TRANSGENERATIONAL PROGRAMMING OF BRAIN AND BEHAVIOUR BY PRENATAL STRESS

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

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ABSTRACT

Exposure to adverse environmental factors such as prenatal stress (PS) can have long-lasting effects on brain health and disease. Through direct and transgenerational genetic and epigenetic influences on healthy development and aging, PS may promote adaptive developmental plasticity, but at the same time also lead to increased health risks. Ultimately, the main goal of this research was to determine if PS-associated alterations of the fetal developmental programing can be transmitted across generations to affect brain development and behaviour, and ultimately increase the susceptibility to disease throughout lifespan. Work in Chapter 2 showed sexually dimorphic effects of multigenerational prenatal stress on behavioural traits, laterality and hemispheric dominance in male and female rats. In Chapter 3, hair elementary analysis was shown to be a sensitive, comprehensive and accurate screening tool of age-related metabolic and overall health status. Chapter 4 determined the manifestations of PS on behavioural and physiological outcomes in aging male rats after exposure to PS in one generation (F1-PS) vs. multiple generations (F4-PS). These results provide evidence that PS-associated alterations of the fetal developmental programming may be transmitted across regenerations altering brain development and inducing behavioural disturbances throughout lifespan.

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LIST OF ABBREVIATIONS

ACC	Anterior Cingulate Cortex
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit/hyperactive disorder
Al	Aluminium
ANOVA	Analysis of Variance
AVP	Arginine vasopressin
Ca/K ratio	Thyroid gland activity
Cd	Cadmium
CORT	Corticosterone
Co	Cobalt
CRH	Corticotrophin releasing hormone
DES	Diethylstilbestol
EPM	Elevated plus maze
FO	Gestating female
F1	Prenatal stress exposure in one generation
F4	Prenatal stress exposure over multiple (four) generations
FST	Forced swim task
GR	Glucocorticoid receptor
11β- HSD2	11β-hydrohxysteroid dehydrogenase type 2
HPA	Hypothalamus-pituitary-adrenal axis
HPC	Hippocampus
K	Potassium
miRNA	micro RNA
MR	Mineralglucocorticoid receptor
mPFC	Medial prefrontal cortex
Na/K ratio	Adrenal gland activity
NAc	Nucleus accumbens
Ni	Nickel
Par 1	Anterior region of parietal cortex
PFC	Prefrontal cortex
PPD	Post-partum depression
piRNA	Piwi-interacting RNA
PS	Prenatal stress
PTSD	Post traumatic stress disorder
PVN	Periventricular nucleus
ppm	Parts per million
OFC	Orbitofrontal cortex
OFT	Open field task
SDN-POA	Medial preoptic area
Se	Selenium
Ti	Titanium

CHAPTER 1

Introduction: Long-Lasting Effects of Prenatal Stress on Brain, Behaviour and Disease

1.1 Abstract

Exposure to adverse environmental factors such as prenatal stress (PS) can have long-lasting effects on brain health and disease. Through direct and transgenerational genetic and epigenetic influences on healthy development and aging, PS may promote adaptive developmental plasticity, but at the same time also lead to increased health risks. Studies have shown that PS induces fetal developmental reprograming of the hypothalamic-pituitary-adrenal axis (HPA axis) through excess glucocorticoids and altered brain development. PS is associated with morphological changes in prefrontal cortex, hippocampus and amygdala that are accompanied by behavioural disturbances, including a greater risk of anxiety, depression, and cognitive impairments. These changes in brain morphology and behaviour may persist to adulthood and may be causally linked to DNA imprints and altered epigenetic regulation of gene expression. A variety of epigenetic signatures of PS have been identified in association with enhanced vulnerability to neurodegenerative diseases in later life, and shorter lifespan. These epigenetic signatures may be transmitted across multiple generations. In addition, PS effects are not limited to epigenetic programing but may also involve telomere biology inducing cellular aging and increased susceptibility to disease over the lifespan. This review will discuss the main physiological components of PS and their effects on development and programming of the fetal brain and behaviour. Additionally, we will examine the epigenetic changes associated with PS, and consider possible processes of how these may be inherited over many generations. Lastly, we will discuss the latest evidence to create a link between PS-induced epigenetic changes and disease onset.

1.2 Introduction

Exposure to maternal stress during early periods can alter the developing brain and cause a subsequent cognitive, emotional, endocrine and neurochemical responses throughout life (Bowman et al., 2004). In humans, prenatal stress (PS) is associated with delayed early motor development, higher incidence of mild intellectual and language impairment in childhood, along with higher incidence of psychiatric disorders such as schizophrenia, minor depression, and asocial disorder in adulthood (Weinstock, 1997; Weinstock, 2008). Similarly, rodent models of PS show increased latency to play (Charil et al., 2010), increased anxiety-like behaviours levels in an elevated plus maze (Harris and Seckl, 2011), increased floating time (Franklin et al., 2010) in a forced swim task and decrease in learning and memory capacity (Lemaire et al., 2004). Interestingly, the type and the severity of impairments depend on the intensity and duration of the stress as well as the developmental time window during which the stress occurs. For example, human studies reported a high incidence of schizophrenia in offspring whose mother experienced stress during the first trimester (Weinstock, 2008; Charil et al., 2010). However, the offspring of mothers who experienced stress during last trimester rather experienced high incidence of depression and anxiety as adults (Welberg and Seckl, 2001; Weinstock, 2008; Lupien et al., 2009; Charil et al., 2010; Harris and Seckl, 2011). This may indicates that different types of cells and brain areas may be sensitive to PS during different times.

The association between environmental challenges such as PS during pregnancy and the fetal growth and development and later incidence of disease can be explained by the concept of developmental programming (Harris and Seckl, 2011, Cottrell and Seckl, 2009). Since the brain undergoes extreme growth and changes during fetal development, it also becomes the region that is most sensitive to PS. During brain development and programming, PS will alter

the developmental trajectories and neuronal connections affecting its function and the susceptibility to disease (Cottrell and Seckl, 2009). For example, PS affects neuronal morphology of the prefrontal cortex (Kolb et al., 2012) and increases the risk of depressive-like behaviours (Franklin et al., 2010). In PS pups there was a decrease in dendritic branching and length of the orbital frontal cortex (OFC; Muhammad et al., 2012). Similarly, Murmu et al., (2006) reported PS associated reduction in spine density in the anterior cingulate cortex (ACC) and OFC.

Importantly, the effects of PS may potentially be transmitted to subsequent generations without further exposure of the F1 generation (Skinner 2008; Cottrell and Seckl, 2011; Skinner et al., 2011). Key components in transgenerational transmission of PS effects are epigenetic mechanisms (Meaney, 2010; Migicovsky and Kovalchuk, 2011; Skinner et al., 2011). PS-induced epigenetic modifications can be maintained through germ cell maturation (Harris and Seckl 2011, Skinner et al., 2011). Transgenerational inheritance involves exposure of the parental generation resulting in transmission to unexposed generations, while direct exposure over multiple generations will result in multigenerational phenotype. Although very little is known about the mechanism by which transgenerational or multigenerational exposure to PS may affect the offspring's development and pathologies, the effects of PS on brain and behaviour in the offspring (F1 generation) have been well studied.

The previous literature indicates that PS alters the HPA axis activity ultimately affecting the fetal development. This review will discuss the main physiological components of PS and their effects on development and programming of the fetal brain, development and behaviour. Additionally, we will summarize the effects of PS that last to adulthood and may affect aging and disease. Lastly, we will examine the epigenetic changes associated with PS, and consider possible processes of how these may be inherited over many generations. The

main focus of this review is that the PS-associated alterations of the fetal developmental programing can be transmitted across generations to affect brain development and behaviour, and ultimately increase the susceptibility to disease throughout lifespan.

1.3 Circulating hormones during gestational stress affect long-term HPA axis programming

Exposure to maternal stress during the gestational period can lead to fetal CNS programming and development. Maternal stress hormones which reach the fetal brain via placenta include catecholamines (epinephrine, norepinephrine and dopamine), corticotrophin releasing hormone (CRH) and adrenal steroids or glucocorticoids (Weinstock, 2005). In humans, CRH is released from the placenta and can induce release of glucocorticoids from the fetal adrenal by activating the CRH type 1 receptor, which is present from mid-gestation (Smith et al., 1998; Weinstock, 2008). Rodents and non-human primates do not have CRH in placenta, however, maternal stress increases fetal circulating stress hormones such as cortisol in monkeys, and aldosterone, catecholamines, adrenocorticotrophic hormone (ACTH), and corticosterone (CORT) in rats (Weinstock, 2008).

1.3.1. Glucocorticoids

The main hormones released in response to stress are glucocorticoids (cortisol in humans; CORT in rodents). Glucocorticoids play a vital role during normal fetal development. Pregnancy results in a natural increase in this circulating maternal hormone that is transmitted to the fetus. Glucocorticoids that cross the placenta influence the developing fetus by binding to glucocorticoid receptors (GR) or mineralocorticoid receptors (MR) which are expressed in most fetal tissue, including the placenta from early embryonic stages (Cole et al., 1995; Lupien et al., 2009; Harris and Seckl, 2011). Thus, circulating glucocorticoids are essential for normal

tissue development. For example, glucocorticoids support lung maturation and production of surfactant necessary for extra-uterine lung function (Weinstock 2008; Charil et al., 2010; Harris and Seckl, 2011; Cottrell and Seckl, 2009). Furthermore, glucocorticoids are needed for healthy brain development by supporting the remodeling of axons and dendrites, initiating terminal maturation and promoting cell survival (Harris and Seckl, 2011). Although glucocorticoids are necessary for normal fetal development, its mode of secretion follow an inverted U-shape curve in that excessive or insufficient amounts of this hormone will have a drastic effects on developmental processes.

1.3.2 Placenta and HSD2

One role of placenta is to moderate the fetal exposure to maternal circulating levels of glucocorticoids (Cottrell and Seckl, 2009). Although the glucocorticoids can pass freely across placenta, the levels are significantly lower in fetus than in the mother. This is because the placental enzyme 11β-hydrohxysteroid dehydrogenase type 2 (11 β-HSD2), which breaks down glucocorticoids, provides a protective barrier for the fetus. This barrier allows only 10-20% of maternal glucocorticoids to reach the fetus. During periods of severe maternal stress, however, excessive maternal glucocorticoid levels may saturate this placental isozyme and cross the placenta (Benediktsson et al., 1997). Indeed, PS offspring show a reduction in placental HSD2 (Harris and Seckl, 2011). Thus, altered basal levels of 11 β-HSD2 in placenta will result in fetal exposure to excess glucocorticoid levels.

The effects of PS on reduction in placental 11 β-HSD2 activity have two corollaries. First, from the evolutionary prospective it may be beneficial that the fetus receives a reliable signal about the environmental adversity it may face after birth (Weinstock, 2008). This will ensure the survival and possibly reproduction by providing resilience and strength; however the

cost may be increased incidence of diseases and short lifespan (Glover 2010). Second, since the placental 11 β-HSD2 mRNA expression drops dramatically toward the end of gestation (Harris and Seckl, 2011), it allows excess glucocorticoids to pass across the placenta and reach the fetus. Importantly, the low levels of placental 11 β-HSD2 activity are correlated with low birth weight in both human and rodent models (Cottrel and Seckl 2009; Weinstock 2008). Excess amounts of glucocorticoids that reach the fetus may have long-lasting consequences on offspring health by means on HPA axis programming.

1.3.3 PS and HPA axis programming

Recent animal studies suggest that PS and excess amount of glucocorticoids are associated with an exaggerated and prolonged stress response in offspring, indicating a reduced negative feedback to regulate HPA axis activity (Cottrell and Seckl, 2009). Activation of the HPA axis results in the release of corticotrophin releasing hormone (CRH) (see Figure 1.1) and arginine vasopressin (AVP) from the hypothalamic periventricular nucleus (PVN). This will trigger the subsequent secretion and release of adrenocorticotropic hormone (ACTH) leading to production and release of glucocorticoids from the adrenal cortex (Lupien et al., 2009; Cottrell and Seckl, 2009). Increased levels of circulating glucocorticoids will now feedback to reduce the HPA axis response, thus preventing excess production of stress hormones and return to a set homeostatic point. These feedback loops involve various levels of regulation, including the adrenal pituitary, the hypothalamus and other brain regions such as hippocampus and frontal cortex (Lupien et al., 2009; Charil et al., 2010; Harris and Seckl, 2011).

The hippocampus has two types of glucocorticoid receptors, mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs; Zhe et al., 2008). These receptors differ in their affinity to glucocorticoids. MRs have a higher affinity for cortisol than GRs (Zhe et al.,

2008). PS is associated with reduction in hippocampal GRs (Sapolsky et al., 2000; Lupien et al., 2009; Charil et al., 2010). Rodent studies have reported altered density of hippocampal GRs in pups exposed to PS (Henry et al., 1994). Furthermore, the reduction in hippocampal GR is believed to be associated with reduced negative feedback of the HPA axis (Zhe et al., 2008; Cottrell and Seckl, 2009; Lupien et al., 2009; Charil et al., 2010). Indeed, the lack of hippocampal GR is altering HPA axis inhibition loop and causing over-activity and elevation of circulating glucocorticoids (Cottrell and Seckl, 2009). Additionally, literature reports suggest that the overactivity of the HPA axis in depressive-like behaviours may be due to an abnormality of GR density at the limbic-hippocampal level (Zhe et al., 2008). Importantly, this elevation in circulating glucocorticoids can have long-lasting effects on brain and behaviour of an individual throughout the lifespan.

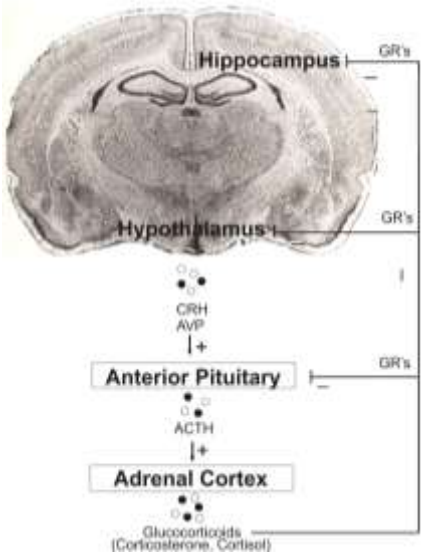

Figure 1.1: Schematic representation of major stress response system: Hypothalamo-pituitary-adrenal (HPA) axis. CRH, corticotrophin-releasing hormones; AVP, arginine vasopressin; GR, glucocorticoid receptor; ACTH, adrenocorticotrophic hormone.

1.4 PS modulates the trajectory of fetal brain development

The fetal brain development is characterized by a high turnover of neuronal connections

that predict the behavioural outcomes (Weinstock, 2008). Therefore, this rapid growth rate makes the fetal brain very vulnerable to the maternal stress hormones such as glucocorticoids (Weinstock, 2008). Exposure to such hormones during the critical window of development could impede the formation of correct neural connections, reduce plasticity and neurotransmitter activity, and ultimately alter the behaviour (see Figure 1.2). Indeed, recent experimental data suggest that the gestational stress predisposes offspring to excess amounts of glucocorticoids during the critical window of development and alters the vulnerable brain regions such as amygdala, prefrontal cortex, hippocampus, and hypothalamus (Weinstock, 2008; Charil et al., 2010; Markam and Koening, 2011; Harris and Seckl, 2011). The specific effects of PS on these brain areas will be discussed below.

1.4.1 Amygdala

The amygdala is central in both mood regulation and the mediation of fear responses (Weinstock 2008; Harris and Seckl, 2011). Experimental evidence suggests that PS affects the development, size and functioning of the amygdala. For example, neurons in the basolateral amygdala (blA) are generated during days 14 and 17 of gestation in rats and are responsive CRH and CORT (Weinstock, 2008; Charil et al., 2010). Studies have shown that the amygdala of PS offspring have higher levels of CRH and its receptors in amygdala (Weinstock, 2008). Furthermore, PS is associated with an expansion of lateral nucleus (Salm et al., 2004). Interestingly, greater anxiety in male PS rats was correlated with fewer BDZ binding sites in the central nucleus (ceA) of amygdala (Weinstock, 2008; Markam and Koening, 2011). This evidence indicates that the development of the fetal amygdala is very sensitive to excess maternal stress hormones, which ultimately may result in permanent alterations in neuronal amygdalar activity and behavioural alterations (Weinstock, 2008; Glover, 2011). These

morphological changes may explain the general observation that excess maternal CRH and CORT levels may permanently elevate anxiety-like behaviours and fear responses in PS offspring (Barros et al., 2006). Furthermore, because the amygdala is bi-directionally related to the frontal cortex and hippocampus, the morphological consequences of PS may relate to other fundamental changes in cognition. For example, altered amygdala activity may send wrong information to frontal lobes affecting decision making and reasoning.

1.4.2 Prefrontal cortex

The prefrontal cortex receives input from all other cortical regions and is implicated in decision making, the regulation of emotional and cognitive behaviours and planning and directing motor movements (Weinstock, 2008; Kolb et al., 2012). Depending on the severity, time and duration, PS may affect these functions as a result of dendritic plasticity of prefrontal cortex subregions, including nucleus accumbens (NAc), medial prefrontal cortex (mPFC), anterior cingulate (ACC) and orbitofrontal cortex (OFC). For example, 21-day old male and female offspring treated with PS revealed significantly increased dendritic branching, length and spine density in both NAc and the mPFC subregions of the prefrontal cortex (Muhammad et al., 2012). Similarly, Murmu et al. (2006) reported a decrease in dendritic spine density in the ACC and OFC in both male and female offspring aged 23 days (Murmu et al., 2006). Additionally, there was a decrease in length and complexity of pyramidal apical dendrites in both ACC and OFC only in males but not in females (Murmu et al., 2006). Furthermore, Kolb and Muhammad et al. (2011) found that the exposure to mild gestational stress during gestational days 12-18 induced a decrease in spine density in mPFC and no effect in OFC when the brains were examined in adulthood. Therefore, the timing of PS exposure, sex of the offspring and the age at which the brain is examined result in different plastic changes in

neuronal circuits of the prefrontal cortex (Kolb et al., 2012).

1.4.3 Hippocampus

The hippocampus plays a vital role in memory formation and cognition. Since the dentate gyrus (DG) region of the hippocampus is a region of adult neurogenesis, major hormonal or neurochemical insults that modulate neurogenesis can affect hippocampal function. In particular, the hippocampus is very vulnerable to glucocorticoid manipulations early in life, as it has highly expressed GR and MR (Weinstock, 2008). Accordingly, rodent studies have suggested a significant decrease in synaptic spine density of the hippocampus and impaired reversal learning in PS offspring on postnatal day 35 (Harris and Seckl, 2011). Furthermore, the exposure of rats to severe PS (bright light and restraint 3x45 min/d in pregnant dams) reduces hippocampal neurogenesis at various ages (Lemaire et al., 2000). For example, neurogenesis decreased by 38% at the age of 28 days, by 59% at 3 months, by 42.3% at 10 months, and 55.2% at 24 months of age (Lemaire et al., 2000; Charil et al., 2010). Similarly, severe PS in rhesus monkeys causes a decrease in neurogenesis and subsequently reduced hippocampal volume (Charil et al., 2010). By contrast, a milder form of PS in rodents revealed an increase in hippocampal neurogenesis (Weinstock, 2008). Therefore, the timing and severity of PS play a crucial role in the severity of changes in neurogenesis and neuronal plasticity. Notably, the increased glucocorticoid activity as a by-product of PS may explain the reduction in hippocampal GR and MR receptors (Charil et al., 2010) and HPA axis dysregulation (Lupien et al., 2009).

1.4.4 Hypothalamus

Both rat and human hypothalamus develops from the diencephalon in the late

embryonic or early fetal periods. The hypothalamus is composed of several nuclei, of which some are sexually dimorphic (Charil et al., 2010). One of those nuclei is the medial preoptic area (SDN-POA), which plays a role in male sexual behaviour in rats, such as mounting and ejaculation (Charil et al., 2010). A cross sectional analysis of the SDN-POA area has revealed that this area is larger in males than in females on postnatal day 20 and 60, but there is no difference at birth (Charil et al., 2010). Similarly, the human SDN-POA contains twice as many cells and is twice larger in males than in females (Charil et al., 2010). This sexually dimorphic difference in size arises around the age of four years when the cell numbers start to decline in girls but remain the same in boys (Charil et al., 2010).

The size of the SDN-POA is responsive to the effects of PS in a sexually dimorphic manner. Recent evidence has indicated that PS rats have smaller SDN-POA at birth than non-stressed offspring, and this is true only for males (Anderson et al., 1985). In particular, the size of the SDN-POA in PS males is two times larger than that of non-stressed males at birth and later at 20 and 60 days of age it is 50% smaller and very similar to values of control, non-PS females (Charil et al., 2010). No difference was found in the size of SDN-POA between PS and non-stressed females at birth at the age of 20 or 60 days. This indicates that the SDN-POA in females is not sensitive to the effects of PS. The observed PS effects on the size of the SDN-POA in males can be linked to alterations in sexual behaviour. Indeed the PS rats that did not copulate showed significantly reduced SDN-POA volumes and were feminized (Hofman 1997; Charil et al., 2010), in comparison to the SDN-POA size of males which copulated. Therefore, PS has differential effects on the morphology of certain hypothalamic nuclei by decreasing or increasing their volume in a sexually dimorphic manner, and these volumetric changes are causally linked to specific behaviours (Anderson et al., 1985; Charil et al., 2010).

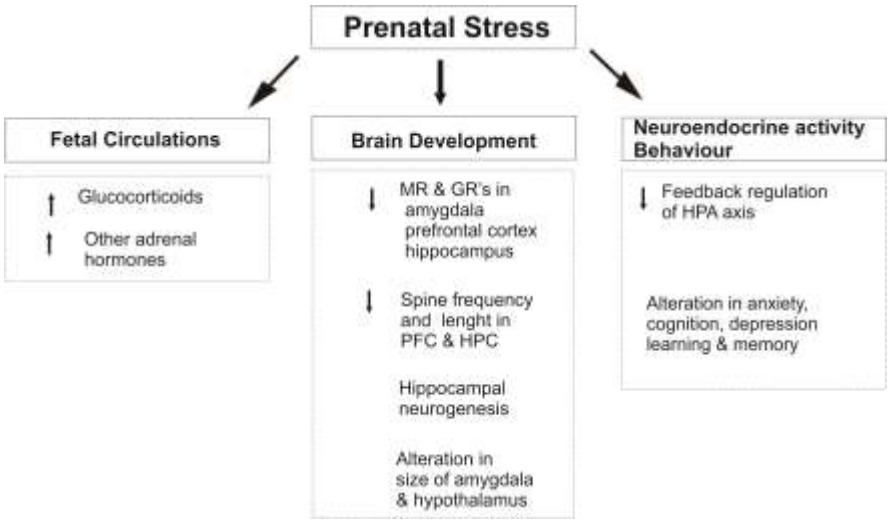

Figure 1.2: Diagram illustrating the routes by which PS can alters fetal programming. Excess levels of glucocorticoids and other adrenal hormones released during stressful experience will pass through placenta and affect fetal brain programming. This will result in alteration of various limbic system structures and function as well as the HPA axis. Ultimately, PS will alter fetal behaviours throughout the lifespan.

1.5. PS alters behaviour in adulthood

In adulthood, PS is associated with affective changes and cognitive impairments. Both human and animal data has linked maternal stress to poor coping behaviours under diversity, increased levels of anxiety-like behaviours, impaired learning and memory, and depression (Lemaire et al., 2000; Darnaudery and Maccari, 2008; Weinstock, 2008; Harris and Seckl, 2011). Interestingly, these disorders share a common causative factor, the dysregulation of the HPA axis along with decreased GR receptor density (Chung et al., 2005; Ishiwata et al., 2005).

1.5.1 Anxiety-like behaviour and impaired coping in adversity

Adult prenatally stressed offspring are often described as having a high degree of emotionality (Franklin et al., 2010; Welberg and Seckl, 2001). For example, a human study has revealed that children exposed to PS were more likely to be emotionally disturbed during adolescence and adulthood than children that experienced no stress during gestational period

(Harris and Seckl, 2011). Furthermore, maternal anxiety during 12-22 weeks but not 32-40 weeks of gestation was significantly linked with self-reported anxiety in 10-year old offspring (Darnaudery and Maccari, 2008; Weinstock 2008; Harris and Seckl, 2011). Additionally, these offspring also exhibited higher baseline cortisol levels than the non-stressed offspring. Although this evidence is convincing it is difficult to imply causation. It is challenging to pinpoint if the anxious offspring are genetically anxious, programmed to be anxious or learn to be anxious (Harris and Seckl, 2011).

To investigate causality, it is important to model anxiety-like behaviours, fear and stress in experimental animals as they allow for better control of confounding maternal factors and genetic influences. Anxiety-like behaviour is a result of threat which is perceived to be uncontrollable while the fear is response to known threat. Both of these behaviours are induced by stressful experience which results in activation of flight-or-fight response by sympathetic nervous system. Commonly used to elicit anxiety-like behaviours in rodents is the exposure to an unfamiliar, brightly illuminated open space, such as an open field. Exposure to such adverse environments are usually accompanied by CORT release (Darnaudery and Maccari, 2008; Weinstock, 2008). Several studies have shown that prenatally stressed rats spend more time freezing and less time in the center of an open field than controls (Harris and Seckl, 2011). Additionally, both male and female adult PS offspring display an increase in ultrasonic vocalization and decreased locomotor activity in the open field (Welberg and Seckl, 2001). When Bowman et al. (2004) measured anxiety-like behaviours in adult male and female that had experienced PS, they found sexually dimorphic responses in anxiety-like behaviours (Bowman et al., 2004). PS increased female latency to enter the open field but did not alter male latency (Bowman et al., 2004).

Another popular test for PS-induced anxiety-liked behaviours is the elevated plus maze

(EPM). The EPM consists of a plus-shape maze with two open and two closed arms. When placed into the EPM, an animal is faced with a conflict of fear of height and open space and the desire to explore a novel situation. Anxiety-like behaviour is indicated by an animal avoiding the open arms and preferring the closed arms. Accordingly, both male and female PS rats spend less time than controls in the open arms and more time in closed arms of the EPM (Darnaudery and Maccari, 2008; Weinstock, 2008). Interestingly, in a similar study a reduced number of visits to open arms of the EPM were positively correlated with CORT levels, indicating that higher anxiety is associated with elevated CORT levels (Welberg and Seckl 2001). The severity of anxiety-like behaviours is related to the severity of the PS. Consequently, when pregnant dams were exposed to restraint stress only once during gestation for 45 min, their adult offspring did not show anxiety-like behaviours in the EPM, not even when the adversity of the EPM was increased by additional bright lights (Weinstock, 2008). Therefore, depending on the time of the exposure to stress, intensity of exposure, frequency of exposure and sex and age of an animal, the axiogenic property of PS may vary.

There are two explanations for the increased hyper-emotional state of the PS rats. Firstly, it is possible that the altered anxiety-like behaviour observed in PS animals is a direct result of an elevated HPA axis response to novelty (Harris and Seckl, 2011). An alternative explanation is altered functioning of the amygdala. The amygdala mediates fear and anxiety-related behaviours, but also contains MR, GR and CRH receptors and CRH producing cells. CRH is believed to be the key neurotransmitter which mediates the effects of PS on anxiety. Therefore, any alterations in CRH levels either via HPA axis activity or increased levels of CRH in the amygdala will elicit anxiety-like behaviours in rats (Darnaudery and Maccari, 2008; Harris and Seckl, 2011). Furthermore, volume and cell numbers of amygdala are altered in PS offspring in different manner depending on the age of measurement (Salm et al., 2004).

For example, Kawamura et al., have reported a 30% decrease in amygdala neurogenesis in 10-day old PS offspring, whereas at postnatal day 45, the lateral amygdalar nucleus was increased by 30% (Salm et al., 2005; Charil et al., 2010). The change in the size of the lateral amygdala nucleus may be due to different stages of development and hormonal influences on this structure. This indicates that PS programs developmental trajectories of amygdala nuclei in different ways and these are time sensitive. Therefore, the age of amygdalar assessment will play an important role on the intensity of behavioural disturbances.

1.5.2 Cognition, impaired learning and memory

Evidence from both human studies and rodent models has linked PS to cognitive impairments in offspring. Similarly to anxiety-like behavioural disturbances, cognitive impairments will vary depending on the time of the exposure to stress, intensity of exposure, frequency of exposure and sex and age of the subject.

Human studies have reported impairments of intellectual activity and language ability in children and young adults of mothers exposed to stress or severe anxiety during gestation (King and Laplante, 2005; Weinstock, 2008). For example, the children of mothers that experienced a freezing ice storm or objective stress during pregnancy had significantly poorer general intellectual and language outcomes than a cohort of non-exposed children (Weinstock, 2008; Charil et al., 2010). Additionally, there was a reduction in the children's school grades at the age of six if their mothers experienced stress during early gestation. Similarly, Hay et al. (2008) reported a higher incidence of cognitive impairment in offspring of mothers with post-partum depression (PPD) than of non-stress mothers. Furthermore, infants born to mothers who were pregnant during the World Trade Center Attack developed post-traumatic stress disorder (PTSD) along with altered cortisol levels (Yehuda and Bierer, 2007). This suggests that

exposure to maternal PTSD may have altered glucocorticoid programming, which may contribute to mental and cognitive problems.

Work in rodent models has also linked gestational stress with impaired cognitive behaviours. Both acute and repeated maternal stress results in reduced spatial learning in a water maze and reduced working spatial memory in the radial arm maze in adult offspring (Welberg and Seckl, 2001; Bowman et al., 2004). For example, daily maternal stress (restraint or foot shock) during the last 6 days of gestation resulted in slowed acquisition of spatial learning (Ishiwata et al., 2005; Son et al., 2006). Furthermore, PS-exposed rats show altered performance in a water maze task, and this alteration is more apparent with advanced age, as it first appears in midlife at about 12-15 months of age (Welberg and Seckl, 2001; Weinstock, 2008; Glover, 2011). Interestingly, PS affects male and female cognitive performance differently. Only in males, PS has been associated with impaired cognitive behaviour in both water maze and radial arm maze tasks (Bowman et al., 2004). On the contrary, PS females displayed improved cognitive abilities in these tasks.

The cognitive impairments observed in PS animals could be due to dysregulation of HPA axis activity and/or due to altered hippocampal volumes as it is the central structure directly involved in spatial learning and memory (Welberg and Seckl, 2001; Bowman et al., 2004; Weinstock, 2008; Glover, 2010).

1.5.3 Depressive-like behaviours

The experience of stress or a threat instantly activates cortical and limbic neuronal circuits to induce a fight-or-flight response (Lupien et al., 2009; Charil et al., 2010). However, PS may result in a hyperactive HPA axis and altered development of the forebrain limbic circuitry, so that these systems overrespond to the stressor thus producing the depressive-like

behaviours (Darnaudery and Maccari, 2008; Weinstock, 2008; Charil et al., 2010).

Human studies have reported high incidences of major depression in young men and women of mothers that were exposed to an earth quake during pregnancy. In a similar study adults exposed to earth quake *in utero* had an 8% higher risk of experiencing major depression than control subjects (Charil et al., 2010). This strong link between PS and major depression is also evident in children born to mothers whose husbands were bereaved of their spouses during pregnancy (Weinstock, 1997). Notably, none of these studies have found a significant difference between the incidence of depression and the gestational trimester during which the stress occurred (Weinstock, 2008). Additionally, although the activity of the HPA axis was not assessed in these subjects, it is believed that the greater risk of depression is associated with impaired regulation of the HPA axis (Darnaudery and Maccari, 2008; Weinstock, 1997). Indeed, individuals with major depression show significantly elevated basal cortisol levels as well as prolonged and increased cortisol levels in response to stress, and down-regulated density of GR receptors (Cottrell and Seckl, 2009; Harris and Seckl, 2011).

In rodent models, a high incidence of depressive-like behaviours has been reported in offspring exposed to gestational stress (Weinstock, 2008; Harris and Seckl, 2011). In rats and mice, behavioural parameters that at least partially reflect the main symptoms of depression in humans have been developed. These measures include loss of active coping, immobility and social withdrawal (Darnaudery and Maccari, 2008; Weinstock 2008). An inescapable stress test known as forced swim test (FST) is used to measure depressive-like behaviours in rodents by quantifying the amount of time spent immobile or floating in a pool of water. Interestingly, PS male and female rats exhibit significantly increased immobility than their control counterparts (Weinstock 2008; Charil et 2010; Harris and Seckl, 2011). Furthermore, PS-exposed females generally spend more time in an immobile state than males. Additionally, aging increases the

immobility time in both sexes, with aged females showing the highest immobility in the FST (Franklin et al., 2010; Charil et al., 2010). Similarly to humans, depressive-like behaviours in rodents are believed to be associated with dysregulation of the HPA axis and down-regulation of hippocampal GRs (Darnaudery and Maccari, 2008; Weinstock, 2008; Charil et al., 2010). Indeed, rodent studies have reported reduced hippocampal volume, neurogenesis and neuronal plasticity in adult rats with depression-like behaviours (Wainwright and Galea, 2013). Additionally, depressive-like behaviour is associated with reduced dendrite length and complexity (Watanabe et al., 1992; Galea et al., 1997; Wainwright and Galea, 2013), spine density (Shors et al., 2001; Wainwright and Galea, 2013) and reduced expression of synaptic proteins (Muller et al., 2011; Wainwright and Galea, 2013). Importantly, programming effects of early stress on depression-like behaviours can be observed across multiple generations (Franklin et al., 2010).

1.6 PS affects epigenetic programming and its effects are transmitted across generations

Mechanisms of transgenerational inheritance include altered gestational endocrine milieu maternal care and epigenetic inheritance. The early physiological processes are largely under the influence of maternally derived factors and intrauterine environment (Ho and Burggren, 2010). For example, chickens whose early-stage embryos were removed from native yolk and explanted to continue development on the yolk of either other bird species or other chicken species, revealed altered growth and heart rate (Ho and Burggren, 2010). Similarly, rats exposed to maternal glucocorticoids during gestation will have altered glucose metabolism (Drake et al., 2005; Ho and Burggren, 2010). As a second mechanism maternal care taking behaviours in rodents provide most convincing evidence of transgenerational transfer (Ho and Burggren, 2010; Zucchi et al., 2013). For examples, alterations in maternal liking and

grooming are associated with modifications in offspring stress response (Weaver et al., 2004; Champagne and Meaney, 2007; Ho and Burggren, 2010). The third mechanism of transgenerational transfer involves epigenetic mechanisms of inheritance. Changes in the epigenome as a result of environment-gene interaction may be transmitted from one generation to next to affect later phenotype, health and disease (Relton and Smith, 2010; Ho and Burggren, 2010; Kilpinen and Dermitzakis, 2012; Aiken and Ozane, 2013).

1.6.1 Epigenetics

The term epigenetics was first coined by Conrad Waddington in the 1940's, who analyzed the role of gene-environment interactions in producing specific phenotypes (Waddington, 1942; Skinner et al., 2011; Guerrero-Bosagna and Skinner, 2011). Based on Waddington's study epigenetics was defined as "the branch of biology which studies the causal interaction between genes and their products which brings phenotypes into beings" (Skinner et al., 2011; Guerrero-Bosagna and Skinner, 2011). This early definition of epigenetics had a developmental basis (Guerrero-Bosagna and Skinner, 2011) and may have contributed to processes of developmental plasticity (Jablonka and Lamb, 2005; Jablonka and Raz, 2009). Since 1970 many contemporary definitions of epigenetics exist reflecting distinctive prospective such as developmental biology, evolution, ecology and genetics (Ho and Burggren, 2010). Epigenetic regulation is most commonly defined as changes in gene expression without altering the primary DNA sequence that are mitotically stable (Painter et al., 2008; Relton and Davey-Smith, 2010; Meaney, 2010; Ho and Burggren, 2010; Skinner et al., 2012). According to literature evidence the environmental factors drive the epigenetic events through alterations in epigenome (Ho and Burggren, 2010).

The major epigenetic events include DNA cytosine methylation, histone modifications,

transcriptional and posttranscriptional control of gene expression through Piwi-interacting RNA (piRNA) and microRNA (miRNA) (Meaney, 2010; Migicovsky and Kovalchuk, 2011). DNA methylation is most extensively studied. The analysis of histone modifications requires the use of DNA while analysis of miRNA requires a source of RNA (Relton and Davey-Smith, 2010; Skinner, 2011). Extremely adverse early environments such as PS may alter miRNA expression resulting in modified physiological processes (Zucchi et al., 2013). For example, Zucchi et al. (2013) reported upregulation of miRNA-323 and miRNA-98 in brains of offspring exposed to PS indicating altered inflammatory functions. Therefore, PS can alter the epigenome and impact the phenotype either through one or all of these mechanisms by creating an abnormal state of cellular differentiation (Guerrero-Bosagna and Skinner, 2011). Such alterations in molecular processes influence genome activity through somatic cell exposure and may result in a multigenerational phenotype (Skinner et al., 2011).

1.6.2 Multigenerational phenotype

Direct exposure over multiple generations to an environmental factor or toxicant will result in a multigenerational phenotype (Skinner et al., 2011). This phenotype results from environmental actions on somatic and germ-line cells that allow tissue-specific toxicology in the individuals exposed (Skinner et al., 2011; Guerrero-Bosagna and Skinner, 2011). For example when a gestating female F0 is exposed to environmental factor, i.e. PS, multiple generations will be affected. The epigenome of both the F1 and the F2 offspring will be altered by PS in response to direct somatic and/or germ line cell exposure.

Multigenerational epigenetic actions of environmental signals included agents such as Bis-phenol-A, DES, maternal depression, food restriction, maternal care, maternal stress etc. (Dunn et al., 2011; Skinner et al., 2011; Kilpinen et al., 2012; Roseboom and Watson, 2012).

The mechanisms through which these factors contribute to change in gene expression likely involves complex interaction between the genes and environment (Meaney, 2010; Dunn et al., 2011). For example, PS may induce epigenome alterations through complex interaction between the maternal environment, placental change and epigenetic programing of embryo (Meaney, 2010; Dunn et al., 2011, Kilpinen et al., 2012). Importantly, following direct somatic or germ-line tissue exposure such epigenome alterations may be transmitted to multiple generations down the line, promoting multigenerational phenotype. However, in the event that only germ-line epimutation is involved then the exposure may promote a transgenerational phenotype and be transmitted to unexposed generations (Skinner et al., 2011).

1.6.3 Transgenerational phenotype

According to Skinner et al., (2010) A truly transgenerational phenotype is transmitted through the germ line in the absence of direct exposure. This involves exposure of the parental generation and an epigenetic modification of the germ line for transmission to multiple generations (Skinner et al., 2011). Mechanisms of transgenerational inheritance include the transfer of genetic imprints (i.e., DNA methylation [DNAm], chromatin modifications) and microRNA (miRNA) changes across multiple generations (Skinner et al., 2008; Relton and Davey-Smith, 2010; Ho and Burggren, 2010; Migicovsky and Kovalchuk, 2011). For example, exposure to adversity during early development was shown to induce epigenetic modifications in F1-F4 generations (Skinner, 2008). Although it was only the F1 and F2 generation that were exposed directly, alterations in the F3 and F4 generations were affected (Skinner, 2008). Thus, these epigenetic alterations were transmitted to unexposed F3 and F4 generation inducing behavioural disturbances and physiological abnormalities. This indicates germ line transmission between generations without direct exposure to the environmental factor (Skinner,

2008), involving transgenerational inheritance

However, according to Kovalchuk (2012) transgenerational epigenetic effects can be both heritable and non-heritable. The transgenerational heritability reflects changes at the cellular level (meiotic and mitotic) population and the behavioural levels (Kovalchuk 2012). This involves mechanisms of transgenerational response, transgenerational response or transgenerational memory (Kovalchuk 2012).On contrary not all transgenerational transfer is heritable in nature (Kovalchuk 2012). One example of non-heritable transfer is the activity of small non-coding RNA's in the ovum and sperm cells (Kovalchuk 2012). For example, altered developmental and phenotypic appearances in response to stress are result of the accumulation of proteins and metabolites in the cytoplasm and organelles of maternal gametes (Kovalchuk 2012). According to Kovalchuk (2012) these events are part of the transgenerational inheritance and are hardly epigenetic in nature.

Understanding the molecular and cellular mechanism of transgenerational phenomena will be critical in understanding the interactions between early environment and genes (Meaney, 2010; Skinner et al., 2011). Transgenerational epigenetics may be an important mechanism in understanding the fetal basis of complex adult disease (Katti et al., 2002, 2007; Ho and Burggren, 2010; Skinner et al., 2011) Studies involving the Dutch famine birth cohort have linked grand-parental nutrition with disease rates in the second generation (Katti et al., 2002, 2007; Harris and Seckl, 2011; Roseboom and Watson, 2012). Importantly, developmental programing of the brain and the behaviour by environmental factors such as PS is not restricted to the immediate offspring of directly exposed, but it may also affect the developmental programing of subsequent generations (Harris and Seckl, 2011). Therefore, there is a potential for epigenetic transgenerational phenomena in explaining disease etiology (Skinner et al., 2011; Roseboom and Watson, 2012; Zucchi et al., 2013).

1.7 PS programs aging, disease and lifespan

A rapidly growing body of empirical evidence from both animal and human studies suggests that an adverse early life environment increase the susceptibility for many common age-related disorders (Entringer et al., 2011; Relton and Smith, 2010; Aiken and Ozanne, 2013). Indeed, in humans low birth weight is associated with hypertension, diabetes, and cardiovascular disease in adulthood (Weinstock, 2008; Lupien et al., 2009; Entringer et al., 2011; Aiken and Ozanne, 2013). Similarly, rodent models have associated low birth weight with an increased risk of affective and cognitive disorders in adulthood such as schizophrenia, attention deficit/hyperactive disorder (ADHD), increased vulnerability to post traumatic stress disorder (PTSD), anxiety, depression and learning impairments (Welberg and Seckl, 2001; Markam and Koening, 2011). Low birth weight is believed to be associated with intrauterine growth restriction, lack of oxygen, lack of nutrition and maternal stress. Importantly, PS through low birth weight may contribute to disease development through complex interaction between maternal environment, placental changes and epigenetic programming of the embryo (Dunn et al., 2011).

Alterations in epigenetic markers have been linked to cancer, Fragile x syndrome, Angelman syndrome and brain disorders such as autism, schizophrenia, Rhett syndrome and neurodegenerative diseases (Relton and Davey-Smith, 2010; Skinner et al., 2011; Babenko et al., 2012; Zucchi et al., 2013). Therefore, PS can alter the epigenome and impact the phenotype by creating an abnormal state of cellular differentiation (Guerrero-Bosagna and Skinner, 2011). Altered genome persists throughout the life of an individual, and as the individual ages the adult phenotype formation of disease onset is more prominent (Guerrero-Bosagna and Skinner, 2011). Additionally, the fetal onset of adult disease can result via somatic cell modification

where a multigenerational phenotype develops via germ line modification which results in development of a transgenerational phenotype (Skinner et al., 2011).

Multigenerational effects of exposure have been mostly studied in terms of the effects of environmental toxicant effects. For example, an environmental endocrine disruptor during gestation resulted in abnormal reproductive tract formation and gonadal dysfunction in F1 male and female offspring (Skinner et al., 2011). In females, these effects transmitted to the F2 generation and led to abnormal reproductive tract formation (Skinner et al., 2011). Interestingly, the exposure of diethylstilbestol (DES) had different effects on the F1 vs. F2 generation. Multigenerational transmission resulted in direct somatic tissue exposure in the F1 generation and germ-line tissue exposure of the F2 generation (Skinner et al., 2011). Furthermore, maternal stress during gestation is associated with anxiety inductions in individuals exposed, which is passed on to multiple generations and can be transmitted to the unexposed generation to produce transgenerational effects (Ho and Burggren, 2010; Relton and Davey-Smith, 2010; Skinner et al., 2011; Zucchi et al., 2013).

A classic example of transgenerational programming is the exposure to vinclozolin in rodents during embryonic gonadal sex determination (Skinner 2008). This treatment resulted in reduced male fertility in F1-F4 generations (Skinner, 2008). For the same reason, exposure to moderate maternal undernutrition during pregnancy resulted in modified heart function and HPA axis in adult offspring in both the F1 and F2 generation, but no effects were found in this case the F3 progeny because the programming was not epigenetically transmitted (Harris and Seckl, 2011). Furthermore, studies involving the Dutch famine birth cohort have linked grand-parental nutrition to increased disease risk in the second generation (Katti et al., 2002, 2007; Harris and Seckl, 2011; Roseboom and Watson, 2012).

Interestingly, the intrauterine environment may also play important role on the

programing of the telomere activity and its later association with disease development (Entringer et al., 2011; Entringer et al., 2012, Haussmann et al., 2012, Reynolds, 2013; Shavel et al., 2013). Telomeres represent DNA-protein complexes that cap chromosomal ends and promote chromosomal stability (Entringer et al., 2011). The telomere length is set at birth and shortens in all replicating somatic cells with age (Entringer et al., 2011; Entringer et al., 2012; Shavel et al., 2013). PS and psychosocial stress are believed to produce variations in telomere length, by reprograming its trajectories at birth which would increase individual's susceptibility for age related common diseases (Entringer et al., 2011) throughout life. For example, human studies have found that offspring exposed to psychosocial intrauterine stress had shorter telomere length in adulthood, and were more susceptible to disease over the lifespan (Entringer et al., 2011; Shavel et al., 2013). Similarly, when Haussmann et al. (2012) injected chick eggs with glucocorticoids in order to mimic the PS exposure, they reported a decrease in telomere length and an increase in oxidative stress in offspring exposed in comparison to non-treated chicks (Haussmann et al., 2012). Therefore, glucocorticoids induced phenotypes either through steroid injection or exposure to PS can accelerate aging and increase mortality (Haussmann et al., 2012). In summary, PS alters both fetal developmental programing and programing of the telomere biology in manner that affects offspring brain, behaviour, cellular aging, morbidity and mortality (Entringer et al., 2011; Haussmann et al., 2012, Reynolds, 2013).

1.8 Conclusions

Early life experiences such as PS are linked to susceptibility to many common age-related disorders throughout the lifespan. PS can alter the fetal developmental programing resulting in hyperactive HPA axis, altered brain morphology, increased vulnerability to develop affective and neurodegenerative diseases in adulthood and old age. Furthermore, effects of PS

can induce epigenetic changes that last long after the initial exposure to PS, predisposing an individual to disease later in life. These alterations in the epigenome induced by PS can be transmitted across multiple generations to affect the health of directly exposed and unexposed individuals. Furthermore, PS alters programing of telomere biology inducing cellular aging and increased susceptibility to disease over the lifespan of exposed individual.

1.9 Thesis Objectives and Rationale

The main objective of the thesis is to investigate if multigenerational or recurrent prenatal stress across four generations will exaggerate well studied effects of prenatal stress in single generation on the brain and behaviour across lifespan

The major objectives of this thesis include:

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1) Determine possible sexually dimorphic effects of multigenerational PS on the brain and behaviour of adult rats.

2) Examine and compare the manifestations of PS on behaviour and physiological outcomes in aging rats after exposure to PS in one generation (F1-PS) vs. multiple generations (F4-PS).

The present thesis is structured into four chapters. Chapter 2 assesses sex specific effects of multigenerational stress on the brain and behavior in adult rats. Chapter 3 will provide necessary evidence that the hair elementary analysis is accurate screening tool for age-related health problems. Chapter 4 will combine hair elementary analysis with other behavioural and physiological tests to determine possible interacting effects between PS in one (F1-PS) vs. four generations (F4-PS) and aging. The experimental chapters are followed by a final discussion and conclusions chapter.

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CHAPTER 2

Experiment 1: The Formation of New Behavioural Traits by Multigenerational Prenatal Stress in Males is Associated with a Functional Hemispheric Dominance Shift

2.1 Abstract

In a continuously stressful environment, the effects of recurrent prenatal stress may accumulate across generations and generate new behavioural traits in the absence of genetic variation. Here we investigated if multigenerational prenatal stress across four generations differentially affects behavioural traits, laterality and hemispheric dominance in male and female rats. Pregnant F0, F1, F2 and F3 dams were either stressed from gestational days 12-18 or served as non-stress control. Their adult male and female F4 progeny was tested in fine motor skills, paw preference and hemispheric dominance. Multigenerational stress reduced skilled reaching and skilled walking ability in males, but promoted skilled movement abilities in females beyond levels of control animals. Skilled movement impairments in multigenerationally stressed males were accompanied by shifts in paw preference towards the right hemisphere. The sexually dimorphic complex sensorimotor behaviours were accompanied by changes in parietal cortex dendritic morphology. Females revealed significantly longer dendrites and more elaborate dendritic branching, while males showed greater spine density in both hemispheres. Compared to non-stressed males, stressed males had longer apical dendrites, shorter basilar dendrites, greater dendritic branching and fewer dendritic spines. Importantly, greater left-paw dominance in stressed males was associated with decreased spine density in the right hemisphere. Thus, cumulative multigenerational stress supports a shift of dominance towards the right hemisphere and left-handedness. These findings partially explain the origins of apparently heritable behavioural traits and the developmental origins of handedness in the absence of major genetic variations.

2.2 Introduction

Early life experience, such as prenatal stress (PS), modifies the developing brain and behaviour in a sexually dimorphic manner with potentially life-long consequences. Severe PS may result in reduced hippocampal volume (Lemaire et al., 2000) and altered function of prefrontal structures such as anterior cingulate cortex (ACC) and orbital frontal cortex (OFC) (Murmu et al., 2006; Mychasiuk et al., 2012; Muhammad et al., 2012). PS offspring exhibit reduced dendritic spine density and dendritic atrophy in both ACC and OFC (Murmu et al., 2006). These anatomical changes of PS are associated with cognitive impairments, such as learning and memory deficits (Lemaire et al., 2000; Welberg and Seckl, 2001; Bowman et al., 2004; Weinstock, 2008; Glover et al., 2011), delayed motor reflex development (Patin et al., 2004) and reduced motor ability and strength in later life (Kofman, 2002; Canu et al., 2007; Cao et al., 2012).

In a continuously stressful environment (similar to our everyday lives), the effects of PS may accumulate across generations and generate new behavioural traits. Studies involving the Dutch famine birth cohort have linked grand-parental undernutrition with altered glucose tolerance and anxiety in the second generation (Roseboom et al., 2001; Roseboom and Watson, 2012). Furthermore, effects of toxins and pharmaceutical agents may later affect behavioral functions across two or even three subsequent generations (Skinner et al., 2011; Vyssotski, 2011). Notably, very little is known about the transgenerational programming by stress. Matthews et al. (2012) reported reduced locomotor activity and blunted cortisol response to swim stress in F2 guinea pig offspring whose mothers received betamethasone (BETA 1) during pregnancy (Igbal and Matthews et al., 2012; Matthews et al., 2012). Additionally, mutigenerational adverse exposure may result in behavioural traits that may very across generations. Behavioural variations across generations may be explained by epigenetic

mechanisms including DNA methylation, histone modifications, chromatin and microRNA (miRNA) changes (Meaney, 2010; Migicovsky and Kovalchuk, 2011; Skinner, 2011; Dunn et al., 2011). The epigenome changes caused by PS may be transmitted and accumulated across generations. Continuous direct exposure to PS over four generations (F0-F4) may produce a multigenerational phenotype with adaptive or very vulnerable characteristics. This may generate new behavioural traits and morphological linkages that may reveal sexual dimorphisms.

Altered behavioural traits caused by multigenerational PS may vary between males and females. PS exposure is associated with reduction in hippocampal and cortical plasticity in males and an increase in females (Schmitz et al., 2002; Muhammad et al., 2011; Mychasiuk et al., 2011). Additionally, PS induces memory and learning impairments in males while it improves these cognitive functions in females (Lemaire, 2000; Bowman et al., 2004; Son et al., 2006; Darnaudery and Maccari, 2008). Human studies have reported greater motor deficits in male than the female PS offspring (Patin et al., 2004; Dipietro and Kivighon, 2009; Cao et al., 2012). Importantly, a particular outcome of PS may affect behavioural laterality in a sexually dimorphic manner, with males being more susceptible to laterality changes than females (Alonso et al., 1991, 1997; Tang and Verstynen, 2002). These experimental data are supported by human findings where maternal stress at 18 weeks of pregnancy predicted atypical handedness (Glover et al., 2004) that was more prominent in males. Specifically, left handedness was observed to increase in response to exposure to PS only in males (Elis, 1991). Handedness and motor laterality were suggested to have a heritable component. Handedness seems to be a heritable trait and associated with genes such as PCSK6, which encodes the enzyme proprotein convertase subtilisin/kexin type 6 (Scerri et al., 2011). Additionally, genetic models have been used to explain greater incidence of left-handedness in males (McManus

1991). Left-handedness seems to rather be passed on from the maternal side than from the paternal side (McManus, 1991; Annette, 1996). However, a recent genome-wide association study found no genetic linkages (Armour et al., 2013).

Transgenerational programming may provide a potential mechanism linking heritable patterns in handedness and laterality to genetic changes by means of epigenetic mechanisms. A continuously stressful environment may lead to cumulative changes in brain laterality that are associated with heritable patterns in epigenetic regulation of gene expression that are reflected by altered neuronal plasticity (Babenko et al., 2012; Muhammad et al., 2011, 2012; Mychasiuk et al., 2012). Here we tested if cumulative effects of adverse experience across generations can lead to life-long changes in motor laterality and altered neuronal morphology in a sexually dimorphic manner. This study assessed the effects of multigenerational PS on fine sensorimotor skills, hand preference, and structural plasticity of parietal cortex (Par 1) in adult (6 month old) male and female rats in the F4 generation. The findings reveal impaired motor skills and a shift of brain lateralization towards the right hemisphere resulting in left handedness in males, but not females. These functional changes were accompanied by decreased spine density in the right parietal cortex.

2.3 Methods

2.3.1 Animals

Fourty-one adult Long-Evans rats raised at the Canadian Centre for Behavioural Neuroscience vivarium were used. These rats were the F4 generation of a line of pregnant dams that were either stressed four successive generations (F4-PS) or left undisturbed to serve as control (F4-C). The offspring from four different litters per groups was used for the present experiments [males: n=21 (F4-C=11, F4-PS=10); females: n=20 (F4-C=10, F4-PS=10); see

Figure 2.1]. All animals were tested in behaviour at the age of 6 months and then divided for processing of brain tissues. miRNA profiling was performed for n=17 [male n=9 (F4-C=5, F4-PS=4) and female n=8 (F4-C=4, F4-PS=4)] and the remaining 24 animals [male n=12 (F4-C=6, F4-PS=6) and female n=12 (F4-C=6, F4-PS=6)] were used for histological analysis. The male animals were housed in pairs and female animals in cages of three under a 12:12 h light/dark cycle with light starting at 07:30 h and the room temperature set at 22 °C. Prior to behavioural training, rats were food deprived to reach 90–95% of their baseline weight. To maintain this weight, rats received standard chow food in their home cages five hours after completion of daily skilled reaching training sessions. Rats were weighed daily. All procedures were approved by the University of Lethbridge Animal Care Committee in compliance with the guidelines of the Canadian Council on Animal Care.

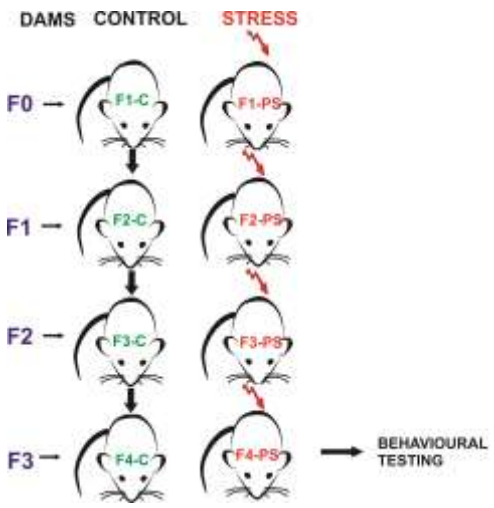

Figure 2.1: Mutigenerational prenatal stress (PS) paradigm. Dams F0-F3 (blue) were exposed to swim and restraint stress during pregnancy. The offspring received PS exposure (red arrow) during gestational days 12-18 over four consecutive generations. This experiment involved the F4 generation stressed offspring F4-PS and non-stressed controls F4-C.

2.3.2 Prenatal Stress

In order to obtain F4 multigenerational stressed rats (see Figure 2.1), each generation of pregnant dams (F0-F3) was subjected to daily stress from gestational days (GD) 12-18.

Stressors included restraint in a Plexiglas cylinder for 20 min and forced swimming in warm water (22 °C) for 5 min. The animals received both stress procedures each day in a semi-random order either in the morning or afternoon hours.

2.3.3 Behavioural Testing

2.3.3.1 Skilled Reaching Task

Animals were trained and tested in skilled reaching according to protocols by Metz and Whishaw (2000). Briefly, rats were pre-trained to reach asymptote levels in skilled reaching success. Percent success was recorded for 14 consecutive days. In each session rats were allowed to reach for 20 food pellets. The success percent (%) was calculated using the following formula:

$$\text{Success percent} = \frac{\text{number of successes}}{\text{number of attempts}} \text{ x } 100$$

On day 15 of behavioural testing, the performance was videotaped with a digital video camcorder (Sony, at the Canadian Center for Behavioural Neuroscience), and a blind experimenter performed the qualitative analysis of video recordings. Qualitative reaching performance was based on 11 main movement components and 35 subcomponents (see Table 2.1-A; Metz and Whishaw, 2000). Three successful reaches per each rat were analysed and averaged for the qualitative features of movements.

2.3.4.2 Skilled Walking Task

The horizontal ladder rung walking apparatus (Metz and Whishaw, 2002) was used to assess skilled walking performance. Briefly, pre-trained animals were tested three times in each

daily session and video recordings were collected. The recordings were analyzed by a blind experimenter according to our previously published rating scale (Metz and Whishaw 2002). The quantitative analysis included the average number of errors for each limb: left forelimb (LFL), right forelimb (RFL), left hind limb (LHL) and right hind limb (RHL) as a ratio of total number of steps. For qualitative analysis the limb placement was scored on a scale of 0 to 6, where 0 was a total miss and 6 represented a correct limb placement (see Table 1-B). An error was defined as each limb placement that received score of 0, 1 or 2 points (see Table 2.1-B).

Behaviour	Characteristics	Score
1. Orient	Head orient to target, sniffing	2
2. Limb Lift	Body weight lift to back, hindlimbs aligned with body, limb moves forward, digits on midline	4
3. Digits Closed	Palm supinated semi in, digits semi flexed	2
4. Aim	Elbow comes in, palm in midline	2
5. Advance	Elbow in, limb forward, limb directed to target, head and upper body raised, body weight shifts front and lateral	6
6. Digits Open	Digits open, discrete limb movement, not fully pronated	3
7. Pronation	Elbow in, palm down in arpeggio	2
8. Grasp	Arm still, digits close, hand lifts	3
9. Supination I	Elbow in, palm medially before leaving slot, palm turned 90^0	3
10. Supination II	Head points down, body horizontal, palm straight up, distal limb movement	4
11. Release	Open digits, puts food in mouth, head and upper body lowered, raise other paw	4

Table 2.1-A

Behaviour	Characteristics	Score
1. Total miss	Deep fall after the limb missed the rung	0
2. Deep slip	Deep fall after the limb slipped off the rung	1
3. Slight slip	Slight fall after the limb slipped of the rung	2
4. Replacement	Limb replaced from one rung to another	3
5. Correction	Limb aimed for one rung but was placed on another Or: Limb position on a same rung was corrected	4
6. Partial placement	Limb placed on a rung with either digits/toes or wrist/heel	5
7. Correct placement	Midportion of a limb placed on rung	6

Table 2.1-B

Table 2.1: A) Table showing the rating scale for the qualitative sequenced movements in the skilled reaching task. The best possible score is 35. B) Table showing the rating scale for foot placement in the skilled ladder walking task. The foot placement accuracy or number of errors was defined as each limb received a score of 0, 1, or 2.

2.3.4 Histological processing for Golgi-Cox staining

After behavioural testing was completed the animals were administered an overdose of pentobarbital and were intracardially perfused with 0.9% saline. The brains were removed and preserved in Golgi-Cox solution for 14 days in a dark location. Then the brains were placed in 30% sucrose for 28 days. A vibratome (Leica, Buffalo Grove, IL) was used to cut the brains at 200 µm and the slices were mounted on gelatin-coated slides. In the final step the brains were stained according to the procedure published by Gibb and Kolb (1998). Distal dendrites of individual neurons were stained. For the analysis dendritic segments met the criteria of being thoroughly stained and without overlapping another dendrite or a blood vessel.

Pyramidal cells from layer three of the parietal cortex (PAR1) were selected for analysis. Parietal cortex cells were analysed due to their role in integration of sensory information from various parts of the body and in the manipulation of objects. A camera lucida mounted on a microscope was used to trace individual neurons from the Golgi-Cox stained brains. A total of 10 cells (5 per hemisphere) were traced at 200× magnification. Neuronal morphological measurements included apical and basilar Sholl analysis (an estimate of dendritic length derived from dendritic branches that intersect concentric circle 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×; Gibb and Kolb, 1998).

2.3.5 Statistical Analysis

A statistical analysis was performed using SPSS 20 for Windows 11.5.0 (IBM Corporation, Armonk, NY). Two-way ANOVA's with stress and sex as factors were run for the behavioural tasks (pellet reaching task and skilled walking task) and the neuronal morphology of the

Parietal cortex (Par 1). Unpaired sample t-tests were used for all parametric data. Pearson correlation was used for behaviour and neuronal morphology, while chi-square was performed to reveal possible association between treatment and paw-preference. The results are shown as the means ± standard error of the mean (±SEM), based on unpaired t-test. Asterisks indicate significances: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

2.4. Results

2.4.1 Male rats respond to multigenerational PS by increased left handedness

The ratio of paw preference in skilled reaching among males and females revealed sexually dimorphic effect of multigenerational PS. A chi-test revealed that multigenerational stress increased left paw preference in males $\chi^2(1)=4.53$, $p<0.05$. Non-stressed male rats (n=21) were more likely to be right handed (81%) than left handed (19%; see Figure 2.2). However, multigenerational PS decreased the proportion of right handedness among males (n=22) to 50%. However, there was no effect of multigenerational PS among females χ^2 (1)= 0.78, p=0.37. The non-stressed females (n=24) were more likely to be right handed (66%) than left handed (34%). Multigenerational PS in females (n=24) changed this ratio slightly to 54% right handed and 36% left handed (see Figure 2.2).

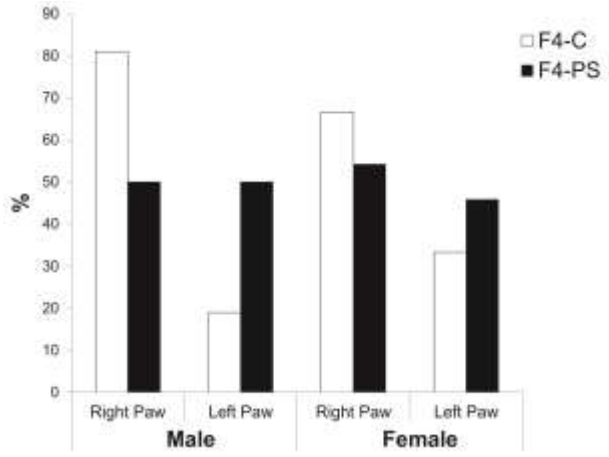

Figure 2.2: Mean percentage of rats showing either right or left paw preference in the reaching

task. Non-stressed males (n=21) had absolute right paw preference (81:19) and the stressed males (n=22) did not prefer (50:50) either paw. Interestingly, neither stressed (n=24) nor non-stressed (n=24) females showed clear right or left paw preferences (F4-PS, 54:46; F4-C, 66:34). It should be noted that multigenerational PS modified only handedness only in males.

2.4.2 Multigenerational PS reduced skilled reaching performance in males but promoted it in females

The average qualitative reaching movement score for the fine movements revealed a main effect of sex ($F(1,35) = 50.368$, $p<0.001$), as the female rats performed better than the male rats ($p\leq0.001$; Figure 2.3). The average score in females was 30 versus score of 24 in males. Further, there was a significant Sex × PS interaction ($F(2,35)=7.302$, $p<0.05$), as stressed F4-PS females performed better than non-stressed F4-C females ($p\leq0.05$), and F4-PS males performed slightly worse than F4-C males ($p=0.50$; see Figure 2.3). However, there was no main effect of PS on the quantitative fine motor skills or % success ($F(1,35)=0.993$, $p=0.327$), possibly due to small group sizes. Notably, PS exerted sex-specific effects on the aim component. F4-PS males revealed impaired aim of the paw to retrieve the pellet, while this component was left intact in F4-PS females in comparison to control.

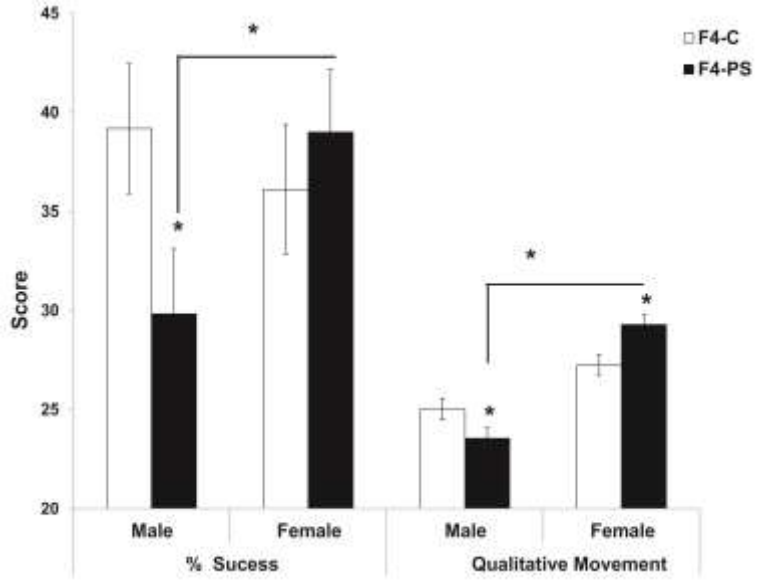

Figure 2.3: Prenatal stress (PS) alters skilled reaching success and movement trajectories in a sexually dimorphic manner. Females performed skilled reaching movements with overall higher success rates and higher qualitative movement scores. Interestingly, PS in males (n=10) reduced success rates and qualitative movement scores compared to non-stressed males (n=11). By contrast, PS in females (n=10) increased reaching success and qualitative movement scores compared to non-stressed females (n=10). Asterisks indicate significances: * $p < 0.05$. All data mean ± SEM.

2.4.3. Multigenerational PS elevates error rates in skilled walking only in males

The error rates in skilled walking revealed a significant main effect of sex, as female rats overall made fewer forelimb (LFL: $F(1,40)=15.369$, $p<0.001$; RFL: $F(1,40)=22.674$, $p<0.001$) and hind limb (LHL: $F(1,40)=5.239$, $p<0.05$; RHL: $F(1,40)=8.784$, $p<0.005$] errors than males (Figure 2.4). Furthermore, RHL errors revealed a main effect of PS, ($F(1,40)=4.584$, $p<0.05$) and a significant Sex × PS interaction for RHL errors ($F(1,40)=8.52$, $p<0.01$) and for LFL errors ($F(1,40)=4.323$, $p<0.05$). PS exhibited dimorphic effects on skilled walking in right hind limb errors as stressed (F4-PS) males made more errors than the non-stressed (F4-C) males ($p\leq0.05$). By contrast, the stressed (F4-PS) females made slightly fewer errors than the non-stressed (F4-C) females ($p= 0.35$; Figure 2.4).

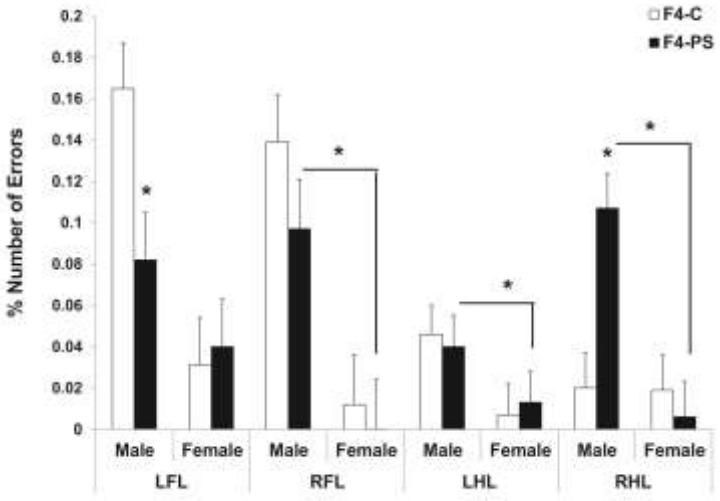

Figure 2.4: Prenatal stress (PS) alters skilled walking ability success in a sexually dimorphic manner. Females showed higher foot placement accuracy than males in all components. PS in

males increased the error rates with reduced error rates in the right hindlimb, indicating that their brain laterality was modified. By contrast, foot placement accuracy in female rats was not affected by PS. Asterisks indicate significances: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. All data mean ± SEM.

2.4.4 Multigenerational PS altered spine density and dendritic complexity of parietal cortex (Par1) neurons in a sexually dimorphic manner

Branch order of the PAR cortex in the right and left apical fields revealed a significant main effect of PS ($F(1, 23)=4.10$, $p\leq0.05$). There was an increase in dendritic branching in both right ($F(1,23)=7.391$, $p<0.05$) and left apical ($F(1,23)=7.438$, $p<0.05$) neurons of stressed male and female animals (see Figure 2.5). Other factors or interactions were not significant for branch order in the apical field.

In the basilar fields, female rats overall showed significantly longer dendrites in the right ($F(1,23)=9.927$, $p<0.005$) and left ($F(1,23)=5.964$, $p<0.05$) hemisphere compared to males (Figure 5B). Furthermore, female rats had significantly longer dendrites in the apical field in the right hemisphere ($F(1,23)=8.962$, $p<0.01$) compared to males. The dendritic length in the basilar field in the left hemisphere revealed a significant Sex × PS interaction ($F(1,23)=4.004$, $p<0.05$). Stress caused a decrease in dendritic length in females and an increase in males. A significant effect of sex was found for dendritic spine density in the basilar field of the right ($F(1,23)=6.569$, $p<0.05$) and left ($F(1,23)=9.742$, $p<0.005$) hemispheres; males had a larger spine density than females ($p\leq0.05$). Similarly, males showed a significantly greater spine density in the left apical field ($F(1,23)=12.226$, $p<0.01$) in comparison to females. Additionally, stress decreased male spine density in the right apical field ($p<0.05$).

2.4.5 Skilled motor performance in multigenerationally stressed males was associated with decreased spine density in the right hemisphere

Correlation analysis revealed a significant relationship between the right-apical spine density and skilled reaching qualitative scores for males in both experimental groups. Right dendritic spine density was negatively related to qualitative reaching movement scores in control males ($r=-0.889$, $p<0.05$; Figure 2.6). Interestingly, there was a positive correlation between reaching movement scores and dendritic spine density in the apical field of the right hemisphere in stressed males ($r=0.896$, $p<0.05$; Figure 2.6). There were no significant correlations in females, however (see Figure 2.6). Additionally, there was a positive correlation between skilled walking scores and dendritic length in the apical field of the right hemisphere in stressed males ($r=0.878$, $p<0.05$; Figure 2.6). These findings suggest that improved skilled reaching and walking ability of the left paw were associated with larger dendritic remodeling of the right hemisphere in males.

2.5. Discussion

The present data confirm our hypothesis that multigenerational PS promotes the development of new behavioural traits and affects brain and behavioural laterality in a sexually dimorphic manner. The present study provides three main findings. First, multigenerational PS shifted paw dominance in males but not in females. Second, multigenerational PS compromised skilled movement trajectories and skilled walking ability in males, but rather improved these abilities in females. Third, dendritic morphology indicated that PS altered multisynaptic plasticity in males and females. Notably, the shift towards left handedness in stressed males was accompanied by increased dendritic complexity in the right parietal cortex. Therefore, in the used measurements, the behaviour and the brains of stressed males were

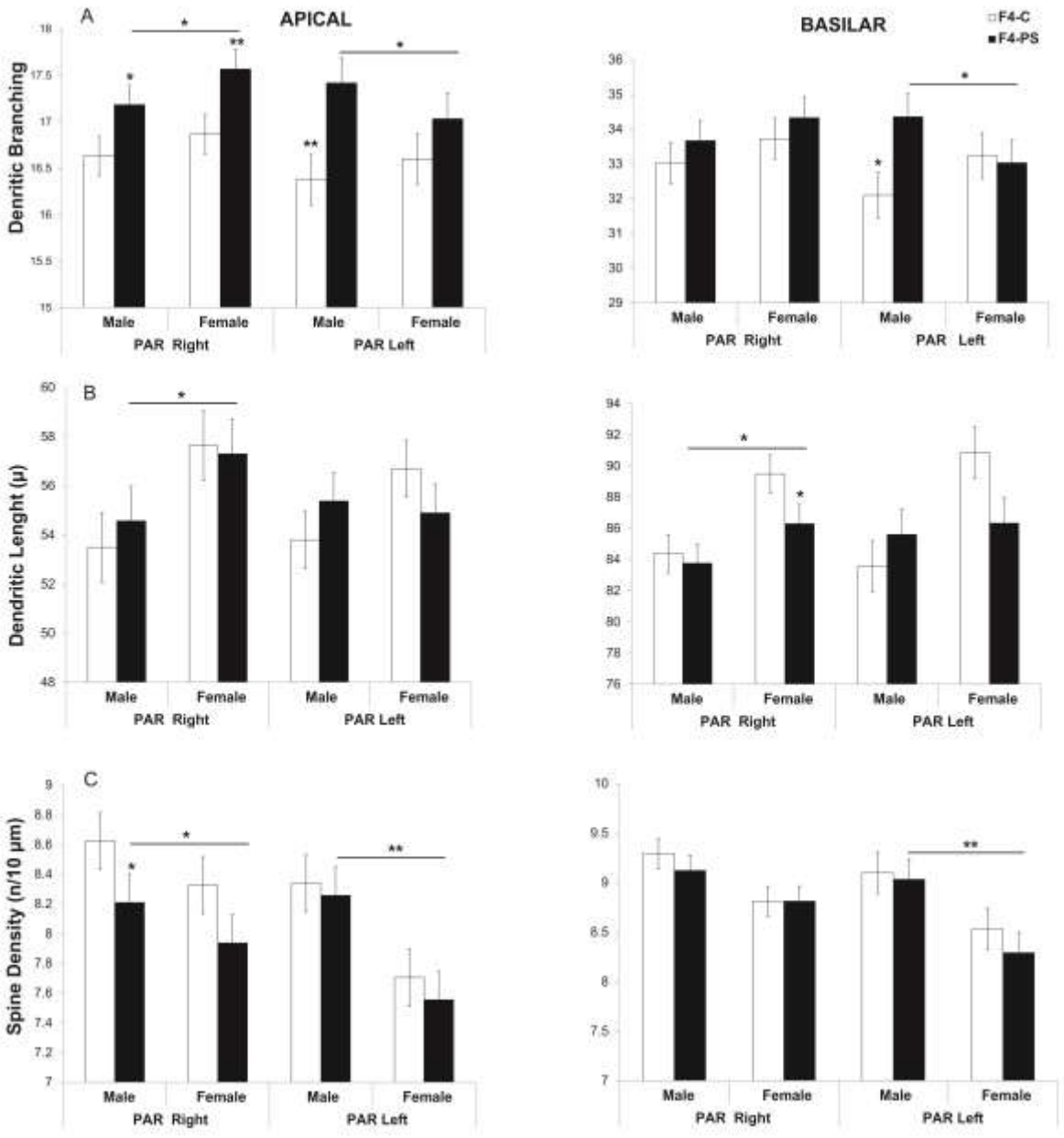

Figure 2.5: Prenatal stress (PS) altered dendritic morphology in the parietal cortex. (A) Dendritic branching (B) dendritic length and (C) spine density in the par1 region of both right and left hemispheres in male and female rats exposed to multigenerational PS. Note that PS altered neuronal morphology of the parietal cortex in sex-specific manner. Asterisks indicate significances: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. All data mean ± SEM.

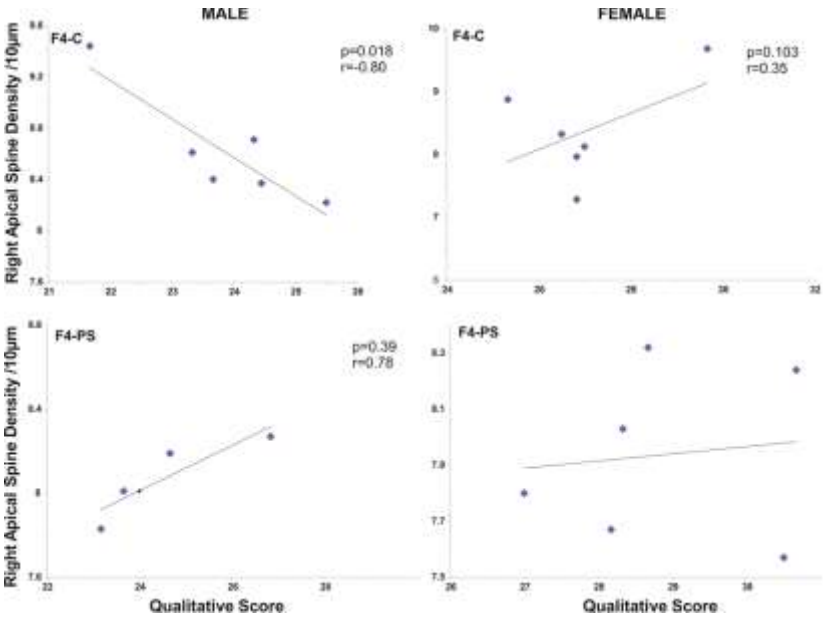

Figure 2.6: Correlation between the mean spine density in apical field of right parietal cortex and mean qualitative movement score for non-stressed (F4-C) males; stressed (F4-PS) males; non-stressed (F4-C) females; and stressed (F4-PS) females. A strong negative correlation was found for non-stressed males and strong positive correlation for stressed males, indicating that PS modified brain laterality in males. No correlation was found in female rats.

were feminized to resemble the control female brains.

Lateralized brains were believed to be a unique human feature for many years (Diamond et al., 1981; Camp et al., 1984; Alonso et al., 1991; Tang and Verstynen, 2002). However, Rogers and colleagues in 2000 reported that the lateralization in humans is not unique neither in nature nor in extent (Rogers, 2000; Gao et al., 2008). Over the last 30 years asymmetries in brain and behaviour were described in many other species, such as rat, cat, rabbit and non-human primates (Kolb et al., 1982; Camp et al., 1984; Alonso et al., 1991; Tang and Verstynen, 2002; Gao et al., 2008). For example, rat studies described behavioural deficits associated with right hemisphere lesions which are not present in left hemisphere lesioned animals (Kolb et al., 1982). Therapeutic benefits after brain lesion also differ depending on hemispheric dominance (Nikkhah et al., 2001). Furthermore, both humans and rats display a high degree of hemispheric specialization within the parietal cortex (Rushworth et al., 1997;

Schluter et al., 2002; Rushworth et al., 2003; Culham et al., 2006; Mento et al., 2010). The right parietal cortex is specialized for spatial processing and attention (Culham et al., 2006). The motor and somatosensory representation of the body, including the limbs is primarily localized in the left parietal cortex (Rushworth et al., 2003; Culham et al., 2006). This lateralization difference supports the overall preference of the right upper limbs (Kolb and Whishaw, 2003). Paw preference and handedness reflect better or more precise use or individual preference of one hand over the other, for which standardized tests exist in humans and rats.

Evidence indicates the rat paw preference is similar to human handedness (Tsai et al., 1930; Tang and Verstynen, 2002; Guven et al., 2003; Vyazovskiy et al., 2008). In the 1930's Tsai and colleagues observed that rats prefer the right paw over the left (Tsai et al., 1930). Distribution of rat paw preference is similar to that of human handedness in males (right 80% vs. 20% left), whereas in females this distribution appears rather equally distributed (Tsai et al., 1930; Tang and Verstynen, 2002; Guven et al., 2003). However, there are inconsistent results in the literature regarding handedness. According to Tang and Verstynen, (2002) these inconsistences can be explained in terms of differences among testing methods. Further, recent evidence indicated that early life experiences can modify handedness, with males and females being affected differently.

A variety of environmental and experiential factors can shift the ratio of handedness and hemispheric dominance. For example, it has been shown that cortical development is very susceptible to a variety of environmental factors (Kolb et al., 2012). Furthermore, the time of exposure, intensity of the experience, developmental stage and sex generally affected neuronal plasticity related to the maturation of hemispheric dominance (Michelsen et al., 2007; Muhammad et al., 2011; Kolb et al., 2012).

The present data confirm these previous observations by showing that stress can alter brain dominance and hand preference. The most profound modifications are generally found in males, where their absolute hand preference may decrease from 80:20 right hand dominance in the control population to 60:40 (Tsai et al., 1930; Tang and Verstynen, 2002; Guven et al., 2003; Gao et al., 2008). In contrast, in rats the female paw dominance tends to be smaller with 60:40 right paw dominance (Tsai et al., 1930; Guven et al., 2003), which is also observed in the human population (Alfonso et al., 1991, Tang and Verstynen, 2002). Such changes can be correlated to neuronal morphology. For example, moderate PS stress exposure was shown to decrease spine density in medial prefrontal cortex and orbitofrontal cortex, while mild PS decreased spine density in medial prefrontal cortex and did not affect spine density in the orbitofrontal cortex (Kolb et al., 2012; Muhammad et al., 2012). Furthermore, early life experiences, such as PS, determine sexual dimorphisms in brain development and hemispheric dominance.

Brain organization and motor functions that depend on handedness may be differentially expressed in males and females (Culham et al., 2006; Tomasi et al., 2012). Evidence suggests that PS can modify synaptic connectivity and neuronal morphology (Mychasiuk et al., 2012) of the prefrontal cortex (PFC) and the hippocampus (HPC) (Mychasiuk et al., 2013). Furthermore, PS may regulate fundamental epigenetic mechanisms and endocrine patterns across generations (Morgan and Bale 2001) that may also shift lateralization in behaviour and brain function. It is possible that early life adversity modifies cerebral asymmetry trough general neuromodulatory systems (Tang and Verstynen, 2002). In particular, testosterone is a likely candidate to influence cortical lateralization and paw preference (Fleming et al., 1986; Steward & Kolb, 1988; Wisniewski, 1998; Guven et al., 2003). Thus, the multigenerational PS in the present study may alter early cerebral

development via reduction in testosterone levels, thus affecting male paw preference more severely than female laterality (Steward and Kolb, 1988; Culham et al., 2006). Similar mechanisms may be responsible for reducing male skilled reaching performance as well.

The present results revealed that multigenerational PS affected male and female skilled reaching and walking abilities differently. While multigenerational PS reduced skilled reaching performance in males, it seemed to promote performance among the stressed females. Notably, the impairments in skilled walking were greater for the non-dominant limb. It is possible that more successful skilled reaching motor performance among females is linked to improved recovery from stress (Jadavji and Metz, 2008), or to coping with PS (Bowman et al., 2004; Marrocco et al., 2012). Furthermore, females in general are believed to be more resistant than males to stress induced impairment (Zuena et al., 2008). Thus, better coping and resistance in females may promote both sensory and motor aspects of skilled movement performance, as observed in our data.

The sexually dimorphic changes in sensorimotor performance were accompanied by striking neuromorphological adaptations in a functionally meaningful area, the parietal cortex. Experience-dependent changes in neuromorphology depend on the type of experience, its time and duration, sex and age of an animal (Murmu et al., 2006; Kolb et al., 2012; Mychasiuk et al., 2013). Although there is no previous data on the effects of multigenerational PS on cortical neuromorphology, earlier studies in PS confirm the present findings. Muhammad et al. (2012) reported that PS caused a decrease in basilar orbitofrontal cortex (AID) in both males and females (Muhammad et al., 2012). Furthermore, PS diminished dendritic growth in males (Bustamante et al., 2013) with reduction in branching of the apical dendrites in layer II/III pyramidal neurons of the parietal cortex (Bustamante et al., 2013). Furthermore, multigenerational PS exposure increased spine density in the parietal cortex neurons, consistent

with data reported that showed an increase in the cortical spine density in PS offspring (Muhammad et al., 2011, 2012). Accordingly, cortical spine density in right hemisphere and skilled reaching movement performance were positively correlated in stressed males but not in females. Other studies have shown that PS exposure modifies the neuronal morphology of hippocampus and nucleus accumbens as well (Mychasiuk et al., 2011).

Thus, the effects of multigenerational stress may not be limited to the parietal cortex and may also modulate the functions of other cortical areas. Alterations in neuronal plasticity may be due to toxic effects of excess circulating glucocorticoids (Takashi 1998; Seckl and Meaney 2004) and prolonged HPA axis response (Koenig et al., 2005). Mechanisms have been proposed that contribute to neuronal damage after exposure to PS. First, increased corticosterone circulation is associated with stress-induced decrease in neurotrophins, particularly brain-derived neurotrophic factor (BDNF) in various brain regions (Smith et al., 1995; Uysal et al., 2011; Bustamante et al., 2013). Second, PS may alter microtubule-associated protein 2 (MAP2) synthesis in the brain (Barros et al., 2006), resulting in impaired synaptogenesis and neurite outgrowth (Bustamante et al., 2013). Additionally, the mechanism which may explain sex differences may involve suppression of late prenatal testicular androgen secretion in males (Bowman et al., 2004). Alteration in androgen hormones during early development can have profound effects on brain development, feminizing males and masculinizing females (Bowman et al., 2004; Zuena et al., 2008). Since recent genome-wide twin sequencing failed to identify any locus associated with handedness (Armor et al., 2013), importance of environmental factors on sex-specific brain development is even is more prominent. Thus, the epigenetic changes resulting from environment gene interaction may be the best explanation of sexually-dimorphic effects of PS on neuronal and behavioural alterations.

In summary, we show that four generations of direct exposure to PS (multigenerational PS) is associated with larger left hemispheric dominance in male offspring. In spite of greater dendritic plasticity, this shift compromises the ability to perform skilled movements. While the current study does not provide conclusions about possible transgenerational programming of hemispheric dominance in the absence of stress, it is possible that multigenerational PS generates new behavioural traits that may be transmitted to subsequent generations. Thus, even in the absence of recognizable PS effects, hemispheric dominance may become altered to produce a left-handed or ambidextrous phenotype. Based on evidence that PS or multigenerational programming of hemispheric dominance may also shift hemispheric dominance in humans, the present findings provide a mechanism for the lack of genetic associations with left handedness in humans.

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CHAPTER 3

Experiment 2: Hair Trace Elementary Profiles in Aging Rodents and Primates: Links to Altered Cell Homeodynamics and Disease

3.1 Abstract

Aging is associated with an increased incidence of pathological conditions such as neurodegeneration, cardiovascular and renal disease, and cancer. These conditions are believed to be linked to a disruption in cell homeodynamics, which is regulated by essential trace elements. In this study we used hair elementary analysis by inductively coupled plasma mass spectrometry (ICPMS) to examine age-related profiles of 47 elements in both rats and common marmoset monkeys. Hair was collected from young adult (6 months) and aged (18 months) Long-Evans male rats, and young adult (2 years), middle-aged (4 years) and aged (>8 years) marmosets. The results revealed that aging reduces content levels of the minerals Co, K and Se while content levels of Al, As, B, Hg, Mo, and Ti were elevated in aged rats. Similarly, aged marmosets showed reduced levels of cobalt and elevated levels of aluminium. Case studies in aged rats revealed that myocardial infarcture is associated with elevated levels of sodium, potassium and cadmium and reduced zinc, while renal failure is linked to elevated content of potassium, chloride and boron and reduced contents of manganese. Carcinoma was linked to elevated arsenic and reduced selenium levels. The findings indicate that hair elementary profiles in healthy aging and age-related diseases reflect altered cell and organ metabolic functions. Cobalt and aluminium in particular may serve as biomarkers of aging in animal models. Thus, elementary deposition in hair may have predictive and diagnostic value in age-related pathological conditions, including cardiovascular and kidney disease and cancer.

3.2. Introduction

Healthy aging refers to the overall physical and mental wellbeing in advanced age (Strawbridge et al., 2002; Gilmer and Aldwin, 2004). According to the concept of homeodynamics, healthy aging results from the cumulative influences of damage, maintenance and repair across the lifespan (Rattan, 2013). The homeodynamic state is determined by gene-environment interactions (Rattan, 2013). Thus, healthy aging and adverse health outcomes are strongly influenced by life style and environmental factors (Merrett et al., 2010; Maestripieri and Hoffman, 2011; Zucchi et al., 2012; Metzler et al., 2013). For example, environmental contamination by toxic metals can affect the risk of metabolic, cardiovascular and neurological diseases (Mezzetti et al., 1998; Zatta et al., 2002; Wilson, 2010; Farina et al., 2013). Further, the prevalence of diabetes and cardiovascular disease grows in populations exposed to high levels of cadmium and low levels of chromium III (Schroeder et al., 1967; Davies et al., 1997; Youker et al., 2007; Shcherbatykh et al., 2007; Lind et al., 2011). Furthermore, soil contamination with arsenic, boron, mercury and nickel increases the incidence of cancer (Cowgil et al., 1983; Eck et al., 1989; Rodriguez et al., 2003; Wright et al., 2006) and mental illness (Rodriguez et al., 2003; Wright et al., 2006). Based on the rapid growth of the aging population in Western societies, there is an urgent need for validated predictive markers of aging and the risk of age-related chronic diseases.

A central feature in the development of age-related chronic diseases is the disruption of cell homeodynamics (Mezzetti et al., 1998) and altered levels of trace elements that regulate homeodynamic processes (Farina et al., 2013). Dysregulation of cell homeodynamics in essential elements during aging may gradually increase the risk of adverse health outcomes. For example, Cass and colleagues correlated the accumulation of iron in the striatum of aged rhesus monkeys with degenerating motor function (Cass et al., 2007). Such associations may be

suitable to predict the age-related motor decline and the risk of neurodegenerative disorders such as Parkinson's disease (Cass et al., 2007). Further evidence indicates that neuropathological conditions are linked to exposure to or accumulation of elements, such as arsenic, mercury, aluminium, boron and titanium in organ tissues or hair (Mezzetti et al., 1998; Kawahara et al., 2001; Zatta et al., 2002; Rodriguez et al., 2003; Domingo, 2006; Wilson, 2010). Elevated levels of circulating heavy metals are linked to oxidative stress, which ultimately accelerates neurodegenerative events in the aging brain and other organs (Farina et al., 2013). An imbalance between transition metal ions, such as zinc and copper may favour oxidative stress, development of atherosclerosis and coronary heart disease (Mezzetti et al., 1998). Furthermore, electrolyte abnormalities associated with aging such as altered potassium levels are common symptoms in kidney failure (Eck et al., 1989; Schlander et al., 2010; Wilson, 2010).

Body hair analysis has been an effective and non-invasive tool to determine environmental exposure and accumulation of trace elements (Brown et al., 1980; Davies et al., 1997; Rahil-Khazen, 2002; Wilson, 2010; Afridi et al., 2013). Characteristic changes in body hair elementary composition may also reflect age-related changes in metabolic profiles across the life span. Furthermore, hair elementary composition may predict the risk of age-related chronic diseases in longitudinal studies. Here, we investigated the validity of hair elemental analysis for aging processes in laboratory rodent and non-human primate models. We compared elemental content across ages (young, middle age, old age). Possible endocrine changes were assessed through Na/K ratios for adrenal gland activity (Schlander et. al. 2010; Wilson, 2010), and Ca/K ratios for thyroid gland activity (Wilson, 2010). To assess predictive value of hair elemental analysis for age-related chronic disease, three case studies of spontaneous renal and cardiovascular failure, and metastatic carcinoma were used. Our data

support the use of hair elemental analysis for prediction of age-related chronic disease.

3.3. Methods

3.3.1 Animals

3.3.1.1. Rats. Fourteen healthy male (young [6 months or 27% of anticipated lifespan completed], n = 7; aged [18 months or 83 % of anticipated lifespan completed], n = 7) Long–Evans hooded rats (*Rattus norvegicus*), raised at the University of Lethbridge vivarium, were used. The animals were housed in pairs under a 12:12 h light/dark cycle with light starting at 07:30 h and the room temperature set at 22° C. Rat chow food and water were available *at libitum*. The rats were left undisturbed except for regular cage cleaning, weekly weighing and handling until the age of 6 months. All procedures involving rats were approved by the University of Lethbridge Animal Care Committee in compliance with the guidelines of the Canadian Council on Animal Care.

3.3.1.2. Marmosets. Twenty-four healthy male and female (young [1.5-2 years or 24% of anticipated average lifespan completed], n = 8; middle aged [4 years or 47% of anticipated lifespan completed], n = 8; aged [> 8 years, or 90% lifespan completed], n = 8) common marmoset monkeys (*Callithrix jacchus*) weighing between 400-700 g, were obtained from the breeding-colony of the German Primate Center (Göttingen, Germany). The animals were housed in same or mixed-sex pairs (as required by the experimental setup) in a temperature- (25 ± 1° C) and humidity-controlled (65 ± 5 %) facility. Illumination was provided by artificial lighting on our standard light cycle, consisting of a 12.5 h light phase with dusk and dawn effects for the first and last 30 min and a 11.5 h dark phase. Light levels measured in the middle of the cage were 600–650 Lux during the day and 350–400 Lux during dim light phases. Each cage (50 × 98 × 70 cm, Ebeco, Castrop-Rauxel, Germany) was furnished with

wooden branches and shelves, and contained either a metal (20 × 20 × 30 cm, entrance 15 × 18 cm) or a wooden (35.5 ×26 ×17 cm) sleeping box. The animals were fed ad libitum with a pelleted marmoset diet (ssniff Spezialdiäten, Soest, Germany). In addition, 20 g mash per animal was served in the morning and they received 30 g clean-cut fruits or vegetables mixed with noodles or rice in the afternoon. Water was always available.

Animals have undergone behavioural testing before they were shaved for hair collection. The study was performed in accordance with the European Communities Council Directive 86/609/EEC and the German Animal Welfare Act and was approved by the Lower Saxony Federal State Office for Consumer Protection and Food Safety, Germany.

3.3.2 Hair Sampling

To reduce potential stress-associated changes in hair composition due to repeated sampling, hair was only collected once per animal. The abdominal and back hair was cut with scissors post-mortem from rats, and shaved from live marmosets. Approximately 0.6 to 0.8 g was collected. Marmoset hair was collected from live animals from the back. Approximately 1.0 g was collected. The length of all collected hair was up to 2.5 cm from skin. To control for metal trace contamination, fabric was cut with the same pair of scissors and used as control for hair sample analysis. The hair samples were stored in 2-ml Eppendorf tubes at room temperature.

3.3.3 Hair Trace Elementary Analysis

Hair sample analysis was performed by CanAlt Health Laboratories (Ontario, Canada). Hair samples were cut into small pieces using clean stainless steel scissors. About 300 ± 5 mg was transferred into tarred, labeled centrifuge tubes, and the exact weight was recorded. To

each sample digestion tube, 3.0 ml of reagent-grade nitric acid (HNO_3) was added. Samples were incubated for 25 minutes. Samples were then subjected to acid microwave digest, in order to stabilize the elements of interest. The digestate solution was analyzed for amounts of mineral element and trace metals by inductively coupled plasma mass spectrometry (ICPMS). Sample results were quantified by comparison with calibration solutions of known concentrations.

3.3.4 Statistical Analysis

The element levels were averaged and analysed for young, middle aged and aged animals. Statistical analysis was performed using SPSS 20 for Windows 11.5.0 (IBM Corporation, Armonk, NY) by one-way Analysis of Variance (ANOVA) and independent sample *t-tests*. In order to adjust for multiple comparisons in the one-way ANOVA Bonferroni *post-hoc* tests were performed. Hair samples were analysed individually, however, data are presented as group means. Data shown are in parts per million (ppm). The graphs show mean ± standard error of the mean (S.E.M) in % content changes.

3.4. Results

3.4.1 Hair Element Content Levels in Rats

Hair elemental analysis in aged rats revealed reduced contents of cobalt ($t(12) = 2.01$, $p < 0.05$), potassium ($t(11) = 2.479$, $p < 0.05$) and selenium ($t(12) = 4.27$, $p < 0.001$) when compared to young rats (see Figure 3.1). There was a 25 % decrease in selenium and cobalt contents in aged animals in comparison to young animals.

In contrast to K, Co and Se levels, the content levels of aluminium ($t(12)=–3.183$, $p < 0.05$), arsenic ($t(12) =–3.282$, $p<0.01$), boron ($t(12) =–2.68$, $p<0.05$), molybdenum ($t(12) =–2.38$, $p<0.05$), titanium ($t(12) =–3.18$, $p<0.05$) and zirconium ($t(12) = –3.19$) $p<0.01$) in aged

animals increased significantly compared to young animals (see Figure 3.2).

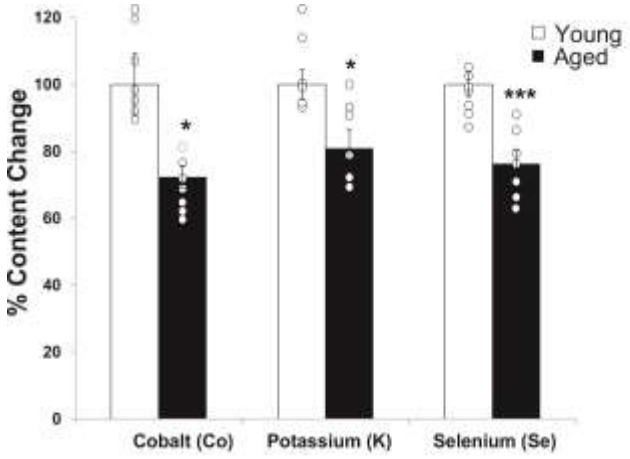

Figure 3.1: Hair elemental % content change in young and aged rats. Aging was associated with a significant decrease in content levels of cobalt (Co), potassium (K) and selenium (Se) when compared to young animals (n = 6-7). Asterisks indicate significances: * $p < 0.05$, *** $p < 0.001$. Error bars represent ±SEM. Scatter plot illustrates individual values.

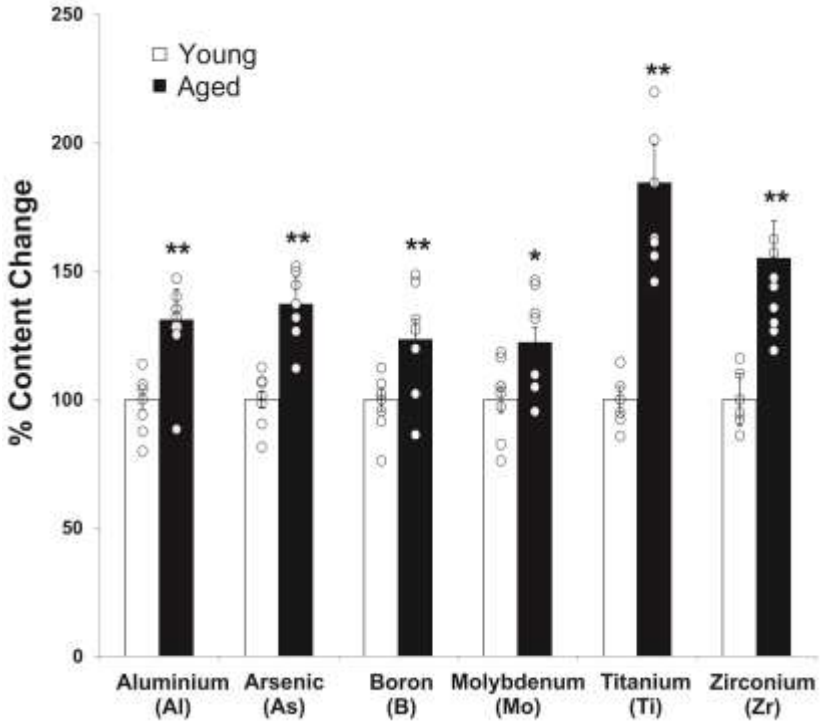

Figure 3.2: Hair metal % content change in young and aged rats. Aging resulted in a significant increase in heavy metal content levels of aluminium (Al), arsenic (As), boron (B), molybdenum (Mo), titanium (Ti), and zirconium (Zr) when compared to young animals (n = 7). Asterisks indicate significances: * $p < 0.05$, ** $p < 0.01$. Error bars represent ±SEM. Scatter plot illustrates individual values.

3.4.2 Adrenal and Thyroid Gland Activity in Rats

The Na/K ratio was used as an indicator of potential alterations in adrenal gland activity. Aging led to a significant increase in the Na/K ratio (Figure 3.3) in comparison to young animals ($t(11) = -2.48$, $p<0.05$). Similarly, the Ca/K ratio was used as an indicator of potential thyroid gland activity. Aging increased the Ca/K ratio when compared to young animals ($t(12) = -2.15$, $p<0.05$).

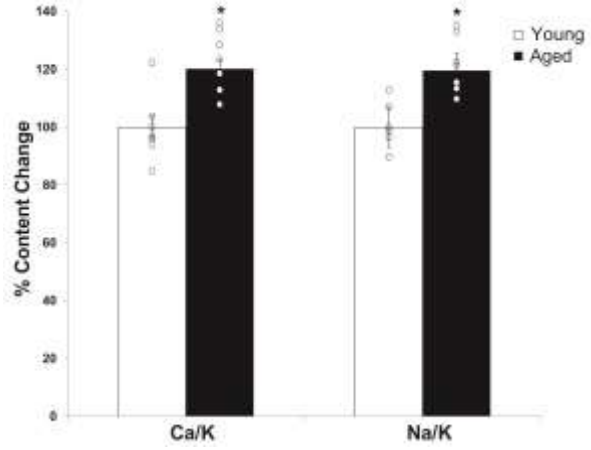

Figure 3.3: Aging alters mean Na/K and Ca/K content level ratios. Aged animals showed significantly higher levels of potassium in relation to sodium or calcium than younger animals. Asterisks indicate significances: * $p < 0.05$. Error bars represent ±SEM. Scatter plot illustrates individual values

3.4.3 Hair Element Content Levels in Marmosets

There were no sex differences and data for males and females were combined for further analysis. Levels of cobalt were assessed across the three age groups ($F(2,21) = 37.07$, $p < 0.001$; Figure 3.4A). Aged marmosets (mean = 0.010) showed a decrease in content levels of cobalt compared to middle-aged (mean = 0.0137) and young animals (mean = 0.0319). A Bonferoni post-hoc test showed a significant decrease in the content levels in aged compared to the young and middle aged ($p < 0.001$). Levels of aluminium were assessed across the three age

groups (F(2,16)=3,950, p<0.01 Figure 3.4B). Aged marmosets (mean = 1.281) showed an increase in aluminium content levels compared to middle aged (mean = 0.251) and young (mean = 0.170) animals, and a Bonferroni post-hoc test showed a significant increase in aged in comparison to middle aged and young animals (p<0.05).

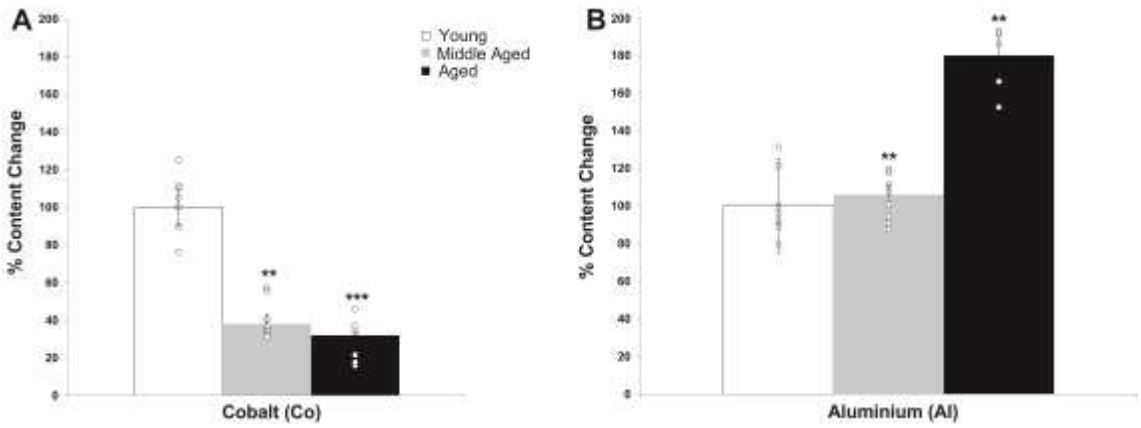

Figure 3.4: Hair trace elementary analysis in marmosets. (A), mean % content change of cobalt and (B) aluminium. Samples were taken from young (n =7-8), middle aged (n = 6-8) and aged (n = 4-8) marmosets. Note the age-related decrease in content levels of the trace mineral cobalt and the increase in the heavy metal aluminium. Asterisks indicate significances: * p < 0.05, ** p < 0.01, *** p < 0.001. Error bars represent ±SEM. Scatter plot illustrates individual values.

3.4.4 Hair Element Content as Predictor of Chronic Disease

Hair of rats with fatal adult onset diseases was investigated. Their profiles differed from those of the healthy cohort of age-matched controls.

3.4.4.1 Myocardial infarct

Compared to healthy age-matched controls (n=6-7) the hair of aged rats with myocardial infarct (n=3) revealed significantly increased content levels of cadmium (t(7) =-4.17, p<0.01, potassium (t(8) = -4.813, p < 0.001), and decreased content levels of zinc (t(8) = 6.75, p<0.001; Figure 3.5A). There was a non-significant increase in sodium levels (t(8) =-1.093, p>0.05) in rats with myocardial infarct in comparison to healthy rats.

3.4.4.2 Renal failure

Compared to age-matched healthy controls (n = 7), rats with fetal renal insufficiency (n = 3) displayed significantly elevated content levels of boron ($t(8)=-4.103$, $p<0.01$), potassium ($t(8) = -5.68$, $p<0.001$) and non-significant elevations in chlorine ($t(8) = -2.0$, $p > 0.05$; Figure 3.5B). These changes were accompanied by non-significant manganese defiency ($t(8) = 0.051$, $p>0.05$).

3.4.4.3 Carcinoma

Compared to age-matched healthy controls (n=6), rats with multiple metastatic carcinoma (n=3) involving the abdominal cavity, bladder and adipose tissue revealed significantly increased content levels of arsenic ($t(7) = -6.60$, $p<0.001$) and decreased content levels of selenium ($t(7) =7.150$, $p<0.001$; see Figure 3.5C).

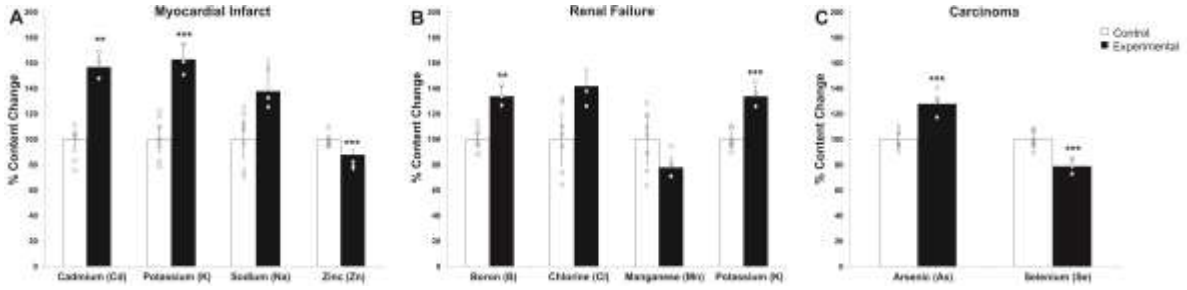

Figure 3.5: Hair trace elementary analysis in case studies. Trace element % content changes associated with (A) fatal myocardial infarcture; (B) fatal renal failure; and (C) fatal carcinoma in the abdominal cavity compared to age-matched healthy controls. Myocardial infarcture was associated with elevated content levels of the electrolytes Na and K, heavy metal Cd and decreased content levels of Zn. Kidney insufficiency was associated with elevated levels of the toxic heavy metal B and altered Mn levels, which contributed to electrolyte imbalance with increased levels of K and Cl. Carcinoma was associated elevated As and reduced Se. E, experimental animal; C, control. Asterisks indicate significances: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars represent ±SEM. Scatter plot illustrates individual values.

3.5 Discussion

Aging is the single most important risk factor for the development of complex chronic diseases. Conditions such as neurodegenerative diseases, heart and kidney failure and

carcinomas are linked to an imbalance in trace elements (Zatta et al., 2009). These imbalances are arguably the result of cumulative metabolic impairments, growing cell homeodynamic imbalance and/or gradual organ insufficiency across the life span (Zatta et al., 2009). Accordingly, the present data show age-related changes in elementary content levels in both a rodent and a primate model. Age-related hair elementary profiles revealed characteristic changes in eight elements (aluminium, arsenic, boron, cobalt, molybdenum, potassium, selenium, titanium, and zirconium) in rats and corresponding changes in two elements (aluminium, cobalt) among marmosets.

Interestingly, the largest differences seem to occur early in life in comparison to middle aged and aged animals. Thus, the most significant changes may occur during the midlife period. Starting in midlife, hair reflects the loss of essential elements, such as cobalt, and the accumulation of metals, such as aluminium. This may indicate that the process of aging exerts vulnerability on cell homeodynamics, and that the midlife period may be particularly susceptible to environmental changes. Therefore, focusing on cellular health maintenance and repair in midlife may help establish therapeutic approaches to support successful aging.

3.5.1 Hair as an Indicator of Homeodynamics

A number of characteristics support the rationale for using hair as a biological indicator of aging processes. Toxins and metabolites are incorporated into the hair during two main growth phases that include proliferation, differentiation and cellular rearrangement (Ebling 1976; Lavker et al., 1993). The phases of hair growth are similar in marmosets and rats. The pattern and rate of growth and rest depend on species and body size (Lavker et al., 1993). The duration of the active growth phase of hair follicles in rats is 7-20 days, while in the marmoset it is 50-130 days, which becomes the time window of potential biomarker deposition.

3.5.2 Aging Alters the Deposition of Trace Elements

Changes in essential elements and heavy metals observed in aged rats confirm earlier reports in humans. Aging in human subjects was shown to be associated with depletion of essential minerals such as cobalt, potassium and selenium (Brown 1980; Takahashi et al., 2001; Andres et al., 2007). By contrast, content levels of aluminium, arsenic, boron, molybdenum, titanium and zirconium exhibited significant increases (Takahashi et al., 2001; Serpa et al., 2006; Farina et al., 2013). Notably, an age-related decrease of cobalt, an essential element in neuronal and glial tissue, which also modulates cobalamin (B12 vitamin) availability (Wolters et al., 2004; Andres et al., 2007; Wilson, 2010; Becker et al., 2012; Simonsen et al., 2012). Interestingly, cobalt and cobalamine deficiencies in aged human patients have been linked to malnutrition, malabsorption, or congenital deficiencies in intracellular enzymes (Lindenbaum et al., 1988; Eck et al., 1989; Andres et al., 2007; Becker et al., 2012). While the variation across individual cases in the present study was rather low, the present data suggest that also normal aging processes may contribute to altered cobalt availability. It remains to be determined if the loss of any elements in the present study are causative or symptomatic of age-related changes in metabolic activity.

3.5.3 Aging Modulates Hair Metal Deposition

Aluminium, as the third most abundant element in the environment (Kawahara et al., 2001, 2005) has been widely recognized as neurotoxin. Significant increases in the content levels of aluminium have been linked with diseases of inner organs and neurological disorders (Kawahara et al., 2001, 2005; Wilson 2010; Lind et al., 2012). Accordingly, it was shown that neurodegenerative disorders in rat and marmoset models are associated with elevated neuronal aluminium levels (Kawahara et al., 2001, 2005; Zatta et al., 2009).

Significant changes associated with aging also concerned arsenic, boron and molybdenum content levels. The present finding of elevated arsenic levels parallels the observation of age-associated accumulation in the liver (Shimamura et al., 2013). High molybdenum levels were also found in the aging murine brain (Nakagawa, 1998). All three elements have biological functions at the cellular level involving the brain and other organs. For example, high arsenic content levels in humans have been linked to neurological disorders and cancer (Cowgil et al., 1983; Rodriguez et al., 2003; Wright et al., 2006; Wilson, 2010) and elevated blood pressure (Mordukhovich et al., 2012). In addition, boron affects oxidative metabolism and modulates rat behaviour (Nielsen and Penland, 2006) and molybdenum in implicated in age-related cognitive functions (Nakagawa, 1998). Interestingly, high molybdenum as well as selenium dietary supply has been positively correlated with longevity (Lv et al., 2011).

3.6.4 Cardiovascular Disease: Link to High Cadmium and Low Zinc/Chromium Levels

While aging is generally associated with elevated content levels of cadmium (Shimamura et al., 2013), further elevations in circulating cadmium levels were found in individuals with myocardial infarction (Eck et al., 1989; Davies et al., 1997; Lind et al., 2012). Similarly, both human patients and rats suffering from cardiovascular disease reveal increased cadmium, potassium levels and decreased zinc levels, respectively (Schroeder et al., 1967; Shcherbatykh et al., 2007; Youkeret al., 2007; Lind et al., 2012; Afridi et al., 2013). Although the present study does not allow causal conclusions regarding these elements, it nevertheless suggests a link between age-related metabolic changes and elevated risk of cardiovascular disease.

3.5.5 Trace Elements May Reveal Electrolyte Imbalances in Renal Failure

The present data suggest that renal failure is associated with the accumulation and/or altered metabolism of metals such as boron and imbalanced electrolytes (sodium and potassium). These observations are in accordance with a previously established link between aging, diabetes, kidney failure and electrolyte imbalance (Kazi et al., 2008; Schlander et al., 2010; Wilson, 2010; Afridi et al., 2013). Notably, electrolyte abnormalities associated with aging such as altered potassium levels are most common causes of kidney failure (Eck et al., 1989; Schlander et al., 2010; Wilson, 2010).

3.5.6 Possible Association between Hair Selenium Deficiency and Elevated Arsenic Levels in Cancer

Multiple carcinomas in aged rats were associated with low levels of the antioxidant selenium and high levels of arsenic. In combination these changes may weaken the immune system and raise the susceptibility to cell-damage and cancer (Cowgil et al., 1983; Rodriguez et al., 2003; Wright et al., 2006; Wilson, 2010). The changes parallel observations in human hair linked to incidence of cancers and the vulnerability to inflammation (Cowgil et al., 1983; Wilson 2010).

3.6 Conclusion

The present study illustrates a profile of *ad lib* fed rodents and non-human primates that reflects a characteristic spectrum of age-related depletion of essential minerals and accumulation of metals. It remains to be explored if the observed changes in major and trace elements are symptomatic or causative of age-related functional changes that are commonly observed (Farina et al., 2012; Shimamura et al., 2013). Notably, heavy metals have the ability

to replace or alter tissue mineral content based on their chemical properties (Gordon 1985; Rahil-Khazen et al., 2002; Farina et al., 2013). Thus, it could be argued that the decrease in essential minerals in aged animals is a result of their interaction with heavy metal levels. Furthermore, it is important to consider the biphasic response or hormesis of trace elements (Calabrese, 2004), which generate a dose-response relationship with mainly beneficial effects at low doses and mainly adverse effects at high doses.

Interestingly, reductions in cobalt and increases in aluminium were observed in both rats and marmosets in parallel to previous human studies. Thus, rats and marmosets may represent useful pre-clinical models in the study of age-related trace elementary content levels. Furthermore, cobalt and aluminium content levels may represent reliable biomarkers of aging. Aluminium in particular is regarded as an indicator of age-related neuropathology of vulnerable brain regions in Alzhheimer's disease (Walton, 2013). In conclusion, the present data and case studies suggest that hair elemental analysis serves as a sensitive, comprehensive and accurate screening tool of age-related metabolic and overall health status.

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Chapter 4

Experiment 3: Transgenerational Programming by Prenatal Stress Increases the Vulnerability to Anxiety and Depression in Old Age

4.1 Abstract

Exposure to stress during early development was shown to affect brain development, hypothalamic-pituitary-adrenal (HPA) axis reactivity and increase susceptibility to mental illness such as anxiety and depression in adulthood. Here we investigated (1) if recurrent prenatal stress (PS) across four consecutive generations of rats (F4) will exaggerate the effects of PS in a single generation (F1); and (2) if aging enhances the vulnerability to recurrent PS on mental health. Prenatally stressed male rats were derived from the F1 generation, in which parental F0 mothers experienced stress, and multigenerationally stressed male rats from the F4 generation, in which F0-F3 mothers were stressed. Non-stressed controls were also tested. Anxiety-like and depression-like behaviours in an open field, elevated plus maze and forced swim task at 12 (middle aged) and 18 (aged) months of age revealed greater anxiety-like and depression-like behaviours among stressed F4 animals compared to F1. Altered programming of HPA axis activity was revealed by reduced adrenal gland activity (Na/K ratio) in F4 rats using inductively coupled plasma mass spectrometry (ICPMS). Radioimmunoassays revealed reduced basal plasma corticosterone levels in PS F4 rats. Elevated affective states and HPA axis activity were most evident in aged F4 animals than in the F1 generation. This suggests that, in the face of persistent stress, multigenerational PS cumulatively exaggerates the risk of affective disorders across generations, and these effects are age sensitive. Interestingly, PS generated bystander effects that also affected controls. These findings suggest transgenerational inheritance of mental health risk and stress resilience. They also provide an explanation for the increasing incidence of common mental illnesses in the elderly.

4.2 Introduction

Exposure to stress during early development represents one of the most prominent risk factors for altered brain development, enhanced hypothalamic-pituitary-adrenal (HPA) axis reactivity and increased susceptibility to mental illness, such as anxiety and depression (Welberg and Seckl, 2001; Markham and Koenig, 2011; Muhammad et al., 2012). The experience of prenatal stress (PS) in children increases the likelihood of emotional disturbances during adolescence and adulthood (Ward, 1991; Van den Berg and Marcoen, 2004; Weinstock, 2008; Sharp et al., 2012). Furthermore, high incidences of anxiety and major depression were reported in young men and women born to mothers that were exposed to an earth quake during pregnancy (Watson et al., 1999; Charil et al., 2010).

Similar to human evidence, experimental animal studies reported increased anxiety-like behaviours in prenatally stressed offspring, such as reduced exploratory behaviours in a new environment, immobility in a forced swim task and avoidance of open areas (Fride and Weinstock, 1988; Murmu et al., 2006; Charil et al., 2010; Harris and Seckl, 2011). These behavioural characteristics are usually accompanied by impaired regulation of HPA axis activity (Weinstock, 1997; Tsigos and Chrousos, 2002; Darnaudery and Maccari, 2008). Moreover, HPA axis dysregulation becomes more prominent in older age (Glover et al., 2012), which may be associated with the greater prevalence of anxiety and depression commonly observed in the elderly population (Djernes, 2006; Alasdair et al., 2006).

It is reasonable to expect that PS will lead to cumulative effects in subsequent generations. PS can modify epigenetic signatures linked to mental illness and these signatures are potentially heritable (Radtke et al., 2011; Kujjo et al., 2011; Zucchi et al., 2013). Accordingly, a previous study showed transgenerational effects of early maternal separation transmit to the adult male F2 and F3 generation in terms of increased anxiety- and depression–

like behaviours along with impaired serotonergic signaling (Franklin et al., 2010). In the face of a continuously stressful environment, the cumulative effects of stress may amplify HPA axis dysregulation and augment the susceptibility to mental illness in exposed individuals. While the effects of a single generation of PS are well established, the consequences of persistent PS across multiple generations have not been shown. Multigenerational programming by PS may cause differential outcomes compared to a single generation of PS (Zucchi et al., 2012), as previously shown in maternal behaviour (Ward et al., 2013).

Here we investigated the manifestations of PS in behavioural and physiological outcomes in adult male rats after exposure to PS in one generation (F1-PS) versus cumulative effects of persistent PS across four generations (F4-PS). To investigate the long-term mental health outcomes and synergistic effects of PS and age, anxiety-like and depression-like behaviours in aging rats were assessed longitudinally. Lastly, we examined the possible effects of transgerational programing by parental stress on the HPA axis reactivity through baseline corticosterone levels and adrenal gland activity. We hypothesized that a continuously stressful environment in multigenerational PS will exceed the effects of a single generation PS. Our data suggest that a continuously stressful environment programs HPA axis regulation and adrenal exhaustion and contributes to the risk and severity of anxiety-like and depressive-like behaviours. These effects are age sensitive.

4.3 Methods

4.3.1 Animals

Twenty-six Long-Evans male rats {[F1: middle aged; n=13 (control=7-9, PS=4-7, aged; n=13 (control=4-7, PS=7-9)] and [F4: middle aged; n=13 (control=7-9, PS=4-7), aged; n=13 (control=4-7, PS=7-9)]} raised at the Canadian Centre for Behavioural Neuroscience,

University of Lethbridge vivarium, were used. The animals were housed in pairs under a 12:12 h light/dark cycle with light starting at 07:30 h and the room temperature set at 22 ℃. Rat chow food and water were available *at libitum*. The rats were left undisturbed except for regular cage cleaning and weekly weighing until they reached middle age (12 months) or old age (18 months) and, at this time the animals were tested in behavioural tasks. All procedures were approved by the University of Lethbridge Animal Care Committee in compliance with the guidelines of the Canadian Council on Animal Care.

4.3.2 Experimental Design

Four successive generations of timed-pregnant female rats were bred under standard conditions. Parental female rats (F0) were exposed to stress during pregnancy, and they carried the first generation (F1-PS) of stressed rats. Their F1 pregnant daughters were also stressed during pregnancy, so were their F2-grandaughters and F3-great granddaughters which gave birth to F4-PS offspring (see Figure 4.1). Yolked controls were bred in parallel for each generation. Groups of male offspring used in this experiment were F1-C non-stressed controls (n=7-9), F1-PS (n=4-7), F4-C (n=7-9) and F4-PS (n=4-7) animals.

4.3.3 Stress Procedure

Pregnant dams were subjected to stress daily GD 12 to GD 18. Stressors included restraint in a Plexiglas cylinder for 20 min and forced swimming in warm water at 21°C for 5 min. The animals received both stress procedures each day in a random alternating order; in the morning between 8:00-9:00 hrs. or in the afternoon between 16:00-17:00 hrs.

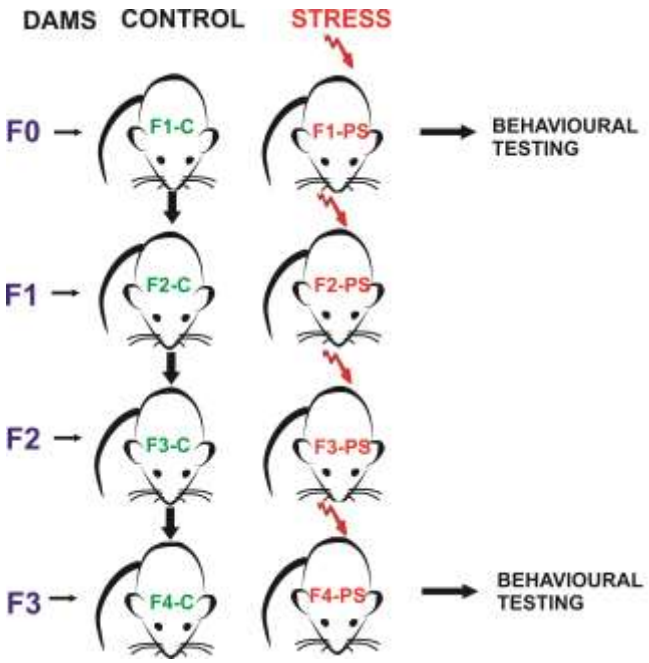

Figure 4.1: The flow chart illustrating stress paradigm. Pregnant dams F0-F3 were either exposed to stress or left undisturbed GD12-18. Offspring exposed to stress in single generation the F1-PS, and across four generations the F4-PS were used for behavioural testing.

4.3.4 Behavioural Testing

4.3.4.1 Open Field Exploration. Open field locomotor activity was used to measure exploratory behaviour. Animals were placed in Accuscan activity monitoring Plexiglas boxes (length 42 cm, width 42 cm, height 30 cm) and recorded for 10 min. The boxes were connected to a computer interface that recorded the activity as the number of sensor beam breaks. Horizontal beam breaks were recorded using the VersaMax™ software and converted to spread sheets using VersaDat™ software (AccuScan Instruments, Inc., Columbus, Ohio, USA).

4.3.4.2 *Elevated Plus Maze (EPM).* Time spent in closed arms of the EPM was used to measure anxiety-like behaviours in rats. The plus '+' shaped maze consisted of two open and two closed arms, each 113 cm in length, 10 cm in width and elevated 88 cm above the ground. Rats were placed in the center of the maze facing a closed arm and exploration was video recorded for 5 min. An experimenter blind to the animal's condition analyzed the filmed video

for the time spent in closed arms and the number of entries into open and closed arms.

3.3.4.3 *Forced Swim Task (FST).* The time spent floating in the FST was used to measure helplessness or depression-like behaviours in rats. The FST was performed by placing an animal into cylinder containing warm water at 21°C and letting it swim or float for 5 min in each session. All sessions were filmed and later analyzed for the time spent floating, swimming and climbing by an experimenter blind to the animal's condition.

4.3.5 Tissue Collection

4.3.5.1 Blood. Blood samples (0.6 ml) were collected from the tail vein at the age of 12 and 18 months. The animals were placed under 4% isoflurane anaesthesia and blood collection took place between 8:00 and 9:00 hrs. Radioimmunoassays used commercial Corticosterone Elisa kit (Cayman Chemical Company, Ann Arbor, MI) to determine plasma corticosterone concentrations.

4.3.5.2 *Hair*. Approximately 0.6-0.8 g of abdominal hair was collected post-mortem. Hair sample analysis was performed using inductively coupled plasma mass spectrometry (ICPMS) by CanAlt Health Laboratories (ON, Canada; see Ambeskovic et al., 2013).

4.3.6 Statistics

Statistical analysis was performed using SPSS 20 software for Windows 11.5.0 (IBM Cooperation, Armonk, NY) by repeated measures factorial analysis of variance (ANOVA), two-way ANOVA, and follow-up with unpaired t-test. All data are presented as mean ± standard error of the mean (SEM).

4.4. Results

4.4.1 Multigenerational PS Alters Exploratory Behaviour in Aging Animals

Animals exposed to single generation PS (F1-PS) spent significantly less time exploring the open field as shown by shorter distances traveled than animals exposed to multigenerational PS (F4-PS; $F(1,20) = 68.14$, $p<0.001$; see Figure 4.2). PS had a non-significant marginal main effect $F(1,20) = 3.77$, $p=0.066$) on the distance travelled, but it significantly decreased the distance travelled in F1-PS rats in comparison to non-stressed controls ($p<0.05$). Furthermore, there was an Age x Generation interaction where total distance traveled increased in F1-PS animals and decreased in F4-PS animals in comparison to their middle aged counterparts ($F(1,20) = 6.175$, $p<0.05$; Figure 4.2). Additionally, PS exposure in F1-PS reduced the distance traveled in comparison to non-stressed F1-C controls, but it had the opposite effect in F4-PS rats by increasing the distance traveled in comparison to non-stressed F4-C controls ($F(1,20)= 13.981$, $p<0.001$; Figure 4.2). Paired comparison revealed that F4-PS significantly decreased the exploratory behaviour in aged rats in comparison to middle aged animals ($p<0.05$). Additionally, F4-PS rats displayed significantly increased exploratory behaviour in both age groups ($p<0.01$). In summary, multigenerational PS exposure increased exploratory behaviours and induced hyperactivity, this effect was reduced by aging.

4.4.2 Multigenerational PS Increases Anxiety-like Behaviours

Aging significantly increased anxiety-like behaviours in the EPM as animals spent more time in closed arms than the middle aged rats ($F(1,21) = 15.858$, $p<0.001$; see Figure 3). This increase in anxiety-levels was independent of the stress exposure ($F(1, 21) = 8.086$, $p= 0.337$; Figure 4.3), as stressed and non-stressed age matched animals spent a similar amount of time in the closed arms.

Interestingly, exposure to F1-PS significantly decreased the time spent in closed arms in middle aged rats ($p<0.05$) and had no effect in aged rats. By contrast, exposure to F4-PS

increased these behaviours in middle aged rats and decreased them in aged animals ($F(1,21) = 11.880$, $p<0.01$; Figure 4.3). Pairwise comparison revealed that the F4-PS exposure significantly increased the time spent in closed arms in aged animals in comparison to F1-PS ($p<0.01$). Therefore, transgenerational stress exposure interacts with aging to enhance anxiety-like behaviours in male rats.

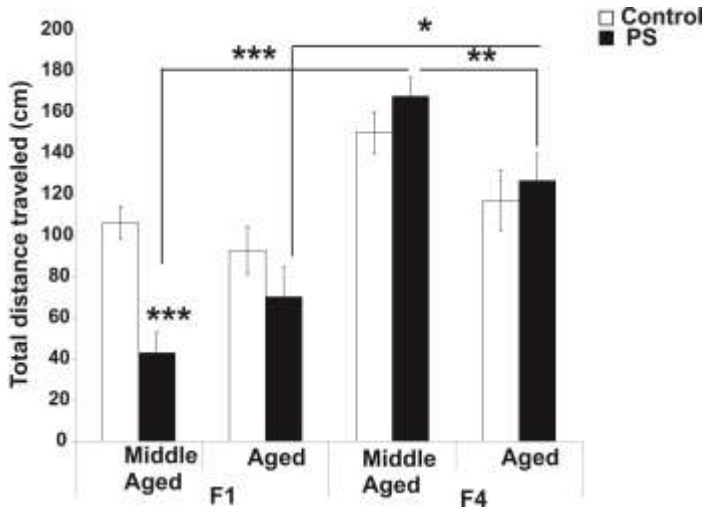

Figure 4.2: Transgerational programing by prenatal stress altered exploratory behaviour in aging animals. Mean ± SEM of total distance traveled in the open field task (OFT). Transgerational stress (F4-PS) increased the exploratory behaviour in middle aged and aged animals in comparison to prenatal stress (F1-PS). Importantly, transgenerational programming had biggest effects in midlife, as these animals experienced the highest increase in the exploration. Asterisks indicate significances: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

4.4.3 Depression-like Behaviours are Altered by Aging and PS, but not by Multigenerational PS

Age is associated with higher susceptibility to develop the depression-like behaviours, which is reflected by the time spent floating in the FST. Accordingly, the aged animals spent significantly more time floating in the FST than middle aged animals from either group ($F(1,19) = 173.0$, $p<0.001$; see Figure 4.4). F1-PS and F4-PS rats did not induce a significant effect in the time spent floating. However, there was an Age x Generation interaction in which

animals exposed to F1-PS in either age group spent less time floating than F4-PS animals or control animals ($F(1,19) = 30.611$, $p<0.001$; Figure 4.4). Paired tests revealed that the F4-PS exposure significantly increased depression-like behaviours in middle age in comparison to animals exposed to F1-PS ($p<0.001$).

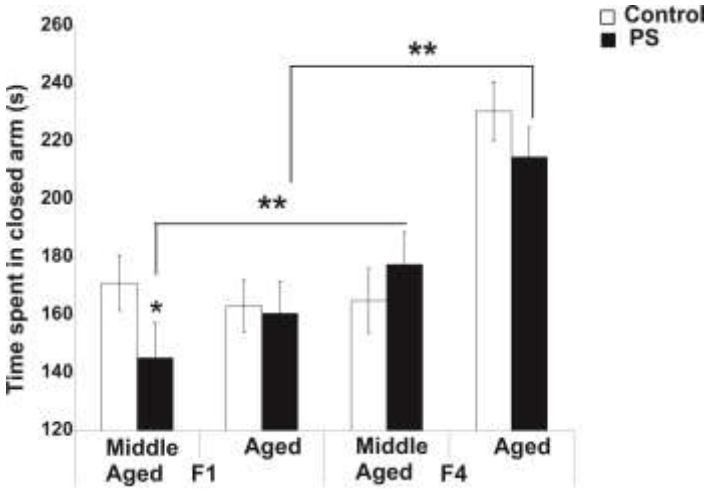

Figure 4.3: Transgenerational stress enhanced age-related increase in anxiety-like behaviour. Mean ± SEM of time spend in closed arms of the elevated plus maze (EPM). Aged animals were more anxious then the middle aged. Both prenatal and transgenerational stress increased the anxious behaviour, except that the increase was two fold in animals the F4-PS. Asterisks indicate significances: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

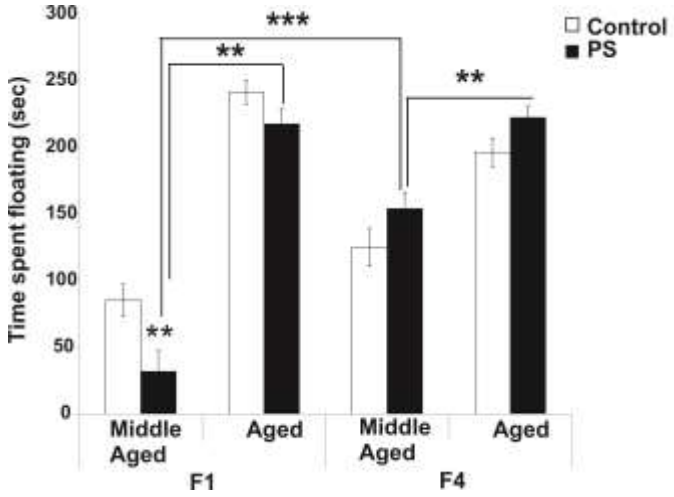

Figure 4.4: Age-related alterations in the depressive-like behaviour were enhanced by transgenerational and prenatal stress. Mean ± SEM of time spend floating in the forced swim task (FST). Aging increased depressive behaviours, as aged animals spend more time floating than the middle aged. Transgenerational stress increased depressive-like behaviours at both aged. The prenatal stress and transgenerational stress had opposite effects on depressive

behaviours, as floating time decreased in the F1-PS and increased in the F4-PS animals. Asterisks indicate significances: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

4.4.4 Basal Corticosterone Levels are Altered by Multigenerational PS in Aged Animals

Animals exposed to F4-PS had significantly higher basal corticosterone levels than those of the F1-PS group ($F(1,19) = 7.143$, $p<0.05$; Figure 4.5). Interestingly, there was a significant Age x Generation interaction ($F(1,19) = 4.142$, $p<0.05$; Figure 4.5) where neither PS exposure nor aging had an effect on basal corticosterone levels. However, F4-PS increased corticosterone levels at middle age, but decreased them at older age. Pairwise comparisons revealed that F4-PS exposure significantly decreased corticosterone levels in aged in comparison to middle aged animals ($p<0.01$). Therefore, elevated basal corticosterone levels as a result of multigenerational stress in middle age are altered by aging.

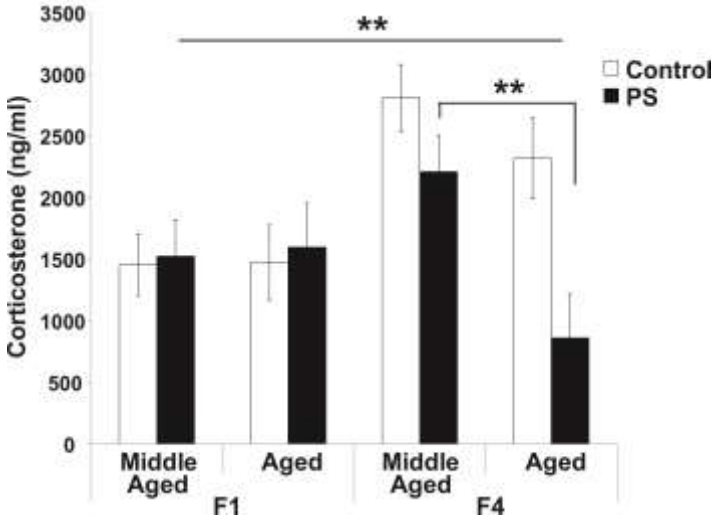

Figure 4.5*:* Transgenerational but not prenatal stress altered the corticosterone (CORT) levels in aged specific manner. Mean ± SEM of baseline CORT levels. Prenatal stress slightly increased CORT levels, with no aging effects. There was an aging decrease in CORT levels in the F4-PS animals. Transgenerational stress increased CORT levels in middle aged and decreased in aged animals. Asterisks indicate significances: ** $p < 0.01$, * $p < 0.05$.

4.4.5 Multigenerational PS Alters Chronic Adrenal Gland Activity

Multigenerational stress significantly altered both adrenal and thyroid gland activity in

aged animals. Two-way ANOVA revealed main effects of Generation ($F(3,28) = 47.272$, $p<0.01$; Figure 4.6) and Group ($F(3,28) = 6.601$, $p<0.05$; Figure 4.6). F1-PS and F4-PS exposures increased Na/K ratio, which is indicative of increased adrenal gland activity. Additionally, animals exposed to F4-PS showed a significant decrease in the Na/K ratio in comparison to F1-PS animals ($p<0.01$). Therefore, it is possible that multigenerational PS compromises adrenal gland function in contributes to adrenal exhaustion.

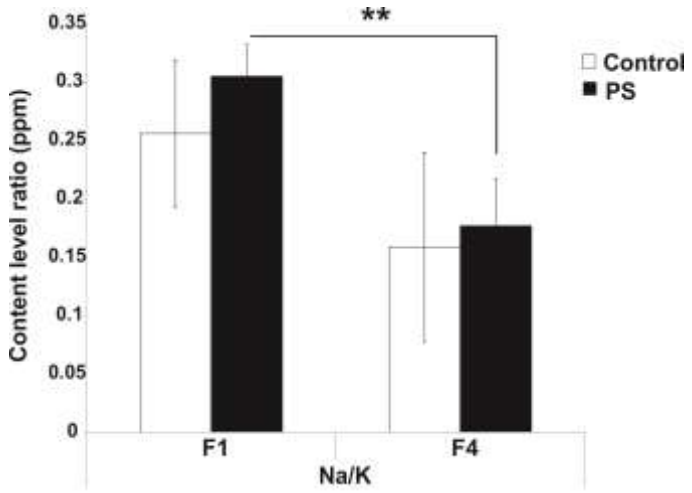

Figure 4.6: Transgenerational stress altered adrenal gland activity. Mean ± SEM of Na/K content level. Aged animals exposed to transgeration stress show a decrease in Na/K content levels. Asterisks indicate significances: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

4.5. Discussion

Although the association between PS and mental health in humans and experimental animals is well-established, no study had yet investigated the consequences of multigenerational PS, resembling offspring being born to a continuously stressful environment. Here we provide evidence that multigenerational programming by PS disrupts HPA axis reactivity and increases the severity of anxiety-like and depression-like behaviours in middle aged and aged male rats. Our data suggest that multigenerational PS through elevated HPA axis activity may contribute to adrenal exhaustion and reduced circulating plasma corticosterone

levels and ultimately alter the general lifetime susceptibility to behavioural alteration, and vulnerability to behavioural changes is increased in older age. Additionally, aging increased anxiety-like and depression-like behaviours in non-stressed and parentally stressed (F1-PS) animals; this effect was exaggerated by multigenerational exposure to PS. While the initial behavioural manifestations of anxiety and depression-like behaviours often start in adolescence and early adulthood, these findings provide a new insight into synergistic effects of transgenerational inheritance and age-related increased risk of anxiety and depression.

A confounding effect of the present stress paradigm was its apparent effects on control animals. While middle aged and aged F4-PS rats revealed distinct changes compared to the F1 generation, these were also evident in the control cohort. Controls and PS rats were housed in the same room that, despite its considerable size, possibly allowed controls to participate in bystander effects of the stress treatment. Similar observations have been described in detail previously (Mychasiuk et al., 2011a, 2011b), demonstrating behavioural, neuromorphological and epigenetic consequences. Indeed, altered dendritic complexity in the prefrontal cortex, orbitofrontal cortex and hippocampus may at least in part explain some of the intergenerational changes among the controls. Although pregnant dams in the present study were housed with a cage mate from the same experimental group, it is likely that through ultrasonic vocalisation (Mychasiuk et al., 2011b) or odor transmission PS may also have disrupted control behaviours and stress response.

The relationship between PS, brain development and the origins of mental disorders in adolescence and adulthood is well established (Lupien et al., 2009). PS is regarded as a predisposing factor for psychopathologies and behavioural disturbances in animals (Coe et al., 2003; Bowman et al., 2004) and humans (Weinstock 1997, 2002; Koenig et al., 2002; Kofman 2002). For example, in humans gestational stress is linked to poor coping behaviour, a greater

risk for hyperactivity disorder, anxiety and depression in children and adults (Weinstock 1997, 2001; Harris and Seckl 2011). While most studies have investigated effects of PS in young rats (Alonso et al., 1991; Takashi and Kalin 1991; Maccari et al., 1995; Seckl 1998), our data provide mechanistic evidence that PS is a risk factor for mental disorders in middle aged and aged male rats.

The present data suggest a synergistic interaction of PS and multigenerational PS with aging. While in the F1-PS generations the effects were visible mainly in middle aged male animals, in the F4-PS generation these effects were comparatively exaggerated, as indicated by the time spent in closed arms of the elevated plus maze. Notably, PS and aging had interactive effects across generations, especially in midlife. One reason for this observation may be an age-associated increase in glucocorticoid secretion and associated neuronal plasticity in hippocampus, hypothalamus and prefrontal cortex (Alasdair et al., 2006), possibly predisposing these individuals to behavioural disturbances (Weinstock et al., 1997, 2008; Glover et al., 2010; Harris and Seckl 2011). Thus, it is possible that PS exposure accelerates aging (Haussmann et al., 2012) resulting in loss of resilience to corticosterone hypersecretion and neuronal changes (Bloss et al., 2010; McEwan and Morrison 2013) in middle age rather than an older age. In the present study, the PS offspring seem more vulnerable to age-related functional decline and may develop psychopathologies earlier in life than the non-stressed individuals.

The present data provide a close correlation between behavioural phenotype and enhanced HPA axis activity. Fetal exposure to excess glucocorticoids during early development was discussed as a risk factor for HPA axis dysfunction (Weinstock 1997; Tsigos et al., 2002; Bowman et al., 2004; Cottrel and Seckl 2009; Gardner et al., 2013). The PS offspring in general exhibits hyper-reactivity to an acute stress challenge or novelty and increased susceptibility to emotional disturbance throughout life (Fride et al., 1986; Weinstock 2008;

Harris and Seckl, 2011). These changes parallel those found in depressed patients with hypercortisolemia (Alasdair et al., 2006). Furthermore, feedback inhibition of the HPA axis by circulating glucocorticoids is impaired in both PS rodents and depressed patients (Holsboer et al., 1984; Maccari et al., 1995; Valle´e et al., 1997).

Effects of PS on depressive-like behaviour are mediated by alterations in neurotransmitters such as serotonin 5-HT and glucocorticoid receptor (GR) density (McEwan 1987; Lupien et al., 2009). PS has been shown to reduce serotonin 5-HT, noradrenaline and dopamine levels in the adult brain (Peters et al., 1982; Takashi et al., 1992; Welberg and Seckl 2001).These changes may modulate hippocampal synaptic density in adult PS rats (Lupien et al., 2009). Furthermore, PS alters GR density in hippocampus and frontal cortex in young and adult rat offspring (Lindsay et al., 1996). This PS-induced reduction in hippocampal GR density impairs the negative axis feedback function through the hippocampus (Weinstock et al., 1997; Lupien et al., 2009). The resulting hyperactive HPA axis will expose limbic regions such as hippocampus, amygdala and frontal cortex to excess glucocorticoids, possibly inducing depressive behaviours (Charil et al., 2010; Harris and Seckl 2011).

The consequences of PS are highly sex specific, which is confirmed by the present study. In general, males seem more sensitive to effects of PS. Rodent studies reported that PS male offspring are more prone to developing learning impairments in water maze than PS female offspring (Szuran et al., 2000; Weinstock, 2007). Additionally, PS male rats display an increased response to anxiogenic stimuli in an open field (Van den Hove et al., 2005) and an elevated plus maze, while females show decreased response to these stimuli (Vallee et al., 1997; Maccari et al., 2007; Zuena et al., 2008). Interestingly, male rats that displayed increased anxiety-like behaviours in the elevated plus maze showed reduction in the survival of newborn cells in the dentate gyrus but an increased level of brain-derived neurotropic factor (Zuena et

al., 2008). By contrast, females seem to show more resilience to the effects of PS. In general, PS females show preserved ability of spatial learning (Szuran et al., 2000) and skilled reaching compared to males (Jadavji and Metz, 2008). This behavioural resilience is in contrast to a tendency of females to display higher corticosterone levels than males (Szuran et al., 2000; Jadavji and Metz, 2008).

In the present study, elevated HPA axis activity induced by PS was associated with a decreased Na/K ratio. Particularly, multigenerational stress exposure decreased the Na/K ratio in aged male F4-PS in comparison to the F1-PS animals. As suggested by Ambeskovic et al. (2013), a low Na/K ratio may represent an indicator of adrenal gland activity and thus a reduction may suggest adrenal exhaustion due to chronic elevated stress reactivity in old age. Furthermore, low Na/K ratio and weaker adrenal glands may be result of lower cellular energy associated with aging (Wilson, 2010). Importantly, low Na/K ratio in F4-PS aged animals may be an indicator of chronic stress accumulated over generations that alters circulating aldosterone and corticosterone (Hilavacova and Jezova, 2008; Kubzansky and Adler, 2010). Thus, emotional and psychological symptoms such as depression observed in our study may be linked to alterations in these hormones.

A novel feature of the present study is the comparison of PS within one and across multiple generations. The result show, aside from bystander stress effects in controls, that transgenerational cumulative experiences affect the risk of anxiety- and depression-like behaviours. Notably, Franklin et al. (2010) reported the propagation of early life stress to the F3 generation, where it results in depression-like behaviours in forced swim task in males, but not in females (Franklin et al., 2010). Thus, programming of stress-related behavioural traits is not restricted to direct effects on the germ line in the immediate offspring exposed to PS, but also transmits to subsequent generations (Matthews and Phillips, 2012).

A continuously stressful environment, as induced by multigenerational PS persisting for four generations, arguably results in a cumulative risk for anxiety-like and depression-like behaviours. Development of these psychologically vulnerable phenotypes may be explained by increased HPA axis activity due to PS (Radtke et al., 2011). DNA methylation is a potential mechanism by which multigenerational PS may program changes in genes operating in the HPA axis (Radtke et al., 2011). The GR gene is a key regulator of HPA axis function and negative feedback regulation (Radtke et al., 2011). In humans, PS is associated with methylation of exon 1F in the GR promoter, indicating a perinatal programming effect that may exert a lifelong influence on HPA-axis regulation (Radtke t al., 2011). Such changes in endocrine regulation, through altered forebrain limbic circuit and catecholamine activity (de Kloet Er et al., 2005), may be linked to onset of anxiety-like and depressive-like behaviours (Ehlert et al., 2001; Welberg and Seckl, 2001; Darnaudery and Maccari, 2008). It is possible that these changes, in an adaptive or maladaptive way, are altered by cumulative PS across generations.

Aside from endocrine programming, the effects of PS across generations may also be passed on by epigenetically induced modification of gene expression (Skinner, 2008). In a rodent study, PS altered expression of 582 hippocampal genes, of which 52% were under-expressed and 48% were over-expressed (Bogoch et al., 2007). Importantly, under-expressed genes were essential for development and axonal growth, function of ion channels and regulations of neurotransmitter release (Bogoch et al., 2007). These changes represent risk factor for development of anxiety-like and depressive-like behaviours.

The present study shows transgenerationally cumulative effects of multigenerational PS exposure in terms of physiological and behavioural consequences. These findings provide novel insights into the transgenerational origins of mental health and suggest increased

vulnerability to mental illness in older age. Thus, while the younger brain may somewhat show resilience to some consequences of multigenerational PS, PS certainly comes at an expense to the aged brain. These findings provide an explanation for the increasing incidence of common mental illnesses in the elderly. Mechanistic insights like these are important in order to advance the discovery of biomarkers of mental health and healthy aging and improve early life interventions.

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Chapter 5: General Discussion on Transgenerational Programing by Prenatal Stress

4.1 Summary

The main objective of this research was to investigate if multigenerational or recurrent prenatal stress across four generations will exaggerate well studied effects of prenatal stress in single generation on the brain and behaviour across lifespan. In Chapter 2, it was examined if multigenerational prenatal stress differentially affects behavioural traits, laterality and hemispheric dominance in male and female rats. Adult male and female F4 progeny were tested in fine motor skills, paw preference, hemispheric dominance and the neuronal morphology of the parietal (Par1) cortex was analyzed. Chapter 3, provided evidence and data which suggest that hair elemental analysis serves as a sensitive, comprehensive and accurate screening tool of age-related metabolic and overall health status. In chapter 4, the manifestations of PS on behavioural and physiological outcomes in adult male rats after exposure to PS in one generation (F1-PS) vs. multiple generations (F4-PS) were examined. To investigate the long-term mental health outcomes and synergistic effects of PS and age, anxiety-like and depression-like behaviours in aging rats were assessed longitudinally. Lastly, possible effects of transgerational programing by parental stress on the HPA axis reactivity trough baseline corticosterone levels and adrenal gland activity were examined. The effects of PS on the brain and behaviour in single generation (F1), recurrent multiple generations (F4-PS) and PS (F1-PS) vs. multigenerational PS (F4-PS) will be discussed below. Lastly, possible mechanism of transgerational transfer across generations will be examined.

4.2. Effects of PS (F1) across lifespan

4.2.1 PS modulates the trajectory of fetal brain development

The fetal brain development is characterized by a high turnover of neuronal connections that predict the behavioural outcomes (Weinstock, 2008). Recent experimental data suggest

that PS predisposes offspring to excess amounts of glucocorticoids during the critical window of development and alters the vulnerable brain regions such as amygdala, prefrontal cortex, hippocampus, and hypothalamus (Charil et al., 2010; Markam and Koeing, 2011). Bellow we will discuss PS induced neuronal changed in prefrontal cortex, hippocampus and hypothalamus.

The prefrontal cortex receives input from all other cortical regions and is implicated in decision making, the regulation of emotional and cognitive behaviours and planning and directing motor movements (Weinstock, 2008; Kolb et al., 2012). PS may affect these functions as a result of dendritic plasticity of prefrontal cortex subregions, including nucleus accumbens (NAc), medial prefrontal cortex (mPFC), anterior cingulate (ACC) and orbitofrontal cortex (OFC).

The hippocampus plays a vital role in memory formation and cognition. Since the dentate gyrus (DG) region of the hippocampus is a region of adult neurogenesis, major hormonal or neurochemical insults that modulate neurogenesis can affect hippocampal function. In particular, the hippocampus is very vulnerable to glucocorticoid manipulations early in life, as it has highly expressed GR and MR (Weinstock, 2008). Rodent studies have suggested a significant decrease in synaptic spine density of the hippocampus and impaired reversal learning in PS offspring (Harris and Seckl, 2011). Importantly, the increased glucocorticoid activity as a by-product of PS may explain the reduction in hippocampal GR and MR receptors (Charil et al., 2010) and HPA axis dysregulation (Lupien et al., 2009). One of the major players in the HPA axis activity is the hypothalamus.

The hypothalamus is composed of several nuclei, of which some are sexually dimorphic (Charil et al., 2010). One of those nuclei is the medial preoptic area (SDN-POA), which plays a role in male sexual behaviour in rats, such as mounting and ejaculation (Charil et al., 2010). A

cross sectional analysis of the SDN-POA area has revealed that this area is larger in males than in females on postnatal day 20 and 60, but there is no difference at birth (Charil et al., 2010). Similarly, the human SDN-POA contains twice as many cells and is twice larger in males than in females (Charil et al., 2010). This sexually dimorphic difference in size arises around the age of four years when the cell numbers start to decline in girls but remain the same in boys (Charil et al., 2010).

4.2.2 PS alters behaviour in adulthood

In adulthood, PS is associated with the dysregulation of HPA axis along with decreased GR receptor density (Chung et al., 2005; Ishiwata et al., 2005) resulting in affective disturbances. Both human and animal data has linked maternal stress to poor coping behaviours under diversity, increased levels of anxiety-like behaviours, depression and impaired learning and memory (Lemaire et al., 2000; Darnaudery and Maccari, 2008; Weinstock, 2008).

Several studies have shown that prenatally stressed rats spend more time freezing and less time in the center of an open field than controls (Harris and Seckl, 2011). Additionally, PS male and female rats spend less time than controls in the open arms and more time in closed arms of the EPM (Darnaudery and Maccari, 2008; Weinstock, 2008). Interestingly, depending on the time of the exposure to stress, intensity of exposure, frequency of exposure and sex and age of an animal, the affective property of PS may vary.

PS may result in a hyperactive HPA axis and altered development of the forebrain limbic circuitry, so that these systems overrespond to the stressor thus producing the depressive-like behaviours (Darnaudery and Maccari, 2008; Weinstock, 2008; Charil et al., 2010). In rodent models, a high incidence of depressive-like behaviours has been reported in offspring exposed to gestational stress (Weinstock, 2008; Harris and Seckl, 2011). PS male and

female rat's exhibit significantly increased immobility than their control counterparts in the forced swim task (Weinstock, 2008; Harris and Seckl, 2011). Furthermore, PS-exposed females generally spend more time in an immobile state than males. Additionally, aging increases the immobility time in both sexes, with aged females showing the highest immobility in the FST (Franklin et al., 2010; Charil et al., 2010).

4.3. Effects of multigenerational PS (F4) in adult male and female rats

In Chapter 2, it was shown that multigenerational PS promotes the development of new behavioural traits and affects brain and behavioural laterality in a sexually dimorphic manner. Three main findings are provided by this study. First, multigenerational PS shifted paw dominance in males but not in females. Second, multigenerational PS compromised skilled movement trajectories and skilled walking ability in males, but rather improved these abilities in females. Third, dendritic morphology indicated that multigenerational PS decreased multisynaptic plasticity in males but increased it in females. Notably, the shift towards left handedness in stressed males was accompanied by increased dendritic complexity in the right parietal cortex. Therefore, in the used measurements, the behaviour and the brains of stressed males were feminized to resemble the control female brains.

In summary, it could be argued that four generations of direct exposure to PS (multigenerational PS) is associated with larger left hemispheric dominance in male offspring. In spite of greater dendritic plasticity, this shift compromises the ability to perform skilled movements. While the current study does not provide conclusions about possible transgenerational programming of hemispheric dominance in the absence of stress, it is possible that multigenerational PS generates new behavioural traits that may be transmitted to subsequent generations. Thus, even in the absence of recognizable PS effects, hemispheric

dominance may become altered to produce a left-handed or ambidextrous phenotype. Based on evidence that PS or multigenerational programming of hemispheric dominance may also shift hemispheric dominance in humans, the present findings provide a mechanism for the lack of genetic associations with left handedness in humans.

4.4 Longitudinal effects of PS (F1-PS) vs. multigenerational PS (F4-PS) on affective disorders

Chapter 4 provides new evidence that multigenerational programming by PS (F4-PS) disrupts HPA axis reactivity and increases the severity of anxiety-like and depression-like behaviours in middle aged and aged male rats. Our data suggest that multigenerational PS (F4-PS) through elevated HPA axis activity may contribute to adrenal exhaustion and reduced circulating plasma corticosterone levels and ultimately alter the general lifetime susceptibility to behavioural alteration, and vulnerability to behavioural changes is increased in older age. Additionally, aging increased anxiety-like and depression-like behaviours in non-stressed and parentally stressed (F1-PS) animals; this effect was exaggerated by multigenerational exposure to PS (F4-PS).

In conclusions, the present study shows transgenerationally cumulative effects of multigenerational PS exposure in terms of physiological and behavioural consequences. These findings provide novel insights into the transgenerational origins of mental health and suggest increased vulnerability to mental illness in older age. Thus, while the younger brain may somewhat show resilience to some consequences of multigenerational PS, PS certainly comes at an expense to the aged brain. These findings provide an explanation for the increasing incidence of common mental illnesses in the elderly. Mechanistic insights like these are important in order to advance the discovery of biomarkers of mental health and healthy aging

and improve early life interventions.

4.5 Possible mechanism of transgenerational transfer across generations

Mechanisms of transgenerational transfer include altered gestational endocrine milieu maternal care and epigenetic inheritance. The early physiological processes are largely under influence of maternally derived factors and intrauterine environment (Ho and Burggren 2010). For instance, rats exposed to maternal glucocorticoids during gestation will have altered glucose metabolism (Ho and Burggren 2010). In our study, excess glucocorticoids during gestation altered HPA axis and GR density in prenatally stressed (F1-PS) offspring affecting behavioural outcomes and this effect was exaggerated by multigenerational PS (F4-PS).

Second, maternal care taking behaviours in rodents provide most convincing evidence of transgenerational transfer (Meaney et al., 2000; Zucchi et al., 2013). Ward et al., (2013) reported that stressed mothers spend more time chasing their tails than grooming their pups (Ward et al., 2013). Importantly, reduction in maternal liking and grooming of the pups are believed to be associated with modifications in offspring stress response (Weaver et al., 2004; Champagne and Meaney 2007; Ho and Burggren 2010). This may be particularly important for PS offspring which are predisposed to alterations in HPA axis trough excess maternal glucocorticoids. Thus, impaired HPA axis function will overreact to stress and induce behaviours such as anxiety and depression.

The third and widely investigated mechanism of transgenerational transfer is the epigenetic inheritance. Changes in the epigenome as a result of environment gene interaction may be accumulated and/or transmitted from one generation to next (Relton and Smith 2010; Ho and Burggren 2010; Kilpinen and Dermitzakis 2012; Aiken and Ozane 2013) altering the phenotypes of exposed and unexposed offspring. The major epigenetic events include DNA

cytosine methylation, histone modifications, transcriptional and posttranscriptional control of gene expression through Piwi-interacting RNA (piRNA) and microRNA (miRNA) (Meaney 2010; Migicovsky and Kovalchuk 2011). These epigenetic changes may produce two phenotypes. Multigenerational phenotype is results from direct environmental exposure over many generations on somatic and germ-line cells that allow tissue- specific toxicology in the individuals exposed (Skinner et al., 2011; Guerrero-Bosagna and Skinner 2011), an example of this is our F4-PS animals. A truly transgenerational phenotype is transmitted through the germ line in the absence of direct exposure (Skinner et al., 2011).

4.6 Conclusion

Multigenerational PS was shown to promote the development of new behavioural traits and affecting brain and behavioural laterality in a sexually dimorphic manner with shifted paw dominance in males but not in females. This shift in laterality was accompanied by impaired skilled movements in males and improved in females. Based on evidence that PS or multigenerational programming of hemispheric dominance may also shift hemispheric dominance in humans, the present findings provide a mechanism for the lack of genetic associations with left handedness in humans. Additionally, multigenerational programming by PS (F4-PS) disrupted HPA axis reactivity and increased the severity of anxiety-like and depression-like behaviours in middle aged and aged male rats. These findings provide an explanation for the increasing incidence of common mental illnesses in the elderly. Mechanistic insights like these are important in order to advance the discovery of biomarkers of mental health and healthy aging and improve early life interventions. Importantly, chapter 3 showed that hair elemental analysis serves as a sensitive, comprehensive and accurate screening tool of age-related metabolic and overall health status. Cobalt and aluminium content

levels may represent reliable biomarkers of aging. Aluminium in particular is regarded as an indicator of age-related neuropathology of vulnerable brain regions in Alzheimer's disease.

4.7 Limitations

The following three weaknesses can be identified in the approach to study the effects of transgenerational programming by prenatal stress on the brain and behaviour across the lifespan. First, this study lacks the true transgenerational effect, as SNNN animals were not available to compare to F4-PS or SSSS. Second, as reported in Chapter 3, our F4-control animals experienced bystander effects of PS. This made it difficult to make conclusive observations on the effects of multigenerational stress (F4-PS) on the affective state. Third, doing longitudinal study meant that the animals had to live and age up to 18 month, as this is considered old age for Long Evans rats. One of consequence of aging is health decline and sometimes death. In our experiment, our male population was decreased by 50% by the time animals would reach 18 months of age, or the last phase of testing. Thus, the small number of aged animals meant low statistical power, and sometimes inconclusive results.

4.7 References

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APPENDIX A: Single Pellet Reaching Task Rating Scale

1. Orient	-Head oriented to target			
	-sniffing			
2. Limb Lift	-body weight shift to back			
	-hindlimbs aligned with body			
	-limb moves forward			
	-digits on midline			
3. Digits Close	-palm supinated, semi-in			
	-digits semiflexed			
4. Aim	-elbows come in			
	-palm in midline			
5. Advance	-elbow in			
	-limb forward			
	-limb directed to target			
	-head and upper body raised			
	-body weight shift front			
	-body weight shift lateral			
6. Digits Open	-digits open			
	-discrete limb movement			
	-not fully protonated			
7. Pronation	-elbow out			
	-palm down in arpeggio			
8. Grasp	-arm still			
	-digits close			
	-hand lifts			
9. Supination I	-elbow in			
	-palm medially before leaving slot			
	-palm turned 90^{o}			
10. Sup. II	-head points down			
	-body horizontally			
	-palm straight up			
	-distal limb movement			
11. Release	-open digits			
	-puts food in mouth			
	-head and upper body lowered			
	-raises other paw			