SEX-SPECIFIC EFFECTS OF ANCESTRAL STRESS ON BRAIN HEALTH AND DISEASE ACROSS THE LIFESPAN

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I dedicate this thesis in memory of my father
Muhamed Ambeskovic.

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ABSTRACT

Early life stress alters fetal brain development with lifetime consequences on individuals exposed and future generations. This thesis investigated the effects of ancestral stress on behaviour, brain aging, and disease incidence of the F1-F4 generation offspring. Two types of ancestral stress offspring were examined: transgenerational stress, where only great-great grandma was stressed and mutigenerational stress where four consecutive F0-F3 generations were stressed during pregnancy. Here were report three main findings: 1) ancestral stress induced sex-specific anxiety-like behaviour and brain plasticity through altered epigenetic regulation; 2) the effects of ancestral stress persisted across the lifespan, altered physical and mental health and increased risk of disease; and 3) social isolation stress altered stress and immune systems and contributed to sex-specific cognitive impairments. These findings contribute to the overall understanding of the perinatal origins of healthy brain aging and disease, and address the urgent need of recommendations to support healthy aging worldwide.

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LIST OF ABBREVIATIONS

ADHD	Attention Deficit Hyperactivity Disorder
AID	Orbital Frontal Cortex
ANOVA	Analysis of Variance
Cg3	Medial Prefrontal Cortex
CORT	Corticosterone
CRH	Corticotropic Releasing Hormone
DEET	N,N-Diethyl-m-toluamide
DOHaD	Developmental Origins of Health & Disease
DNMT	DNA Methyltransferase
ELISA	Enzyme-Linked Immunosorbent Assay
EPM	Elevated Plus Maze
ER alpha	Estrogen Receptor Alpha
FST	Forced Swim Task
GR	Glucocorticoid Receptor
HPA Axis	Hypothalamic-Pituitary-Adrenal Axis
HPC	Hippocampus
IL-18	Interleukin-18
IL-5	Interleukin-5
LG	Licking and Grooming
LFL	Left Forelimb

LHL	Left Hind Limb
M-CSF	Macrophage Colony-Stimulating Factor
MCP-1	Monocyte Chemoattractant Protein 1
Map1A	Microtubule Associated Protein 1A
MGV	Mean Gray Value
miRNA	MicroRNA
mPFC	Medial Prefrontal Cortex
MPS	Multigenerational Prenatal Stress
MRI	Magnetic Resonance Imaging
MWT	Morris Water Task
NTRK1	Neurotrophic Receptor Tyrosine Kinase1
NGF1-A	Nerve Growth Factor-Induced Protein A
OFC	Orbital Frontal Cortex
OFT	Open Field Task
PAR	Parietal Cortex
PFC	Prefrontal Cortex
PGCs	Primordial Germ Cells
PTSD	Post-Traumatic Stress Disorder
RFL	Right Forelimb
RHL	Right Hind Limb
ROI	Region of Interest
ROS	Reactive Oxygen Species
RS	Restraint Stress

SEM	Standard Error of Mean
TGS	Trans-Generational Stress
TPS	Transgenerational Prenatal Stress

CHAPTER 1: Transgenerational Effects of Early Environmental Insults on Aging and Disease Incidence

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1.1 Abstract

Adverse early life experiences are major influences on developmental trajectories with potentially life-long consequences. Prenatal or early postnatal exposure to stress, undernutrition or environmental toxicants may reprogram brain development and increase risk of behavioural and neurological disorders later in life. Not only experience within a single lifetime, but also ancestral experience affects health trajectories and chances of successful aging. The central mechanism in transgenerational programming of a disease may be the formation of epigenetic memory. This review explores transgenerational effects of early adverse experience on health and disease incidence in older age. First, we address mechanisms of developmental and transgenerational programming of disease and inheritance. Second, we discuss experimental and clinical findings linking early environmental determinants to adverse aging trajectories in association with possible parental contributions and sex-specific effects. Third, we outline the main mechanisms of age-related functional decline and suggest potential interventions to mitigate negative effects of stress. Thus, strategies that support healthy development and successful aging should take into account the potential influences of transgenerational inheritance.

1.2 Introduction

The global aging population continues to grow very rapidly. The United States Census Bureau predicted that very soon the number of individuals aged 65 will exceed the number of children aged 5 years and younger (Bureau, 2016). In 2016, the elderly made up 8.5% of the world's population, and this percentage is expected to rise to 17% by the year 2050 (Bureau, 2016). This rapid growth of an aging population presents both opportunities and challenges. Individuals aging successfully may benefit the society by remaining longer in the workforce, while individuals aging unsuccessfully may constitute an economic burden to the society due to increased disability and disease incidence. Thus, defining what constitutes healthy or successful aging has become a top priority across all medical disciplines.

Although an unambiguous definition of successful aging is still being debated, the most accepted concept comes from the Rowe and Khan model (Rowe & Kahn, 1997; Rowe & Kahn, 2015). In this model, successful aging refers to better-than-usual aging and consists of three principle components: 1) low disease risk; 2) high mental and physical functioning; and 3) continued social engagement (Bowling & Dieppe, 2005; Rowe & Kahn, 1997; Rowe & Kahn, 2015). On the contrary, reversal of these principle components such as low physical and mental state and increased risk of disease would be considered as unsuccessful aging or accelerated aging. This concept provides the fundamental definition for the present review.

The incidence of chronic diseases typically associated with aging, such as cardiovascular, autoimmune and neurodegenerative diseases and metabolic disorders has risen sharply over the past decades. However, aging is not a disease; rather, diseases

associated with aging are the result of accumulated DNA and cell damage and altered protein function that are acquired throughout a lifetime (Bender et al., 2006; Kennedy & Herr, 2012; Tarry-Adkins et al., 2008). McLaughlin et al., (2010) estimated that each year roughly 12% of the older population age successfully while the remaining population still faces age-associated health issues (McLaughlin et al., 2010). Understanding the determinants and mechanisms that influence aging processes, therefore, represents an unmet and urgent medical need.

The origins of adverse health outcomes associated with aging and disease risk in most cases remain unknown. Striking evidence, however, suggests that lifestyle and adverse experiences such as stress play a critical role in onset and severity of these conditions. A substantial body of literature in clinical and experimental studies suggests that experience in early life influences health trajectories and chances for healthy aging. According to the hypothesis of developmental origins of health and disease (DOHaD) both fetal and early childhood exposures to extreme environments such as stress, nutritional challenges and infection may "program" risks for diseases in later life (Barker, 2007; Vaiserman, 2015). For example, children born to mothers who were exposed to famine, war, natural disasters or infection during pregnancy have a higher incidence of cardiac disease, diabetes, neurological and psychiatric diseases as adults (Barker et al., 1993; Devakumar et al., 2014; Meyer et al., 2011; Roseboom, 2000; Roseboom et al., 2001; Yehuda et al., 1998). Similarly, animal studies showed that offspring exposed to adverse environments such as stress, undernutrition, and endocrine disruptors during prenatal or early postnatal development are more likely to show signs of cardiovascular and metabolic disease, anxiety-like and depression-like behaviours in adulthood (Anway et al., 2006;

Franklin et al., 2010; Huck et al., 1987; Long et al., 2013; Manikkam et al., 2012; Rechavi, 2014; Stern et al., 2012). In addition, recent findings suggest that not only experiences within one lifetime, but also experiences of preceding generations of ancestors, affect the chances of healthy aging and disease risk via epigenetic modifications.

Epigenetic mechanisms, such as DNA methylation, histone modification and the action of small non-coding RNAs, play a significant role in the programming of health across a lifetime and across generations. The mechanisms of transgenerational programming are very complex and arguably vary with the severity and the type of exposure, lineage of transmission (maternal or paternal), time of exposure (window of vulnerability of an organ), the sex of an organism and possible germline exposure (Bale & Epperson, 2015; Barker et al., 2012; Jirtle & Skinner, 2007; Lupien et al., 2009; Manikkam et al., 2013; Weinstock, 1997). Furthermore, the severity and the timing of an environmental exposure may determine the specific or broad health consequences ranging from acute to chronic.

Germ cell exposure to an adverse environment during critical developmental periods results in reprogramming and transmission of epigenetic marks across generations. To enable transgenerational inheritance an epigenetic mark has to escape reprogramming in the germline to persist in the absence of re-exposure or discontinued exposure (Bale & Epperson, 2015; Kovalchuk, 2012; Migicovsky & Kovalchuk, 2011; Skinner, 2008). In this review, we use the term transgenerational inheritance to refer to the persistence of an epigenetic change to the unexposed F2 offspring born to exposed preconception males, and the unexposed F3 offspring born to exposed gestating females (Figure 1.1; Skinner, 2008; Zucchi et al., 2012). Intergenerational transmission refers to the propagation of an

epigenetic component from one generation to the next which may not always involve genuinely heritable components (Figure 1.1). For example, studies by Pembrey et al. (2006), reported that the mortality rate of grandsons was associated with their paternal father's food supply during mid-childhood, whereas the mortality rate of the granddaughters was associated solely with the paternal mother's food supply (Bygren et al., 2001; Pembrey et al., 2006). Moreover, Gapp et al., showed that early life stress in fathers improves the behavioural flexibility in their F1 male and F2 female offspring (Gapp et al. 2014). These phenotypic changes were causally associated with altered microRNA (miRNA) expression (Gapp et al., 2014). These observations suggest a role for epigenetic regulation of gene expression in behavioural and emotional phenotype that may propagate from one generation to another.

The epigenetic mechanisms of transgenerational programming of a phenotype may help uncover the causal link between early life stressors and health trajectories from early development to old age. Animal and human studies have shown that environmental insults such as stress, undernutrition, and chemical toxicants can have adaptive or maladaptive consequences on the phenotype (Morley-Fletcher et al., 2011; Zuena et al., 2008), with the latter facilitating transgenerational programming of disease risks. In addition, the sex of an offspring seems to play a major role in the transgenerational inheritance of disease. Although both male and female offspring may have higher disease incidence after ancestral exposure to stress or an environmental toxin, the types of disease and timing of disease onset may vary (Anway et al., 2006). Understanding the factors and mechanisms involved in stress and sex-specific transgenerational programming is necessary to identify robust predictors and new diagnostic biomarkers of lifetime health risks and longevity.

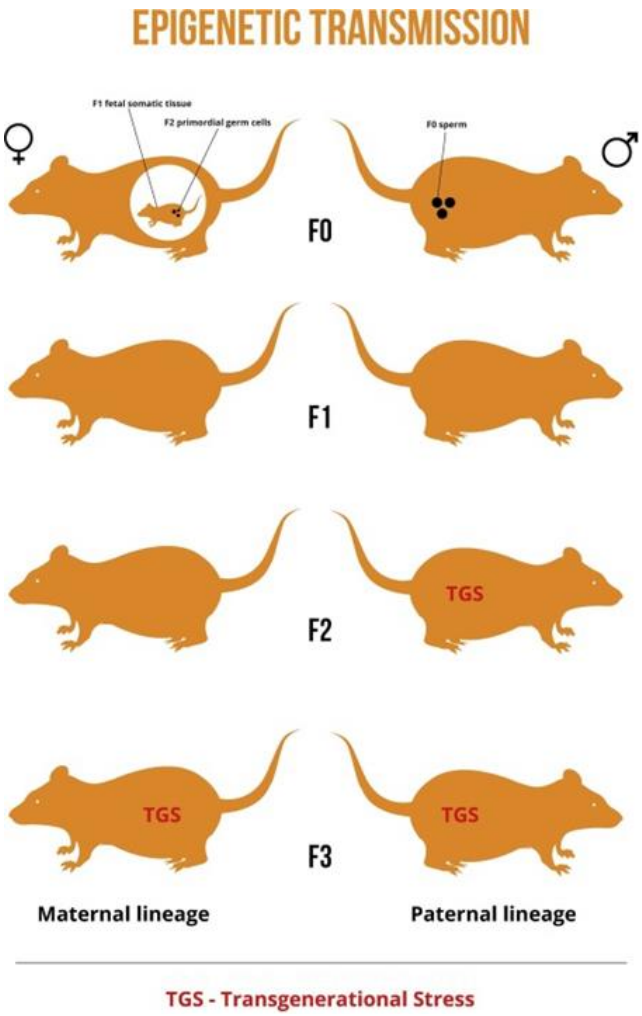

Figure. 1.1. Epigenetic programming by stress in maternal and paternal lineages. Maternal lineage: Exposure of pregnant female rats (F0) to adverse environments, such as stress, alters the somatic and germ cells of the F1 generation. The F2 generation that originates from the F1 germ cells thus was directly exposed to stress. The F3 generation is the first generation not directly exposed to stress that then can be considered to have a transgenerational stress (TGS) phenotype. Paternal lineage: Exposure of F0 fathers will epigenetically alter their sperm, which may then fertilize the oocyte to produce the F1 generation. The F1 generation is directly exposed to stress. When the F1 generation breeds it generates the F2 offspring which represents the first unexposed generation. Therefore, to generate a truly transgenerational phenotype, programmed by heritable epigenetic mechanisms, it takes three generations in the maternal lineage, while it takes only two generations in the paternal lineage.

In this review, we propose the hypothesis that prenatal and transgenerational stressors, through epigenetic regulation, program the lifetime health trajectory, chances of

healthy aging and disease incidence in an individual. We will discuss the main experimental and clinical findings suggesting that the early environment is a critical determinant of the chances of successful aging, and how parental contributions may affect offspring disease risk. Sex-specific epigenetic mechanisms contributing to aging will be examined, with the aim to shed light on why males and females are affected differently by early environmental stressors. Lastly, we will explore the mechanisms of aging and how they may be affected by experiences within a lifespan and across generations. We note that this review is based on the assumption that the foundation for successful aging is laid early in life. Thus, ancestral stress that threatens successful aging may be indicated at a younger age in terms of higher disease risk, greater stress vulnerability or other health complications. We are required to infer such linkages in many cases due to the circumstance that very few life-long causal studies exist in animal and human cohorts. Indeed, the majority of experimental aging studies use young adult animals due to practical, time and financial constraints. Prospective lifetime health studies and transgenerational considerations in experimental and human cohorts are urgently needed in order to further the understanding of complex and age-associated disease etiology.

1.3. Developmental Programming of the Risk of Chronic Disease

Seminal studies by the British epidemiologist David Barker in the 1980s initiated a new era of health research by highlighting that the early environment is a major influence on developmental trajectories with potential life-long consequences (Barker et al., 1989; Barker & Osmond, 1986). Barker (1989, 1993) and his team from the University of Southampton, UK, recognized a striking geographical relationship between prenatal environmental experience and postnatal mortality from heart disease (Barker et al., 1993,

1989). Specifically, a study followed men living in different districts in England to investigate if low fetal growth due to undernutrition contributes to higher risk of ischemic heart disease (Barker et al., 1993). It was found that the incidence of death from heart disease was three times greater in males who weighed 18 pounds or less during their first year of life than those who weighed more. This link between early developmental weight and adult disease risk was termed Barker's fetal origins hypothesis (Barker et al., 1993; Barker & Osmond, 1986). This hypothesis proposes that early developmental reprogramming in the womb induces adaptations in anticipation of a similar postnatal environment (Bale, 2015; Bale et al., 2010; Barker et al., 1993).

Furthermore, Anders Forsdahl and his team observed a high mortality rate in Finnmark, a region in Norway, linked to poor living conditions such as starvation during childhood and adolescence during World War I and the economic downturn (Forsdahl, 2002). The poor living conditions in this region during these times may be responsible for higher infant and adult mortality rates (Forsdahl, 2002). Thus, an adverse early environment during critical periods of development may pose a greater risk of disease in later life and reduce the chances of successful aging in this population (Figure 1.2).

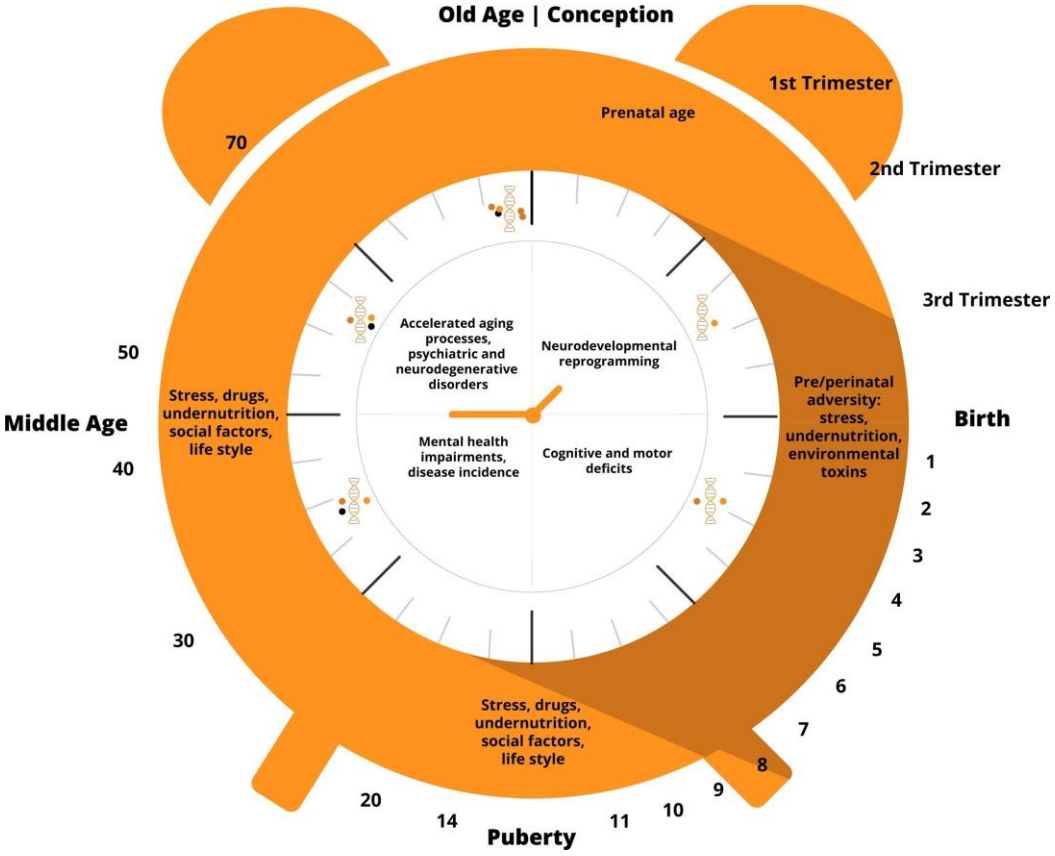

Figure. 1.2. Lifetime clock determining the health trajectory. This model illustrates that the timing of an impact, such as exposure to inherited or acquired adverse experience, is critical in generating a disease phenotype from birth to old age. The timing of exposure is an important variable in determining the risk of disease, with early exposure being the most potent.

It may be argued that undernutrition during intrauterine or early neonatal life prepares the offspring's metabolism to anticipate a low nutrient availability after birth and promote postnatal survival. The functional and structural adaptations in the developing organs resulting from prenatal undernutrition then may not meet the metabolic requirements if, instead of inadequate nutritional supply, the organism is exposed to an abundance of food in later life. This concept proposes a mismatch between the intrauterine and extrauterine environments, in which reprogramming increases disease risk in later life (Bale, 2015; Barker et al., 1993). Hence, the risk of disease is heightened for children with

low birth weight who may then experience a rapid increase in body fat mass later in life (Barker et al., 1993). Accordingly, it was shown that low birth weight was associated with increased risk of coronary heart disease, hypertension, and diabetes later in life (Ashman et al., 2016; Barker et al., 1993). The mismatch hypothesis is also supported by observations in individuals born around the time of the Dutch hunger winter in 1944-45 (Roseboom et al. ,2001) and the Swedish Överkalix study (Kaati et al., 2002; Pembrey et al., 2006). As discussed in more detail later, these studies reported that the type and severity of a disease observed in children and older individuals were related to adverse environment exposure at certain gestational and postnatal ages. The mechanisms of such intergenerational influences on disease risk are complex and not well understood yet. However, recent research has strengthened its focus on such topics and shed light on the mechanisms of inter- and transgenerational programming of health and disease.

1.4 Mechanisms of Transgenerational Programming and Inheritance of Disease

The information from the mother and the father can be passed on to the offspring via three main modes of transmission (Bohacek & Mansuy, 2013; Jirtle & Skinner, 2007; Matthews & Phillips, 2012). First, maternal exposure to an adverse environment such as stress or caloric restriction during pregnancy may affect maternal endocrine function and subsequently induce changes in stress responsiveness in the offspring. Second, altered maternal behaviour and care of the offspring may have long-term consequences on the progeny's development and stress response. Both endocrine changes and maternal care can modify epigenetic regulation of gene expression, leading to an up-stream mechanism of inter- and transgenerational programming. Third, experience-dependent modifications of the epigenome may be passed on to the offspring and potentially to subsequent generations.

Thus, epigenetic modifications can alter the phenotype of the unexposed offspring. Altered epigenetic regulation, in turn, has feedback implications on regulating endocrine function and behaviour. Furthermore, epigenetic regulation of gene expression can alter cellular metabolic processes in response to stress, which directly affects organ function and disease pathways.

1.4.1 Programming by Endocrine Function

Pregnancy represents a period of altered maternal endocrine function. Late gestation and transition to labor is characterized by increased release of maternal stress hormones and hypothalamic-pituitary adrenal (HPA) axis activation (Charil et al., 2010; Glover, 2011). Normally, the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (11 β-HSD2; Benediktsson et al., 1997) which breaks down the maternal stress hormone cortisol, protects the fetus from direct effects of maternal HPA activation. But, when mothers are exposed to severe and prolonged distress during late pregnancy, excessive maternal glucocorticoid levels may saturate the activity of the protective placental enzyme 11 β-HSD2 (Benediktsson et al., 1997; Duthie & Reynolds, 2013), thus making the placenta partially permeable for stress hormones, such as cortisol. Thus, severe maternal distress that leads to the release of excess glucocorticoids may ultimately program fetal HPA axis activity and long-term stress responsiveness (Cole et al., 1995; Harris & Seckl, 2011).

New evidence indicates that fetal programming of HPA axis activity via endogenous glucocorticoids may not only affect the next generation of offspring (F1) but their F2 and even F3 offspring (Yao et al., 2014; Zucchi et al., 2012). For example, Long et al. (2013) reported increased levels of adrenocorticotropic hormone (ACTH) and cortisol with blunted HPA axis reactivity in F2 generation sheep following maternal exposure to

synthetic glucocorticoids during pregnancy (Long et al., 2013). Moreover, synthetic glucocorticoid injection into pregnant guinea pigs raised negative HPA feedback and induced changes in hippocampal glucocorticoid receptor (GR) mRNA expression of their F2 generation male offspring (Iqbal et al., 2012).

Severe distress or undernutrition may also alter the function of major organs (Seki et al., 2012; Sohi et al., 2011). In a rat model, undernutrition during pregnancy raised hepatic cholesterol levels along with increased methylation of histone H3 lysine 9 (Sohi et al., 2011) and hypomethylation of the hepatic GR 1–10 promoter in the liver of offspring (Lillycrop et al., 2007). Thus, maternal undernutrition during pregnancy can determine the metabolic phenotype of the offspring via epigenetic regulation (Lillycrop et al., 2007; Sohi et al., 2011). Moreover, Bertram et al. (2008) reported transgenerational effects of prenatal undernutrition on heart and HPA axis function (Bertram et al., 2008). In this study, pregnant Guinea pigs were placed on food restriction either during early or late pregnancy. The results included drastic changes in heart morphology and HPA axis function in both F1 and F2 generation males, with the largest effects in the F2 generation that was subjected to grandmaternal food restriction during late gestation (Bertram et al., 2008). These F2 male guinea pigs revealed increased basal HPA function, as highlighted by high ACTH and cortisol levels and abnormal heart growth with increased left ventricular wall thickness and mass (Bertram et al., 2008). The findings in the male F2 generation indicate that maternal undernutrition during pregnancy regulates the endocrine and metabolic phenotype via epigenetic alteration.

1.4.2 Programming by Maternal Care

An inter- and transgenerational phenotype can also be induced through a behavioural mode of generational transfer, especially via the critical interactions between mother and her offspring. Maternal care during early fetal development represents a sensitive predictor of psychological development and long-term health in the offspring (Belsky et al., 1991). Accordingly, human and animal data indicate that the quality of maternal care affects both emotional and cognitive development of an offspring (Belsky et al., 1991; Champagne et al., 2003; Champagne & Meaney, 2006; Francis & Meaney, 1999). In rodents, a common paradigm used to assess maternal care is the time a dam spends licking and grooming her pups, and this behaviour is particularly susceptible to stress (Champagne et al., 2003). In seminal experiments, the behaviour of dams was categorized as either high licking-grooming (high LG) or low licking-grooming (low LG; Champagne, 2008; Champagne et al., 2003; Champagne & Meaney, 2006; Francis & Meaney, 1999; Meaney, 2001; Weaver et al., 2004). For example, gestational stress in high LG mothers reduced the quality and frequency of maternal care to that of low LG behavioural patterns (Champagne et al., 2003; Champagne & Meaney, 2006; Meaney, 2001). Moreover, a classic cross fostering study has shown that the patterns of maternal behaviour may be transmitted across generations (Francis et al. 1999). After biological offspring of low LG mothers were reared by high LG dams, they exhibited fewer fearful behaviours and improved HPA axis regulation than any of those raised by low LG mothers (Francis & Meaney, 1999).

A causal relationship between maternal care and stress reactivity was also affirmed by studies examining early postnatal handling in relation to HPA axis reactivity. A study by Macrì et al. (2008) demonstrated that active maternal care is a more salient stimulus in

regulating HPA axis reactivity than maternal separation (Macrì et al., 2008). The development of stress reactivity and HPA axis function in response to altered maternal care were proposed to be mediated by changes in gene expression in brain regions that regulate endocrine responses to stress (Francis & Meaney, 1999; Francis et al., 1999). For example, cross-fostered offspring of low LG mothers reared by high LG mothers had a normal expression of both GR mRNA and corticotrophin-releasing hormone (CHR), which was similar to levels found in high LG offspring.

Growing evidence suggests that the epigenetic changes leading to differential GR gene expression may represent a mechanism of phenotypic transgenerational programming by maternal behaviour (Liu et al., 1997; Weaver et al., 2004). Weaver et al. (2004) demonstrated an increase in DNA methylation of the GR gene at a single CpG site within the nerve growth factor-inducible protein A (NGFI-A) in low LG offspring, following both one week after birth and throughout adulthood (Weaver et al., 2004). In addition, low LG offspring show reduced binding of NGFI-A binding site to the GR promotor, which may contribute to hypomethylation at this site (Weaver et al., 2004). Interestingly, when low LG offspring are adopted by a high LG mother, these epigenetic effects are reversed. Moreover, maternal behaviour may be transmitted across multiple generations via an epigenetic transmission (F1 to F3; Francis et al., 1999). These GR promotor methylation patterns can be maintained across generations and may contribute to transgenerational inheritance of a behavioural phenotype through the maternal lineage (Bohacek & Mansuy, 2013). Accordingly, a study by Liu et al. (1997) demonstrated that the offspring (F1 and F2) of low LG mothers has decreased GR mRNA expression in hippocampus and increased CRH mRNA in the hypothalamus (Liu et al., 1997). The relative contribution of direct

maternal care transmission versus its epigenetic corollary in the transgenerational formation of a behavioural phenotype remains to be determined.

1.4.3 Programming by Epigenetic Regulation

Salient experiences due to a changing environment may reflect in epigenetic modifications in somatic and germ cells (Hajkova et al., 2002). To be considered a transgenerationally inherited trait an epigenetic modification in the germline must be associated with phenotypic alterations (Skinner et al., 2010). Exposure to adverse environmental factors during early development may induce epigenetic changes in the F1 generation germline through direct exposure, but only when the phenotypic alteration is maintained to the F2 generation in the paternal lineage or to the F3 generation in the maternal lineage the modification is considered a truly transgenerational phenotype (Figure 1.1; Anway et al., 2005; Anway et al., 2006; Jirtle & Skinner, 2007; Skinner, 2008; Skinner et al., 2010). Recent studies have demonstrated that the majority of DNA methylation marks is erased during early embryogenesis, but some marks appear to exhibit meiotic stability and are then transferred across generations through early embryo reprogramming (Matthews & Phillips, 2012; Migicovsky & Kovalchuk, 2011). Importantly, germline transmission of DNA methylation across generations may be promoting DNA sequence variation and thus facilitate genetic mutations (Skinner et al., 2015).

The purpose of erasing and re-installing epigenetic signatures via DNA methylation in the primordial germ cells (PGCs) is to establish parental genomic imprints where epimutations are erased, and totipotent and multipotent cells are generated (Hajkova et al., 2002; Reik et al., 2001; Skinner et al., 2013). The PGCs are derived from the epiblast during embryonic development, and by embryonic day (E) 7.2 the embryo contains approximately

45 PGCs (Hajkova et al., 2002). On E13 in rats, the PGCs proliferate and migrate into developing genital ridges (Anway et al., 2005; Hajkova et al., 2002; Manikkam et al., 2013; Mochizuki & Matsui, 2010; Seisenberger et al., 2013). The PGCs will stay in the developing gonads until sex differentiation into male or female germline is initiated at E13 (Hajkova et al., 2002; Skinner et al., 2013). Moreover, PGS undergo an active erasure of the methylome at E13 in the rat and the methylome will be re-established by new DNA methylation at E16 (Hajkova et al., 2002).

Recent research has shown that exposure to stressful events early in life can alter DNA methylation in the germline (Franklin et al., 2010). In a study by Franklin et al. (2010), male mice subjected to early postnatal maternal separation presented with altered methylation of the CpG island surrounding the transcription initiation sites of MeCP2, CB1, and CRFR2 genes in sperm. Similarly, a ground-breaking study by Skinner et al. (2013) showed that exposure to adverse environments early in development alters the DNA methylation marks of the germline and that this epigenetic reprograming can be transmitted transgenerationally. In this rodent study, the germline of F3 generation male offspring whose great-grandmothers were exposed to an agricultural fungicide (vinclozolin) during pregnancy were analyzed for alterations in the germline transcriptome and epimutations at E13 and E16 (Skinner et al., 2013). The results showed disruptions in DNA methylation patterns with larger alterations in the E13 germ cells. In vinclozolin F3 rats a total of 592 genes were differently expressed in the E13 germ cells, and only 148 genes in the E16 germ cells (Skinner et al., 2013).

Although the above studies confirm experience-induced epigenetic alteration in the male germline and research on epigenetic regulation in female germline is still scarce, it

does not mean that female germline programming is less important. The male and female germ cells embark on different sex differentiation processes. In males, gametic methylation is acquired during early spermatogenesis, and methylation occurs during early prenatal life (Bourc'his & Proudhon, 2008). Briefly, germ cells of developing male gonads will proliferate mitotically, and organize into a developing cord that will mature into the seminiferous tubules as part of the testes after birth (Skinner et al., 2013; Wilhelm et al., 2007). In contrast, in the female germ line, gametic methylation occurs in postnatally growing oocytes (Lucifero et al., 2007). A considerable time after somatic methylation, female germ cells will undergo a few mitotic events followed by phase 1 of meiosis before becoming the primary oocytes (Pepling, 2006; Skinner et al., 2013).

In addition to the normal mitotic proliferation of male germ cells during development, subsequent mitotic unrest or meiotic events may take place before full spermatogenic maturation. Moreover, during the migration phase, there is a random X chromosome inactivation in XX germ cells (Tam et al., 1992). It has been demonstrated that the majority but not all inactive X chromosomes are activated when the PGCs are present in the genital ridge, indicating that they potentially play a role in shaping sex differences (Tam et al., 1992). Sex-specific epigenetic programming may also be affected by the line of transmission, via either maternal or paternal lineage. The maternal genome appears epigenetically static while paternal genome exchanges protamines for histones, acquires histone modifications and undergoes DNA methylation (Bourc'his & Proudhon, 2008; Gabory et al., 2009). For example, DNA methylation marks caused by a synthetic GC injection in the paternal lineage were still found in F3 sperm (Franklin et al., 2010),

thus potentially affecting the progeny's health trajectories and disease incidence (Tam et al., 1992).

In general, causal studies linking epigenetic variants and phenotype across generations have been challenging. This challenge is posed mainly by experimental limitations, inabilities to identify if single or global changes in epigenome contribute to transgenerational inheritance and limitations in the available methods for engineering changes to the epigenome. Nevertheless, a few causal connections are documented, such as those linking DNA methylation marks or short non-coding RNAs, mainly miRNAs to the transgenerational transmission of a specific, functionally relevant phenotype (Gapp et al., 2014; Skinner, 2011; Stegemann & Buchner, 2015). For example, Waterland et al. (2008) showed in Agouti viable yellow mice that maternal (F0 generation) obesity could be transmitted to their F2 generation grand-offspring to induce diabetes, and this effect was reversed by a methyl-supplemented diet (Waterland et al., 2008). This finding indicates that epigenetic mechanisms such as a specific DNA methylation mark may be associated with transgenerational inheritance of a complex disease. Two distinct approaches have been used to disentangle transgenerational epigenetic programming from direct influences: 1) investigating the mechanisms of disease transmission to offspring by assessment of the epigenome in germ cells; and 2) investigating the physiological mechanisms of disease through measurements of the epigenome in relevant somatic tissues such as liver, pancreas, adipose tissue and the brain (Stegemann & Buchner, 2015).

Epigenome analyses of the germline have mainly focused on DNA methylation patterns. Interestingly, previous investigations have successfully identified variations in the level of DNA methylation in sperm that is correlated with disease susceptibility in

subsequent generations (Stegemann & Buchner, 2015). For example, in a study where gestating rat mothers were exposed to endocrine disrupting compounds, DNA methylation assays of all promoter regions in the genome of F3 offspring identified 197 promotor regions that were differently methylated (Manikkam et al., 2013). Although these promotor regions were distributed throughout the genome, their functional clusters were located in the glial cell line-derived neurotrophic factor (GDNF) and neurotrophin-3 (NTF-3) signaling pathways and able to induce five obesity-related genes [Tnfrsf12a, Esrra, Fgf19, Wnt10b, Gdnf, (Manikkam et al., 2013)]. For example, DNA methylation marks in the Gdnf and Esrra genes linked to diabetes risk were found in the sperm of the F3 generation generated by a maternal lineage that was exposed to plastic-derived endocrine disrupting chemicals in the parental F0 generation (Manikkam et al., 2013). Moreover, these changes in DNA methylation patterns in F3 offspring were also related to more frequent occurrences of testis and ovarian disease (Manikkam et al., 2013). Thus, exposure to an adverse environment in utero during germline programming may induce disease phenotypes in subsequent generations via maternal ovaries or paternal sperm (Manikkam et al., 2013; Skinner, 2014).

A study by Wei et al. (2014) examined changes to both the germline and the somatic tissue via genome-wide DNA methylation profiling of sperm and pancreatic islets obtained from F1-F3 generation offspring whose gestating mothers were treated with the pesticide dichlorodiphenyltrichloroethane (DDT). They identified 6000 intragenic differently methylated regions (DMRs) in sperm and 7000 DMRs in islets, of which over 35% or 2269 DMRs were in common (Wei et al., 2014). To further investigate the functional role of these DMRs, 542 sperm and 782 islets DMRs were compared. Of 16 DMRs that were

shared between the two tissues in the F1 generation, ten showed consistent differences in the F2 generation as well (Wei et al., 2014).

DNA methylation marks linked to aging, health and disease may be differentially transmitted through the paternal or maternal lineages. A process termed genomic imprinting involves DNA methylation of imprinted control regions (ICRs) to regulate gene expression according to a parent-of-origin pattern (Wilkinson et al., 2007). Depending on the parental route of transmission, one allele may be expressed and the other one will be silenced. A classic example is the gene encoding insulin-like growth factor 2 (IGF2/Igf2), which is only expressed from the allele inherited from the father. The allele coming from the mother can be imprinted and thus silenced (Wilkins & Haig, 2003). The process of genomic imprinting only concerns about 1% of the genome in mammals (Wilkins & Haig, 2003). More recent evidence from animal studies shows that not only DNA methylation but also miRNA expression and histone modification are implicated in the transgenerational inheritance of disease (Migicovsky & Kovalchuk, 2011).

1.5 Animal Studies

Experimental studies investigating transgenerational inheritance have been performed in model organisms such as the nematode, fruit fly, rodents, sheep and other species. Some advantages of using model organisms to study environmental effects across generations include invasive phenotypes, short generation times and relatively short lifespans. Also, model organisms allow for full experimental control over precise aspects of an animal's genetic makeup and environmental exposure. For example, it is possible to control for the timing, dosage and transmission lineage of a specific experience or environmental exposure.

1.5.1 Simple Animal Models

Vital progress in understanding the transgenerational effects of adverse environments on the pathophysiology of diseases and the lifespan have come from studies in the nematode Caenorhabditis elegans, which shares many conserved molecular pathways of neuronal plasticity and disease with higher species. A recent study by Rechavi (2014) demonstrated that starvation alters the metabolism in three subsequent generations via transgenerational propagation of miRNAs. Food restriction in L1C. elegans larvae during early development increased the lifespan of the F3 progeny by 37% (Rechavi, 2014; Rechavi et al., 2011), indicating that a single metabolic challenge can modify the phenotype throughout the lifespan and also transgenerationally (Stegemann & Buchner, 2015). Similarly, Benayoun and Brunet (2012) proposed that environmental cues can affect the longevity of multiple generations through the formation of an epigenetic memory (Benayoun & Brunet, 2012). They hypothesized that transgenerational inheritance of longevity may result from heritable depletion of histone lysine four trimethylations (H3K4me3; Benayoun & Brunet, 2012). Indeed, the progeny of mice that are mutant for the COMPASS H3K4 trimethylation complex show extended lifespan over three generations (Greer et al., 2010, 2011). Thus, deficiency in highly conserved COMPASS complex members seems to be critical in extending the lifespan in a germline-dependent manner.

Recent studies have also indicated RNA interference (RNAi) mechanisms in transgenerational inheritance. Vastenhouw et al. (2006) showed that a single exposure to RNAi at P0 silenced the target genes across twenty generations (Vastenhouw et al., 2006). Notably, this was true only for genes expressed in the germline, such as ceh-20 (Vastenhouw et al., 2006). Later, Buckley et al. (2012) examined which specific genes may

be involved in gene silencing in germ cells in multiple generations. A green fluorescent protein (GFP) transgene was used to confirm its silencing response to RNAi (Buckley et al., 2012), and identify a molecule that directs gene silencing. This group identified the Argounate heritable RNAi defective (HRDE)-1 protein as a responsible element for gene silencing events in germ-cell nuclei, suggesting that the Argonaute HRDE-1 may be driving the transgenerational RNA inheritance and possibly promoting germ cell immortality (Buckley et al., 2012). In addition, Ashe et al., demonstrated that through a piwi-interacting RNA (piRNA), stable RNAi could be induced for at least 20 generations through convergent pathways (Ashe et al., 2012). Similarly, earlier studies via screening of RNAi mechanisms revealed that more than 100 inactivated genes might be involved in generating longevity (Hamilton et al., 2005; Hansen et al., 2005). Most of these genes were linked to very specific pathways, such as those involved in insulin signaling, caloric restriction and endocrine regulation (Hansen et al., 2005). Importantly, exposure to an adverse environment such as stress has been linked to these pathways as well (Cottrell & Seckl, 2009; de Rooij & Roseboom, 2013; Kiss et al., 2016).

Other model organisms including the fruit fly Drosophila have demonstrated that an adverse environment can modify the trajectory of normal development along with epigenetic changes that may be passed on from one generation to the next. For example, a study by Stern et al. (2012) demonstrated that toxic stress produces epigenetically heritable alterations during fly development, and these changes are epigenetically inherited across subsequent (F1–F6) generations of unchallenged offspring. Accordingly, even memory impairments in the fruit fly may be programmed by ancestral experience (Burns & Mery, 2010). Further, they showed that the age of parents influences the severity of memory

impairments at least across two subsequent generations (F1–F2), and offspring memory impairment was linked to elevated oxidative stress in the aged parental generation (Burns & Mery, 2010). These examples illustrate the significant value of simple animal models in the study of complex transgenerational phenomena underlying lifetime health trajectories.

1.5.2 Rodent Models

1.5.2.1 Maternal Lineage Transmission

Challenges and promises of studying transgenerational inheritance were highlighted by the pioneering studies in mice carrying the Agouti viable yellow allele (Duhl et al., 1994; Morgan et al., 1999). In this model, treatment with dietary compounds can be used to study mechanisms of transgenerational inheritance of metabolic disease (Morgan et al. 1999). In particular, the phenotypes of coat colour, adiposity risk, and DNA methylation status, are maintained when inherited through the female germline (Duhl et al., 1994; Miltenberger et al., 1997; Morgan et al., 1999). The severity of the phenotype in this model is variable and correlates with DNA methylation, as confirmed by supplementation with a methyl donor-enriched diet (Duhl et al., 1994; Morgan et al., 1999).

Research on the transmission of environmental effects using rodent models started about a decade earlier. In the early 1980s, Kahn studied mouse offspring born to mothers who were kept in small ventilated cages during gestation and were provided with a solution of yeast RNA in their drinking water (Kahn, 1982). Across multiple generations, adult offspring were tested for hemoglobin concentration. The study demonstrated that the transmission of alteration of hemoglobin synthesis persisted across more than one generation (F1–F3), even when the experimental lineage was no longer exposed to the RNA supplement (Kahn, 1982). A few years later Huck et al., reported that a caloric restriction

early in life can influence sex ratios of future generations (F1–F3) in hamsters (Huck et al., 1987). In this study, female hamsters that were exposed to a restricted diet as juveniles weighed less than controls at the end of their pregnancy, reared fewer young and weaned male young that weighed less than their female siblings across more than one generation (Huck et al., 1987). This phenomenon was later confirmed by studies in young female sheep which demonstrated that prenatal overnutrition induces metabolic dysfunction in their female offspring. Thus, abnormal food intake during pregnancy can determine offspring survival, growth, metabolic function, and sex ratio with potentially long-term consequences for multiple generations.

Adverse environmental exposure during early development not only affects the sex ratio but may change mate preference as well. A study by Crews et al. (2012) in laboratory rats demonstrated that transgenerational endocrine disruptors affect the mate preference in a sexually dimorphic manner (Crews et al., 2012). Exposure of pregnant dams to endocrine disrupting chemicals, such as the fungicide vinclozolin, during gestational days 8–14 stimulated mate preference in their F3 female progeny, but not in the males (Crews et al., 2007). Consequently, the F3 female rats whose great-grandmothers had been exposed to vinclozolin preferred to mate with males from an unexposed lineage (Crews et al., 2007). Females from an unexposed lineage also showed a similar preference for unexposed control males. This study concluded that the sex specific phenotype of mate preference was causally linked to epigenetic imprinting based on the remote effects in the ancestrally exposed F3 generation (Crews et al., 2007). Thus, phenotype alterations involved the ability of an environmental toxin to promote epigenetic reprogramming through DNA methylation marks in the germ line that were transmitted to the progeny. This permanent modification

of the epigenome altered gene expression patterns in the brain and reproductive success (Crews et al., 2007). Some alternative mechanisms which may play a role in the transgenerational origins of mate preference may have been the allelic differences in the highly polymorphic major histocompatibility complex (MHC) genes, and alterations in odor discrimination by inhibition of the c-fos-activated vomeronasal organ (Crews et al., 2007).

A follow-up study from the same group investigated the epigenetic mechanisms involved in the transgenerational actions of environmental compounds and their consequences on reproductive function and adult onset disease (Manikkam et al., 2012a). This study assessed the F1, F2, and F3 generation offspring whose F0 gestating mothers were exposed during gestational days 8–14 of pregnancy to common environmental toxins such as plastic compounds (bisphenol A), dioxin (TCDD) or jet fuel. The results demonstrated that these toxins promoted early-onset puberty in the F3 generation female offspring (Manikkam et al., 2012a; Manikkam et al., 2014). Moreover, spermatogenic cell apoptosis was affected, and ovarian primordial follicle pool size was significantly decreased in all groups whose great-grandmothers were exposed to one of the toxin (Manikkam et al., 2012b). DMRs were identified in all exposed male lineages of the F3 generation sperm. Furthermore, several genomic features of DMR such as low-density CpG content (Manikkam et al., 2012b) and 363 differentially methylated DNA regions termed epimutations (Manikkam et al., 2012a) were identified in the sperm of F3 adult offspring.

The epigenetic changes induced by exposure to environmental toxins may be the contributing factor to adult onset diseases. Indeed, a study by Manikkam and Skinner reported that adult offspring whose grandmothers were exposed to the insect repellant N,

N-diethyl-metatoluamide (DEET) during E8-14 of their fetal development caused a higher incidence of pubertal abnormalities, testicular and ovarian (Manikkam et al., 2012a). The transgenerational exposure to environmental toxins during early development, however, affects more than just the reproductive system. Previous studies using vinclozolin have associated transgenerational epigenetic inheritance with adult onset diseases such as mammary and prostate tumors, kidney and immune disorders with disease onset usually during adulthood (6–12 months of age in rats; Anway et al., 2006). Arguably, experiences that have such drastic consequences on lifetime health trajectories will also affect brain function and neurological disorders.

A number of studies have shown that transgenerational programming by early stressors affects brain function, such as mental health. Xu et al. (2018) demonstrated that prenatal glucocorticoid exposure programs CRH responses thereby promoting depression-like behaviour in F1 and F2 generation rat offspring (Xu et al., 2018). The depressive-like phenotypes were linked to hypomethylation of the crhr1 promoter and contributed to programming of the stress response via differential activation of the CRH receptor 1 (Xu et al., 2018). Furthermore, anxiety-like behaviours were observed in F3 and F4 offspring subjected to prenatal stress (Erickson et al., 2014; Kiss et al., 2016; McCreary et al., 2016b). Adult F4 offspring who were subjected to cumulative prenatal stress reported altered connectivity in manganese-enhanced magnetic resonance imaging (McCreary et al., 2016b) and lower electrophysiological field potentials across multiple brain regions (Skelin et al., 2015). In addition, along with elevated HPA axis activity and anxiety-like behaviours these animals showed reduced neuronal density in the hippocampus and prefrontal cortex along with altered expression of neurotrophic factors (McCreary et al., 2016b).

In a line of separate studies, adult male and female F4 offspring whose mothers, grandmothers and great-grandmothers were exposed to stress during pregnancy showed sexually dimorphic effects of stress (Ambeskovic et al., 2017). These behavioural changes were associated with metabolomic alterations in pathways related to mental health demonstrating that both transgenerational (one generation exposed) and multigenerational (multiple generations exposed) stress programs central metabolic pathways linked to psychiatric disorders (Kiss et al., 2016). Specifically, F4 male offspring of multi- or transgenerationally stressed lineages exhibited changes in histamine, hippurate, tyrosine and 1-methylnaphthalene levels which are involved in stress-related metabolic adaptations such as aminoacyl-tRNA biosynthesis, methane metabolism and glycine metabolism (Kiss et al., 2016). These results indicate that male offspring whose ancestors were exposed to prenatal stress during early development seem to be more susceptible to affective behaviour changes than females (Dias & Ressler, 2014; Franklin et al., 2010; Gapp et al., 2014a).

Sexually dimorphic effects of prenatal stress can propagate across generations to generate a variety of behavioural phenotypes along with corresponding changes in neuromorphology. For example, cumulative multigenerational stress in a rat model induced new behavioural traits, shifted laterality, and altered neuronal morphology in the parietal cortex (PAR) only in males (Ambeskovic et al., 2017). In this study, prenatally stressed F1 and the multigenerationally and transgenerationally stressed adult male and female F4 generation were tested for paw preference (Ambeskovic et al., 2017). Both transgenerational and multigenerational stress affected paw preference in F4 adult male offspring, as the dominance shifted from right to left paw compared to their F1 ancestors or the non-stress control lineage (Ambeskovic et al., 2017). However, significant paw

preference shifts from right to left dominance were observed in multigenerationally stressed males only. Furthermore, left-handedness in multigenerationally stressed F4 males was accompanied by greater dendritic complexity and larger spine density in the right parietal cortex (Ambeskovic et al., 2017). In addition, multigenerational stress increased fine motor skill abilities in F4 females, without affecting their paw preference or neuronal plasticity (Ambeskovic et al., 2017). However, when a second stressor such as brain damage was experienced in adult female rats which had a history of recurrent prenatal stress, the initial movement performance benefits were diminished (Faraji et al., 2017).

Moreover, McCreary et al. (2016a) found that the corticospinal tract, the main pathway to control skilled hand and digit function, revealed reduced axonal density in ancestrally stressed lineages. In both multi- and transgenerationally stressed males, diminished axonal density was associated with significant impairments in skilled walking (McCreary et al., 2016a). Thus, convincing evidence supports the contribution of maternal transmission of stress across multiple generations and its effects on disease and health outcomes across the lifespan in offspring. Furthermore, knowledge has accumulated over the past few years suggesting a similarly prominent role for fathers in shaping offspring development and brain function.

1.5.2. Paternal Lineage Transmission

While transgenerational inheritance of disease through the maternal lineage has been relatively well characterized, less is known about the possible role of paternal modes of transmission (Bale, 2015). Studies of paternal transmission across generations include mice or rats being subjected to maternal separation, social defeat, chronic stress, a high-fat diet or aversive olfactory cues prior to breeding (Bale, 2015).

In a highly cited study by Franklin et al. (2010), F1 generation offspring were subjected to unpredictable maternal separation in combination with unpredictable maternal stress (MSUS) for 3 h daily from postnatal day 1–14 (Franklin et al., 2010). MSUS males were mated with non-stressed females to generate F2 and F3 generation offspring. When male and female F1–F3 generations were tested as adults, they displayed negative consequences of stress on affective behaviours (Franklin et al., 2010). Specifically, MSUS males of the F1 and F3 generations exhibited increased depressive-like behaviours, while in the females only the MSUS F2 generation showed depressive-like behaviours (Franklin et al., 2010). The study concluded that depressive like symptoms can be transmitted through the germline across several generations but with a complex sex-specific mode of transmission (Franklin et al., 2010). Yet another study demonstrated that the paternal trauma during early adulthood might be inherited transgenerationally with recognizable consequences at the behavioural, neuroanatomical and epigenetic level. Dias and Ressler (2013) subjected F0 male mice to odor fear conditioning by acetophenone before conception. The study showed that exposed F1 and F2 male offspring had increased behavioural sensitivity to the F0 conditioned odor and altered expression of the Olfr151 gene, leading to more neurons and larger dorsal and medial glomeruli in the olfactory bulb (Dias & Ressler, 2014). It was proposed that DNA methylation explains the inherited effects, as in the F2 generation mice the acetophenone sensing gene in sperm cells showed fewer methylation marks (Dias & Ressler, 2014). Therefore, fearful memories can be transmitted across generations through epigenetic marks.

The transmission of memories or early adverse experience in fathers may be beneficial for survival and wellbeing of the offspring. Indeed, Gapp et al. (2014a)

demonstrated that traumatic experiences in early life can be passed on across generations to promote adaptive responses in their adult offspring (Gapp et al., 2014a). In this study newborn male mice (F1) were subjected to MSUS for postnatal days 1–14. Their F2 male and female offspring, when tested as adults at 3–6 months of age, showed that exposure to ancestral MSUS favoured goal-directed behaviour and enhanced behavioural flexibility (Gapp et al., 2014a). This effect included epigenetic changes involving histone post-translational modifications at the mineralocorticoid receptor (MR) gene and decreased MR expression in the hippocampus (Gapp et al., 2014a). A follow-up study on the same cohort showed that sperm RNA is implicated in transgenerational inheritance of early trauma in mice (Gapp et al., 2014a; Gapp et al., 2014b). The authors demonstrated that traumatic stress in early life altered mouse miRNA expression and behavioural and metabolic responses in the progeny (Gapp et al., 2014). Specifically, the study reported that miR-375, miR-200b, miR-673 and miR-466 were upregulated in sperm and serum, and only miR-375 was upregulated in the hippocampus of F1 offspring (Gapp et al., 2014b). Interestingly, in F2 offspring only miR-375 and miR-466 were upregulated in the hippocampus, and no changes were observed in sperm or serum. Nevertheless, both F1 and F2 MUSU offspring showed increased prevalence of depressive and anxiety-like behaviours and altered metabolic responses (Gapp et al., 2014b).

Convincing evidence on the role of epigenetic mechanisms in the transmission of environmentally induced phenotypic trait from father to offspring has motivated studies involving multiple generations. For example, in a transgenic mouse model Siklenka et al. (2015) demonstrated that overexpression of the histone H3 lysine 4 (H3K4) demethylase KDM1A during spermatogenesis reduced H3K4 demethylation in sperm. Such disruption

in developing sperm is believed to impair offspring health across generations. Here, the F1 generation offspring born to F0 fathers with altered histone methylation and RNA content in sperm increased the incidence of abnormal development (growth retardation, limb abnormalities, craniofacial anomalies, edema and hemorrhagic gut) which significantly hampered survival (Siklenka et al., 2015). Moreover, an increased incidence of abnormal development was observed in both F2 and F3 offspring, indicating transgenerationally heritable effects (Siklenka et al., 2015). Thus, this study illustrates the complexity of transgenerational epigenetic inheritance which involves factors such as the establishment of chromatin states in spermatogenesis and sperm RNA (Siklenka et al., 2015).

Significant progress has been made in showing that adverse experience prior to reproduction can be passed down multiple generations via both maternal and paternal lineages to affect the epigenome, brain development, behavioural and disease outcomes. While developmental studies suggest consequences of transgenerational programming in aging processes, there still is a dearth in the use of aged animals in the field. Early development and old age both represent the most vulnerable ages displaying behavioural changes, while phenotypic differences may not always be obvious at reproductive ages (Erickson et al., 2014). Thus, the use of young adult animals bears considerable limitations for inferences about aging, emphasizing the need for long-term lifespan studies to gain clinically relevant insights.

1.6 Human Populations – Clinical Studies

1.6.1 Transgenerational Studies

New advancements in epigenetic epidemiology have enabled an exponential growth in human studies of transgenerational programming of health and disease. Classic clinical studies of transgenerational inheritance come from the study of Prader-Willi Syndrome, which results from imprinting abnormalities on chromosome 15 leading to a higher risk of metabolic and cognitive disturbances (Stegemann & Buchner, 2015). Furthermore, exposure to adverse environments early in life, such as stress, undernutrition and inflammation, can also have negative effects on long-term health outcomes. A rapidly expanding research field investigates clearly recognizable stressful events during a lifetime, including natural disasters, war, migration and other forms of trauma. Due to the long-term nature of these studies, most prospective human cohorts thus far include the F0–F1 generations, with a few cohorts allowing observations in the F2 generations. Nevertheless, these and a number of retrospective studies provide exciting new mechanistic insights into the inter-generational and transgenerational transmission of phenotypes. As indicated earlier, both the parental line of transmission and the sex of an offspring are vital determinants of the behavioural phenotype and the disease incidence across the lifespan.

1.6.2 Maternal Lineage Transmission

Compelling evidence supporting the intergenerational transmission of stress phenotype through the maternal lineage stems from Rachel Yehuda's work. Her group has shown that traumatic experiences in Holocaust survivors may be a risk factor for the development of posttraumatic stress disorder (PTSD) in their offspring (Yehuda & Bierer, 2007). In earlier studies, Yehuda et al. (1998) demonstrated that parental (mothers and fathers) PTSD resulted in decreased basal cortisol levels in male and female offspring indicating intergenerational transmission of clinical hallmarks of PTSD (Yehuda et al.,

1998). Later studies showed a negative correlation with neuroendocrine markers and severity of paternal PTSD. For example, paternal PTSD decreased mean urinary cortisol excretion and salivary cortisol in F1 adult offspring (Yehuda & Bierer, 2007). Based on these findings, GC-mediated programming involving the HPA axis was proposed as a possible mechanism of transmission of parental traumatic experience and the child's stress response.

Further studies have investigated the possible transmission of stress or trauma across generations (Braga et al., 2012). In a study by Braga et al. (2012), adult men and women (F1 generation) whose parents were exposed to trauma during the Holocaust completed a series of comprehensive questionnaires, to investigate emotional trauma. Their analysis revealed that the traumatic dimensions of stressful experience could be conveyed intergenerationally, by overcoming trauma, through the development of resiliency mechanisms (Braga et al., 2012). On the contrary, a study by Jelinek et al. (2013) investigated neuropsychological performance in a trauma sample of older adults displaced during World War II and the transgenerational effects in their offspring (F2) with and without PTSD (Jelinek et al., 2013). The study found no differences in displaced individuals (F1) or their offspring (F2), providing the first evidence for a rather resilient PTSD population (Jelinek et al., 2013). These somewhat mixed responses to stress being observed by various researchers may be due to the fact that the transmission of trauma and/or resilience is occurring through both maternal and paternal lineages. Clinical findings in which a phenotype of ancestral stress disappears in the F2 paternal lineage generation or the F3 maternal lineage generation would suggest that the phenotype was induced by direct exposure rather than by truly heritable epigenetic regulation (Skinner, 2008; Zucchi et al., 2012).

Various cohorts have focused on consequences of maternal stress and trauma on fetal development in the womb in an effort to deduce any causal mechanistic pathways. The hypothesis that certain types of chronic disease may originate through adaptations made by the fetus in response to maternal undernutrition has been extensively investigated in the Dutch famine birth cohort (de Rooij & Roseboom, 2013; Painter et al., 2008; Roseboom et al., 2000; Roseboom et al., 2001; Veenendaal et al., 2013). Over the past two decades, the Dutch famine birth cohort has provided convincing evidence that ancestral prenatal undernutrition has a lifelong impact on health and disease incidence. The Dutch famine originated from a post-World War II disaster, theHungerWinterin1944–45, during which acute shortages of food resulted in severe starvation in the Netherlands. The first series of studies in afflicted families suggested that maternal malnutrition during pregnancy permanently affects adult offspring (F1) health outcomes across the lifespan (Roseboom et al., 2001). A series of investigations in this cohort compared the effects of caloric restriction at different gestational ages in relation to the organs developing at these particular stages. For example, exposure to famine during early gestation was associated with increased rates of cardiovascular disease; suggesting lasting effects of famine exposure on the development of the heart in early gestation (Roseboom, 2000).

Also, exposure to famine in early gestation was associated with structural changes in the brain. Exposure to famine during mid-gestation was associated with micro-albuminuria which may suggest that the numbers of nephrons in the kidney, which is laid down in mid gestation, was affected by undernutrition during that specific critical period of development (Painter et al., 2005). Moreover, exposure to famine in mid-gestation was associated with chronic obstructive airways disease, which might reflect interference with

the rapid growth of the bronchial tree in mid-gestation (Roseboom et al., 2001). Lastly, exposure to famine during any period of gestation, especially late gestation was associated with reduced glucose tolerance, which might suggest lasting effects of undernutrition on the beta cells which are laid down in late gestation (de Rooij et al., 2006).

The effects of famine exposure do not seem to be limited to one generation only. There were indications that the effects of famine passed down to the next generation through both the maternal and paternal line. Children whose mothers had been exposed to famine prenatally increased adiposity at birth (Painter et al., 2008) while at adult age, offspring of prenatally exposed fathers seemed to be more obese (Veenendaal et al. 2012) suggesting they might be at increased risk of developing cardiovascular disease in later life. Moreover, Veenendaal et al. (2012) found increased methylation status of the PPARy promotor, a site coding for a peroxisome proliferator-activated receptor, and hypermethylation of the GR promotor in the peripheral blood of adult offspring exposed to famine during perinatal development (Veenendaal et al., 2012). It was concluded that changes in the PPARy promotor were associated with lower plasma triglyceride and higher HDL levels, while the hypermethylation of the GR promotor may have contributed to low scores observed on the anxiety and depression test and perceived wellness.

The findings from the Dutch Famine Study emphasize that the stage of development during which exposure to environmental adversity occurs is a prominent modulator of health trajectories and disease risk. Adding to these observations is a growing body of evidence showing that early pregnancy is a particularly sensitive period for fetal development depending on the nature or type of the challenge. For example, children born to mothers who experienced death in the family during their first trimester of pregnancy

were more likely to suffer from schizophrenia than offspring whose mothers experienced death in their second or third trimesters (Khashan et al., 2008). A mechanistic study pursued a genome-wide analysis and showed striking changes in 181 DMRs associated with prenatal nutrition (Tobi et al., 2014). A differential profile of prenatal malnutrition-associated DMRs (P-DMRs) extends along the pathways related to growth and metabolism (Tobi et al., 2014). Here, differential methylation of P-DMRs located in the genes of insulin receptor precursor (INSR) and carnitine O-palmitoyltransferase 1 (CPT1A) was associated with altered birth weight and LDL cholesterol (Tobi et al., 2014). The mechanisms of intergenerational inheritance in disease risk are complex and likely involve multiple levels of epigenetic regulation.

Similar findings linking maternal adverse experiences to fetal metabolic programming also stem from studies in prenatal maternal stress (PNMS). In January 1998 Quebec was hit with an Ice Storm, which led to extended power outages in numerous households for as long as a month. Suzanne King and her team formed the Project Ice Storm cohort, which consists of mothers who were pregnant during the ice storm. Both mothers and children subjected to PNMS during the storm are followed longitudinally and undergo regular behavioural and physiological testing. Consistent with other human studies, exposure to PNMS induced epigenetic modifications in their offspring. For example, when a genome-wide DNA methylation approach in PNMS 13-year old children was performed, the authors showed a significant correlation between PNMS and methylation at 1657 CpG sites affiliated with 957 genes (Cao-Lei et al., 2016a; Cao-Lei et al., 2016b). The latter were predominantly related to immune function and metabolism, which may explain the higher body mass index (BMI), the risk of adiposity and type-1 and −2 diabetes mellitus in this

cohort (Cao-Lei et al., 2015; King et al., 2012). Interestingly, the methylation patterns were predominantly linked to objective PNMS assessments, rather than subjective stress reported by mothers during pregnancy (Cao-Lei et al., 2016a; Cao-Lei et al., 2016b). The drastic metabolic and physical phenotype of PNMS exposure at the age of 13 years suggests that the traumatic stress caused by a natural disaster can have a lifetime impact and potential consequences for chances of successful aging.

Moreover, intriguing evidence from the Gomerol Gaarynggal study in Australia demonstrates the impact of ancestral stress on early development and lifetime health. This study is investigating the origins of chronic diseases among Indigenous Australians (Ashman et al., 2016a; Ashman et al., 2016b). Their preliminary data show that indigenous children whose mothers and grandmothers were exposed to adversity such as residential schooling, infection, smoking, psychological stress and/or poor nutrition are more likely to have their life expectancy shortened by ten years (Ashman et al., 2016a). Furthermore, the exposed individuals may be at a higher risk of developing renal disease, diabetes, cardiovascular disease, hypertension and stroke (Rae et al., 2016; Ashman et al., 2016a, 2016b). Although exploration of these rich datasets is in the early stages, these studies provide much-needed evidence for the transgenerational determinants of aging and the potential risks of disease. Importantly, Rae et al. (2016) designed an intervention that overcame some of the consequences of ancestral adverse experience by offering individuals at risk an expressive arts therapy (Rae et al., 2016) . It can be expected that both stress and beneficial interventions in later life through biological channels, such as epigenetic mechanisms, affect health trajectories.

Currently, the largest effort to provide systematic insights into the biological mechanisms of transgenerational programming involves the Avon Longitudinal Study of Parents and Children (ALSPAC). This cohort was developed based on pregnant women residing in the South West of England during the early 1990′s (Golding, 1990). Almost 12,000 pregnancies were recruited, and the mother-child dyads were followed by questionnaires and medical records ever since. Intriguing evidence from this cohort showed that grandsons, but not granddaughter who's maternal grandmothers smoked to be on average 61 g heavier at birth (Miller et al., 2014; Pembrey et al., 2014). No fetal growth differences were observed if the father's grandmother had smoked (Miller et al., 2014). Others have also reported intergenerational effects of smoking on asthma. Li et al. (2005) reported that the childhood (F1 and F2) risk of asthma was enhanced by both prenatal maternal and grandmaternal smoking (Li et al., 2005). The interaction between maternal smoking and newborn birth weight was causally related to the genotype, such as a variation at rs1051730 which was robustly associated with smoking status during pregnancy (Tyrrell et al., 2012). Thus, interventions at the population level that aim to effectively reduce exposure to first- or second-hand smoke during pregnancy may directly promote a better start for newborn babies and future generations.

1.6.3 Paternal Lineage Transmission

A seminal systematic retrospective study of transgenerational epigenetic inheritance in humans comes from the Överkalix population in Sweden (Pembrey et al., 2006; Pembrey et al., 2014). Health records of men and women born in 1890, 1905 and 1920 were examined to determine a possible association between early life food availability, longevity and later disease incidence in their 1818 children and grandchildren.

Food availability was assessed by rating historical records of regional harvests statistics and food prices on a three-point scale. The results showed that longevity, morbidity and mortality were linked to food availability in previous generations (Bygren et al., 2001; Bygren et al., 2014; Kaati et al., 2002; Pembrey et al., 2006). In particular, the availability of food during a child's slow growth period (SGP), which occurs prior to their pre-pubertal peak in growth, was shown to be associated with longevity in grandchildren in a sex-specific manner (Bygren et al., 2001; Pembrey et al., 2006). While the risk of mortality in grandsons was linked to their paternal father's available food resources in mid-childhood, the risk of mortality in the granddaughters was linked to the paternal mother's food resources (Bygren et al., 2001; Pembrey et al., 2006). Furthermore, the risk of death in grandchildren because of cardiovascular disease or diabetes was also linked to paternal food supply during the SGP (Kaati et al., 2002). Thus, food abundance in paternal fathers during their SGP raised the risk of diabetes in the grandchildren by four times, whereas undernutrition during their SGP reduced cardiovascular mortality (Kaati et al., 2002). In a follow-up study, Kaati et al. (2007) demonstrated that when childhood social circumstances such as father's ownership of land, maternal literacy and death were taken into account, proper nutrition in grandfathers was associated with reduced survival of their male but not female grand offspring (Kaati et al., 2007). Moreover, Bygren's team investigated if pre-migration status of individuals moving away from the Överkalix area is associated with mortality rates. The observations revealed that higher pre-migration socioeconomic status favorably affects mortality risks in females (Tinghög et al., 2011). Specifically, female migrants living under better pre-migration conditions were ultimately healthier and experienced more positive health trajectories than females who came from low

socioeconomic background. This relationship was not observed in males (Tinghög et al., 2011).

In the early 1990′s the ALSPAC cohort was established in the UK to confirm the Överkalix hypotheses of mid-childhood sensitive periods to disease inheritance, such as metabolic disorders. Northstone et al. (2014) investigated the effects of paternal smoking on their children's birth weight and BMI at the age of 7–9 years (Northstone et al., 2014). It was demonstrated that the earlier the father took up smoking, the higher was the BMI at nine years in their sons but not their daughters. The strongest association was found in sons of fathers who started smoking before the age of 11, and the greatest increase in weight was observed in boys from 13 years onward (Northstone et al., 2014). On the contrary, paternal smoking was only associated with a reduction in total lean mass in daughters (Northstone et al., 2014). Examining multiple anthropometric measurements in male and female grandchildren at birth and during childhood in the ALSPAC cohort, another study confirmed sex-specific transgenerational inheritance (Golding et al., 2014). When the paternal grandmother smoked during pregnancy, the grandsons had greater bone and lean mass, while their granddaughters had also increased mass but were also taller (Golding et al., 2014). Moreover, a family study of the Keelung Community investigated the possible association between paternal chewing of betel quid and the risk of metabolic syndrome in their children. The results showed that paternal betel quid use increased the incidence of metabolic syndrome in their children in a dose-dependent relationship.

Other studies have reported sex-specific programming of metabolic diseases, and mental health outcomes in offspring whose ancestors were subjected to adverse environments during prenatal and early postnatal periods. A sex-specific transgenerational

inheritance of mental health has been reported in a German famine cohort, which originated in 1916–17 from a British blockade of food supplies to Germany during the middle of World War I. This study examined height, mental health and educational achievement in the adult descendants (F1 and F2) of boys and girls exposed to undernutrition at the ages of 9–12 and 8–10 years, respectively

(Pembrey et al., 2014; van den Berg & Pinger, 2014). The authors reported a negative association between pre-pubertal nutrition and mortality in the (F3) grand-offspring. Interestingly, the third generation (F3) adult male offspring tended to have higher mental health scores if their paternal grandfather was exposed, whereas adult female offspring showed higher mental health scores if the maternal grandmother was exposed (van den Berg and Pinger, 2014). Thus, exposure during a critical mid-childhood period may determine lifetime health outcomes at least across three generations (van den Berg and Pinger, 2014). Furthermore, paternal transgenerational effects of adverse environments were also examined in the Dutch famine birth cohort. Veenendaal et al. (2013) reported that F2 generation adult offspring (32–35 years of age) of fathers, who experienced malnutrition prenatally, had higher BMIs than offspring who had not been undernourished prenatally. These findings support experimental studies confirming transgenerational non-genomic mechanisms of inheritance in the paternal lineage.

1. 7 Sex-Specificity of Epigenetic Mechanisms and Transgenerational Programming

Most diseases that are potentially influenced by early life experiences such as diabetes, cardiovascular disorder, autoimmune and psychiatric diseases exhibit partial sex bias. Moreover, animal and human studies have repeatedly indicated that epigenetic responses to early life adversity can propagate across multiple generations in a sex-specific

manner. Interestingly, transgenerational studies indicate that the incidence of disease in the offspring depends on both the sex of a parent and the offspring (Gabory et al., 2009). Thus, sex-dependent programming by environmental factors may arise in parental gametes, and during prenatal and early postnatal development. The following discusses four lines of evidence suggesting sex-dependent epigenetic mechanisms involved in transgenerational inheritance.

First, adverse environmental influences may exert sex-specific differences via the germline transmission of parentally imprinted genes. During early embryonic development and sexual differentiation, the DNA methylation marks in the germline undergo erasure and re-establishment. The DNA methyltransferase (DNMT) family of enzymes catalyzes the transfer of methyl groups to DNA. DNA methylation homologs (DNMT3) coordinate a parent-specific expression of the germline-acquired DNA methylation marks at imprinted control regions. They also play a critical role in parental imprint acquisition of sexual dimorphisms in the offspring (Bourc'his & Proudhon, 2008; Lucifero et al., 2007). For example, in males, DNMT3L is not needed for paternally imprinted methylation, but it is required for retrotransposon methylation. By contrast, DNMT3L is necessary for maternally imprinted methylation with limited requirements for the retrotransposon methylation (Bourc'his et al., 2001; Okano et al., 1999).

Through these and other mechanisms, environmental factors can directly affect gametogenesis in a sex-specific manner. For example, F1 male rats exposed to the fungicide vinclozolin during the prenatal period exhibit subfertility in later life due to a spermatogenic cell defect (Anway et al., 2006). In another study, these authors demonstrated that this subfertility was associated with DNA methylation patterns in genes that are inherited via

the male germline, as observed across four consecutive generations (Anway et al., 2005). In addition, male rats in the F2–F4 generations displayed increased incidence of testicular and kidney disease, whereas in females vinclozolin increased the incidence of kidney disease and tumors only when disease incidence was combined across all generations (Anway et al., 2006). Thus, environmental factors can stimulate transgenerational disease programming via the maternal or paternal germline in one sex but not the other. In addition to germline epigenetic processes, placental programming during early life may also produce sex-specific phenotypes in the offspring.

Second, adverse environmental exposure may have different effects on the epigenetic regulation of the male versus female placentas. The placenta represents the nexus between the mother and her offspring during early development and plays a role in the transfer of nutrients, hormones and other information from the mother to the fetus in a sex specific manner. Recent studies have demonstrated that maternal exposure to an adverse environment during gestation may exert long lasting sex-dependent effects on the offspring via placental mechanisms (Donnell et al., 2009; Rossant & Cross, 2001). It appears that the placentas attached to male offspring were more likely affected by maternal adverse experiences than those attached to female offspring (Mueller & Bale, 2008). In a mouse model, Mueller and Bale (2008) demonstrated that prenatal stress alters expression of growth- and development-specific genes in the placentas of male offspring only. Significantly, higher levels of gene expression were reported for peroxisome proliferator-activated receptor alpha, insulin-like growth factor binding protein 1 (IGFB-1), glucose transporter type 4 and hypoxia inducible factor 3-alpha in males when compared to female placentas (Mueller & Bale, 2008). Moreover, these male offspring also displayed

maladaptive stress responsiveness and depressive-like behaviours. Thus, alterations in growth factor gene expression and placental methylation patterns may be linked to a higher risk for neurodevelopmental and psychiatric disorders with lifelong consequences especially in male offspring (Mueller & Bale, 2008; Myatt, 2006; Rivera et al., 2008). One of the mechanisms responsible for sex-specific programming may be the protective mechanisms of genes on the X chromosome in placentas. The X chromosome in placenta seems to escape some of the gene inactivation (Carrel & Willard, 2005; Dunn et al., 2011) leading to elevated expression of more genes particularly in the female placenta. The increased expression of a large number of genes may protect the female fetus from adverse health outcomes (Carrel and Willard, 2005).

Recent transgenerational studies are beginning to provide evidence in support of sex-specific programming by adverse environmental exposure across generations. A report by Franklin et al. (2010) demonstrated that postnatal separation altered methyl CpG binding protein expression in the germline of the first and second generation male offspring (Franklin et al., 2010). In addition, the first and second generation male offspring displayed increased depressive-like behaviours in adulthood, while no changes were observed in females (Franklin et al., 2010).

Third, the regulation of gonadal steroids during early development via maternal stress, diet or steroid exposure alters fetal metabolism, neurodevelopment and endocrine function with lifetime consequences (Challis et al., 2002; Timmerman et al., 2000). Steroid hormones prepare vital regulators of brain organization and sex determination in early development. For instance, the male brain is organized by a surge in testosterone before and shortly after birth (Phoenix et al., 1959). However, it is the conversion of testosterone

to estradiol by the enzyme aromatase that will alter gene expression and influence brain sex differentiation (McCarthy et al., 2009). Convincing evidence has identified several estrogen receptors (ER; ER alpha and ER beta) as major players in sex differentiation (Kudwa et al., 2005). The process may involve estrogen binding to ER to generate a ligand-activated transcription factor which will bind to DNA to control gene expression (as reviewed by McCarthy et al., 2009). The function of ERs as nuclear transcriptional factors enables them to induce epigenetic changes with profound consequences for fetal brain development, behaviour and brain disorder risk. For example, in a study by Murray et al. (2009) postnatal administration of a histone deacetylase inhibitor was able to disrupt dimorphic sexual development of bed nucleus and stria terminalis in males only (Murray et al., 2009). The authors concluded that histone deacetylase may induce lifetime changes in gene expression by blocking masculinization via testosterone in these brain areas (Murray et al., 2009). Thus, through DNA methylation and histone modification, steroid hormones and receptors can regulate brain development in males and females differently (Champagne et al., 2006; Issa et al., 1996; Nugent & McCarthy, 2011).

Recently, it was demonstrated that early developmental experience may alter not only adult sexual behaviour but ER expression as well (Champagne et al., 2006; Kurian et al., 2010; Morgan & Bale, 2011). Kurian et al. (2010) demonstrated that sexually dimorphic behaviour where mothers groom anogenital regions of male more than female offspring along with estradiol exposure may induce epigenetic modification to organize lasting sex differences in the brain (Kurian et al., 2010). The tactile stimulation of the anogenital area on postnatal days 5–7 or estradiol injection on a postnatal day 1 elicited an increase in ER alpha promotor CpG methylation in the preoptic area in female offspring,

thus indicating masculinization via epigenetic mechanisms (Kurian et al., 2010). Considering that stress during pregnancy alters maternal behaviour, we would expect altered methylation patterns on the ER alpha promotor in the preoptic area in male and female offspring, resulting in sex-specific disease incidence.

Moreover, exposure to adverse environments during early gestation was shown to alter expression of brain ER regulatory miRNAs in a sex dependent manner (Morgan and Bale, 2011). For example, approximately 250 highly expressed miRNAs in the brain were found to be differentially expressed in male and female offspring that were exposed to an adverse environment prenatally (Morgan & Bale, 2011). Moreover, adverse early environments such as maternal stress during pregnancy can be passed on across generations to affect ER expression. Thus, transgenerational stress via the paternal lineage dysmasculinized male F2 offspring as indicated by upregulation of ER (alpha and beta), shorter anogenital distance and reduced expression of miR-322, miR574 and miR-873 in the brain (Morgan & Bale, 2011). Notably, the epigenetic and ER changes observed in F2 males were similar to control females, while transgenerational stress had no effect on ER or miRNA expression in females. However, gonadal hormones are not the only player in determining sexually dimorphic characteristics, but sex chromosomes have an impact as well.

Fourth, exposure to adverse environments early in life such as maternal stress and caloric restriction may affect the expression of the sex determining region of Y (SRY) gene and influence long-term programming of sexually dimorphic brain and aging processes. Normally, sexual differentiation is initiated by the presence or absence of SRY encoded on the Y chromosome (Wilhelm & Koopman, 2006). Its presence would initiate a cascade of

male secondary characteristics such as hormone secretion and brain organization (Wilhelm & Koopman, 2006). In contrast, both X chromosomes are needed to induce female sex-determination (Blecher & Erickson, 2007). Importantly, sex-determining genes found on sex chromosomes can influence the development of many systems such as brain and other organs, and any changes in expression or activation of these genes may induce sex specific phenotypes (Blecher & Erickson, 2007).

Some genes may be expressed differently depending on their status or position on the X or Y chromosomes (Gabory et al., 2009). These parentally imprinted genes may be influenced by adverse external environments and passed on to offspring. Recent evidence for sex-specific transmission through differential X and Y chromosome reading has been provided by the Överkalix study (Pembrey et al., 2014). Here, the authors hypothesized that the no combining part of the Y chromosome could more easily retain epigenetic marks in the gametes or carry a genomic stress 'sensor' where DNA damage can trigger a noncoding DNA response (Pembrey et al., 2014). Furthermore, sex-specific transgenerational responses may be one of the determinants of common complex diseases (Pembrey et al., 2014). Indeed, Pembrey et al. (2006) showed that the food supply of the paternal grandfather was linked to higher mortality risk among grandsons, but not granddaughters (Pembrey et al., 2006). There are a number of suspected mechanisms associated with increased disease incidence in aging that will be addressed next.

1.8 Possible Mechanisms Contributing to Accelerated Aging and Disease Incidence in Response to Environmental Insults

Aging is a major risk factor for many chronic diseases. Hallmarks of aging include reduced telomere length, impaired function of antioxidant systems, reduced somatic repair,

altered stress response and chronic inflammation (Figure. 1.3; de Rooij & Roseboom, 2013). In successful aging, these processes and the pathogenic processes of disease are slow. However, exposure to adverse environmental factors such as stress, starvation or endocrine disruptors early in life or for extended periods of time can reprogram these systems, resulting in accelerated initiation and progression of aging (Figure. 1.3). For example, early environmental insults are associated with shorter telomere length at birth (Marchetto et al., 2016), increased production of reactive oxygen species (ROS; Głombik et al., 2015), altered gene expression, and increased presence of inflammatory cytokines such as interleukin (IL)-18 and alpha1 AGP (Murgatroyd et al., 2016) early in adulthood. Furthermore, studies have shown that insults to early developmental programming can also have lifetime consequences, affecting the individual course of aging and disease incidence (de Rooij & Roseboom, 2013; Pembrey et al., 2006; Pembrey et al., 2014).

1.8.1 Reduced Cellular Repair

Naturally, over time, an organism loses some of its functional capacity to repair itself at the cellular level. One of the markers of cellular aging, disease and longevity is telomere length (Desai et al., 2009; Finch, 2010). Telomeres are DNA repeat sequences found at the end of a chromosome (Hallows et al., 2012; Lu et al., 2013) which decrease in length with age (Hallows et al., 2012). Telomere shortening is the leading cause of reduced cellular activity and disease (Lu et al., 2013; Price et al., 2013). For instance, a cell with shortened telomeres can no longer reproduce, resulting in degeneration or death of tissue (Calado & Young, 2009; Hallows et al., 2012; Jennings et al., 1999; Price et al., 2013; Shalev et al., 2013). Recent studies have shown that telomere shortening or damage due to insults during early development may be the initiator of major degenerative diseases and

reduced lifespan (Hallows et al., 2012; Jennings et al., 1999; Shalev et al., 2013). For example, offspring of mothers who were fed a calorie restricted diet during pregnancy had shortened telomere length in kidney, aortic tissue and pancreatic islet cells and shorter lifespan (Jennings et al., 1999; Tarry-Adkins et al., 2008; Price et al., 2013). Because the telomere length begins to shorten during germ cell development, one can speculate that an adverse intrauterine environment can already negatively affect telomere length, thus ultimately compromising longevity. These and other hypotheses remain to be investigated.

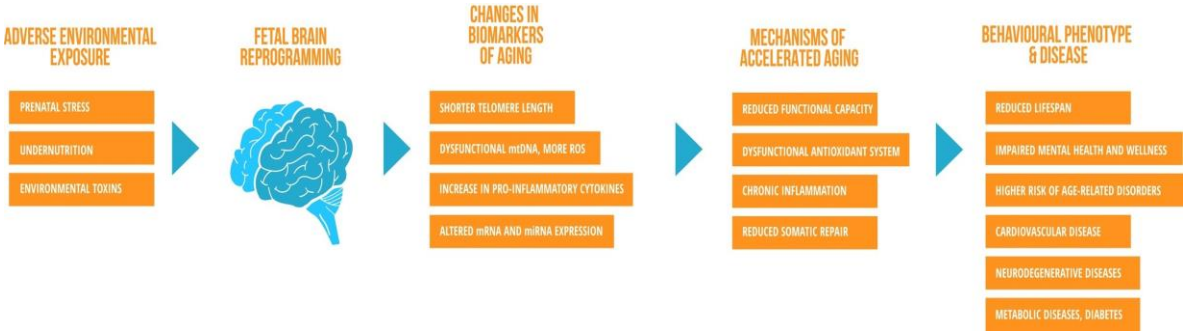

Figure. 1.3. Flow diagram of environmental factors leading to disease phenotype. Early life adversity, such as prenatal stress, undernutrition or endocrine disruptors, reprogram the developing brain and stress susceptibility that alter the aging trajectory. Alterations in biomarkers of aging such as telomere length, epigenetic expression, inflammatory cytokines and reactive oxygen species are hallmarks of early adversity and may impair biochemical and physiological pathways leading to accelerated aging. Ultimately, adverse environmental factors early in life generate the risk of abnormal behavioural phenotypes and disease in later life.

1.8.2 Reactive Oxygen Species and Mitochondria

One of the most widely accepted explanations for the mechanistic basis of aging is the free radical theory. This theory is based upon the disparity between the production of ROS or free radicals and the inability of the body to neutralize the ROS ultimately elevating oxidative stress (Cui et al., 2012). An overload of oxidative stress will cause damage to cells, tissues and organs and even reduce telomere length, which then becomes implicated

in many age-associated diseases such as cancers, cardiovascular and neurodegenerative disorders (Cui et al., 2012; Uttara et al., 2009). In addition, free radicals may accelerate neurodegenerative processes and contribute to neuronal loss in cerebral ischemia or lead to earlier onset of Parkinson's disease (Uttara et al., 2009). New evidence demonstrates that the exposure to adverse environmental factors during early development may affect ROS production and reduce the oxidative defense capacity, resulting in accelerated aging. Accordingly, the progeny of rats who were exposed to a protein-restricted diet in utero showed weakened oxidative defense and increased production of ROS (Tarry-Adkins et al., 2008). ROS was also shown to cause damage to the mitochondria, the "powerhouses" of a cell. Damage or loss of mitochondria may play a central role in aging processes and even be a key pathological feature in neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases (Kennedy et al., 2012).

A strong body of evidence suggests that mitochondrial DNA (mtDNA) mutations contribute to aging processes. Human studies reported that an increase in mtDNA deletion mutations (Cao et al., 2001; Corral-Debrinski et al., 1992; Kennedy et al., 2012) leads to a pathological loss of the cellular respiratory capacity (Cao et al., 2001; Lezza et al., 1994). A similar, age-dependent increase in mtDNA mutations was observed in an animal model, indicating that the mtDNA is of universal importance for aging (Kennedy et al., 2012; Schwarze et al., 1995). Furthermore, an increase in mtDNA mutations was observed in various cancer types (Copeland et al., 2002; Fliss et al., 2000) and Parkinson's and Alzheimer's diseases (Bender et al., 2006; Coskun et al., 2004; Kennedy et al., 2012).

Early experience may also affect mitochondrial function in later life. Glombik et al., demonstrated that maternal stress during pregnancy can alter the profile of mitochondrial

proteins in the brain of their adult offspring, and heighten psychiatric disease incidence (Głombik et al., 2015). In this study, prenatally stressed adult male rats were tested for depressive-like behaviours using sucrose preference and elevated plus maze tests prior to evaluating the expression of mitochondrial biogenesis proteins. The results showed that the prenatal stress increased depressive-like behaviours and reduced levels of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) protein, which is the principal regulator of mitochondrial biogenesis in the frontal cortex and hippocampus (Głombik et al., 2015). In addition, exposure to stress during early development resulted in upregulation of the pro-apoptotic protein Bax, down-regulation of the mitochondrial ribosomal protein L12 (Mrpl12) and mitochondrial NADH dehydrogenase (ubiquinone) flavoprotein 2 (NDUFV2) in the frontal cortex (Głombik et al., 2015).On the contrary, maternal stress down-regulated mitochondrial pyruvate dehydrogenase E1 component subunit beta, and the voltage-dependent anion-selective channel protein 2 (VDAC2) levels (Głombik et al., 2015). Such reduced biosynthesis capacity of mitochondria and the resulting disruption in the expression of mitochondrial proteins caused by early life stress will likely accelerate aging processes in later life. In addition, it may be speculated that the changes in the mitochondrial protein synthesis may be passed on to multiple generations much like the depressive-like behaviours in rodent studies (Franklin et al., 2010). Future studies on this topic will help shed light on the transmission of stress memory and its effect on mitochondrial component involved in aging processes and disease incidence.

1.8.3 Chronic Inflammation and the Phenomenon of "Inflammaging"

The growing understanding that aging is closely entwined with inflammatory processes led to the concept of inflammaging. In general, the processes coined

inflammaging refer to an age-related upregulation of the inflammatory response (Baylis et al., 2013; Franceschi & Campisi, 2014). Inflammaging results from changes in the immune system such as excessive production of cytokines over time (Baylis et al., 2013). Normally, acute inflammation represents a beneficial and adaptive response that is tailored to protect the body from harmful conditions such as an infection by invading pathogens (Franceschi & Campisi, 2014). In contrast, chronic low-grade inflammation may be harmful over time and trigger tissue degeneration and apoptosis (Franceschi & Campisi, 2014). It is during persistent and low-grade inflammation that chronic upregulation of pro-inflammatory mediators is implicated in the aging processes and several age-related diseases such as diabetes, dementia and cardiovascular disease (de Rooij & Roseboom, 2013). For instance, renal fibrosis is associated with alterations in pro-inflammatory mediators such as the elevated presence of the cytokines TGF-β, TNF-α, IL-6 and IL-1b, which can contribute to tumor formation (Franceschi & Campisi, 2014).

Recent reports suggested that the exposure to adverse environments such as stress, undernutrition and chemical factors may chronically upregulate pro-inflammatory cytokines and thus contribute to pathogenetic processes. Desai et al. (2009) administered the proinflammatory agent lipopolysaccharide to offspring born to mothers who experienced 50% diet reduction during pregnancy. The authors reported that this combined treatment led to increased basal inflammation but a decreased inflammatory response in the offspring (Desai et al., 2009). In addition, several studies have reported an association between reduced fetal growth and augmented levels of basal C-reactive protein (CRP), a major inflammatory factor in adulthood (de Rooij & Roseboom, 2013; Sattar et al., 2004; Tzoulaki et al., 2008). For example, a large epidemiological study reported a correlation between a birth weight reduction by 1 kg with an 11% increase in CRP in adults age 30–

59 years (Sattar et al., 2004). Canoy et al. (2009) further reported an association between low birth weight and a number of leukocytes, where a total number of leukocytes at the age of 31 years was higher in the low birth weight individuals(Canoy et al., 2009). Interestingly, there is evidence from rodent studies showing that the effects of early environments such as stress may be passed across multiple generations to affect inflammatory processes that may ultimately contribute to disease pathologies.

Murgatroyd et al. (2016) argue that the immune system may represent an important inter- and transgenerational etiological factor in disorders such as comorbid immune disorders and psychiatric disorders (Murgatroyd et al., 2016). This study investigated F1 and F2 male and female rats whose mothers were exposed to a novel male intruder to induce chronic social stress (CSS) during days 2–16 of lactation. Subsequently, the animals were tested for emotionality, grooming behaviours and immune response (Murgatroyd et al., 2016). The aim was to study if the changes in offspring behaviours were associated with basal peripheral levels of immune and neurotrophic factors. Here, CSS decreased acid glycoprotein (alpha1AGP), intracellular adhesion molecule-1(ICAM-1) and brain-derived neurotrophic factor (BDNF) in F2 females, and increased microphage stimulating factor (GM-CSF), IL-18, tissue inhibitor of metalloproteinase and vascular endothelial growth factor in F2 males (Murgatroyd et al., 2016). Furthermore, the study demonstrated that the changes in alpha1AGP, GM-CSF and progesterone correlated with decreased grooming in the F2 offspring of stressed mothers. Thus, this study supports the idea that transgenerational social stress, even when passed via maternal behaviour, affects both social behaviour and immune system activation (Murgatroyd et al., 2016).

The results by Murgatroyd et al. (2016) also support previous evidence on the adverse effects of early life stress on later immune function and stress-related immunological disorders. Exposure to both prenatal and postnatal early life stress can induce brain alterations (Howerton & Bale, 2012; Murgatroyd et al., 2016) and these effects may be regulated by the immune system (Howerton & Bale, 2012). Immune factors can alter brain programming to affect the risk of diseases in later life. For example, neurodegenerative disorders have been recognized to be associated with inflammation (Howcroft et al., 2013). Furthermore, it can be speculated that the exposure to early life stress may speed up the processes underlying the phenomenon of inflammaging.

According to Franceschi and Campisi (2014) four main sources of inflammaging can be distinguished: 1) damaged macromolecules and cells that accumulate with age-associated increased production and their inadequate elimination; 2) increase in harmful products produced by microbial constituents of the body such as oral or gut microbiota, 3) cellular senescence in response to accumulated damage and stress; and 4) increased activation of the coagulating system with age (Franceschi & Campisi, 2014). First, the functional integrity of a cell may be compromised in response to damage to macromolecules, damage to cellular and organelle components, the formation of free radicals from oxidative stress, metabolites such as extracellular ATP, ceramides, urate crystals, succinate, and others. The molecules may be recognized as danger signals and initiate an immune response for repair (Franceschi & Campisi, 2014). Over time as damage accumulates, such uncertain responses may result in chronic inflammation. Interestingly, a rodent study by Kiss et al. (2016) demonstrated that the exposure to ancestral gestational stress alters metabolite accumulation along with impaired psychiatric health in the F4

generation (Kiss et al., 2016). This study stated that both trans-and multigenerational exposure to prenatal stress alter metabolite accumulation such as, formate, tyrosine, histamine, and hippurate in male offspring along with an elevated affective state (Kiss et al., 2016). Furthermore, metabolite set enrichment analysis of involved pathways revealed that the altered metabolites are involved in immune response and microbial-host interaction (Kiss et al., 2016).

In line with the above concept (Franceschi & Campisi, 2014), the second source of inflammation might be due to products generated by microbial constituents of the body (Franceschi & Campisi, 2014). Extensive research over the last few years has been examining gut microbiota, as it may leak into surrounding tissues and the circulatory system. It is believed that with age, the ability of the gut to isolate leaking microbes declines, but also the gut microbiota itself may change the inflammatory capacity (Franceschi & Campisi, 2014). Accordingly, host-microbial interactions may critically influence inflammatory factors in the gut and cytokine production which, in the case of an aberrant immune response, may precipitate a specific disease. For example, levels of interleukin-6 (IL-6) and soluble tumor necrosis factor alpha (TNF-α) were identified as predictors of 10-year mortality (Franceschi & Campisi, 2014). Interestingly, prenatal stress can also induce alterations in IL-6 levels in adult offspring (Ślusarczyk et al., 2015). One mechanism responsible for the changes in this proinflammatory cytokine may be the direct transmission of maternal microbes to offspring which then inoculates the organism with a certain immune status (Jašarević et al., 2015). Furthermore, it was hypothesized that the transfer of maternal bacteria to offspring may be an emerging factor in transgenerational disease risk (Jašarević et al., 2015) which may be susceptible to stress (Prenderville et al.,

2015). Indeed, in a rodent study, chronic social stress altered levels of IL-18 and was associated with the psychiatric disease in adult male offspring (Murgatroyd et al., 2016).

The third source of inflammaging may be produced by cellular senescence (Franceschi & Campisi, 2014). Senescence aids in wound healing and may prevent cancer by inhibiting the proliferation of cells (Baker et al., 2011). The major problem is that the senescent cells accumulate with advancing age and can not be eliminated properly. Moreover, sensence may disrupt cell homeodynamics, which is regulated by essential trace elements to result in cancer and neurodegenerative diseases (Ambeskovic et al., 2013). Interestingly, the Dutch famine birth cohort study investigating aged men and women that were exposed to undernutrition during early development reported that these individuals age quicker due to senescence (de Rooij & Roseboom, 2013). Senescent cells accumulate to high levels especially in fat tissue and potentially have systemic effects on health (Barker et al., 2012). Hence, individuals with adverse childhood experiences and high rates of obesity at the same time may be at increased risk of accelerated aging (de Rooij & Roseboom, 2013). Furthermore, obesity may also be linked to a greater risk of an impaired coagulation system.

The fourth source of inflammaging is the increased activation of the coagulation system with advanced age (Franceschi & Campisi, 2014). Due to shared components and interactions, coagulation may be considered a part of inflammation because both serve as critical host-defense systems. Systemic inflammation usually activates coagulation through the action of pro-inflammatory cytokines (Levi et al., 2003). Moreover, an augmented hypercoagulation state is usually observed with advanced age and accounts for a greater incidence of arterial disease in the elderly (Franceschi & Campisi, 2014). Furthermore,

alterations in the coagulation system may affect brain function, considering that the brain requires major blood supply. However, further mechanistic studies and translation of these findings to human populations are still necessary to disentangle the many different levels of molecular and physiological interactions involved in inflammaging.

1.8.4 Epigenetic Regulation of Aging and Lifespan

Epigenetic influences are dynamic regulators of gene expression, especially during early development. These changes are biologically programmed and functionally appropriate (Jung & Pfeifer, 2015). Epigenetic changes during adulthood are usually brought about by experiences and aging-associated events (Jung & Pfeifer, 2015). Small non-coding RNAs (sncRNAs) are believed to contribute to aging-associated events. A study by Kato et al. (2011) showed that sncRNAs, including miRNAs, may play a role in lifespan regulation in the nematode C. elegans (Kato et al., 2011). By profiling small RNAs by deep sequencing over the course of aging in C. elegans, the authors found a significant number of miRNAs that changed their expression during aging (Kato et al., 2011). For example, miR-38 and miR-47, let-7 and lin-4 were downregulated, while miR-239a and miR-34 were upregulated (Kato et al., 2011). These signatures may represent characteristic biomarkers for aging in C. elegans with potential clinical value.

Another epigenetic marker that has been studied extensively in association with aging is DNA methylation, which is characterized by adding one or more methyl groups at the 5-position of cytosine at specific DNA locations (Jung & Pfeifer, 2015). Significant changes in 5-methylcytosine (5mC) occur depending on the organ and life stage and sometimes there is an inverse relationship with a decrease of 5mC DNA methylation and lifespan (Jung & Pfeifer, 2015). This finding indicates that a faster decline in DNA

methylation is linked to shorter life expectancy. Moreover, a systems approach in investigating genes undergoing age-associated changes in DNA methylation in the context of protein interaction networks reported that age-associated epigenetic drift may be occurring in the specific networks of genes (Zannas & West, 2014). Notably, topographical properties of many of the affected gene classes include longevity, age-associated expression and those implicated in disease overlap (Zannas & West, 2014). Exploring the amount of DNA methylation from various tissues and cell types can also be used to predict the biological age (Horvath, 2013). Steve Horvath's epigenetic clock predicts age using 353 CpG sites which combined form an aging clock in regard to chromatin states and tissue variance (Gibbs, 2014; Horvath, 2013). Thus, measuring the cumulative effect of epigenetic maintenance can provide useful information about aging processes.

Recent observations indicate that exposure to an adverse environment in utero may be a major contributor to alterations in DNA methylation (Cao-Lei et al., 2014; Cao-Lei et al., 2016). For example, in Project Ice Storm, a human study of exposure to natural disaster, prenatal stress altered DNA methylation levels in CpGs affiliated with 957 genes related to immune function in 8-year-old offspring (Cao-Lei et al., 2014). Furthermore, these authors found that maternal objective stress, but not subjective stress was correlated with these DNA methylation levels in the children (Cao-Lei et al.,2014). Similar findings were reported in the Dutch Famine Study, where F1 offspring exposed to maternal undernutrition showed reduced DNA methylation of the imprinted IGF2 gene sixty years later (Heijmans et al., 2008). Importantly, exposure to adverse environments early in life not only affects the directly exposed offspring but their unexposed descendants as well.

Rodent studies have demonstrated that exposure to stressors during early development alter DNA methylation in sperm of male F3 offspring (Manikkam et al., 2012a), DNA methylation in germline of F2 and F3 offspring along with induction of depressive-like behaviours (Franklin et al., 2010), and the number of DNA methylation marks in F2 male mice (Dias & Ressler, 2013). Of note are also epigenetic changes in the miRNA expression after exposure to early stressors. Notably, work by Gapp et al., demonstrated that both prenatal and transgenerational stress could induce upregulation on miRNAs 375, 200b, 673 and 466 in both sperm and brain of male mice offspring (Gapp et al., 2014a, 2014b). Importantly, altered miRNA expression patterns are implicated in stress response and metabolic regulation (Babenko et al., 2012; Babenko et al., 2015; Metz et al., 2015), which may make them more vulnerable to accelerated aging an disease incidence. However, further research is needed to causally link changes in these epigenetic responses to disease incidence or aging trajectories.

1.9 Potential Interventions to Reverse the Negative Effects of Adverse Life Experience

Enriched environments (EE) may reduce the adverse effects of stress and reverse epigenetic changes. EE modulates HPA axis activity and essential immune functions likely through epigenetic regulation of gene expression (Kolb et al., 2012; McCreary et al., 2016c). EE may enhance beneficial endocrine and behavioural changes associated with epigenetic re-programming throughout the life span, thus overcoming transgenerational and early life adverse endocrine programming. Animal studies have shown that negative behavioural effects resulting from prenatal stress may be reduced or even eliminated by EE. For example, increased levels of anxiety-like behaviour in prenatally stressed rats (Cottrell & Seckl, 2009; Harris & Seckl, 2011; Lupien et al., 2009; Weinstock, 1997) were mitigated

when subjected to EE (Laviola et al., 2004). Moreover, stress-induced learning and memory impairments may be minimized or offset with EE (for review, see McCreary & Metz, 2016). In addition, EE ameliorates not only adverse cognitive effects of stress but also impaired neuronal function. A study by Yang et al. (2007) demonstrated that EE was able to restore impaired long-term potentiation and long-term depression in the hippocampus of stressed rats (Yang et al., 2007).

Recently it was shown that EE can be used to rescue the adverse consequences of transgenerational stress (Gapp et al., 2016; McCreary et al., 2016c). This work demonstrated that postnatal paternal trauma alters coping behaviours, increases GR expression along with reduced DNA methylation of the GR promoter in adult male mouse offspring (Gapp et al., 2016). However, when these offspring were placed in EE, there was a reversal of alterations in GR expression and DNA methylation in hippocampus accompanied by prevention of transgenerational behavioural symptoms (Gapp et al., 2016). Furthermore, EE promoted miRNA-mediated expression of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) along with improved affective behaviour (McCreary et al., 2016c). Thus, environmental intervention can partially overcome the consequences of adverse ancestral experiences. Although transgenerational aging studies on the effects of EE are lacking, much can be learned from clinical aging studies.

Based on clinical evidence, other interventions that may be used to enhance chances of successful aging include: 1) stimulating social lifestyle, 2) cognitive training, 3) expressive therapies such as writing or painting, 4) dietary regimens, and 5) physical activity and aerobic exercise (Hertzog et al., 2008; Niles et al., 2014; Rae et al., 2016). For example, the literature shows that social interactions at any age are protective against the

incidence of diseases such as cardiovascular and immune disorders (Ford et al., 2000). Moreover, individuals who had more social interactions were happier and lived longer (Berkman & Syme, 1979; Smith & Christakis, 2008). Recently, Hobbs et al. (2016) demonstrated that online social interactions also had benefits to overall health and longevity (Hobbs et al., 2016). Moreover, adults who have meaningful social engagement also have a better cognitive function (Hertzog et al., 2008). In addition, recently it was shown that when older adults undergo cognitive training such as a visual search dual task and divided attention tests, improvements in their cognitive processing and memory were reported (Oei & Patterson, 2013). Expressive writing may also be ideally suited to reduce the impact of emotional stress and promote effective coping strategies. Dietary regimens are also important factors that affect aging across species. In primates, the moderate caloric restriction may reduce age-related mortality (Colman et al., 2014). In this study, only 30% restriction in diet from young adulthood into old age improved survival in rhesus monkeys (Colman et al., 2014). Exposure of the developing primate fetus to moderate caloric restriction during pregnancy, however, can cause adverse outcomes for brain development (Antonow-Schlorke et al., 2011). Lastly, both physical activity and aerobic exercise are believed to enhance cognitive aging. Both animal and human data show that exercise improves cognitive function, especially aspects of executive function (Gill et al., 2015) and reduces the impact of oxidative damage (Radák et al., 2001). Thus, the uncontrollable impact of ancestral adverse stress and trauma can potentially be offset by a beneficial experience throughout life.

1.10 Conclusions

Exposure to adverse environments in early life, such as psychological stress, undernutrition, and endocrine-disrupting chemicals, may reprogram organ development with potentially lifelong consequences. The disruption of various cellular functions may contribute to accelerated aging and the potential risk of disease in older age, all of which may be regulated by epigenetic mechanisms. Recent epigenetic findings indicate that even unexposed generations of descendants born to stressed ancestors may be at risk of accelerated aging and higher disease incidence. Thus, it is critical to recognize that the foundation for successful aging is laid early in life or even in previous generations. Fortunately, while the stressful lives of ancestors cannot be reversed, adverse consequences on brain and physical health in their descendants may be offset by beneficial experiences and life style changes possibly at any time in life. Thus, exposure to EE or other beneficial experiences should be considered as therapeutic intervention to support healthy aging. Moreover, the study of inter- and transgenerational origins of age-related diseases offers the opportunity to identify predictive or diagnostic biomarkers. These biomarkers are central to guide the development of new interventions according to a personalized medicine approach that supports the chances of healthy aging in today's population and future generations.

1.11 Thesis Objectives and Outline

The main objective of the proposed thesis research is to study the effect of ancestral stress (MPS and TPS type) on healthy brain aging and disease incidence in a rat model using comprehensive behavioural, neuroanatomical and physiological assessment.

The specific goals of this thesis include:

1) To study the behavioural, neuroanatomical, epigenetic and physiological effects of ancestral prenatal stress on mental health by comparing F1-F4 MPS, TPS offspring and non-stress CONTROL rats;

2) To study the effect of the MPS on aging by comparing behavioural, epigenetic and physiological outcome, disease incidence and longevity across the lifespan in rats;

3) To study the effects of maternal social stress during pregnancy on the stress response, immune system, motor and cognitive function and brain morphology in the F4 (MPS, TPS and CONTROL) generation offspring across the lifespan.

This will be accomplished in three chapters, each presenting one set of experiments, which make up the body of the thesis. Chapter 1 contains a manuscript published in Neurosci Biobehav Rev by Ambeskovic, Roseboom & Metz in which we reviewed the main experimental and clinical studies suggesting that adverse early environment is critical determinant of brain development with long term behavioural outcomes and disease incidence in offspring exposed and future generations. Chapter 2 contains a manuscript published in Scientific Reports by Ambeskovic, Metz et al., in which we studied whether ancestral stress exposure in a single generation (TPS) vs. multiple generations (multigenerational prenatal stress; MPS) produces a recognizable behavioural and endocrine phenotype in the F4 generation rat offspring. Specifically, we investigated sexual dimorphic patterns in stress reactivity and neuroplastic adaptations in the medial prefrontal cortex (mPFC) and orbital frontal cortex (OFC) during early and late adulthood. In this study, we hypothesized that MPS will induce sex-specific effects on anxiety-like behaviours, endocrine phenotypes and neuroplasticity of the PFC regions. In Chapter 3, we studied the impact of recurrent cumulative stress (MPS) in the fourth (F4) generation on age-dependent physical and mental health trajectories, stress response and epigenetic

regulation by miRNA deep sequencing as a function of sex. In this study, we hypothesized that MPS will alter the stress response and accelerate biological aging and exhibit sex-specific disease incidence. In Chapter 4, we studied the effects of maternal social stress during pregnancy on the stress response, immune system, motor and cognitive function and brain morphology in the F4 generation offspring (MPS, TPS and CONTROL) across the lifespan. We hypothesized that ancestral stress will alter health outcomes, cognitive function and induce accelerated aging trajectories across the lifespan in a sex-specific manner in both MPS and TPS F4 generation offspring. Lastly, in Chapter 5 contains the general discussion of findings, the implications and whether the type of stressor (restrain or social isolation) induces different brain and behavioural changes in male and female rats across the lifespan.

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CHAPTER 2: Ancestral Stress Alters Lifetime Mental Health Trajectories and Cortical Neuromorphology via Epigenetic Regulation

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2.1 Abstract

Experiences during early development are powerful determinants of lifetime mental health. Prenatal stress increases the risk of mental disorders in later life and even affects unexposed offspring. Here we investigated if ancestral stress regulates the brain's epigenetic memory to alter neuromorphology and emotionality in the progeny. Pregnant female dams were exposed to restraint stress on gestational days 12-18 to generate F1 stress lineage. Transgenerational stressed lineage was generated by stressing only dams of the parental F0 generation, while F1-F3 offspring were left undisturbed. Stressing dams of each consecutive (F1-F3) generation during pregnancy generated a multigenerational stress lineage. A lineage of yoked controls was bred with each generation. Results indicate that both trans- and multigenerational stress were characterized by an altered affective state at postnatal day 90, however the F4 multigenerational stressed offspring were most affected and therefore underwent further behavioural, physiological, neuromorphological and epigenetic testing in adulthood. Multigenerational stress increased anxiety-like behaviours and stress response in adult F4 generation male but not female rats. Functional changes were accompanied by reduced spine density in the male medial prefrontal cortex with opposite effects in the orbital frontal cortex. Furthermore, stress regulated miR-221 and miR-26 expression and their target genes, thus downregulating *Ntrk2* and *Map1a* genes in males while downregulating *Crh* and upregulating *Map1a* genes in females. These

epigenetic miRNA-dependent pathways are candidates for developmental programming of mental health and stress vulnerability. Thus, multigenerational stress critically determines sexually dimorphic predisposition to adverse mental health outcomes and stress sensitivity.

2.2 Introduction

The prevalence of mental health disorders worldwide has surged to 36.7 % in the last decade (Whiteford et al., 2013). Evidence suggests that the exposure to adverse environments during early development plays a role in programming mental illness, such as attention deficit hyperactive disorder (ADHD), anxiety, depression, and vulnerability to post-traumatic stress disorder (PTSD; (Lewis et al., 2014). The developmental origins of health and disease (DOHaD) theory posits that the exposure to adverse environments during the fetal period and early childhood can program the vulnerability to disease in later life (Barker et al., 1993). Accordingly, numerous studies have shown that prenatal stress, undernutrition, and perinatal inflammation may alter the stress response and behavioural phenotype leading to increased risk of psychiatric disorders later in life (Lewis et al., 2014; Nätt et al., 2017; Welberg & Seckl, 2008; Yehuda & Bierer, 2007).

Prenatal exposure to stress in particular alters fetal brain development which manifest in behavioural alterations associated with mental health disorders later in life. For example, human studies have reported increased incidence of anxiety and depression in children born to mothers who experienced distress, such as war, family violence, natural disaster or death of a close relative, during pregnancy (Gillott & Standen, 2007; King et al., 2012; Lewis et al., 2014). Similarly, animal models of prenatal stress have shown increased latency to play (Charil et al., 2010), and anxiety-like behaviours (Vallée et al., 1997) in offspring. Stress programming is likely associated with altered neuronal plasticity

(Muhammad & Kolb, 2011; Murmu et al., 2006). Reports demonstrated alterations in spine density and dendritic branching in both medial prefrontal cortex (mPFC) and orbital frontal cortex (OFC), two frontal regions associated with emotional behaviours (Muhammad & Kolb, 2011; Welberg & Seckl, 2008) with significant sexual dimorphisms (Muhammad et al., 2012; Mychasiuk et al., 2012).

Human and animal studies support the notion of inter-generational programming by prenatal stress. Prenatal undernutrition has life-long impact on health and disease incidence of children and grand-children (Ambeskovic et al., 2017; Roseboom et al., 2001). Moreover, ancestral prenatal exposure to elevated steroid hormones and toxins alters behaviour and disease incidence in unexposed grand offspring (Anway et al., 2006; Iqbal et al., 2012). We have shown that ancestral prenatal stress alters maternal behaviour by reduced maternal tail chasing activity post-partum and offspring sensory motor development at P7 and fine motor development in adulthood across generations (Ambeskovic et al., 2017; Yao et al., 2014). Furthermore, ancestral stress induced distinct metabolic profiles with consistent upregulation of hippurate and downregulation of tyrosine, threonine, and histamine (Kiss et al., 2016) and altered neuronal epigenetic regulators such as miR-200 and miR-181 expression (McCreary et al., 2016; Yao et al., 2014) in a mainly sex-specific manner. Ancestral epigenetic programming has been ascribed an important role in disease risk (Anway et al., 2006; Gapp et al., 2014). Thus, we hypothesized that through epigenetic regulation of neuronal microRNA (miRNA) expression, a stressful environment may result in a transgenerational and multigenerational neuromorphological, endocrine and emotional phenotype. This mechanism may explain why the mental illness may occur in the absence of genetic risk factors, such as indicated

for the *Ntrk2* single nucleotide polymorphisms in PTSD (Bremer et al., 2007; Rothbaum et al., 2014).

The present study investigated if ancestral stress exposure in a single generation vs. multiple generations produces a recognizable behavioural and endocrine phenotype in the F4 generation unexposed rat offspring. The experiment was designed to investigate sexual dimorphic patterns in stress reactivity and neuroplastic adaptations in the medial prefrontal cortex (mPFC) and orbital frontal cortex (OFC) during early and late adulthood. The findings show that recurrent prenatal stress exposure over four generations (multigenerational stress) exceeded the effects of single generation stress (transgenerational) and induced anxiety-like behaviours in adult male but not female offspring. The present findings suggest miRNA dependent pathways may play role in sex-specific transgenerational programming of lifelong stress vulnerability and resiliency.

2.3 Methods

2.3.1 Animals

Five generations of Long-Evans hooded rats were bred and raised at the Canadian Centre for Behavioural Neuroscience vivarium under carefully controlled conditions. For the present study 280 adult rats were housed in groups (males in pairs, females three per cage) under a 12:12 h light/dark cycle with light starting at 07:30h and the room temperature set at 22°C. All rats were tested on postnatal day (P90; early adulthood) and left undisturbed except for weekly weighing and cage changes until P180. A subset of animals was tested in mid adulthood at six months of age as indicated. All procedures were approved by the University of Lethbridge Animal Care Committee in compliance with the guidelines by the Canadian Council on Animal Care.

2.3.2 Experimental Design

Under standardized conditions, two different lineages of timed pregnant rats were bred (see Figure 1), prenatally stressed (S) and non-stressed (N) conditions. In the stress condition, female rats were stressed on gestational days 12-18 by a semi-random daily sequence of 5 min swim and 20 min restraint in a Plexiglas cylinder to generate F1-S offspring. In the non-stress control condition, dams remained unstressed (N) to generate F1-N offspring. A transgenerationally stress lineage was generated by stressing only dams of the parental F0 generation, while F1 daughters (F1-S), F2 granddaughters (F2-SN) and F3 great-granddaughters (F3-SNN) were not stressed during pregnancy. In addition, stressing dams of each consecutive (F1-F3) generation during pregnancy generated multigenerational stress lineage (F1-S; F2-SS; F3-SSS; F4-SSSS; Figure 2.1).

A lineage of yoked controls was bred with each generation (non-stress pregnant F0, F1-N, F2-NN, F3-NNN). Each generation F1-F4 was outcrossed or outbred. F1, F2, F3 and F4 generation animals from different litters within each exposure group were mated to each other, avoiding sibling and family inbreeding which went back at least past grand mother. A maximum of three offspring per litter of each sex was randomly selected to be included in the experiments. Each experimental group included offspring from at least 3-4 different litters.

By including the F1 generation with prenatal stress (F1-S), the F3 generation with transgenerational stress (F3-SNN) and multigenerational stress (F3-SSS), and the F4 generation with transgenerational stress (F4-SNNN) and multigenerational stress (F4-SSSS), the present study differentiates the direct impact of stress from truly heritable epigenetic phenotypes (Figure 2.1; Ambeskovic et al., 2017). The experiments included

both male and female F1 animals [males: n=20 (F1C=10, F1-S=8); females: n = 17: (F1-C=9, F1-S=8], F3 animals [males: n=56 (F4-NNNN=21, F4-SNNN=18, F4-SSSS=17); females: n=60 (F4-NNNN=29, F4-SNNN=15, F4-SSSS=16)] and F4 animals [males: n=58 (F4-NNNN=23, F4-SNNN=11, F4-SSSS=24); females: n=66 (F4NNNN=24, F4-SNNN=18, F4-SSSS=24)]. All groups were tested for exploratory activity and anxiety-like behaviours at P90. F4 animals (SSSS and SNNN) only were also tested for long term anxiety-like behaviours at six months of age because the largest effects of ancestral stress were observed in this generation [male n=21 (F4-NNNN=11, F4-SSSS=10); female n=24 (F4NNNN=13, F4-SSSS=11]. Since the multigenerational stress (F4-SSSS) induced more profound effects on anxiety-like behaviours than transgenerationally stressed offspring further neuromorphological [male n=12 (F4-NNNN=6, F4-SSSS=6); female n=12 (F4-NNNN=6, F4SSSS=6)] and epigenetic [male n=6 (F4-NNNN=3, F4-SSSS=3); female n=6 (F4-NNNN=3, F4SSSS=3)] analyses focused on the F4 generation.

2.3.3. Prenatal Stress

Pregnant dams were stressed daily by forced swimming in warm water (22 °C) for 5 min and 20 min restraint in a Plexiglas cylinder daily on gestational days 12-18 (Ambeskovic et al., 2017; Erickson et al., 2014; Faraji et al., 2017; Yao et al., 2014). Stressors were administered each day in semi-random order either in the morning (9:00) or afternoon (15:00) hours.

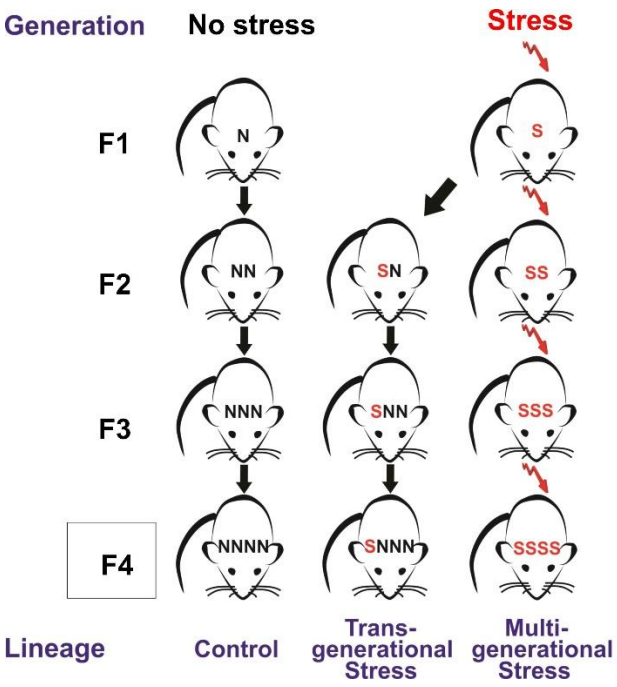

Figure 2.1. Trans- and multigenerational stress lineages. Naïve dams were stressed during timed pregnancy to generate the F1 prenatally stressed offspring (F1-S). The F3 transgenerationally stressed offspring (F3-SNN) were generated from prenatally stressed F1 grandmothers, whereas multigenerationally stressed offspring (F3-SSS) were produced by stressing pregnant mothers in 3 consecutive generations. For the F4 generation, transgenerationally stressed (F4-SNNN) offspring came from a lineage where only great-great grandmothers were exposed to stress during pregnancy, while multigenerationally stressed offspring (F4-SSSS) came from a lineage where 4 consecutive generations of pregnant mothers were subjected to stress. Each generation F1-F4 was outcrossed or outbred. F1, F2, F3 and F4 generation animals from different litters within each exposure group were mated to each other, avoiding sibling and family inbreeding which went back at least past grand mother.

2.3.4. Behavioural Testing

2.3.4.1 Open Field

Exploratory activity and anxiety-like behaviours were recorded by open field locomotor activity, a standard measurement of emotional states in rats (Denenberg, 1969). Briefly, animals were placed individually into Accuscan activity monitoring Plexiglas boxes (length 42 cm, width 42 cm, height 30 cm) and monitored for 10 min using

VersaDatTM software (AccuScan Instruments Inc., OH, USA). Total distance traveled (cm) per 10 minutes was used to measure overall activity, and the total time (sec) spent in margins was used to indicate anxiety-like behaviours.

2.3.4.2 Elevated Plus Maze

Anxiety-like behaviour was assessed using the elevated plus maze. The '+' shaped maze consisted of two open and two closed arms (each 40 cm long and 10 cm wide) and was elevated 90 cm above the ground. The open arms had no side or end walls, and the closed arms had side and end walls (40 cm high). Briefly, rats were placed individually in the central square (10 cm × 10 cm) facing either closed arm and were allowed to explore the apparatus for 5 min while being video-recorded. Following each test, the apparatus was thoroughly cleaned with 10% clinicide (Vetoquinol, Lavaltrie, QC, Canada) to eliminate the odor trace. Anxiety-related behaviour in terms of time spent risk assessing was evaluated by an experimenter blind to the experimental conditions (Waldherr & Neumann, 2007).

2.3.5 Blood Collection and Analysis

Blood samples were obtained three days prior behavioural testing. On average 0.6 ml of blood was collected from the tail vein between 8:00 and 10:00 AM under 4% isoflurane anesthesia (Faraji et al., 2017). The blood was transferred to centrifuge tubes and plasma was obtained by centrifugation at 5,000 rpm for 10 minutes. The samples were stored at –86° C. Plasma corticosterone (CORT) levels were determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits (Cayman Chemical, Ann Arbor, MI, USA).

2.3.6 Histological Processing for Golgi-Cox Staining

Following behavioural testing, animals were treated with an overdose of pentobarbital (Euthansol 100 mg/kg; CDMV Inc., Québec, Canada) and intracardially perfused with 0.9% saline. Brains were removed and preserved in Golgi-Cox solution for 14 days then placed in 30% sucrose for 28 days. Brains were sectioned on a vibratome (Leica, Buffalo Grove, IL, USA) at 200 µm, and slices were mounted on gelatin-coated slides. Sections underwent a Golgi-Cox staining protocol 39 for visualization of distal dendrites. Only dendritic segments that met the criteria of being thoroughly stained and without overlap with another dendrite or blood vessel were included in the examination (20).

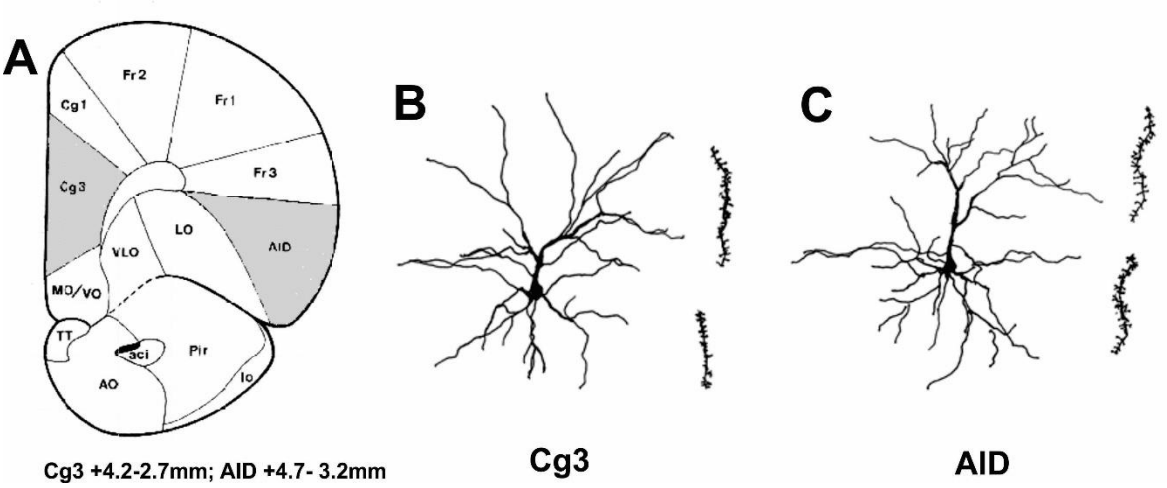

Figure 2.2. Morphometry of Golgi-stained pyramidal neurons in the medial prefrontal cortex (Cg3) and the orbital frontal cortex (AID). (A) Schematic diagram of a coronal section illustrating the location of Cg3 and AID (shaded areas). (B) Representative Cg3 pyramidal neuron showing apical and basilar dendrites and dendritic segment with spines. (C) Representative AID neuron showing basilar dendrites and dendritic segment with spines.

Pyramidal cells from cortical layer III, of the orbital frontal cortex (OFC/AID) and medial prefrontal cortex (mPFC/Cg3) were analyzed (Figure 2.2). Individual neurons were traced from the Golgi-Cox stained brains, using a camera lucida mounted on a microscope. A total of 10 cells (5 per hemisphere) were traced at 200× magnification per animal. Neuromorphological measurements obtained from the AID and Cg3 included apical and basilar Sholl analysis (an estimate of dendritic length derived from dendritic branches that intersect concentric circles spaced 25 µm apart), apical and basilar dendritic branch order (an estimation of dendritic complexity based on the number of branch bifurcations) and spine density (the number of spine protrusions on a 40 µm-segment of dendrite traced at 1000× magnification (Ambeskovic et al., 2017; Gibb & Kolb, 1998).

2.3.7 MiRNA and mRNA Deep Sequencing

Following behavioural testing, the subgroup of animals (n=3) was treated with an overdose of pentobarbital (Euthansol 100 mg/kg; CDMV Inc., Québec, Canada), and once the vital signs were discontinued animals were decapitated. The brains were rapidly removed, dissected and flash-frozen (-80C) for miRNA and mRNA analysis. The TRI Reagent Solution was used to extract total RNA from the frontal cortices (Invitrogen, Carlsbad, CA, USA). MicroRNA expression analysis was performed by Illumina GAIIx genomic analyzer (Illumina, CA, USA). Briefly, using default setting the base calling and demultiplexing was completed by the CASAVA 1.8.1 software pipeline (Illumina, CA, USA). To examine short read quality FastQC software was used. Adapters were trimmed using Cutadapt software (https://cutadapt.readthedocs.org/; Anders & Huber, 2010). Another FastQC quality check was performed after trimming. MicroRazerS version 1.0 (Emde et al., 2010) short read aligner was used to perform miRNA mapping. Reads

mapping to mature miRNAs were counted using an ad hoc bash script. Potential targets of selected miRNA of interest were predicted using the 3′ UTR available for Rat rn5 (UCSC) genome. An algorithm (miRanda v.3.3a; Computational Biology Center of Memorial Sloan-Kettering Cancer Center, NY, USA) was used for miRNA target prediction.

mRNA analysis was also performed by a Illumina GAIIx genomic analyzer (Illumina 462 Inc., San Diego, CA, USA), using multiplex. Every library was sequenced across 3 separate lanes. Base calling and demultiplexing was performed by Illumina CASAVA 1.8.1 with default settings using Rat - Rnor 5.0 (Ensembl) as reference, and sequence and annotation information were downloaded from iGENOME (Illumina). Raw count data were uploaded into R, initial data exploration and outlier detection were performed using array Quality Metrics and DESeq2 bioconductor packages. First raw counts underwent normalization and variance stabilization procedure as described in DESeq2 manual. Hierarchical clustering of transcriptional profiles based on top 100 most variable genes, pre-selected from the subset of highly expressed genes (higher than the median expression). Clustering was performed using heatmap.2 function from g plots package with default clustering algorithm, gene expression values were displayed as heatmap. In addition to hierarchical clustering, similarity between samples were visualized as PCA plots built using plot PCA function implemented in DESeq2. Outlier detection and transcriptional profile quality control was performed using array Quality Metrics package.

2.3.8 Statistical Analysis

Statistical analysis was performed using SPSS 20 for Windows 11.5.0 (IBM Corporation, Armonk, NY, USA). Three-way ANOVA with sex, stress, and generation as

factors was run for behavioural tasks at P90. Similarly, three-way ANOVA with sex, stress and hemisphere as factors was run for neuromorphology of the orbital frontal cortex (OFC; AID) and the medial prefrontal cortex (Cg). A two-way ANOVA was completed for open field activity at P180 and elevated plus maze task at P180. Tukey's test was used for all behavioural and neuromorphological posthoc analyses when possible. Otherwise, independent sample t-test was run. For miRNA and mRNA analysis, raw count data was first normalized and regularized with log transformation using statistical routines implemented in the DESeq2 bioconductor package (Anders & Huber, 2010) as described in the DESeq2 user manual. Default settings were used to perform normalization and statistical analyses. Pairwise comparisons between experimental groups (stress and non-stress) were performed using DESeq2. To be considered differently expressed, miRNA's and mRNA's with a false discovery rate adjusted p-values <0.1 were used. All results are shown as the means ± standard error of the mean (±SEM).

2.4 Results

2.4.1 Transgenerational and Multigenerational Stress Increased Levels of Anxiety-Like Behaviours in Male but not Female F1-F4 Generation Offspring at P90 in Open Field Test

Ancestral prenatal stress across generations cumulatively induced anxiety-like behaviours in F3 and F4 generation male offspring that were subjected to transgenerational stress (SNNN) or multiple repeated stress or multigenerational stress (SSSS; $P<0.05$). Females showed overall higher level of anxiety-like behaviours than males across all generations, however no significant effects of ancestral stress were observed in females. A three-way ANOVA with Sex, Generation, and Stress as factors revealed a main effect of

all three factors individually, but no interactions. The time spent in margins of the open field revealed a significant main effect of Sex ($F(1,261)=39.87$, $P<0.001$, Figure 2.3A), as female rats overall spent more time in margins than males when F1-F4 generation offspring were combined. Moreover, when each generation was observed separately, significant sex differences were found in F1 and F3 generations ($P<0.001$), while smaller, but significant, differences were found between male and female offspring in the F4 generation ($P<0.05$; Figure 2.3A). These data indicate that multiple exposures to stress either through transgenerational or multigenerational transmission has fewer effects on anxiety-like behaviours in females than males and may demasculinize male behaviour. For instance, multigenerational stress increased the levels of anxiety in males to nearly comparable levels observed in females, as indicated by increased time spend in margins in F4 generation males. Increased margin time at the expense of centre time generally indicates higher anxiety-like behaviour.

Time spent in margins of the open field revealed a significant main effect of Generation ($F(2,260)=3.15$, $P<0.05$; Figure 2.3A), as the time in margins increased in later generations. For example, the offspring belonging to F1 and F3 generations spent significantly less time ($P<0.05$) in margins than the F4 generation rats. Moreover, time spent in margins revealed a main effect of Stress (($F2,260)=3.15$, $P<0.05$; Figure 2.3A), as overall stressed male and female rats spent significantly more time in margins of an open field than controls. Notably, when the effects of stress were observed within each generation individually, the stress had most profound effect on the time spent in margins in the F4 generation as suggested above. Stress has slightly but nonsignificant decreased time spent in margins in both males ($P>0.05$) and females ($P>0.05$) when compared to non-

stressed rats. Stress-induced a non-significant decrease in the time spent in margins in the F3 multigenerational and transgenerational males (P>0.05) and an increase in females (P>0.05; Figure 3A) in comparison to non-stressed rats. Although not significant, the largest effect of stress was observed in the F4 generation, where stress increased the time spent in margins in both males (P>0.05) and females (P>0.05). Since the largest effect was observed in F4 generation offspring at P 90, these animals were aged and tested again to determine if differences persist.

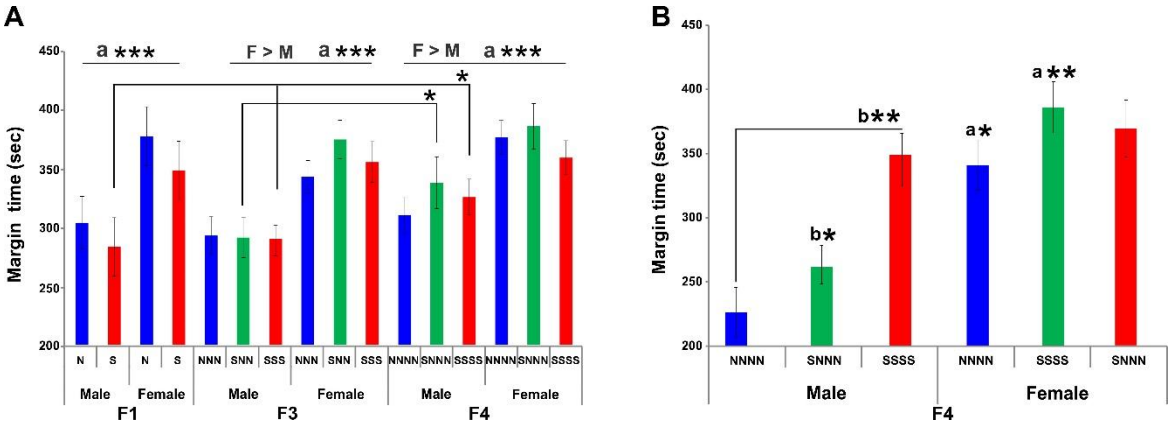

Figure 2.3. Effects of prenatal stress across generations on anxiety-like behaviour. Anxiety-like behaviour was indicated by the amount of time a rat spent in the margins of an open field task.
(A) Both trans- and multigenerational stress increased levels of anxiety-like behaviours in F3 and F4 generation male but not female rats at postnatal day (P) 90. (B) Multigenerational stress cumulatively exacerbated anxiety-like behaviours, as F4-SSSS males spent more time in margins than SNNN (transgenerational) or NNNN (non-stressed) rats at P180. Asterisks indicate significances: *P<0.05, **P<0.01, ***P<0.001. All data presented as mean ± SEM. “a” indicates sex effect; “b” indicates stress effect.

2.4.2 Multigenerational but not Transgenerational Stress Cumulatively Increased Levels of Anxiety-Like Behaviours in Male F4 Offspring at P180 in Open Field Test

Ancestral exposure to stress across four generations significantly increased time spent in margins in multigenerationally stressed (SSSS) but not transgenerationally stressed

(SNNN) male rats or females. Interestingly, multigenerationally stressed males show similar behaviour profiles as compared to females.

A two-way ANOVA revealed a main effect of Stress and Sex, and their interaction. The time spent in margins of the open field revealed a significant main effect of Sex ((F(1,53)=17.9, P<0.000; Figure 2.3B), as female rats overall spent more time in the margins than male offspring. Moreover, stress had an almost significant (F(2,52)=2.94, P=0.062; Figure 3B) effect on the time spent in margins. A pairwise comparison showed that both transgenerationally (F4-SNNN, P<0.05) and multigenerationally (F4-SSSS, P<0.05) stressed rats spent more time in margins than non-stressed ones (F4-NNNN). However, a Tukey posthoc test comparing the effect of stress within their respective sex revealed a significant effect in multigenerationally stressed (F4-SSSS) males only. When compared to non-stressed males (F4-NNNN), transgenerationally stressed (F4-SNNN, P=0.95) males did not spend more time in the margins, while multigenerationally stressed ones did (F4-SSSS, P>0.05; Figure 2.3B). Neither transgenerationally (F4-SNNN, P=0.37) nor multigenerationally (F4-SSSS, P=1) stressed females spent significantly more time in margins than non-stressed (F4-NNNN) rats. Time spent in margins also showed a significant interaction Sex x Stress (F(2,52)=4.0, P<0.05; Figure 2.3B), as transgenerational stress prolonged the time spent in margins in females (F4-SNNN), while decreasing it in males (F4SNNN) when compared to multigenerationally stressed rats.

2.4.3 Multigenerational Stress Increased Anxiety-Like Behaviours in F4 Generation Males at P180 in the Elevated Plus Maze Test

Because cumulative effects of multigenerational stress here showed the largest impact at P90, additional analyses on aging animals at P180 were performed for the F4-

SSSS generation to further discover sexual dimorphisms. Recurrent stress across multiple generations in males decreased their latency to enter closed arms, while it had no effect on females. Two-way ANOVA revealed a main effect of Stress as overall stressed male and female offspring were significantly ($F_{1,35}=3.9$, $P=0.05$; Figure 2.4) faster to enter closed arms than non-stressed rats. Moreover, latencies in stressed males were significantly shorter than non-stressed males ($t(16)=2.11$, $P<0.05$; Figure 2.4), stressed ($t(16)=3.88$, $P<0.001$) and non-stressed ($t(16)=3.16$, $P<0.01$; Figure 2.4) females. Sex X Stress interaction showed a trend ($F(1,35)=3.5$, $P=0.069$; Figure 2.4) with stress moderately increasing latencies in females but decreasing it in males.

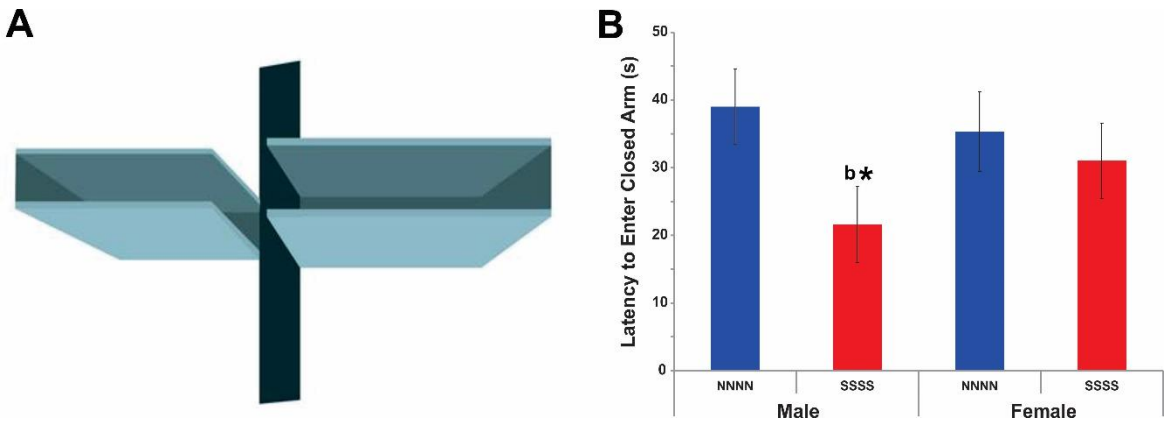

Figure 2.4. Effects of multigenerational prenatal stress on anxiety-like behaviours in the elevated plus maze (EPM) at P180. Anxiety-like behaviour was indicated by the latency to enter or escape into a closed arm. (A) A representative photograph of a rat escaping into a closed arm of the EPM. (B) Multigenerational stress decreased latency to enter closed arm in F4-SSSS males in comparison to non-stressed males. Asterisks indicate significances: *$P<0.05$. All data are presented as mean ± SEM. "b" indicates stress effect.

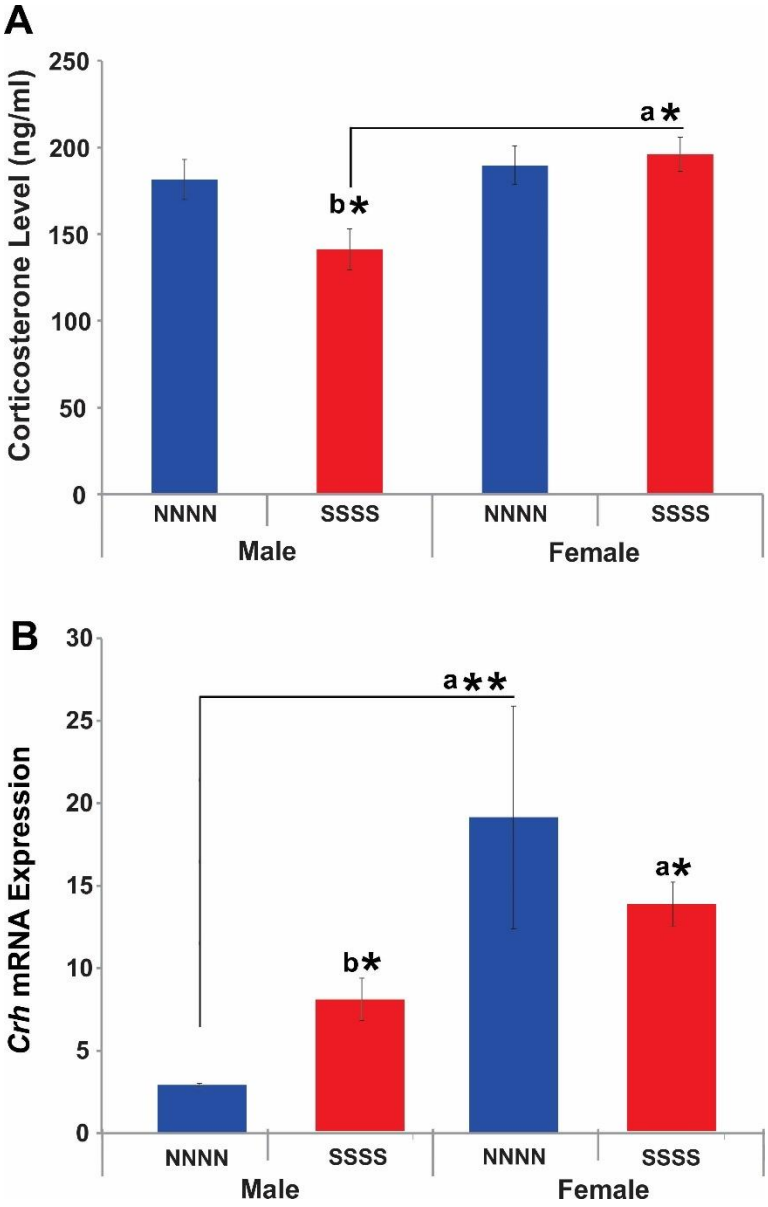

Figure 2.5. Effects of multigenerational stress on stress response systems. (A) Multigenerational stress reduced circulating corticosterone levels in males, while no changes were observed in female rats. (B) Deep sequencing of frontal cortex revealed that multigenerational stress upregulated Crh mRNA expression in males, with slight but non-significant downregulation in female rats. Asterisks indicate significances: *P < 0.05, **P<0.01. All data are presented as mean ± SEM. "a" indicates sex effect; "b" indicates stress effect.

2.4.4 Multigenerational Stress Blunted Basal Circulating Corticosterone Levels in F4 Generation Males Only

A two-way ANOVA with Sex and Stress as factors revealed a main effect of Sex. Overall significantly higher levels of circulating corticosterone were found in female rats ($F(1,35)=5.34$, $P<0.05$; Figure 2.5A). Moreover, a trend of interaction between Sex and Group ($F(1,35)=3.77$, $P=0.061$; Figure 2.5A) indicated moderately higher corticosterone levels in female and reduced levels in males rats. An independent sample-test revealed increased corticosterone levels in stressed males $t(16)=2.61$, $P<0.05$) but not non-stressed ($t(16)=2.61$, $P<0.05$; Figure 2.5A) male controls, non-stressed females ($t(16)=2.86$, $P<0.05$) or stressed female ($t(16)=-2.66$, $P<0.05$).

2.4.5 Multigenerational Stress Altered Neuromorphology of Orbital Frontal Cortex (OFC) and Medial Prefrontal Cortex (mPFC) in F4 Generation Males and Females

Repeated exposure to stress across multiple generations induced opposite effects in medial prefrontal cortex (mPFC or Cg3) and orbitofrontal cortex (OFC or AID) neuromorphology. A three-way ANOVA revealed a main effect of Sex ($F(2,46)=25.0$, $P<0.001$), Stress ($F(2,46)=4.2$, $P<0.01$), and nearly significant effect of Hemisphere ($F(2,46)=2.2$, $P=0.066$) for the Cg3. Spine density in males was higher than in females ($F(1,47)=52.9$, $P<0.001$), while dendritic length and branching were unaffected by sex. Stress decreased branch order and spine density. Dendritic branching in the basilar field revealed a significant main effect of Hemisphere ($F(1,47)=4.7$, $P<0.05$) as the right hemisphere had more elaborate branching than the left.

Moreover, multigenerational stress induced a non-significant increase in dendritic branching ($F(1,47)=3.4$, $P=0.07$) in the apical field of both hemispheres and sexes in comparison to non-stressed controls.

	Male			Female	
	Right Hemisphere	Left Hemisphere		Right Hemisphere	Left Hemisphere
	SSSS	SSSS		SSSS	SSSS
Branching					
Cg3 apical	↑	↓		↑	↑
Cg3 basilar	↓	↓		↓	↑
AID basilar	↓	↓		↑	↓
Length					
Cg3 apical	↓	↓*		↑	↑
Cg3 basilar	↓	↓		↓	↑
AID basilar	↓	↓		↑	↑
Spine Density					
Cg3 apical	↓	↓		↓	↓
Cg3 basilar	↓*	↓*		↓*	↓
AID basilar	↑	↑		↑**	↑

Table 2.1. Summary of dendritic branching, length and spine density results in the Cg3 region of the mPFC and the AID region of the OFC. The arrows represent the direction of effects in response to stress (relative to controls). Asterisk indicate significant differences, *p<0.05, **p<0.01.

Dendritic length in the apical field exhibited a significant Sex x Stress interaction ($F(1,47)=6.3$, $P<0.05$), as stress diminished dendritic length in the left hemisphere of males but exacerbated it in females. For spine density, apical dendrites exhibited a significant effect of Hemisphere ($F(1,47)=3.85$, $P=0.057$; Figure 2.6A & B), as the right hemisphere

had longer dendrites than the left. A significant effect of Sex was found in both apical ($F(1,47)=52.9$, $P<0.001$) and basilar ($F(1,47)=138.6$, $P<0.001$; Figure 2.6A & B) fields, as males had larger spine density than females. Importantly, multigenerational stress significantly reduced basilar spine density in both right (Male: $t(10)=2.14$, $P<0.05$; Female: $t(10)=3.17$, $P<0.05$; Figure 2.6, A & B; Table 2.1) and left (Male: $t(10)=2.73$, $P<0.05$; Female: $t(10)=2.25$, $P<0.05$; Table 2.1) hemisphere of F4 male and female rats when compared to non-stressed (NNNN).

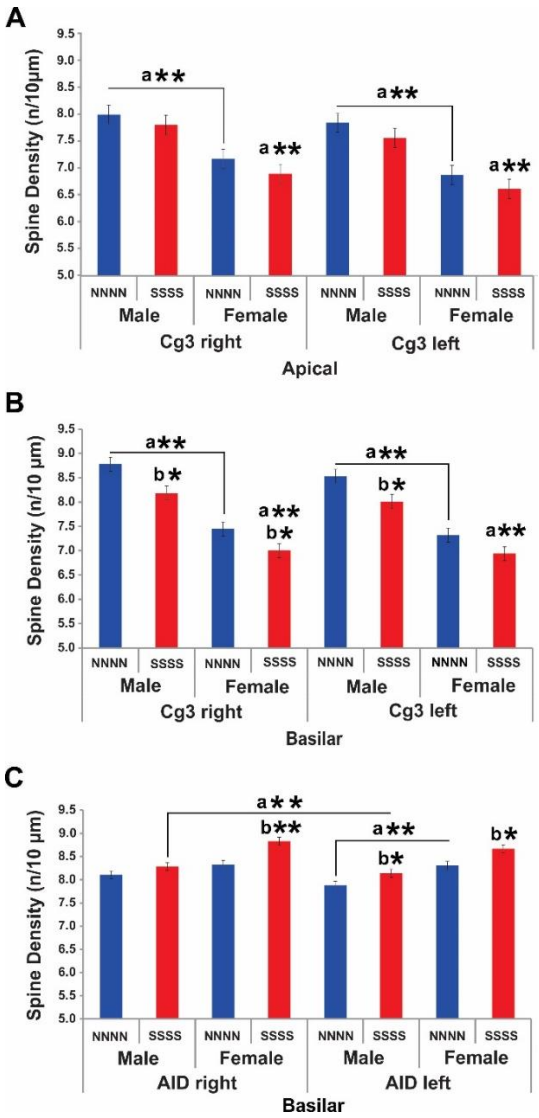

Figure 2.6. Dendritic spine organization of the medial prefrontal cortex (Cg3) and orbital frontal cortex (AID) in response to multigenerational stress. Multigenerational stress induced sexually dimorphic effects in Cg3 and AID dendritic spine density. (A) Cg3 spine density in the apical field was higher in non-stressed and stressed males compared to females. (B) Multigenerational stress decreased the number of spines in the Cg3 basilar field in male and female rats. Males had higher spine density than stressed (SSSS) and non-stressed (NNNN) females. (C) Mutigenerational stress increased the number of spines in the AID in males and females. Cg3 dendritic spine density in females were higher than in males. Multigenerationally stressed females had most dendritic spines in the right hemisphere of the AID. Asterisks indicate significances: *P < 0.05, **P<0.01. All data are presented as mean ± SEM. “a” indicates sex effect; “b” indicates stress effect.

For the orbital frontal cortex (AID), a three-way ANOVA revealed main effects of Sex ($F(2,46)=18.3$, $P<0.001$; Figure 2.6C) and Stress ($F(2,46)=9.21$, $P<0.001$; Figure 2.6C). Females exhibited predominantly longer dendrites with more spines than males. Stress overall slightly decreased complexity and length of dendrites in AID, while increasing the number of spines. Both right and left hemispheres had similar dendritic branching and length; however, the right hemisphere of both males and females had more spine density. Specifically, spine density in AID exhibited a main effect of Hemisphere ($F(1,47)=5.1$, $P<0.05$; Figure 2.6C), Sex ($F(1,50)=50.1$, $P<0.001$; Figure 2.6C) and Stress ($F(1,47)=28.24$, $P<0.001$; Figure 2.6C) but no significant interactions. Importantly, multigenerational stress increased spine density in both right (Male: $t(10)=-2.18$, $P<0.05$; Female: $t(10)=-3.86$, $P<0.05$) and left (Male: $t(10)=-2.32$, $P<0.05$; Female: $t(10)=-2.32$, $P<0.05$; Figure 2.6C, Table 2.1) AID hemispheres of both F4 generation males and females.

2.4.6 Multigenerational Stress Altered Epigenetic Regulation Through miR-221 and miR-26 and its Target Genes in Prefrontal Cortex Tissue in F4 Generation Males

Deep sequencing revealed that two miRNAs of interest linked to anxiety-like behaviours through regulation of neuronal proliferation and plasticity were differentially

expressed in the prefrontal cortex in response to stress (Hamada et al., 2012; Gu et al., 2015). Stress downregulated miR-221 and upregulated miR-26 expression in multigenerational stress males (SSSS; FDR P<0.05 adjusted using the Benjamini and Hochberg correction; Figure 2.7B). Expression of these miRNAs was not significantly affected in females. Thus, miR-26 and miR-221 expression showed sexually dimorphic effects. Moreover, multigenerational stress upregulated gene expression for corticotrophin releasing hormone *(Crh;* Figure 5B*),* and downregulated neurotrophic receptor tyrosine kinase 2 (*Ntrk2;* Figure 2.7C*),* and microtubule-associated protein 1a *(MAP1a;* Figure 2.7D*)* gene expression in male (SSSS) offspring. Interestingly, miR-26 is an up-stream regulator of *Ntrk2* and Map1a expression (Becks et al., 2016; https://mpd.bioinf.uni-sb.de).

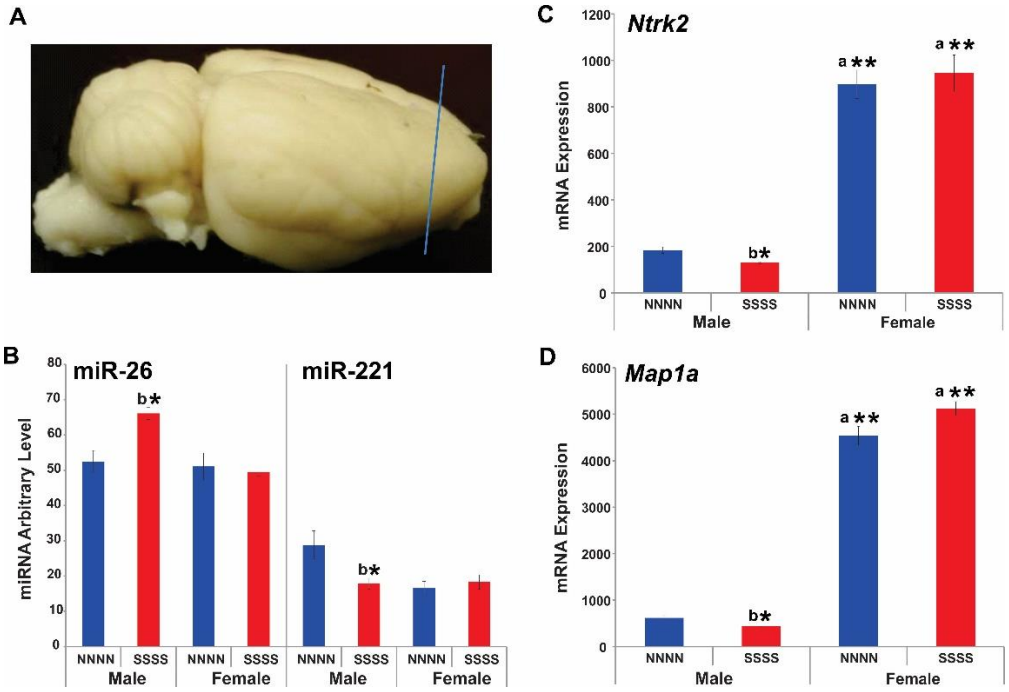

Figure 2.7. MiRNA and mRNA expression in the frontal cortex. Multigenerational stress altered epigenetic regulation of miR-221 and miR-26 and mRNA (Ntrk2, Map1a) in a sex-specific manner. (A) Location of frontal cortex tissue sample used for deep sequencing. (B) Multigenerational stress downregulated miR-221expression and upregulated miR-26 expression in males. (C) Multigenerational stress significantly decreased the Ntrk2 mRNA expression in males, while a slight upregulation was observed

in female rats. Females showed four times higher expression of Ntrk2 than males. D) Multigenerational stress downregulated Map1a expression in males. Asterisks indicate significances: *P < 0.05, **P<0.01. All data are presented as mean ± SEM. “a” indicates sex effect; “b” indicates stress effect.

2.5 Discussion

The present findings support the notion that ancestral stress may program mental health outcomes via epigenetic regulation of neuroendocrine activity and neuronal spine density in males. Here we provide four main supportive arguments. First, both transgenerational and multigenerational ancestral stressors were characterized by an altered affective state in early adulthood. However, multigenerational stress exceeded the effects of transgenerational stress by increasing anxiety-like behaviours in older adult male but not female rats. Second, multigenerational stress altered stress responsiveness, and reduced corticosterone levels in males only. Third, multigenerational stress-related functional and physiological changes were accompanied by changes to dendritic branching and spine density of the prefrontal brain regions (mPFC and AID). Multigenerational stress reduced spine density in the mPFC but increased it in the AID in both male and female offspring. Fourth, deep sequencing revealed that stress altered miR-221, miR-26 and mRNA expression of some of their target genes (*Crh, Ntrk2, and Map1a*) in frontal cortices. The latter findings indicate that epigenetic regulation may be causally related to behavioural, neuroendocrine and morphological pathophysiologies of mental health (Figure 2.8).

The present observations corroborate the idea that adverse early life experiences, such as prenatal stress, are major determinants of brain development and mental health. Human studies have demonstrated that children exposed to prenatal stress are more vulnerable to emotional disturbances in adolescence and adulthood than non-exposed

children (Glover, 2011). Similarly, rodent studies revealed that exposure to prenatal or chronic postnatal stress raises levels of anxiety-like behaviours, such as spending less time than controls in the open arms of the EPM (Darnaudéry & Maccari, 2008) and enhanced freezing (Wilber et al., 2011).

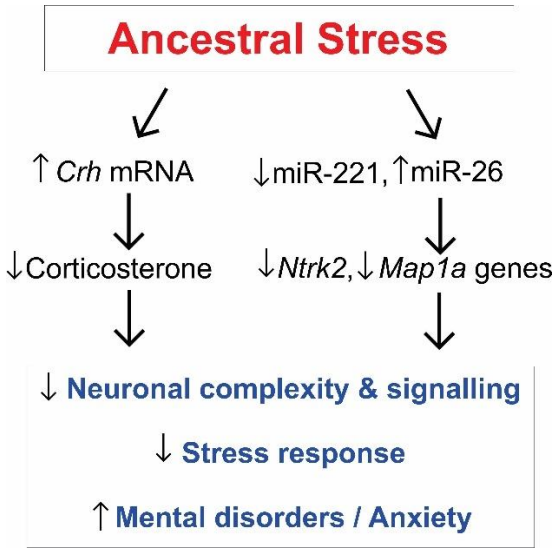

Figure 2.8. Diagram illustrating potential epigenetic mechanisms by which ancestral stress may regulate neuromorphology and mental health. In pathway #1 ancestral stress alters expression of miR-221 and miR-26. Upregulated mir-26 results in downregulated Ntrk2 and Map1a expression which ultimately reduces neuronal complexity and adaptive stress response. In pathway #2 ancestral stress alters Crh mRNA expression in frontal cortex that leads to HPA axis impairment with blunted corticosterone levels, as found in PTSD. This ultimately alters neuronal morphology and stress system as hallmarks of mental health.

Recent experimental and clinical studies have demonstrated that exposure to adverse environments early in life may propagate across generations to alter emotionality in the unexposed offspring (Harper, 2005; Kim et al., 2009; Kiss et al., 2016). Here we show that exposure of mothers, grandmothers and even great-grandmothers to stress during pregnancy is transmitted to their indirectly (transgenerational) or directly (multigenerational) exposed offspring in terms of phenotype traits. Specifically, both trans- and multigenerational stress increased anxiety-like behaviours in F3 and F4 generation

male offspring only. Similarly, Franklin et al, (Franklin et al., 2010) showed that exposing great-grandfathers to unpredictable postnatal maternal separation increased emotionality in F3 transgenerationally stressed male and F2 female mice. Moreover, Dias and Ressler, (2014) reported that F1 and F2 generation offspring whose fathers and grandfathers were subjected to odor fear conditioning during adult life exhibited increased sensitivity to the same odor, even if they never experienced it themselves. The present findings add and expand on these observations by suggesting that both transgenerational and multigenerational ancestral stress can affect the affective state of adult offspring in a sexually dimorphic manner. In addition, recurrent prenatal stress in the multigenerationally stress lineage seems to exacerbate the effects of transgenerational stress. As emotionality is a prominent behavioural trait with evolutionary importance, ancestral transmission of stress to future progeny may serve as a way to build resilience (Faraji et al., 2017), and improve fitness and survival via epigenetic memory.

Our results indicate that each additional generation of gestational stress via maternal lineage incrementally exacerbated anxiety-like behaviours, with the F4 multigenerationally stressed male offspring experiencing the largest change. Our previous animal cohorts support vulnerability of multigenerationally stressed lineages to anxiety-like states (Kiss et al., 2016) and hyperactivity (Erickson et al., 2014) in adult F4 males. The compounding effects of prenatal stress across generations seem to be very profound and sex-specific. For example, our earlier study reported impairments in skilled reaching movements in male and improvements in female multigenerationally stressed F4 rats (Ambeskovic et al., 2017). Multigenerationally stressed F4 females commonly display higher stress resiliency unless faced by another challenge (Faraji et al., 2017) suggesting a dysfunctional stress response

system as the core issue (McCreary et al., 2016; Yao et al., 2014). It is well established that ancestral stress elevates hypothalamic-pituitary adrenal axis activity due to impaired negative feedback loop (Koehl et al., 1999) permanently altering brain morphology and behaviour (Ambeskovic et al., 2017; Franklin et al., 2010; Gapp et al., 2014; Long et al., 2013).

Here we report changes in the anxiety-like behavior and the stress responsiveness via corticosterone levels and *Crh* expression observed in the multigenerationally stressed males were accompanied by the dendritic reorganization of functionally meaningful areas, the medial prefrontal cortex (mPFC) and the orbital frontal cortex (OFC). Multigenerational stress increased dendritic spine density in both right and left OFC hemisphere in female and only in left OFC in male offspring. On contrary, stress-induced decrease in dendritic spine density of the basilar field mPFC in both sexes. Earlier findings showed a decrease in dendritic spine density in the parietal cortex of multigenerationally stressed offspring (Ambeskovic et al., 2017). Accordingly, prenatal and chronic stress can have lasting effects on the neuromorphology of the prefrontal cortex (Bloss et al., 2011; Muhammad & Kolb, 2011). Mychasiuk et al. (2011) found that prenatal stress decreased dendritic length in the OFC in juvenile male and female rats but had no effects on the mPFC. Moreover, prenatal stress increased spine number in mPFC and decreased it in OFC in males, while the opposite was observed in females (Mychasiuk et al., 2012). Prenatal stress resulted in decrease in cortical spine density in the OFC in both males and females (Muhammad et al., 2012). These observations indicate that stress particularly programs basilar dendritic spine density which may then reflect in the behavioural phenotype (Bloss et al., 2011) which may be due to vulnerability of thin spines to stress (Cook & Wellman,

2004). Decreased spine density might be related to multigenerational stress altering the functional signaling among cortical regions such as mPFC (Skelin et al., 2015) and reflect homeostatic reaction of the dendritic arbour to stress during early stages of connectivity (Tripodi et al., 2008).

The multigenerational stress across four generations altered stress responsiveness especially in males. Specifically, stress diminished circulating corticosterone levels and upregulated expression of corticotrophin-releasing hormone *(Chr)* gene in the frontal cortex of males, while corticosterone levels and *Chr* expression were not affected in females. Accordingly, McCreary and colleagues (McCreary et al., 2016) demonstrated that multigenerational stress incrementally elevated corticosterone levels in young adult female (F1-F3) offspring. In a sheep study where pregnant mothers were injected with synthetic glucocorticoids during pregnancy, Long and colleagues (Long et al., 2013) reported increased levels of cortisol and adrenocorticotropic hormone in the F1 and F2 generation female offspring (Long et al., 2013). Similarly, a synthetic glucocorticoid injection to pregnant guinea pigs altered concentrations of cortisol, corticotropic releasing hormone (CRH) and hippocampal glucocorticoid receptor expression in F2 male and female offspring (Iqbal et al., 2012). These changes may functionally explain phenotypic differences across ages, sexes and generations, with males generally being more susceptible (Ambeskovic et al., 2017; McCreary et al., 2016; Welberg & Seckl, 2008; Wilber et al., 2011).

Epigenetic regulation of the *Crh* gene may serve as a mechanism of ancestral programming of HPA axis activity. CRH plays a prominent role in HPA axis regulation and downstream adrenal corticotropin-releasing hormone (ACTH; Bowman et al., 2004;

Butler et al., 2016; Gai et al., 2016). *Crh* gene expression in both the frontal cortex and amygdala can promote anxiety-like behaviours (Lee & Davis, 1997). In turn, microinjecting a CRH antagonist (Samaco et al., 2012) or knocking out the hypothalamic *Crh* gene (Zhang et al., 2017) suppress anxiety-like behaviour.

miRNAs have been classified epigenetic modulators as they affect the protein levels of the target genes without modifying their DNA sequence. Moreover, miRNAs may be involved in upstream regulation of stress response. We showed here that multigenerational stress downregulated expression of miR-221 and upregulated expression of miR-26 in males, while no significant changes were observed in females. Mir-221 is found in distal axons where it targets mRNAs involved in neuronal communication and differentiation, neurite outgrowth and neurogenesis pathways (Hamada et al., 2012; Loohuis et al., 2012). Upregulation of miR-221 facilitates formation of neurite networks and synapses (Gu et al., 2015; Hamada et al., 2012). Similarly, miR-26 is enriched within neuronal dendrites and spines in the forebrain where it regulates synaptic plasticity associated with long-term potentiation (LTP) (Gu et al., 2015; Loohuis et al., 2012; Shi, 2015). Reduced miR-26 expression is required for LTP maintenance, spine formation and enlargement (Gu et al., 2015). Consistent with the present findings studies have also found that stress downregulates miR-221 (Hollins & Cairns, 2016a) and upregulates miR-26 (Eipper-Mains et al., 2011; Rinaldi et al., 2010). Moreover, adverse experience such as exposure to cocaine also downregulates miR-221 expression in striatum but this change was reversed by enriched environment (Emoto, 2011). Importantly, miR-26 targets genes such as neurotrophic receptor tyrosine kinase 2 (*Ntrk2*), and microtubule associated protein 1a

(*Map1a*), further regulating neuronal growth, maintenance and communication (Backes et al., 2017; Hamada et al., 2012; Loohuis et al., 2012; Shi, 2015).

Multigenerational stress previously downregulated the expression of neurotropic tyrosine kinase receptor 2 (*Ntrk2*), and microtubule-associated protein 1a (*Map1a*) in male offspring only. The *Ntrk2* or *TrkB* receptor for BDNF is critical in mediating activity-dependent synaptic plasticity and LTP (Eipper-Mains et al., 2011; Shi, 2015). Downregulation of *Ntrk2* expression may decrease BDNF activity thus diminishing chances to generate LTP and synaptic plasticity. Similarly, downregulation of Map1a gene would result in reduced LTP, as it is involved in functional maintenance and LTP related plasticity in mature neurons (Emoto, 2011). Moreover, reduced expression of *Map1a* may result in remodeling of dendritic arbours and reduced density of active spines and synaptic surface density (Emoto, 2011; Hollins & Cairns, 2016; Szebenyi et al., 2005). Thus, altered regulation of the target genes *Ntrk2, Map1a* and miR-221 and miR-26 may explain the sexually dimorphic neuromorphological findings and functional consequences on behaviour. Moreover, change in *Ntrk2* expression is believed to be involved in synaptic plasticity and mental processes underlying psychopathology. Indeed, multiple studies have demonstrated association between *Ntrk2* and psychiatric disorders (Deo et al., 2013; Ernst et al., 2011; Gupta et al., 2013). Previous findings have linked abnormal *Ntrk2* expression to higher PTSD risk and suggested that it serves as one of the predictive biomarkers for risk for developing PTSD following trauma (Kiss et al., 2016) or as predictor of therapeutic response (Bremer et al., 2007). The present findings in rats show certain parallels to human PTSD, including abnormal HPA axis regulation and blunted stress response, and suggest that in the absence of DNA sequence variations epigenetic mechanisms may contribute to

PTSD risk. Multigenerational stress across four generations affected anxiety-like behaviours, neuronal re-organization and HPA axis activity likely via epigenetic regulation in a sex-specific manner. Sex specific effects were reported previously by showing region- and sex-specific changes in dendritic organization and spine density (Ambeskovic et al., 2017; Murmu et al., 2006; Mychasiuk et al., 2012), including decreased length and spine density in the mPFC (Muhammad et al., 2012). Prenatal stress can induce anxiety-like behaviours especially in male rats in association with HPA axis dysfunction (Bowman et al., 2004; Welberg & Seckl, 2008). Mechanisms for these sexual dimorphisms may include interaction between *Crh* with estrogen and its receptors (ER alpha and ER beta) (Vamvakopoulos & Chrousos, 1993). Because estrogen receptors are found in various brain regions, the *Crh* gene has been shown to be important target of these steroids and potential mediator of sexually dimorphic stress response (Vamvakopoulos & Chrousos, 1993). Mueller and Bale, (Mueller & Bale, 2008) showed that estradiol regulates *Crh* expression via ER alpha and ER beta pathways. Transgenerational stress can epigenetically promote dysmasculinization and upregulation of ER alpha and ER beta receptors in male mice (Mueller & Bale, 2008). Moreover, estrogen receptors regulate both miR-221 (Di Leva et al., 2010) and miR-26 expression (Yang & Wang, 2011). For example, ER-alpha can directly bind to the promotor region of miR-221 to suppress its expression (Di Leva et al., 2010). Thus, transgenerational programming of steroid hormone actions may provide further insights into sex-specific emotional resilience or sensitivity to stress.

In conclusion present findings are the first to show that the prenatal stress phenotype is epigenetically transmitted and accumulated across generations to resemble features of PTSD as indicated by altered Ntrk2 expression, abnormal HPA axis regulation and blunted

stress response, especially in males. Downstream regulation of *Ntrk2* and *Map1a* by miR-26 resulted in impaired neuronal connectivity and increased incidence of symptoms associated with mental illness. We propose that epigenetic mechanisms such as miR-221 and miR-26 may be regulating the male phenotype and anxiety-like pathology and serve as potential biomarkers of stress vulnerability and mental illness. Furthermore, the data suggest that epigenetic mechanisms, in the absence of DNA sequence variations, contribute to PTSD risk and other adverse health outcomes.

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CHAPTER 3: Epigenetic Programming by Multigenerational Prenatal Stress Determines Age-Related Health Trajectories in Sex-Specific Manner

3.1 Abstract

Biological age is indicated by the rate of mental and physical health decline. Early life stress is among the most significant risk factors of premature aging and higher disease risk. Prenatal stress not only affects the lifetime health trajectory, but also propagates across multiple generations to compromise the chances of healthy aging in the unexposed offspring. Here we investigated if multigenerational prenatal stress (MPS) affects age-dependent profiles of physical and mental health, stress response, and epigenetic regulation by microRNA (miRNA) expression. The fourth (F4) generation male and female MPS offspring whose great-grandmothers, grandmothers, mothers and themselves were exposed to prenatal stress gestational days 12-18 were tested across the lifespan at 6 (young), 12 (middle-aged) and 18 (aged) months in multiple behavioural and physiological tests. The findings indicate that aging increases the incidence of anxiety- and depression-like behaviours and impairs sensorimotor behaviours in a sex-specific manner. MPS further exacerbated emotional and physiological impairments and increased incidence of disease such as kidney failure, inflammatory disease and tumours in a sex-specific manner. Unbiased deep sequencing revealed that MPS altered cortical synaptic plasticity and stress regulators miR-34a, miR-124 and miR-129, DNA methylation regulators miR-29b, immune function B and T cell-regulators miR-150-5p and miR-181a, and the senescence biomarker miR-21. These findings suggest that programming by MPS is a significant determinant of lifetime mental health trajectories, physical wellbeing and risk of common age-related diseases through altered epigenetic regulation. Disease incidence may be

regulated by sex-specific pathways. miRNAs may play a role in the transmission of generational stress and represent predictive biomarkers of age-related diseases.

3.2 Introduction

The world's aging population is rapidly growing and by 2020 the number of individuals 60 years and older is expected to exceed the number of children and youth (Bureau, 2016; WHO). The sharp increase in aging individuals highlights the urgent need to identify strategies to support healthy aging. Approximately 88% of aged individuals in North America experience dramatic physical and mental health decline (McLaughlin et al., 2010), which mainly results from accumulated cell and DNA damage acquired across the lifespan and through interactions with the environment (Ambeskovic et al., 2017; Bender et al., 2006; Kennedy et al., 2012). Experiences in early life also play a critical role in programming risk of disease and the chances of successful aging.

The developmental origins of health and disease (DOHaD) hypothesis postulates that many common diseases originate in utero, and that exposure to an adverse prenatal environment through maternal stress, infection, endocrine disruptors or malnutrition may re-program fetal physiological and metabolic responses to cause lifelong changes in organ and tissue function (Ambeskovic et al., 2017; Barker et al., 1993; Barker 1989; Barker, 2007; Painter et al., 2008). Offspring exposed to an adverse prenatal environment may show exacerbated hypothalamic-pituitary-adrenal (HPA) axis responsiveness potentially followed by abnormal heart and kidney morphology, increased blood pressure, high cholesterol, insulin sensitivity and impaired mental health trajectories (Bertram et al., 2008; Peixoto-Silva et al., 2011; Weiss et al., 2004; Zambrano et al., 2005). Clinical studies also suggest impaired immune function, increased risk of heart and renal disease, diabetes,

obesity, behavioural problems, anxiety and depression and shorter lifespan (Ashman et al. 2016; de Rooij et al., 2007; Entringer et al., 2008).

The biological signatures linked to adverse early life experiences can be transmitted across generations and influence offspring health trajectories. In fact, ancestral stress such as multigenerational prenatal stress (MPS) was shown to be more potent in programming the stress response than early life stress (McCormick et al., 2017). Intriguingly, cumulative impacts of MPS impair fine motor function, alter neuromorphology and induce hemispheric dominance shift in males (Ambeskovic et al., 2017), while improving fine function and promoting resilience in MPS female (Ambeskovic et al., 2017; Faraji et al., 2017). Moreover, MPS altered locomotor function across the lifespan in males (Erickson et al., 2014). Accordingly, natural disaster and nutritional birth cohorts (Bygren et al., 2001; de Rooij et al., 2006; Painter et al., 2008; Pembrey et al., 2014) and experimental studies (Crews et al., 2012a; Franklin et al., 2010; Gapp et al., 2014; Kiss et al., 2016; McCreary et al., 2016) have demonstrated that ancestral adverse experiences increase the risk of developing metabolic, cardiac and renal disease, and mental illness across multiple generations with a sex-specific bias (Ambeskovic et al., 2017; Anway et al., 2006; Franklin et al., 2010; Mueller & Bale, 2008; Veenendaal et al., 2013). Ancestral biological memories of adverse experiences are generally linked to epigenetic modification, such as DNA methylation, histone modification and microRNA (miRNA) expression (Babenko et al., 2015; Kovalchuk, 2012; Zucchi et al., 2012). The consequences of ancestral stress become particularly visible during development and old age (Erickson et al., 2014), the impact of ancestral stress on physical and mental health decline during aging has not been demonstrated yet, however.

Here, we investigated the impact of cumulative ancestral stress in the fourth (F4) generation on age-dependent physical and mental health trajectories, stress response and epigenetic regulation by miRNA deep sequencing as a function of sex. The findings show that aging and MPS synergistically disturb the stress response and accelerate age-associated decline in health and longevity with sex-specific disease incidence. Thus, programming by multigenerational prenatal stress may be a significant determinant of lifetime physical and mental wellbeing through altered epigenetic regulation by sex-specific pathogenic pathways.

3.3 Methods

3.3.1 Animals

In this study, 85 Long-Evans hooded rats (41 males, 44 females) were used. All animals were bred and raised in-house at the Canadian Centre for Behavioural Neuroscience vivarium. Subjects were housed in groups (males in pairs, females three per cage) under a 12:12 h light/dark cycle with light starting at 07:30 h and the room temperature set at 22 °C. Animals were observed and tested at different ages across the lifespan. To reduce the risk of rapid health decline and disease incidence such as urinary tract infection after midlife (as seen in previous cohorts), all animals were placed on cranberry juice from 13 months of age onward (Fleet, 2009; High & High, 2001). In addition, with increased frailty animals experienced appetite and weight loss, additional rat food powder mixed with Ensure (Abbott) was fed as needed. All procedures were approved by the University of Lethbridge Animal Care Committee in compliance with the guidelines by the Canadian Council on Animal Care.

3.3.2 Experimental Design

This study used F4 generation male and female offspring derived from two lineages bred under standardized conditions: a multigenerational prenatal stress lineage and a non-stress lineage. Prenatal stress consisted of subjecting pregnant dams to semi-random daily 5 min swim and 20 min restraint stress in a Plexiglas cylinder from gestational days 12-18 (Ambeskovic et al., 2017; Erickson et al., 2014; Faraji et al., 2017; Kiss et al., 2016). To generate the multigenerational prenatal stress (F4-MPS) lineage (see Figure 3.1A), pregnant F1 daughters, F2 granddaughters and F3 great-granddaughters were stressed during pregnancy (Yao et al., 2014). A lineage of yoked controls was bred with each generation to generate a lineage of non-stress controls (F4-CONTROL).

The F4 generation offspring were tested at 6, 12 and 18 months of age (Figure 3.1B). One group of offspring was tested at 6 months and euthanized for tissue collection. Another group of animals was tested longitudinally at 12 and 18 months and euthanized at the age of 18 (aged) months, or as recommended by veterinary advice. Males showed generally higher disease incidence and their population declined more rapidly than the female population. The groups comprised the following F4 generation animals: (1) young [males: n=20 (CONTROL=10, MPS=10); females: n=18: (CONTROL=10, MPS=9], (2) middle-aged [males: n=21 (CONTROL=9, MPS=12); females: n=26 (CONTROL=12, MPS=14)] and (3) aged [males: n=13 (CONTROL=6, MPS=7); females: n=18 (CONTROL=10, MPS=8)].

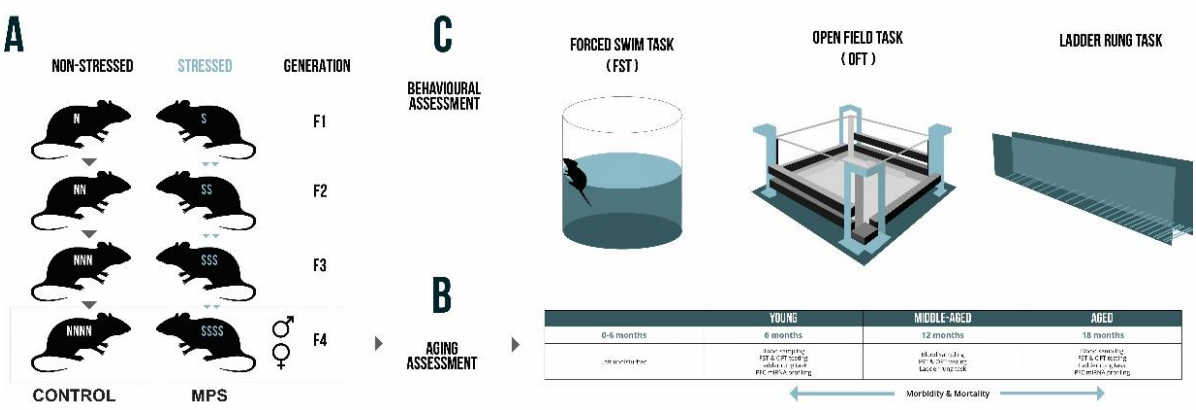

Figure 3.1. Multigenerational prenatal stress paradigm and experimental design. (A) Pregnant dams were either stressed to generate an ancestral stress lineage or left undisturbed to generate non-stressed lineage. MPS was generated by stressing (red arrows) pregnant dams over four consecutive generations (F0, F1, F2, F3) to produce multigenerationally stressed F4 offspring. In parallel non-stress CONTROL offspring were generated. This experiment used the CONTROL and MPS F4 generation. (B) Aging timeline illustrating a mixed longitudinal experiment, where one group of animals was tested at 6 months and tissue collected and a second set of animals was tested at 12 and 18 months of age to record morbidity and mortality. The data acquisition consists of open field (OFT), forced swim task (FST) and the ladder rung walking task. C) Illustrative images of behavioural assessment tasks including OFT, FST and ladder rung apparatus.

Behavioural testing included open field, ladder rung walking and Porsolt swim tasks (see Figure 3.1C) at 6, 12 and 18 months of age (Figure 1B) pursued by an experimenter blind to the experimental conditions. Glucose levels and plasma corticosterone levels were determined at each time point and animals were weighed weekly. Overall health status and disease incidence were noted. A subset of F4 animals was randomly assigned for epigenetic analyses [male n=12 (CONTROL=6, MPS=6); female n=12 (CONTROL=6, MPS=6)] at 6 and 18 months of age (n=3 per time point).

3.3.3 Behavioural and Physiological Testing

3.3.3.1 Exploratory Activity and Anxiety-Like Behaviour

Exploratory activity and anxiety-like behaviours were assessed in an open field task (OFT; Figure 3.1C; Denenberg, 1969; Erickson et al., 2014). Briefly, animals were placed individually into Accuscan (AccuScan Instruments Inc., OH, USA) activity monitoring Plexiglas boxes (length 42 cm, width 42 cm, and height 30 cm) and recorded for 10 min. Horizontal distance travelled (cm) and total time (sec) spend in margins were recorded based on horizontal beam breaks via a computer interface with the VersaMaxTM program and converted to spreadsheets using the VersaDatTM software.

3.3.3.2 Depression-Like Behaviour

Self-helplessness and depression-like behaviours were recorded using the Porsolt swim task (FST; Figure 3.1C; Porsolt et al., 1977). Animals were placed in a cylinder containing warm water (21°C) and their swimming was filmed for 5 min. Analysis included the time spent floating, swimming and climbing.

3.3.3.3 Skilled Walking

Skilled fore- and hind limb placements were assessed by the ladder rung walking task (Figure 3.1C; Metz & Whishaw, 2009). Animals were pre-trained and the next day tested three times at each time point. Video recordings were analyzed for qualitative placement scores [left forelimb (LFL); right forelimb (RFL); left hind limb (LFL) and right hind limb (RHL)].

3.3.3.4 Blood Collection, Corticosterone Analysis and Blood Glucose Level Measurements

Blood samples were obtained three days prior to behavioural testing at the 6, 12 and 18 month time points (Figure 3.1B). On average 0.6 ml of blood was collected from the tail vein during morning hours between 8:00 and 10:00 AM under 4% isoflurane anaesthesia (Faraji et al., 2017; Yao et al., 2014). In addition, blood glucose was measured using an Ascensia Breeze Blood Glucose Meter (Bayer, Toronto, ON, Canada) with test strips. From the remaining blood plasma was obtained by centrifugation at 5,000 rpm for 10 minutes. The samples were stored at -80°C. Plasma corticosterone (CORT) levels were determined by enzyme-linked immunosorbent assays (ELISA) using commercial kits (Cayman Chemical, Ann Arbor, MI, USA).

3.3.3.5 Brain and Organ Collection

Animals were euthanized with an overdose of pentobarbital (Euthansol 100 mg/kg; CDMV Inc., Québec, Canada). The brains were rapidly removed, dissected and flash-frozen in -80°C. Portions of kidney, liver and lungs were also rapidly removed, dissected and flash-frozen in -80°C, while the remaining organs and tissues were saved for disease diagnosis. Post-mortem diagnoses of disease and organ pathologies were identified by the University of Lethbridge veterinarian. The veterinarian was blind to animal identification and treatment group.

3.3.4 MiRNA Deep Sequencing

The TRI Reagent Solution (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from the frontal cortices. miRNA expression analysis was performed by Illumina GAIIx genomic analyzer (Illumina, CA, USA). Briefly, base calling and demultiplexing was completed using CA SAVA 1.8.1 software pipeline with default settings. FastQC software was used to examine short read quality. Adapters were trimmed using cutadapt

software (https://cutadapt.readthedocs.org/; Anders & Huber, 2010). After trimming FastQC quality, the check was performed. Standalone MicroRazerS version 1.0 (Emde, Grunert, Weese, Reinert, & Sperling, 2010) was used to perform miRNA detection and counting. Potential targets of selected miRNA of interest were predicted using the 3′ UTR available for Rat rn5 (UCSC) genome. An algorithm (miRanda v.3.3a; Computational Biology Center of Memorial Sloan-Kettering Cancer Center, NY, USA) was used for miRNA target prediction.

3.3.5 Statistical Analysis

Statistical analysis was performed using SPSS 20 for Windows 11.5.0 (IBM Corporation, Armonk, NY, USA). Three-way ANOVA with sex, stress, and age as factors was run for behavioural tasks (OFT, ladder rung, FST), corticosterone levels, body weight and glucose levels. Two-way ANOVA was run for the overall lifespan in days. Tukey's test was used for all behavioural and physiological post hoc analyses whenever possible. Otherwise, independent sample t-test was run. Survival probability was assessed using Kaplan-Meier survival curves when significant Cox Regression was performed to calculate the hazard ratios (HR). Survival rate at 14-15 months of age was assessed using Fisher's exact test to determine significance. Statistical analysis of morbidity and mortality and specific disease incidence in the MPS vs. Control F4 generation were performed by Fisher's exact test analysis. Also, relative risk (RR) and confidence intervals (CI) were calculated for each disease (with MPS vs CONTROL comparison) using standard epidemiology equations. For miRNA analysis, raw count data was first normalized and regularized with log transformation using statistical routines implemented in the DESeq2 bioconductor package (Anders & Huber, 2010) as described in the DESeq2 user manual. Then,

normalized relative miRNA expression values were analysed by two-way ANOVA with age and treatment as independent factors, separately for male and female rats. When significant, independent sample t-test was conducted to examine specific group differences. Small RNAs with false discovery rate adjusted p-values <0.1 were considered differentially expressed. Results are shown as the means ± standard error of the mean (±SEM). Asterisks indicate significances: ***p<0.001, **p<0.01, *p<0.05. Letters represent a-age, b-stress, and c-sex differences.

3.4 Results

3.4.1 Physical Health Outcomes

MPS and aging synergistically altered exploratory activity in the open field. Exploratory activity across all ages revealed sexually dimorphic characteristics. A three-way ANOVA revealed main effects of AGE ($F(2,102)=54$, $p=0.001$), SEX ($F(2,102)=132$, $p=0.000$), STRESS ($F(2,102)=6.43$, $p=0.013$) and AGE x SEX interaction ($F(2,102)=7.7$, $p=0.001$) in OFT exploratory activity. Aging reduced locomotor activity, as aged rats on average travelled significantly shorter distances than young ($p<0.001$) or middle-aged ($p<0.05$; Figures 3.2A, 3.2B) rats. Moreover, females were twice as active as males and consistently travelled about twice as far as males (Figure 3.2A). MPS animals across all ages travelled longer distances than age-matched non-stressed animals (Figure 3.2A, 3.2B). In particular, significantly longer distance travelled was observed in young MPS ($p<0.05$, Figure 3.2B) and aged MPS females ($p<0.05$; Figure 3.1B), while no significant effects of MPS were found in males (Figure 3.2A). Thus, MPS showed a sexually dimorphic exploratory profiles, and females were more physically active than males across the lifespan.

3.4.1.2 MPS Exacerbated Age-Associated Impairments in Skilled Walking in Males but not in Females

MPS and aging altered both forelimb (FL) and hind limb (HL) paw placement in the ladder rung walking task with sex-specific outcomes. A three-way ANOVA revealed a main effect of SEX, as females had significantly more precise forelimb [average FL: $F(2,125)=27.5$, $p<0.0001$] and hind limb [average HL: $F(2,125)=24.3$, $p<0.001$] placement than males. A main effect of AGE was found for forelimb [$F(2,125)=10.4$, $p<0.001$; Figures 3.2C, 3.2D] and hind limb [$F(2,125)=5.31$, $p<0.01$; Figures 3.2E, 3.2F] placement, as younger animals had the highest limb (FL and HL) placement score, and this score decreased with age in males. By contrast, in females, the limb placement score was the lowest in middle-aged animals compared to older ages. In addition, FL limb placement scores were significantly diminished in MPS groups ($F(2,125)=3.69$, $p<0.05$; Figures 3.2C, 3.2D) with no changes in HL placement ($p>0.05$; Figures 3.2E, 3.2F). An interesting AGE x SEX interaction was observed for both forelimbs ($F(2,125)=7.17$, $p<0.001$; Figures 3.2C, 3.2D) and hind limbs ($F(2,125)=7.60$, $p<0.001$; Figures 3.2E, 3.2F) as females had higher placement scores than males at young and old age, while males had higher limb placement score at middle-age.

Importantly, MPS altered limb placement and motor coordination in the ladder rung walking task differently in males and females, and these effects were age-dependent. MPS animals had higher forelimb placement scores in males at 6 (young, $p<0.05$) and decreased at 12 months of age (middle-age) ($p<0.05$; Figures 3.2C, 3.2D), while no changes were observed in females. Hind limb placement was impaired in males across all ages, and most severely in aged males ($p<0.05$), while in females MPS non-significantly reduced limb placement in middle and aged animals.

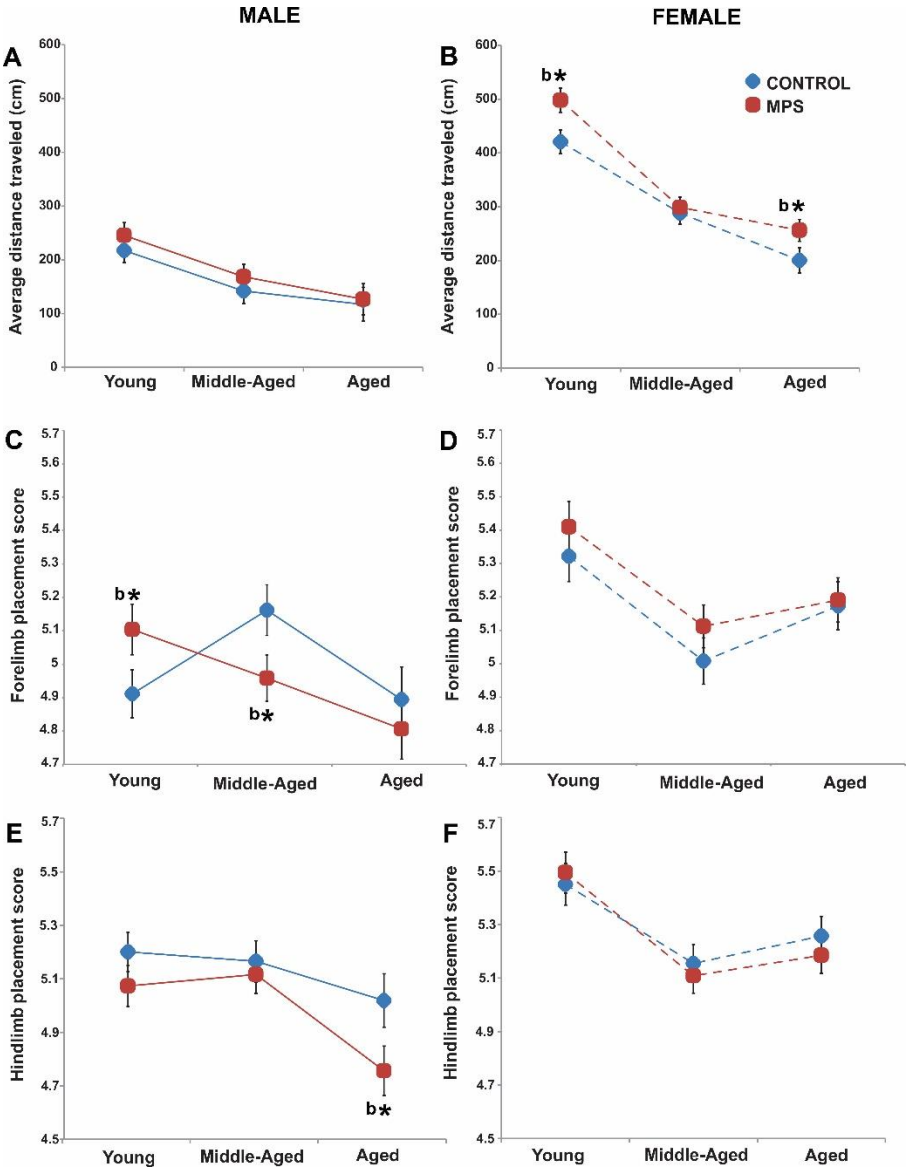

Figure 3.2. MPS in the F4 generation modifies locomotor activity and skilled walking across the lifespan.

Physical health indicators were measured by overall locomotor activity in the open field, and by forelimb and hind limb placement scores while crossing the ladder rung walking apparatus. (A & B) MPS increased the exploratory locomotor activity in young and aged females. Although females were overall more active than males, age decreased the overall locomotor activity across the lifespan in both sexes. (C & D) MPS induced sex- and age-specific effects on forelimb placement in skilled walking across the lifespan. Stress impaired limb placement in middle-aged males, and improved it in young. (E & D) MPS exacerbated age-associated impairments in hindlimb placement in males (E), while it had no effects in females (D). Asterisks indicate significances: *P<0.05. All data are presented as mean ± SEM. "a" indicates age effects, "b" indicates MPS effect, and "c" indicates sex effects.

3.4.2 Mental Health Outcomes

3.4.2.1 MPS Induced Sex-Specific Vulnerability to Age-Associated Depressive-Like Behaviours

MPS induced loss of motivation as a symptom of depression-like behaviours after midlife in males, while it provided resiliency in middle-aged females. A three-way ANOVA revealed a main effect of AGE ($F(2,108)=29.8$, $p<0.001$), SEX ($F(2,108)=16.45$, $p<0.001$), AGE x SEX interaction ($F(2,108)=3.7$, $p<0.05$) and SEX x STRESS interaction ($F(2,108)=4.51$, $p<0.05$). Aging promoted depressive-like behaviours as indicated by less time spent climbing in the FST (Figures 3.3A, 3.3B). Tukey's Post-Hoc test revealed significant differences between young and middle-aged ($p<0.01$) and middle-aged and aged ($p<0.001$) rats. Moreover, males were more motivated and spent more time climbing than females (Figures 3.3A, 3.3B). Although not significant, stressed rats spent slightly less time climbing than their non-stressed counterparts.

Overall, stress exerted sex-specific effects as it decreased depressive-like behaviours in females and increased it in males, an effect that varied across aging trajectories (Figures 3.3A, 3.3B). MPS reduced climb time in aged male rats ($p<0.01$; Figure 3.3A). Thus, MPS had the most impact on emotional wellbeing after midlife in males, while MPS females mainly showed resilience.

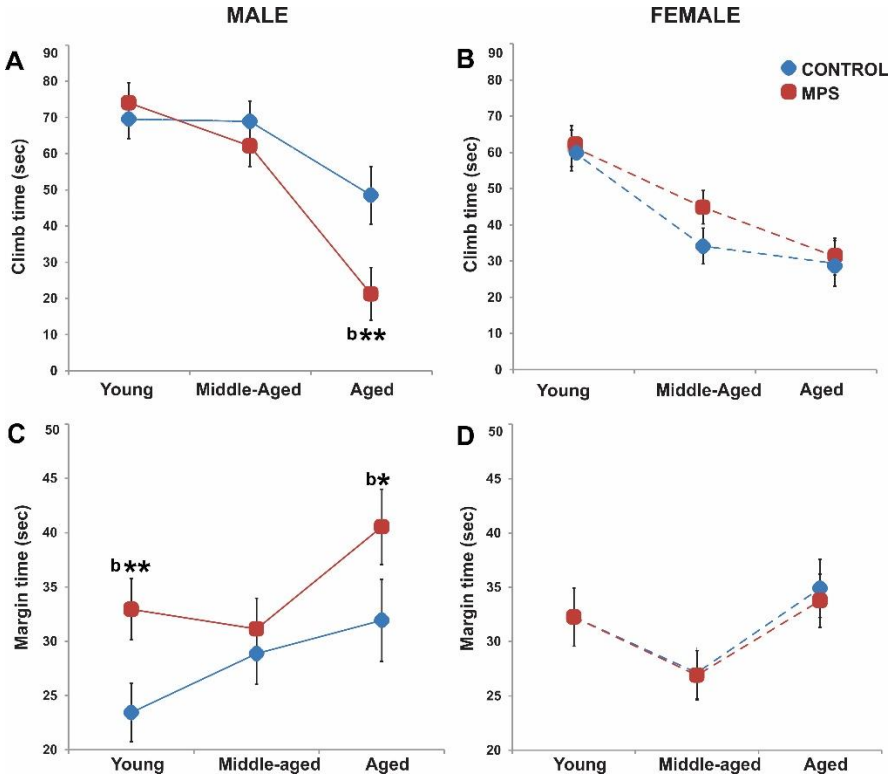

Figure 3.3. MPS in the F4 generation shapes mental health outcomes across the lifespan.

Mental health was measured by the time spent climbing in the forced swim task as an indication of depressive-like behaviours and time spent in the margins of the open field as an indication of anxiety-like behaviours. (A & B) MPS exacerbated an age-associated increase in depressive-like behaviours in males, especially in old age, while only slightly decreasing depressive-like behaviours in females as indicated by more time spent climbing. (C & D) MPS heightened age-related increases in anxiety-like behaviours in young and aged males (C), while it had no effect in females (D). All data are presented as mean ± SEM. "a" indicates age effects, "b" indicates MPS effect, and "c" indicates sex effects.

3.4.2.2 MPS Induced Anxiety-Like Behaviours in Young and Aged Males

Stress increased arousal as a symptom of anxiety-like behaviours, in males as a function of age. A three-way ANOVA revealed a main effect of AGE ($F(2,116)=5.37$, $p<0.01$), STRESS ($F(2,116)=3.8$, $p<0.05$) and a significant SEX x STRESS interaction ($F(2,116)=4.9$, $p<0.05$). Aged animals spent significantly more time in open field margins than the young ($p<0.001$) or middle-aged ($p<0.05$), while no differences were observed

between young and middle-aged male and female rats ($p>0.05$; Figures 3.3C, 3.3D). MPS animals spent more time in margins than CONTROL counterparts with a larger stress effect observed in males than in females. Independent sample t-test demonstrated that stressed males spent more time in margins at 6 months ($p<0.01$; Figure 3.3C) and 18 months ($p<0.05$) than CONTROL rats. On the contrary, time spent in margin remained the same in females, with only a slight decrease in aged MPS females ($p>0.05$; Figure 3.3D). Thus, MPS altered life trajectories of anxiety-like behaviours in males with larger emotional changes early and later in life while females were less affected.

3.4.3 Physiological Health Outcomes

3.4.3.1 MPS Induced Sex- and Age-Specific Alterations in Endocrine Stress Response

A three-way ANOVA revealed a main effect of AGE ($F(2,104)=50.3$, $p<0.001$), STRESS ($F(2,104)=7.18$, $p<0.01$), but no effects of SEX on plasma corticosterone levels. The highest levels were observed in middle-aged animals and the lowest in aged animals. Moreover, MPS reduced basal corticosterone levels compared to CONTROL animals. A significant interaction between AGE x SEX ($F(2,104)=4.21$, $p<0.05$), SEX x STRESS ($F(2,104)=4.5$, $p<0.05$) and AGE x SEX x STRESS ($F(2,104)=6.7$, $p<0.05$) indicates that MPS affected males and females differently depending on age. MPS reduced circulating corticosterone levels in aged male rats ($p<0.01$; Figure 3.4A) while MPS females showed significantly reduced corticosterone levels at middle age ($p<0.01$; Figure 3.4B).

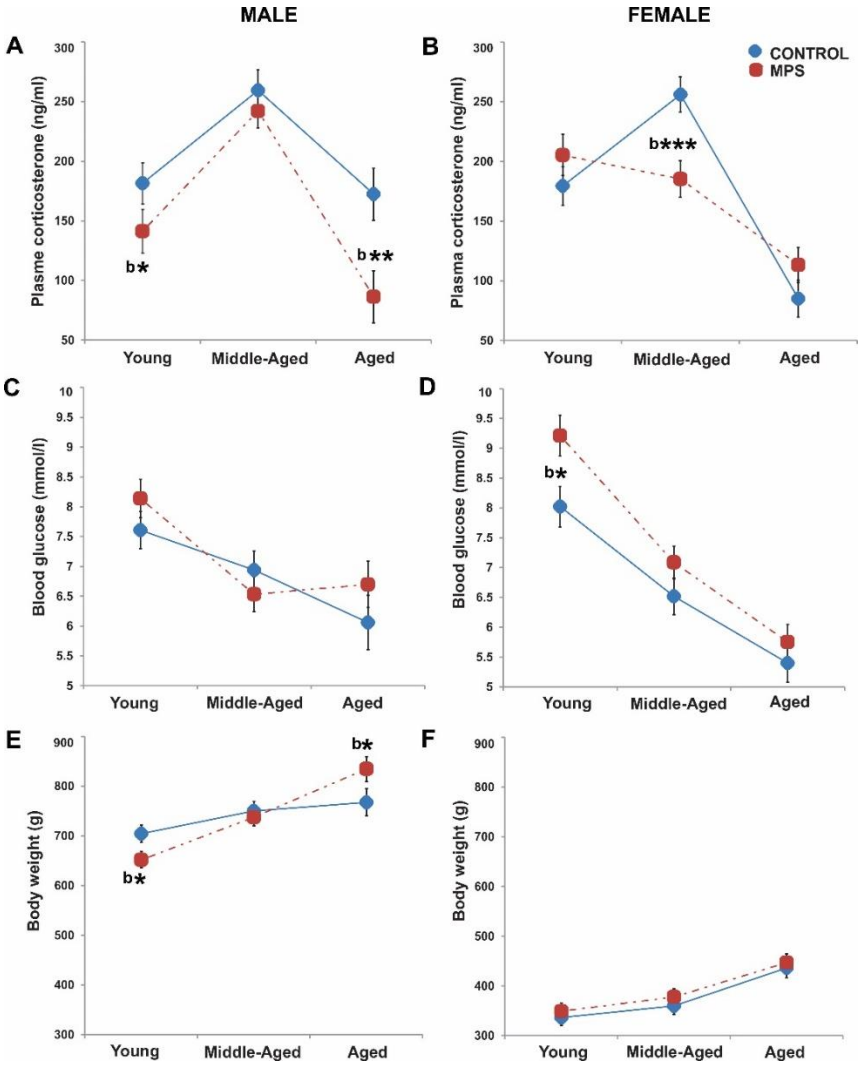

Figure 3.4. MPS in the F4 generation determined physiological health across the lifespan.

Physiological health was measured by plasma corticosterone levels, blood glucose levels and body weight across the lifespan in male and female rats. (A & B) MPS induced sex- and age-specific effects on the stress response as indicated by reduced plasma corticosterone levels in young and aged males (A), and middle-aged females (B). (C & D) MPS elevated non-fasting blood glucose levels especially in young females. (E & F) Ancestral stress decreased body weight in young and increased it in old males (E), while it had no effect on females (F). Aging increased the body weight in both males and females, while males weighed twice as much as females. All data are presented as mean ± SEM. "a" indicates age effects, "b" indicates MPS effect, and "c" indicates sex effects.

3.4.3.2 MPS Reversed Age-Associated Effects on Circulating Blood Glucose Levels

A three-way ANOVA revealed a main effect of AGE ($F(2,108)=44.9$, $p<0.001$), STRESS ($F(2,108)=6.13$, $p<0.05$), and a SEX x AGE interaction ($F(2,108)=4.87$, $p<0.01$). Aging decreased circulating blood glucose levels, as young animals had significantly higher glucose levels than middle-aged and aged animals ($p<0.001$; Figures 3.4C, 3.4D). Notably, MPS animals had higher glucose levels than CONTROL ones (Figures 3.4C, 3.4D) across males and females and all ages except for middle-aged males. MPS females had higher glucose levels across all ages than CONTROL females, and an independent t-test revealed a significant increase in young ($p<0.01$; Figure 3.4D).

3.4.3.3 MPS Showed Sex-and Age-Specific Growth in Body Weight

A three-way ANOVA revealed a main effect of AGE ($F(2,135)=38.9$, $p<0.001$), and SEX ($F(2,135)=12.94$, $p<0.001$), but no effect of STRESS. Aging on average increased body weight by about 50 g, a growth that was significant across ages ($p<0.001$, Tukey post hoc test; Figures 3.4E, 3.4F). Females on average weighed less than male rats (Figure 3.4E and 3.4F).

Interestingly, MPS males had lower body weight at 6 ($p<0.05$) and 12 months of age ($p>0.05$; Figure 3.4E) than CONTROL males. However, at 18 months of age, MPS males experienced substantial body weight gain ($p<0.05$), so much so that it surpassed the body weight of CONTROL rats. Although non-significant ($p>0.05$; Figure 3.4F), MPS females on average weighed more than their CONTROL counterparts across the lifespan.

3.4.5. Mortality and Disease Incidence

3.4.5.1 MPS Generated Sex-Specific Midlife Mortality and Lifetime Survival Probability

MPS animals showed sex-specific survival probability in midlife. MPS males had heightened premature mortality compared to any other group. The observational and qualitative data demonstrate that MPS males were 33% more likely to die by the age of 14 months as opposed to only 7% of MPS females. However, this effect was non-significant according to Fisher's Exact Test ($p=0.1$), Phi co-efficient (0.90) and Kaplan Meiere and Cox-regression possibly due to small sample size (using 14 months as an endpoint; Figure 3.5A). Post-mortem examination revealed that the premature mortality in middle-aged (12-14.5-month-old) MPS males was linked to multiple pathologies. MPS males (n=12) showed multiple morbidities such as renal failure (2/12) (Figure 3.5B), heart disease (1/12), respiratory disease (1/12) or tumors (1/12), whereas among CONTROL males (n=10) the premature death was induced by sudden heart attack (1/10) or respiratory disease (1/10; Figure 3.5B). In females (n=28) 100% of premature deaths were due to renal failure, afflicting one MPS (1/14) and two CONTROL females (2/14).

MPS did not have a significant effect on the overall survival probability in males nor females when compared to CONTROL animals across the 18-month lifespan. The Kaplan Meiere and Cox regression tests ($p<0.05$; HR=0.33) revealed that the probability of dying was 67% lower in females than males over the 18-month time course. MPS males had a higher likelihood of dying in middle age (14 months) (33%; 4/12) than CONTROL males (20%; 2/10). At 14 months of age, only 67% (n=8/12) of MPS males and 80% (n=8/10; Figure 5A) of CONTROL males were still alive. By old age around 17-18 months (530 days) only 50 % of MPS and 50% of CONTROL animals were still alive (Figure 3.5A). MPS had only slight effects on female mortality at middle age (alive CONTROL, n=12/14; MPS, n=13/14) and somewhat positive effects in the later age of 18 months (alive CONTROL, n=10/14; MPS, n=13/14). Importantly, increased incidence of multiple

diseases in MPS animals during midlife in comparison to incidence of two specific diseases in CONTROLS (one renal failure and one heart disease) indicate the magnitude and unpredictability of ancestral stress effects.

When lifespan data were analyzed for the total number of days alive, a two-way ANOVA revealed a main effect of SEX ($F(1,46)=5.7$, $p<0.05$ (Figure 3.5A, 3.5C) as females overall had a longer lifespan than males. No STRESS effects were observed. Interestingly, t-test revealed no difference ($p>0.05$) between CONTROL male and females. MPS males and MPS females were significantly different ($t(26)=-2.8$, $p<0.01$) indicating sex-specific programming by stress (Figure 5C). Compared to MPS males (n=5, at the end of experiment), while 13 MPS females lived to reach the experimental endpoint of 530 days. Thus, not sex differences but MPS accounted for shorter lifespan in MPS males.

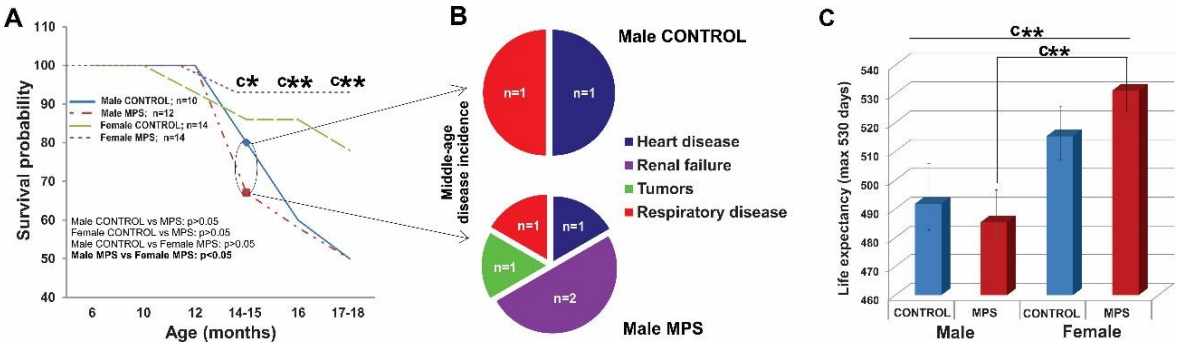

Figure 3.5. MPS altered survival probability, midlife disease incidence and overall longevity.
(A) MPS male offspring were more likely to die prematurely at (14-15 months) than CONTROL males or MPS and CONTROL females. Although the risk of premature mortality in midlife was non-significant in males, the data shows a relevant trend. (B) Midlife premature death in MPS males was linked to various diseases such as renal failure, heart and respiratory disease and tumors, while CONTROL animals (n=2) died from either renal failure or heart disease in midlife. Moreover, premature mortality in male and female animals showed opposing effects. C) Overall life expectancy with 530 days experimental endpoint. MPS raised life expectancy in females but lowered it in males induces. Although MPS did not induce significant changes in life when compared to CONTROLS, the rates were significantly different when compared to each other.

3.4.6 MPS Increased Risk of Inflammatory, Renal and Respiratory Disease

Relative risk (RR) analysis demonstrated increased risk of inflammatory, renal and respiratory disease in MPS males and a higher risk of respiratory disease and tumours in MPS females. Hence, MPS males were 1.66 times more likely to suffer from inflammatory disease (RR=1.66; CI: 0.85-3.25; Figures 3.6B, 3.6C) and 1.88 times more likely to get renal failure (RR=1.88; CI: 0.82-4.28; Figure 3.6B) than CONTROL animals. The risk of respiratory disease was 2.5 more likely in MPS than in CONTROL males (RR=2.5; CI: 0.305-20.4; Figure 6B). MPS females were 7.14 times more likely to suffer from respiratory illness compared to CONTROL females (RR=7.14; CI: 1.10-49.6; Figures 3.6B, 3.6C). MPS females were also 6.14 times more likely to suffer from tumours than CONTROL females (RR=6.14; CI: 0.825-43.5; Figure 3.6B & 3.6C). Thus, MPS increased the sex-specific risk of disease across the lifespan.

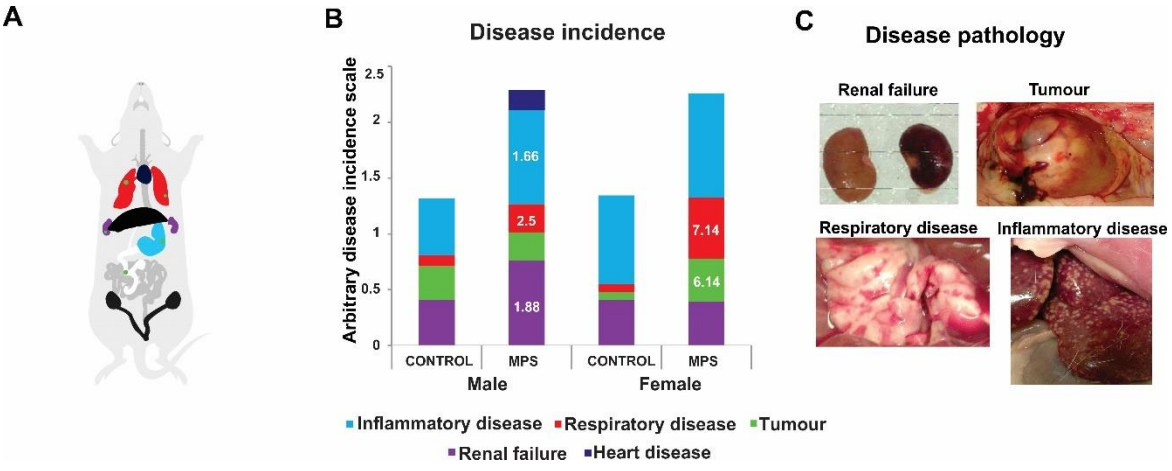

Figure 3.6. MPS altered disease incidence and pathology. A) Diagram illustrating the colour code of pathophysiological changes. B) Disease incidences as represented by respective colours. The relative risk (RR) value found in white letters on the bars of MPS males and females indicate the risk of developing specific disease in relations to CONTROL animals. C) Photographs of disease pathology in MPS animals, illustrating the

kidneys linked to renal failure, abdominal tumour, spotty lungs or diseased lungs and inflamed and enlarged spleen as representation of inflammatory disease.

3.4.7 Epigenetic Regulation

3.4.7.1 MPS Induced Sex- and Age-Specific Epigenetic Programming by miRNA

Here deep sequencing revealed nine miRNAs were differentially expressed in the prefrontal cortex of MPS rats when compared to CONTROLS. Altered miRNAs included: 1) miR-150-5p,miR-181a-5p and miR-181c-5p that regulate immune function through B and T cell regulation; 2) miR-34a-5p, miR-34c-5p, and miR-124-3p that regulate synaptic plasticity and influence mental health; 3) miR-29a-3p and miR-29b-3p that determines DNA methylation; 4) the senescence and aging biomarker miR-21-5p (Figure 3.7).

The male and female relative miRNA expression changes were statistically analysed sperate with AGE and STRESS as independent variables within each analysis. Two-way ANOVA revealed an effect of AGE and STRESS on specific miRNA in both males and females. In CONTROL males, a two-way ANOVA revealed a main effect of AGE, as indicated by upregulated miR-29a ($F_{1,12}=11.9$, $p<0.05$; Figure 3.7A) and miR-21 ($p<0.05$; Figure 3.7A and downregulated miR-150 ($F_{1,12}=6.2$, $p<0.05$; Figure 3.7A) and miR-181c ($F_{1,12}=16.7$, $p<0.01$; Figure 3.7A). In MPS males, aging upregulated expression of miR-34a (near significant, $p=0.06$), and downregulated expression of miR-124 (near significant, $p=0.07$) and miR-181c ($p<0.01$). At any age, MPS upregulated expression of mir-181a ($F_{1,12}=6.7$, $p<0.05$; Figure 3.7A) and miR-181c ($F_{1,12}=5.6$, $p<0.05$; Figure 3.7A) and nearly significantly downregulated expression of miR-124 ($F_{1,12}=3.7$, $p=0.06$; Figure 7A). Independent sample t-test revealed significantly upregulated expression of miR-181a ($p<0.05$) in aged and miR-181c ($p<0.05$) and miR-21 (near significant $p=0.08$) in young MPS males. Lastly, near significant AGE x STRESS

interaction was observed for miR-21 (F(1,12)=3.7, p=0.07), miR-34a (F(1,12)=4.6, p=0.06) and miR-150 (F(1,12)=4.8, p=0.055).

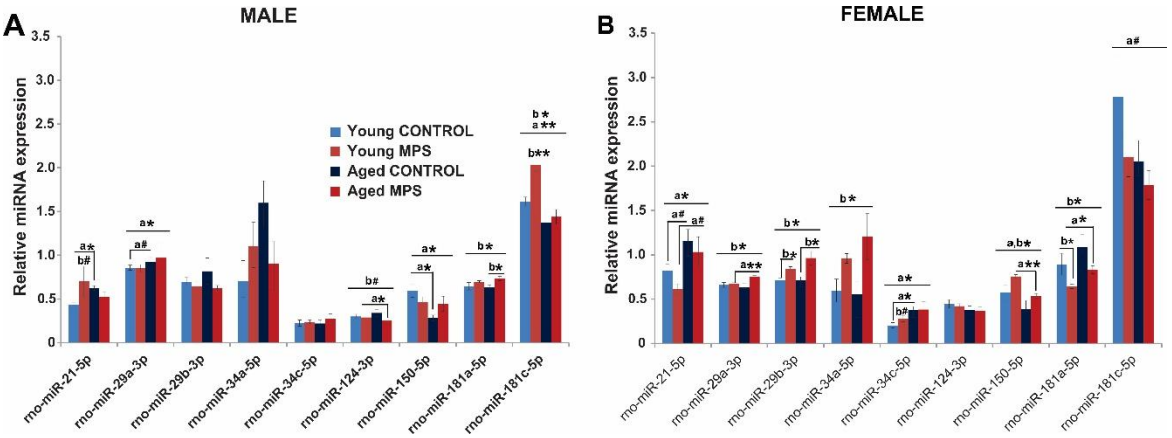

Figure 3.7. MPS altered miRNA expression in the frontal cortex tissue in young and aged rats.
(A & B) MPS and aging altered expression of multiple miRNAs (miR-21-5p, miR-29a-3p, miR-29b-3p, miR-34a-5p, miR-34c-5p, miR-124-3p, miR-129-5p, miR-150-5p, miR-181a-5p and miR-181c-5p) involved in emotional regulation, stress and immune responses, and longevity. Asterisks indicate significances: *P < 0.05, **P<0.01, #: near significance (P=0.06-0.07). All data are presented as mean ± SEM. "a" indicates age effects, "b" indicates MPS effect, and "c" indicates sex effects.

In females, a two-way ANOVA revealed a main effect of STRESS (F(1,12)=221, p<0.01; Figure 3.7B), STRESS x AGE interaction (F(1,12)=5.6, p<0.05; Figure 3.7B) but no effect of AGE, when all nine miRNA are combined. Further analysis demonstrated that AGE upregulated expression of miR-21 (F(1,12)=9.87, p<0.05; Figure 3.7B) and miR-34c (F(1,12)=6.8, p<0.05; Figure 3.7B) and downregulated expression of miR-150 (F(1,12)=9.2 , p<0.05; Figure 3.7B) and miR-181c (near significant F(1,12)=4.1, p=0.07) when MPS and CONTROL animals were combined. In CONTROL females aging upregulated expression of miR-21 (near significant, p=0.09) and miR-34c (p<0.05; Figure

3.7B). In MPS females, aging upregulated expression of miR-21 (near significant, $p=0.08$), miR-29a ($p<0.01$; Figure 3.7B) and miR-181a ($p<0.05$; Figure 3.7B) while downregulating expression of miR-150 ($p<0.01$; Figure 3.7B). At any age, MPS females revealed upregulated expression of miR-29a ($F(1,12)=5.2$, $p=0.05$), miR-29b ($F(1,12)=16.9$, $p<0.01$), miR-34a ($F(1,12)=8.9$, $p<0.05$), miR-150 ($F(1,12)=6.3$, $p<0.05$) and downregulated expression of miR-181a ($F(1,12)=7.1$, $p<0.05$). Aged MPS animals in general revealed upregulated expression of miR-29b ($p<0.05$) and miR-34a (near significant, $p=0.06$) in young and miR-29b ($p<0.05$; Figure 3.7B). Taken together, males seem to be more severely affected by age and MPS; MPS males showed the largest alteration in key epigenetic markers of biological aging.

3.5 Discussion

Through epigenetic programming, environment and lifestyle are the main determinants of lifelong health. The present findings for the first time confirm and support the notion that mutigenerational ancestral adverse experiences accelerate age-associated mental and physical health decline. While aging increased the risk of anxiety- and depression-like behaviours and impaired locomotion and coordination, these deficits were further exacerbated by multigenerational stress reaching back across five generations (F0-F4). Moreover, the impact of MPS coincided with miRNA markers for emotion, immune and stress vulnerability in prefrontal cortex, a critical regulator of emotion and stress response. miRNAs such as those responsible for emotional regulation (miR-124 and miR-34a), HPA axis function and stress response (miR-34a) and B and T cell regulators of immune function (miR-150 and miR-181a, c) were differentially expressed in multigenerational stress males and females. Furthermore, aging and stress synergistically

disturbed the stress response and accelerated age-associated morbidity and mortality especially in males in association with regulation of senescence markers miR-21 and miR-29. The findings suggest that epigenetic programming by stress experienced by previous generations is a determinant of sex-specific lifetime health trajectories and risk of common age-related diseases.

Here we demonstrate that aging induces sexually dimorphic patterns of mental and physical health decline, altered stress response and metabolic profiling along with vulnerability to disease. Our results show that aging increases the risk of anxiety-like behaviours as both male and female rats spent more time in margins of the open field. Moreover, aged rats spend less time climbing the walls during the forced swim task indicating self-helplessness and a depression-like phenotype. These altered mental and cognitive phenotypes were exacerbated by exposure to MPS. The latter findings are in line with clinical and animal studies showing that offspring exposed to perinatal stress, undernutrition, infection or environmental chemicals are more likely to show signs of motor and cognitive decline early in childhood along with increased risk of anxiety, depression and cardiovascular disease later in life (Anway et al., 2006; Crews et al., 2012; Meyer et al., 2011; Roseboom, 2000; Yehuda et al., 1998). In general, males appear more vulnerable to stress as widely described in the literature (Bale, 2011; Goel et al., 2014; Kudielka & Kirschbaum, 2005).

As aging represents a risk factor for declining physical and mental health and incidence of disease, most age-associated disorders and health outcomes seem to exhibit a sex bias (Bowling & Dieppe, 2005; Matthews & Phillips, 2010). The sexual dimorphisms were displayed in phenotypic features such as impaired inter-limb coordination and hind limb placement accuracy on the rung by aged males. Forelimb placement accuracy was

higher in females early and later in life while middle-aged CONTROL males performed better than CONTROL females, but as good as MPS females. Moreover, MPS animals overall were hyperactive, than CONTROLS across the lifespan independent of sex. Depressive-like behaviours were most pronounced in females at middle-age, while MPS males showed the highest levels of these behaviours. Although no sex differences were observed for anxiety-like behaviours, a prominent sex-by-stress-by-age interaction was observed, as stressed males show slightly lower levels of depression-like behaviour than even females when young, and the levels increased with aging. Moreover, MPS induces age-specific effects in males and females. Specifically, young and older MPS females had higher corticosterone and glucose levels than MPS males. However, body weight in males was twice as high as in females along with shorter lifespan resulting from premature midlife morbidity. Morbidity and mortality were linked to a higher incidence of renal failure and heart disease especially in MPS animals. Thus, MPS is associated with psychopathologies, premature aging and increased risk of disease along with sex-specific epigenetic regulation.

Here we demonstrate that ancestral stress across four generations in interaction with aging synergistically altered stress vulnerability mainly in males. The present data show that the HPA axis and biomarkers of stress are particularly sensitive to display the impact of stress at young and old age in accordance to earlier findings (Erickson et al., 2014b). The reduced corticosterone levels at young and old ages indicates the chronic impact of MPS on stress response similar to reduced basal corticosterone levels seen in post-traumatic stress disorder (PTSD; Yehuda & Bierer, 2007). Hence MPS in males may blunt basal HPA axis activity thus compromising adaptive stress response at particularly vulnerable times in life. Accordingly, we also demonstrated in chapter 2 that MPS upregulates cortical chr gene expression in adult males, but not in females. These findings suggest a characteristic age-

dependent profile of stress markers to be considered in the prediction and diagnosis of stress-related disease.

Our unbiased deep sequencing approach revealed core epigenetic regulatory pathways altered by ancestral stress. In the present study, two miRNAs stood out as regulators of the consequences of MPS, upregulated miR-21 in males and down-regulated in females, and upregulated miR-34 in both sexes. MiR-21 is regarded as a marker of longevity via regulation of down-stream biomarkers of senescence and aging (ElSharawy et al., 2012). Clinical studies demonstrated that individuals who age successfully to 80+ years display down-regulated miR-21 (ElSharawy et al., 2012; Haramati et al., 2011). The present miR-21 down-regulation in young and aged females provides a mechanistic link to lower mortality, which by regulating the p53 pathway, may prevent tumorigenesis and maintain genomic integrity during aging (ElSharawy et al., 2012; Hou & Zhao, 2013). On the contrary, upregulated miR-21 may result in premature aging and associated pathologic conditions (ElSharawy et al., 2012; Hou & Zhao, 2013), as is the case in the present male rat population. Accordingly, antagomirs directed against miR-21 were suggested as a preventive treatment for pulmonary disease, cardiac fibrosis and renal fibrosis (Liu et al., 2010; Thum et al., 2008).

In addition, miR-34 as a marker of stress response and emotional regulation was upregulated in young males and females but downregulated in aged males. miR-34 is implicated in anti-apoptotic actions in differentiated and aged neurons, with enrichment particularly in aged cells, as revealed by PC12 cell culture (Jauhari et al., 2018). miR-34 may also facilitate adaptation to environmental stressors as in nematodes miR-34 deletion or overexpression was linked to impaired stress response (Isik et al., 2016). As miR-34 regulates the expression of corticotropin-releasing hormone (CRH) receptor 1 gene (crhr1;

Haramati et al., 2011; Heinrichs et al., 2004; Miñones-Moyano et al., 2011), its upregulation may indicate reduced CRH expression contributing to higher anxiety-like behaviour (Haramati et al., 2011). The present upregulation in young males and females coincided with heightened arousal, but downregulation in aged males when again arousal was elevated. Thus, the present findings provide new insight into age-associated dichotomy of miRNA-expression patterns, which indicate that age-related arousal and anxiety-like behaviours may become independent of miR-34 levels.

The programming of aging trajectories and accelerated aging phenotypes by prenatal stress may not only impact one generation of exposed offspring but may also be transmitted and accumulate across multiple generations. The present observations for the first time demonstrate that ancestral prenatal stress alters lifetime mental health trajectories. In young animals, the present data affirm previous research demonstrating that prenatal stress across multiple generations alters anxiety-like (Ambeskovic et al., 2017; Dias & Ressler, 2014; Erickson et al., 2014; Kiss et al., 2016) and depressive-like behaviours (Franklin et al., 2010) in adulthood. The changes occurred in a sex-specific manner, with increased anxiety-like behaviours in young males and little consequences in females. In addition, longitudinal testing through 18 months revealed that multigenerational stress males exhibited exacerbated levels of anxiety- and depressive-like behaviours in old age while aged females remained resilient along with reduced physical frailty.

The causes for age-related health decline may stem from dysfunctional cellular and DNA repair, altered immune and neuroendocrine functions, and changes in epigenetic regulation, such as reduced DNA methylation (Ambeskovic et al., 2017; Bender et al., 2006; Kennedy et al., 2012). Moreover, the present data demonstrate that miRNAs represent not only sex- but also age-specific biomarkers. Deep sequencing revealed that

upregulated miR-150 serves as the most age-sensitive immune marker in MPS males. In females, however, this marker was upregulated by MPS independently of age. In addition, in MPS females at any age, miR-181 was generally downregulated. Both miR-150 and miR-180 are involved in fine-tuning adaptive immune response, with miR-150 being mostly expressed in mature B and T cells (Hollins & Cairns, 2016; Hou & Zhao, 2013; Sayed & Abdellatif, 2011) suggesting that stress-related miR-150 overexpression may indicate abundant B and T cells and risk of autoimmune dysfunction (Zhou et al., 2007). On the other hand, stress downregulated miR-181 in females while similar changes were reported following exposure to environmental contamination (Zhang et al., 2018) and cocaine exposure (Hollins & Cairns, 2016). Overall the miRNA profiles indicate susceptibility to diseases associated with inflammation, which applies to the majority of neurological and psychiatric disorders according to the concept of "inflammaging" (Deleidi et al., 2015; Gabuzda & Yankner, 2013; Giunta et al., 2008; Hagberg et al., 2015; Margaretten et al., 2011).

The epigenetic data suggest that ancestral stress influences disease susceptibility through a pro-inflammatory state. The pro-inflammatory miRNA profiles were accompanied by stress-related increased incidence of inflammatory disease in males, but not females. MPS males were 1.66 times more likely to develop inflammatory disorders over the life course than non-stressed. Interestingly, observed heightened risk of inflammatory disease in MPS males was similar to the average risk of morbidity in females independently of stress. It may be suggested that ancestral stress may have dysmasculinized males via reduced androgen expression (Morgan & Bale, 2011) and altered their disease phenotype accordingly. The interactive nature between the immune system and androgens (Khan & Ahmed, 2015; Kissick et al., 2014) may explain sex-specific disease incidence.

MPS males were 1.88 times more likely to suffer from renal failure and heart disease and 2.5 times more likely to contract respiratory disease than females. In turn, as estrogen modulates T cell activity, and promotes T-helper 2 differentiation (Khan & Ahmed, 2015), females become more prone to autoimmune disease (Kissick et al., 2014). Here we show a higher incidence of respiratory disease and tumors in females with a relative risk of 7.1 for respiratory disease and 6.1 for tumors in the MPS lineage.

Aside from epigenetic regulation, down-stream consequences of ancestral stress on aging and disease may be linked to sex-specific metabolic homeodynamics (Iozzo et al., 2014; Maccari et al., 2014) via altered glucose levels (Yokoyama et al., 2015). Glucose levels were slightly lower in older compared to younger males and females, an effect opposite to clinical reports (Kalyani & Egan, 2013; Ko et al., 2006). Dysregulated blood glucose levels associated with higher risk of diabetes have been observed following early life adversity in clinical and experimental studies (Ashman et al., 2016; Barker et al., 1993; de Rooij et al., 2006; Tamashiro et al., 2009). Aside from altered miR-34 expression, epigenetic mediators of this phenomenon may include differently methylated regions (DMRs) in DNA of pancreatic islet cells that was shown to be transgenerationally heritable (Wei et al., 2014). In addition, F4 trans- and multigenerational stress adult males show altered histamine and hippurate levels (Kiss et al., 2016) which are involved in glucose and insulin regulation (Greene et al., 1987; Pini et al., 2016). Moreover, MPS exaggerated aging effects on body weight (Altun et al., 2007; Pellizzon et al., 2000), as stressed females were slightly heavier at each age than non-stressed. Stressed males, however, had lower body weight at a young age and then grew significantly heavier by aging. Abdominal obesity, diabetes and heart disease may result from stress-associated changes in feeding behaviour and caloric intake (Foster et al., 2009). The present data are the first to show

multigenerational programming of blood glucose levels across the lifespan, suggesting a central role for ancestral programming in susceptibility to diabetes and potentially other metabolic diseases.

The present data suggest that repeated generational stress increases premature morbidity and mortality in F4 offspring. Stressed animals had higher prevalence of kidney failure, tumours, respiratory and inflammatory diseases and others. At 14 months of age, 33 % of MPS males had died compared to non-stressed males, while 95% of females lived to the end of the experiment at 18 months of age. Similarly, exposure to the environmental endocrine disruptor vinclozolin promoted mammillary and prostate tumour, and kidney and immune disorders in 6-12-month-old F1-F4 rat offspring (Anway et al., 2006). In the Swedish Överkalix cohort, ancestral food availability affected morbidity and disease prevalence along with longevity (Bygren et al., 2001; Kaati et al., 2007). Thus, a transgenerationally inherited epigenotype and physiological phenotype acquired prenatally may have phenotypic consequences later in life (Anway et al., 2005; Morita et al., 2013) potentially due to accelerated aging, impaired cellular repair and increased oxidative stress.

Mechanisms that determine successful aging, in addition to miRNA changes, arguably include stress-induced DNA methylation, involving DNA methyltransferase 3 alpha (DNM3a) or DNA methyltransferase 3 beta (DNMT3b) of primordial cells and sperm to provide heritable marks (Anway et al., 2005). Although the present research did not examine germ cells, sex-specific changes in brain miR-29 expression, known to modulate DNMT3a and DNMT3b expression, may underlie transgenerational inheritance (Hollins & Cairns, 2016; Okano et al., 1999). Importantly, miR-29 regulates DNMT3 activity in primordial cells of females rather than males (Takada et al., 2009). Here, MPS promoted

upregulation of miR-29 expression in females but downregulation in males, which may indicate programming by stress in early development particularly in the female lineage. It remains to be tested if these differences may have protected female offspring against adverse health outcomes. As miR-29 alterations regulate somatic mutations of DNMT3a, which is associated with pathological states, in particular immunodeficiency syndrome (Baubec et al., 2015; Morita et al., 2013; Xu et al., 1999), manipulating miR-29 expression may have therapeutic applications to treat heart fibrosis and related diseases (Hou & Zhao, 2013; van Rooij et al., 2008).

3.6 Conclusion

The present data demonstrate that repeated ancestral stress represents a main determinant of lifetime health trajectories. MPS in males resembled phenotype features of human PTSD proposing its value as a new animal model for this condition. MPS accelerated aging processes in males and partially spared females. Mechanisms of stress vulnerability in males and potential resilience in females include dysregulated stress response and altered inflammatory status. miRNAs may play a key role programming stress vulnerability through the maternal lineage and provide predictive biomarkers of age-related diseases suitable for consideration in precision medicine approaches. The present data emphasize that aging is not only influenced by genetics and lifestyle but also by experiences in previous generations.

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CHAPTER 4: Ancestral Social Stress Alters Aging-Dependent Changes in Cognition, Motor Function and Brain Volume Through Sex-Specific Stress and Immune Response Activation

4.1 Abstract

The aging population worldwide is growing and so are the incidences of aging-associated diseases including cognitive disorder and neurodegeneration. Early life stress represents a robust challenge that can reprogram brain development with lifelong behavioural and physiological consequences on the offspring and future generations. Due to inextricably linked stress and immune systems, abnormal activation of one system will activate the other, altering health outcomes and aging trajectories across the lifespan. In the current study, we investigated the effects of maternal social stress during pregnancy on the stress response, immune system, motor and cognitive function and brain morphology in the F4 generation offspring across the lifespan. Male and female transgenerational stress (TPS, great-grandmother stressed), multigenerational stress (MPS, four consecutive generations of stress), and a non-stressed (CONTROL, left undisturbed) offspring were assessed in clinically relevant tasks of exploratory activity, skilled reaching and skilled walking, learning and memory and underwent MRI scans longitudinally at 6, 12 and 18 months of age. Here, we report transgenerational transmission of behavioural phenotypes up to the 4th generation in a rat model of maternal social stress. Our data demonstrate three main findings. First, ancestral stress exacerbated aging-associated immune and stress response activation, behavioural impairments and brain volume decay in the F4 generation offspring. Second, ancestral stress programmed aging trajectories in a sex-specific manner, as stress related changes are observed in earlier ages in male than female offspring. Third, sex-specific physiological and behavioural effects were induced by specific stress type;

transgenerational and multigenerational stress had similar negative effects on male offspring, while multigenerational stress prompted resilience in females. Thus, the present data demonstrate for the first time that maternal social stress during pregnancy is transmitted to their distant great-grandchildren, either via transgenerational or multigenerational transmission to alter health outcomes, cognitive function and induce accelerated aging trajectories across the lifespan in a sex-specific manner.

4.2 Introduction

The global aging population continues to grow very rapidly. In 2017 the number of seniors in North America for the first time exceeded the number of children aged 5 years and younger (WHO). Biological aging rather than chronological aging is associated with increased risk of physical and mental health frailty (Ambeskovic et al., 2017; Bale & Epperson 2015; Rowe & Kahn 1997; McLaughlin et al. 2010). Biological aging, however, is not always closely correlated with chronological age and it may be possible to slow or accelerate the biological aging processes and pathologies by adverse experiences (Peters, 2006; Rowe & Kahn, 2015). In particular, adverse early life environments are potential causes for accelerated biological aging processes and adverse health trajectories (de Rooij & Roseboom, 2013; Franke et al., 2017; Painter et al., 2005; Roseboom et al., 2011).

Experimental and clinical evidence has shown an association between adverse early life experiences such as pre- and postnatal stress, undernutrition, and exposure to environmental chemicals permanently alters the stress response and hypothalamic-pituitary-adrenal (HPA) axis feedback regulation (Glover et al., 2010; Murgatroyd & Spengler, 2011). The consequences influence early brain development, behaviour, temperament and the risk of disease across the lifespan (de Rooij & Roseboom, 2013). For

example, prenatal stress raises the lifetime risk of psychological illnesses, including depression and anxiety (Ravelli et al., 1998; Zhu et al., 2014), cognitive and motor deficits (King et al., 2012) and metabolic homeodynamics (Bateson et al., 2004; Gluckman et al., 2008). Increased risk for neuropsychiatric and neurodevelopmental disorders is also observed in offspring whose mothers have been exposed to infection, indicating that stress and inflammation may share converging pathways in modulating brain development (Allswede et al., 2016; Brown et al., 2005; Buka et al. 2001; Howerton & Bale 2012a; Gray et al., 1991).

Recent evidence suggests that transgenerational stress activates peripheral pro-inflammatory cytokine, including IL-18 (Babb et al., 2014a; Murgatroyd et al., 2016) which may contribute to transgenerational phenotypes of psychopathologies (Veenema et al., 2008; Slopen et al., 2013; Murgatroyd et al., 2016; Grassi-Oliveira et al., 2016; Ambeskovic, et al., 2017; Kiss et al., 2016; McCreary et al., 2016). Pro-inflammatory cytokines are associated with social behavioural deficits, depressive- and anxiety-like behaviours (Murgatroyd et al., 2015; Nephew et al., 2017). Furthermore, ancestral early life stress may induce behaviorally relevant changes in brain development which are immune mediated (Bale, 2015; Howerton & Bale, 2012; Murgatroyd et al., 2016; Nephew et al., 2017). Thus, ancestral early life stress through altered inflammatory activation or inflammation alters neural plasticity and neurogenesis with lifelong behavioural consequences (Musaelyan et al., 2014a). Adult neurogenesis seems to be particularly responsive to stress-related immune responses (Musaelyan et al., 2014a). Moreover, activation of microglia and cytokines play a causal role in depression and neurodegenerative diseases including Alzheimer disease (Musaelyan et al., 2014b; O'Connor et al., 2014).

While cumulative ancestral stress has been recognized as a major risk factor for adult disease, it has not yet been shown if it is linked to immune activation to explain complex neuropathologies associated with old biological age. Previous research demonstrated that multigenerational ancestral stress, compared to single generational stress, induces the most pronounced changes in behavioural phenotype (Yao et al., 2014) and leads to new behavioural traits (Ward et al., 2013; Ambeskovic et al., 2017). These changes are likely linked to epigenetic inheritance (Dancause et al., 2012; Yao et al., 2014). GR resistance may explain the failure to downregulate inflammatory responses in chronic stress conditions (Cohen et al., 2012) and prenatal stress (Lopez-Duran et al., 2009). Our previous research indicated the multigenerational stress induces a complex phenotype displaying both stress vulnerability and resilience in its behavioural and metabolic phenotype (Kiss et al., 2016; Faraji et al., 2017).

The objective of this study was to investigate biological aging trajectories in animals exposed to maternal social stress five generations removed. We compared the impact of trans- versus multigenerational stress on immune status associated with the multidimensional phenotype of stress vulnerability and resilience in terms of motor and cognitive function and in vivo neuroanatomy. Since immune system function depends on age this study also investigated the immune status across the lifespan. The present data indicate for the first time that multigenerational prenatal stress (MPS) through sex-specific stress and immune response activation alters physical health, motor and cognitive function, hippocampal and cortical morphology and accelerates biological aging. The data broaden the concept of inflammaging (Franceschi et al., 2018) and open new venues to predict and mitigate the risk of aging-related disorders.

4.3 Methods

4.3.1 Animals

Four generations of Long-Evans hooded rats were bred and raised at the Canadian Centre for Behavioural Neuroscience vivarium under carefully controlled conditions. In this study, we used 48 rats that were housed in groups (males in pairs, females three per cage except for isolation stress) under a 12:12 h light/dark cycle with light starting at 07:00h and the room temperature set at 22°C. All rats were tested longitudinally in various behavioural tasks and underwent magnetic resonance imaging (MRI) scans at 6, 12 and 18 months of age. Body weight was measured weekly. All procedures were approved by the University of Lethbridge Animal Care Committee in compliance with the guidelines by the Canadian Council on Animal Care.

4.3.2. Experimental Design

Under standardised conditions, three different lineages of rats were bred (Figure 1). In the transgenerational prenatal stress (TPS) and the multigenerational prenatal stress (MPS) lineages females in the parental (F0) generation underwent chronic social stress by isolation housing while the third lineage served as yoked handled, non-stress control. In the TPS lineage, 90-day old female rats in the F0 generation were separated from their companions and housed alone starting two weeks before pregnancy throughout the entire duration of pregnancy. In the MPS lineage, female rats in the F0, F1, F2, and F3 generations underwent this type of stress. A maximum of three offspring per litter of each sex was randomly selected to be included in the experiments. Each experimental group included offspring from at least 3-4 different litters. The experiments included both male and female F4 animals [males: n=25 (F4-TPS=9, F4-MPS=8, F4-CONTROL=8); females: n=22 (F4-

TPS=9, F4-MPS=5, F4-CONTROL=8)]. By comparing the F4 generation of TPS and MPS with non-stressed animals the present study differentiates the direct impact of stress from truly heritable epigenetic phenotypes (Skinner, 2008; Zucchi et al., 2012; Figure 4.1).

The offspring were tested in exploratory activity, skilled fore- and hind limb function and spatial learning and memory longitudinally at 6, 12 and 18 months of age. Blood was also collected at these ages for assays of blood glucose and corticosterone. A subset of F4 animals from each lineage were assessed using in vivo magnetic resonance imaging (MRI) at 6, 12 and 18 months of age [male n=18 (F4-TPS=6, F4-MPS=6, F4-CONTROL=6); female n=18 (F4-TPS= 6, F4-MPS=6, F4-CONTROL=6] to examine hippocampal and prefrontal cortex mean gray value.

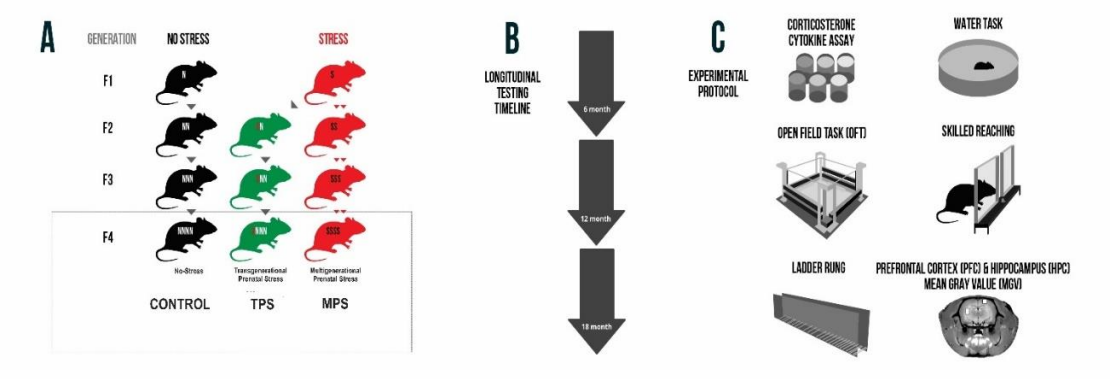

Figure 4.1. Ancestral stress paradigm and longitudinal experimental design
Pregnant dams were either stressed to generate ancestral stress lineage or left undisturbed to generate non-stressed lineage. Ancestral stress lineage was generated by stressing (red arrows) pregnant dams over four consecutive generations (F0, F1, F2, F3) to produce F4 multigenerational stress (MPS; SSSS) or only in first generation (F0) to produce transgenerational stress (TPS; SNNN) offspring. Stress consisted of subjecting dams to social isolation from two weeks before pregnancy until after pup weaning. During the same time non-stressed CONTROL offspring were generated. This experiment used CONTROL MPS and TPS fourth generation offspring. (B) Animals were assessed across the lifespan longitudinally at 6, 12 and 18 months of age. (C) Experimental protocol consisted of blood sampling from the tail vein for the corticosterone and cytokine assays, open field task (OFT), ladder rung task, skilled reaching task, Morris water task (MWT) and magnetic resonance imaging (MRI) across the lifespan. Illustrative images of corticosterone and

cytokine assays, behavioural OFT, MWT, reaching task, ladder rung apparatus, and an MRI brain slice with ROI (prefrontal cortex (PFC) and hippocampus (HPC)).

4.3.3 Blood Collection, Corticosterone and Cytokine Assays

Blood samples were obtained on days without behavioural testing. On average 0.6 ml of blood was collected from the tail vein between 8:00 and 10:00 AM under 4% isoflurane anesthesia (Faraji et al., 2017). Plasma was obtained by centrifugation at 5,000 rpm for 10 minutes. The samples were stored at –80° C. Plasma corticosterone (CORT) levels were determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits (Cayman Chemical, Ann Arbor, MI, USA). A Rat Cytokine 23-Plex assay (Bio-Rad Laboratories, Montreal, Quebec) panel consisting of pro- and anti-inflammatory cytokines (G-CSF, GM-CSF, GRO/KC, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, IL-17A, IL-18, M-CSF, MCP-1, MIP-1α, MIP-3α, RANTES, TNF-α, and VEGF) was conducted on a Luminex Bio-Plex Platform (Bio-Rad Laboratories, Montreal, Quebec). Peripheral plasma cytokine levels were determined using Bio-Rad Pro rat cytokine multiplex commercial kit (Bio-Rad, Montreal, QC).

4.3.4 Behavioural Testing

4.3.4.1 Exploratory Activity Task

Exploratory activity was assessed using the open field task, a standard measurement of locomotion and emotional states in rats (Denenberg, 1969; Erickson et al., 2014; Kiss et al., 2016). Briefly, rats were placed individually into AccuScan activity monitoring Plexiglas boxes (length 42 cm, width 42 cm, height 30 cm) and monitored for 10 min using VersaDatTM software (AccuScan Instruments Inc., OH, USA). Total distance traveled (cm) per 10 minutes was used to measure the overall activity.

4.3.4.2 Skilled Reaching Task

To asses fine motor function, animals were tested in a single pellet reaching task according to established protocols (Metz & Whishaw, 2000). Animals were pretrained to reach asymptote levels in skilled reaching success. Then they were tested for fine motor skills as quantified by percent success and qualitative score over the next 13 days. On day 13 reaching performance was videotaped with a digital video camcorder (WV-BP330, Panasonic, Minato-ku, Tokyo, Japan), and the videos were later analyzed by an experimenter blind to the experimental conditions. Three successful reaches per each rat were analyzed and averaged to assess the qualitative features of movements. The maximum reaching movement score was 35 points (Metz & Whishaw, 2000; Ambeskovic et al., 2017).

4.3.4.3 Skilled Walking Task

To assess skilled walking performance, animals were tested in the horizontal ladder rung walking apparatus (Metz & Whishaw, 2009). After pre-training was completed, three crossings per animal were video recorded. The recordings were analyzed by a blind experimenter according to our previously published rating scale (Metz & Whishaw, 2009). The quantitative analysis of skilled walking scores included the average right forelimb (RFL) and left forelimb (LFL) and right and left hind limb (RHL; LFH, respectively) scores. For qualitative analysis, the limb placement was scored on a scale ranging from 0 to 6, where 0 represented a total miss and 6 represented a correct limb placement (Metz & Whishaw, 2009).

4.3.4.4 Learning and Memory Task

A hidden platform version of the water maze (WM) task was used to asses for spatial performance (Faraji et al., 2011; Morris, 1984). The apparatus consisted of a large pool filled with opaque water (20° C), coloured by white non-toxic paint. The pool was located in a room decorated with distal cues on the walls that were kept consistent throughout the experiment. Rats were tested over nine consecutive days. Days 1-8 tested for spatial learning and memory by moving the hidden platform (1.5 cm under the surface of the water) every two days. On odd days (1, 3, 5, and 7) learning performance was measured as the location of platform moved to a different quadrant on these days while even (2, 4, 6, and 8) days examined memory while the location of the platform stayed constant with the previous learning day. Rats were tested in eight trials per day, and maximum duration of each trial was 60 seconds.

Each trial began with the animal being placed in the pool in pseudorandom sequence, where the swim time, speed and distance to find the platform was tracked by a camera connected to a computer (HVS Image 2020; HVS Image Ltd, UK). The ninth day (probe day), the hidden platform was removed, and animals were assessed for recall of located on the previous two days. The probe day consisted of one 30-second trial, and it measured the percent of time spent in the quadrant of interest. The location of quadrant of interest in this experiment was in NE or quadrant 1 as per tracking system software (HVS Image 2020 Plus Tracking System, 1998–2002; HVS Image Ltd, UK) and an Acer computer (Travel Mate 225X, Lethbridge). Here we examined average learning and memory path length and the percent time spent in the quadrant of interest on the probe day. The path length was used as an indication of cognitive performance, to account for confounding impact by the speed or motor deficits in aging animals. The percent time spent in probe trial was used as a true measurement of spatial memory.

4.3.5 In Vivo MRI Imaging

Images for the mean gray value of hippocampus (HPC) and prefrontal cortex (PFC) were acquired using a 4.7 T Oxford magnet (Oxford, UK). Animals were imaged at 6, 12 and 18 months of age. The imaging protocol consisted of: a) localizer images (SE-TR/TE 700/16 ms, 0.2x0.2x1.5 mm3), b) T2 measurements (TE/ TR 22, 26, 35, 60, 90/3000 ms, 0.23x0.23x2 mm3) and c) T2-weighted images (TE/TR 22/3000ms, 0.23x0.23x2 mm3). Mean Gray Values (MGV) were measured using quantitative cytoarchitectonic (SEMS images) analyses and corresponding to an ROI measuring HPC (area 0.30 mm2, -4.80mm relative to bregma) and PFC (area 7 mm2, 3.70 mm relative to bregma; Figure 4.1C) were performed with ImageJ (https://fiji.sc/). Then mean brightness or mean gray values of the ROIs were calculated and averaged for left and right hemispheres.

4.3.6 Statistical Analysis

Statistical analysis was performed using SPSS 20 for Windows 11.5.0 (IBM Corporation, Armonk, NY, USA). Repeated measures ANOVA with age (6, 12 and 18 months) as within-subject factors over time, and sex and stress as between-subject factors was run for behavioural tasks, corticosterone, cytokine levels, and mean gray volume measurements. Bonferroni post hoc test was used for within factor to adjust for multiple comparisons and whenever possible posthoc tests (Bonferroni and LSD) were run. For age-specific stress effects either one-way ANOVA or t-test were performed. All results are shown as the means ± standard error of the mean (±SEM).

4.4 Results

4.4.1 Ancestral Stress Alters Aging-Dependent Changes in Stress Response

Stress altered aging-dependent increase in corticosterone levels at different times across the lifespan in males and females. Repeated measures ANOVA revealed a main effect of AGE ($F(2,86)=40.16$, $p<0.001$), AGE x STRESS interaction ($F(4,86)= 3.61$, $p<0.01$). Circulating corticosterone levels increased with age, as young animals (M=48.13; Figure 4.2) had the lowest levels, and these increased in middle-aged (M=67.3) and aged (M=76.9). Bonferroni post hoc tests revealed a significant increase in corticosterone levels in middle-aged ($p<0.001$; Figure 4.2) and aged ($p<0.001$) when compared to young, while a smaller but still significant ($p<0.05$) increase was observed from middle-aged to aged animals. In contrast, stress reduced corticosterone levels in aged animals, showing opposite effects to the age-dependent increase in corticosterone levels observed in non-stressed animals. Although no overall differences in the corticosterone levels were observed in male and female animals, further analysis of each sex revealed sexual dimorphisms. One-way ANOVA revealed a near significant ($F(2,24)=2.8$, $p=0.07$; Figure 4.2A) effect of stress on corticosterone levels in middle-aged males. The LSD posthoc test revealed a significant ($p<0.05$; Figure 4.2A) increase in circulating corticosterone levels in multigenerational (MPS) in comparison to non-stressed (CONTROL; Figure 4.2A) animals. On the contrary, in females stress had significant ($F(2,20)=3.54$, $p<0.05$; Figure 4.2B) effect on the corticosterone levels in old age. Post-hoc tests (Bonferroni, $p<0.05$; LSD, $p<0.05$) revealed a large decrease in corticosterone levels in TPS when compared to CONTROL animals (Figure 4.2B).

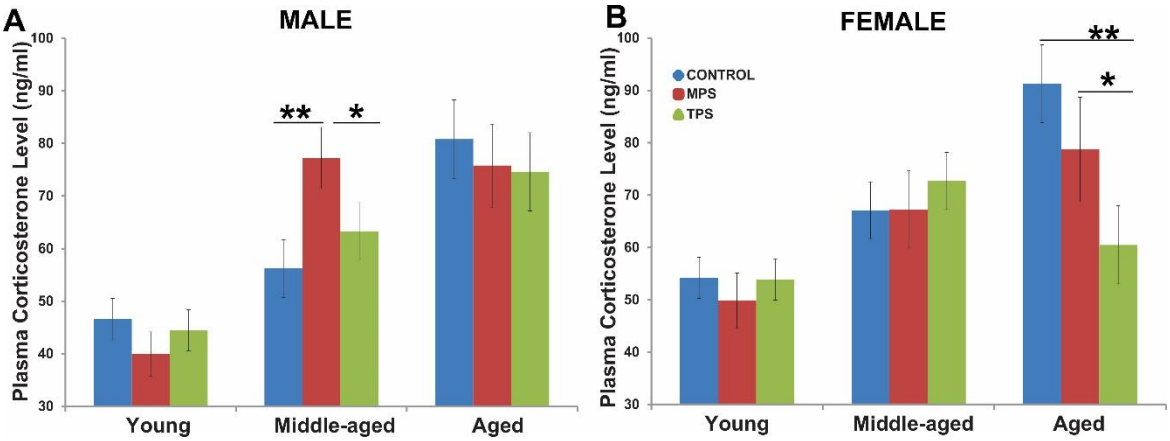

Figure 4.2. Ancestral stress alters aging-dependent changes in stress response. Changes in circulating plasma corticosterone levels across the lifespan. (A) MPS male offspring showed increased circulating corticosterone levels in middle-age, in comparison to CONTROL and TPS offspring. (B) TPS female offspring had decreased circulating corticosterone levels in comparison to CONTROL and MPS aged animals. Asterisks indicate significances: *P < 0.05, **P<0.01. All data are presented as mean ± SEM.

4.4.2 Ancestral Stress Altered Immune System Activation: Sex-Specific Increase in Proinflammatory Cytokines and Chemokines Across the Lifespan

The plasma values for immune markers (G-CSF, GM-CSF, GRO/KC, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, IL-17A, MIP-1α, MIP-3α, RANTES, TNF-α, and VEGF) fell below or above the standard curve of the Luminex Bio-Rad. Nevertheless, stress and aging induced sex-specific effects on the circulating peripheral levels of IL-18, IL-5, GM-CSF and MCP-1 (Figure 4.3).

Repeated measures mixed ANOVA with AGE as within factor and SEX and STRESS as between factors revealed various effects on the IL-18, M-CSF, and MCP-1 and a near significant effect on IL-5. Specifically for IL-18, repeated measures mixed ANOVA with AGE as within factor and SEX and STRESS as between factors revealed effects on AGE ($F(2,70)=7.1$, $p<0.01$), AGE x SEX x STRESS interaction ($F(4,70)=2.81$, $p<0.05$), SEX ($F(1,35)=4.07$, $p=0.05$; Figure 4.2A&B). Briefly, IL-18 cytokine levels increased with

age, as young (M=556) animals had significantly lower levels than middle-age (M=764, $p<0.01$; Figure 4.3) and aged (M=749, $p<0.05$), while no differences were observed between middle-aged and aged. Males (M=781; Figure 4.3A) on average had higher IL-18 levels than females (M=599; Figure 4.3B), and TPS animals had the highest levels in females, while both TPS and MPS stress males had higher levels that were age-specific. Although no main effects were observed for the IL-5 concentration levels, LSD post-hoc test revealed significant Il-5 increase ($p<0.05$; Figure 4.3C) in young MPS males, while both MPS and TPS aged females had lower IL-5 levels than CONTROL ($p<0.05$; Figure 3D).

The chemokine M-CSF showed a main effect of AGE ($F(2,68)=5.34$, $p<0.01$), AGE x SEX interaction ($F(2, 68)=3.88$, $p<0.05$), STRESS ($F(2,34)=3.15$, $p=0.05$), and SEX ($F(1,34)=4.06$, $p=0.05$; Figure 4.3G & H). Aging decreased levels of M-CSF, as young (M=399) animals had significantly higher levels ($p<0.05$; Figure 4.3G & H) than middle-aged (M=326) animals, and aged (M=341). Moreover, males (M=385; Figure 4.3G) had higher levels overall than females (M=326; Figure 4.3H). Interestingly, ancestral stress increased M-CSF levels, as TPS (M=387) and MPS (M=372) animals had higher levels than CONTROLs (M=307; Figure 4.3G & H). Bonferroni post hoc tests revealed a significant increase in circulating M-CSF in TPS animals when compared to CONTROL. For the MCP-1 levels, a main effect of AGE ($F(2, 53.3)= 2.67$, $p=0.05$) and SEX ($F(1, 32) =16.7$, $p<0.001$; Figure 4.3E & F) was observed while no interactions were observed. Aging overall increased the levels, with young (M=334) animals having the lowest levels, then middle-aged (M=384) and aged with the highest levels (M=404). A Bonferroni post-hoc test revealed near significant ($p=0.08$) differences between young and aged animals.

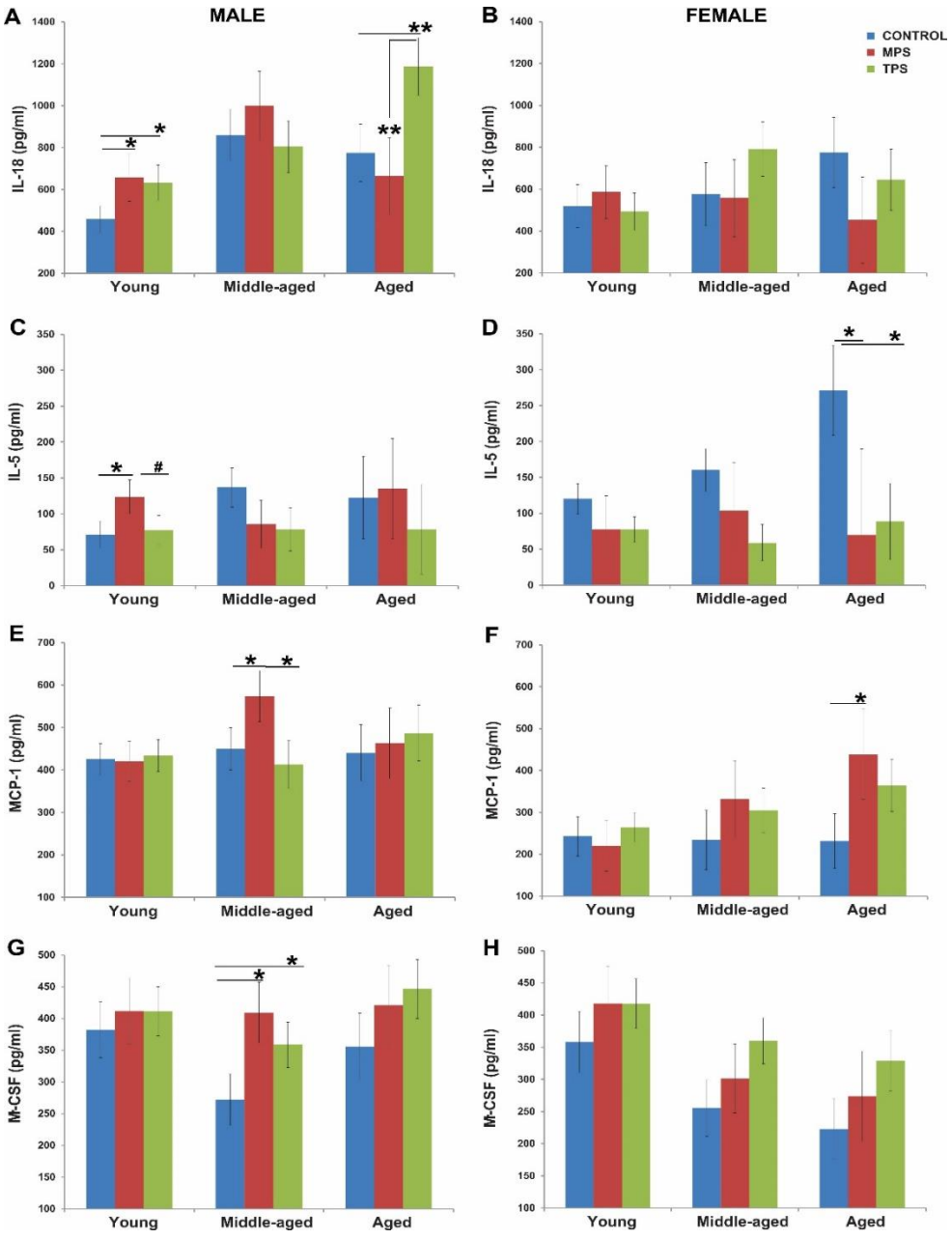

Figure 4.3. Ancestral stress altered immune system activation: sex-specific increase in proinflammatory cytokines and chemokines across the lifespan.

Plasma cytokine levels across the lifespan as measured by commercial multiplex cytokine kit. (A, C, E, G) MPS male offspring showed upregulated pro-inflammatory cytokine (IL-18), neuroinflmmatory chemokine (MCP-1), and B and T cell regulators (IL-5, M-CSF) in young and middle-age, while in IL-18 was upregulated in aged TPS males. (B, D, F, H). TPS female offspring had upregulated neuroinflmmatory chemokine (MCP-1), while downregulated B and T cell regulators (IL-5) was observed in aged MPS and TPS animals. Asterisks indicate significances: *P < 0.05, **P<0.01. All data are presented as mean ± SEM.

4.4.3 Ancestral Stress Induced Sex- and Age-Specific Alterations in Hippocampal and Prefrontal Cortex Mean Gray Value (MGV)

Age-dependent decrease in the hippocampus and prefrontal cortex MGV was stress- and sex-specific. For hippocampal mean grey volume (Figure 4.4A), repeated measures ANOVA revealed main effect of AGE ($F(2,54)= 11.16$, $p<0.000$; Figure 4.4B & C) and AGE x STRESS interaction ($F(4,54)=2.86$, $p<0.05$), while no other effects nor interactions were observed. Briefly, aging decreased MGV across the lifespan, as young (M=2270) animals had higher MGV ($p<0.05$) than middle-aged (M=2048) and aged (M=1895; Figure 4.4). Interestingly, Bonferroni posthoc test did not reveal significant differences ($p>0.05$; Figure 4.4B & C) between middle-aged and aged animals, indicating that most brain volume changes occurred during early adulthood with a slowdown from middle-aged to aged, potentially due to decreased neurogenesis. In addition, aging consistently reduced MGV from young to middle-aged and aged, while ancestral stress increased MGV in aged animals in comparison to middle-aged. The latter was especially marked in male offspring (Figure 4B). While no overall sex differences were observed, ancestral stress induced significant MGV changes in the middle-aged females ($F(2,17)=6.89$, $p<0.01$; Figure 4.4C). A Bonferroni post-hoc revealed significant differences ($p<0.01$) between TPA and CONTROL groups and near significant effects ($p=0.078$) between MPS and CONTROL animals (Figure 4.4B & C).

When the MGV of the prefrontal cortex (Figure 4.4D) was analyzed, repeated measures ANOVA revealed a main effect of AGE ($F(2,48)=13.6$, $p<0.0001$; Figure 4.4E & F), while no other effects nor interactions were observed. Briefly, aging decreased MGV, as the young (M=2627) had the largest volume, with a decrease in middle-aged (M=2302) and aged (M=2335) animals. Bonferroni post hoc test revealed a significant decrease in

MGV in middle-aged ($p<0.001$; Figure 4.4E & F) and aged ($p<0.001$; Figure 4.4) animals when compared to young, while no significant differences ($p>0.05$) were observed between middle-aged and aged rats.

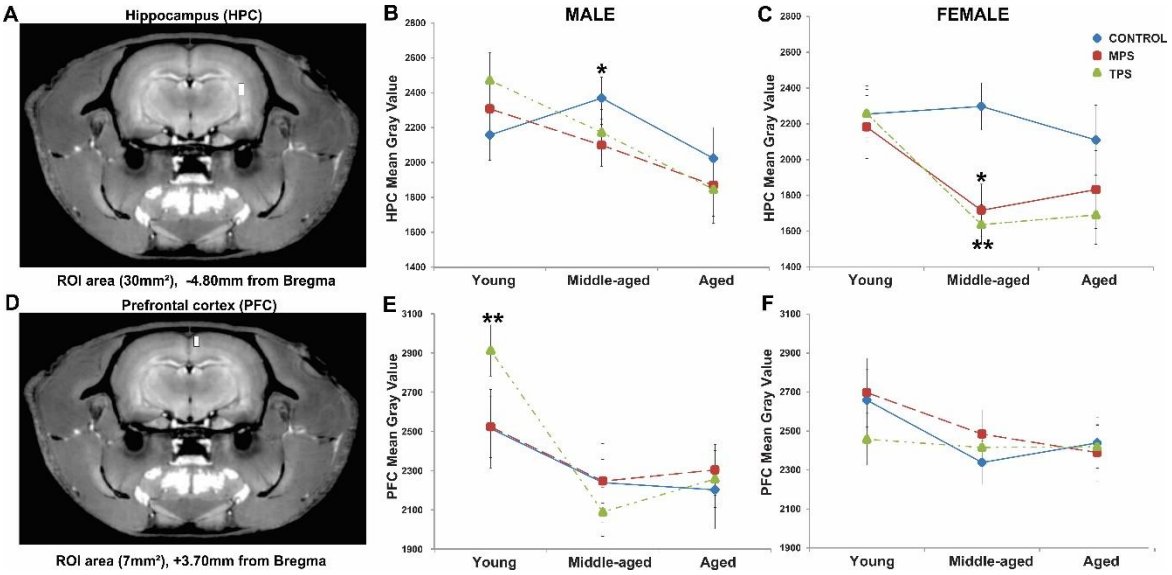

Figure 4.4. Ancestral stress induced sex- and age-specific alterations in hippocampal and prefrontal cortex mean gray value (MGV).
Mean gray value measurements were recorder longitudinally across the lifespan by 3T MRI.
(A, D) SEMS Images of the brain slice showing the HPC-ROI and PFC-ROI respectively. (B&C) MPS male offspring had lower HPC-MGV in middle-aged than CONTROL or TPS, while TPS male offspring had higher PFC-MGV in young age. (E&F) MPS and TPS middle-aged female offspring had much lower HPC-MGV than (CONTROL). Asterisks indicate significances: *P < 0.05, **P<0.01. All data are presented as mean ± SEM.

Moreover, although ancestral stress and sex did not significantly affect MGV of the PFC, a significant effect of stress ($F(2,18)=8.7$, $p<0.01$; Figure 4.4E) was observed in young males only. Bonferroni post hoc test revealed that TPS offspring had significantly higher MGV than CONTROL and MPS young male rats ($p<0.01$; Figure 4.4F). Thus, aging decreased the MGV as expected, while ancestral stress did not exhibit overall stress effects, but instead induced sex- and age-specific alterations. It may be suggested that males are more sensitive to ancestral stress than females, as the largest MGV effects are observed in

adult males, while in females this happened in middle-age. Ancestral stress may initiate accelerated brain aging in males earlier than in females.

4.4.4 Ancestral Stress Exacerbated Age-Associated Locomotor Activity Loss in a Sex-Specific Manner

A repeated measure mixed ANOVA revealed a main effects of AGE ($F(1.55,61.86) = 59.25$, $p<0.001$), SEX ($F(1,40)=15.97$, $p<0.001$), SEX x AGE interaction ($F(1.55,61.86)=17.67$, $p<0.0001$) and no effect of STRESS (Figure 4.5B & C) as measured in the open field task (Figure 4.5A). Briefly, aging reduced locomotor activity across the lifespan across all groups. Young animals (6 months; Bonferroni, M=3233) were significantly less active ($p<0.001$) than middle-aged (12 months; M=2490) and aged rats (18 months; M=1787). Similarly, middle-aged rats had significantly ($p<0.001$) higher locomotor activity than aged rats ($p<0.001$; Figure 4.5C). Females were significantly more active ($p<0.001$; M=2159; Figure 4.5C) than males (M=1786, Figure 4.5B). Males displayed a rapid, significant decrease in activity at every age (Figure 4.5B), while females showed an increase at middle-age and reduction at old age. Bonferroni post hoc test revealed significant reduction ($p<0.001$; Figure 4.5C) in aged and a slight increase in middle-aged rats when compared to young females. This increase in locomotor activity at middle age was specific to CONTROL and MPS females, and post hoc tests revealed a significant decrease in activity levels of TPS females at this age ($p<0.05$). Thus, aging and ancestral stress induced sex-specific effects on locomotor activity across the lifespan.

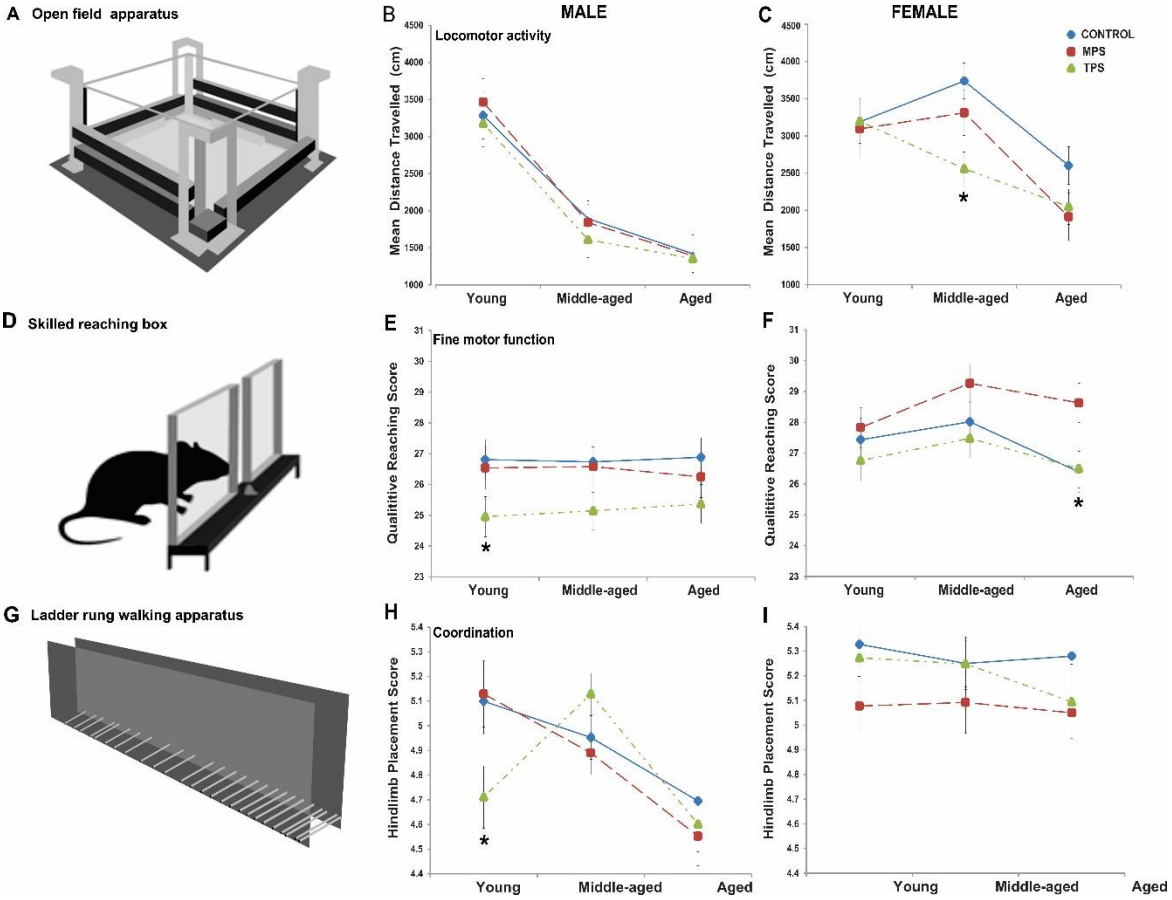

Figure 4.5. Ancestral stress exhibits age and sex-specific effects on motor function. Motor performance outcomes across the lifespan as measured by the open field (A), skilled reaching box (D) and ladder rung apparatus (G).
(B, E&H) Note that TPS male offspring showed impaired fine motor function and hindlimb placement at young age when compared to MPS and CONTROL rats, while everyone displaced age-associated impairments in locomotor activity across the lifespan. (C, F&I) TPS female offspring showed decreased locomotor activity and impaired fine motor function in middle and older age, while aged MPS females showed improved fine motor function and impaired hindlimb placement when compared to CONTROL animals. Asterisks indicate significances: *P < 0.05, **P<0.01. All data are presented as mean ± SEM.

4.4.5 Ancestral Stress and Aging Altered Fine Forelimb Motor Control

A repeated measure mixed ANOVA revealed no main effects or interactions qualitative fine motor control (Figures 4.5E-D). Females (M=27.6) had higher total qualitative reaching scores (p<0.01) than males (M=26.2) across the lifespan independent

of stress exposure. MPS (M=27.51) animals performed significantly better ($p<0.05$) than TPS (M=26.03) or CONTROL rats (M=27.04, $p>0.05$). Males displayed an effect of STRESS ($F(1,23)=4.09$, $p<0.01$; Figure 4.5E), as the TPS rats had significantly lower reaching scores ($p<0.05$; Figure 4.5E) than CONTROL animals. In females, the qualitative reaching score was significantly changed ($F(2,18)=4.49$, $p<0.01$; Figure 4.5F) across the lifespan when all groups were examined together. A Bonferroni post-hoc revealed a significant increase in qualitative reaching performance between young and middle-aged ($p<0.05$), a decrease between middle-aged and aged ($p<0.05$), and a slight non-significant decrease between young and aged female rats ($p>0.5$; Figure 4.5F). Thus, ancestral stress impaired fine motor function in males especially in the TPS condition while it had mainly no effects on females, other than improvement at middle-age. Aging affected qualitative reaching performance in females only, and this could be linked to females performing better than males at young age.

4.4.6 Ancestral Stress and Aging Synergistically Impaired Skilled Hind Limb Movement

Ancestral stress and aging synergistically decreased hind limb placement scores. Mixed repeated measures ANOVA revealed main effects of AGE ($F(2,80)=7.57$, $p<0.001$), SEX ($F(1,40)=26.28$, $p<0.001$), and AGE x SEX interaction ($F(2,80)=3.55$, $p<0.05$; Figure 5.5G, H & I). Young (M=5.1) and middle-aged animals (M=5.09; Figure 4.5H & I) had similar hind limb placement scores while aged (M=4.87) had the lowest score. Bonferroni posthoc test revealed significantly lower hind limb scores in aged than in middle-aged animals independent of sex and stress ($p<0.05$; Figure 4.5H & I. When the analysis was split by sex, a significant age effect ($p<0.001$) was observed in males, but not in female

rats. Females showed significantly higher hind placement accuracy ($p<0.001$; M=5.19; Figure 4.5I) than males (M=4.86; Figure 4.5H). Although aging decreased overall limb placement accuracy in both sexes, an increase was observed in middle-aged TPS stress males and CONTROL aged females. TPS males showed lower scores than any other group at six months of age (Figure 4.5H).

4.4.7 Ancestral Stress and Aging Induce Sex-Specific Effects on Spatial Learning and Memory

Mixed repeated measures ANOVA revealed main effects of AGE ($F(2,82)=61.84$, $p<0.001$), AGE x SEX interaction ($F(2,82)=37.3$, $p<0.001$), effect of DAYS ($F(4.6,287)=56.7$, $p<0.001$), AGE x DAYS interaction ($F(7.6,394)=56.03$, $p<0.001$), effect of SEX ($F(1,41)=39.1$, $p<0.001$) and AGE x DAYS x SEX interaction ($F(7.54,394)=3.4$, $p<0.001$; Figure 4.6). Briefly, the path length to locate the platform decreased with age, as young animals took longer to learn the task (M=2.94) than middle-aged (M=2.28) and aged animals (M=2.23). Young animals were significantly slower at locating the escape platform than middle-aged ($p<0.001$) and aged animals ($p<0.001$; Bonferroni). This indicates that animals learned the task after the first session in young adulthood and aging did not alter spatial navigation overall. Males were faster at finding the platform than females. Over time spatial navigation performance improved as indicated by shorter latency to locate the platform, and Bonferroni revealed a significant difference ($p<0.05$) between learning (odd days, 1, 3, 5, 7) and memory (even, 2, 4, 6, 8) days.

Learning days: Mixed repeated measures ANOVA of path length revealed a main effect of AGE ($F(1.74,71.5)=79.7$, $p<0.001$), AGE x STRESS interaction ($F(3.48,71.5)= 2.53$, $p=0.05$; Figure), effect of SEX ($F(1.74,71.5)=24.9$, $p<0.001$), and AGE x SEX interaction

(F(1.74, 71.5)=24.9, p<0.001). Young animals (M=3.69) travelled longer distances (p<0.001; Figure 4.6A & B) than middle-aged and aged animals (M=2.65). Middle-aged females performed better than aged (Figure 4.6B). Males had shorter path lengths than females.

Memory days: Mixed repeated measures ANOVA with average path length as dependent measurement revealed a main effect of AGE (F(1.75,71.3)=10.0, p<0.001), SEX (F(1,41)=28.8, p<0.001; Figure 4.6C & D) and AGE x SEX interaction (F(1.74,71.3)=19.2, p<0.001). The path length to locate the hidden platform diminished with age, as young animals (M=2.2) were slower than middle-aged (M=2.03) and aged rats (M=1.8). Bonferroni posthoc test revealed that young and middle-aged animals had significantly longer path length than young (p<0.001; Figure 4.6C & D). Males (M=1.71; Figure 4.6C) had a shorter path length than females (M=2.32; Figure 4.6D).

Probe trial: Mixed repeated measures ANOVA revealed a main effect of SEX (F(1,41)=13.02, p<0.001; Figure 4.6E & F). Males spent a higher percentage of time than females (M=27.95) in the quadrant where the platform was last located (M=34.37, Figure 4.6E & F). When analysis was split by sex, in males the type of ancestral stress had age-specific effects. A t-test revealed that young TPS offspring spent significantly more time in the quadrant of interest than MPS (p<0.05). Similarly, MPS offspring spent less time in a quadrant of interest than CONTROLs. In middle-aged females, the preference for the quadrant where the platform was last found was nearly significantly (p=0.06; Figure 4.6F) altered by ancestral stress. Specifically, t-tests revealed that MPS animals spent more time in the quadrant of interest than CONTROLs at middle-age (p<0.05; Figure 4.6F). At the young age the TPS females spent more (p<0.05) time in a quadrant of interest than non-stressed.

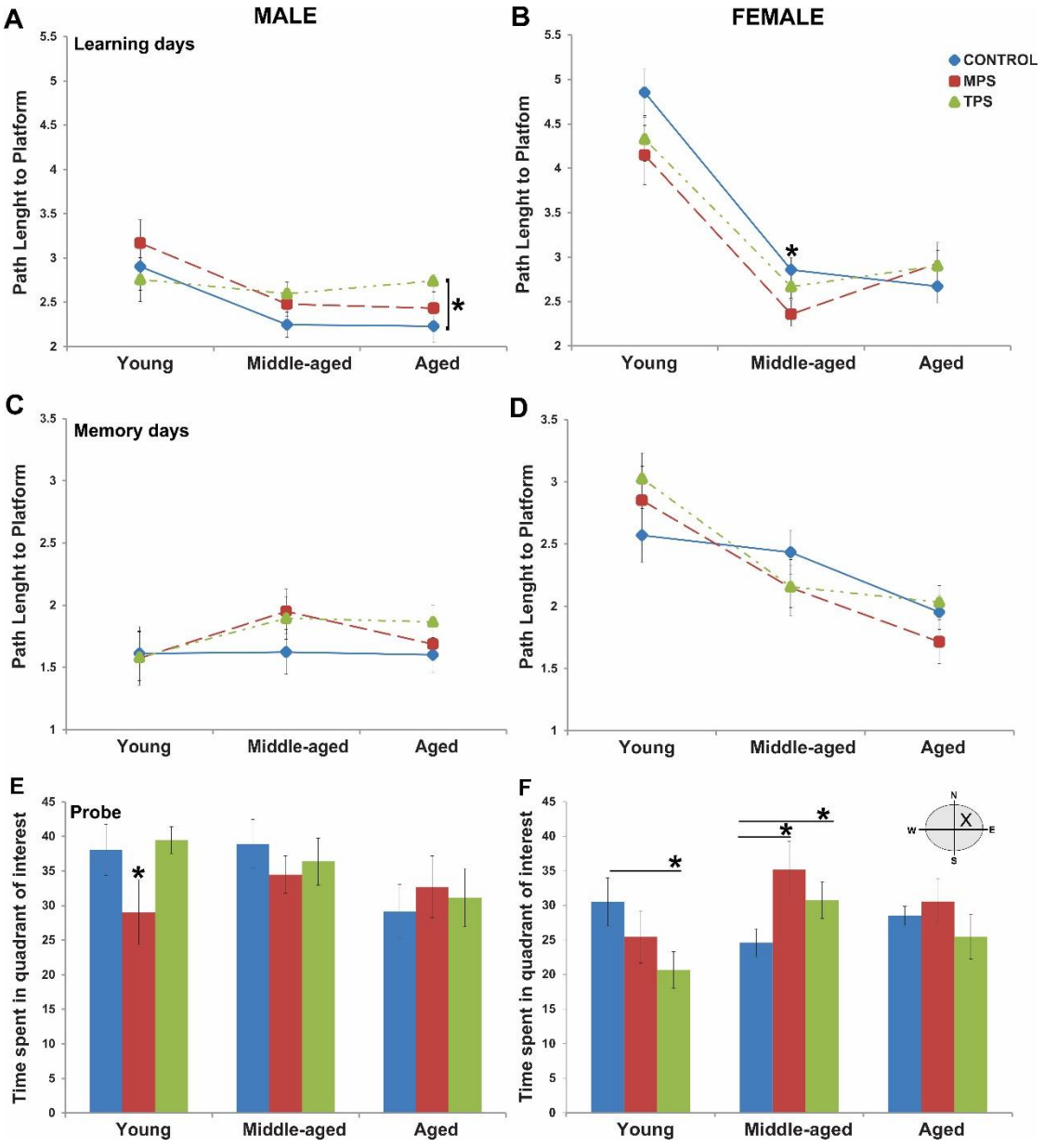

Figure 4.6. Ancestral stress and aging induce sex-specific effects on spatial learning and memory. Spatial learning and memory performance as measured by the Morris water maze task across the lifespan. (A, E) TPS male offspring showed impaired learning in old age as indicated by the longer path length, while young MPS males had impaired memory as indicated by spending smaller percentage of time in the quadrant of interest than CONTROL and MPS offspring on probe day. (B, F) MPS middle-aged females had the shortest path length on the learning days, thus improved spatial learning when compared to TPS and CONTROL animals. Interestingly TPS female offspring showed memory impairments in young and memory improvements in middle-age when compared to CONTROL group. Similarly, MPS females had improved memory in middle-age in comparison to non-stressed (CONTROL) females. Asterisks indicate significances: *P < 0.05, **P<0.01. All data are presented as mean ± SEM.

4.5 Discussion

Inter-generational early life stress alters brain development and behavioural phenotypes in adult offspring potentially through immune-mediated activation (Babb et al., 2014b; Howerton & Bale, 2012; Murgatroyd et al., 2016). Here for the first time we provide evidence for life-long, sex-specific alterations in stress and immune activation, neuromorphology and cognitive function in the F4 ancestrally stressed offspring. The present study shows three main findings. First, ancestral stress exacerbated aging-dependent immune, brain and behavioural changes in F4 generation offspring, as indicated by heightened stress and immune activation, altered locomotor, fine motor and cognitive functions in both trans- and multigenerational stress F4 offspring. Second, ancestral stress induced sex-specific biological aging trajectories, as stress-induced morbidity and mortality occurred earlier in males than in females. Third, ancestral stress exhibited stress type (trans- vs multigenerational) specific effects across the lifespan. While in males trans- and multigenerational stress had equally adverse impact, in females multigenerational stress induced partial resilience and least impairments early in life, while transgenerational stress had adverse consequences across the entire lifespan. Thus, the present data indicate for the first time that maternal social stress during pregnancy is transmitted to their distant great-great-grand offspring to alter lifetime health outcomes and accelerate biological aging.

The present study corroborates previous evidence that adverse experiences early in life can propagate across multiple generations to alter hypothalamic-pituitary-adrenal (HPA) axis sensitivity (Faraji et al., 2017; Faraji et al., 2018; McCreary et al., 2016; McCreary et al., 2016; Babb et al., 2014b). The present data are the first to show that even remote ancestral stress effects persist across the lifespan and exhibit sex-specific changes.

Maternal social stress during pregnancy induced heightened stress sensitivity particularly in middle-aged males and aged females. Since the HPA axis cross-communicates with the immune system (Howerton & Bale, 2012) through corticotropin-releasing factor (CRF), adreno-corticotropic hormone (ACTH) and glucocorticoids (Eskandari et al., 2003; Howerton & Bale, 2012; Tait et al., 2008), peripheral and central interactions between stress and immune systems may activate a pro-inflammatory or immunosuppressive cascade (Steinman, 2004; Tait et al., 2008). Here we report that aging promoted stress and immune response activation by raising corticosterone levels and pro-inflammatory cytokine secretion, such as IL-5, IL-18, M-CSF and MCP-1. These findings are in line with previous research demonstrating that early life stress alters systemic immune response and raises pro-inflammatory cytokine levels such as the IL-1 family, IL-6 and TNF-alpha and IL-18 in young animals (Coussons-Read et al., 2007; O'Mahony et al., 2011; O'Mahony et al., 2009).

Our findings confirm the previous report by Murgatroyd and colleagues (2016), where transgenerational social stress impaired immune response in early adulthood (postnatal day 70), by increasing levels of IL-18 and GM-CSF in F2 male offspring only (Murgatroyd et al., 2016). This is not surprising considering that the pro-inflammatory cytokines such as IL-18 and chemokines are affected by stress (Alboni et al., 2010; Murgatroyd et al., 2016; Ojala & Sutinen, 2017; Sugama & Conti, 2008; Swartz et al., 2017) and influence brain development, behavioural pathologies and disease. Aging was shown to represent a significant risk factor for altered immune and stress functions (Ostan et al., 2017) which seems to synergistically interact with ancestral stress. Specifically CRF as a upstream mediator of stress, acts as an immune stimulant enhancing B and T cell proliferation (Karalis et al., 1995; Wei et al., 1986). Our previous studies showed that

ancestral early life stress alters both expression of the CRH gene (Ambeskovic et al., 2017) and the B and T cell proliferation regulator miR-150.

Many behavioural pathologies observed in prenatally stressed offspring have been linked to stress-induced maternal or ancestral immune activation (Eskandari et al., 2003; Howerton & Bale, 2012; Steinman, 2004; Tait et al., 2008). The reciprocal interaction between the immune activation and HPA axis responses offers a causal mechanism by which the maternal stress during pregnancy can affect fetal brain development, cortical neuromorphology and behaviour (Howerton & Bale, 2012; Murgatroyd et al., 2016).

Maternal social stress induced sex- and age-specific effects on gross and fine motor function in the F4 offspring, with the largest deficits observed after transgenerational stress exposure. Specifically, stress diminished locomotor activity in middle-aged transgenerational stress females, fine motor function and hind limb placement precision in young transgenerationally stressed males, while it enhanced reaching skills in aged multigenerational stress females. This confirms previous findings indicating heightened stress sensitivity in young males (Ambeskovic et al., 2017; Erickson et al., 2014; Franklin et al., 2010), and stress resilience in females (Faraji, et al., 2017). The early life motor impartments observed in these F4 transgenerational stress male offspring arguably reflects genuine epigenetic inheritance of stress impact and suggests that similar impairments in children exposed to gestational stress (Grace et al., 2016; Huizink, et al., 2003) or prenatally stressed rodents (McCreary et al., 2016) may represent the precursors of this phenomenon.

Motor impairments were accompanied by cognitive decline in both trans- and multigenerationally stressed male offspring. Cognitive deficits occurred earlier in life in stressed males compared to females, indicating greater vulnerability to stress- and sex-mediated accelerated aging processes in males. For example, transgenerational stress male

offspring experienced learning impairments in old age (18 months), as opposed to facilitated cognitive functions in multigenerational stress middle-aged females. In addition, transgenerational stress aged males displayed a sharp increase in the pro-inflammatory cytokine IL-18, which was found to be a marker of cognitive deficits (Andreotti et al., 2015; Bossù et al., 2008; Lee et al., 2018; Leite-Almeida et al., 2009). Spatial learning impairments also coincided with aging-associated increases in pro-inflammatory cytokines of IL-5, M-CSF, and MCP-1 in young and middle-aged males to reach equivalent levels found in middle-aged and aged non-stress offspring. However, spatial memory improved in middle-aged transgenerational and multigenerational stress females, indicating a potential midlife advantage. Although no other data exist on the transgenerational effect on cognitive function, other human (Buitelaar et al., 2003; de Rooij et al., 2010; Huizink et al., 2003; Laplante et al., 2004; Robinson et al., 2011; Sandman et al., 2012) and animal studies (Faraji et al., 2018; Grassi-Oliveira et al., 2016; Griffiths & Hunter, 2014; Wong et al., 2016) showed similar cognitive impairments in offspring exposed to early life adversity.

Exacerbated stress and immune responses may have contributed to HPC atrophy giving rise to hyperactivity and cognitive deficits. Both trans- and multigenerational stress reduced the HPC mean gray value in middle-aged F4 males and females to resemble the MGV of non-stressed aged males and females. Interestingly, aging-associated PFC atrophy was highest in transgenerational males, and lowest in young females. This PFC volume change may be associated with stress-related impairments in physical activity and memory of young males. The PFC atrophy and stress related behavioural impairments may have resulted from stress induced reduction in the brain derived neutrophic factor (BDNF) (Murgatroyd et al., 2016; Ślusarczyk et al., 2015; McCreary et al., 2016; Lupien et al.,

2009), and reduced synaptic density (McCreary et al., 2016; Mychasiuk et al., 2012; Ambeskovic et al., 2017; Hunter & McEwen, 2013) in response to stressful environments.

HPC and PFC morphology may have been affected by a reciprocal relationship with stress and immune response, as the presence of GRs renders them susceptible to HPA axis hyperactivity and capable of negative feedback regulation. Their atrophy will therefore provide a mechanism by which intergenerational programming by stress may affect immune function and behaviour. Accordingly, stress effects are usually more severe in the brain regions closely regulated by the HPA axis (Glover et al., 2010; Goel et al., 2014; Kudielka & Kirschbaum, 2005; Murgatroyd & Spengler, 2011) and GRs (Gassen et al., 2017; Sorrells et al., 2014) such as HPC than PFC. This potentially indicates weaker inhibitory regulation of immune response and the HPA axis to stress. Nonetheless, any morphological changes will have long-term effects on the affective state (Faraji et al., 2018, 2017; Franklin et al., 2010; McCreary et al., 2016a), and behavioural phenotypes.

Converging evidence has also demonstrated that IL-18 has an interconnected relationship with the HPA axis which also affects brain and behaviour (Alboni et al., 2010; La Fratta et al., 2018; Sugama & Conti, 2008). IL-18 expression has been suggested to affect long-term potentiation (LTP) (Cumiskey et al., 2007), NMDA receptor affinity (Curran & O'Connor, 2001) and activity of amygdalar glutamatergic neurons (Francesconi et al., 2016). Similarly, M-CSF and MCP-1 play a role in brain development and functioning (Luo et al., 2013; Smith et al., 2013; Yao & Tsirka, 2014). Therefore, it is not surprising then that various human and animal studies have shown IL-18 association with emotional behaviours, motor function, and cognitive deficits (Oztürk et al., 2007; Rubio-Perez & Morillas-Ruiz, 2012; Sacchinelli et al., 2018; Yaguchi et al., 2010). Moreover, upregulated MCP-1 levels excite dopaminergic neurons producing hyperactive behaviour

(Guyon et al., 2009; Semple et al., 2010), while abnormal IL-5 and M-CSF levels are associated with cognitive impairments in children and elderly, and neuroinflammatory disease (Andreotti et al., 2015; Laske et al., 2010; Luo et al., 2013; Smith et al., 2013). These data suggest that maternal social stress remodels the innate immune system (Baylis et al., 2013) and aging trajectories according to the concept of inflammaging (Franceschi et al., 2018).

Inflammaging refers to early life adversity that influences later life health outcomes with increased risk of age-related disorders via accelerated aging processes (Gabuzda & Yankner, 2013). Franceschi and colleagues (2018) proposed that inflammaging is unpredicted by evolution, so that intergenerational programming by stress confers fitness early in life but accelerates biological aging later in life. A potential mediator of the accelerated aging in ancestral stress offspring may be NF-kappa B via activation of IL-18 and M-CSF (Franceschi et al., 2018; Sun et al., 2016; Waterland & Michels, 2007; Xia et al., 2016). Here stress revealed sex and age-specific effects on PFC volume, confirming notions that early life stress induces sex-specific alterations in brain density of directly exposed offspring (Kolb & Gibb, 2011; McEwen et al., 2016; Muhammad & Kolb, 2011) and future generations (Ambeskovic et al., 2017; McCreary et al., 2016a). Similar findings were demonstrated in the Dutch famine study, where prenatally exposed F1 generation males but not females, showed smaller intracranial and total brain volumes compared to unexposed subjects at middle-age or 68 years of age (de Rooij et al., 2016).

Adverse early life experiences induce sex-specific behavioural pathologies and disease incidence in exposed offspring (Bale & Epperson, 2015; McEwen et al., 2015; Mueller & Bale, 2008), and future generations (Anway et al., 2006; Anway et al., 2005;

Franklin et al., 2010; Ambeskovic et al., 2017; Murgatroyd et al., 2016). Males seem to be more sensitive to ancestral stress effects at a younger age, while females showed either no deficits or fewer deficits in middle or old age. Potential mechanism that may explain sexually dimorphic effects of stress are alterations in androgen hormone secretion during early development (Bowman et al., 2004), and epigenetic regulation (Morgan & Bale, 2011). Early life stress, including transgenerational stress may dysmasculinize or feminize males and masculinizing females (Bowman et al., 2004; Morgan & Bale, 2011; Zuena et al., 2008). These sex-differences may program early life stress via altered epigenetic regulatory markers such as CRH and miR-34. The interactions between Crh with estrogen and its receptors (ER alpha and ER beta), may affect HPA function (Vamvakopoulos & Chrousos, 1993) and alter overall brain development and long-term health outcomes of the offspring.

4.6 Conclusion

The aging population worldwide is growing and so are the incidences of aging-associated diseases including cognitive disorder and neurodegeneration (Ambeskovic, et al., 2017; Bale & Epperson, 2015; McLaughlin et al., 2010; Rowe & Kahn, 1997). This rapid growth of an aging population presents both opportunities and challenges. The present data for the first time demonstrate that maternal social stress during pregnancy is transmitted to their distant great-great-grandchildren and accelerates biological aging trajectories. Identifying mechanisms of sex-specific programming of the immune and endocrine response and behavioural outcomes by ancestral stress may be helpful in developing a new animal models of mental illness such as post-traumatic stress disorder. In agreement with our data, transgenerational stress was shown to mimics physiological

facets of human PTSD (Yehuda and Brier, 2007) with reduced basal corticosterone levels, while multigenerational stress offers a means to study adaptive responses to recurrent stress. Hence, ancestral stress may provide ecologically valid models to study the origins of PTSD, the biological foundations of resilience and the discovery of new biomarkers and interventions.

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CHAPTER 5: General Discussion & Conclusions

5.1. Cumulative or Singular Adverse Early Life Experiences as Determinants of Stress Vulnerability and Resilience in Generations of Offspring

In accordance with other animal and human studies, we demonstrated that adverse early life experiences such as prenatal stress alter brain development with long-term behavioural consequences to the offspring directly exposed and future generations (Ambeskovic et al., 2017; Bale, 2015a; Constantinof et al., 2016; Roseboom et al., 2011; Skinner, 2014; Yao et al., 2014). Transgenerational programming of offspring in distant generations by stress may occur through three main mechanisms including altered maternal stress response, altered maternal post-partum behaviour and transmission of epigenetic marks (Ambeskovic et al., 2017; Bale, 2015b; Matthews & Phillips, 2012).

Stressful experiences during pregnancy may modify the maternal stress response via hypothalamic-pituitary-adrenal (HPA) axis regulation. Excessive and chronic maternal stress may result in excess exogenous glucocorticoid release which may cross the placenta, possibly programming fetal HPA axis activity and influencing the developing brain (Iqbal et al., 2012; Whittle et al., 2001). Indeed, maternal excess glucocorticoids during pregnancy have been linked to altered HPA axis activity and impaired negative feedback regulation in her F2 offspring (Iqbal et al., 2012). In line with this evidence in Chapters 2, 3 and 4 we demonstrated that either MPS or a single generation of ancestral maternal stress (TPS) altered the HPA axis function in F4 offspring independent of the type of stressors.

A second mechanisms by which experience that may be transmitted across generations is modified maternal care. Convincing evidence has linked maternal exposure

to stress during pregnancy to impaired licking and grooming of her pups (Francis et al., 2002; Turecki & Meaney, 2016; Weaver et al., 2004). Impaired maternal care during the early postnatal period alters HPA axis function and feedback loop in the offspring and future generations via epigenetic regulation of steroid receptors such as glucocorticoid receptors (GR's; Constantinof et al., 2016; Iqbal et al., 2012; Turecki & Meaney, 2016; Weaver et al., 2004). Although this thesis did not investigate the quality of maternal postpartum care, previously published studies from our lab showed that stressed mothers and grandmothers showed a significant reduction in tail chasing behaviour in F1-SS, F2-SNN and F2-SSS generations, which may be indicative of impaired postnatal care (Ward et al., 2013). These findings are in agreement with other research showing that maternal care can alter offspring's stress response and later maternal care (Nestler, 2011), and demonstrate that pre-partum maternal behaviour in rats is programmed by inter-generational inheritance of stress response (Ward et al., 2013) via both multigenerational (MPS) and transgenerational stress (TPS) lineages. This observation indicates that our F4 restraint stress (Chapters 2 and 3) offspring may have been subject to altered maternal care, which may influence the activity of their stress response system and resilience.

Lastly, a third mode of transgenerational inheritance is the transmission of epigenetic marks via germline. The transmission of epigenetic marks most likely occurs during early embryogenesis, when most DNA methylation is erased, however following the exposure to adverse experiences a few epigenetic marks escape the erasure and are passed to future generations (Constantinof et al., 2016; Okano et al., 1999; Kovalchuk, 2012; Siklenka et al., 2015). Because the objective of this thesis was to investigate the transgenerational inheritance of health outcome later in life, the early embryogenetic effects

were not investigated. However, we did report in Chapter 3 altered miR-29 expression in F4 MPS offspring. This miRNA that plays an important role in regulation of the DNA methyltransferase 3 gene (DNM3A and DNM3B) (Morita et al., 2013; Takada et al. 2009). This gene encodes a DNA methyltransferase which is thought to function in de novo methylation and its expression is essential for normal imprinting and embryonic development (Morita et al., 2013; Okano et al., 1999). It is possible that DNA methylation during early embryogenesis may have been altered in our F4 offspring, and that these markers may have been transferred across generations in form of miRNA expression change. It is also possible that these epigenetic marks were transmitted from great-grandmother or acquired from each generation of stress in the MPS lineage. Unfortunately, we did not perform miRNA profiling of TPS animals, nor did we do miRNA analysis of the social isolation animals (TPS nor MPS). It would have been interesting to determine if similar miRNA alterations are observed in those cohorts.

It is important to note that the MPS offspring represent cumulative impact of repeated stress across generations. In addition to carrying the burden of stress from remote generations, this group also received direct stress over four consecutive generations. Thus, stress effects in F4 offspring may have been transmitted and accumulated across multiple generations and interact with stress-induced epigenetic marks being transmitted across generations. Because there has been no other laboratory investigating this type of stressor in a controlled environment the existing literature on their characteristic vulnerability and resilience is limited. In general, the multigenerational cohort is uniquely suited to investigate the impact of multiple hits, which may promote epigenetic and stress response adaptation or resilience.

5.2. Ancestral Stress Affects Brain, Behaviour and Disease Incidence in the F4 Generation Across the Lifespan

Adverse experiences early in life reprograms brain development with lifelong behavioural consequences in offspring directly exposed and remote generations. This is especially true if the environmental insults occur during critical pre- and post-natal periods, during which the brain is most sensitive to the external environment. Previously, we showed that exposure to prenatal stress over four consecutive generations can alter neuronal complexity and dendritic spine density in the parietal cortex in the adult F4 multigenerational stress (MPS) offspring (Ambeskovic et al., 2017). Moreover, in Chapter 2 of this thesis the results demonstrated altered dendritic spine density in frontal cortex regions (medial prefrontal cortex and orbital frontal cortex) of F4 MPS offspring. Furthermore, prenatal stress alters mean gray values and volumes of various brain regions including the hippocampus of F3 generation multigenerational stress female offspring (McCreary et al., 2016) and prefrontal cortex and hippocampus in F4 MPS and TPS offspring (Chapter 4). Neuromorphological and neuroanatomical modifications are generally observed in association with behavioural pathologies in offspring whose ancestors were exposed to stress during pregnancy.

In line with other work (Babb et al., 2014; Franklin et al., 2010; Kiss et al., 2016; Murgatroyd et al., 2016) we demonstrated that prenatal stress propagates across generations to induce depressive- and anxiety-like behaviours in F2-F4 generation

		Male			Female	
Measurement	Young	Middle-Aged	Aged	Young	Middle-Aged	Aged
Baseline Corticosterone Levels	↓MPS, TPS; ↓MPS*,	↑MPS**, TPS*; nc	↓MPS, TPS; ↓MPS**	↓MPS; ↑MPS	↑TPS; ↓MPS***	↓MPS; TPS**; ↑MPS
Interleukin 18	↑MPS*, TPS*;	↑MPS, ↓TPS	↓MPS, ↑TPS**	↑MPS	↑TPS	↓MPS, TPS
Interleukin 5	↑MPS*	↓MPS, TPS	↓TPS	↓MPS, TPS	↓MPS, TPS	↓MPS*, TPS*
MCP-1	no change	↑MPS*	↑MPS, TPS	↓MPS, ↑TPS	↑MPS, ↓TPS	↑MPS*, TPS
M-CSF	↑MPS, TPS	↑MPS*, TPS*	↑MPS, TPS	↑MPS, TPS	↑MPS, TPS	↑MPS, TPS
Prefrontal Mean Gray Value	↑TPS*	↓TPS	n/c	↓TPS	↑MPS, TPS	n/c
Hippocampal Mean Gray Value	↑MPS, TPS	↓MPS*, TPS	↓MPS, TPS	no change	↓MPS**, TPS*	↓MPS, TPS
Locomotion (OFT)	↑MPS, ↓TPS; ↑MPS	↓TPS; ↑MPS	n/c; n/c	no change; ↑MPS*	↑MPS, ↓TPS*	↑MPS; ↑MPS*
Forelimb Fine Motor Function (SPRT)	↓TPS* ↓MPS; ↓MPS	↓TPS	↓MPS, TPS	↑MPS, ↑MPS; ↓TPS	↑MPS, ↓TPS	↑MPS*; ↑MPS
Hindlimb Coordination (Ladder)	↓TPS*; ↓MPS	↓MPS, ↑TPS; n/c	↓MPS, TPS; ↓MPS*	↓MPS*, TPS; n/c	↓MPS; n/c	↓MPS, TPS; ↓MPS
Learning (MWT, path length to platform)	↓MPS, n/c; ↑TPS	↓MPS, n/c; TPS	↓MPS, TPS*	↑MPS, ↓TPS	↓MPS, TPS	↑MPS
Memory (MWT, probe)	↓MPS*	↓MPS, TPS	no change	↓MPS, TPS*	↑MPS, TPS	n/c
Anxiety-like behaviour (EPM)	↑MPS ↑MPS*			↓MPS ↓MPS*		

Table 5.1. Overall behavioural and physiological changes in ancestral stress offspring. The black label denotes the F4-generation offspring subject to gestational social isolation, while the red label denotes the F4-generation offspring whose mothers were exposed to restraint and swim stress during pregnancy. ↑increase, ↓decrease, * significant at $p<0.05$, ** significant at $p<0.01$, n/c- no change.

offspring. In Chapters 2 and 3, we observed increased levels of depressive- and anxiety-like behaviours in F4 MPS offspring across the lifespan. In addition to changes in mental

functioning we report various physical alterations including impaired forelimb and hind limb fine motor function in Chapters 3 and 4, as observed in F4 TPS and MPS offspring across the lifespan. Furthermore, prenatal isolation stress both TPS and MPS altered cognitive function including spatial memory in males and females (Table 5.1)

Exposure to adverse environments early in life affects not only brain development and brain function but also the health and function of other organs (Crews et al., 2012) and can contribute to shorter lifespan. Furthermore, literature has shown that gestational exposure to adverse environment can reprogram organ development (Ashman et al., 2016). Moreover, that stress can induce tear and wear effects on the body, thus affecting not only brain functioning but also the health and function of other organs including kidneys and heart (Hunter & McEwen, 2013; McEwen et al., 2015). For example, Skinner and his lab have demonstrated that exposure to chemical agents early in life results in transgenerational epigenetic inheritance of disease such as mammary tumors, prostate, kidney and immune disorders (Anway et al., 2006). Similarly, in Chapter 3 we are the first to report increased incidence of renal failure, tumors and decreased lifespan in F4 MPS offspring due to stress (Table 5.3).

5.3. Ancestral Stress Induces Few Similar Behavioural and Physiological Phenotypes Independently of Stressor Type

Exposure to adverse environments during the prenatal period reprograms brain development with lifelong behavioural consequences and increased risk of disease (Ambeskovic et al., 2017; Anway et al., 2006; Bale, 2015a; Champagne & Meaney, 2006; Skinner, 2008; Yao et al., 2014). Previous research has demonstrated that stress type, severity, duration and the developmental window during which stress occurs have different

effects on brain plasticity and behavioural outcomes in subsequent generations of offspring (Bale, 2015a). Although the timing of stress exposure and developmental programing has shown some specific effects, there are also general commonalities observed independent of stress type and duration.

		Male				Female	
Measurement	**Young**	**Middle-Aged**	**Aged**		**Young**	**Middle-Aged**	**Aged**
Baseline Corticosterone Levels	↓MPS, ↓MPS*		↓MPS ↓MPS**				
Locomotion (OFT)	↑MPS, ↑MPS ↓TPS; ↓TPS		n/c; n/c				↑MPS; ↑MPS*
Forelimb Fine Motor Function (SPRT)	↓MPS ↓MPS				↑MPS, ↑MPS		↑MPS* ↑MPS
Hindlimb Coordination (Ladder)			↓MPS, ↓MPS*				↓MPS, ↓MPS
Anxiety (EPM)	↑MPS ↑MPS*				↓MPS ↓MPS		

Table 5.2. Same phenotypic changes independent of stress type in ancestral stress offspring. The black label denotes the F4-generation offspring subject to gestational social isolation, while the red label denotes the F4-generation offspring whose mothers were exposed to restraint and swim stress during pregnancy. ↑increase, ↓decrease, * significant at $p<0.05$, ** significant at $p<0.01$, n/c- no change.

In this thesis, we investigated the effects of restraint stress experienced during a time that is equivalent to the third trimester of human pregnancy (Bale, 2015b) in Chapters 2 and 3 and social isolation experienced during the entire pregnancy in Chapter 4. Stress occurred in the great-great-grandmother of the transgenerational stress (TPS) lineage or for four consecutive generations of mothers in the multigenerational stress (MPS) lineage. In both cases, stress started four generations removed from the F4 generation. These two

cohorts were bred under carefully controlled conditions and tested at different timepoints (2 years difference), but they were all housed in the same rooms, underwent same testing procedures and were in most cases tested by a same experimenter under blind conditions.

Based on multiple behavioural and physiological measures taken, we observed that prenatal ancestral stress exposure altered the stress response and behavioural phenotypes independently of the stress type. The physiological stress response and HPA axis regulation were similarly altered in F4 multigenerational (MPS) male offspring by both prenatal restraint stress and prenatal social isolation (Table 5.2). Interestingly, both young and aged male offspring revealed reduced baseline corticosterone levels, indicating dysfunctional HPA axis regulation. This is not surprising, as the literature has demonstrated that male offspring are more sensitive to adverse environmental exposure early in life (Mueller & Bale, 2008) and to ancestral stress (Dunn et al., 2011a). Moreover, both prenatal ancestral stress types altered physical and emotional outcomes including overall exploratory activity and locomotion, fine motor function, hindlimb coordination and anxiety-like behaviours predominantly in multigenerational stress (MPS) offspring (Table 5.2). We observed increased locomotion or hyperactive behaviour in the open field task in MPS young male offspring and MPS aged female offspring, while a decrease in overall activity was observed in TPS males. Both restraint and social isolation prenatal ancestral stress impaired fine motor function in young MPS males, while stress improved fine motor function in young and aged MPS females. However, impaired hindlimb coordination was observed in both aged MPS male and female offspring. In addition to altered physical wellbeing, sex-specific emotional effects were observed, with increased levels of anxiety-like behaviours in F4-young male offspring, and a slight decrease in MPS young female offspring.

Similar behavioural and physiological alterations observed in multigenerational stress cohorts may be explained by sex-specific evolutionary adaptation. Exposure to stress over multiple generations may better prepare the offspring for postnatal life than a single-generation stress and enhance coping with stressful experiences once they happen. Thus, a hyper-sensitive stress response system may be helpful for survival of male offspring, especially when it comes to hypervigilance (Glover, 2011; Glover et al., 2010) or by increasing the ability to stay away from danger in early years or during the age of high reproductive success. The latter is reflected by depressive-like behaviours in the laboratory test situation. Sometimes the cost of surviving and increased hyperactivity can be detrimental to other functions such as forelimb fine motor function and later life hindlimb coordination (Table 5.2), as observed in males. It may be suggested that the evolutionary adaptation may be improve fine motor function and decrease anxiety-levels in female offspring, enabling them to provide good maternal care, seek social support and raise healthy offspring (Ambeskovic et al., 2017; Faraji et al., 2018, 2017).

It should be noted that the TPS offspring were available for comparisons only at young age (restraint stress cohort), and therefore only a limited number of comparisons were made between TPS offspring exposed to restraint stress and social isolation during prenatal periods. This may have skewed the observations made, and it is possible that some phenotypes in transgenerational offspring may not have been too different or would have been very different depending on the type (restraint vs social isolation) of prenatal stress exposure.

5.4. Different Types of Prenatal Ancestral Stressors (Restraint vs Social Isolation) Produce Sex-Specific Health Outcomes Across the Lifespan

Severity, duration and timing of prenatal stress exposure have been shown to have specific or broad sex-specific effects on health outcomes in the offspring (Bale, 2015). Moreover, transgenerational disease inheritance, arguably via epigenetic factors, determines health outcomes of future generations (Bale, 2015). As reported in previous sections, two types of stress examined in this thesis (restraint and social isolation) resulted in mainly similar stresses response and locomotor, particularly in MPS male offspring when compared to non-stressed CONTROLS (Table 5.2). Interestingly, different prenatal stressors induced sex-specific effects in MPS F4 offspring in terms of stress response, disease incidence and longevity.

The HPA axis activity was influenced by the stress type in a sex-specific manner. Male offspring (young and aged) showed reduced levels of corticosterone independently of stressor type (restraint or social isolation). On contrary, female offspring exposed to multigenerational prenatal social isolation stress revealed decreased levels of corticosterone, while exposure to multigenerational prenatal restraint stress increased corticosterone levels in young and aged females. This observation suggests that the male offspring's stress response system is more sensitive to any environmental stress early in life, while different types of stress have different effects on females. Specifically, social isolation seems to affect the female offspring most profoundly. A recent study by Faraji and colleagues (2018) showed that socially enriched housing conditions enhance the levels of circulating oxytocin in females only. Thus, it may be suggested that females are particularly responsive to social isolation, which may induce the opposite effects by decreasing levels of oxytocin (Faraji et al., 2018), thus impairing the stress response through oxytocin-HPA axis binary effects. Considering the bidirectional communication between oxytocin and the HPA axis system (Gibbs, 1986; Goodson et al., 2015; Parker et

al., 2005) it is possible that the social isolation stress in females may contribute to further alterations in the HPA axis, with potential lifelong consequences on their health outcomes and longevity.

		Male				Female	
	CONTOL	MPS	TPS		CONTROL	MPS	TPS
Life expectancy (max 530 d)	490; 536	482; 524	530		512; 530	530; 502	530
Mortality due to disease incidence	HD & RF; HD & Stroke x 2 (paralysis)	RF x 5; Brain tumor	n/c		RF x 3; Cervical tumor	RF; RF x 4	n/c

Table 5.3. Lifespan and disease incidence (before the experimental endpoint) in ancestral stress offspring. The black label denotes the F4-generation offspring subject to gestational social isolation, while the red label denotes the F4-generation offspring whose mothers were exposed to restraint and swim stress during pregnancy. ↑increase, ↓decrease, * significant at $p<0.05$, ** significant at $p<0.01$, n/c- no change/incidence, HD- heart disease and RF- renal failure.

Although not reported in Chapter 4, we observed (Table 5.3) that concurrent social isolation stress altered the longevity and induced disease incidence in MPS female offspring but not male offspring. According to Faraji and colleagues, social enrichment increases the telomere length in females but not in males (Faraji et al., 2018). Since increased telomere length is associated with increased longevity (Iozzo et al., 2014; Tarry-Adkins et al., 2008) it may suggest reduced morbidity and increased lifespan in females. Although not directly measured here, this association between health and telomere length, may have resulted in shorter longevity of social isolation lineages compared to non-stressed (CONTROL) and TPS females (Table 5.3). By contrast, MPS males of the restraint stress cohort exhibited reduced longevity and increased risk of disease. Interestingly, the shorter

longevity in both restraint stress MPS male offspring and social isolation stress MPS female offspring resulted in premature renal failure.

In conclusion, our data suggest that different types of prenatal ancestral stress and the duration of stress exposure have sex-specific effects on the health outcomes and longevity of offspring. This suggests that male and female offspring have different sensitive periods during early development when they are most susceptive to environmental insults and that androgen hormones may play a major regulatory role in developmental programming (Bale, 2015a; Bale & Epperson, 2015; Dunn et al., 2011).

5.5. Significance of Ancestral Stress Models and Implications for Human Studies

Ancestral stress models can uncover the secret epigenetic memory passed to us by our ancestors without even knowing it. Animal models can help pave the way into investigations of true epigenetic modification in response to adverse environments. We now know that our health outcomes and wellbeing depend on gene-environment interactions, therefore we should make every effort to understand such interactions and the specific epigenetic modifications at play, so we can promote risk prediction, targeted intervention and improve our health outcomes. Using animal models to study the epigenetic modifications in ancestral stress cohorts is an excellent way to control for confounding factors which are hard to control for in human studies and ultimately investigate the true environmental influence on our health via epigenetic regulation.

Understanding the mechanisms behind the epigenomic changes and transgenerational inheritance of health and disease is becoming ever so important in today's environment. Understanding how the experiences of our ancestors many years ago, and our own experiences can interact to affect our health and wellbeing today will help us

understand and treat individuals with physical and mental illnesses differently. For example, ancestral stress studies can help understand the risk of diabetes, mental health and addiction in Indigenous populations. Considering that most Indigenous populations have an ancestral history of trauma through colonization and residential school systems, along with a lifetime of stressful experiences through discrimination and often poor living conditions, the multigenerational stress model is very helpful for understanding the causes of health outcomes of these populations and design potential interventions. Convincing literature had demonstrated that adverse experiences can change the epigenome in ancestors, and that these epigenetic marks can then be transmitted across generations to their distant offspring to interact with their environment and induce certain phenotypes (Ambeskovic et al., 2017; Crews et al., 2012; Mannironi et al., 2013; Morgan & Bale, 2011; Painter et al., 2012). However, behavioural adversity may be mitigated by enriched environments within that generation either trough parent (Leshem & Schulkin, 2012), early postnatal (Leshem & Schulkin, 2012) or adolescent (McCreary et al., 2016) offspring's direct exposure to enriched environments. Thus, enriched environments and beneficial experiences can mitigate the negative effects of ancestral adversity at any time in life.

Moreover, ancestral stress models such as transgenerational lineages may help us discover the Western nations' problem of high disease incidences such as Alzheimer's disease and mental illnesses. Migration of populations following the world wars and current migration crisis may be explained or understood better through the Barker hypothesis of mismatch in the prenatal and postnatal environments and its links to risk of disease and adverse health outcomes not seen before (Barker et al., 1993; Barker, 2007). This mismatch may result when the prenatal adaptions of biological systems such as neuro-endocrine

(HPA axis) and immune systems do not match actual environments that are activating them, resulting in altered functioning and activity of these systems.

5.6. Limitations and Future Directions

There are few limitations of our ancestral stress models that need to be considered. First, when studying the true effects of transgenerational stress inheritance, it would have been ideal to include all generations, F1-F4 and all lineages (F1-N, S; F2-NN, F2-SN, F2-SS; F3-NNN, F3-SNN, F3-SSS, and F4-NNNN, F4-SNNN (TPS), and F4-SSSS (MPS)). Since the objective of this thesis was to investigate the aging effects of ancestral stress, which took over 18 months of just animal testing for one generation in each of two cohorts, for a total of 36 months of data collection for 2 cohorts. Thus, time, personnel and financial constraints made it impossible to test all generation for the graduate thesis dissertation.

Second, although we planned to perform more comparisons across cohorts we were not able to perform entirely the same tissue collections in both social isolation and restraint stress cohorts. We believed that the health outcomes would not be too different, so we tried to diversify the data collection to provide a novelty to the experiments. In addition, since these two cohorts were raised and tested at different times, two years apart from each other, from the epigenetic perspective the expected epigenetic signatures were expected to be quite different.

Third, it would have been ideal to include a transgenerational stress group (in restrain stress cohort) to study the differences between trans- and multigenerational stress (MPS) in disease and lifespan changes, particularly for males. However, again due to the planning of the experiments and time, man power and financial constraints this was not feasible.

Fourth, it would have been desirable to include larger animal groups especially for the aging experiment, considering that 50% of the ancestral stress offspring did not reach the experimental endpoint due to various chronic diseases. Unfortunately, this was the very first time that a trans- and multigenerational rodent cohort was examined in an aging study and the considerable morbidity and mortality rate of ancestrally stressed rats was not anticipated at the beginning of the experiment. The aim was for n=12-14 per group, based on statistical power calculations and other research.

The fifth limitation would be that we used cell populations within the frontal cortex in the epigenetic analysis. Since each cell type has different epigenetic profile, and there is more one type of cells than other within any specific brain region, our results may be somewhat generalized or skewed. Thus, in future it would be ideal to do single cell epigenetic profile for each brain region, and pick the cell which makes up most of that region.

The present data demonstrate that ancestral stress studies are critical to the understanding of mental and physical health manifestations, and risk of disease in the absence of genetic determinism. We are the first to show that early life adverse experiences of our ancestors influence our health outcomes across the lifespan and longevity trough epigenetic memory. Moreover, ancestral experiences passed to us trough epigenetic transmission may interact with our experiences and contributes to diverse health outcomes. Interestingly, the severity, type and the duration of early life adversity exhibited sex-specific effects on some health outcomes, disease incidence and longevity, with females being more sensitive to prenatal social stress while males were more affected by prenatal restraint stress.

We anticipate that these findings will facilitate new interventions and treatments to target specific miRNA's involved in mental and physical health manifestations across the lifespan. Furthermore, the identification of miRNAs involved in inheritance of ancestral epigenetic memory may help advance the development of predictive biomarkers of disease and new therapeutic targets. We hope that over next 5-10 years, our data will change the course of new investigations of complex health outcomes by taking a wholistic approach by incorporating ancestral experiences, early developmental programing, sex and the age of the individual when designing and prescribing therapies/treatments. In addition, this research may benefit precision medicine treatments leading to increased treatment success rates and better health outcomes. The ultimate goal of this type of research is that one day these studies will help facilitate development of social and health policies targeting prevention of disease and adverse health outcomes later in life by promoting healthy/enriched early brain development in children.

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