychopathologies. For example, until recently, theories regarding schizophrenia indicated that the first hit may be genetic predisposition with the second hit being environmental (Bayer et al., 1999). Another study proposes that the first hit may be an early environmental insult, such as inflammation, during early development, with the second hit being subsequent latent or chronic inflammation (Meyer et al., 2011). Overall, the "two-hit" hypothesis indicates that two adverse events cumulate to cause allostatic overload and ensuing deficits are worse than those caused by an individual "hit".

A clinically relevant two-hit model is one where inflammation and stress both occur during pregnancy. It is possible that women experiencing high AL, such as stress during

pregnancy, need only another insult or "hit", such as catching a flu, to reach the tipping point that increases their offspring's vulnerability to neurological deficits (Olson et al., 2015). Inflammation, which may be caused by viral, bacterial, systemic infections or other immune events during pregnancy have been associated with an increased risk of psychopathologies, including schizophrenia, depression (Miller et al., 2009), bipolar disorder (Goldstein et al., 2009), and anxiety (Hou and Baldwin, 2012), among others (Khansari and Sperlagh, 2012). Specifically, studies have demonstrated that lipopolysaccharides (LPS), an endotoxin, as well as Polyinosinic-polycytidylic acid (Poly(I:C)), which is an immunostimulant associated with viral infection, cause neuroanatomical alterations and abnormalities in learning and behavior (Shi et al., 2003, Zuckerman et al., 2003, Golan et al., 2005, Meyer et al., 2006), with increased risk for schizophrenia and autism (Brown et al., 2000, Beversdorf et al., 2005, Feigenson et al., 2014).

In this study, the original disturbance applied is prenatal stress, which is known to synergistically aggravate inflammatory processes, thus exacerbating potential adverse effects on brain development. Mutual interactions between neuroendocrine stress response pathways and the immune system are well documented, and studies suggests that inflammation occurs downstream of stress in pregnancy (Arck, 2001, Coussons-Read et al., 2005, Butts and Sternberg, 2008). In this study, we chose to use IL-1β as the inflammatory "hit", the second hit, to exacerbate the stress response further. Because LPS and poly (I:C), most commonly used in inflammatory studies, stimulate the production and release of many pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, (Fortier et al., 2004, Meyer et al., 2006, Cunningham et al., 2007), suggesting that IL-1β injected on its own would simulate similar immediate effects.

We hypothesized that both prenatal stress and IL-1β administered together represent stressors that cumulatively increase AL in pregnant dams which will lead to more severe deficits in the offspring than any single event alone. We propose a "two-hit" hypothesis whereby each significant "hit" by a stressful or inflammatory insult may cumulatively challenge allostasis causing allostatic overload and subsequent consequences in the offspring. Additionally, the purpose of this study was to investigate the effects of maternal inflammation and stress on offspring neurodevelopmental trajectories and mental health in later life. Neurodevelopmental trajectories were investigated at 4 time points after weaning to establish a time course of brain maturation. The present study was based on *in vivo* T_2-relaxometry to provide a quantitative measure of brain tissue changes in an animal model of maternal stress and pro-inflammatory processes induced by IL-1β treatment.

5.3 Materials and Methods

5.3.1 Animals and Experimental Design

The study involved eighteen male Long-Evans hooded rats, bred and housed at the Canadian Centre for Behavioural Neuroscience. The animals were housed under a 12 h light/dark cycle with lights on at 7:30 AM. The room temperature was maintained at 20^{o}C with relative humidity at 30%.

Rats were divided into three groups; Control (n =6), Stress only (n=6), and IL-1β and stress combined with inflammation (IL-1β +S; n=6). Pregnant rat mothers were stressed on gestational day (G) 12-18 by restraint and forced swimming applied in a semi-random sequence (Yao et al., 2014). Groups of animals received daily intraperitoneal

injections of either 5 mg/kg IL-1β (IL-1β +S) or saline (Stress only) from G17 until delivery. Control mothers were administered a saline injection from G17 to delivery. At the age of postnatal day 30 (P30), P45, P60 and P90, the offspring were imaged using a 4.7 T Oxford magnet (Oxford, UK). Animals were weighed each day prior to imaging. Animals were euthanized on P90 after final imaging, and brain tissue was collected for histological analysis. All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Lethbridge Animal Welfare Committee.

5.3.2 MR Imaging

Imaging was performed using a 4.7 T 330 mm horizontal bore magnet (Oxford, UK) and a MR6000 console (MR Solutions, UK). A 2-RF coil system consisting of a quadrature birdcage transmit coil (10 cm in diameter, 10 cm in length; Morris Instruments, ON, Canada), a homebuilt receive-only butterfly coil (2 cm in diameter) and PIN diode switching unit (Morris Instruments, ON, Canada), was used. The imaging protocol consisted of: a) Localizer images (Spin-Echo- Repetition time (TR)/ Echo Time (TE) 700/16 ms, 0.2x0.2x1.5 mm^3) and b) T_2 measurements (TE/ TR 22, 26, 35, 60, 90/3000 ms, 0.23x0.23x2 mm^3) and c) T_2-weighted images (TE/TR 22/3000ms, 0.23x0.23x2 mm^3). Each imaging session lasted less than 60 minutes. All images were co-registered. T_2 values were extracted from regions of interest (ROI's) corresponding to the prefrontal cortex (PFC) from the fitted maps with Image J (NIH, USA). T_2 relaxometry was quantified during post-processing using a train of five images with their respective echo times. Images were fitted on a pixel by pixel basis to obtain the T_2 maps (Figure 5.2) using customized software in IDL (ITT, USA). T_2 data values diminish as a

function of reduced free water content and can be interpreted as elevated regional density of larger macromolecules or brain matter (Figure 5.1).

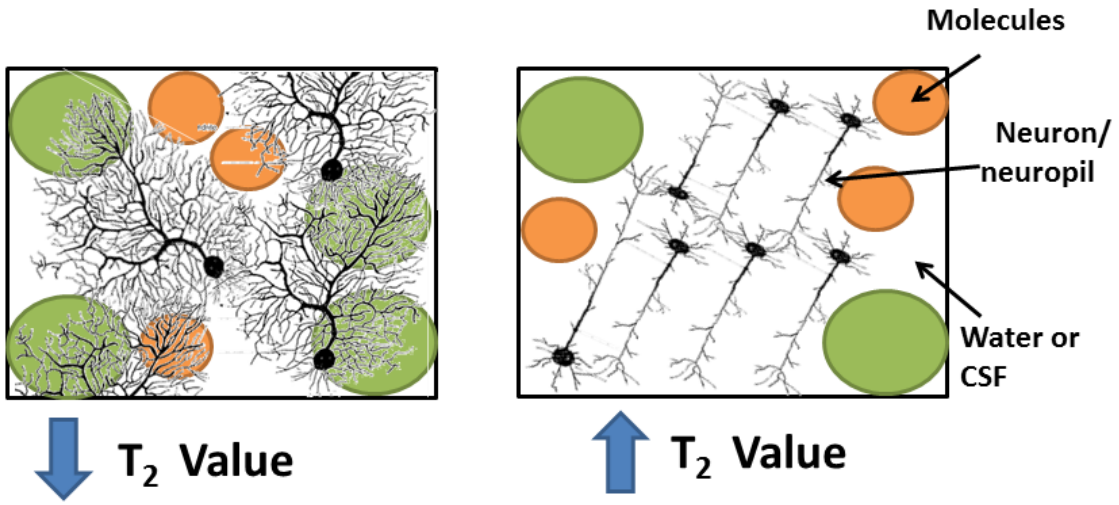

Figure 5.1. Illustration showing how neuronal density, macromolecules, water and cerebrospinal fluid (CSF) affect the T_2 value. T_2 relaxation values decrease as a function of reduced free water content and elevated regional density of larger macromolecules or brain matter.

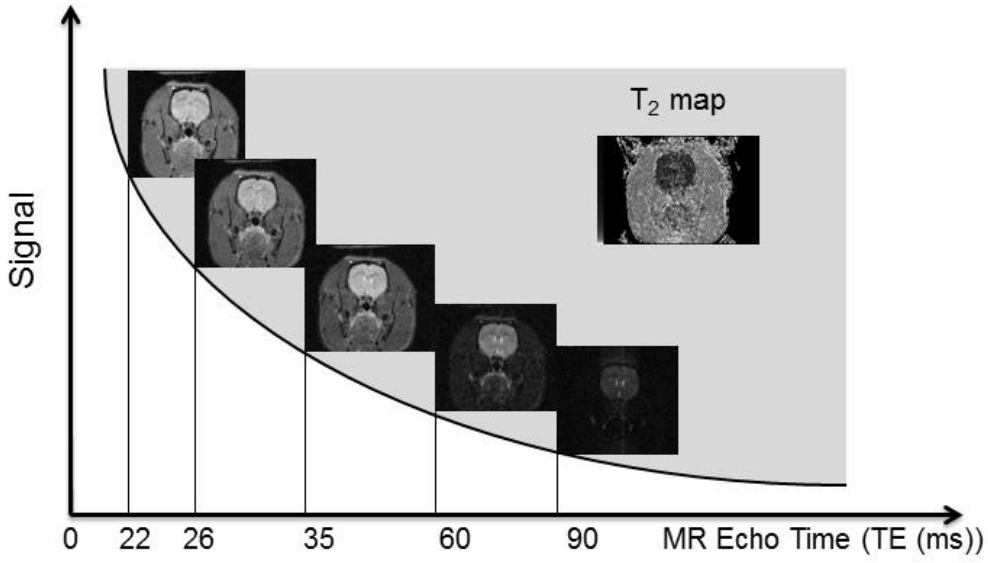

Figure 5.2. Graph illustrating the transverse decay of the MRI signal as a function of five different echo times used to create the T_2 map. T_2 relaxometry was quantified during post-processing where a train of 5 images, depicted here with their respective echo times, were fitted on a pixel-by-pixel basis to obtain the T_2 maps, shown top right.

5.3.3 Tissue Collection and Histology

At the age of 90 days rats were euthanized with an overdose of Euthanosol® (Merck, QC, Canada) and perfused transcardially with phosphate buffer solution (PBS; approximately 200 ml) followed by a transcardial injection of approximately 200 ml of 4% Paraformaldehyde (PFA; Sigma-Aldrich, MO, USA). Brains were extracted, stored in 4% PFA and refrigerated for 24 h and then transferred to sucrose solution for at least three days.

Brains were cut with a microtome in coronal sections at a thickness of 40 µm and 12 series interval. Sections were stored in 0.01 M PBS with a 1:1000 concentration of sodium azide. Every third series of sections was mounted and stained with cresyl violet to detect Nissl bodies. Photographs of the sections were captured using a motorized Zeiss AxioImager M1 microscope (Zeiss, Jena, Germany) at 1X magnification.

5.3.3.1 Mean Grey Value

Quantitative cytoarchitectonic analyses were performed for cresyl violet-stained sections corresponding to an ROI measuring 0.64 mm^2 on slides from 3.20 mm to -0.40 mm relative to Bregma using Image J V1.36 (http://rsb.info.nih.gov/ij/download.html). The "absolute grey level index" was ascertained as the measured parameter (Zilles et al., 1980). A step tablet was used (http://rsb.info.nih.gov/ij/download.html) to calibrate the optical density in the 8-bit images.

5.3.3.1 Cortical Thickness

Cortical thickness was measured on cresyl stained sections at Bregma level 3.20 mm and -0.04 mm using ImageJ software (NIH). Brain coordinates were chosen based on Paxinos and Watson (Paxinos, 1998). Three measurements were made that were

positioned medially, centrally and laterally along the cortex with respect to the corpus callosum, which are illustrated in Figure 5.6.

5.3.4 Statistical Analysis

Analyses were conducted using the statistical software StatView (version 5.0). Data were analyzed using parametric analysis of variance (ANOVA), followed by Fisher's LSD *post hoc* comparisons whenever a main effect or interaction reached statistical significance. Statistical significance was set at $p < 0.05$.

5.4 Results

5.4.1 Body Weight

Results for weight of animals across all groups at P30, P45, P60 and P90 are shown in Figure 5.3. There was a significant effect of treatment at P30 ($F(2,15)=4.470$, $p=0.0300$). IL-1β+S weight was significantly lower than the Controls ($p=0.0114$) and Stress group was lower than Controls, although this was not significant ($p=0.0503$). At P45, there was no overall effect of treatment, ($F(2,15)=3.389$, $p=0.0610$). However, *post hoc* tests revealed that the IL-1β+S group and the Stress group were significantly lower than Controls ($p<0.05$). At P60 body weight of IL-1β+S animals was still significantly lower than Controls ($p<0.05$), and at P90, the IL-1β +S group was lower but no longer significantly different ($p=0.06$).

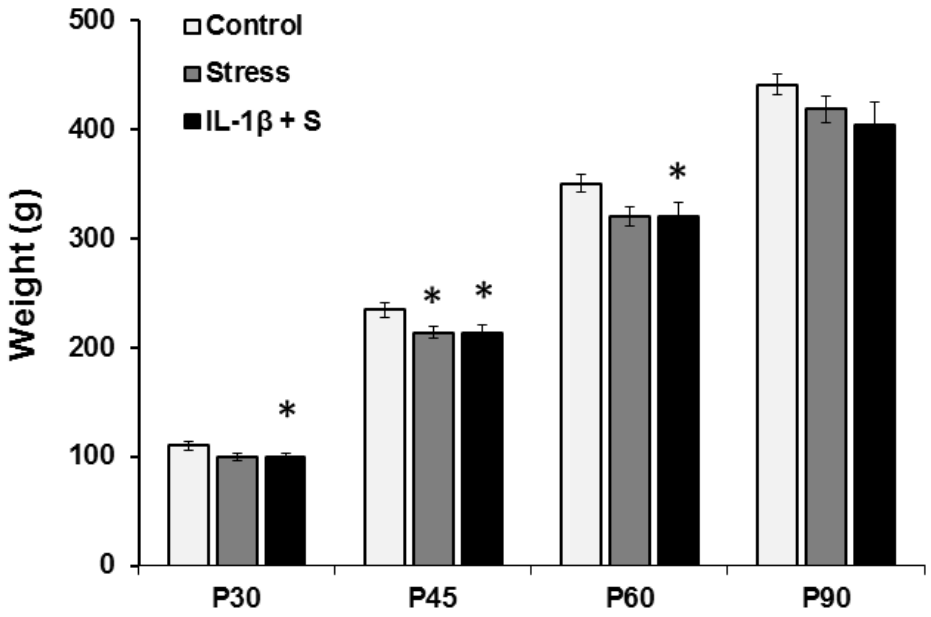

Figure 5.3. Body weight of offspring at P30, P45, P60 and P90. The Il-1β+S group was significantly lower at P30-P60. Stress caused a significantly lower body weight at P45. Values represent means +/- SEM. *denotes a significance (*p<0.05).

5.4.2 T_2-Relaxometry

Results for the mean T_2 value measured in areas corresponding to the PFC across all groups are illustrated in Figure 5.4. There was a significant difference between T_2 values in the Stress and IL-1B+S treated groups compared to Controls in the ROI drawn on a slice 1.70 mm relative to Bregma. Compared to Controls T_2 values were significantly higher ($p<0.05$) in the Stress group at P30, P45 and P60 with the largest effect at P30. The T_2 values were significantly larger in the IL-1β+S rats compared to the Stress group at P45 and was significantly larger than Controls at P45 and P60. At P90, the effect of IL-1β+S was related to major changes in tissue density as measured by cresyl violet histology.

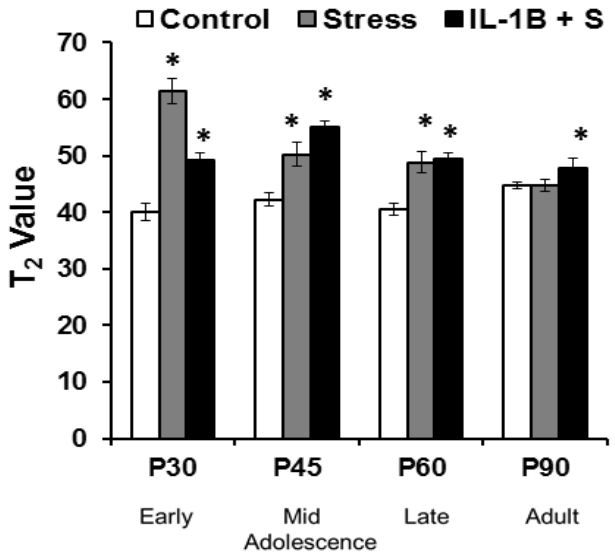

Figure 5.4. T_2 measurements at developmental milestones. T_2 values were significantly larger in the IL-1β+S than in animals treated with stress alone at P45 or control animals at P45 and P60. T_2 values were significantly increased in the stress group at P30, P45 and P60 with the largest effect at P30. At P90, only T_2 values in the IL-1β+S group were significantly higher. Values represent means +/- SEM. * denotes a significance (*$p<0.05$).

5.4.3 Mean Gray Value

The density of tissue in the ROI drawn on the coronal sections corresponding to Bregma level 3.20 mm (PFC) was significantly lower in the Il-1β+stress group compared to the control group ($p<0.05$). There were no differences in slices corresponding to Bregma level -0.40 mm (motor cortex; MC; Figure 5.5).

5.4.4 Cortical Thickness

Figure 5.6 illustrates the measurements (A) and differences in cortical thickness across groups (B). There were no significant differences in cortical thickness across groups in the slice corresponding to Bregma level -0.04 mm (MC). There was a significant effect of treatment on the slice corresponding to Bregma level 3.20 mm (PFC;

F(2,144)=4.395, p<0.05). Specifically, there was a significant decrease in cortical thickness in the medial measurement of the PFC of the IL-1β+S rats compared to Stress animals (p<0.05).

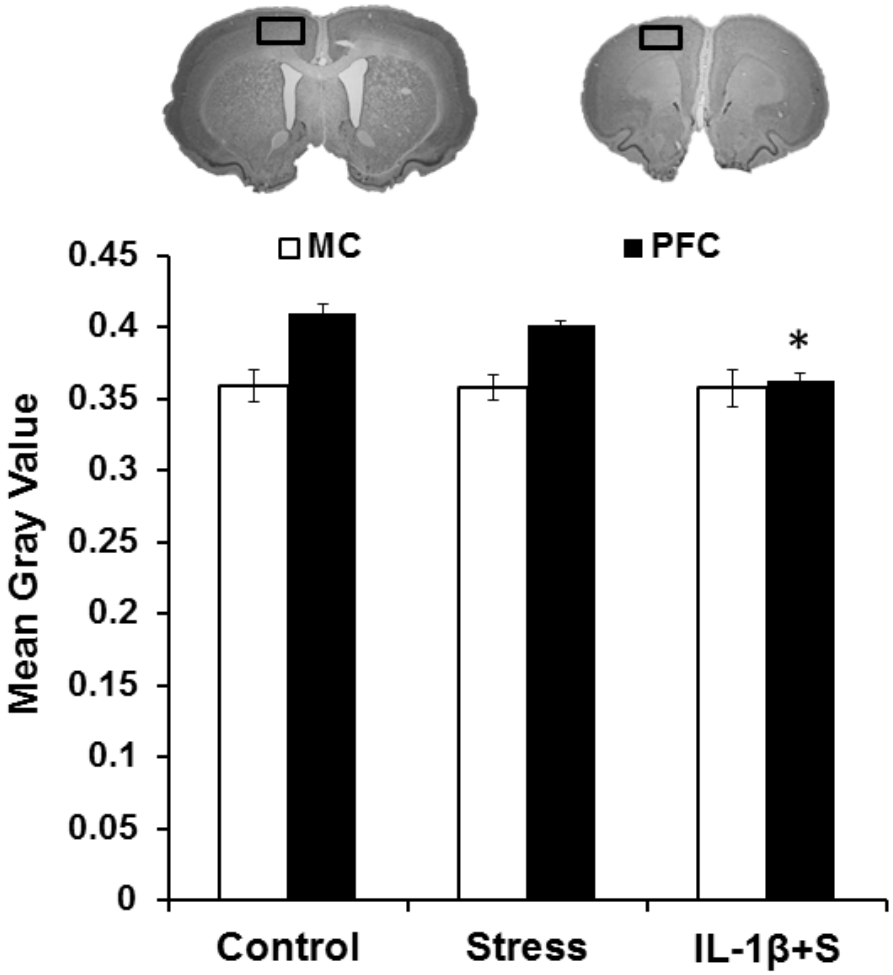

Figure 5.5. Graphic representation of the mean gray value (MGV) collected from the ROI drawn on the coronal sections corresponding to Bregma level 3.20 mm (prefrontal cortex, PFC) and Bregma level -0.40 mm (motor cortex). MGV in the PFC was significantly lower in the Il-1β+S group compared to the control group. There were no differences in slices corresponding to MC. Values represent means ± SEM. * denotes a significance (*p<0.05).

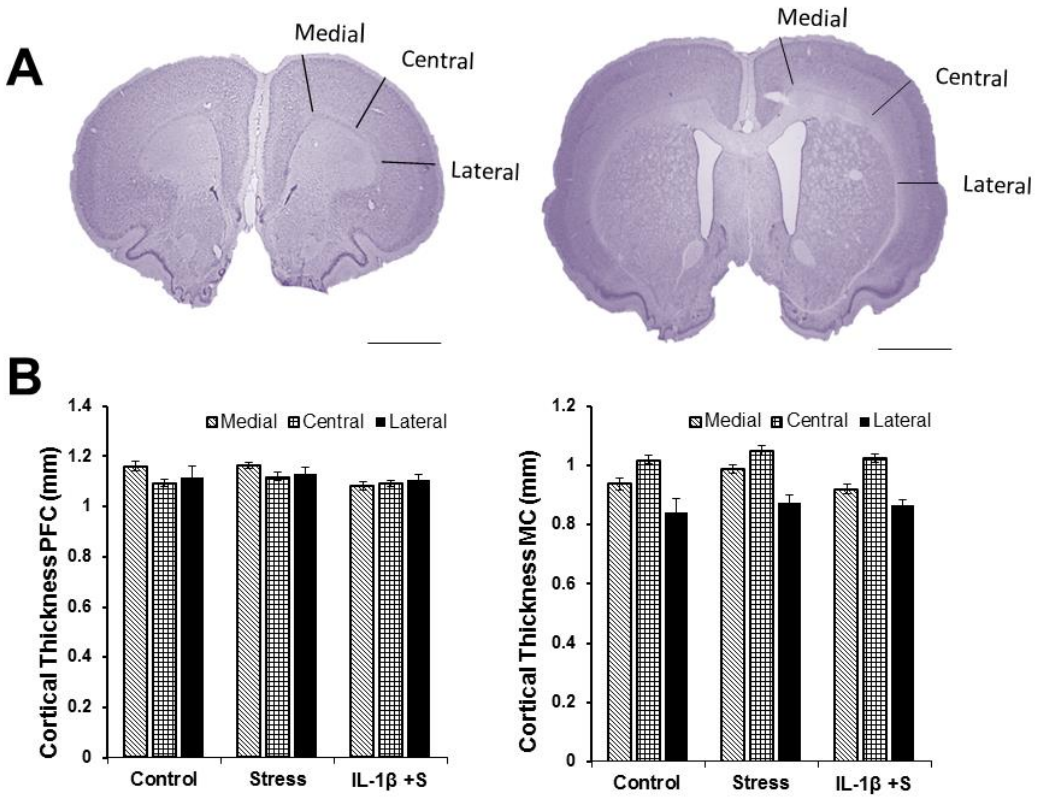

Figure 5.6. Graphic representation of the differences in cortical thickness measured medially, laterally and centrally (A). There were no significant differences in cortical thickness across all groups in the section corresponding to Bregma level -0.04 mm. There was a significant effect of treatment on the section corresponding to Bregma level 3.20 mm in the medial area of the prefrontal cortex of the IL-1β+S rats compared to stressed animals. Values represent means ± SEM. * denotes a significance (*$p<0.05$).

5.5 Discussion

Prenatal stress, in particular maternal stress, has been linked to an increased risk of neurodevelopmental disorders and psychopathologies, including schizophrenia, autism, and hyperactivity (van Os and Selten, 1998, Koenig et al., 2002, Ronald et al., 2010). Related consequences arise following prenatal immune activation, suggesting that these deficits of maternal stress and infection or inflammation may influence common pathways (Boksa, 2010, Howerton and Bale, 2012, Hsiao and Patterson, 2012). We

predicted that a combination of prenatal stress and inflammation, or the "two-hit" model, would lead to more severe deficits than a single hit of stress alone. The results from this study confirm the hypothesis that multiple hits during pregnancy synergistically exacerbate the consequences of a single stressor with regards to offspring brain development.

The present study presents three main findings. First, prenatal inflammation, in combination with prenatal stress, reduced body weight during early and late adolescence and in adulthood. Second, T_2 values were altered across development, with both stress and stress plus inflammation resulting in higher T_2 values. Third, increased T_2 values were only seen in the combined stress and inflammation group. Lastly, T_2 measurements in MRI were confirmed by reduced neural density in adulthood. Overall, the present findings demonstrate synergistic effects of stress with pro-inflammatory processes disturbing trajectories of normal brain development.

The results of the present study revealed decreased body weight gain due to a combined impact of stress and inflammation, which was evident from early to late adolescence. Stress has long been recognized as a potential cause for intrauterine growth restriction in animals and humans (Barlow et al., 1978, Grimm and Frieder, 1987, Weinstock et al., 1988, Veenendaal et al., 2013), whereas prenatal inflammation does not seem to strongly affect offspring morphogenic parameters (Golan et al., 2005). However, Golan et al. (2005) focused on prenatal inflammation in mice and its consequences during the first month of life. The present findings suggest that prenatal stressors may affect offspring weight not in the early postnatal period but later in life. Growth restriction due to prenatal insults have been associated with anatomical and functional deficiencies of the developing brain (Kramer et al., 1990). Evidence for abnormal brain development in

stressed animals, especially those affected by two hits, also derives from findings of T_2 relaxometry.

Both prenatal stress and inflammation alone have been shown to cause alterations in brain anatomy and function (eg. Golan et al., 2005; Meyer et al., 2006; Mychasiuk et al., 2012). In the present study, MRI T_2 relaxometry was used longitudinally to detect changes during development in the brains of offspring treated with prenatal stress and inflammation. T_2 relaxometry is an MRI method which utilizes the quantitative data provided from T_2 maps. The T_2 value is affected by water mobility and has been suggested to represent a particularly valuable marker of brain tissue changes in health and disease states by assessing "free" tissue water content (Kalviainen et al., 1995, Laakso et al., 1996, Raz et al., 2005). T_2 relaxation measurements have been used previously to detect tissue abnormalities in Alzheimer's disease (Laakso et al., 1996), epilepsy (Kalviainen et al., 1995) and aging (Raz et al., 2005). Only one other study has investigated the effects of prenatal stress on T_2-weighted MRI signals, and found an increased hippocampus T_2 time at approximately 120 days of age (Lui et al., 2011a), indicating decreased tissue density.

In the present study, T_2 relaxometry was measured from P30-P90 which is analogous to early childhood to adulthood in humans (Kolb et al., 2012), to detect changes in the prefrontal cortex, which is a central structure to the regulation of the stress response and mental health. Our results found that prenatal stress along with inflammation induced by maternal IL-1β treatment caused the most significant impairments as measured by a reduced tissue density at 45 days of age with lasting impairments in adulthood. The effect in the stress group was largest at P30 but diminished with older age. In a healthy developing rat brain, excessive synapse

production occurs between P0-P40, while cortical (more posterior or sensory regions) synaptic pruning occurs from P25-P100, while prefrontal cortex (more anterior) pruning (synapse elimination) occurs from P40-P160 before myelination occurs from P10-P180 (Kolb et al., 2012).

The present data suggest that the impact of prenatal stress on brain development is most recognizable at P30, whereas combined IL-1B+stress treatment caused the largest effect at P45. This observation indicates that different types of stressors may affect different stages of brain development, i.e., stress may largely influence synapse production, and inflammation may influence synaptic pruning. Overall, the present results confirm previous studies demonstrating reduced neuronal density, or other mechanisms leading to functional deficits, in the prefrontal cortex after stress (Mychasiuk et al., 2012) and inflammation (Zhou, 2015), which may occur as a consequence of abnormal synapse generation or pruning. The present MRI findings were confirmed by histology in adulthood, indicating that exposure to stress and IL-1β during gestation caused a decrease in neuronal density in the prefrontal cortex that lasted until adulthood. Thus, multiple hits during pregnancy are more likely to cause lasting deficits than any single stressor alone.

Although the present study did not investigate the exact mechanisms of abnormal neuronal development, multiple studies have indicated roles of glucocorticoids and cytokines in neural development. In addition to their prominent function as regulators of the immune system, cytokines also modulate neuronal survival (Marx et al., 2001) and differentiation (Potter et al., 1999), as well as dendrite development and complexity (Gilmore et al., 2004), thereby exerting multiple effects on the developing brain. Maternal inflammation has been shown to increase pro-inflammatory cytokines in fetal brains along with other molecules responsible for neuronal organization such as the glycoprotein

reelin (Meyer et al., 2006), possibly altering neuronal connectivity and pruning. Moreover, glucocorticoids, released as early as gestational day 13, have been shown to act on neuronal maturation and all stages of the cell cycle, including replication and even programmed cell death (Flagel et al., 2002, Harris and Seckl, 2011). It can be expected that abnormal levels of glucocorticoids and cytokines during critical periods of early brain development *in utero* may adversely affect neurodevelopmental trajectories. This ultimately contributes to increased susceptibility to complex brain disorders with a developmental origin, such as schizophrenia and autism.

5.5.1 Limitations

There are a few limitations of this study in need of discussion. First, one limitation to this study is the lack of animals which were treated with prenatal IL-1β only. This group of rats was included in the larger cohort, however, they were not available for an imaging study. It could be asked whether the effects seen in the IL-1β+S group is due to the effects of IL-1β only, and if IL-1β has a more powerful impact on brain development than prenatal stress. In addition to the imaging studies, behavioural data were also collected from all animals. These data are mentioned by Olson et al. (2015) and further details will be published elsewhere. However, it should be mentioned that the behavioural deficits in the offspring were largest in the IL-1β+S group.

The second limitation to the present study is its focus on males. Sex differences are a significant factor in onset, symptomology, and treatment of abnormal brain development. For instance, boys present with attention deficit hyperactivity disorder 2–3 times more frequently than girls, and autism has a rate of 4–5 times higher in boys than in girls (Froehlich et al., 2007, Bloom et al., 2013).

Lastly, the contributing mechanisms by which exposures such as stress and inflammation program brain developmental trajectories in the present study remain to be determined. Additional brain tissue samples from these animals were collected and will be analyzed in the future. It is hypothesized that the mechanisms of stress and inflammatory cascades may involve converging pathways and will likely include alterations in the fetal placental barrier. This should provide a better understanding of how maternal stress and inflammation contributes to offspring development and maturation.

5.6 Conclusions

Growing evidence suggests that stress and maternal infection affect the developing fetal brain and future mental health trajectories. The combination of inflammation and stress may be key to understanding the prevalence and susceptibility to psychopathologies and affective disorders. Critical mechanism in the priming of brain development may be the transition of pro-inflammatory cytokines, particularly, IL-1β, and glucocorticoids across the fetal-placental barrier. Here we show that multiple hits by a combination of maternal stress and inflammation, both which individually cause neuronal deficits, may aggravate the effects, increasing allostatic load, and cause lasting changes. Overall, results reflect disturbed neuronal development during maturation, which represents a prominent neuropathological finding in human neurodevelopmental disorders including autism and schizophrenia. Moreover, the present findings also confirm T_2-relaxometry as a valid method for determining the effects of inflammation and stress during pregnancy on the developing brain with implications for the origins of mental illness.

Chapter 6

6.1 Summary

The objectives of this thesis were to investigate the manifestations of ancestral prenatal stress across three generations in a rat model and to create an allostatic load index to assess and interpret the consequences on stress resiliency and disease vulnerability. Moreover, we designed a study to test how stress, combined with inflammation, may synergistically influence offspring development. Furthermore, this thesis investigated if postnatal enrichment is able to attenuate the effects of prenatal stress transmitted through the maternal ancestral lineage.

Findings revealed that ancestral prenatal stress, either occurring once in the F0 parental generation (TPS) or occurring repeatedly in each generation (MPS) results in drastic alterations in many systems compared to unstressed controls. Additionally, our results demonstrated that EE reduced the adverse consequences of ancestral prenatal stress on hypothalamic-pituitary-adrenal (HPA) axis activation, central nervous system (CNS) morphology, epigenetic regulators and related behaviours. Using a new allostatic load index developed for animal models, we determined that cumulative ancestral stress has a higher overall allostatic load than transgenerational stress, and EE decreases this score in both models. Moreover, we developed a novel tool, the cumulative animal allostatic load measure (CAALM) index, which proved useful in predicting future health deficits induced by ancestral stress along with verifying the positive impact of EE.

Lastly, observations show that endocrine and behavioural impairments linked to prenatal stress can be aggravated by additional insults, such as a second hit by inflammation, with consequences that last into adulthood. Summarized below are the neuromorphological, physiological, behavioural, and epigenetic changes programmed

from ancestral stress, and the effects of stress and inflammation in one generation. An interesting point of discussion arising from the topic of allostatic load and stress is the concept of individual resilience and vulnerability to related deficits, which will be discussed afterward. Lastly, future work and final conclusions will be presented.

6.2 Adverse Effects of Ancestral Stress and Their Mitigation by Enrichment Therapy

According to our hypothesis, the present results indicate that stress response can indeed be programmed generationally, and can be alleviated by EE in animals (Figure 6.1). In this study, stress was found to have a significant effect on the number of glucocorticoid receptors (GRs) in the hippocampus, which was accompanied by an increase in glucocorticoids, or corticosterone in this study. Our results are in line with other studies that have found lower GR density in the hippocampus (HPC) (Henry et al., 1994, Maccari et al., 1995) and associated changes in circulating glucocorticoid levels due to prenatal stress. Changes in hypothalamic-pituitary-adrenal (HPA) axis functionality and epigenetic alterations due to the programming of maternal stress, led to many morphological changes in cortical and subcortical brain regions, including the corticospinal tract (CST).

The present study investigated the influence of ancestral stress on neuromorphology in three distinct regions of the brain. These areas included the HPC, which in ancestrally stressed animals displayed lower GR density, the prefrontal cortex (PFC) and the parietal cortex, both displaying reduced cortical thickness and reduced dendritic complexity as measured by the mean number of intersections. These morphological changes have potentially complex consequences on behaviour and endocrinology. For example,

reduced thickness of the PFC may significantly affect higher cognitive functions. The PFC is particularly important in higher-order executive functions, sensory perception and social reasoning (Kolb et al., 2012). Moreover, studies have investigated the relation between PFC function and HPA axis activation and concluded that the PFC is a part of the regulatory circuitry involved in the stress response (Kern et al., 2008). The decrease in cortical thickness and density in the present study may therefore diminish the cortical negative feedback on HPA axis activity and contribute to the observed slight increase in CORT levels. Furthermore, ancestral stress decreased dendritic length or complexity of neurons in the parietal cortex. Lastly, and possibly most intriguing, ancestral stress significantly decreased fiber density in the thoracic region of the CST.

Programming of the developing brain and HPA-axis with prenatal stress leading to neuromorphological adjustments resulted in changes in motor activity and affective behaviour similar to those seen in previous studies (Holmes et al., 2006, Lupien et al., 2009, Harris and Seckl, 2011). Results from the open field and the elevated plus maze tests showed that animals that descended from ancestral stress lineages, both TPS and MPS, were more vulnerable to anxiety-like as well as hyperactive behaviours. These findings agree with current literature that investigated PS (Li et al., 2007, Metz, 2007, Field et al., 2008, Gatzke-Kopp, 2011).

The mainly adverse consequences of ancestral stress were mitigated by exposing animals to beneficial postnatal experience in an EE. EE rats spent more time in the open arms of the elevated plus maze, suggesting decreased anxiety-related behaviour in EE-treated groups. Motor behaviour as assessed using the skilled walking task showed that ancestral stress led to motor deficits. This deficit was also improved with enrichment. The neuromorphological and behavioural consequences of ancestral stress were linked to

altered epigenetic regulation involving microRNAs (miRNAs). The potentially mitigating role of miRNAs was found to possibly play a role in influencing cortical neuromorphology, CST development, as well as associated locomotor and affective behaviours.

It is apparent that the mechanism through which stress contributes generationally involves a complex interaction between genes and environment, which is mediated by epigenetic regulators (Meaney, 2010, Dunn et al., 2011a). Accordingly, this study has found the transfer of generational stress include the programming of the HPA axis and altered behaviour linked to miRNA alterations or epigenetic modifications. The results revealed that functionally appropriate miRNAs were expressed that are associated with the respective phenotype of each group. MiRNAs that were differentially expressed in the PFC due to stress were found in the literature to be relevant biomarkers in psychiatric diseases such as depression (Wan et al., 2015), and schizophrenia (Kocerha et al., 2009), as well as memory impairments (Griggs et al., 2013). Moreover, enrichment therapy in all cases reversed the effect of stress by either up-regulating or down-regulating the miRNA expression in the opposite direction as had been induced by ancestral stress. However, although approximately the same numbers of miRNAs were activated due to TPS and MPS, the number of miRNAs with respect to enriched conditions differed in the enriched TPS and MPS groups. The MPS group had three times the number of miRNAs differentially expressed compared to TPS, possibly indicating that the burden of MPS causes more miRNA activation in order to alleviate the stress effects by enrichment therapy.

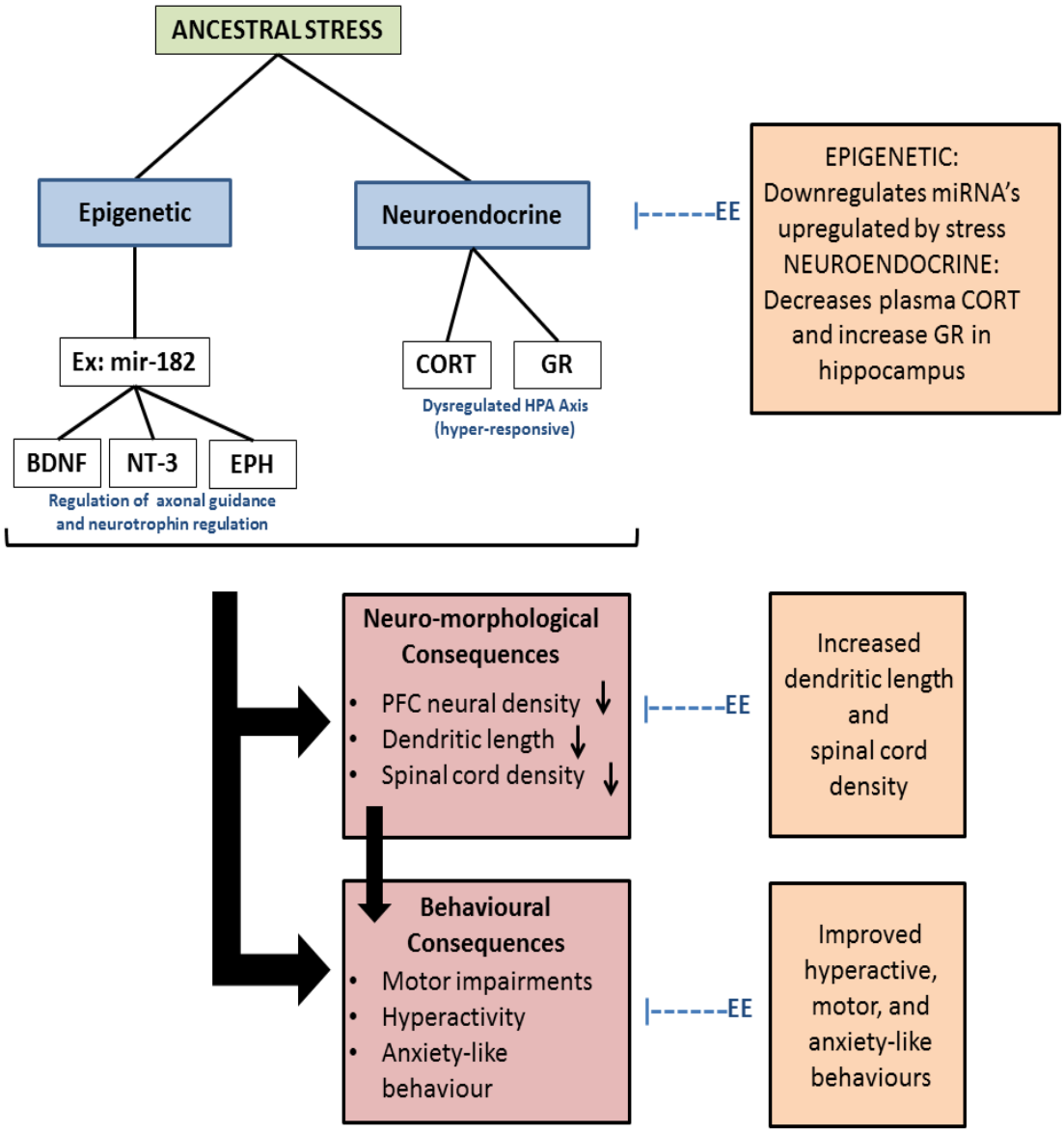

Figure 6.1. An illustration of the overall effect of ancestral stress and mitigation by enrichment. Ancestral stress leads to changes in the epigenome and neuroendocrine system, which leads to an overall change in central nervous system morphology and rat behaviour. Enrichment acts on each level in an attempt to reverse the effects of stress. Corticosterone (CORT), glucocorticoid receptors (GR), brain derived neurotrophin factor (BDNF), neurotrophin-3 (NT-3), ephrin (EPH), pre frontal cortex (PFC).

6.3 The Two-Hit Hypothesis: Exacerbating Stress with Inflammation

Just as multiple generations of cumulative stress seem to exacerbate the stress response and overall allostatic load, inflammation during pregnancy, in combination with stress, also has this effect. In this study, we examined this proposition by combining prenatal stress with an inflammatory stressor induced by injections of the pro-inflammatory interleukin IL-1β.

The present study involved *in vivo* magnetic resonance imaging (MRI) to gain clinically relevant insights into further mechanisms of stress-related changes in brain plasticity. The experiments used the analysis of maps of transverse relaxation times (T_2) to provide a quantitative measure of brain tissue changes (Kalviainen et al., 1995, Laakso et al., 1996). T_2 relaxometry proved useful in determining the effects of ancestral stress and inflammation on brain development. The MRI findings indicate that exposure to IL-1β and stress during gestation caused a decrease in neuronal density in the PFC beginning in infancy and lasting into adulthood, as validated by histological examination. This observation may indicate disturbed neuronal pruning during early brain development, which represents a prominent neuropathological finding in human neurodevelopmental disorders. This concept of stress acting in aggregation with other insults is an area of research that is in the early stages, and worthy of future research.

6.4 Development of the CAALM Index: Insights in to Individual Vulnerability and Resilience

The degree of interactions between different pathways and the outcomes of ancestral stress are complex, and are based on potentially accumulative, oppositional or antagonistic mechanisms among individual animals. Due to the complexity and non-

linearity of the consequences of ancestral stress, we created an allostatic load index, similar to that used in humans (Juster et al., 2010), to determine the cumulative burden of stress. We used our established ancestral stress model to test the cumulative animal allostatic load measure (CAALM) index and found that cumulative ancestral stress in the MPS group led to higher allostatic load values as opposed to transgenerational stress lineage or non-stress control conditions. The results also showed that rats with higher multivariable CAALM scores had an increased vulnerability to lower neuronal density. Moreover, enrichment therapy reduced the allostatic load as indicated by single and combined allostatic load scores. Surprisingly, some animals were variable in terms of their allostatic load scores independent of their treatment, indicating a role for individual resilience or vulnerability in determining the CAALM index outcomes.

Stress resilience and stress vulnerability is influenced by sex (Van den Hove et al., 2013) and environmental factors, such as prenatal and postnatal experiences (Bergstrom et al., 2008, Castro et al., 2012). Vulnerability refers to the inability to adapt to stressors that can become persistent states of stress, whereas resilience refers to the adaptive mechanism where one perceives adversity as minimally threatening, developing adaptive physiological and psychological responses (Franklin et al., 2012). In the present studies, although the transgenerational and multigenerational stress lineages showed similar trends with respect to endocrine, behavioural, epigenetic and morphological modifications, the CAALM index indicated that some animals, unique in their groups, exhibit either higher resilience or higher vulnerability to stress. For example, two multigenerational stress rats showed allostatic load scores below 2, whereas the remainder of the group were above a score of 5. The two animals of lower allostatic load scores had arguably acquired or inherited traits that led to decreased CAALM scores. Such

individual differences point to an intrinsic cause to the variation in the severity of deficits due to stress, which may be based on ancestral experiences, as supported by the present line of research.

Individual differences in stress resilience and stress vulnerability may stem from neurochemical, neuropeptide and hormonal mediators, along with behavioural traits of the psychobiological response of stress coping (Charney, 2004). The question of why some individual animals gain stress resilience and others do not addresses a timely topic which may provide new avenues for the treatment of stress-associated mental health disorders and complex diseases. The etiology of many psychopathologies and addiction remain largely unknown and an individual's vulnerability to these conditions has become a focus area of current research. Our approach using the CAALM index allows for the individual assessment of stress resilient and stress vulnerable traits and offers a promising potential for translating the biomarkers and mechanisms of stress research in animal models to the clinical environment.

6.5 Potential Limitations of the Present Studies

There are some potential limitations to the interpretation of the data presented in this thesis. First, the present research only included male animals, and as gender appears to be a critical factor with known sex biases in onset, symptomology, and treatment of stress-associated diseases, it would be important to investigate both male and female offspring in future research. Second, the ancestral studies included in this thesis only consider the third generation offspring. It would be useful to compare the effects size seen in the F3 generation to F1 and F2 generation phenotypes in order to understand the gradual impact of stress across generations. However, both of these limitations will be

addressed ultimately as this study is part of a larger study which addresses sex differences and the effects of stress in F0-F4 generations. These aspects of research will be able to further elaborate on stress resiliency and stress vulnerability traits that may develop across generations. Third, a limitation is that the development of the CAALM index was based on retrospective data, where the biomarkers were chosen after data collection was completed. The measures of allostatic load were designed to summarize dysregulation across multiple physiological systems induced by stress. Biomarkers were selected based on theoretical grounds from those assessed in clinical populations and from those presented in previous literature and were constrained within the project itself. I suggest, for more accurate comparisons to the human allostatic load index, to include a larger variety of biomarkers of stress for future studies involving CAALM.

Lastly, with respect to the use of animal models in general, and more specifically, to investigate ancestral stress and environmental enrichment, it is important to note the challenge in translating results to human populations. For example, in our studies, our control animals are housed in standard laboratory environments, which may be considered impoverished conditions. The enriched environments may just be simulating what animals encounter in the wild, and thus when interpreting these results, this factor should be taken in to consideration.

6.6 Future Work

The present data provide insights into the mechanisms of transgenerational programming by stress with potential implications to the human population. Comparative studies will be possible in the future based on transgenerational human cohorts such as the Dutch Famine Birth Cohort (Veenendaal et al., 2013), Project Ice Storm from Quebec

(Laplante et al., 2008) and studies of the World Trade Center attacks trauma (Yehuda et al., 2005) based on studies of mothers exposed to adverse experiences during pregnancy and their children along with follow-up studies considering the multigenerational impacts of stress on future generations, including holocaust survivors' offspring (Dekel et al., 2013) and traumatic stress (Roth et al., 2014, Saile et al., 2014). Except for the Dutch Famine Birth Cohort, the above cohorts are based on prospective study design and therefore will take time to reach three generations. Animal models offer particular value for the isolated investigation of stress, transgenerational programing of disease and associated predictive biomarkers. Animal studies allow mechanistic studies and manipulations of biomarkers to identify potential therapeutic approaches, however, an iterative process of knowledge translation to the human cohorts will be the most critical step for the development of new predictive, diagnostic and therapeutic strategies in personalized medicine approaches.

Additionally, it is important to continue the investigation of longitudinal health trajectories in the transgenerational animal cohort to learn about health risks from early development to old age in relation to predictive and causative biomarkers. Moreover, future studies should continue to elaborate on the ability to represent cumulative effects of stress in animals using the CAALM index or a new comparable index. The CAALM or similar indices should be applied to other animal models of stress or trauma, including aging populations to determine the longitudinal effects of stress.

6.7 Conclusions

Growing evidence suggests that many psychopathologies and neurodevelopmental disorders have their origins, at least in part, in a troubled prenatal environment. The

present findings and previous data suggest that stressful experiences during pregnancy can cause transgenerationally heritable phenotypes with altered endocrine status, modified neuroplasticity and contribute to associated pathological conditions. However, because the effects of prenatal and transgenerationl stress are complex, a method of quantifying the cumulative and often antagonistic effects of stress was introduced.

The concept that ancestral stress may influence disease etiology for generations to come is an important revelation in regards to the increasing rate of neurological and psychiatric diseases in our society and a rapidly growing exposure to environmental risks, such as domestic violence and war, poverty and pollutants. Further, the knowledge that environmental enrichment can effectively reverse some detrimental effects of ancestral stress will need to be seriously considered in future approaches for prevention, intervention and therapies in health management. It is my hope that the awareness and warnings seen with regards to prenatal alcohol and nicotine exposure, may begin to include those of prenatal stress as well.

7. References

Ahima RS, Bjorbaek C, Osei S, Flier JS (1999) Regulation of neuronal and glial proteins by leptin: implications for brain development. Endocrinology 140:2755-2762.

Allen PJ (2012) Creatine metabolism and psychiatric disorders: Does creatine supplementation have therapeutic value? Neurosci Biobehav Rev 36:1442-1462.

Ambeskovic M (2013) Transgenerational programming of brain and behaviour by prenatal stress.

Ambeskovic M, Soltanpour N, Falkenberg E, Zucchi F, Kolb B, Metz G (2015) Ancestral exposure to stress generates new behavioural traits and a functional hemispheric dominance shift. Cereb Cortex.

Andersen SL (2003) Trajectories of brain development: point of vulnerability or window of opportunity? Neurosci Biobehav Rev 27:3-18.

Arck PC (2001) Stress and pregnancy loss: role of immune mediators, hormones and neurotransmitters. Am J Reprod Immunol 46:117-123.

Arnsten AFT (2009) Stress signalling pathways that impair prefrontal cortex structure and function. Nature Reviews Neuroscience 10:410-422.

Babb JA, Carini LM, Spears SL, Nephew BC (2014) Transgenerational effects of social stress on social behavior, corticosterone, oxytocin, and prolactin in rats. Horm Behav 65:386-393.

Babenko O, Kovalchuk I, Metz GA (2012) Epigenetic programming of neurodegenerative diseases by an adverse environment. Brain Res 1444:96-111.

Babenko O, Kovalchuk I, Metz GA (2015) Stress-induced perinatal and transgenerational epigenetic programming of brain development and mental health. Neurosci Biobehav Rev 48:70-91.

Baldini S, Restani L, Baroncelli L, Coltelli M, Franco R, Cenni MC, Maffei L, Berardi N (2013) Enriched Early Life Experiences Reduce Adult Anxiety-Like Behavior in Rats: A Role for Insulin-Like Growth Factor 1. J Neurosci 33:11715-11723.

Barlow SM, Knight AF, Sullivan FM (1978) Delay in postnatal growth and development of offspring produced by maternal restraint stress during pregnancy in the rat. Teratology 18:211-218.

Bayer TA, Falkai P, Maier W (1999) Genetic and non-genetic vulnerability factors in schizophrenia: the basis of the "two hit hypothesis". J Psychiatr Res 33:543-548.

Belz EE, Kennell JS, Czambel RK, Rubin RT, Rhodes ME (2003) Environmental enrichment lowers stress-responsive hormones in singly housed male and female rats. Pharmacol Biochem Behav 76:481-486.

Benediktsson R, Calder AA, Edwards CR, Seckl JR (1997) Placental 11 beta-hydroxysteroid dehydrogenase: a key regulator of fetal glucocorticoid exposure. Clin Endocrinol (Oxf) 46:161-166.

Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I (2001) Controlling the false discovery rate in behavior genetics research. Behav Brain Res 125:279-284.

Bennett EL, Diamond MC, Krech D, Rosenzweig MR (1964) Chemical and Anatomical Plasticity Brain. Science 146:610-619.

Bergstrom A, Jayatissa MN, Mork A, Wiborg O (2008) Stress sensitivity and resilience in the chronic mild stress rat model of depression; an in situ hybridization study. Brain Res 1196:41-52.

Beversdorf DQ, Manning SE, Hillier A, Anderson SL, Nordgren RE, Walters SE, Nagaraja HN, Cooley WC, Gaelic SE, Bauman ML (2005) Timing of prenatal stressors and autism. J Autism Dev Disord 35:471-478.

Bindu B, Alladi PA, Mansooralikhan BM, Srikumar BN, Raju TR, Kutty BM (2007) Short-term exposure to an enriched environment enhances dendritic branching but not brain-derived neurotrophic factor expression in the hippocampus of rats with ventral subicular lesions. Neuroscience 144:412-423.

Bizik G, Picard M, Nijjar R, Tourjman V, McEwen BS, Lupien SJ, Juster RP (2013) Allostatic load as a tool for monitoring physiological dysregulations and comorbidities in patients with severe mental illnesses. Harv Rev Psychiatry 21:296-313.

Bloom B, Jones LI, Freeman G (2013) Summary health statistics for U.S. children: National Health Interview Survey, 2012. Vital Health Stat 10 1-81.

Boksa P (2010) Effects of prenatal infection on brain development and behavior: a review of findings from animal models. Brain Behav Immun 24:881-897.

Bosch OJ, Musch W, Bredewold R, Slattery DA, Neumann ID (2007) Prenatal stress increases HPA axis activity and impairs maternal care in lactating female offspring: implications for postpartum mood disorder. Psychoneuroendocrinology 32:267-278.

Bracht T, Schnell S, Federspiel A, Razavi N, Horn H, Strik W, Wiest R, Dierks T, Muller TJ, Walther S (2013) Altered cortico-basal ganglia motor pathways reflect reduced volitional motor activity in schizophrenia. Schizophr Res 143:269-276.

Brennan AR, Arnsten AF (2008) Neuronal mechanisms underlying attention deficit hyperactivity disorder: the influence of arousal on prefrontal cortical function. Ann N Y Acad Sci 1129:236-245.

Brooks KP, Gruenewald T, Karlamangla A, Hu P, Koretz B, Seeman TE (2014) Social relationships and allostatic load in the MIDUS study. Health Psychol 33:1373-1381.

Brosnan JT, Brosnan ME (2007) Creatine: endogenous metabolite, dietary, and therapeutic supplement. Annu Rev Nutr 27:241-261.

Brown AS, van Os J, Driessens C, Hoek HW, Susser ES (2000) Further evidence of relation between prenatal famine and major affective disorder. Am J Psychiatry 157:190-195.

Buitelaar JK, Huizink AC, Mulder EJ, de Medina PG, Visser GH (2003) Prenatal stress and cognitive development and temperament in infants. Neurobiol Aging 24 Suppl 1:S53-60; discussion S67-58.

Bustamante C, Henriquez R, Medina F, Reinoso C, Vargas R, Pascual R (2013) Maternal exercise during pregnancy ameliorates the postnatal neuronal impairments induced by prenatal restraint stress in mice. Int J Dev Neurosci 31:267-273.

Butts CL, Sternberg EM (2008) Neuroendocrine factors alter host defense by modulating immune function. Cell Immunol 252:7-15.

Caldji C, Tannenbaum B, Sharma S, Francis D, Plotsky PM, Meaney MJ (1998) Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proc Natl Acad Sci U S A 95:5335-5340.

Cannon WB (1932) The wisdom of the body: W. W. Norton and Co., Inc., New York.

Cao W, Duan J, Wang X, Zhong X, Hu Z, Huang F, Wang H, Zhang J, Li F, Zhang J, Luo X, Li C-Q (2014a) Early enriched environment induces an increased conversion of proBDNF to BDNF in the adult rat's hippocampus. Behav Brain Res 265:76-83.

Cao X, Laplante DP, Brunet A, Ciampi A, King S (2014c) Prenatal maternal stress affects motor function in 5(1/2)-year-old children: project ice storm. Dev Psychobiol 56:117-125.

Castro JE, Diessler S, Varea E, Marquez C, Larsen MH, Cordero MI, Sandi C (2012) Personality traits in rats predict vulnerability and resilience to developing stress-induced depression-like behaviors, HPA axis hyper-reactivity and brain changes in pERK1/2 activity. Psychoneuroendocrinology 37:1209-1223.

Champagne F, Diorio J, Sharma S, Meaney MJ (2001) Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. Proc Natl Acad Sci U S A 98:12736-12741.

Champagne F, Francis DD, Mar A, Meaney MJ (2003a) Variations in maternal care in the rat as a mediating influence for the effects of environment on development. Physiol Behav 79:359-371.

Champagne FA (2011) Maternal imprints and the origins of variation. Horm Behav 60:4-11.

Champagne FA, Meaney MJ (2006) Stress during gestation alters postpartum maternal care and the development of the offspring in a rodent model. Biol Psychiatry 59:1227-1235.

Champagne FA, Meaney MJ (2007) Transgenerational effects of social environment on variations in maternal care and behavioral response to novelty. Behav Neurosci 121:1353-1363.

Champagne FA, Weaver IC, Diorio J, Sharma S, Meaney MJ (2003c) Natural variations in maternal care are associated with estrogen receptor alpha expression and estrogen sensitivity in the medial preoptic area. Endocrinology 144:4720-4724.

Chao HM, McEwen BS (1994) Glucocorticoids and the expression of mRNAs for neurotrophins, their receptors and GAP-43 in the rat hippocampus. Brain Res Mol Brain Res 26:271-276.

Charney DS (2004) Psychobiological mechanisms of resilience and vulnerability: implications for successful adaptation to extreme stress. Am J Psychiatry 161:195-216.

Clarke RS, Heron W, Fetherstonhaugh ML, Forgays DG, Hebb DO (1951) Individual differences in dogs: preliminary report on the effects of early experience. Can J Psychol 5:150-156.

Cottrell EC, Seckl JR (2009) Prenatal stress, glucocorticoids and the programming of adult disease. Front Behav Neurosci 3:19.

Coussons-Read ME, Okun ML, Schmitt MP, Giese S (2005) Prenatal stress alters cytokine levels in a manner that may endanger human pregnancy. Psychosom Med 67:625-631.

Cunningham C, Campion S, Teeling J, Felton L, Perry VH (2007) The sickness behaviour and CNS inflammatory mediator profile induced by systemic challenge of mice with synthetic double-stranded RNA (poly I:C). Brain Behav Immun 21:490-502.

Danese A, McEwen BS (2012) Adverse childhood experiences, allostasis, allostatic load, and age-related disease. Physiol Behav 106:29-39.

Dekel S, Mandl C, Solomon Z (2013) Is the Holocaust implicated in posttraumatic growth in second-generation Holocaust survivors? A prospective study. J Trauma Stress 26:530-533.

Diorio D, Viau V, Meaney MJ (1993) The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. J Neurosci 13:3839-3847.

Douaud G, Mackay C, Andersson J, James S, Quested D, Ray MK, Connell J, Roberts N, Crow TJ, Matthews PM, Smith S, James A (2009) Schizophrenia delays and alters maturation of the brain in adolescence. Brain 132:2437-2448.

Dunn GA, Morgan CP, Bale TL (2011a) Sex-specificity in transgenerational epigenetic programming. Horm Behav 59:290-295.

Dunn GA, Morgan CP, Bale TL (2011c) Sex-specificity in transgenerational epigenetic programming. Horm Behav 59:290-295.

Ehlert U, Patalla U, Kirschbaum C, Piedmont E, Hellhammer DH (1990) Postpartum blues: salivary cortisol and psychological factors. J Psychosom Res 34:319-325.

Eigler N, Sacca L, Sherwin RS (1979) Synergistic interactions of physiologic increments of glucagon, epinephrine, and cortisol in the dog: a model for stress-induced hyperglycemia. J Clin Invest 63:114-123.

Emde AK, Grunert M, Weese D, Reinert K, Sperling SR (2010) MicroRazerS: rapid alignment of small RNA reads. Bioinformatics 26:123-124.

Erickson ZT, Falkenberg EA, Metz GA (2014) Lifespan psychomotor behaviour profiles of multigenerational prenatal stress and artificial food dye effects in rats. PLoS One 9:e92132.

Falkenberg T, Mohammed AK, Henriksson B, Persson H, Winblad B, Lindefors N (1992) Increased expression of brain-derived neurotrophic factor mRNA in rat hippocampus is associated with improved spatial memory and enriched environment. Neurosci Lett 138:153-156.

Farr TD, Liu L, Colwell KL, Whishaw IQ, Metz GA (2006) Bilateral alteration in stepping pattern after unilateral motor cortex injury: a new test strategy for analysis of skilled limb movements in neurological mouse models. J Neurosci Methods 153:104-113.

Feigenson KA, Kusnecov AW, Silverstein SM (2014) Inflammation and the two-hit hypothesis of schizophrenia. Neurosci Biobehav Rev 38:72-93.

Field T, Diego M, Hernandez-Reif M, Figueiredo B, Deeds O, Ascencio A, Schanberg S, Kuhn C (2008) Prenatal dopamine and neonatal behavior and biochemistry. Infant Behav Dev 31:590-593.

Flagel SB, Vazquez DM, Watson SJ, Jr., Neal CR, Jr. (2002) Effects of tapering neonatal dexamethasone on rat growth, neurodevelopment, and stress response. Am J Physiol Regul Integr Comp Physiol 282:R55-63.

Forns J, Torrent M, Garcia-Esteban R, Caceres A, Gomila MP, Martinez D, Morales E, Julvez J, Grimalt JO, Sunyer J (2012) Longitudinal association between early life socio-environmental factors and attention function at the age 11 years. Environ Res 117:54-59.

Fortier ME, Joober R, Luheshi GN, Boksa P (2004) Maternal exposure to bacterial endotoxin during pregnancy enhances amphetamine-induced locomotion and startle responses in adult rat offspring. J Psychiatr Res 38:335-345.

Francis D, Diorio J, Liu D, Meaney MJ (1999) Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science 286:1155-1158.

Franklin TB, Russig H, Weiss IC, Graff J, Linder N, Michalon A, Vizi S, Mansuy IM (2010) Epigenetic Transmission of the Impact of Early Stress Across Generations. Biol Psychiatry 68:408-415.

Friedman JM, Halaas JL (1998) Leptin and the regulation of body weight in mammals. Nature 395:763-770.

Frodl T, Reinhold E, Koutsouleris N, Reiser M, Meisenzahl EM (2010) Interaction of childhood stress with hippocampus and prefrontal cortex volume reduction in major depression. J Psychiatr Res 44:799-807.

Froehlich TE, Lanphear BP, Epstein JN, Barbaresi WJ, Katusic SK, Kahn RS (2007) Prevalence, recognition, and treatment of attention-deficit/hyperactivity disorder in a national sample of US children. Arch Pediatr Adolesc Med 161:857-864.

Galani R, Berthel MC, Lazarus C, Majchrzak M, Barbelivien A, Kelche C, Cassel JC (2007) The behavioral effects of enriched housing are not altered by serotonin depletion but enrichment alters hippocampal neurochemistry. Neurobiol Learn Mem 88:1-10.

Galea L, Brummelte S (2008) Prolonged exposure to high levels of corticosterone during gestation and/or postpartum as an animal model of post partum stress and depression causes changes in maternal care and mood and alters offspring behaviour. Canadian Journal of Experimental Psychology-Revue Canadienne De Psychologie Experimentale 62:319-320.

Gapp K, Jawaid A, Sarkies P, Bohacek J, Pelczar P, Prados J, Farinelli L, Miska E, Mansuy IM (2014) Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. Nat Neurosci 17:667-669.

Garcia-Amado M, Prensa L (2012) Stereological analysis of neuron, glial and endothelial cell numbers in the human amygdaloid complex. PLoS One 7:e38692.

Gardiner E, Beveridge NJ, Wu JQ, Carr V, Scott RJ, Tooney PA, Cairns MJ (2012) Imprinted DLK1-DIO3 region of 14q32 defines a schizophrenia-associated miRNA signature in peripheral blood mononuclear cells. Mol Psychiatry 17:827-840.

Gatzke-Kopp LM (2011) The canary in the coalmine: The sensitivity of mesolimbic dopamine to environmental adversity during development. Neurosci Biobehav Rev 35:794-803.

Gauthier-Campbell C, Bredt DS, Murphy TH, El-Husseini Ael D (2004) Regulation of dendritic branching and filopodia formation in hippocampal neurons by specific acylated protein motifs. Mol Biol Cell 15:2205-2217.

Gelfo F, De Bartolo P, Giovine A, Petrosini L, Leggio MG (2009) Layer and regional effects of environmental enrichment on the pyramidal neuron morphology of the rat. Neurobiol Learn Mem 91:353-365.

Gibb RL, Gonzalez CL, Kolb B (2014) Prenatal enrichment and recovery from perinatal cortical damage: effects of maternal complex housing. Front Behav Neurosci 8:223.

Gilmore JH, Fredrik Jarskog L, Vadlamudi S, Lauder JM (2004) Prenatal infection and risk for schizophrenia: IL-1beta, IL-6, and TNFalpha inhibit cortical neuron dendrite development. Neuropsychopharmacology 29:1221-1229.

Giovanoli S, Engler H, Engler A, Richetto J, Voget M, Willi R, Winter C, Riva MA, Mortensen PB, Feldon J, Schedlowski M, Meyer U (2013) Stress in puberty unmasks latent neuropathological consequences of prenatal immune activation in mice. Science 339:1095-1099.

Gitau R, Cameron A, Fisk NM, Glover V (1998) Fetal exposure to maternal cortisol. Lancet 352:707-708.

Gitau R, Fisk NM, Glover V (2001a) Maternal stress in pregnancy and its effect on the human foetus: an overview of research findings. Stress 4:195-203.

Gitau R, Fisk NM, Teixeira JM, Cameron A, Glover V (2001b) Fetal hypothalamic-pituitary-adrenal stress responses to invasive procedures are independent of maternal responses. J Clin Endocrinol Metab 86:104-109.

Glenn TC, Martin NA, Horning MA, McArthur DL, Hovda DA, Vespa P, Brooks GA (2015) Lactate: brain fuel in human traumatic brain injury: a comparison with normal healthy control subjects. J Neurotrauma 32:820-832.

Glover V, Bergman K, Sarkar P, O'Connor TG (2009) Association between maternal and amniotic fluid cortisol is moderated by maternal anxiety. Psychoneuroendocrinology 34:430-435.

Glover V, O'Connor TG, O'Donnell K (2010) Prenatal stress and the programming of the HPA axis. Neurosci Biobehav Rev 35:17-22.

Gogtay N, Lu A, Leow AD, Klunder AD, Lee AD, Chavez A, Greenstein D, Giedd JN, Toga AW, Rapoport JL, Thompson PM (2008) Three-dimensional brain growth abnormalities in childhood-onset schizophrenia visualized by using tensor-based morphometry. Proc Natl Acad Sci U S A 105:15979-15984.

Golan HM, Lev V, Hallak M, Sorokin Y, Huleihel M (2005) Specific neurodevelopmental damage in mice offspring following maternal inflammation during pregnancy. Neuropharmacology 48:903-917.

Goldstein BI, Kemp DE, Soczynska JK, McIntyre RS (2009) Inflammation and the phenomenology, pathophysiology, comorbidity, and treatment of bipolar disorder: a systematic review of the literature. J Clin Psychiatry 70:1078-1090.

Gomez-Palacio-Schjetnan A, Escobar ML (2013) Neurotrophins and synaptic plasticity. Curr Top Behav Neurosci 15:117-136.

Grace T, Bulsara M, Robinson M, Hands B (2015) The Impact of Maternal Gestational Stress on Motor Development in Late Childhood and Adolescence: A Longitudinal Study. Child Dev.

Gramsbergen A (2003) Clumsiness and disturbed cerebellar development: insights from animal experiments. Neural Plast 10:129-140.

Griggs EM, Young EJ, Rumbaugh G, Miller CA (2013) MicroRNA-182 regulates amygdala-dependent memory formation. J Neurosci 33:1734-1740.

Grimm VE, Frieder B (1987) The effects of mild maternal stress during pregnancy on the behavior of rat pups. Int J Neurosci 35:65-72.

Gruenewald TL, Seeman TE, Ryff CD, Karlamangla AS, Singer BH (2006) Combinations of biomarkers predictive of later life mortality. Proc Natl Acad Sci U S A 103:14158-14163.

Gundersen HJG, V. JB, Kie K, Nielsen J (1999) The efficiency of systematic sampling in stereology -reconsidered. Journal of Microscopy 193:199-211.

Halbreich U, Karkun S (2006) Cross-cultural and social diversity of prevalence of postpartum depression and depressive symptoms. J Affect Disord 91:97-111.

Hamann JJ, Kelley KM, Gladden LB (2001) Effect of epinephrine on net lactate uptake by contracting skeletal muscle. J Appl Physiol (1985) 91:2635-2641.

Hands B, Kendall G, Larkin D, Parker H (2009) Perinatal Risk Factors for Mild Motor Disability. International Journal of Disability, Development and Education 56:317-331.

Harris A, Seckl J (2011) Glucocorticoids, prenatal stress and the programming of disease. Horm Behav 59:279-289.

Hawkley LC, Cole SW, Capitanio JP, Norman GJ, Cacioppo JT (2012) Effects of social isolation on glucocorticoid regulation in social mammals. Horm Behav 62:314-323.

Hebb DO (1947) The effects of early experience on problem solving at maturity. Am Psychol 2:306-307.

Henry C, Kabbaj M, Simon H, Le Moal M, Maccari S (1994) Prenatal stress increases the hypothalamo-pituitary-adrenal axis response in young and adult rats. J Neuroendocrinol 6:341-345.

Herzog A, Brosamle C (1997) 'Semifree-floating' treatment: a simple and fast method to process consecutive sections for immunohistochemistry and neuronal tracing. J Neurosci Methods 72:57-63.

Ho DH, Burggren WW (2010) Epigenetics and transgenerational transfer: a physiological perspective. J Exp Biol 213:3-16.

Holloway T, Moreno JL, Umali A, Rayannavar V, Hodes GE, Russo SJ, Gonzalez-Maeso J (2013) Prenatal stress induces schizophrenia-like alterations of serotonin 2A and metabotropic glutamate 2 receptors in the adult offspring: role of maternal immune system. J Neurosci 33:1088-1098.

Holmes MC, Abrahamsen CT, French KL, Paterson JM, Mullins JJ, Seckl JR (2006) The mother or the fetus? 11beta-hydroxysteroid dehydrogenase type 2 null mice provide evidence for direct fetal programming of behavior by endogenous glucocorticoids. J Neurosci 26:3840-3844.

Hou R, Baldwin DS (2012) A neuroimmunological perspective on anxiety disorders. Hum Psychopharmacol 27:6-14.

Howerton CL, Bale TL (2012) Prenatal programing: at the intersection of maternal stress and immune activation. Horm Behav 62:237-242.

Hsiao EY, Patterson PH (2012) Placental regulation of maternal-fetal interactions and brain development. Dev Neurobiol 72:1317-1326.

Hu YS, Long N, Pigino G, Brady ST, Lazarov O (2013) Molecular mechanisms of environmental enrichment: impairments in Akt/GSK3beta, neurotrophin-3 and CREB signaling. PLoS One 8:e64460.

Huizink AC (2012) Prenatal substance use, prenatal stress and offspring behavioural outcomes: Considerations for future studies. Nordic Journal of Psychiatry 66:115-122.

Hurd C, Weishaupt N, Fouad K (2013) Anatomical correlates of recovery in single pellet reaching in spinal cord injured rats. Exp Neurol 247:605-614.

Ickes BR, Pham TM, Sanders LA, Albeck DS, Mohammed AH, Granholm AC (2000) Long-term environmental enrichment leads to regional increases in neurotrophin levels in rat brain. Exp Neurol 164:45-52.

Jadavji NM, Kolb B, Metz GA (2006) Enriched environment improves motor function in intact and unilateral dopamine-depleted rats. Neuroscience 140:1127-1138.

Jadavji NM, Metz GA (2009) Both Pre- and Post-Lesion Experiential Therapy Is Beneficial in 6-Hydroxydopamine Dopamine-Depleted Female Rats. Neuroscience 158:373-386.

Jadavji NM, Supina RD, Metz GA (2011) Blockade of mineralocorticoid and glucocorticoid receptors reverses stress-induced motor impairments. Neuroendocrinology 94:278-290.

Johansson BB, Belichenko PV (2002) Neuronal plasticity and dendritic spines: effect of environmental enrichment on intact and postischemic rat brain. J Cereb Blood Flow Metab 22:89-96.

Johnson AR, Milner JJ, Makowski L (2012) The inflammation highway: metabolism accelerates inflammatory traffic in obesity. Immunol Rev 249:218-238.

Jung CKE, Herms J (2014) Structural Dynamics of Dendritic Spines are Influenced by an Environmental Enrichment: An In Vivo Imaging Study. Cereb Cortex 24:377-384.

Juster RP, McEwen BS, Lupien SJ (2010) Allostatic load biomarkers of chronic stress and impact on health and cognition. Neurosci Biobehav Rev 35:2-16.

Kalviainen R, Laakso MP, Partanen K, Soininen H, Riekkinen PJ, Pitkanen A (1995) Elevated Magnetic-Resonance T2 Relaxation-Time as a Marker of Hippocampal Pathology in Temporal-Lobe Epilepsy - a Comparison to Alzheimers-Disease. Neurology 45:A403-A403.

Kamiyama T, Kameda H, Murabe N, Fukuda S, Yoshioka N, Mizukami H, Ozawa K, Sakurai M (2015) Corticospinal tract development and spinal cord innervation differ between cervical and lumbar targets. J Neurosci 35:1181-1191.

Kao TJ, Law C, Kania A (2012) Eph and ephrin signaling: lessons learned from spinal motor neurons. Semin Cell Dev Biol 23:83-91.

Karlamangla AS, Singer BH, McEwen BS, Rowe JW, Seeman TE (2002) Allostatic load as a predictor of functional decline. MacArthur studies of successful aging. J Clin Epidemiol 55:696-710.

Kern S, Oakes TR, Stone CK, McAuliff EM, Kirschbaum C, Davidson RJ (2008) Glucose metabolic changes in the prefrontal cortex are associated with HPA axis response to a psychosocial stressor. Psychoneuroendocrinology 33:517-529.

Khan M (2008) Role of Cytokines. In: Immunopharmacology, pp 33-59: Springer US.

Khansari PS, Sperlagh B (2012) Inflammation in neurological and psychiatric diseases. Inflammopharmacology 20:103-107.

Kiess W, Meidert A, Dressendorfer RA, Schriever K, Kessler U, Konig A, Schwarz HP, Strasburger CJ (1995) Salivary cortisol levels throughout childhood and adolescence: relation with age, pubertal stage, and weight. Pediatr Res 37:502-506.

Kim MJ, Lyoo IK, Kim SJ, Sim M, Kim N, Choi N, Jeong DU, Covell J, Renshaw PF (2005) Disrupted white matter tract integrity of anterior cingulate in trauma survivors. Neuroreport 16:1049-1053.

Knieling M, Metz GA, Antonow-Schlorke I, Witte OW (2009) Enriched Environment Promotes Efficiency of Compensatory Movements after Cerebral Ischemia in Rats. Neuroscience 163:759-769.

Kocerha J, Faghihi MA, Lopez-Toledano MA, Huang J, Ramsey AJ, Caron MG, Sales N, Willoughby D, Elmen J, Hansen HF, Orum H, Kauppinen S, Kenny PJ, Wahlestedt C (2009) MicroRNA-219 modulates NMDA receptor-mediated neurobehavioral dysfunction. Proc Natl Acad Sci U S A 106:3507-3512.

Koene S, Kozicz TL, Rodenburg RJ, Verhaak CM, de Vries MC, Wortmann S, van de Heuvel L, Smeitink JA, Morava E (2009) Major depression in adolescent children consecutively diagnosed with mitochondrial disorder. J Affect Disord 114:327-332.

Koenig JI, Kirkpatrick B, Lee P (2002) Glucocorticoid hormones and early brain development in schizophrenia. Neuropsychopharmacology 27:309-318.

Kolb, Metz (2003) Animal models of brain plasticity and behavioral change. . In: Handbook of Neuropsychology, vol. 9 (Grafman J, R. I., ed), pp 139-152 Amsterdam: Elsevier.

Kolb B, Forgie M, Gibb R, Gorny G, Rowntree S (1998) Age, experience and the changing brain. Neurosci Biobehav Rev 22:143-159.

Kolb B, Gibb R (1991) Environmental enrichment and cortical injury: behavioral and anatomical consequences of frontal cortex lesions. Cereb Cortex 1:189-198.

Kolb B, Mychasiuk R, Muhammad A, Li Y, Frost DO, Gibb R (2012) Experience and the developing prefrontal cortex. Proc Natl Acad Sci U S A 109 Suppl 2:17186-17193.

Kondo DG, Hellem TL, Sung YH, Kim N, Jeong EK, Delmastro KK, Shi X, Renshaw PF (2011) Review: magnetic resonance spectroscopy studies of pediatric major depressive disorder. Depress Res Treat 2011:650450.

Koo JW, Park CH, Choi SH, Kim NJ, Kim HS, Choe JC, Suh YH (2003) Postnatal environment can counteract prenatal effects on cognitive ability, cell proliferation, and synaptic protein expression. FASEB J 17:1556-+.

Koolhaas JM, Bartolomucci A, Buwalda B, de Boer SF, Flugge G, Korte SM, Meerlo P, Murison R, Olivier B, Palanza P, Richter-Levin G, Sgoifo A, Steimer T, Stiedl O, van Dijk G, Wohr M, Fuchs E (2011) Stress revisited: a critical evaluation of the stress concept. Neurosci Biobehav Rev 35:1291-1301.

Korte SM, De Boer SF (2003) A robust animal model of state anxiety: fear-potentiated behaviour in the elevated plus-maze. Eur J Pharmacol 463:163-175.

Korte SM, Koolhaas JM, Wingfield JC, McEwen BS (2005) The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. Neurosci Biobehav Rev 29:3-38.

Kramer MS, Olivier M, McLean FH, Willis DM, Usher RH (1990) Impact of intrauterine growth retardation and body proportionality on fetal and neonatal outcome. Pediatrics 86:707-713.

Kubera B, Hubold C, Otte S, Lindenberg AS, Zeiß I, Krause R, Steinkamp M, Klement J, Entringer S, Pellerin L, Peters A (2012) Rise in Plasma Lactate Concentrations with Psychosocial Stress: A Possible Sign of Cerebral Energy Demand. Obesity Facts 5:384-392.

Laakso MP, Partanen K, Soininen H, Lehtovirta M, Hallikainen M, Hanninen T, Helkala EL, Vainio P, Riekkinen PJ, Sr. (1996) MR T2 relaxometry in Alzheimer's disease and age-associated memory impairment. Neurobiol Aging 17:535-540.

Langelaan S, Bakker AB, Schaufeli WB, van Rhenen W, van Doornen LJ (2007) Is burnout related to allostatic load? Int J Behav Med 14:213-221.

Laplante DP, Brunet A, Schmitz N, Ciampi A, King S (2008) Project Ice Storm: prenatal maternal stress affects cognitive and linguistic functioning in 5 1/2-year-old children. J Am Acad Child Adolesc Psychiatry 47:1063-1072.

Laviola G, Rea M, Morley-Fletcher S, Di Carlo S, Bacosi A, De Simone R, Bertini M, Pacifici R (2004) Beneficial effects of enriched environment on adolescent rats from stressed pregnancies. Eur J Neurosci 20:1655-1664.

Leahy R, Crews DE (2012) Physiological dysregulation and somatic decline among elders: modeling, applying and re-interpreting allostatic load. Coll Antropol 36:11-22.

Leggio MG, Mandolesi L, Federico F, Spirito F, Ricci B, Gelfo F, Petrosini L (2005) Environmental enrichment promotes improved spatial abilities and enhanced dendritic growth in the rat. Behav Brain Res 163:78-90.

Lenroot RK, Giedd JN (2008) The changing impact of genes and environment on brain development during childhood and adolescence: initial findings from a neuroimaging study of pediatric twins. Dev Psychopathol 20:1161-1175.

Leshem M, Schulkin J (2012) Transgenerational effects of infantile adversity and enrichment in male and female rats. Dev Psychobiol 54:169-186.

Levine ES, Dreyfus CF, Black IB, Plummer MR (1995) Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors. Proc Natl Acad Sci U S A 92:8074-8077.

Li M, Wang M, Ding S, Li C, Luo X (2012) Environmental Enrichment during Gestation Improves Behavior Consequences and Synaptic Plasticity in Hippocampus of Prenatal-Stressed Offspring Rats. Acta Histochemica Et Cytochemica 45:157-166.

Li Q, Lu G, Antonio GE, Mak YT, Rudd JA, Fan M, Yew DT (2007) The usefulness of the spontaneously hypertensive rat to model attention-deficit/hyperactivity disorder (ADHD) may be explained by the differential expression of dopamine-related genes in the brain. Neurochem Int 50:848-857.

Li Y, Li S, Yan J, Wang D, Yin R, Zhao L, Zhu Y, Zhu X (2015) miR-182 (microRNA-182) suppression in the hippocampus evokes antidepressant-like effects in rats. Prog Neuropsychopharmacol Biol Psychiatry 65:96-103.

Li YJ, Xu M, Gao ZH, Wang YQ, Yue Z, Zhang YX, Li XX, Zhang C, Xie SY, Wang PY (2013) Alterations of Serum Levels of BDNF-Related miRNAs in Patients with Depression. PLoS One 8.

Liao M, Yang F, Zhang Y, He Z, Su L, Li L (2014) White matter abnormalities in adolescents with generalized anxiety disorder: a diffusion tensor imaging study. BMC Psychiatry 14:41.

Lister RG (1987) The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 92:180-185.

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550.

Lui CC, Wang J-Y, Tain Y-L, Chen Y-C, Chang K-A, Lai M-C, Huang L-T (2011a) Prenatal stress in rat causes long-term spatial memory deficit and hippocampus MRI abnormality: Differential effects of postweaning enriched environment. Neurochem Int 58:434-441.

Lui CC, Wang JY, Tain YL, Chen YC, Chang KA, Lai MC, Huang LT (2011b) Prenatal stress in rat causes long-term spatial memory deficit and hippocampus MRI abnormality: Differential effects of postweaning enriched environment. Neurochem Int 58:434-441.

Lupien SJ, McEwen BS, Gunnar MR, Heim C (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat Rev Neurosci 10:434-445.

Maccari S, Piazza PV, Kabbaj M, Barbazanges A, Simon H, Le Moal M (1995) Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress. J Neurosci 15:110-116.

Mak CS, Waldvogel HJ, Dodd JR, Gilbert RT, Lowe MT, Birch NP, Faull RL, Christie DL (2009) Immunohistochemical localisation of the creatine transporter in the rat brain. Neuroscience 163:571-585.

Marques AH, Silverman MN, Sternberg EM (2009) Glucocorticoid dysregulations and their clinical correlates. From receptors to therapeutics. Ann N Y Acad Sci 1179:1-18.

Marques MR, Stigger F, Segabinazi E, Augustin OA, Barbosa S, Piazza FV, Achaval M, Marcuzzo S (2014) Beneficial effects of early environmental enrichment on motor development and spinal cord plasticity in a rat model of cerebral palsy. Behav Brain Res 263:149-157.

Martinez-Tellez RI, Hernandez-Torres E, Gamboa C, Flores G (2009) Prenatal stress alters spine density and dendritic length of nucleus accumbens and hippocampus neurons in rat offspring. Synapse 63:794-804.

Marx CE, Jarskog LF, Lauder JM, Lieberman JA, Gilmore JH (2001) Cytokine effects on cortical neuron MAP-2 immunoreactivity: implications for schizophrenia. Biol Psychiatry 50:743-749.

Matrisciano F, Tueting P, Maccari S, Nicoletti F, Guidotti A (2012) Pharmacological activation of group-II metabotropic glutamate receptors corrects a schizophrenia-

like phenotype induced by prenatal stress in mice. Neuropsychopharmacology 37:929-938.

Matthews SG, Phillips DI (2012) Transgenerational inheritance of stress pathology. Exp Neurol 233:95-101.

McCreary JK, Truica LS, Friesen B, Yao Y, Olson DM, Cross AR, Metz GAS (2015) Recurrent prenatal stress exacerbates stress sensitivity and vulnerability to mental illness across generations. Submitted.

McEwen BS (2000a) Allostasis and allostatic load: implications for neuropsychopharmacology. Neuropsychopharmacology 22:108-124.

McEwen BS (2000g) Allostasis, allostatic load, and the aging nervous system: role of excitatory amino acids and excitotoxicity. Neurochem Res 25:1219-1231.

McEwen BS (2003) Interacting mediators of allostasis and allostatic load: towards an understanding of resilience in aging. Metabolism 52:10-16.

McEwen BS, De Kloet ER, Rostene W (1986) Adrenal steroid receptors and actions in the nervous system. Physiol Rev 66:1121-1188.

McEwen BS, Gianaros PJ (2011) Stress- and allostasis-induced brain plasticity. Annu Rev Med 62:431-445.

McEwen BS, Seeman T (1999) Protective and damaging effects of mediators of stress. Elaborating and testing the concepts of allostasis and allostatic load. Ann N Y Acad Sci 896:30-47.

McEwen BS, Stellar E (1993) Stress and the individual. Mechanisms leading to disease. Arch Intern Med 153:2093-2101.

Meaney MJ (2001) Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu Rev Neurosci 24:1161-1192.

Meaney MJ (2010) Epigenetics and the biological definition of gene x environment interactions. Child Dev 81:41-79.

Meaney MJ, Szyf M (2005) Maternal care as a model for experience-dependent chromatin plasticity? Trends Neurosci 28:456-463.

Metz GA (2007) Stress as a modulator of motor system function and pathology. Rev Neurosci 18:209-222.

Metz GA, Jadavji NM, Smith LK (2005) Modulation of motor function by stress: a novel concept of the effects of stress and corticosterone on behavior. Eur J Neurosci 22:1190-1200.

Metz GA, Ng JW, Kovalchuk I, Olson DM (2015a) Ancestral experience as a game changer in stress vulnerability and disease outcomes. Bioessays 37:602-611.

Metz GA, Whishaw IQ (2002) Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. J Neurosci Methods 115:169-179.

Metz GA, Whishaw IQ (2009) The ladder rung walking task: a scoring system and its practical application. J Vis Exp.

Metz GAS, Ng JWY, Kovalchuk I, Olson DM (2015d) Ancestral experience as a game changer in stress vulnerability and disease outcomes. Bioessays 37:602-611.

Metzler MJ, Saucier DM, Metz GA (2013) Enriched childhood experiences moderate age-related motor and cognitive decline. Front Behav Neurosci 7.

Meyer C, Saar P, Soydan N, Eckhard M, Bretzel RG, Gerich J, Linn T (2005) A potential important role of skeletal muscle in human counterregulation of hypoglycemia. J Clin Endocrinol Metab 90:6244-6250.

Meyer U (2013) Developmental neuroinflammation and schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 42:20-34.

Meyer U, Feldon J, Dammann O (2011) Schizophrenia and autism: both shared and disorder-specific pathogenesis via perinatal inflammation? Pediatr Res 69:26R-33R.

Meyer U, Nyffeler M, Engler A, Urwyler A, Schedlowski M, Knuesel I, Yee BK, Feldon J (2006) The time of prenatal immune challenge determines the specificity of inflammation-mediated brain and behavioral pathology. J Neurosci 26:4752-4762.

Migeon CJ, Prystowsky H, Grumbach MM, Byron MC (1956) Placental passage of 17-hydroxycorticosteroids: comparison of the levels in maternal and fetal plasma and effect of ACTH and hydrocortisone administration. J Clin Invest 35:488-493.

Miller AH, Maletic V, Raison CL (2009) Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biol Psychiatry 65:732-741.

Moisiadis VG, Matthews SG (2014) Glucocorticoids and fetal programming part 2: mechanisms. Nature Reviews Endocrinology 10:403-411.

Monk C, Spicer J, Champagne FA (2012) Linking prenatal maternal adversity to developmental outcomes in infants: the role of epigenetic pathways. Dev Psychopathol 24:1361-1376.

Moriceau S, Roth TL, Okotoghaide T, Sullivan RM (2004) Corticosterone controls the developmental emergence of fear and amygdala function to predator odors in infant rat pups. Int J Dev Neurosci 22:415-422.

Morley-Fletcher S, Rea M, Maccari S, Laviola G (2003) Environmental enrichment during adolescence reverses the effects of prenatal stress on play behaviour and HPA axis reactivity in rats. Eur J Neurosci 18:3367-3374.

Muhammad A, Carroll C, Kolb B (2012) Stress during development alters dendritic morphology in the nucleus accumbens and prefrontal cortex. Neuroscience 216:103-109.

Muhammad A, Hossain S, Pellis SM, Kolb B (2011) Tactile Stimulation During Development Attenuates Amphetamine Sensitization and Structurally Reorganizes Prefrontal Cortex and Striatum in a Sex-Dependent Manner. Behav Neurosci 125:161-174.

Murmu MS, Salomon S, Biala Y, Weinstock M, Braun K, Bock J (2006) Changes of spine density and dendritic complexity in the prefrontal cortex in offspring of mothers exposed to stress during pregnancy. Eur J Neurosci 24:1477-1487.

Mychasiuk R, Gibb R, Kolb B (2012) Prenatal stress alters dendritic morphology and synaptic connectivity in the prefrontal cortex and hippocampus of developing offspring. Synapse 66:308-314.

Mychasiuk R, Muhammad A, Kolb B (2014) Environmental enrichment alters structural plasticity of the adolescent brain but does not remediate the effects of prenatal nicotine exposure. Synapse 68:293-305.

Nacci DE, Gleason TR, Munns WR (2002) Evolutionary and ecological effects of multi-generational exposures to anthropogenic stressors. Hum Ecol Risk Assess 8:91-97.

Nakamura H, Kobayashi S, Ohashi Y, Ando S (1999) Age-changes of brain synapses and synaptic plasticity in response to an enriched environment. J Neurosci Res 56:307-315.

Nordling CO (1953) A new theory on cancer-inducing mechanism. Br J Cancer 7:68-72.

Nugent KL, Chiappelli J, Rowland LM, Hong LE (2015) Cumulative stress pathophysiology in schizophrenia as indexed by allostatic load. Psychoneuroendocrinology 60:120-129.

Olson D, Severson E, Verstraeten B, Ng J, McCreary J, Metz G (2015) Allostatic Load and Preterm Birth. Int J Mol Sci 16:26209.

Pallares ME, Adrover E, Baier CJ, Bourguignon NS, Monteleone MC, Brocco MA, Gonzalez-Calvar SI, Antonelli MC (2013) Prenatal maternal restraint stress exposure alters the reproductive hormone profile and testis development of the rat male offspring. Stress 16:429-440.

Pascual R, Valencia M, Bustamante C (2015) Purkinje Cell Dendritic Atrophy Induced by Prenatal Stress Is Mitigated by Early Environmental Enrichment. Neuropediatrics 46:37-43.

Patin V, Vincent A, Lordi B, Caston J (2004) Does prenatal stress affect the motoric development of rat pups? Brain Res Dev Brain Res 149:85-92.

Paxinos G, and Watson, C. (1998) The rat brain in stereotaxic coordinates: San Diego, Academic Press.

Pena Y, Prunell M, Dimitsantos V, Nadal R, Escorihuela RM (2006) Environmental enrichment effects in social investigation in rats are gender dependent. Behav Brain Res 174:181-187.

Pena Y, Prunell M, Rotllant D, Armario A, Escorihuela RM (2009) Enduring effects of environmental enrichment from weaning to adulthood on pituitary-adrenal function, pre-pulse inhibition and learning in male and female rats. Psychoneuroendocrinology 34:1390-1404.

Peng Y, Jian X, Liu L, Tong J, Lei D (2011) Influence of environmental enrichment on hippocampal synapses in adolescent offspring of mothers exposed to prenatal stress. Neural Regeneration Research 6:378-382.

Perez-Iglesias R, Tordesillas-Gutierrez D, McGuire PK, Barker GJ, Roiz-Santianez R, Mata I, de Lucas EM, Rodriguez-Sanchez JM, Ayesa-Arriola R, Vazquez-Barquero JL, Crespo-Facorro B (2010) White matter integrity and cognitive impairment in first-episode psychosis. Am J Psychiatry 167:451-458.

Phypers B, Pierce JT (2006) Lactate physiology in health and disease. Continuing Education in Anaesthesia, Critical Care & Pain 6:128-132.

Potter ED, Ling ZD, Carvey PM (1999) Cytokine-induced conversion of mesencephalic-derived progenitor cells into dopamine neurons. Cell Tissue Res 296:235-246.

Prins SA, Przybycien-Szymanska MM, Rao YS, Pak TR (2014) Long-Term Effects of Peripubertal Binge EtOH Exposure on Hippocampal microRNA Expression in the Rat. PLoS One 9.

Quirk GJ, Mejia WR, Hesse H, Su H (1995) Early malnutrition followed by nutritional restoration lowers the conduction velocity and excitability of the corticospinal tract. Brain Res 670:277-282.

Ranger M, Chau CM, Garg A, Woodward TS, Beg MF, Bjornson B, Poskitt K, Fitzpatrick K, Synnes AR, Miller SP, Grunau RE (2013) Neonatal pain-related stress predicts cortical thickness at age 7 years in children born very preterm. PLoS One 8:e76702.

Raz N, Lindenberger U, Rodrigue KM, Kennedy KM, Head D, Williamson A, Dahle C, Gerstorf D, Acker JD (2005) Regional brain changes in aging healthy adults: general trends, individual differences and modifiers. Cereb Cortex 15:1676-1689.

Reiner A, Veenman CL, Medina L, Jiao Y, Del Mar N, Honig MG (2000) Pathway tracing using biotinylated dextran amines. J Neurosci Methods 103:23-37.

Ronald A, Pennell CE, Whitehouse AJ (2010) Prenatal maternal stress associated with ADHD and autistic traits in early childhood. Front Psychol 1:223.

Rosenzweig MR, Krech D, Bennett EL, Zolman JF (1962) Variation in environmental complexity and brain measures. J Comp Physiol Psychol 55:1092-1095.

Roth M, Neuner F, Elbert T (2014) Transgenerational consequences of PTSD: risk factors for the mental health of children whose mothers have been exposed to the Rwandan genocide. Int J Ment Health Syst 8:12.

Roy V, Chapillon P (2004) Further evidences that risk assessment and object exploration behaviours are useful to evaluate emotional reactivity in rodents. Behav Brain Res 154:439-448.

Saile R, Ertl V, Neuner F, Catani C (2014) Does war contribute to family violence against children? Findings from a two-generational multi-informant study in Northern Uganda. Child Abuse Negl 38:135-146.

Schinder AF, Poo M (2000) The neurotrophin hypothesis for synaptic plasticity. Trends Neurosci 23:639-645.

Schneider ML, Coe CL (1993) Repeated social stress during pregnancy impairs neuromotor development of the primate infant. J Dev Behav Pediatr 14:81-87.

Schuff N, Zhang Y, Zhan W, Lenoci M, Ching C, Boreta L, Mueller SG, Wang Z, Marmar CR, Weiner MW, Neylan TC (2011) Patterns of altered cortical perfusion and diminished subcortical integrity in posttraumatic stress disorder: an MRI study. Neuroimage 54 Suppl 1:S62-68.

Seeman, McEwen BS, Rowe JW, Singer BH (2001) Allostatic load as a marker of cumulative biological risk: MacArthur studies of successful aging. Proc Natl Acad Sci U S A 98:4770-4775.

Seeman T, Glei D, Goldman N, Weinstein M, Singer B, Lin YH (2004a) Social relationships and allostatic load in Taiwanese elderly and near elderly. Soc Sci Med 59:2245-2257.

Seeman TE, Crimmins E, Huang MH, Singer B, Bucur A, Gruenewald T, Berkman LF, Reuben DB (2004d) Cumulative biological risk and socio-economic differences in mortality: MacArthur studies of successful aging. Soc Sci Med 58:1985-1997.

Seeman TE, Singer BH, Rowe JW, Horwitz RI, McEwen BS (1997) Price of adaptation--allostatic load and its health consequences. MacArthur studies of successful aging. Arch Intern Med 157:2259-2268.

Segerstrom SC, Miller GE (2004) Psychological stress and the human immune system: a meta-analytic study of 30 years of inquiry. Psychol Bull 130:601-630.

Selye H (1984) The Stress of Life. New York McGraw-Hill.

Seplaki CL, Goldman N, Glei D, Weinstein M (2005) A comparative analysis of measurement approaches for physiological dysregulation in an older population. Exp Gerontol 40:438-449.

Seplaki CL, Goldman N, Weinstein M, Lin YH (2004) How are biomarkers related to physical and mental well-being? J Gerontol A Biol Sci Med Sci 59:201-217.

Sharp H, Pickles A, Meaney M, Marshall K, Tibu F, Hill J (2012) Frequency of Infant Stroking Reported by Mothers Moderates the Effect of Prenatal Depression on Infant Behavioural and Physiological Outcomes. PLoS One 7.

Shaw P, Kabani NJ, Lerch JP, Eckstrand K, Lenroot R, Gogtay N, Greenstein D, Clasen L, Evans A, Rapoport JL, Giedd JN, Wise SP (2008) Neurodevelopmental trajectories of the human cerebral cortex. J Neurosci 28:3586-3594.

Shi L, Fatemi SH, Sidwell RW, Patterson PH (2003) Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. J Neurosci 23:297-302.

Sima A, Sourander P (1976) The effect of pre- and postnatal undernutrition on the calibre growth of central motor fibres: a morphometric ultrastructural study on rat cortico-spinal tract. Acta Neuropathol 34:105-114.

Sima A, Sourander P (1978) The effect of pre- and postnatal undernutrition on axonal growth and myelination of central motor fibers. A morphometric study on rat cortico-spinal tract. Acta Neuropathol 42:15-18.

Skinner MK (2008) What is an epigenetic transgenerational phenotype? F3 or F2. Reprod Toxicol 25:2-6.

Skinner MK (2014) Environmental stress and epigenetic transgenerational inheritance. BMC Med 12:153.

Skinner MK, Manikkam M, Guerrero-Bosagna C (2011) Epigenetic transgenerational actions of endocrine disruptors. Reprod Toxicol 31:337-343.

Smit-Rigter LA, Champagne DL, van Hooft JA (2009) Lifelong impact of variations in maternal care on dendritic structure and function of cortical layer 2/3 pyramidal neurons in rat offspring. PLoS One 4:e5167.

Smith D, Pernet A, Hallett WA, Bingham E, Marsden PK, Amiel SA (2003) Lactate: a preferred fuel for human brain metabolism in vivo. J Cereb Blood Flow Metab 23:658-664.

Smith LK, Jadavji NM, Colwell KL, Katrina Perehudoff S, Metz GA (2008) Stress accelerates neural degeneration and exaggerates motor symptoms in a rat model of Parkinson's disease. Eur J Neurosci 27:2133-2146.

Spinillo A, Stronati M, Ometto A, Fazzi E, Lanzi G, Guaschino S (1993) Infant neurodevelopmental outcome in pregnancies complicated by gestational hypertension and intra-uterine growth retardation. J Perinat Med 21:195-203.

Spivey JM, Shumake J, Colorado RA, Conejo-Jimenez N, Gonzalez-Pardo H, Gonzalez-Lima F (2009) Adolescent female rats are more resistant than males to the effects of early stress on prefrontal cortex and impulsive behavior. Dev Psychobiol 51:277-288.

Sterling P, Eyer J (1989) ALLOSTASIS A NEW PARADIGM TO EXPLAIN AROUSAL PATHOLOGY.

Stork C, Renshaw PF (2005) Mitochondrial dysfunction in bipolar disorder: evidence from magnetic resonance spectroscopy research. Mol Psychiatry 10:900-919.

Sullivan RM, Gratton A (2002) Prefrontal cortical regulation of hypothalamic-pituitary-adrenal function in the rat and implications for psychopathology: side matters. Psychoneuroendocrinology 27:99-114.

Takahashi LK (1998) Prenatal stress: consequences of glucocorticoids on hippocampal development and function. Int J Dev Neurosci 16:199-207.

Tanebe K, Nishijo H, Muraguchi A, Ono T (2000) Effects of chronic stress on hypothalamic lnterleukin-1beta, interleukin-2, and gonadotrophin-releasing hormone gene expression in ovariectomized rats. J Neuroendocrinol 12:13-21.

Tannenbaum B, Tannenbaum GS, Sudom K, Anisman H (2002) Neurochemical and behavioral alterations elicited by a chronic intermittent stressor regimen: implications for allostatic load. Brain Res 953:82-92.

Thallmair M, Metz GA, Z'Graggen WJ, Raineteau O, Kartje GL, Schwab ME (1998) Neurite growth inhibitors restrict plasticity and functional recovery following corticospinal tract lesions. Nat Neurosci 1:124-131.

Thoenen H (1995) Neurotrophins and neuronal plasticity. Science 270:593-598.

Ublinskii MV, Semenova NA, Lukovkina OV, Sidorin SV, Lebedeva IS, Kaleda VG, Barkhatova AN, Akhadov TA (2015) Characteristics of Diffusion in the Corticospinal Tract of Patients with Early Stage of Schizophrenia: Diffusion Tensor Magnetic Resonance Imaging. Bull Exp Biol Med 159:29-31.

Ulupinar E, Erol K, Aya H, Yucel F (2015) Rearing conditions differently affect the motor performance and cerebellar morphology of prenatally stressed juvenile rats. Behav Brain Res 278:235-243.

Van den Hove DL, Kenis G, Brass A, Opstelten R, Rutten BP, Bruschettini M, Blanco CE, Lesch KP, Steinbusch HW, Prickaerts J (2013) Vulnerability versus resilience to prenatal stress in male and female rats; implications from gene expression profiles in the hippocampus and frontal cortex. Eur Neuropsychopharmacol 23:1226-1246.

Van der Meer E, Van Loo PL, Baumans V (2004) Short-term effects of a disturbed light-dark cycle and environmental enrichment on aggression and stress-related parameters in male mice. Lab Anim 38:376-383.

Van Eekelen JA, Jiang W, De Kloet ER, Bohn MC (1988) Distribution of the mineralocorticoid and the glucocorticoid receptor mRNAs in the rat hippocampus. J Neurosci Res 21:88-94.

van Os J, Selten JP (1998) Prenatal exposure to maternal stress and subsequent schizophrenia. The May 1940 invasion of The Netherlands. Br J Psychiatry 172:324-326.

van Praag H, Kempermann G, Gage FH (2000) Neural consequences of environmental enrichment. Nature Reviews Neuroscience 1:191-198.

Vavrek R, Girgis J, Tetzlaff W, Hiebert GW, Fouad K (2006) BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats. Brain 129:1534-1545.

Veenendaal MV, Painter RC, de Rooij SR, Bossuyt PM, van der Post JA, Gluckman PD, Hanson MA, Roseboom TJ (2013) Transgenerational effects of prenatal exposure to the 1944-45 Dutch famine. BJOG 120:548-553.

Veenman CL, Reiner A, Honig MG (1992) Biotinylated dextran amine as an anterograde tracer for single- and double-labeling studies. J Neurosci Methods 41:239-254.

Vidal AC, Benjamin Neelon SE, Liu Y, Tuli AM, Fuemmeler BF, Hoyo C, Murtha AP, Huang Z, Schildkraut J, Overcash F, Kurtzberg J, Jirtle RL, Iversen ES, Murphy SK (2014) Maternal stress, preterm birth, and DNA methylation at imprint regulatory sequences in humans. Genet Epigenet 6:37-44.

Wagner G, Schultz CC, Koch K, Schachtzabel C, Sauer H, Schlosser RG (2012) Prefrontal cortical thickness in depressed patients with high-risk for suicidal behavior. J Psychiatr Res 46:1449-1455.

Wan Y, Liu Y, Wang X, Wu J, Liu K, Zhou J, Liu L, Zhang C (2015) Identification of differential microRNAs in cerebrospinal fluid and serum of patients with major depressive disorder. PLoS One 10:e0121975.

Ward ID, Zucchi FCR, Robbins JC, Falkenberg EA, Olson DM, Benzies K, Metz GA (2013) Transgenerational programming of maternal behaviour by prenatal stress. BMC Pregnancy Childbirth 13.

Weinstock M, Fride E, Hertzberg R (1988) Prenatal stress effects on functional development of the offspring. Prog Brain Res 73:319-331.

Whiteford HA, Degenhardt L, Rehm J, Baxter AJ, Ferrari AJ, Erskine HE, Charlson FJ, Norman RE, Flaxman AD, Johns N, Burstein R, Murray CJ, Vos T (2013) Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. Lancet 382:1575-1586.

Wood SJ, Yucel M, Pantelis C, Berk M (2009) Neurobiology of schizophrenia spectrum disorders: the role of oxidative stress. Ann Acad Med Singapore 38:396-396.

Wyss M, Schulze A (2002) Health implications of creatine: can oral creatine supplementation protect against neurological and atherosclerotic disease? Neuroscience 112:243-260.

Yang J, Hou C, Ma N, Liu J, Zhang Y, Zhou J, Xu L, Li L (2007) Enriched environment treatment restores impaired hippocampal synaptic plasticity and cognitive deficits induced by prenatal chronic stress. Neurobiol Learn Mem 87:257-263.

Yang J, Li W, Liu X, Li Z, Li H, Yang G, Xu L, Li L (2006) Enriched environment treatment counteracts enhanced addictive and depressive-like behavior induced by prenatal chronic stress. Brain Res 1125:132-137.

Yao Y, Robinson AM, Zucchi FC, Robbins JC, Babenko O, Kovalchuk O, Kovalchuk I, Olson DM, Metz GA (2014) Ancestral exposure to stress epigenetically programs preterm birth risk and adverse maternal and newborn outcomes. BMC Med 12:121.

Yehuda R, Engel SM, Brand SR, Seckl J, Marcus SM, Berkowitz GS (2005) Transgenerational effects of posttraumatic stress disorder in babies of mothers exposed to the world trade center attacks during pregnancy. J Clin Endocrinol Metab 90:4115-4118.

Yildiz-Yesiloglu A, Ankerst DP (2006) Review of 1H magnetic resonance spectroscopy findings in major depressive disorder: a meta-analysis. Psychiatry Res 147:1-25.

Zeybel M, Hardy T, Wong YK, Mathers JC, Fox CR, Gackowska A, Oakley F, Burt AD, Wilson CL, Anstee QM, Barter MJ, Masson S, Elsharkawy AM, Mann DA, Mann J (2012) Multigenerational epigenetic adaptation of the hepatic wound-healing response. Nat Med 18:1369-+.

Zhang Z, Zhang H, Du B, Chen Z (2012a) Enriched environment upregulates growth-associated protein 43 expression in the hippocampus and enhances cognitive

abilities in prenatally stressed rat offspring. Neural Regeneration Research 7:1967-1973.

Zhang Z, Zhang H, Du B, Chen Z (2012c) Neonatal handling and environmental enrichment increase the expression of GAP-43 in the hippocampus and promote cognitive abilities in prenatally stressed rat offspring. Neurosci Lett 522:1-5.

Zhou H (2015) Region Specific Effects of Maternal Immune Activation on Offspring Neuroimmune Function. Open J Immunol 5:51-63.

Zhu SW, Yee BK, Nyffeler M, Winblad B, Feldon J, Mohammed AH (2006) Influence of differential housing on emotional behaviour and neurotrophin levels in mice. Behav Brain Res 169:10-20.

Zilles K, Zilles B, Schleicher A (1980) A quantitative approach to cytoarchitectonics. VI. The areal pattern of the cortex of the albino rat. Anat Embryol (Berl) 159:335-360.

Zubedat S, Aga-Mizrachi S, Cymerblit-Sabba A, Ritter A, Nachmani M, Avital A (2015) Methylphenidate and environmental enrichment ameliorate the deleterious effects of prenatal stress on attention functioning. Stress 1-9.

Zucchi FC, Yao Y, Metz GA (2012) The secret language of destiny: stress imprinting and transgenerational origins of disease. Frontiers in genetics 3:96.

Zucchi FC, Yao Y, Ward ID, Ilnytskyy Y, Olson DM, Benzies K, Kovalchuk I, Kovalchuk O, Metz GA (2013) Maternal stress induces epigenetic signatures of psychiatric and neurological diseases in the offspring. PLoS One 8:e56967.

Zucchi FCR, Yao YL, Ilnytskyy Y, Robbins JC, Soltanpour N, Kovalchuk I, Kovalchuk O, Metz GAS (2014) Lifetime Stress Cumulatively Programs Brain Transcriptome and Impedes Stroke Recovery: Benefit of Sensory Stimulation. PLoS One 9.

Zucchi., Matthies NF, Badr N, Metz GA (2010) Stress-induced glucocorticoid receptor activation determines functional recovery following ischemic stroke. Exp Transl Stroke Med 2:18.

Zucchi., Yao Y, Ward ID, Ilnytskyy Y, Olson DM, Benzies K, Kovalchuk I, Kovalchuk O, Metz GA (2013) Maternal stress induces epigenetic signatures of psychiatric and neurological diseases in the offspring. PLoS One 8:e56967.

Zuckerman L, Rehavi M, Nachman R, Weiner I (2003) Immune activation during pregnancy in rats leads to a postpubertal emergence of disrupted latent inhibition, dopaminergic hyperfunction, and altered limbic morphology in the offspring: a novel neurodevelopmental model of schizophrenia. Neuropsychopharmacology 28:1778-1789.

Zulman DM, Vijan S, Omenn GS, Hayward RA (2008) The relative merits of population-based and targeted prevention strategies. Milbank Q 86:557-580.

Zuluaga MJ, Agrati D, Uriarte N, Ferreira A (2014) Social aversive stimuli presented to the mother produce the precocious expression of fear in rat pups. Dev Psychobiol 56:1187-1198.

Zwicker JG, Grunau RE, Adams E, Chau V, Brant R, Poskitt KJ, Synnes A, Miller SP (2013) Score for neonatal acute physiology-II and neonatal pain predict corticospinal tract development in premature newborns. Pediatr Neurol 48:123-129 e121.

APPENDIX A:

Supplemental Figures and Tables

Chapter 2:

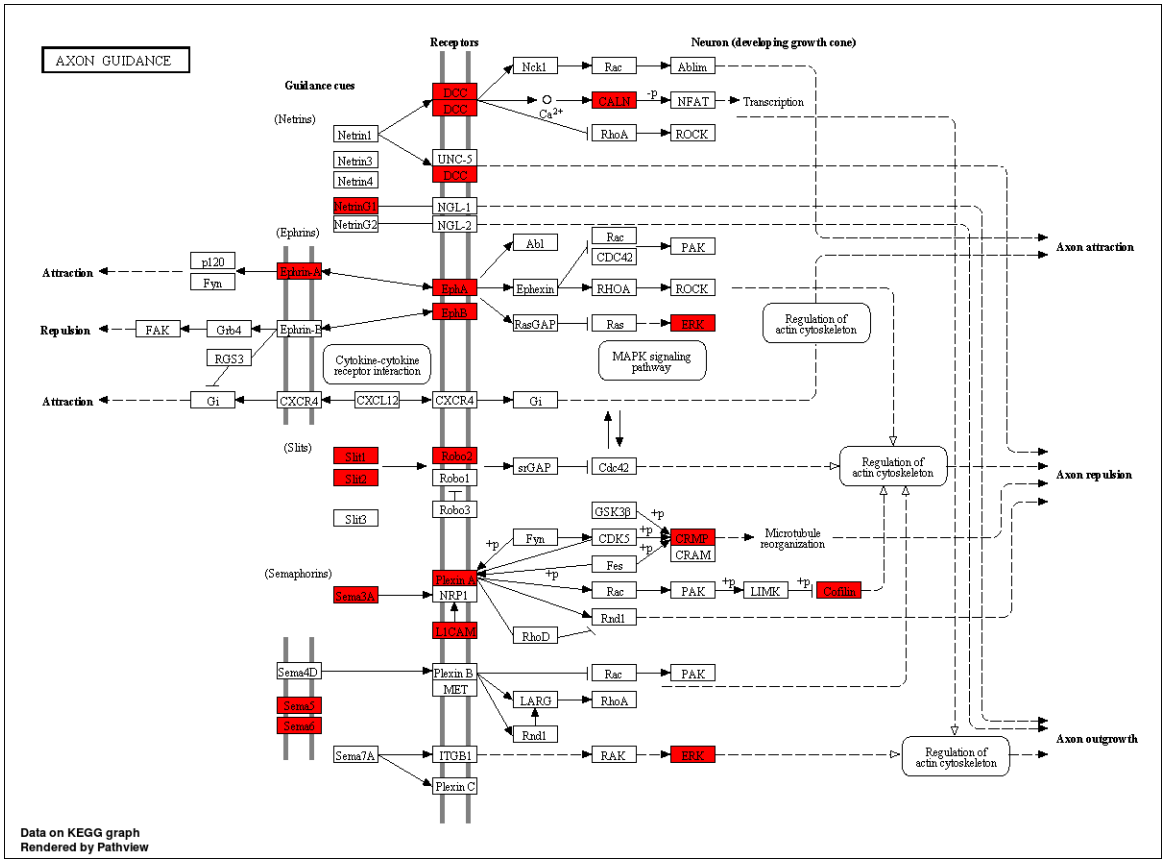

Figure S1: Axon guidance signaling pathway as target for environmentally-regulated miR-182 expression. Predicted direct targets of miR-182 are shown in red and include netrin, semaphorin, ephrin and their receptors. The 3'UTRs of these genes show up as high scoring targets in miR-182 binding prediction. The pathway suggests that miR-182 regulates axonal pathfinding during brain development and maturation, which is altered by transgenerational stress and housing conditions.

Supplemental Table 1. Small RNA expression fold change with raw and adjusted p-value. Included are values with a p-value<0.1. C, control; MPS, multigenerational prenatal stress; TPS, transgenerational prenatal stress; EE, Enriched Environment housing condition. In the order of smallest p-adj. values.

Treatment	small RNA	log2FoldChange	SE	Wald Statistic	p-value	p-adj.
MPS vs C	Rattus_norvegicus_chr2.trna4358-GlyCCC	2.935006	0.38612	7.60117	2.93E-14	4.7E-12
TPS vs C	FR0403295|C/D	3.474859	0.81971	4.23911	2.24E-05	1.2E-02
TPS vs C	rno-miR-10a-5p	7.276458	0.61994	-4.15206	3.29E-05	1.2E-02
TPS vs C	Rattus_norvegicus_chr2.trna4358-GlyCCC	1.638738	0.40991	3.99779	6.39E-05	1.6E-02
TPS vs C	rno-miR-182	1.499731	0.48585	-3.47908	0.000503	2.5E-02
TPS vs C	rno-miR-124-3p	3.576407	0.18801	-3.10761	0.001886	2.8E-02
MPS vs C	rno-miR-124-3p	2.938383	0.19486	-3.28492	0.00102	8.2E-02
MPS vs C	rno-miR-182	1.55158	0.49857	-2.76248	0.005736	3.1E-01
TPS vs C	rno-miR-3553	1.351694	0.77179	-2.71747	0.006578	8.1E-01
TPS vs C	rno-miR-486	8.211783	0.16830	-2.62079	0.008773	9.2E-01
MPS vs MPS-EE	rno-miR-199a-3p	1.40123	0.43517	3.21993	0.001282	9.7E-01
MPS vs C	rno-miR-486	2.758066	0.18923	-2.03387	0.041964	9.9E-01
MPS vs C	rno-miR-10a-5p	4.505499	0.59672	-2.02394	0.042977	9.9E-01
MPS vs C	rno-miR-24-3p	1.108923	0.21318	-1.98813	0.046797	9.9E-01
MPS vs C	rno-let-7b-3p	2.90867	0.29584	1.96806	0.049061	9.9E-01
MPS vs C	rno-miR-411-5p	0.746015	0.20241	1.96534	0.049375	9.9E-01
MPS vs MPS-EE	rno-miR-204-5p	1.161981	0.41687	2.78737	0.005314	9.9E-01
MPS vs MPS-EE	rno-miR-219a-5p	1.462179	0.61631	2.37245	0.01767	9.9E-01
MPS vs MPS-EE	rno-miR-375-3p	1.072906	0.45396	2.36340	0.018108	9.9E-01
MPS vs MPS-EE	rno-miR-21-5p	0.912857	0.38922	2.34534	0.01901	9.9E-01
MPS vs MPS-EE	rno-miR-211-5p	1.437792	0.62700	2.29312	0.021841	9.9E-01
MPS vs MPS-EE	rno-miR-7a-5p	0.956106	0.45649	2.09444	0.036221	9.9E-01
MPS vs MPS-EE	FR0024423|Piwi-interacting	-1.28556	0.66814	-1.92410	0.054342	9.9E-01
MPS vs MPS-EE	rno-miR-3544	1.183926	0.62827	1.88440	0.059511	9.9E-01
MPS vs MPS-EE	rno-miR-26b-5p	0.626912	0.34044	1.84143	0.065558	9.9E-01

MPS vs MPS-EE	rno-miR-450a-5p	0.910405	0.49587	1.83596	0.066364	9.9E-01
MPS vs MPS-EE	FR0077443|Piwi-interacting	0.901941	0.50513	1.78555	0.074173	9.9E-01
MPS vs MPS-EE	rno-miR-200c-3p	1.091507	0.61228	1.78269	0.074637	9.9E-01
MPS vs MPS-EE	FR0059851|Piwi-interacting	1.029639	0.59966	1.71701	0.085977	9.9E-01
MPS vs MPS-EE	rno-miR-106b-5p	0.936773	0.55127	1.69927	0.089268	9.9E-01
MPS vs MPS-EE	rno-let-7d-3p	0.473828	0.27892	1.69879	0.089359	9.9E-01
MPS vs MPS-EE	rno-miR-145-5p	0.552868	0.32931	1.67885	0.093182	9.9E-01
MPS vs MPS-EE	rno-let-7e-3p	0.988459	0.59301	1.66684	0.095545	9.9E-01
MPS vs MPS-EE	rno-miR-136-3p	-0.33601	0.20291	-1.65596	0.097731	9.9E-01
MPS vs MPS-EE	rno-miR-200b-5p	0.987452	0.59807	1.65104	0.09873	9.9E-01
MPS vs MPS-EE	rno-miR-187-3p	1.019519	0.62172	1.63981	0.101044	9.9E-01
MPS vs MPS-EE	rno-miR-152-3p	1.076068	0.66893	1.60862	0.1077	9.9E-01
C-EE vs C	rno-miR-582-5p	1.01799	0.50192	2.02819	0.042541	9.9E-01
TPS vs TPS-EE	rno-miR-582-5p	1.01799	0.50192	2.02819	0.042541	9.9E-01
C-EE vs C	rno-miR-29c-5p	-1.08965	0.56240	-1.93748	0.052686	9.9E-01
TPS vs TPS-EE	rno-miR-29c-5p	-1.08965	0.56240	-1.93748	0.052686	9.9E-01
C-EE vs C	FR0192132|Piwi-interacting	1.049282	0.59089	1.77575	0.075775	9.9E-01
TPS vs TPS-EE	FR0192132|Piwi-interacting	1.049282	0.59089	1.77575	0.075775	9.9E-01
C-EE vs C	rno-miR-1306-5p	1.045943	0.59091	1.77005	0.076719	9.9E-01
TPS vs TPS-EE	rno-miR-1306-5p	1.045943	0.59091	1.77005	0.076719	9.9E-01
C-EE vs C	rno-miR-125a-3p	1.033198	0.58526	1.76535	0.077505	9.9E-01
TPS vs TPS-EE	rno-miR-125a-3p	1.033198	0.58526	1.76535	0.077505	9.9E-01
C-EE vs C	FR0313594|C/D	-1.05611	0.62807	-1.68151	0.092664	9.9E-01
TPS vs TPS-EE	FR0313594|C/D	-1.05611	0.62807	-1.68151	0.092664	9.9E-01
C-EE vs C	rno-miR-1843b-5p	-0.33416	0.20298	-1.64622	0.099719	9.9E-01
TPS vs TPS-EE	rno-miR-1843b-5p	-0.33416	0.20298	-1.64622	0.099719	9.9E-01
TPS vs C	rno-miR-429	-1.82056	0.80683	-2.25641	0.024045	1.0E+00
TPS vs C	rno-miR-3577	1.746913	0.78483	2.22582	0.026026	1.0E+00
TPS vs C	rno-miR-3102	1.222738	0.55617	2.19846	0.027916	1.0E+00
TPS vs C	ENSRNOG00000034753|5S	-1.86927	0.85373	-2.18950	0.02856	1.0E+00

TPS vs C	ENSRNOG00000034421|5S	-1.80827	0.83891	-2.15550	0.031123	1.0E+00
TPS vs C	rno-miR-30b-3p	1.761104	0.81981	2.14819	0.031699	1.0E+00
TPS vs C	ENSRNOG00000034612|5S	-1.68545	0.83485	-2.01885	0.043503	1.0E+00
TPS vs C	rno-miR-667-3p	0.82052	0.40800	2.01104	0.044322	1.0E+00
TPS vs C	ENSRNOG00000034413|5S	-1.7104	0.85375	-2.00339	0.045136	1.0E+00
TPS vs C	ENSRNOG00000041881|5S	-1.67308	0.84934	-1.96985	0.048855	1.0E+00
TPS vs C	rno-miR-128-2-5p	1.645695	0.84239	1.95360	0.050748	1.0E+00
TPS vs C	rno-miR-135b-5p	-0.90651	0.46417	-1.95296	0.050825	1.0E+00
TPS vs C	ENSRNOG00000041036|5S	-1.5774	0.82762	-1.90595	0.056657	1.0E+00
TPS vs C	ENSRNOG00000035238|5S	-1.54693	0.84730	-1.82570	0.067896	1.0E+00
TPS vs C	rno-miR-30d-3p	0.754134	0.42095	1.79150	0.073212	1.0E+00
TPS vs C	rno-miR-541-5p	0.565428	0.31675	1.78506	0.074252	1.0E+00
TPS vs C	rno-miR-151-3p	0.442087	0.25216	1.75318	0.079572	1.0E+00
TPS vs C	rno-miR-25-5p	1.420034	0.82786	1.71530	0.086291	1.0E+00
TPS vs C	rno-miR-141-3p	-1.24529	0.74211	-1.67802	0.093344	1.0E+00
TPS vs C	rno-miR-671	0.776569	0.46780	1.66002	0.096909	1.0E+00
TPS vs C	FR0062876|Piwi-interacting	-1.36453	0.82368	-1.65660	0.0976	1.0E+00
TPS vs C	ENSRNOG00000041010|5S	-1.36884	0.84180	-1.62607	0.103934	1.0E+00
TPS vs C	ENSRNOG00000040834|5S	-1.37447	0.84557	-1.62552	0.104051	1.0E+00
MPS vs C	rno-miR-103-3p	0.313884	0.17247	1.81985	0.068781	1.0E+00
MPS vs C	rno-miR-132-3p	0.280005	0.15908	1.76012	0.078388	1.0E+00
MPS vs C	rno-miR-873-3p	-0.60339	0.35892	-1.68111	0.092742	1.0E+00
MPS vs C	rno-miR-541-5p	0.466462	0.28692	1.62571	0.104011	1.0E+00
MPS vs C	rno-miR-191a-5p	0.371252	0.23036	1.61156	0.107057	1.0E+00
MPS vs C	rno-miR-434-5p	-0.78145	0.48660	-1.60591	0.108295	1.0E+00
MPS vs C	rno-miR-27a-3p	-0.43036	0.26869	-1.60169	0.109224	1.0E+00
MPS vs C	Rattus_norvegicus_chr1.trna2048-SeC(e)TCA	1.765595	0.61820	2.85598	0.00429	NA
MPS vs C	FR0024423|Piwi-interacting	2.16298	0.76316	2.83423	0.004594	NA
MPS vs C	FR0403295|C/D	2.070462	0.76411	2.70962	0.006736	NA
MPS vs C	rno-miR-3553	1.289105	0.68387	-2.48192	0.013068	NA

MPS vs C	rno-miR-3577	1.728112	0.74481	2.32018	0.020331	NA
MPS vs C	rno-miR-342-5p	-1.59533	0.76788	-2.07758	0.037748	NA
MPS vs C	FR0392512|H/ACA	1.485154	0.75781	1.95978	0.050022	NA
MPS vs C	FR0249489|Piwi-interacting	1.200728	0.63571	1.88880	0.058919	NA
MPS vs C	FR0327571|Piwi-interacting	1.391373	0.76610	1.81616	0.069346	NA
MPS vs C	ENSRNOG00000040834|5S	-1.25947	0.72337	-1.74111	0.081665	NA
MPS vs C	rno-miR-30d-3p	0.786133	0.45271	1.73648	0.08248	NA
MPS vs C	FR0107401|Piwi-interacting	0.998947	0.60326	1.65591	0.09774	NA
MPS vs C	FR0234140|C/D	-1.23158	0.74590	-1.65112	0.098713	NA
MPS vs C	rno-miR-1306-5p	1.145874	0.70816	1.61810	0.105641	NA

Chapter 3:

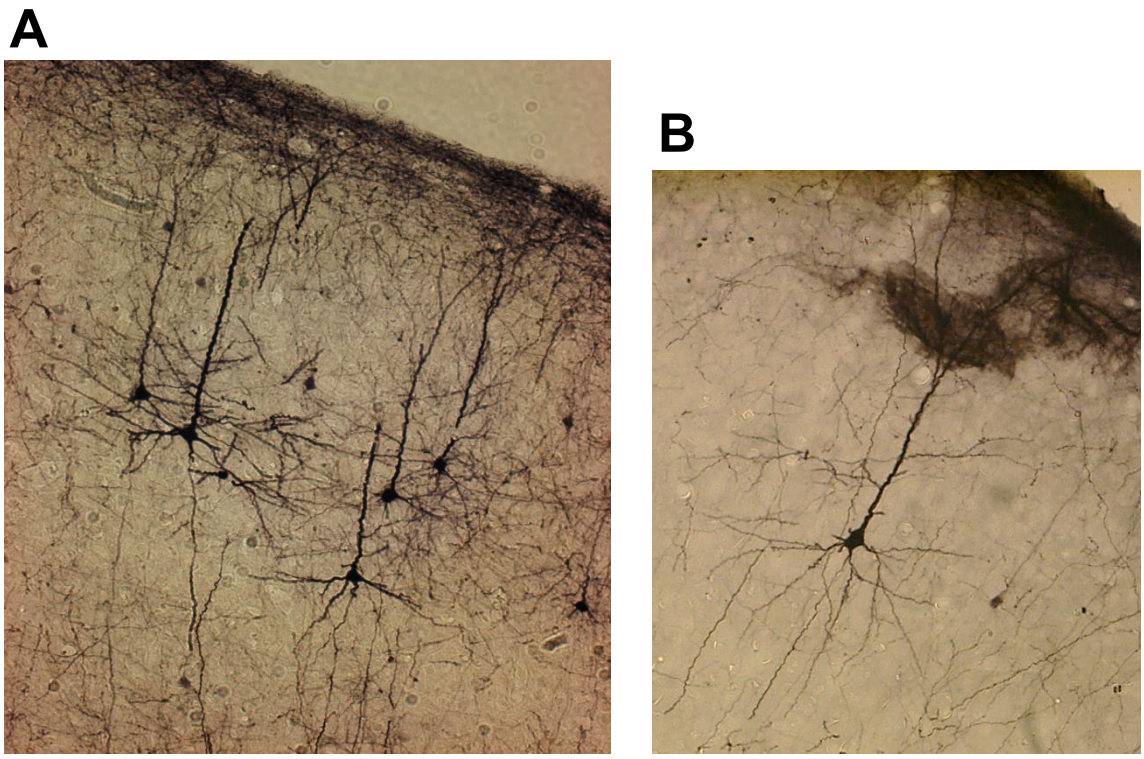

Figure S2. Individual photomicrographs of A) grouping and B) individually stained pyramidal neurons stained with BDA.

Chapter 4:

Supplemental Table 2: Correlations coefficients and significance

	R	P-Value
OF, EPM	2.84E-04	0.9989
OF, CORT	0.08	0.7051
OF, BLOOD GLUCOSE	0.13	0.5237
OF, IL-1β	0.18	0.3652
OF, IL-2	0.03	0.892
OF, Leptin	0.12	0.5456
OF, IL-6	0.37	0.0542
OF, Weight	0.19	0.3253
OF, Lactate	0.09	0.6412
OF, Creatine	0.12	0.5379
OF, MWT	0.42	0.0265*
OF, MGV	0.36	0.0613
OF, CAALM	-0.51	0.0047*
EPM, CORT	-2.57E-03	0.9897
EPM, BLOOD GLUCOSE	-0.03	0.8766
EPM, IL-1β	0.01	0.9527
EPM, IL-2	0.01	0.9439
EPM, Leptin	0.09	0.6596
EPM, IL-6	-4.40E-03	0.9824
EPM, Weight	0.09	0.636
EPM, Lactate	-0.15	0.4571
EPM, Creatine	-0.14	0.4887
EPM, MWT	0.14	0.4829
EPM, MGV	0.23	0.2365
EPM, CAALM	-0.33	0.0818
CORT, BLOOD GLUCOSE	0.2	0.3063
CORT, IL-1β	-0.08	0.7017
CORT, IL-2	0.2	0.3155
CORT, Leptin	0.05	0.7871
CORT, IL-6	0.16	0.4238
CORT, Weight	0.14	0.4695
CORT, Lactate	-0.06	0.7809
CORT, Creatine	-0.08	0.6819
CORT, MWT	-0.08	0.6709
CORT, MGV	-0.19	0.3403
CORT, CAALM	-0.13	0.5063
BLOOD GLUCOSE, IL-1β	0.11	0.5856

BLOOD GLUCOSE, IL-2	-0.12	0.5535
BLOOD GLUCOSE, Leptin	0.22	0.2702
BLOOD GLUCOSE, IL-6	0.1	0.6205
BLOOD GLUCOSE, Weight	0.03	0.8831
BLOOD GLUCOSE, Lactate	0.02	0.9179
BLOOD GLUCOSE, Creatine	0.02	0.9029
BLOOD GLUCOSE, MWT	-0.05	0.8042
BLOOD GLUCOSE, MGV	-0.17	0.3932
BLOOD GLUCOSE, CAALM	0.22	0.2662
IL-1β, IL-2	0.18	0.2155
IL-1β Leptin	-0.17	0.2582
IL-1β, IL-6	0.19	0.1901
IL-1β, Weight	0.18	0.2186
IL-1β, Lactate	0.27	0.0684
IL-1β, Creatine	0.27	0.0684
IL-1β, MWT	-0.21	0.2911
IL-1β, MGV	-0.32	0.0903
IL-1βIL-1β, CAALM	0.46	0.0143*
IL-2, Leptin	-0.11	0.4725
IL-2, IL-6	-0.09	0.5537
IL-2, Weight	0.08	0.5932
IL-2, Lactate	0.23	0.1162
IL-2, Creatine	0.23	0.1162
IL-2, MWT	0.2	0.1799
IL-2, MGV	-0.29	0.0879
IL-2, CAALM	0.35	0.0147*
Leptin, IL-6	0.47	0.0108*
Leptin, Weight	0.15	0.4644
Leptin, Lactate	-0.31	0.1115
Leptin, Creatine	-0.31	0.1041
Leptin, MWT	0.06	0.7675
Leptin, MGV	0.14	0.4844
Leptin, CAALM	-0.12	0.5434
IL-6, Weight	-0.23	0.2358
IL-6, Lactate	-0.22	0.2701
IL-6, Creatine	-0.23	0.2424
IL-6, MWT	0.28	0.1515
IL-6, MGV	0.18	0.3533
IL-6, CAALM	-0.12	0.5532
Weight, Lactate	0.17	0.3914
Weight, Creatine	0.18	0.3538

Weight, MWT	0.01	0.9568
Weight, MGV	-0.25	0.2025
Weight, CAALM	-0.15	0.4611
Lactate, Creatine	0.98	<0.0001*
Lactate, MWT	0.27	0.1728
Lactate, MGV	-0.26	0.0249*
Lactate, CAALM	-0.02	0.9171
Creatine, MWT	0.27	0.1702
Creatine, MGV	-0.25	0.0329*
Creatine, CAALM	-0.05	0.8054
MWT, MGV	-0.09	0.6548
MWT, CAALM	-0.34	0.0769
MGV, CAALM	-0.56	0.0016*
*denotes significance ($p<0.05$)		

APPENDIX B:

Systematic Review

Environmental Enrichment as an Intervention for Adverse Health Outcomes: Postnatal Therapy for Prenatal Stress

Manuscript has been submitted in its entirety

Abstract

Prenatal stress has complex neurological, behavioural and physiological consequences for the developing offspring. The phenotype linked to prenatal stress usually lasts into adulthood and may even propagate to subsequent generations. The mainly uncontrollable exposure to stress and the lasting consequences emphasize the urgent need for treatment strategies that effectively reverse stress programming. Exposure to complex beneficial experiences, such as environmental enrichment (EE), is one of the most powerful therapies to promote neuroplasticity and behavioural performance at any time in life. A small number of studies have previously used EE to postnatally treat consequences of prenatal stress in the attempt to reverse deficits that were primarily induced *in utero*. This review discusses the available data on postnatal EE exposure in prenatally stressed individuals to determine if EE is a suitable treatment option to reverse the adverse consequences of stress programming and enhance stress resiliency. Moreover, This review addresses the cumulative stress hypothesis, which proposes that EE mitigates adverse consequences of PS, versus the mismatch hypothesis, which proposes that EE is inefficient to reverse adverse fetal programming associated with PS. From the articles included, it is clear that EE reverses most behavioural, physiological and neural deficits due to prenatal stress. Differing responses may be dependent on the timing and variability of stress and EE, exercise, and the potential vulnerable or resilient phenotypes to prenatal stress. Results from this study indicate that enrichment may provide an effective therapy for clinical populations suffering from the effects of prenatal stress.

Keywords: Maternal stress, pregnancy, prenatal stress, enriched environment, mismatch hypothesis, HPA axis, vulnerability, resilience

1. INTRODUCTION

Stress during pregnancy, in particular psychosocial stress, is felt by more than 80 percent of women (Woods et al., 2010). Prenatal stress (PS), induced by exposure to social, physical or environmental distress during pregnancy, threatens the natural regulatory capacity of the endocrine, immune and nervous systems in the developing fetus (Koolhaas et al., 2011). A stressful situation experienced by the pregnant mother activates the hypothalamic-pituitary-adrenal (HPA) axis to initiate the release of stress hormones, such as cortisol in humans or corticosterone in rats. The corticosteroids may enter the fetal circulation (Migeon et al., 1956) and program the offspring`s HPA axis activity and stress response in later life (Bosch et al., 2007, Glover et al., 2009). In addition, the effects of maternal stress can be passed on to the offspring through epigenetic mechanisms (Mueller and Bale, 2008, Oberlander et al., 2008) and through changes in maternal behaviour and care (Meaney, 2001, Champagne et al., 2006). Furthermore, paternal effects of stress on maternal behaviour (Mashoodh et al., 2012) and offspring development (Mychasiuk et al., 2013) have also been investigated . Epigenetic changes transmitted from the father by stressing male rats during spermatogenesis, found that stress led to decreased stress reactivity and slowed the development of motor function (Mychasiuk et al., 2013). In addition, the paternal stress altered DNA methylation patterns in offspring which was visible in adolescence. While programming by PS may primarily have the purpose to better prepare the offspring for survival in adverse environmental conditions, the adaptive benefit may come at a physiological and metabolic expense, thus generating adverse health outcomes.

Adverse health outcomes linked to PS concern the endocrine and immune systems, with secondary health outcomes manifesting in the elevated risk of psychopathologies and neurological disease (Glover, 2011), reproductive health and pregnancy outcomes (Arck, 2001, Yao et al., 2014), along with hypertension and diabetes (Lindsay et al., 1996). For example, PS, mainly through dysregulation of the HPA axis, may alter neurodevelopment and lead to altered dendritic morphology (Mychasiuk et al., 2012), and the susceptibility to affective and hyperactive behaviours [(Ronald et al., 2010) for review on PS and brain development see Charil et al., 2010)]. These phenotypes have been shown to persist to future generations (Zucchi. et al., 2013, Yao et al., 2014). Based on the evidence that PS adversely effects health and is passed across multiple generations, treatments to prevent or reverse the detrimental effects of PS are of significant interest.

Enriched environment (EE) is a non-invasive treatment that produces robust changes in neuronal morphology and behaviour. In experimental studies, housing animals in an EE provides them with rich social, motor, cognitive and sensory stimulation. EE was first studied in the 1940's by Donald O. Hebb when he brought rats to his home and had his children play with them, essentially "enriching" their environment. He noted that rats reared as pets in his house performed better on memory tasks compared to rats reared in standard conditions (Hebb, 1947). Further study by Hebb's team found that laboratory dogs that were treated as pets are superior in problem-solving than those reared in simple or deprived environments (Clarke et al., 1951). In addition, the authors suggested that social behaviour and motivation appeared increased in the enriched dogs (Clarke et al., 1951).

Since Hebb's first experiments, the combination of a multimodal stimulation in EE in animal studies has proven to produce many beneficial anatomical, molecular, and

behavioral changes. Neuroanatomical studies have shown an increase in cortical weight, thickness and an increase in dendritic organization in EE rats (Rosenzweig et al., 1962, Bennett et al., 1964, Jung and Herms, 2014). Molecular studies have shown that EE causes an increase in brain-derived neurotrophic factor (BDNF), which is involved in hippocampal (HPC) neuroplasticity and cortex (Falkenberg et al., 1992). EE effectively promotes recovery from neurological disorders, such as stroke (Kolb and Gibb, 1991, Knieling et al., 2009), traumatic brain and spinal cord injury (Fischer and Peduzzi, 2007) and has protective effects in neurodegenerative disorders, such as Parkinson's disease (Jadavji et al., 2006, Jadavji and Metz, 2009). Notably, EE has considerable translational value for preclinical studies. Enriching an environment has significant ecological validity for the human population and better reproduces the human lifestyle than a standard shoebox cage with pair housing. Furthermore, EE strategies have also been successfully applied to the human population. Although enrichment in a complex human environment can hardly be standardized, a wealth of studies has shown that specific experiential treatments promote endocrine, neuronal and behavioural functions. Exposure to mindfulness meditation (Lutz et al., 2008), music lessons (Metzler et al., 2013) or physical activities (Anderson and Shivakumar, 2013) represent just a few of the intriguing examples. Despite these successes, only few studies have used EE to reverse the consequences of PS.

Given the extensive use of EE in animal studies of brain plasticity, it is somewhat surprising that relatively few studies have applied this treatment to PS. One reason for this lack of studies may be the expectation that EE is insufficient to address the drastic impact of PS. For example, the complex environment offered by EE encourages extensive physical exercise, which may elevate the stress response (Larson et al., 2002). Furthermore, EE houses animals in larger groups compared to regular housing conditions in standard shoebox cages. The interaction in a large social setting may at times generate aversive behaviours, depending on the space provided (Marashi et al., 2003). Accordingly, EE has been reported to cause neuroendocrine effects that are similar to chronic stress (Moncek et al., 2004). In addition, Larrson et al. (2002) suggested that the rich physical and sensory stimulation generated by EE may represent a mild, recurring stressor due to the repeated introduction of novel objects (Larsson et al., 2002).

The use of an EE to treat consequences of PS requires a cautionary note. Two hypotheses, the hypothesis of cumulative stress and the mismatch hypothesis, create concern over the appropriate postnatal environment for prenatally stressed individuals. The cumulative stress hypothesis states that aversive experiences in early life predispose individuals to be more vulnerable to aversive challenges later in life (Nederhof and Schmidt, 2012). In this hypothesis, cumulative or chronic stress causes an individual to be unable to cope with stressors and as a result, the wear and tear of stress takes a toll on health. Here, one could expect that EE would have a beneficial effect, essentially reversing the adverse health outcomes promoted by PS. On the other hand, the mismatch hypothesis states that aversive experiences early in life trigger adaptive processes, which render an individual to be better adapted to aversive challenges in later life (Nederhof, 2012, Nederhof and Schmidt, 2012, Santarelli et al., 2014). If this hypothesis proves correct, one would expect that prenatally stressed offspring, who are essentially being prepared for a stressful environment, would not benefit from being placed in an EE. Due to these two contradicting hypothesis, it is important to review the current findings on PS

and EE to determine if EE is a suitable treatment option to reverse the adaptive or detrimental effects of PS.

This review will summarize the current literature addressing the effectiveness of postnatal EE to treat adverse outcomes associated with PS. Specifically, the review will focus on the question of whether deficits due to PS can be reversed through exposure to EE in later life with the goal of supplying a comprehensive overview of the current literature in the field.

2. METHODS

In March 2015, Web of Science and Pub Med were searched for "Enrichment" or "Enriched Environment" and "Prenatal Stress". After the independent captures were merged and duplicates removed, the initial search yielded a total of 183 articles. Eligibility criteria included English articles, non-conference proceedings and research articles. There were no research-based articles which involved human subjects, so all articles reported in the following pertain to animal studies. A final capture of 15 articles was used for full analysis and data extraction. Studies that focused on multiple treatment groups in addition to PS and offspring enrichment were included, but only the groups of interest were included in the data extraction.

Summarized here are the types of PS and EE used in the studies, and the overall behavioural, morphological and molecular changes induced by PS and EE. Emphasis was placed on studies relating to brain and behaviour, i.e., the effectiveness of EE in reversing behavioural and neuronal deficits due to PS.

3. RESULTS

3.1. Forms of Prenatal Stress and Environmental Enrichment

A variety of techniques can be used to induce PS in animal models. The studies that investigated EE used prenatal stressors that ranged from maternal social and psychological stress (i.e., predator stress and social isolation) to physical stress (i.e., foot shocks, restraint and swim stress). In addition to these stressor types, the time at which the stressor was given (gestational day (G) 12-18 or G13-15), the duration of the stressor (between 3-6 hrs, every day or every other day), the order of stressors (if more than one, random or non-random) and the time of day at which the stressors were given, differed as well. These factors contribute to the overall effect size and severity of PS in animals (Charil et al., 2010) and influence the efficacy of EE therapy.

Along with stressor variability, postnatal enrichment paradigms also differed vastly amongst the included studies. In general, there are two categories of enrichment in the literature included in this review; physical enrichment and physical plus social enrichment. Physical enrichment is defined as enrichment using physical or sensory stimuli to enrich the environment. This type of enrichment may include toys, elevated platforms, exercise wheels and novel objects that change on a regular basis. Social enrichment involves the housing of multiple animals in one large condominium in order to promote social interactions and play. These categories are further divided into the cage dynamics (size of cage, platform levels etc.) as shown in Figure 1. Other manipulations to the environment not included in the illustrations include novel food, various materials for bedding, sand or other material for somatosensory stimulation, the time spent in the EE and the frequency of introducing novel items (every day, once a week, etc.). Also, in

some cases, food was frequently moved to different locations to encourage foraging and explorative behaviour.

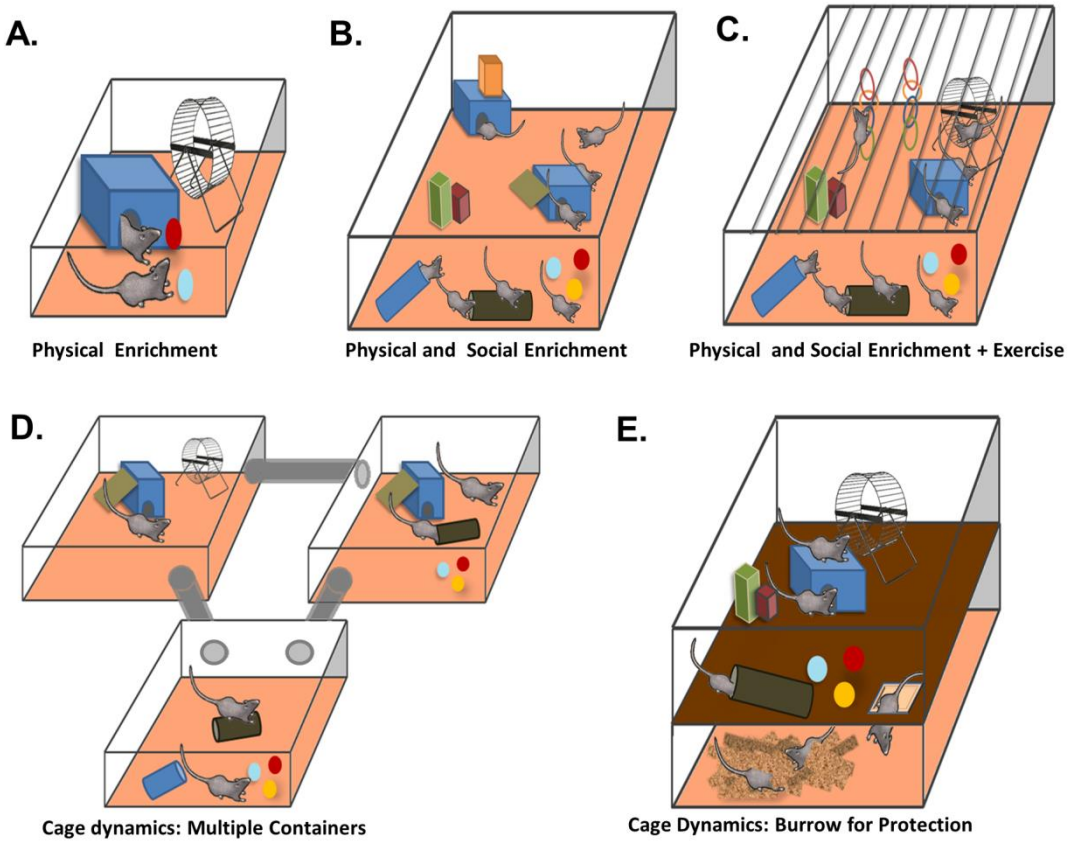

Figure 1: Schematic overview of types of enriched environments. This illustration depicts the variability in enrichment paradigms used across studies, including variation in the physical and sensorimotor components and the social dynamics.

3.2. Reversibility of Prenatal Stress with Enriched Environment

The effects of PS and the impact of EE in the respective animal models of PS were recorded for three categories; behavioural, morphological and molecular. A summary of the effects of PS and EE is provided in the following.

3.2.1. Influence of EE on Behavioural Outcomes Induced by Prenatal Stress

Fourteen of the 15 studies reported behavioural outcomes of PS and EE. Many behavioural paradigms were used to measure changes in affective disorders, fear, addiction, learning and memory, and motor abilities. The particular behavioural changes are summarized in Table 1.

3.2.1.1. Affective Disorders, Fear and Addiction

Anxiety-like behaviours were measured using Open Field, Elevated Plus Maze, Y-maze, social behaviour test, and the Defensive Withdrawal task. In all cases, PS had an anxiogenic effect (Laviola et al., 2004, Pascual et al., 2015, Zubedat et al., 2015), with increased emotionality, as indicated by elevated locomotor activity, increased time spent in closed arms and reduced frequency of age typical rough and tumble play (Table 1). EE had an anxiolytic effect and lowered emotionality. PS was found to increase depressive-like behaviour as measured using the Forced Swim task which was restored with EE (Table 1). Moreover, the amount of fear, as measured by the defensive withdrawal task after acute stress was increased in PS rats whereas animals placed in an EE showed no effect (Qian et al., 2008). Lastly, addiction and attention was measured using Paired Pulse Inhibition (PPI), Object Recognition Task (ORT), and Conditioned Place Preference (CPP). PS increased addictive behaviour in terms of greater preference for morphine (Yang et al., 2006) and impaired selective attention as measured by ORT and impaired partial sustained attention as measured by PPI (Zubedat et al., 2015). Behavioural measurements of addiction were sex-specific, with females showing decreased PPI due to PS and EE, whereas males showed an increase in PPI due to EE only (Emack and Matthews, 2011).

3.2.1.2. Learning and Memory

Learning and memory measured by the Morris water task (or maze), showed impaired performance due to PS in all included studies (Koo et al., 2003, Yang et al., 2007, Lui et al., 2011c, Li et al., 2012, Zhang et al., 2012a, e). The effects of EE were either restorative, eliminating any impairments, as measured by the time to find platform that was similar in both controls and PS+EE, or were additive, which was reflected by improved learning and memory performance as indicated by a decrease in the time to find the hidden platform compared to controls. In all cases, EE seemed to reverse any adverse effect of PS on learning and memory performance (see Table 1 for overview).

It should also be mentioned that one study used electrophysiology (Yang et al., 2007) to study neuroplasticity associated with learning and memory functions. The study reported that long-term potentiation (LTP) and long-term depression (LTD) were altered by both PS and EE; PS impaired LTP and facilitated LTD in the hippocampus, and EE treatment counteracted the PS effect on LTP and LTD (Yang et al., 2007).

3.2.1.3. Motor Skills

Behavioural tests for motor skills in PS- and EE-treated animals included rotorod, string suspension and skilled reaching tasks. PS animals across all studies showed superior performances in motor learning tasks (Ulupinar et al., 2015, Zubedat et al., 2015). EE however, showed differing effects, with one study showing a negative effect in motor learning tasks and reaching performance (Ulupinar et al., 2015), and the other showing superior performance in motor tasks (Zubedat et al., 2015). The major differences between these two studies were the duration of stress (G13-15 versus G14-21) and the type of stressor, one using restraint and the other using a combination of restraint, swim and mirror strength (see Table 1). Notably, EE had a larger benefit when the stress regimen used variable stressors and for a shorter period of time (Zubedat et al., 2015). The two reports of motor performance both provided full access to exercise with the

inclusion of a running wheel within the enriched housing (Ulupinar et al., 2015, Zubedat et al., 2015).

3.2.2. Influence of EE on Neuromorphological Changes Associated with Prenatal Stress

Four of the 15 studies pursued neuromorphological analyses including spine density of granular cells in hippocampus (Peng et al., 2011), Purkinje cell morphology (dendritic area and dendritic perimeter) and granule to Purkinje cell ratio (Pascual et al., 2015, Ulupinar et al., 2015) and T_2 relaxometry (Lui et al., 2011a). All but one study reported that PS induced neuromorphological changes. Regions of interested included the cerebellum and the hippocampus, in particular CA1 and dentate gyrus. Overall, PS decreased dendritic area and perimeter, spine density, granular cells in the HPC, and these consequences were rescued by EE (see Table 1). PS did not affect cerebellar morphology; however, it should be noted that there was no control for the PS condition (Ulupinar et al., 2015). In cerebellum, EE significantly increased the density of granule cells and the granule to Purkinje cell ratio in males. Females showed no significant alterations in cerebellar morphology.

One study used MRI T_2 relaxometry as a measure of neuronal density (Lui et al., 2011a). T_2 relaxometry measures free water concentration where an increased T_2 signal implies increased neuronal loss or possibly increased fiber density or anisotropy (Roch et al., 2002; Fabene and Sbarbati, 2004; Eriksson et al., 2007; Jansen et al., 2008). This study was in agreement with previous reports, showing an increase T_2 signal in the hippocampus, indicating neuronal loss (Kalviainen et al., 1995, Kalviainen et al., 1997).

3.2.3. Effects of EE on Molecular Manifestations of Prenatal Stress

Ten of the 15 studies assessed molecular markers as a function of PS and EE. In the ten studies, 14 markers were used including markers of synaptic plasticity and development, neurogenesis and neuronal growth, stress response, immune regulation, attention, and fear-related learning. These changes are summarized in Table 2.

3.2.3.1. Synaptic Plasticity and Synapse Development

Markers of synaptic plasticity in the cortex and hippocampus included neural cell adhesion molecule (NCAM), synaptophysin (SYP), N-Methyl-D-aspartate receptor (NMDAR), B1-integrin and Tissue plasminogen activator (t-PA). NCAM is a reliable index of synaptic density as it plays a pivotal role in neuronal development, regeneration, and synaptic plasticity (Koo et al., 2003). SYP is a synaptic vesicle protein that is used as an index of synaptic numbers and density and indirectly as a measure of neuronal transmission (Nakamura et al., 1999). Integrins are important in early programming (Huang et al., 2006) and regulate long term potentiation and synaptic efficacy through the activation of NMDAR and calmodulin-dependent protein kinase II signaling cascades (Shi and Ethell, 2006). tPA is highly expressed in brain regions involved in learning and memory, fear and anxiety, motor learning, and stress response (Melchor and Strickland, 2005). Moreover, tPA is thought to increase synaptic strength, as it is elevated after long-term potentiation. Overall, markers of synaptic plasticity in the cortex and hippocampus revealed downregulation in PS and upregulation after EE exposure (Koo et al., 2003, Lui et al., 2011c, Peng et al., 2011, Li et al., 2012). One exception however, showed that a decreased expression of hippocampus b1-integrin and tPA could not be reversed by EE

(Liu et al., 2011). Both are associated with learning and memory consolidation in the adult brain, while mediating synaptic stabilization and strength (Koo et al., 2003). These molecular changes support the behavioural findings related to learning and memory.

3.2.3.2. Neurogenesis and Neuronal Growth

Markers of neurogenesis and neuronal growth included 5-Bromodeoxyuridine (BrdU), BDNF and growth-associated protein 43 (GAP-43). BrdU is incorporated into the DNA during the cell cycle and is widely used as a mitotic marker during development and to identify newly generated neurons in the adult brain (Kolb et al., 1999). BDNF is a neurotrophic protein which regulates activity-dependent dendritic and axonal neuroplasticity along with synapse generation and transmission (Levine et al., 1995, Thoenen, 1995, Schinder and Poo, 2000). BDNF is a prominent marker of structural and functional changes in neuronal populations. GAP-43 is involved in neurite formation, regeneration and plasticity and has shown to play a key role in stress-induced damage to the hippocampus and to regulate dendritic branching *in vitro* (Chao and McEwen, 1994, Gauthier-Campbell et al., 2004). Overall, markers of neurogenesis and neuronal growth were downregulated due to PS, with the exception of one measurement at P15, where upregulation was found (Zhang et al., 2012a). By contrast, these markers were upregulated by EE treatment (Koo et al., 2003, Zhang et al., 2012a, e).

3.2.3.3. Components of the Stress Response

In response to acute and chronic stress, the brain activates many hormonal pathways including the HPA axis. This response leads to the release of corticosteroid hormones from the adrenal glands, which then feedback on the brain by binding to glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs). GRs are of primary interest when investigating PS because they are activated by high levels of corticosteroids or cortisol, and they assist in terminating the stress response through negative feedback regulation. Moreover, GRs participate in memory consolidation and various other behavioural responses (de Kloet et al., 2005).

When comparing levels of cortisol in maternal and fetal plasma samples, researchers have shown that fetal concentrations of cortisol are linearly related to maternal concentrations (Gitau et al., 1998, Gitau et al., 2001) indicating a transfer of HPA programming from the mother to her offspring (Bosch et al., 2007, Glover et al., 2009). Out of the studies investigated here, only two measured stress-related physiological changes. In these two studies, baseline markers of the stress response, CORT and GR, were downregulated due to PS and upregulated due to EE (Emack and Matthews, 2011). In one case, CORT was upregulated in the PS group, but this occurred after an acute bout of stress, and EE further reversed this effect (Morley-Fletcher et al., 2003).

3.2.3.4. Other Relevant Measures

Other measures included markers of immune regulation as well as molecular markers linked to fear-related learning, activity and attention. Markers of immune regulation included CD4 and CD8 T Lymphocytes and interleukin (IL)-1B, IL-10 and IL-2 cytokines. These markers show an increase in inflammatory immune activity due to PS and a reversal due to EE. The measure of activity and attention included dopamine

receptors (DRD 1 and 2) in the nucleus accumbens (NAc). Dopamine receptor D1 (DRD1) and D2 (DRD2) are key receptors that regulate growth and differentiation of dopaminergic neurons (Cabib and Puglisi-Allegra, 2012) and are known to have a decreased expression during stress (Scheggi et al., 2011).

Fear-related learning was also measured using gastrin-releasing peptide receptors (GRPR). GRP and the GRP receptor (GRPR) are distributed throughout the central nervous system and play an important role in regulating amygdala-dependent fear-related learning (Shumyatsky et al., 2002). Previous studies revealed that GRP modulates the expression of corticotropin-releasing hormone (CRH) in the amygdala (Kent et al., 2001a). Markers of activity and attention, and fear-related learning were not affected by PS in these studies, but were positively modulated by EE (Qian et al., 2008).

4. DISCUSSION

The present review investigated if environmental enrichment offers an effective treatment option to reverse adverse behavioural, physiological, and neurological consequences associated with early programming by stress *in utero*. This review provides an aggregated summary of the effect of PS and EE to address the cumulative stress hypothesis, which proposes that EE mitigates adverse consequences of PS, versus the mismatch hypothesis, which proposes that EE is inefficient to reverse adverse fetal programming associated with PS. We reviewed the current findings on PS and postnatal EE to determine if EE provides a suitable treatment option to reverse adverse effects of PS.

Of the initial number of captured studies, 15 were eligible for review. These 15 studies specifically investigated behavioural, physiological, neuromorphological or molecular aspects of PS and postnatal EE therapy. Across all studies, EE seems to have overall beneficial effects on animals that were prenatally stressed. This review revealed that EE, for the most part, reverses behavioural, morphological and molecular consequences of PS. The efficacy of EE, however, may depend on the timing and variability of stress and EE exposure, susceptibility and resilience to PS, and is especially dependent on the application of exercise. Thus, the findings of this report support the cumulative stress hypothesis suggesting that EE offsets the cumulative wear and tear induced by PS and subsequent HPA axis programming and elevated stress sensitivity. Thus, EE may present an effective and clinically relevant experience-based therapy to treat stress-associated disorders and consequences of early trauma.

4.1. The Influence of Variability, Timing, Vulnerability and Resilience

It is apparent that differences in the nature and intensity of PS affect the severity of symptoms and therefore the effectiveness of enrichment therapy. In terms of timing, one study found that enrichment did not reverse the main effects of PS, however, the stress paradigm used in this study was unpredictable and could be considered severe (Emack and Matthews, 2011). The results of this study suggest that shorter bouts of stress, or acute stress late in pregnancy, may have greater long-term effects on HPA activity and related behaviours. Where stress was unpredictable and variable, enrichment did not improve learning and memory or alter SYP levels in the hippocampus (Emack and Matthews, 2011). On the other hand, another study found that enrichment had a larger positive effect on motor abilities when stress was more variable for an acute time period (Zubedat et al., 2015). This could indicate that the timing and variability may affect

behavioural functions and systems differently (motor versus memory). In terms of severity, the most severe stressor included in this review was 6 hours of restraint stress daily for 8 days during late pregnancy (Lui et al., 2011). This study found that EE was not able to reverse the molecular deficits in β-1 Integrin and tPA in the HPC that were generated by PS.

Another consideration is that individual animals, depending on their gender, species and/or age may respond differently to PS based on transgenerationally programmed stress resilience and stress vulnerability (Franklin et al., 2010; Yao et al., 2014). Evidence points to large variations in phenotype observed among PS individuals in a study that suggests differences in vulnerability and resilience to PS (Boersma and Tamashiro, 2015). Stress resilience and vulnerability, according to the mismatch hypothesis, may be related to stress imposed during the prenatal environment as well as to the possible stress induced by enrichment. The stress associated with enrichment may pertain to extensive physical exercise, the encounter of novel objects, presence of social competitors and larger, open spaces which the animals may perceive as threatening.

Vulnerable individuals may present with a recurrent maladaptive response to stressors and resilient individuals may recognize a stressful situation as minimally threatening enabling the development of a more appropriate, adaptive physiological and psychological response (Franklin et al., 2012). Although these changes were not addressed directly by the articles subject to this review and there was limited work that included females, the data suggest a slight gender difference. It seems that PS causes females to become more vulnerable to stress of enrichment, and males to be more affected by the PS itself. For example, one study showed that enrichment had an opposite effect in males and females where enrichment in females diminished the ability of sensorimotor gating in PPI tests. Furthermore, in another study, prenatally stressed females showed no significant alterations in cerebellar morphology indicating greater resilience to stress due to PS (Pascual et al., 2015). These results help confirm the role of sex steroids in stress vulnerability and resilience (Emack and Matthews, 2011). However, the number of studies including both males and females was only 3 of 15 articles. It is therefore important to include the underrepresented females in future studies to more thoroughly understand sexual dimorphisms in the response to PS and EE.

It should be noted that although previous animal studies have indicated variability in stress resilience or vulnerability as a function of strain, there were no strain differences noted in the studies included in this review. Studies have suggested that Wistar rats may be more “anxious” compared to the Sprague Dawley strain (Rex et al., 2004). These results could suggest that intra-strain differences may play a role in stress response and the effectiveness of EE. According to the mismatch hypothesis, it is possible that the susceptibly anxious Wistar rats would be less likely to benefit from EE exposure.

The cumulative stress and the mismatch hypotheses may also play variable roles at different time periods in life. Therefore, an important consideration concerns the age of the animals at which they were housed in an EE. It has been suggested that individual differences in early programming and the postnatal environment encountered during critical periods of brain development determine whether the cumulative stress hypothesis or the mismatch hypothesis is more applicable (Boersma and Tamashiro, 2015). In the majority of studies in rats, EE was applied at weaning, on or around postnatal day (P) 21, and measurements were collected approximately in adolescence (at P60). However, one group investigated GAP-43 expression at 3 time points; pre-weaning, pre-puberty and

early adolescence, with EE subjected at P10-P30 (Zhang et al., 2012a, e). Here, GAP-43 expression increased significantly on P15 and then decreased from P30-P50. This indicates that during earlier stages, the response to PS may be restorative or protective for the nervous system, and later ages may indicate a rather maladaptive response to PS.

4.2. Exercise as an Essential Component in Enrichment

Each of the reported studies, except one, involved enrichment paradigms that were designed to stimulate physical exercise. This particular aspect of enrichment may have the largest influence on functional improvements in prenatally stressed animals (Harburger et al., 2007). Supporting studies have been designed to measure the effect of the physical exercise component of EE. One such study compared voluntary and forced activity with or without the addition of a learning task in hippocampal neurogenesis of adult mice (van Praag et al., 1999). Their results showed that neurogenesis was only increased in mice that had voluntary access to running wheels (van Praag et al., 1999). In line with this finding, the one study included in this review that did not result in beneficial effects used an EE that lacked an exercise paradigm (Emack and Matthews, 2011). Thus, the inclusion of an exercise facility in the EE may be vital for its benefit. Moreover, in paradigms that lacked social enrichment, there seemed to be no less beneficial effect of enrichment, further emphasizing the positive benefits of physical enrichment. Recently, a group of researchers proposed a standardized EE design, which may assist in obtaining more consistent results across studies (Fares et al., 2012).

4.3. FUTURE IMPLICATIONS

4.3.1. Enriched Environment as a Therapy for Ancestral Stress

Growing evidence indicates that programming by PS not only affects the F1 generation, but also propagates to further generations of offspring (Zucchi et al., 2012, Yao et al., 2014, Babenko et al., 2015). Transgenerational programming by prenatal stress or postnatal trauma across two or three filial generations of the paternal and maternal lineages have been suspected to alter stress response (Morgan and Bale, 2011, Zucchi et al., 2012), elevate risk of metabolic disorders (Yehuda et al., 2005) and psychopathologies (Babenko et al., 2015) and promote pregnancy complications (Yao et al., 2014). Since exposure to ancestral stress is uncontrollable to the filial generations, a vital question is whether EE presents an effective therapy to protect or even reverse adverse health outcomes in a stress lineage. Indeed, our unpublished findings suggest that EE is able to restore phenotypic and epigenetic manifestations linked to ancestral PS (McCreary et al., 2015). This work supported a central role for epigenetic modifications as one of the primary mechanisms of stress transfer and reversal by environmental enrichment. Epigenetic changes can be brought on by prenatal experiences prenatally, during development, or be passed on from the parent to the offspring (Meaney and Szyf, 2005; Franklin et al., 2010). Because epigenetic regulators readily respond to environmental conditions and so allow rapid modifications to a changing environment, these processes may advance the discovery of predictive epigenetic signatures linked to disease and initiate the discovery of new diagnostics and therapeutic interventions for future generations.

4.3.2. Implications for EE Therapy in Human Populations

The results from previous studies and this review spark a discussion about the potential use of enrichment therapies in human populations affected by prenatal or ancestral stress. Surmounting evidence from human epidemiological studies suggest that PS raises risk of metabolic diseases and stress-related psychopathologies (Entringer, 2013). Earlier reports indicated that specific experiential treatments, such as mindfulness meditation (Lutz et al., 2008), music lessons (Metzler et al., 2013) or physical activities (Anderson and Shivakumar, 2013) promote a healthy human lifestyle. Based on the promising findings from such specific experiential therapies, it is reasonable to assume that a more complex experiential therapy, which combines aspects of social and physical enrichment, has beneficial effects on health outcomes in prenatally stressed individuals or other populations at risk. For example, strategies that involve Centering Pregnancy® to form social support groups for prenatal care were proven successful in promoting maternal and newborn health outcomes (Benediktsson et al., 2013, Trotman et al., 2015). Community support groups or policies that facilitate access to lifestyle enrichment may be central prerequisites to develop an effective strategy that targets improved health outcomes in communities.

5. CONCLUSIONS

The last decade is highlighted by considerable efforts to unravel the mechanisms of how maternal stress impacts offspring lifetime health trajectories. Although the consequences of maternal stress can be complex and long-lasting, there still is no recognized therapeutic approach to offset the consequences of adverse programming by PS. In this review, the articles included suggest effectiveness of postnatal EE to treat adverse outcomes associated with PS in terms of behavioural, physiological and neural deficit as long as physical exercise is a part of the enrichment paradigm. Differing responses to EE may be dependent on the timing and variability of stress and postnatal enrichment, and the resiliency or vulnerability of the phenotypes to PS. The advanced understanding of the mismatch hypothesis and the vulnerability to postnatal environments may help us understand how EE can be catered towards individuals at risk.

The present analysis illustrates the relatively small number of studies that addressed EE therapy in the context of PS and recommend cautionary conclusions due to the variability in stress and enrichment procedures, and the lack of data in females. Future studies therefore should consider sex differences and also include advanced ‘omics technologies, such as epigenomics, in order to identify potential prognostic biomarkers and therapeutic targets. Since adverse experience linked to PS is reflected by or even causally linked to epigenetic marks, EE may provide a suitable therapy to reverse stress-associated epigenetic changes through beneficial experience. The present analysis presents EE as a powerful, clinically relevant multi-modal experience-based therapy to treat stress-associated disorders and consequences of early trauma in populations put at risk by poverty, violence, limited opportunity and lack of social support.

Ref.	Prenatal Stressor	Type of EE	Animals	Measures	Behavioural Outcomes of PS	Behavioural Outcomes of PS+EE
Emack & Matthews 2011	G32–66 1 of 4 stressors every 2nd day 1. Novel environment (2/day) 2. Social stress (2/day) 3. Forced foraging 4. Intermittent food	N=6-10 P25 Physical enrichment and social enrichment	Guinea Pig ♂ & ♀ P35, P50 & P70	• Open Field • PPI	Male: PS elevated locomotor activity **PPI:** Females: PS decreased PPI	Females: decrease in locomotor activity **PPI:** Males: EE increased PPI in both control and PS offspring Female offspring: both PS and EE decreased PPI
Koo et al., 2003	G4-5 to G20-21 Immobilization stress for 6 h/day	N= 10-12 P21 Physical enrichment and social enrichment exercise	SD Rats ♂ P60 & P90	• Y-maze test • Water-maze	PS impaired learning & memory performance	EE enhanced cognitive functions
Laviola et al., 2004	G11-G21 Restraint and white light for 45 min 3X a day	N=2 P22 Physical enrichment and exercise	SD rats ♂ P38-P43	• Open field • Social behaviour test	PS reduced age-typical rough-and-tumble play PS increased emotionality	Increase in the amount of positive species-typical behaviour (i.e. rough-and-tumble play) and reduced emotionality
Li et al., 2012	G13- G19 Restraint 3/day at 45mins long	N=4 P21-P34 Physical enrichment and social enrichment exercise	SD rats ♂ & ♀ P35	• EPM • Water-maze	PS impaired learning & memory performance PS displayed more anxiety like behaviour	EE reduced anxiety-like behavior in PS offspring Learning & memory was partially increased
Lui et al., 2011	G14-21 Restraint 6h/day	N=6-8 P22 Physical enrichment and social enrichment exercise	SD rats ♂ P22-120	• Water-maze	PS adult offspring showed cognitive deficit	EE improved cognition in PS-EE group.
Morley-Fletcher et al., 2003	G11-22 Restraint and bright halogen light for 45 minutes 3X/day	N=2 P22 Physical enrichment and exercise and suspended items	SD rats ♂ P22-P60	• Social interaction	PS decreased social play	EE markedly increased social play in PS rats
Pascual et al., 2015	G14-G21 Restraint stress for 45 minutes 3X /day	N=6-8 P22 Physical enrichment and social enrichment exercise and suspended items (EE for 2hrs/day)	CF-1 Mice ♂ P22-P52 or P82	• EPM • Open field	At P82, PS increased anxiety-like behaviour	EE decreased anxiety like behaviour due to PS
Qian et al., 2008	G11-21 Bright light stress for 45 minutes 3X/ day	N= 10 P21 Physical enrichment and social enrichment exercise and suspended items	Wistar Rat ♂ P21-P60	• Defensive with-drawal	Acute Stress increase amount of fear in DWT	Acute stress had no effect on fear in DWT in EE animals

Ulupinar et al., 2015	G14-G21 Restraint 3 h/day	N=12 P21 Physical enrichment and social enrichment with multiple containers and extra exercise	Wistar Rat ♂ & ♀ P49-P60 *no controls	• Rotarod • String suspension • Skilled reaching	Males: PS positively affected reaching performance compared to EE animals	Male: EE negatively affected performance in the Motor-learning tests and reaching performance Females: EE positively affected reaching performance
Yang et al., 2006	G13 - 19 10 foot-shocks (0.8 mA for 1 s, 2–3 min apart for 30 min/day.	N= 10 P22 Physical and social enrichment with multiple containers and exercise	Wistar Rats ♂ P22, P52	• CPP to morphine • Forced Swim	CPP: PS increases addictive behavior (greater preference to morphine) Forced Swim: PS caused higher depressive-like behavior	CPP:EE counteracted PS-induced addictive behavior changes Forced Swim: EE restored PS-elicited depressive-like reactivity in offspring
Yang et al., 2007	G13 - 19 10 foot-shocks (0.8 mA for 1 s, 2–3 min apart for 30 min/day.	N= 10 P22 Physical and social enrichment with multiple containers and exercise	Wistar Rats ♂ P60	• Water-maze • Electro-physiology HPC	Water-maze: Prenatal stress impaired the spatial learning task (longer latencies to escape) Electrophysiology: PS impaired LTP and facilitated LTD	Water-maze: EE rescued spatial learning deficits Electrophysiology: EE treatment counteracted PS effect on LTP and LTD
Zhang et al., 2012A	G13-G19 Restraint 45min 3X/day	Neonatal handling: P4 to P10 (15 mins/day) EE: N=12 P11 Physical and social enrichment exercise and suspended items	SD Rats ♂ P10, P20, P45 (MWM at P45)	• Water-maze	PS impaired the spatial learning and memory ability	EE with neonatal handling promoted the spatial learning memory ability of PS rats
Zhang et al., 2012B	G13-G19 Restraint 45min 3X/day	N=12 P10 Physical and social enrichment exercise and suspended items	SD Rats ♂ P15, P30 & P50	• Water-maze	PS impaired spatial learning & memory	EE enhanced spatial learning and memory compared to controls and PS group
Zubedat et al., 2015	G13–15 G13: Swim stress (5min) G14: Mirror stress (5min) G15: Restraint stress: 3X 30 mins	N=8 P30 Physical and social enrichment with exercise (added sandbox in order to diversify the cage texture, diet enriched)	Wistar Rats ♂ P95	• Open field • Object recog. • Rotor-rod • PPI & startle response	PS had a clear anxiogenic effect. PS increased startle response and immobility in startle reflex test. PS group showed superior performance in the Motor-learning task. PS impaired selective attention (ORT) as well as partial sustained attention (PPI)	EE had an anxiolytic effect. EE decreased startle response and immobility in startle reflex test. EE increased both PPI and ORT performance. EE group showed superior performance in the Motor-learning task

Table 2. Summary of the molecular markers of prenatal stress and environmental enrichment and related outcomes (Hippocampus: HPC, Dentate Gyrus: DG, Prefrontal Cortex: PFC, Nucleus accumbens: NAcc)

References	Marker	Area of interest	PS	EE
Synaptic Plasticity and Development				
Koo et al., 2003	**NCAM**	HPC, Cortex	↓	↑
Koo et al, 2003 Li et al., 2012, Lui et a., 2011, Peng et al., 2011	**SYP**	HPC, Cortex	↓	↑
Lui et al., 2011	**NMDAR**	HPC	↓	↑
Lui et al., 2011	**β-1 Integrin**	HPC	↓	-
Lui et al., 2011	**t-PA**	HPC	↓	-
Neurogenesis and Neuronal Growth				
Koo et al., 2003	**BrdU**	HPC (DG)	↓	↑
Koo et al., 2003	**BDNF**	HPC, cortex	↓	↑
Zhang et al., 2012A, B	**GAP-43**	HPC (P15 and P30-50)	**P15 ↑ P30- P50 ↓**	↑
Stress Response				
Emack and Matthews, 2011 Laviola et al., 2004 Morley-Fletcher et al., 2003	**CORT**	Peripheral	♂ ↓ ↓ ↑	♂ ↑ ♀↑ ↑ ↓
Li et al., 2012	**GR**	HPC	↓	↑
Immune Regulation				
Laviola et al., 2004	**CD 4, CD8**	Spleen, PFC	↓	↑
Laviola et al., 2004	**Il-1b**	Spleen, PFC	↑	↓
Activity and Attention				
Emack and Matthews, 2011	**DRD-1 and DRD-2**	NAcc	-	↓
Fear Related Learning				
Qian et al., 2008	**GRPR**	Amygdala	-	↑

References

Anderson E, Shivakumar G (2013) Effects of exercise and physical activity on anxiety. Front Psychiatry 4:27.

Arck PC (2001) Stress and pregnancy loss: role of immune mediators, hormones and neurotransmitters. American journal of reproductive immunology 46:117-123.

Babenko O, Kovalchuk I, Metz GA (2015) Stress-induced perinatal and transgenerational epigenetic programming of brain development and mental health. Neuroscience and biobehavioral reviews 48:70-91.

Benediktsson I, McDonald SW, Vekved M, McNeil DA, Dolan SM, Tough SC (2013) Comparing CenteringPregnancy(R) to standard prenatal care plus prenatal education. BMC pregnancy and childbirth 13 Suppl 1:S5.

Bennett EL, Diamond MC, Krech D, Rosenzweig MR (1964) Chemical and Anatomical Plasticity Brain. Science 146:610-619.

Boersma GJ, Tamashiro KL (2015) Individual differences in the effects of prenatal stress exposure in rodents. Neurobiology of Stress 1:100-108.

Bosch OJ, Musch W, Bredewold R, Slattery DA, Neumann ID (2007) Prenatal stress increases HPA axis activity and impairs maternal care in lactating female offspring: implications for postpartum mood disorder. Psychoneuroendocrinology 32:267-278.

Cabib S, Puglisi-Allegra S (2012) The mesoaccumbens dopamine in coping with stress. Neuroscience and biobehavioral reviews 36:79-89.

Champagne FA, Weaver IC, Diorio J, Dymov S, Szyf M, Meaney MJ (2006) Maternal care associated with methylation of the estrogen receptor-alpha1b promoter and estrogen receptor-alpha expression in the medial preoptic area of female offspring. Endocrinology 147:2909-2915.

Chao HM, McEwen BS (1994) Glucocorticoids and the expression of mRNAs for neurotrophins, their receptors and GAP-43 in the rat hippocampus. Brain research Molecular brain research 26:271-276.

Charil A, Laplante DP, Vaillancourt C, King S (2010) Prenatal stress and brain development. Brain research reviews 65:56-79.

Clarke RS, Heron W, Fetherstonhaugh ML, Forgays DG, Hebb DO (1951) Individual differences in dogs: preliminary report on the effects of early experience. Canadian journal of psychology 5:150-156.

de Kloet ER, Joels M, Holsboer F (2005) Stress and the brain: from adaptation to disease. Nature reviews Neuroscience 6:463-475.

Emack J, Matthews SG (2011) Effects of chronic maternal stress on hypothalamo-pituitary-adrenal (HPA) function and behavior: No reversal by environmental enrichment. Hormones and Behavior 60:589-598.

Entringer S (2013) Impact of stress and stress physiology during pregnancy on child metabolic function and obesity risk. Curr Opin Clin Nutr Metab Care 16:320-327.

Falkenberg T, Mohammed AK, Henriksson B, Persson H, Winblad B, Lindefors N (1992) Increased expression of brain-derived neurotrophic factor mRNA in rat hippocampus is associated with improved spatial memory and enriched environment. Neurosci Lett 138:153-156.

Fares RP, Kouchi H, Bezin L (2012) Standardized environmental enrichment for rodents in Marlau cage.

Fischer FR, Peduzzi JD (2007) Functional recovery in rats with chronic spinal cord injuries after exposure to an enriched environment. J Spinal Cord Med 30:147-155.

Franklin TB, Saab BJ, Mansuy IM (2012) Neural mechanisms of stress resilience and vulnerability. Neuron 75:747-761.

Gauthier-Campbell C, Bredt DS, Murphy TH, El-Husseini Ael D (2004) Regulation of dendritic branching and filopodia formation in hippocampal neurons by specific acylated protein motifs. Mol Biol Cell 15:2205-2217.

Gitau R, Cameron A, Fisk NM, Glover V (1998) Fetal exposure to maternal cortisol. Lancet 352:707-708.

Gitau R, Fisk NM, Teixeira JM, Cameron A, Glover V (2001) Fetal hypothalamic-pituitary-adrenal stress responses to invasive procedures are independent of maternal responses. The Journal of clinical endocrinology and metabolism 86:104-109.

Glover V (2011) Annual Research Review: Prenatal stress and the origins of psychopathology: an evolutionary perspective. J Child Psychol Psychiatry 52:356-367.

Glover V, Bergman K, Sarkar P, O'Connor TG (2009) Association between maternal and amniotic fluid cortisol is moderated by maternal anxiety. Psychoneuroendocrinology 34:430-435.

Harburger LL, Nzerem CK, Frick KM (2007) Single enrichment variables differentially reduce age-related memory decline in female mice. Behav Neurosci 121:679-688.

Hebb DO (1947) The effects of early experience on problem solving at maturity. Am Psychol 2:306-307.

Jadavji NM, Kolb B, Metz GA (2006) Enriched environment improves motor function in intact and unilateral dopamine-depleted rats. Neuroscience 140:1127-1138.

Jadavji NM, Metz GA (2009) Both Pre- and Post-Lesion Experiential Therapy Is Beneficial in 6-Hydroxydopamine Dopamine-Depleted Female Rats. Neuroscience 158:373-386.

Jung CKE, Herms J (2014) Structural Dynamics of Dendritic Spines are Influenced by an Environmental Enrichment: An In Vivo Imaging Study. Cerebral cortex 24:377-384.

Kalviainen R, Laakso MP, Partanen K, Soininen H, Riekkinen PJ, Pitkanen A (1995) Elevated Magnetic-Resonance T2 Relaxation-Time as a Marker of Hippocampal Pathology in Temporal-Lobe Epilepsy - a Comparison to Alzheimers-Disease. Neurology 45:A403-A403.

Kalviainen R, Partanen K, Aikia M, Mervaala E, Vainio P, Riekkinen PJ, Sr., Pitkanen A (1997) MRI-based hippocampal volumetry and T2 relaxometry: correlation to verbal memory performance in newly diagnosed epilepsy patients with left-sided temporal lobe focus. Neurology 48:286-287.

Knieling M, Metz GA, Antonow-Schlorke I, Witte OW (2009) Enriched Environment Promotes Efficiency of Compensatory Movements after Cerebral Ischemia in Rats. Neuroscience 163:759-769.

Kolb B, Gibb R (1991) Environmental enrichment and cortical injury: behavioral and anatomical consequences of frontal cortex lesions. Cerebral cortex 1:189-198.

Koo JW, Park CH, Choi SH, Kim NJ, Kim HS, Choe JC, Suh YH (2003) Postnatal environment can counteract prenatal effects on cognitive ability, cell proliferation, and synaptic protein expression. Faseb J 17:1556-+.

Larsson F, Winblad B, Mohammed AH (2002) Psychological stress and environmental adaptation in enriched vs. impoverished housed rats. Pharmacology, biochemistry, and behavior 73:193-207.

Laviola G, Rea M, Morley-Fletcher S, Di Carlo S, Bacosi A, De Simone R, Bertini M, Pacifici R (2004) Beneficial effects of enriched environment on adolescent rats from stressed pregnancies. European Journal of Neuroscience 20:1655-1664.

Levine ES, Dreyfus CF, Black IB, Plummer MR (1995) Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors. Proceedings of the National Academy of Sciences of the United States of America 92:8074-8077.

Li M, Wang M, Ding S, Li C, Luo X (2012) Environmental Enrichment during Gestation Improves Behavior Consequences and Synaptic Plasticity in Hippocampus of Prenatal-Stressed Offspring Rats. Acta Histochemica Et Cytochemica 45:157-166.

Lindsay RS, Lindsay RM, Waddell BJ, Seckl JR (1996) Prenatal glucocorticoid exposure leads to offspring hyperglycaemia in the rat: studies with the 11 beta-hydroxysteroid dehydrogenase inhibitor carbenoxolone. Diabetologia 39:1299-1305.

Lui CC, Wang J-Y, Tain Y-L, Chen Y-C, Chang K-A, Lai M-C, Huang L-T (2011a) Prenatal stress in rat causes long-term spatial memory deficit and hippocampus MRI abnormality: Differential effects of postweaning enriched environment. Neurochemistry International 58:434-441.

Lui CC, Wang JY, Tain YL, Chen YC, Chang KA, Lai MC, Huang LT (2011c) Prenatal stress in rat causes long-term spatial memory deficit and hippocampus MRI abnormality: Differential effects of postweaning enriched environment. Neurochemistry International 58:434-441.

Lutz A, Slagter HA, Dunne JD, Davidson RJ (2008) Attention regulation and monitoring in meditation. Trends in cognitive sciences 12:163-169.

Marashi V, Barnekow A, Ossendorf E, Sachser N (2003) Effects of different forms of environmental enrichment on behavioral, endocrinological, and immunological parameters in male mice. Horm Behav 43:281-292.

Mashoodh R, Franks B, Curley JP, Champagne FA (2012) Paternal social enrichment effects on maternal behavior and offspring growth. Proceedings of the National Academy of Sciences of the United States of America 109:17232-17238.

McCreary JK, Erickson ZT, Babenko A, Ilnytskyy Y, Olson DM, Kovalchuk I, Metz GAS (2015) Environmental enrichment reverses transgenerational programming by early trauma In: International Behavioural Neuroscience Society Annual Meeting Victoria, Canada.

Meaney MJ (2001) Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annual review of neuroscience 24:1161-1192.

Melchor JP, Strickland S (2005) Tissue plasminogen activator in central nervous system physiology and pathology. Thromb Haemost 93:655-660.

Metzler MJ, Saucier DM, Metz GA (2013) Enriched childhood experiences moderate age-related motor and cognitive decline. Frontiers in Behavioral Neuroscience 7.

Migeon CJ, Prystowsky H, Grumbach MM, Byron MC (1956) Placental passage of 17-hydroxycorticosteroids: comparison of the levels in maternal and fetal plasma and effect of ACTH and hydrocortisone administration. The Journal of clinical investigation 35:488-493.

Moncek F, Duncko R, Johansson BB, Jezova D (2004) Effect of environmental enrichment on stress related systems in rats. Journal of neuroendocrinology 16:423-431.

Morgan CP, Bale TL (2011) Prenatal stress and transgenerational epigenetic programming via the paternal lineage. Faseb J 25.

Morley-Fletcher S, Rea M, Maccari S, Laviola G (2003) Environmental enrichment during adolescence reverses the effects of prenatal stress on play behaviour and HPA axis reactivity in rats. European Journal of Neuroscience 18:3367-3374.

Mueller BR, Bale TL (2008) Sex-specific programming of offspring emotionality after stress early in pregnancy. J Neurosci 28:9055-9065.

Mychasiuk R, Gibb R, Kolb B (2012) Prenatal stress alters dendritic morphology and synaptic connectivity in the prefrontal cortex and hippocampus of developing offspring. Synapse 66:308-314.

Mychasiuk R, Harker A, Ilnytskyy S, Gibb R (2013) Paternal stress prior to conception alters DNA methylation and behaviour of developing rat offspring. Neuroscience 241:100-105.

Nakamura H, Kobayashi S, Ohashi Y, Ando S (1999) Age-changes of brain synapses and synaptic plasticity in response to an enriched environment. Journal of neuroscience research 56:307-315.

Nederhof E (2012) The mismatch hypothesis of psychiatric disease. Physiology & behavior 106:689-690.

Nederhof E, Schmidt MV (2012) Mismatch or cumulative stress: toward an integrated hypothesis of programming effects. Physiology & behavior 106:691-700.

Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM (2008) Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics : official journal of the DNA Methylation Society 3:97-106.

Pascual R, Valencia M, Bustamante C (2015) Purkinje Cell Dendritic Atrophy Induced by Prenatal Stress Is Mitigated by Early Environmental Enrichment. Neuropediatrics 46:37-43.

Peng Y, Jian X, Liu L, Tong J, Lei D (2011) Influence of environmental enrichment on hippocampal synapses in adolescent offspring of mothers exposed to prenatal stress. Neural Regeneration Research 6:378-382.

Qian J, Zhou D, Pan F, Uu C-x, Wang Y-w (2008) Effect of environmental enrichment on fearful behavior and gastrin-releasing peptide receptor expression in the amygdala of prenatal stressed rats. Journal of neuroscience research 86:3011-3017.

Rex A, Voigt JP, Gustedt C, Beckett S, Fink H (2004) Anxiolytic-like profile in Wistar, but not Sprague-Dawley rats in the social interaction test. Psychopharmacology 177:23-34.

Ronald A, Pennell CE, Whitehouse AJ (2010) Prenatal Maternal Stress Associated with ADHD and Autistic Traits in early Childhood. Frontiers in psychology 1:223.

Rosenzweig MR, Krech D, Bennett EL, Zolman JF (1962) Variation in environmental complexity and brain measures. Journal of comparative and physiological psychology 55:1092-1095.

Santarelli S, Lesuis SL, Wang XD, Wagner KV, Hartmann J, Labermaier C, Scharf SH, Muller MB, Holsboer F, Schmidt MV (2014) Evidence supporting the match/mismatch hypothesis of psychiatric disorders. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology 24:907-918.

Scheggi S, Marchese G, Grappi S, Secci ME, De Montis MG, Gambarana C (2011) Cocaine sensitization models an anhedonia-like condition in rats. Int J Neuropsychopharmacol 14:333-346.

Schinder AF, Poo M (2000) The neurotrophin hypothesis for synaptic plasticity. Trends Neurosci 23:639-645.

Shi Y, Ethell IM (2006) Integrins control dendritic spine plasticity in hippocampal neurons through NMDA receptor and Ca2+/calmodulin-dependent protein kinase II-mediated actin reorganization. J Neurosci 26:1813-1822.

Thoenen H (1995) Neurotrophins and neuronal plasticity. Science 270:593-598.

Trotman G, Chhatre G, Darolia R, Tefera E, Damle L, Gomez-Lobo V (2015) The Effect of Centering Pregnancy versus Traditional Prenatal Care Models on Improved Adolescent Health Behaviors in the Perinatal Period. J Pediatr Adolesc Gynecol 28:395-401.

Ulupinar E, Erol K, Aya H, Yucel F (2015) Rearing conditions differently affect the motor performance and cerebellar morphology of prenatally stressed juvenile rats. Behavioural Brain Research 278:235-243.

van Praag H, Kempermann G, Gage FH (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nature neuroscience 2:266-270.

Woods SM, Melville JL, Guo Y, Fan MY, Gavin A (2010) Psychosocial stress during pregnancy. American journal of obstetrics and gynecology 202:61 e61-67.

Yang J, Hou C, Ma N, Liu J, Zhang Y, Zhou J, Xu L, Li L (2007) Enriched environment treatment restores impaired hippocampal synaptic plasticity and cognitive deficits

induced by prenatal chronic stress. Neurobiology of Learning and Memory 87:257-263.

Yang J, Li W, Liu X, Li Z, Li H, Yang G, Xu L, Li L (2006) Enriched environment treatment counteracts enhanced addictive and depressive-like behavior induced by prenatal chronic stress. Brain research 1125:132-137.

Yao Y, Robinson AM, Zucchi FC, Robbins JC, Babenko O, Kovalchuk O, Kovalchuk I, Olson DM, Metz GA (2014) Ancestral exposure to stress epigenetically programs preterm birth risk and adverse maternal and newborn outcomes. BMC medicine 12:121.

Yehuda R, Engel SM, Brand SR, Seckl J, Marcus SM, Berkowitz GS (2005) Transgenerational effects of posttraumatic stress disorder in babies of mothers exposed to the world trade center attacks during pregnancy. Journal of Clinical Endocrinology & Metabolism 90:4115-4118.

Zhang Z, Zhang H, Du B, Chen Z (2012a) Enriched environment upregulates growth-associated protein 43 expression in the hippocampus and enhances cognitive abilities in prenatally stressed rat offspring. Neural Regeneration Research 7:1967-1973.

Zhang Z, Zhang H, Du B, Chen Z (2012e) Neonatal handling and environmental enrichment increase the expression of GAP-43 in the hippocampus and promote cognitive abilities in prenatally stressed rat offspring. Neuroscience Letters 522:1-5.

Zubedat S, Aga-Mizrachi S, Cymerblit-Sabba A, Ritter A, Nachmani M, Avital A (2015) Methylphenidate and environmental enrichment ameliorate the deleterious effects of prenatal stress on attention functioning. Stress 1-9.

Zucchi FC, Yao Y, Metz GA (2012) The secret language of destiny: stress imprinting and transgenerational origins of disease. Frontiers in genetics 3:96.

Zucchi., Yao Y, Ward ID, Ilnytskyy Y, Olson DM, Benzies K, Kovalchuk I, Kovalchuk O, Metz GA (2013) Maternal stress induces epigenetic signatures of psychiatric and neurological diseases in the offspring. Plos One 8:e56967.