Effect of Prenatal Exposure to Glucocorticoids and Ionizing Radiation on Programming of Adaptive Behaviour and Neural Genetic Dysregulation in Adult Offspring

by

Christine Nancy Lalonde

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy (PhD) in Biomolecular Sciences

The Faculty of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
Laurentian Université/Université Laurentienne
Faculty of Graduate Studies/Faculté des études supérieures

Title of Thesis Titre de la thèse	Effect of Prenatal Exposure to Glucocorticoids and Ionizing Radiation on Programming of Adaptive Behaviour and Neural Genetic Dysregulation in Adult Offspring
Name of Candidate Nom du candidat	Lalonde, Christine Nancy
Degree Diplôme	Doctor of Philosophy
Department/Program Département/Programme	Biomolecular Sciences
Date of Defence Date de la soutenance	July 23, 2020

APPROVED/APPROUVÉ

Thesis Examiners/Examinateurs de thèse:

Dr. TC Tai
(Supervisor/Directeur(trice) de thèse)

Dr. Doug Borham
(Co-supervisor/Co-directeur(trice) de thèse)

Dr. Aseem Kumar
(Committee member/Membre du comité)

Dr. Kabwe Nkongolo
(Committee member/Membre du comité)

Approved for the Faculty of Graduate Studies
Approuvé pour la Faculté des études supérieures
Dr. David Lesbarrères
Monsieur David Lesbarrères
Dean, Faculty of Graduate Studies
Doyen, Faculté des études supérieures

Dr. Tony Hooker
(External Examiner/Examinateur externe)

Dr. Steven Arnocky
(Internal Examiner/Examinateur interne)

ACCESSIBILITY CLAUSE AND PERMISSION TO USE

Abstract

Early life exposure to stress can lead to physiological and behavioural adaptations in offspring. Adaptive changes do not always benefit the organism, as it may result in adult diseases such as hypertension and diabetes in response to prenatal nutritional deficiencies. This thesis investigates the behavioural and genetic profiles of offspring exposed to two pathways of oxidative stress and DNA methylation: synthetic glucocorticoids and ionizing radiation. Synthetic glucocorticoids are able to bypass the placental enzymatic barrier and directly interfere with fetal gene expression by binding to glucocorticoid binding elements to either promote or inhibit expression as well as inducing changes in methylation of CpG islands. Ionizing radiation induces reactive oxygen species that will initiate DNA damage and oxidative stress leading to epigenetic modifications of gene regulation. The exposure to synthetic glucocorticoids induced adaptive phenotypical changes in Wistar-Kyoto offspring, inducing a stress-coping strategy and increased exploratory activity in combination with gene dysregulation in the prefrontal cortices. Exposure to ionizing radiation in C57Bl/6J mice did not induce significant behavioural changes; however, did elicit a few changes in gene expression in the prefrontal cortices, cerebral cortices, hippocampi, and cerebella that were sexually dimorphic. In contrast, the same radiation exposure study replicated in BALB/c mice induced extra-activity in offspring when faced with stress, arguably an adaptive response that may pose a risk to the animal. Significant gene dysregulation of oxidative stress and neuronal proliferation pathways was discovered in the prefrontal cortices, cerebral cortices, and cerebella of the BALB/c offspring. In consideration of the literature and the results of these studies, fetal programming of adult behavioural profiles may be accomplished through stress-induced genetic modifications.

Keywords Ionizing radiation, glucocorticoids, behaviour, adaptation, fetal programming

Acknowledgements

This doctoral thesis is dedicated to my grandparents, Ignatius and Lillian Flynn. This PhD represents the formal start of my academic career, not the end of a goal. My grandparents were pivotal in my love for reading, mystery, and science. They were unwavering in their support of education—always presenting me with more material and never telling me I should pause or that my goals were unobtainable. I hope they realize how much those moments mattered to me and that they are always in my thoughts.

I would like to formally thank my committee, Drs Kumar, Nkongolo, Boreham, and Tai for their positivity, humour, and guidance throughout the past four years. It was always a pleasure and never a struggle to work with you on this project.

This work was not possible without familial support. My mother and father-in-law, Sara and Eugene, have always been there to support me with childcare, to ensure I met deadlines and was not under extra stress. Without them, this would most definitely have taken a decade. And to my parents, André and Michelle, who have always supported me since I was little, knowing nothing other than studying science would make me content, thank you for always cheering me on—even when you did not understand it.

Drs Khurana, Tharmalingam, Thome, and Pirkkanen have always been there, each providing different kinds of mentorship, criticism, and friendship. You have made these four years some of the most memorable in my adult life and have prepared me for things I would never have considered, such as laboratory swordsmanship. Because of all of you, planning and preparation have become key components to all my goals (1).

(Dis)Honorable mentions go to my friends, Amy and Ash, who were there in the beginning. You were and always will be, my first teachers. This is all your fault (2). I love you.

No research seems possible without the most valuable resources a laboratory can offer: technicians and coordinators. Lisa, Mary Ellen, Taylor, and Dr. LeFort, nothing ever happens without all of you and I will never forget that.

My lab mates, Jeremy, Shayen, and Alyssa—thank you for all of the arguments, the scut work, and the laughter, you have all furthered my goals for world domination (3,4).

The last person I would like to acknowledge deserves his name next to mine on this thesis. This work is not just mine, but also Ben's. The time and effort in completing this body of work was possible because my husband stood by me. He worked extra hours so I did not have to, took care of our children and our house to ensure that every turn I was not over-tired, burdened, or alone. Every small and large gesture that was made by him supported every word of this thesis, allowing me to follow a dream. A gift I will never forget. Thank you for the love and support, Ben.

Table of Contents

Thesis Defense Committee ii
Abstract iii
Acknowledgements iv
Table of Contents vi
List of Tables ix
List of Figures x
List of Appendices xi
List of Abbreviations xii
Chapter 1 – Introduction 14
1.1. Brain and Behaviour 14
1.2. Fetal Programming of Brain and Behaviour 15
1.2.1. The Prefrontal Cortex 17
1.2.2. The Cerebral Cortex 18
1.2.3. The Hippocampus 19
1.2.4. The Cerebellum 20
1.2.5. The Hypothalamic-Pituitary-Adrenal Axis 20
1.4. Hypotheses 24
1.5. Objectives 24
Chapter 2 – Late Gestational Exposure to Dexamethasone and Fetal Programming of Abnormal Behaviour and Genetic Dysregulation in Wistar-Kyoto Rats 26
2.1.1. Introduction 27
2.1.2. Methods 27
2.1.3. Results 27
2.1.4. Conclusion 28
2.2. Introduction 29
2.3.1. Ethics 31
2.3.2. Animals and Housing 31
2.3.3. Breeding 32
2.3.4. Glucocorticoid Treatment 32
2.3.5. Offspring 32
2.3.6. Behavioural Tasks 33

2.3.7. Brain Dissections....34
2.3.8. Primer Design....35
2.3.9. RNA Extraction and Complimentary DNA Synthesis....35
2.3.10. RT-qPCR....36
2.3.11. Statistical Analysis....36
2.4. Results....36
2.4.1. Behavioural Tasks....36
2.4.2. Genetic Analysis....39
2.5. Discussion....43
Chapter 3 – Absence of Depressive and Anxious Behaviour Adult C57Bl/6J Mice After Prenatal Exposure to Ionizing Radiation....47
3.1. Abstract....48
3.2. Introduction....49
3.3. Materials and Methods....52
3.3.1. Ethics....52
3.3.2. Animals and Housing....52
3.3.3. Irradiation....52
3.3.4. Offspring....53
3.3.5. Behavioural Tests....53
3.3.6. Brain Dissections....55
3.3.7. Primer Design....55
3.3.8. RNA Extraction and Complimentary DNA Synthesis....55
3.3.9. RT-qPCR....56
3.3.10. Statistical Analysis....57
3.4. Results....57
3.4.1. Behavioural Tests....57
3.4.2. Genetic Analysis....58
3.5. Discussion....62
Chapter 4 – Prenatal exposure to ionizing radiation induces adaptive behaviour and altered gene expressions in the brain of *Mus musculus* offspring....67
4.1. Abstract....68
4.2. Introduction....69
4.3. Results....72

4.3.1. Behavioural Tasks 72
4.3.2. Genetic Analysis 76
4.4. Discussion 79
4.5. Materials and Methods 83
4.5.1. Ethics 83
4.5.2. Animals and Housing 83
4.5.3. Irradiation 83
4.5.4. Offspring 84
4.3.5. Behavioural Tests 84
4.3.6. Brain Dissections 86
4.5.7. Primer Design 86
4.5.8. RNA Extraction and Complimentary DNA Synthesis 86
4.5.9. RT-qPCR 86
4.5.10. Statistical Analysis 87
Chapter 5 – General Discussion, Conclusions and Future Directions 88
Conclusions 92
Future Directions 94
References 96
Appendices 128

List of Tables

Table 1. Average behaviour on elevated plus maze and Porsolt swim task.....37
Table 2. Fold change of DEX males and females compared to naïve controls.....40
Table 3. Observational Data on Open Field Task.....57
Table 4. Observational Data on Social Anxiety Task.....58
Table 5. Relative Gene Expression in 1000 mGy Offspring.....60
Table 6. Relative Gene Expression in BALB/c 1000 mGy Offspring.....77

List of Figures

Figure 1: Various Maternal Stressors....22
Figure 2. Behavioural tasks used to examine the anxiety and stress-coping strategies....34
Figure 3. Treatment main effect of the average number of 4-paw entries into the open arm of the EPM....38
Figure 4. Treatment main effect of the average time (s) spent immobile on testing day 1 in the PST....39
Figure 5. Interaction of Sex by Treatment in gene expression of ryanodine receptor 2....41
Figure 6. Interaction of Sex by Treatment in gene expression of DNA methyltransferase 3-β...42
Figure 7. Interaction of Sex by Treatment in gene expression of membrane-bound transcription factor peptidase site 1....43
Figure 8. Related gene functions in relation to ROS....59
Figure 9. Epigenetic modification pathways....71
Figure 10. Behavioural tasks for Mus musculus....73
Figure 11. Total time (s) spent immobile in a Porsolt swim task....74
Figure 12. Average number of rears during the social anxiety task....75
Figure 13. Average number of approaches to a stranger mouse during the social anxiety task...76
Figure 14. Differentially expressed genes in 1000 mGy male offspring versus 1000 mGy female offspring....77

List of Appendices

Appendix A – Supplementary Information: Primer Design for Dexamethasone Study 128
Appendix B – Supplementary Information 2: Primer Design for Radiation Study 129

List of Abbreviations

ANOVA – Analysis of variance

AP – Anterior posterior

APAF1 – Apoptosis protease activating factor 1

BAX – BCL-2 associated x protein

BDNF – Brain-derived neurotrophic factor

BLA – Basolateral amygdala

BORIS – Behavioural observation research initiative software

BTG2 – B-cell translocation gene 2

cDNA – Complimentary DNA

COMT – Catechol-o-methyltransferase

DCX – Doublecortin

DDB2 – DNA binding protein 2

DNMT1 – DNA methyltransferase 1

DNMT3a – DNA methyltransferase 3a

DNMT3b – DNA methyltransferase 3b

DREADD – Designer receptor exclusively activated by designer drugs

EPM – Elevated plus maze

GAD – Glutamate decarboxylase

GADD45 – Growth arrest and DNA damage

GC – Glucocorticoid(s)

GPX1 – Glutathione peroxidase 1

GR – Glucocorticoid receptor(s)

GRE – Glucocorticoid response element

HPA – Hypothalamus-pituitary-adrenal

HSD – Honestly significant difference

11β1HSD – hydroxysteroid 11βdehydrogenase type 1

11β2HSD – hydroxysteroid 11βdehydrogenase type 2

IGF-1 – Insulin growth factor 1

LSAMP – Limbic system associated membrane protein

MBTPS1 – Membrane bound transcription factor peptidase site 1

NeuroD – Neuronal differentiation

NOS3 – Nitric oxide synthase 3

PER2 – Period 2

PFC – Prefrontal cortex

PSD95 – Post-synaptic density protein 95

PST – Porsolt swim task

ROS – Reactive oxygen species

RT-qPCR – Real time quantitative polymerase chain reaction

RYR2 – Ryanodine receptor 2

SAT – Social anxiety task

SEM – Standard error of the mean

sGC – Synthetic glucocorticoids

SNAP25 – Synaptosome associated protein 25

SOD1,2,3 – Superoxide dismutase 1, 2, or 3

Chapter 1 – Introduction

1.1. Brain and Behaviour

Behaviour is simply defined as an organism's activity that can be observed or measured (5). Behaviours are internally coordinated and are produced in response to internal and external stimuli. Sexual reproduction, resource gathering, predation, and survival rely on appropriate behavioural responses to stimuli and those responses have the ability to change within individuals of most species (6–9). Modifications in normative behaviour can occur during an organism's lifespan through experiential learning. Organisms learn to avoid or carefully approach high-risk situations through first-hand or observational experiences (10,11). In contrast, rewards lead to increased likelihood of repetitive behavioural responses (12). This is an operant conditioning paradigm of learning; classical conditioning may also modify behaviour through association and reinforcement (5). Phenotypical behaviour changes may occur through epigenetic mechanisms, serving as evolutionary adaption and plasticity to enhance survival and fitness; however, may also predispose an organism towards maladaptive impulses and pathophysiology (13).

Changes in behaviour may lead to increased chances of survival and enhanced fitness; however, not all modifications lead to a positive result for the individual. Modifications may lead to the progression of disease and are considered maladaptive (14,15). Reward-seeking behaviour influencing addictions can develop from short-term reward based and disrupted learning processes (14,16). The ability to inhibit responses and focus on long-term rewards instead of short-term rewards are less likely to influence addictions (17). Inhibition and impulsivity have been linked to poor academic performance and risk-taking behaviours, such as gambling (18,19). Obsessive compulsive disorder, post-traumatic stress disorder, major depressive disorder, and schizophrenia

area all psychological disorders with significant maladaptive behaviours that disrupt the ability for individuals to function properly (20,21). These disorders not only highlight maladaptive behaviour, but also neurological dysfunction in various regions and organizational levels (22).

The brain is comprised of specialized electrochemical cells called neurons, that influence behaviour and cognition. Neurons were first introduced to the world in detail in the 1800s by Camillo Golgi and Santiago Ramón y Cajal, who was also the founder of the concept of plasticity (23,24). Plasticity refers to environmentally-dependent phenotype expression; an organism's ability to adapt and change to its environment (25). Neurons are physiologically diverse and function in circuits that are separated regionally. During fetal development and for a period after birth, mammalian neural circuitry is undergoing significant modifications to structure and connectivity (26). This period of development is sensitive to environmental factors and may lead to neurological and behavioural disorders later in life if exposed to stressful and inadequate conditions (27,28). Most mammals have functional homology, making comparative biology a reasonable experimental process for understanding both human and animal behaviour and disease.

1.2. Fetal Programming of Brain and Behaviour

Fetal programming explains the process whereby early environments influence the physiological phenotype of the offspring (29,30). Sometimes referred to as the Barker hypothesis and originally referred to as the thrifty phenotype hypothesis, Hales and Barker described the phenomenon in 1992 (31). The researchers associated early life nutritional deprivation with the development of adult metabolic syndrome. Through replication and further study, developmental plasticity became the forefront of consideration of evolutionary processes; hypothesizing that the adaptation to deficient environments would lead to development of disease (32,33).

Over the progress of fetal programming research, it became clear that nutritional deprivation was not the only prenatal stressor that could lead to maladaptive phenotypes. Malnutrition has been hypothesized to influence programming through a number of mechanisms, such as inflammation, oxidative stress, dysregulated metabolism, a decrease in placental enzymes, and an increase in steroidal hormones (34,35). Maternal plasma glucocorticoid levels have been shown to be increased with food restriction, along with the activation of the hypothalamic-pituitary-adrenal axis, and a decrease of glucocorticoid binding factors, leading to higher concentrations of active hormones free to interact with the fetus (36,37). Through the administration of exogenous steroids, glucocorticoids could stimulate the same physiological phenotype as malnutrition which displays hypertension, hyperglycemia, hyperinsulinemia, and changes in behaviour (38). Active endogenous glucocorticoids are prevented from interacting with the fetus via an enzymatic barrier present in the placenta, the 11β-hydroxysteroid dehydrogenase type 2 (11β2HSD) which catalyzes the conversion of cortisol into cortisone (Figure 8, Chapter 4). If the endogenous hormone concentrations are high, or if the mother is exposed to synthetic hormones which are poor substrates, the enzymatic barrier is inefficient and glucocorticoids will interact with the fetus (38). Exogenous hormones are often given for organ maturation for mothers at risk of preterm labour and lead to alterations in behaviour, including hyperactivity, reduced cognitive function, and anxiety-related behaviour in the offspring (39–41). Endogenous hormones, circulating due to maternal stress, such as anxiety, has also been linked to increased anxiety in their children (42). Once glucocorticoids bind to their receptors, they are able to change gene expression directly, by enabling DNA methylation, and by production of reactive oxygen species (ROS) (43,44).

1.2.1. The Prefrontal Cortex

The prefrontal cortex (PFC) is new on the scale of neurological evolution and is responsible for complex behaviour and cognition; however, it is not distinctly defined in structure or function (45). Research links the region to emotion, social interaction, decision making—all components of cognition (46). Reflexive behaviours are stereotyped and the focal point of other neural structures, but the PFC is known for top-down processing, whereby behaviour may be influenced by stimuli input, providing contextually relevant responses.

In order for the PFC to produce contextually appropriate responses, it requires environmental feedback from other neural regions. A key pathway connects the PFC to the basolateral amygdala (BLA), a structure that regulates emotion. The BLA provides significant positive feedback in early development that gradually declines with maturity; however, this decline is accelerated under stress, such as maternal deprivation (47,48). Disruption of BLA input, through optogenetic manipulation, induces synaptic depletion and interferes with fear-cue learning processes (49). Chronic stress to rodents, such as random exposure to restraint, forced swim in cool water, tilted cages, and shaking cages for fourteen days leads to decline of inhibition feedback, inducing abnormal aggression, inability to recognize novel objects, and increased locomotive activity (50). Reversal of all abnormal behaviours was possible with the introduction of designer receptor exclusively activated by designer drugs (DREADD) to reactivate connectivity between the PFC and the BLA.

Another brain region providing feedback to the PFC is the hippocampus, a structure foremost known for learning and memory, which is also connected to the amygdala. The connection between the PFC and the hippocampus provides experiential context based on episodic memory

formation and retrieval (51). Multiple early life insults from nutritional deficiencies to smoking and alcohol exposures have been associated with cognitive disorders and memory impairments in the hippocampus and PFC (52). Without normal development and connectivity from the PFC to these regions, behavioural responses are modified and adversive.

1.2.2. The Cerebral Cortex

The cerebral cortex in primates is convoluted to increase surface area, in rodents it is smooth, but is still located on the surface and is divided into functional regions called lobes. Often, the cerebral cortex and the prefrontal cortex are used interchangeably in literature and may seem ambiguous; however, the neural function of each region are distinct (53–55). Here we focus on the region involved in stimuli processing. Research into this region has correlated impoverished environments that are reduced in sensory and motor stimulation with decline in stimuli processing development (56). To explore the modification of the cerebral cortex's visual processing, pre-weaned rat pups and their mothers were placed in opaque cages to limit visual input. The effects of rearing pups in a diminished environment lead to delay in weight gain, visual maturity, motor activity, and a reduction of the brain-derived neurotropic factor (BDNF), insulin-growth factor-1 (IGF-1), and glutamate decarboxylase (GAD) gene expression. Prenatally malnourished rats show diminished β-adrenoceptor and BDNF expression levels, as well as impaired learning through long-term-potentiation deficiencies and visuospatial issues (57). Expression levels and other functions were restored with exposures to environmental enrichment, lending to the concept of adaptive modifications and plasticity in brain function. Late gestational exposure to synthetic glucocorticoids increased the regulation of early growth response 1 (EGR1) in the cerebral cortices and hippocampi of guinea-pigs (58,59). EGR1 expression is linked to neuronal activation and is indicative of physiological changes in the cerebral cortices. The ambiguity and interchangeability

of the terms prefrontal cortex and cerebral cortex throughout literature add a level of uncertainty to distinct regional changes with respect to fetal programming. A clear separation of anatomical regions is required to provide a clear representation of genetic phenotypes.

1.2.3. The Hippocampus

As previously mentioned, the hippocampus is well-known for learning and memory function, which are key to appropriate behavioural responses (60,61). The hippocampus is dense with glucocorticoid receptors and is the site of neurogenesis which is linked to cognitive flexibility and inhibition of depression and anxiety (62). Multiple connections with other brain regions support the memory and learning function of the hippocampus; particularly, fear cues and inhibition with feedback to the amygdala and prefrontal cortex (63). Maternal stress has been linked to a decline in neurogenesis, correlated to decreased neuronal functionality, language delays, and decreased cognition (35). Female rats born to dams who were restrained in late gestation had decreased levels of glucocorticoid receptors and impaired spatial learning and memory, implicating significant disruption of hippocampal physiology and function due to prenatal stress (64). Multiple generations of female offspring exposed to glucocorticoids have been shown to have dysregulated gene expression and DNA methylation within the hippocampus (65). Sexual dimorphism in brain regions is not uncommon; 21-day-old Long-Evans rat females have been noted to have significant gene dysregulation related to growth factors in the hippocampus after exposure to prenatal stress (66). Pregnant rats were stressed by placing their cages on an elevated platform for a total of 20 minutes per day during gestational days 12-16 and this environment was sufficiently stressful to show substantial gene regulation changes on a microarray analysis with 200 dysregulated genes in the female hippocampus compared to 167 in the males. Rat pups are not born fully developed and maternal care during early life also has been implicated in hippocampal plasticity, gene

methylation, and gene expression in offspring aged 7-17 weeks (67–72). It is clear that the hippocampus is a sensitive region to early life stress and is a common focus in neural experiments.

1.2.4. The Cerebellum

The cerebellum was originally thought to only control motor function; however, is more recently known to be involved in cognition, addictions, and depression (73). Exposure to prenatal stress, such as a diet insufficient in zinc and fatty acids, leads to decreased cerebellar volume linked to attention deficiencies, poor impulse control, and behavioural disorders (52). C57Bl/6J offspring, prenatally exposed to high levels of folic acid, displayed significant gene dysregulation in the cerebellum in both male and females (74). Genes differentially expressed were linked to autism disorder and neurodevelopment. In contrast to the high levels of folic acid, a study with vitamin deficiencies showed proteomic, cellular, molecular, and behavioural support of cerebellar disruption due to the early life stress (75). In humans, a significant reduction in grey matter within multiple brain regions, notably the prefrontal cortex, cerebral cortex, and cerebellum, was associated with mid-gestation self-reported anxiety (76,77). Cerebellar weight and volume in rat offspring were also sensitive to a single exposure to a synthetic glucocorticoid, betamethasone, in late gestation (78). The single exposure was also associated with anxiety-related behaviour and increased expression of calbindin-D28K, a neuroprotective protein whose expression is associated with levels of glucocorticoids. Similar to the previously highlighted neural regions, the cerebellum is clearly sensitive to early-life stressors.

1.2.5. The Hypothalamic-Pituitary-Adrenal Axis

The hypothalamic-pituitary-adrenal (HPA) axis is pivotal in allostasis—adapting to stressors in order to maintain homeostasis—by utilizing glucocorticoids, hormones produced in the adrenal

cortex, as a biofeedback system (79). It is a fundamental process in driving adaptive or maladaptive responses from an organism and disruption has been associated with mental and neurological disorders, such as schizophrenia, depression, addiction, and mood disorders (79,80). Just as with the other neural regions, the HPA axis is sensitive to early life stress, where deprivation can lead to modification of behavioural phenotypes (81). For example, nine day old Wistar rat pups deprived of maternal care have showed increases in serotonergic activity and anxiety, indicating changes in the hypothalamus (81). Furthermore, synthetic glucocorticoid administration has been associated with HPA modification, hyperactivity, and metabolic impairments (42,82).

Neural regions interact with each other to provide feedback, control, and modulation. The hippocampus and prefrontal cortex have both been implicated in HPA modulation (83,84). Glucose metabolism research shows prefrontal cortex activation during stressful events that is inversely related to salivary cortisol levels (84). Hyperactivity of the HPA axis also leads to decreased prefrontal cortex activity and depressive behaviours (85). Through lesioning, the hippocampus has been implicated as an HPA modulator due to functional changes when compared to lesions in other regions (83). The cerebellum has reciprocal pathways connected to the hypothalamus and has been implicated in depressive symptoms, implicating HPA involvement as well (86). The strength in the relationships between these regions and the HPA axis may implicate a strong response to prenatal stress. Unfortunately, there is a paucity of literature on the involvement of the cerebral cortex, which may be due to the ambiguity of anatomical delineation with the prefrontal cortex.

1.3. Ionizing Radiation

Stress is defined as an internal or external stimulus with a perceived threat to our survival and health (87). Stress may be an illness, injury, familial loss, mental health issues, or exposure to xenobiotics (Figure 1). Once exposed to a stressor, the body may react via the production of glucocorticoids, which may produce ROS (43,44,88). ROS may also be produced through direct and indirect mechanisms of ionizing radiation. Ionizing radiation is the emission or transmission of energy, either through a particle or a wave, that has sufficient energy to remove an electron from an atom or molecule producing a highly reactive ion, or free radical (89). Free radicals, also known as ROS, can induce direct DNA damage and cellular oxidative stress (90).

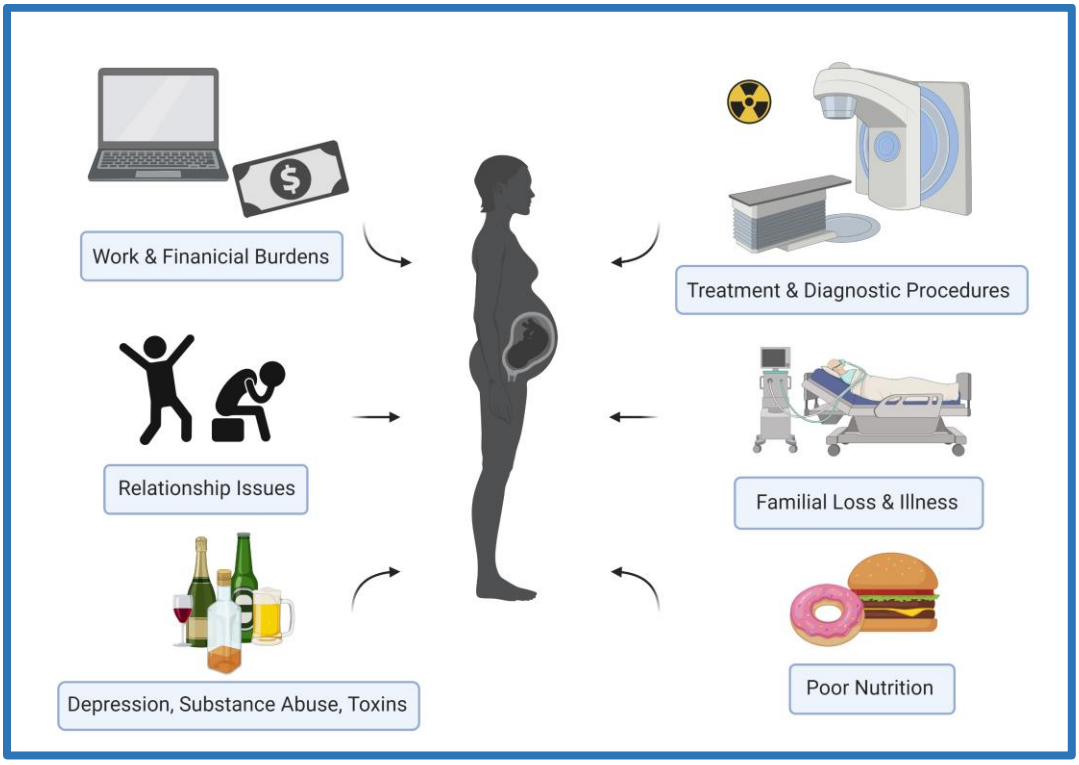

Figure 1: Various Maternal Stressors

Prenatal exposures to ionizing radiation may come from diagnostic imaging or workplace exposures (91). In early gestation, exposures to radiation can lead to fatality and significant organogenesis disruption. Malformities in appendages, growth restriction, and neuronal death are a few of the postnatal effects listed in a review conducted by Sreetharan and colleagues (91).

Exposures of 100-3000 mGy in late gestation led to neuronal cell loss, decrease in overall neural volume, and behavioural changes (91).

Fetal programming through irradiation is supported by previous research involving various radiation sources, multiple doses, and different gestational timing. In one study, whole body x-ray irradiation exposure to doses ranging between 0 and 1000 mGy to C57Bl/6J mice during early neurogenesis on gestational days 11 and 12 has shown locomotor and spatial memory deficits in the offspring (92). In addition, forty-one genes were differentially expressed related to p53 signaling, DNA damage, apoptosis, and signal transduction and there was a significant reduction in cortical thickness and hippocampus proliferation. X-ray exposure to C57Bl/6J mice on gestational day 11 altered post-synaptic density protein 95 (PSD95) in the hippocampi, with 1000 mGy, supporting research implicating the sensitivity of the hippocampus to stress and modifications (93). Swiss albino mice were exposed to 500 mGy of gamma radiation between gestational days 11 and 19 and offspring were tested for a variety of behavioural responses, where exposures to all gestational day treatment groups produced activity, anxiety, and memory deficiencies in the 3-month-old offspring (94). The various behavioural and genetic changes across the literature supports the idea that ionizing radiation may induce behavioural adaptations in response to the maternal stress. Adaptations that may not be preferential in particular circumstances.

Early gestational exposures to ionizing radiation show clear and significant damage to multiple organs and molecular processes; however, late gestational changes are varied and behavioural phenotypes are not always present, which may be due to the choice of testing paradigms, and are not always investigated (95). The neural regions previously discussed have clear sensitivities to exposures to early life stress and, together, are implicated in important behavioural responses.

Considering the regional influences on behaviour: inhibition, sensory input, cognition, fear, and memory consolidation, this thesis focuses on providing a portrait of behavioural and neural genetic phenotypes with respect to early life stress. Inhibition of risk-taking behaviour and stress-coping strategies are valuable to survival and different stressors may bring about adaptive phenotypes affecting similar behavioural and genetic expression patterns.

1.4. Hypotheses

Exposure to early-life stress leads to adaptive phenotypes in offspring that may or may not be adversive in novel situations. Neural regions responsible for complex behaviour will be affected by stress leading to contextual behavioural modifications. Specifically:

1 - Exposure to exogeneous synthetic glucocorticoids (sGC) in late gestation will affect adult offspring, such that: offspring will exhibit adverse behaviour in response to challenging environments. Key brain areas involved in anxiety and depression will display genetic dysregulation in stress and synaptic plasticity pathways.

2 - Exposure to ionizing radiation in late gestation will affect offspring, such that: offspring will elicit similar behavioural response to the sGC study, including pathway dysregulation at a threshold-dose of radiation. Low doses of radiation exposure may provide an adaptive mechanism in challenging environments.

H_0: There will be no genetic or behavioural differences with in-utero exposure to exogenous glucocorticoids or ionizing radiation.

1.5. Objectives

The following objectives were designed to investigate the proposed hypotheses:

1. Determine the behavioural and genetic phenotypes of offspring exposed to prenatal glucocorticoids.
2. Determine the behavioural and genetic phenotypes of offspring prenatally exposed to ionizing radiation.
3. Compare and contrast the adaptive or maladaptive nature of the phenotypes presented by the offspring in all experiments.

Chapter 2 – Late Gestational Exposure to Dexamethasone and Fetal Programming of Abnormal Behaviour and Genetic Dysregulation in Wistar-Kyoto Rats

Christine Lalonde, Julie Grandbois, Sandhya Khurana, Alyssa Murray, Sujeenthar Tharmalingam, Douglas Boreham, and T.C. Tai

In preparation to submit to *Brain and Behaviour*

Acknowledgments

This research was supported by grants from the Canadian Institutes of Health Research and the NOSMFA Research Development Fund.

2.1. Abstract

2.1.1. Introduction

Fetal programming was characterized a few decades ago, explaining the correlation of physiological phenotypes of offspring exposed to early life stress. High acute or chronic prenatal stress can overwhelm the enzymatic placental barrier, inducing transcriptional changes in the fetus that can result in different adverse behavioural and physiological phenotypes. The current study investigates the impact of exposure to the synthetic glucocorticoid, dexamethasone, during late gestation on behavioural outcomes.

2.1.2. Methods

Pregnant Wistar-Kyoto rats were given daily subcutaneous injections from gestational day 15-21 of either dexamethasone (0.9% NaCl, 4% EtOH, 100 µg/kg/day) or were physically manipulated as naïve controls. Pups were raised normally until 17 weeks of age and underwent the Porsolt swim task and elevated plus maze for depressive and anxiety-like behaviours respectively. Neural tissue was preserved for genetic analysis using quantitative real-time polymerase chain reaction.

2.1.3. Results

Statistical analyses show significant disruption of behaviour and genetic profiles of offspring exposed to dexamethasone in-utero. Exposed animals spent more time immobile on the swim task and entered open arms of the elevated plus maze more often than their naïve counterparts. In the prefrontal cortex, there was a sex by treatment interaction on gene expression relevant to neural transmission in ryanodine receptor 2, as well as increased gene expression in SNAP25, COMT,

and LSAMP in males prenatally exposed to dexamethasone compared to controls. Both dysregulated genes and behaviour are linked to decreased anxiety and fear inhibition.

2.1.4. Conclusion

Our results indicate adult offspring exposed to dexamethasone in-utero have a tendency towards passive stress-coping strategies and an inhibition of anxiety on behavioural tasks. Methyltransferase activity, synaptic activity, and cellular processes were disrupted in the prefrontal cortices of these animals. Specifically, genes involved in emotional response pathways were overexpressed, supporting the link between the behavioural and genetic profiles. Combined, we determine that dexamethasone offspring have considerably adverse predispositions when faced with novel situations, with increased immobility in the swim task and increased exploration on the elevated plus maze.

Keywords: Glucocorticoids, Fetal Programming, Prefrontal Cortex

2.2. Introduction

In 1992, Barker and Hales introduced the research community to the concept that early-life environmental conditions can influence the physiological phenotype of the offspring (31). They coined their early work, the 'thrifty phenotype hypothesis,' focusing on metabolism and diet. Their initial research showed that fetuses raised in a nutrient-deficient environment would be predisposed to type 2 diabetes (29,31). Multiple studies over the past decades have supported this hypothesis, implicating insufficient nutritional intake as a driver for adult onset obesity, type 2 diabetes, hypertension, and overall metabolic syndrome markers (96–98). An evolutionary theory proposes that the fetal environment predicts conditions outside, providing an adaptive mechanism whereby offspring will increase fat stores, but this mechanism is maladaptive in a nutritionally dense environment (98,99). Expansion on the thrifty phenotype hypothesis has compelled research in other stressful uterine conditions, such as hypoxia, illness, and the introduction of exogenous glucocorticoid levels (42,100–102). The impact of various maternal stress conditions that influence fetal development, continue to support the theory behind the development of metabolic phenotypes, as well as adverse diseases and behaviour.

In response to a stressful environment, the mother will endogenously produce increased levels of stress hormones, glucocorticoids. The placenta has an enzymatic barrier to reduce a high concentration of glucocorticoids from gaining access to the fetus. The enzyme 11-β hydroxysteroid dehydrogenase 1, or 11β1HSD, bidirectionally converts cortisol and corticosterone into inactive cortisone and 11-dehydrocorticosterone, while 11β2HSD is unidirectional in inactivation and prevents about 80% of glucocorticoids from interacting with the fetus (103). 11β2HSD knockout animal models demonstrate adverse behaviour related to depression and cognition, implicating glucocorticoid (GC) action on the nervous system (103). At high

concentrations, glucocorticoids can overwhelm the enzymatic barrier and bind to glucocorticoid receptors (GR) in the fetus, which are expressed in the cytoplasm of most cells, especially within different regions of the brain. This complex may then translocate to the nucleus and bind to glucocorticoid response elements (GRE) on various genes, initiating transcriptional activation and allowing for change in methylation of CpG islands of the GRE promoter regions (100,104–108). Hypothesized mechanisms for the change in local methylation status near GRE sites are by a decrease in methyltransferases and an increase in ten-eleven translocation methylcytosine dioxygenase (TET) in conjunction with the influx of GC (108). Methylation can then activate or silence genes depending on the number and location of methyl groups (109,110). Synthetic glucocorticoids (sGC), such as betamethasone and dexamethasone are often administered to pregnant mothers who are at risk for preterm birth (111). The sGC mature the fetal lungs, increasing survivability of prematurely born offspring. Synthetic GC bypass the enzymatic barrier because of low binding efficiencies, and bind to GR in high concentrations within the fetus. This process has been linked to fetal programming of metabolic disorder and changes in normative behavioural response to stressful situations in adult offspring (41,112,113).

Much research into methylation and fetal programming has focused on the hypothalamic-pituitary-adrenal (HPA) axis and the hippocampus (59,114,115). GR density, binding rate studies, and adrenalectomies have made clear associations in neurodevelopmental deficiencies (40,42,116,117). The HPA axis is involved in GC feedback and the hippocampus has high concentrations of GR, making both relevant targets of scientific research. When considering other aspects of mental health, however, other neural regions also play key roles and may be affected by GC concentrations

The prefrontal cortex (PFC) has been implicated in many adverse behaviours and neural diseases. Early research involving neural imaging techniques that investigated structural changes, such as regional volume, and functional changes in activity patterns show a correlation in abnormalities in the PFC and clinical depression (118). Abnormal PFC activity is pronounced in individuals with depression and schizophrenia, and further research has linked the PFC to emotional control under stress, where abnormalities within the PFC are correlated to depressive-like symptoms (119–121). Treatment of depression is effective when the PFC is stimulated with transcranial magnetic stimulation, supporting the link between the PFC and depression (122). Additionally, the PFC is also known to be sensitive to high concentrations of GC and increased levels of stress, where exposure leads to impaired working memory and a reduction in grey matter (123). In consideration of the literature, we hypothesized that prenatal exposure to sGC would lead to the fetal programming of abnormal mental health inclinations and PFC dysregulation, the focus of this study.

2.3. Materials and Methods

2.3.1. Ethics

All experimental protocols were approved by the Animal Care Committee of Laurentian University (AUP: 6013917) and were in accordance with the Canadian Council on Animal Care guidelines.

2.3.2. Animals and Housing

Eight-week-old Wistar-Kyoto rats (Charles River Laboratories, Canada) were housed in either pairs or triplets in Innocage® IVC disposable cages (Innovive Inc., San Diego, USA). Cages

contained cob bedding (Harklan, Madison, USA) and enrichment tubes (Bio-serv, Frenchtown, USA) and were placed into a HEPA filter Innorack® Rat airflow system (Innovive Inc., San Diego, USA). Food (Teklad 22/5 Rodent Diet, Harklan, USA) and water were available *ad libitum*. Animals were place on a 12:12 light-dark cycle, with the light cycle starting at 6:00 am. Room temperature was maintained at 25°C and humidity at 53%.

2.3.3. Breeding

Male rats were introduced to three females for a period of five days. After this period, females were checked for the presence of a vaginal plug and were then singly housed and weighed daily for the duration of their pregnancies.

2.3.4. Glucocorticoid Treatment

On gestational day fifteen and onwards, pregnant dams were placed in one of two treatment conditions: daily subcutaneous injections of DEX (0.9% sodium chloride, 4% ethanol, and 100 µg/kg dexamethasone) or naïve controls (physical manipulation only).

2.3.5. Offspring

Pups were weaned at three weeks of age, sexed, and housed in pairs or triplicates. Pups were raised under normal conditions until seventeen weeks of age, whereby they underwent behavioural testing (n = 8 per sex/group). At nineteen weeks, they were euthanized via intraperitoneal injection of 75 mg of ketamine (100 mg/ml, Ketalean, CDMV Inc., Canada) and 5 mg xylazine (100 mg/mL, Sigma, USA) per kg of body weight. Relevant tissues were harvested immediately and frozen on dry ice. Brains were stored at -80°C until genetic analysis.

2.3.6. Behavioural Tasks

All behaviour was recorded utilizing video-cameras and stored for scoring at a later time. Experimenters and observers were blind to treatment groups. Experimenters remained behind a black curtain to prevent distraction of the animals. All tasks were conducted during the light period, between 9:00 am and 4:00 pm. Animals underwent testing for a total of three days; the first task presented was the elevated plus maze (EPM) and then the Porsolt swim task (PST) on the following two days (Figure 2a and b).

(1) Elevated Plus Maze

The EPM task is designed to measure general anxiety associated with thigmotaxic behaviour and exploration (124). Animals were individual transported into the testing room in a normal plexiglass cage with cob bedding. Each animal was placed in the center of the platform, 10.4 cm x 10.1 cm, facing the same open arm. The open arms measured 112 cm x 10.4 cm and the closed arms were 113 cm x 10.1 cm x 36.5 cm. Each animal was tested for a total of five minutes. After each animal, the maze was cleaned with 70% ethanol. Observed behaviours were the total number of two and four paw entries into the open and closed arms, as well as the total time spent in each arm or the central platform.

(2) Porsolt Swim Task

A task designed to measure depressive-like behaviour and learned helplessness, animals were placed in buckets of water, at 25°C and were observed for a total of 5 minutes each day (125). Water depth was maintained at 17 inches in 22 x 18-inch plastic cylinder. Animals were placed in the center of the bucket and were dried off with paper towel and placed back into their home

cages for approximately 24 hours prior to the second day of PST testing. Observed behaviour included latency-to-float and the total time spent immobile. Immobility was defined as swimming cessation—any movement involved in keeping the animal's head above water or to push itself from bumping into the wall were excluded.

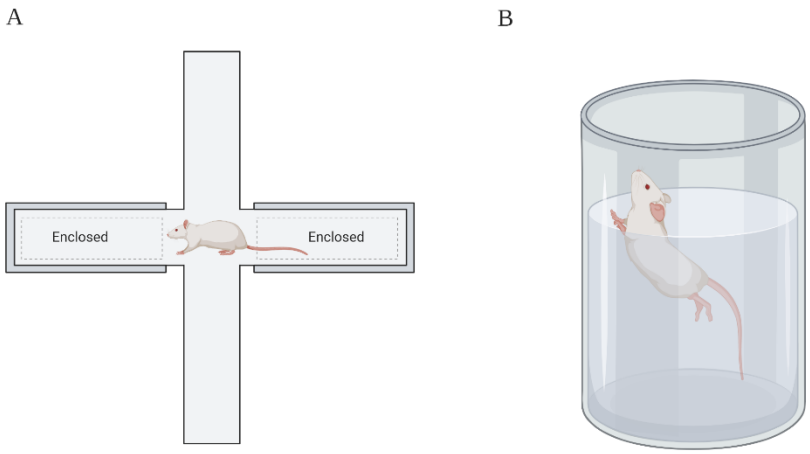

Figure 2. Behavioural tasks used to examine the anxiety and stress-coping strategies. A) Elevated plus maze. B) Porsolt swim task. Figure created with biorender.com.

2.3.7. Brain Dissections

Brain regions were determined utilizing Paxinos and Watson's Rat Brain Atlas (126). The prefrontal cortex was delineated by a +2.2 mm anterior-posterior (AP) position from bregma. All dissections were conducted in sterile petri dishes on top of ice.

2.3.8. Primer Design

Primers were designed using Primer3 and BLAST; sequences and accession numbers are listed in Supplementary Information, Table 1. Primer validation was conducted utilizing serial dilutions of cDNA, and specificity was analyzed using melt curves post amplification (127). Genes were chosen based on a whole genome microarray analysis that were relevant to the metabotropic glutamate receptor pathway; β 1, 2, and 3 adrenergic receptor signaling; serotonin receptor signaling; methylation; neural differentiation and growth; and glucocorticoid receptors (66).

2.3.9. RNA Extraction and Complimentary DNA Synthesis

Each PFC was weighed and then mechanically homogenized for two 2-minute cycles at 30 Hz in a TissueLyser (Qiagen) with TRI Reagent (Sigma-Aldrich; 1 mL/50 mg of tissue). Supernatant was added to 200 µL of chloroform, vortexed, and incubated at room temperature (RT) for 15 minutes (100,128,129). After centrifugation, the top aqueous phase was removed and added to 500 µL of isopropanol. Samples were then vortexed, incubated at RT for 10 minutes, centrifuged, and pellets were washed with 1 mL of 70% ethanol. After a final centrifugation, RNA pellets air dried and then resuspended in 20 µL of diethylpyrocarbonate (DEPC) treated nuclease-free water and were then placed on a Thermomixer R (Eppendorf) for 10 minutes at 1,000 rpm and 37°C. Concentration of total RNA was measured using the spectrophotometric measurement of the absorbance at 260 nm (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA) (129). RNA suspensions were stored at -80°C for long term storage.

Two µg of total RNA was treated with DNAse I (Sigma-Aldrich) and complimentary DNA (cDNA) was synthesized by adding 1 µL of 1 µg/µL of random primers (Roche Diagnostics). According to manufacturer's guidelines, 2U/µL Mu-MLV reverse transcriptase (Promega, 200

U/µL), mixed dNTPs (200µM), M-MuLV 5x Reaction Buffer (Promega), and DEPC water were added to each sample. A negative control was prepared with no reverse transcriptase. Final concentration of cDNA samples was 0.04 µg/µL.

2.3.10. RT-qPCR

Each gene was analyzed utilizing the QuantStudio5 Real-Time PCR System (Applied Biosystems) to compare samples from the naïve and DEX animals (n = 5 to 8). 15 µL reaction volumes were used with final concentrations of 0.4 ng/µL of cDNA of each sample, DEPC water, 1.2 µM/µL of forward and reverse primers, and SYBR green master-mix (SensiFAST SYBR Lo-ROX, Bioline).

2.3.11. Statistical Analysis

All statistical analyses for behavioural and genetic comparisons were carried out using IBM SPSS v20.0. Datasets were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene's test, $p > 0.05$. General linear model analysis of variance (ANOVA) was conducted, otherwise Welch's test was utilized with alpha levels set to $p = 0.05$. All results are presented in mean ± standard error of the mean (S.E.M). Post-hoc analyses were conducted where appropriate, using Tukey's Honestly Significant Difference (HSD).

2.4. Results

2.4.1. Behavioural Tasks

Observational data for the EPM measured exploratory activity as an indicator for general anxiety. A main effect of sex was found on several measures: total time spent in the open arms, F (1, 28) = 6.58, $p = 0.016$; total time spent on the central platform, F (1, 28) = 4.68, $p = 0.039$; total number

of 2-pawed entries into the open arms, F (1, 28) = 9.25, p = 0.005; and the total number of 4-pawed entries into the open arms, F (1, 28) = 6.03, p = 0.021 (Table 1). A main effect of treatment showed fewer 2-pawed entries from the DEX offspring (1.00 ± 0.31) into the closed arms compared to naïve offspring (2.00 ± 0.36), F (1, 28) = 4.24, p = 0.049. A main effect of treatment also showed the DEX offspring entered the open arms with 4-paws more often than the Naïve controls, Welch's F (1, 27.995) = 7.33, p = 0.011 (Figure 3). Within the open arms with 4-paws measure, there was also a main effect of sex F (1, 38) = 6.06, p = 0.018.

Table 1. Average behaviour on elevated plus maze and Porsolt swim task

Open Arms	**Central**	**Open Arms-2 Paws**	**Open Arms-4 Paws**	**Immobility Day 2**
21.4 ± 7.45	104.54 ± 11.28	7.13 ± 0.89	1.93 ± 2.43	174.68 ± 7.10
53.89 ± 10.25	*73.47 ± 8.90*	*3.80 ± 0.64*	*4.13 ± 2.47*	*212.96 ± 6.78*

The mean and S.E.M. of measured behaviour with sex main effects on EPM and PST measures. Open arms and closed arms are measured in seconds, 2 and 4-paw entries are measured in total number of entries, and immobility day 2 is measured in seconds. Males are presented on top and females are presented in italics.

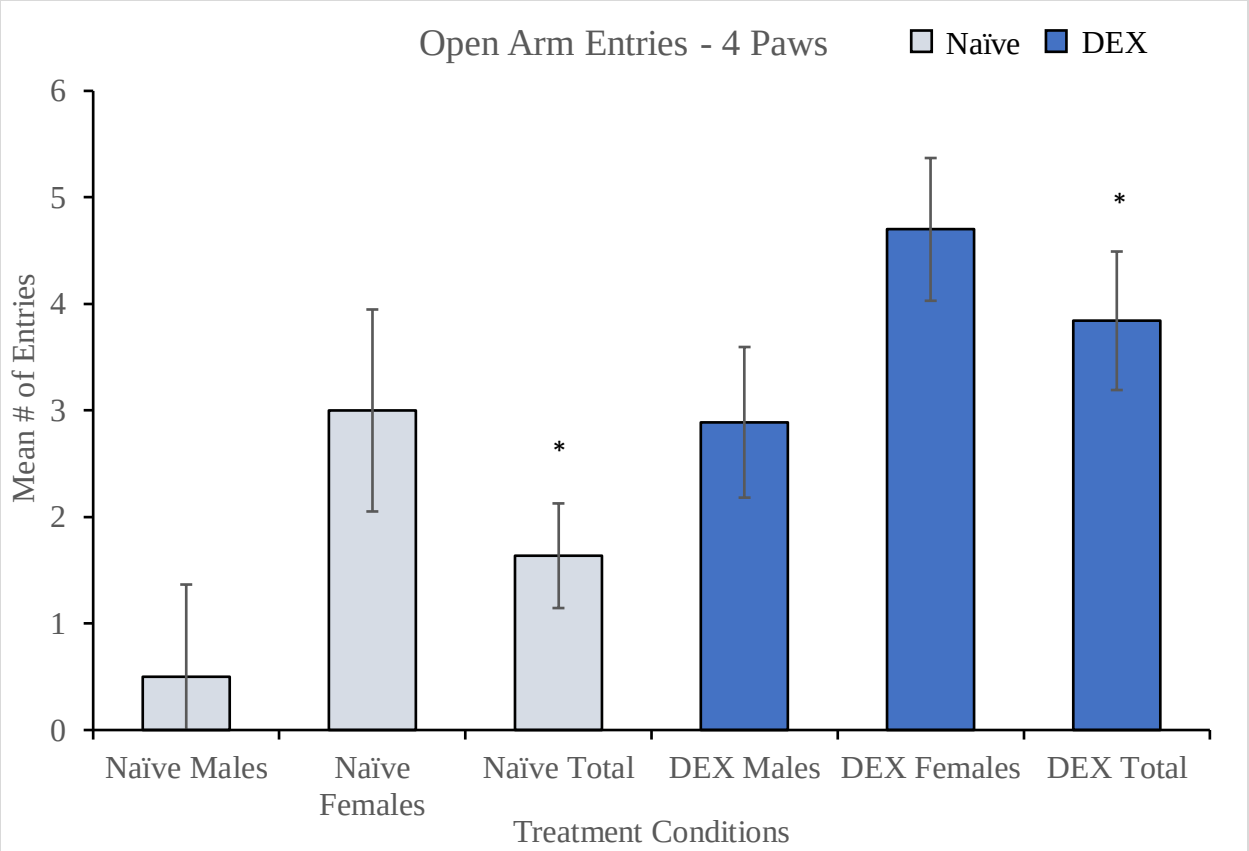

Figure 3. Treatment main effect of the average number of 4-paw entries into the open arm of the EPM. DEX offspring (3.84 ± 0.60) were more active than Naïve controls (1.64 ± 0.49), * denotes $p < 0.05$.

In the PST, animals' immobility and latency-to-float were measured in relation to despondency. There was a main effect of sex on immobility in day 2, F (1, 28) = 15.20, p = 0.001 (Table 1). A main effect of treatment was also shown, where DEX offspring were significantly more immobile than naïve offspring on day 1 of testing, F (1, 28) = 10.57, p = 0.003 (Figure 4), whereas sex effects were trending, but not significant at $p < 0.05$.

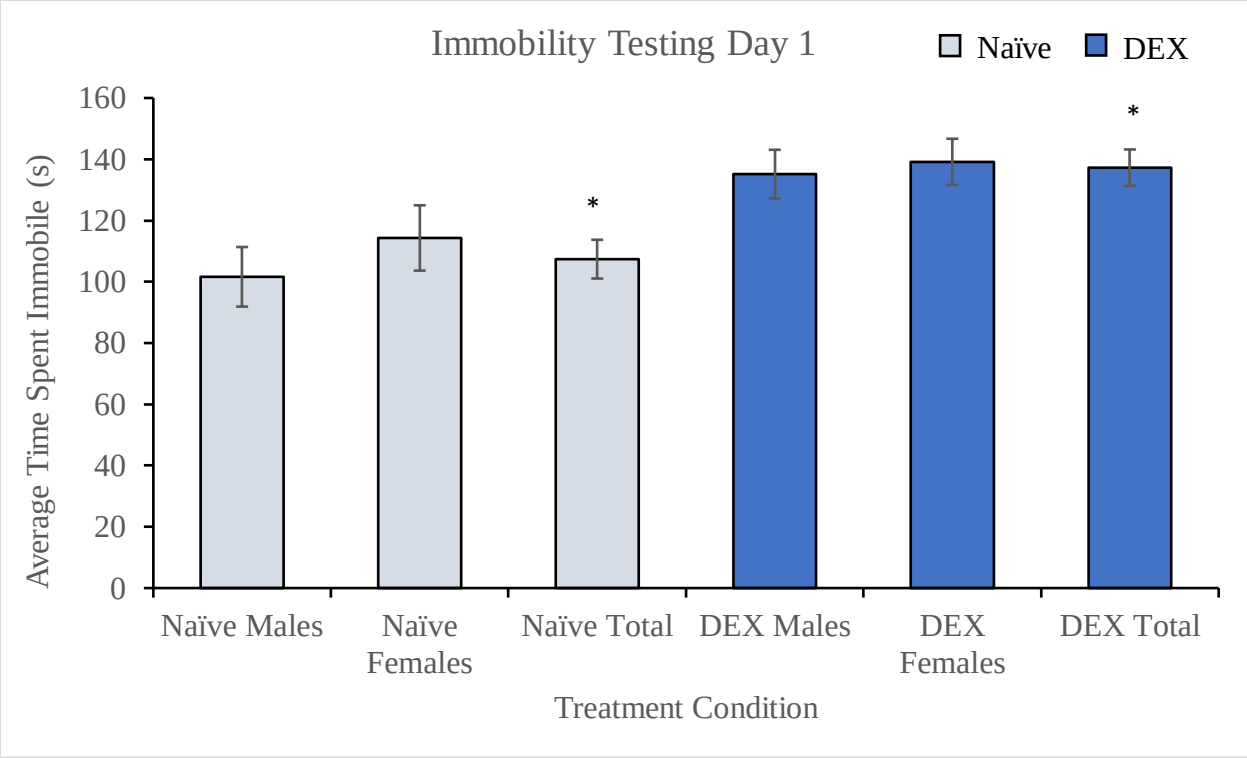

Figure 4. Treatment main effect of the average time (s) spent immobile on testing day 1 in the PST. DEX offspring (137.25 ± 5.94) were significantly more immobile than their Naïve counterparts (107.38 ± 6.32), * denotes $p < 0.05$.

2.4.2. Genetic Analysis

Seventeen genes were analyzed and several genes were dysregulated in the PFC associated with neurotransmission and methylation (Table 2). Synaptosome Associated Protein 25 (SNAP25) was increased in DEX males compared to naïve controls with a fold change of 1.62 ± 0.20 (2^ΔΔCQ ± S.E.M.). Ryanodine Receptor 2 (RYR2) had a sex by treatment interaction, F (1, 28) = 8.61, p = 0.009, η^2 = 0.324 (Figure 5). Limbic System Associated Membrane Protein (LSAMP) had increased expression in DEX males (1.45 ± 0.20), as did the catechol-O-methyltransferase (COMT; 1.28 ± 0.11). DNA-methyltransferase 3 Beta (DNMT3b) displayed a sex by treatment interaction, F (1, 17) = 5.56, p = 0.031, η^2 = 0.246 (Figure 6). As well, Membrane Bound

Transcription Factor Peptidase Site 1 (MBTPS1) also displayed an interaction, F (1, 18) = 11.90, p = 0.003, η^2 = 0.398 (Figure 7).

Table 2. Fold change of DEX males and females compared to naïve controls.

Relative Gene Expression in the Prefrontal Cortices of DEX Offspring		
GENE	**SEX**	**FOLD CHANGE 2^ΔΔCT ± SEM**
Glucocorticoid Receptors		
NR3C1	Males Females	1.17 ± 0.16 1.02 ± 0.15
NR3C2	Males Females	0.97 ± 0.21 1.07 ± 0.34
Methylation		
COMT	Males Females	**1.28 ± 0.11** 0.84 ± 0.17
DNMT3b	Males Females	***1.80 ± 0.32*** ***1.12 ± 0.08***
Glutamate Signaling		
GRM4	Males Females	1.64 ± 0.43 1.53 ± 0.69
SLC1A2	Males Females	1.00 ± 0.06 0.95 ± 0.14
GRIA2	Males Females	1.29 ± 0.19 1.15 ± 0.19
GRM2	Males Females	1.57 ± 0.41 1.00 ± 0.22
Calcium Signaling		
RYR2	Males Females	***1.57 ± 0.25*** ***0.81 ± 0.09***
CACNB2	Males Females	1.12 ± 0.11 0.89 ± 0.16
CACNA1B	Males Females	1.23 ± 0.14 0.95 ± 0.16
PLCH2	Males Females	1.55 ± 0.38 1.08 ± 0.12
RYR1	Males Females	1.34 ± 0.31 1.20 ± 0.14
Neural Transmission		
SNAP25	Males Females	**1.62 ± 0.20** 1.14 ± 0.26
Neuronal Growth & Differentiation		
MYT1L	Males Females	1.00 ± 0.13 1.18 ± 0.28
LSAMP	Males Females	**1.45 ± 0.20** 1.06 ± 0.10
Lysosomal Homeostasis		

MBTPS1	Males Females	***1.40 ± 0.13*** ***0.86 ± 0.09***

Bolded numbers, highlight in red, are significant compared to controls. Sex by treatment interactions are in italics and are shaded blue. All significances are $p \leq 0.05$.

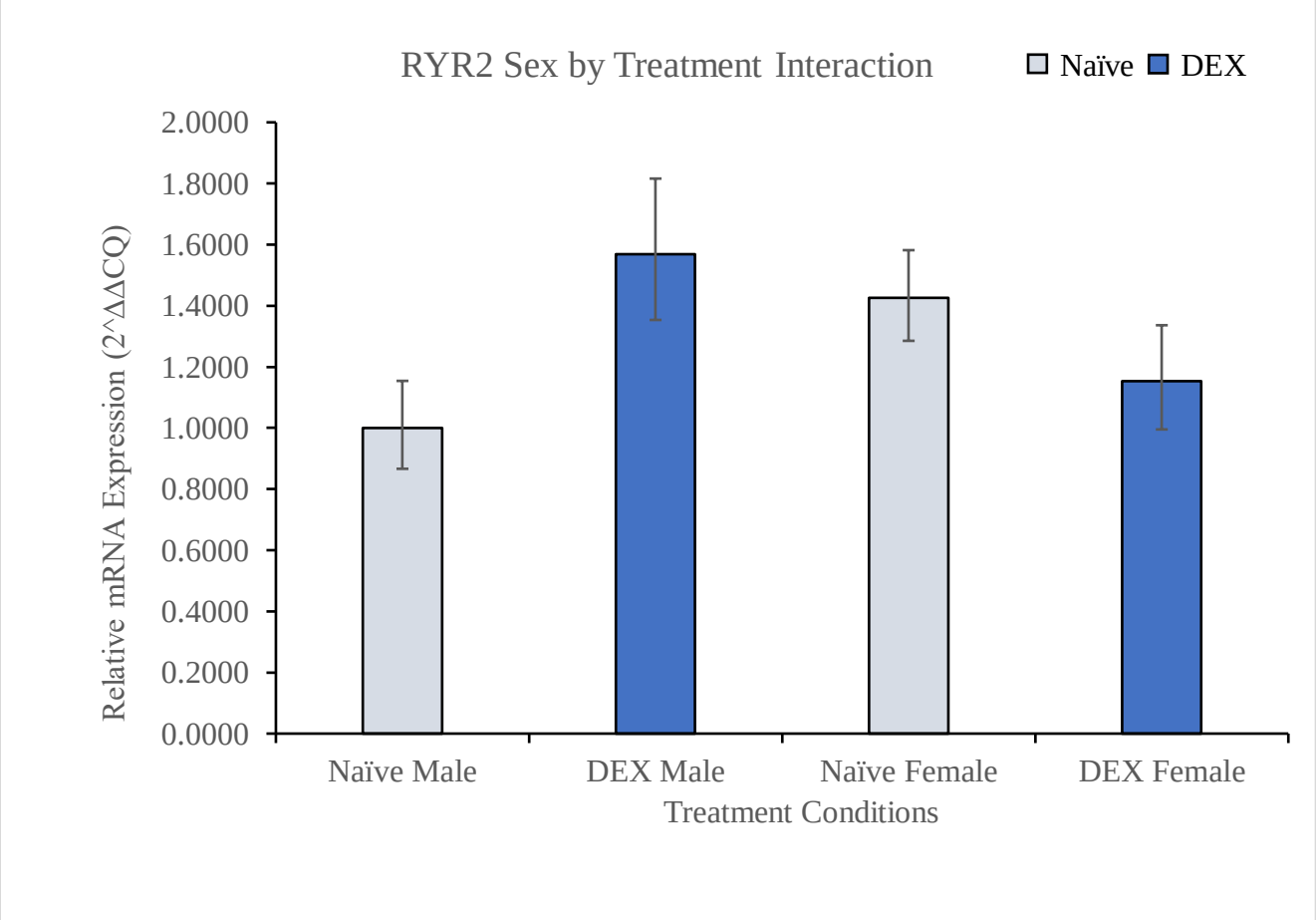

Figure 5. Interaction of Sex by Treatment in gene expression of ryanodine receptor 2. DEX male offspring have an increase in gene expression compared to controls, whereas DEX females show a decrease in overall expression.

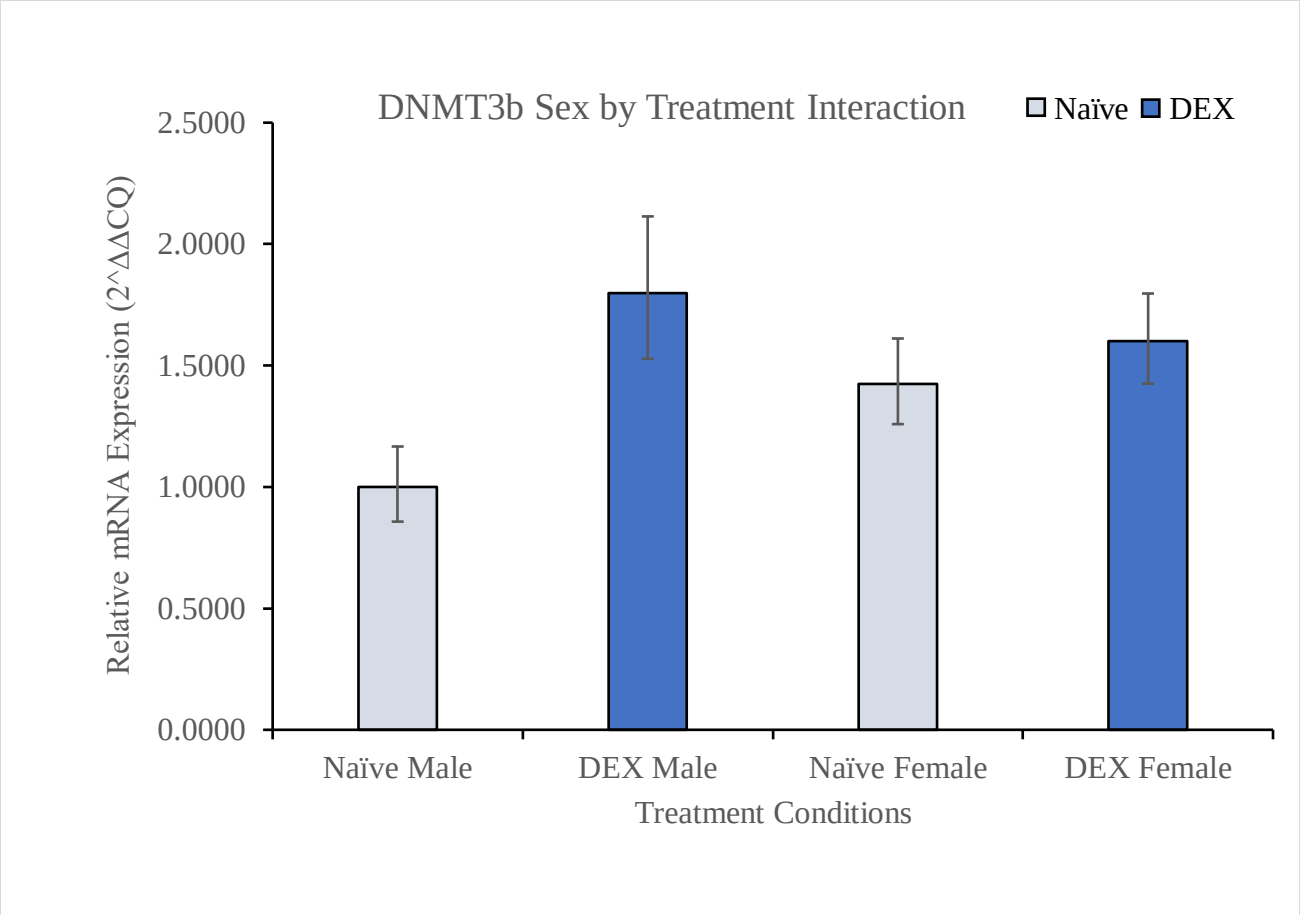

Figure 6. Interaction of Sex by Treatment in gene expression of DNA methyltransferase 3-beta. The gene expression of DEX male offspring is significantly increased in comparison to Naïve males. DEX females do not show a significant change in expression; however female animals overall show higher expression to Naïve males.

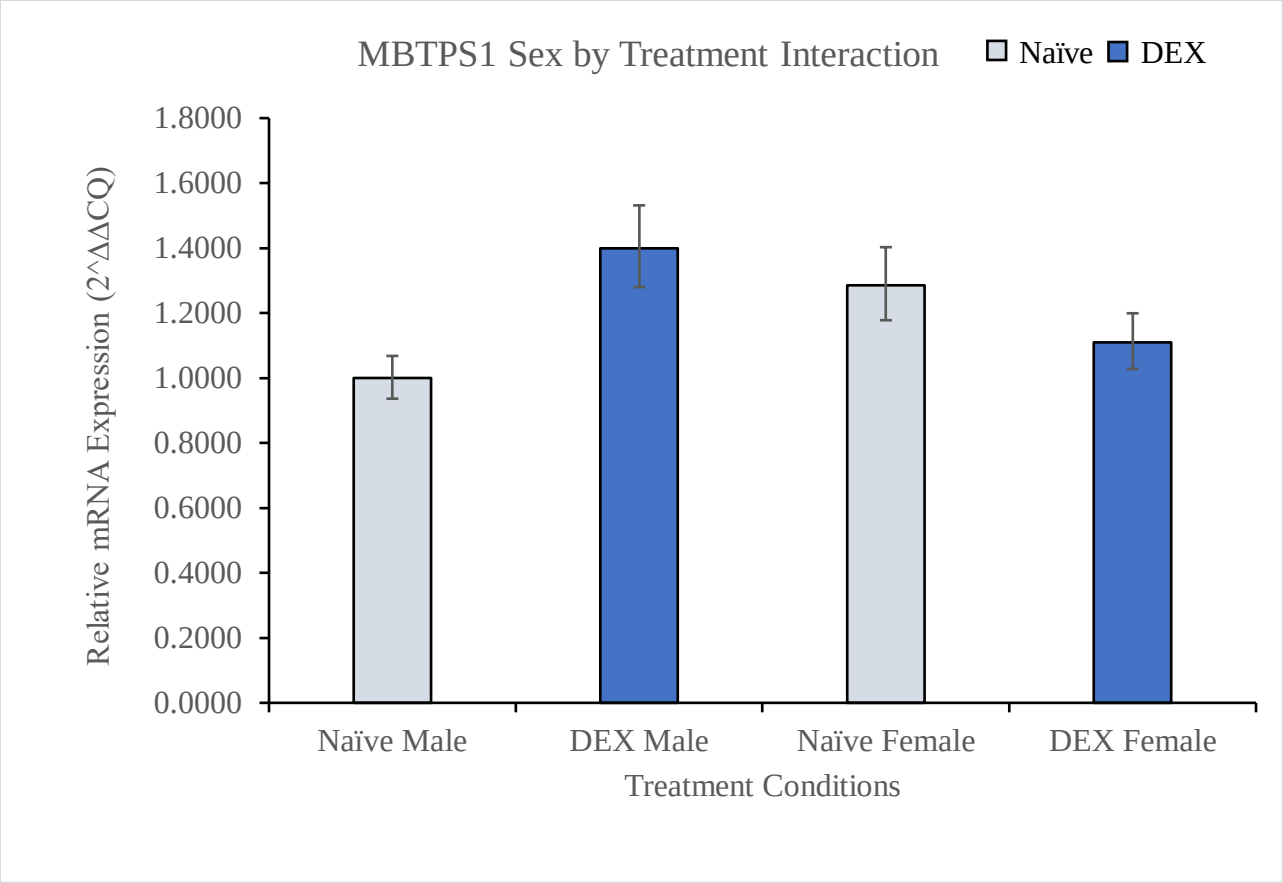

Figure 7. Interaction of Sex by Treatment in gene expression of membrane-bound transcription factor peptidase site 1. Naïve females show baseline levels of higher gene expression; however, DEX females show lower levels of gene expression and DEX males show an increase.

2.5. Discussion

The prefrontal cortex is linked to inhibition and decision making, often referred to as cognitive control, when faced with novel and stressful situations (46,130). It permits for a change in behavioural response upon evaluation of the situation and applying context (131). Provided normal neural development, the PFC is key to making appropriate decisions and responses.

Here, we evaluate the potential for chronic late-gestational prenatal stress to influence PFC function in offspring. Our results lead us to the conclusion that stress can program behavioural changes, considering the increase in immobility in DEX animals in the PST. Historically, the PST has been an indicator of learned helplessness and despondency (132). Recently, however, the

research community has shifted its focus of the PST as a depressive measure to that of adaptive coping strategies when faced with a stressful situation (133). DEX offspring also provided indication of increased exploratory activity, with significant increases in the number of 4-paw entries into the open arms of the EPM, which goes against the animals' natural tendency towards thigmotaxis (134). In consideration of the adaptive theory of fetal programming, these behaviours indicate fetal programming of the PFC leads to changes in typical response to novel situations that may increase survival. Arguably, these behaviours are not always to the benefit of the offspring, such as in the thrifty phenotype, but rather are situationally dependent. Exploratory behaviour may indicate the animal is not anxious, however, poses a risk of predation on the animal. Conservation of energy as a coping strategy may lead to loss of life through hypothermia or a missed opportunity for escape. Similar to the results of the current study, Sprague Dawley rats exposed to late gestation glucocorticoids displayed passive coping strategies on the PST at the age of 9 weeks (135). Another study with Sprague Dawley rats produced a sex-specific effect on the PST, with females spending significantly more time immobile than other treatment conditions (136). In one study utilizing the same dosage and timing of DEX treatment as the current study, the authors found no significant behaviour during the EPM trial; however, their animals were aged 10 weeks, which could implicate behavioural differences may be age-dependent in manifestation (137). Hypothalamic-pituitary-adrenal axis function and behavioural responses in glucocorticoid-exposed adult offspring tend to vary in literature depending upon sex, age, and dosage, but also the testing paradigms (41,138).

In combination with the behavioural changes, overexpression of multiple genes was reported. Changes in methylation and genetic expression within the prefrontal cortex and other brain regions have been linked to adverse behaviour that include depression, anxiety, locomotor activity, and

cognition (65,114,115). Proper regulation and balance of DNA methylation is required for normal neural function and behavioural responses (109). In the current study, COMT was overexpressed in DEX males, a gene that encodes for methyltransferase that commonly inactivates neurotransmitters such as dopamine and noradrenaline. COMT adds methyl groups to the neurotransmitters, preventing their ability to bind to their respective receptors, thereby limiting neural response to emotional stimuli, such as fear and anxiety. In another methyltransferase gene, the DNMT3b interaction shows baseline sex variation and a significant increase in expression of DEX male offspring. This gene regulates neuronal processes, development, and plasticity (139). SNAP25 was also overexpressed in DEX males, a gene involved in neurotransmitter release from vesicles into the synapse; combined with increased expression of COMT, the neurotransmitter signaling pathways in the PFC of DEX animals, particularly males, is overactive. LSAMP, a third neuronally relevant gene, was overexpressed in DEX males and is involved in neuronal growth and tumor suppression. Complete deletion of LSAMP in genetic models leads to significant disruption of anxiety-related behaviour in the EPM, similar to hyperactivity (140). Also involved in neural transmission and synaptic plasticity is calcium receptor RYR2. RYR2 is link to memory processing through calcium release, whereby an increase in calcium concentrations can activate long-term potentiation and solidify memories (141). The RYR2 interaction seen in our results indicates a reversal in sex-specific gene expression. Naïve females have increased relative expression than naïve males, whereas DEX males are overexpressed and DEX females drop in expression to levels closer to the naïve males. The last dysregulated gene was MBTPS1, which followed same pattern as RYR2, where the sex difference in naïve animals switches its pattern in the DEX offspring with DEX males overexpressing and DEX females lowering expression. MBTPS1 cleaves pro-BDNF (brain-derived neurotrophic factor) into a truncated form (142).

Results from the current study show that the prefrontal cortex is a relevant area of interest when exploring prenatal stress and fetal programming. Chronic glucocorticoid exposure during late gestation influences the genetic profile of this cognitively-relevant region and influences adaptive behavioural phenotypes in novel situations in the adult offspring.

Conflict of Interest

The authors declare there are no conflicts of interest.

Chapter 3 – Absence of Depressive and Anxious Behaviour Adult C57Bl/6J Mice After Prenatal Exposure to Ionizing Radiation

Christine Lalonde, Shayenthiran Sreetharan, Alyssa Murray, Lisa Stoa, Mary Ellen Cybulski, Allison Kennedy, Nicholas Landry, Amy Stillar, Sandhya Khurana, Sujeenthar Tharmalingam, Joanna Wilson, Neelam Khaper, Simon Lees, Douglas Boreham, T.C. Tai

In preparation to submit to *Radiation Research*

3.1. Abstract

The exposure of ionizing radiation, during early gestation often leads to deleterious and even lethal effects; however, few extensive studies have been conducted on late gestational exposures. This research examined the behavioural effects of C57Bl/6J mouse offspring exposed to low-dose ionizing gamma irradiation during the equivalent third trimester. Pregnant dams were randomly assigned to sham or exposed to low dose or sublethal dose radiation (50, 300, or 1000 mGy) at gestational day 15. Adult offspring underwent behavioural and genetic analysis after being raised under normal murine housing conditions. Our results indicate very little change in the behavioural tasks measuring general anxiety, social anxiety, and stress-management in animals exposed prenatally across the low dose radiation conditions. Quantitative real-time polymerase chain reactions were conducted on the prefrontal cortex, cerebral cortex, hippocampus, and cerebellum of each animal; results indicate some dysregulation in markers of DNA damage, synaptic activity, reactive oxygen species (ROS) regulation, and methylation pathways in the offspring. Together, our results provide evidence in the C57Bl/6J strain, that exposure to low dose radiation (<1000 mGy) during the last trimester of gestation leads to no observable changes in behaviour when assessed as adults, although some changes in gene expression were observed for specific brain regions. These results indicate that the level of oxidative stress occurring during late gestation for this mouse strain is not sufficient for a change in the behavioural phenotype assessed, but results in some modest dysregulation of the genetic profile of the brain.

Keywords: Radiation, fetal programming, late gestation, depression, anxiety

3.2. Introduction

Ionizing radiation, defined as sufficient incident energy to remove an electron from an atom or molecule, can create a cascade of physiological effects at the molecular level, leading to organ and system dysfunction or failure at sufficient dosage exposures (91). Catastrophic incidents such as nuclear warhead detonation and nuclear power plant failures have propagated fear and avoidance of radiation through workplace, medical intervention, and even of personal devices, regardless of mortality rates and statistics surrounding these exposures (143–146). Dosimetry and exposure-rate research has provided evidence that early embryonic and fetal exposures can lead to termination, significant adverse birth defects, or low birth weights (26,91,147,148).

During the third trimester neural development is characterized by increased neuronal organization, migration, dendritogenesis, and synaptogenesis; all pertinent to healthy cognition and behavioural processes (149,150). Medical diagnostic interventions and therapies that involve radiation during pregnancy are, arguably, of concern to the health and development of the fetus, even during the third trimester, which may not be critical for survival, but is highly sensitive and relevant for positive mental health outcomes (91,151,152). Prenatal exposure to x-ray doses up to 1000 mGy in mice during early gestational has shown deficiencies in locomotor activity and spatial memory (92). Forty-one genes related to p53 signaling, DNA damage, apoptosis, and cell signaling were differentially expressed. Further, x-ray exposures of 1000 mGy to C57Bl/6J mice on gestational day 11 altered post-synaptic density protein 95 (PSD95) in the hippocampi (93). Swiss albino mice were exposed to low levels of gamma radiation between gestational days 11 and 19 and offspring were tested for a variety of behavioural responses, and it was reported that 500 mGy exposures produced activity, anxiety, and memory deficiencies in the 3-month-old offspring (94).

Fetal programming describes the impact of the in-utero environment on the phenotype of the offspring, whereby adverse environments lead to predispositions for adult diseases (30,153,154). Deficient maternal diets, chronic exposures of stress hormones (glucocorticoids), and exposures to low doses of ionizing radiation have been linked to adulthood metabolic disorders which are associated with low birth-weight, changes in blood-pressure and abnormal adult behavioural function (91,113,138,155–158). Fetal programming may occur through multiple mechanisms. First, activation of stress hormones may alter methylation of the genome; when glucocorticoids (GC) are circulating in high levels and overcome the placental enzymatic barrier, they bind to glucocorticoid response elements (GRE) in GC receptors, allowing changes in the methylation of CpG islands (90,104). Depending on the location of methylation, number of methyl groups, and the nucleic acid methylated, a gene may be silenced or activated, affecting physiologic processes that may adversely affect the metabolic and behavioural phenotype of the offspring (159,160). Arguably, this process may be described as an adaptive response to a deficient uterine environment; low prenatal nutrition, for example, predicts a low-caloric diet for the offspring. A "thrifty phenotype" would be considered an adaptive response to a low-nutrient environment; conversely, when faced with a nutrient-rich environment, the response is adversive, leading to the development of adiposity, hypertension, and diabetes (32,161–163). Second, stressors that induce DNA damage and increase levels of reactive oxygen species (ROS) stimulate a series of cellular responses, such as DNA repair and apoptosis. ROS may also alter genetic expression through multiple processes, such as protein adducts, DNA methylation, histone interactions, and through small noncoding RNAs (90). Ionizing radiation interacts with DNA both directly and indirectly by providing energy to the system and thereby releasing electrons from their atoms (90). Free electrons may then interact with other atoms and molecules to form ROS that will lead to DNA

damage inducing cellular damage and repair systems (89). As well as the production of ROS through ionizing radiation exposures, GCs in sufficient quantity to overwhelm the placental barrier may increase levels of ROS and induce genetic changes, providing multiple potential processes for phenotypical changes (44,164).

Adverse states, such as depressive and anxious tendencies, have been associated with late gestational prenatal stress. Critical periods of neural sensitivity to radiation lie between gestational days 11 and 17 in mice, where neurons are still growing and differentiating (26). The hippocampal region is dense with GC receptors and is highly involved in learning and memory, including reward and fear systems by receiving input directly from the emotional processing center, the amygdala. In early gestation, significant hippocampal damage lead to memory and motor deficits in offspring exposed to radiation (165). The prefrontal cortex is linked to risk aversion, social anxiety, and higher order cognition, whereas the cerebral cortex receives sensorimotor input and is responsible for perceptive processing. Early research showed structural damage to both the prefrontal and cerebral cortices from x-irradiation on GD 15 (166). The cerebellum has recently been noted to be more involved in cognition, addiction, and depression. Late gestational exposure at GD 21 to 2.5 Gy x-irradiation and 1.5 Gy cyclotron irradiation both resulted in significant changes in developing cerebella (167).

The current study examined the impact of low dose radiation during the late gestation on adult behavioural outcomes and environmental experiences (such as perception and learning), with a focus on relevant regions of the brain (prefrontal cortex, cerebral cortex, hippocampus, and cerebellum). Gene analysis examined the effects of prenatal radiation exposure on DNA damage, ROS regulation, and synaptic activity to investigate different neural regions and potential genetic modifications.

3.3. Materials and Methods

3.3.1. Ethics

All experimental protocols were approved by the Animal Care Committee of McMaster University (AUP-15-11-26) and are in accordance with the Canadian Council on Animal Care guidelines.

3.3.2. Animals and Housing

Male and female C57Bl/6J mice (8-12 weeks) were obtained from Jackson Laboratories (Bar Harbor, ME) and bred at McMaster University (Hamilton, ON). Two females were placed in a cage overnight with a single male. Pregnancies were confirmed with the presence of a vaginal plug in the morning, which was considered gestational day (GD) 0. Pregnant females were then singly housed for the duration of the pregnancy. Normal rodent chow and water was provided ad-libitum (Teklad Diets Envigo, Madison, WI); and animals were maintained on a 12:12h (7am-7pm) light-dark cycle. Animals were housed in standard, non-vented cages.

3.3.3. Irradiation

Pregnant C57Bl/6 mice were exposed to ionizing radiation with a 662 keV, ^{137}Cs γ-radiation Taylor Radiobiology source at McMaster University. Animals were transported in a temperature-controlled vehicle from the housing facility to the irradiation building in their home cages. Upon arrival, the cages were placed under shielding for acclimatization to the new location for 1 hour prior to exposures. Cages were place equidistant from the source and they received 10 mGy per minute dose on gestational day 15; fetal doses were measured at 8.9 mGy per minute (168). Dam and fetal dose rates were obtained using transplanted thermoluminescent dosimeters as previously

described (168). Animals were randomly assigned to either sham, 50, 300, or 1000 mGy exposure conditions.

3.3.4. Offspring

Two male and two female pups from each dam (n = 8) were utilized for behavioural and genetic testing. Pups were raised under normal conditions, 2-3 per cage, food and water ad-libitum until the age of 17-18 weeks. Pups then underwent behavioural testing and were euthanized by cervical dislocation within 24 hours of the last task. Relevant tissues were harvested, frozen immediately with liquid nitrogen, and then stored at -80°C until genetic analysis.

3.3.5. Behavioural Tests

All behaviour was recorded utilizing video-cameras. Experimenters and observers were blind to treatment groups. Behavioural tasks were conducted in random order to prevent ordering effects and were completed over the course of 1.5 days (169,170). Animals were brought into the testing rooms at least one hour prior to testing to acclimatize. All tasks were conducted during the light period and each task was washed between animal testing with 70% ethanol and water (171). All behaviour was scored using Behavioural Observation Research Initiative Software (BORIS) (172).

(1) Open Field Task

As a measure of general anxiety, four open field boxes (40 x 40 x 30 cm) were placed on the floor (173). Mice were placed in the center of the task and were allowed to explore the novel environment for 5 minutes. Behaviour measured by the observer included number of grid crossings to the center of the filed, total time spent within the center of the field, and the total number of rears. A grid was placed over the computer screen while utilizing BORIS software to

create a central square measuring 20 x 20 cm. Grid crossings were defined as three or more paws across the line and rearing was defined as standing on two rear paws.

(2) Social Anxiety Task

To measure social anxiety, animals were placed in a clean, normal housing cage for a total of 5 minutes with a stranger mouse (174). The stranger mouse was matched for sex, but was not an experimental animal and was replaced with a new stranger after a few trials to prevent stress responses. The stranger was placed behind a plexiglass divider for protection and identification. The stranger mouse had reduced area (3 inches wide) to explore in order to prevent it from hiding from interactions with the experimental animal. Observed behaviour included rearing, as defined above, number of approaches, and total time in approach defined as being within 1.5 inches of the plexiglass, facing the stranger mouse.

(3) Porsolt Swim Task

Originally designed as a measure of despondency and learned helplessness, animals were placed in buckets of water, at 32°C and were observed for a total of 5 minutes (125). Water depth was maintained at 9 inches in 11 x 11-inch plastic buckets. Animals were placed in the center of the bucket and were dried off with paper towel and placed back into their home cages for at least an hour prior to participating in any other behavioural task. Animals were monitored by observers nearby, but out-of-sight in case a rescue was required. No animals required rescuing. Observed behaviour included latency-to-float and the total time spent immobile or floating. Immobility was defined as swimming cessation—movement involved in keeping the animal's head above water or to push itself from bumping into the wall were included in the immobility measure.

3.3.6. Brain Dissections

Brain regions were determined utilizing Paxinos and Franklin's mouse brain atlas (175). The prefrontal cortex was delineated by a +2.2 mm anterior-posterior (AP) position from bregma. The cerebellum was removed using a scalpel, and the remaining cortex and hippocampus were removed using forceps and a scalpel (176). All dissections were conducted in sterile petri dishes on top of ice.

3.3.7. Primer Design

Primers were designed using Primer Bank, Primer3, Primer3Plus, or Gemi; sequences and accession numbers are listed in Supplementary Information, Table 2, Appendix A. The primer for BDNF was designed based on previous literature (177). Primers were validated utilizing serial dilutions and specificity was analyzed using melt curves post amplification (127). Genes were chosen relevant to pathways in ROS regulation, cortisol regulation, cell cycling, DNA damage and tumor suppression, DNA methylation, circadian rhythm, synaptic activity, apoptosis, and microglia activity.

3.3.8. RNA Extraction and Complimentary DNA Synthesis

Neural tissue was weighed and then mechanically homogenized with 1 mL of TRI Reagent (Sigma-Aldrich) per 50 mg of tissue in a TissueLyser (Qiagen) for two 2-minute cycles at 30 Hz. Supernatant was transferred to a fresh tube and 200 µL of chloroform per 1 mL of TRI Reagent was added and vortexed (128,129,156). After incubation at room temperature for 15 minutes, samples were then centrifuged at 12,000 g for 20 minutes at 4°C. The top aqueous phase was removed and added into a fresh tube with 500 µL of isopropanol per 1 mL of TRI Reagent.

Samples were then vortexed and incubated at room temperature for 10 minutes. After incubation, samples were again centrifuged, at 12,000 g for 8 minutes at 4°C and pellets were washed with 1 mL of 70% ethanol per 1 mL of TRI Reagent. A final centrifugation at 7,500 g for 5 minutes was conducted and RNA pellets air dried prior to resuspension in 20 µL of diethylpyrocarbonate (DEPC) treated nuclease-free water. Samples were then placed on the Thermomixer R (Eppendorf) for 10 minutes at 112 g and 37°C. Total RNA concentrations were measured using the spectrophotometric measurement of the absorbance at 260 nm (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA) (129). RNA suspensions were stored at -80°C for long term storage.

2 µg of total RNA was treated with DNAse I (Sigma-Aldrich) according to manufacturer guidelines prior to complimentary DNA (cDNA) synthesis. For cDNA synthesis, random primers (1 µg/1 µL, Roche Diagnostics) were added to the DNAse-treated RNA. Samples were vortexed, spun down, and then heated at 70°C for 5 minutes in a thermal cycler (MJ Mini, Bio-Rad) and then placed on ice. According to manufacturer's guidelines, 2U/µL Mu-MLV reverse transcriptase (Promega, 200 U/µL), mixed dNTPs (200µM), M-MuLV 5x Reaction Buffer (Promega), and DEPC water were added to each sample. A negative control was prepared with no reverse transcriptase. Samples were mixed, spun down, and incubated for 60 minutes at 37°C. Final concentration of cDNA samples was 0.04 µg/µL.

3.3.9. RT-qPCR

Utilizing the QuantStudio5 Real-Time PCR System (Applied Biosystems), genes were analyzed comparing samples from the Sham and 1000 mGy animals (n = 3 to 8). 12 µL reaction volumes were used with 0.3 ng/µL of cDNA of each sample, DEPC water, 2 ng/µL of forward and reverse

primers, and SYBR green master-mix (SensiFAST SYBR Lo-ROX, Bioline). Gene expression was only determined for the 1000 mGy and control animals because no relevant behavioural outcomes were observed at lower doses.

3.3.10. Statistical Analysis

All statistical analyses for behavioural and genetic comparisons were carried out using IBM SPSS 20.0. Datasets were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene's test, $p > 0.05$. Inter-rater reliability was calculated using Pearson's Correlation, $r = 0.993$. General linear model analysis of variance (ANOVA) was conducted, otherwise Welch's test was utilized with alpha levels set to $p = 0.05$. All results are presented in mean ± standard error of the mean (S.E.M). Post-hoc analyses were conducted where appropriate, using Tukey's Honestly Significant Difference (HSD).

3.4. Results

3.4.1. Behavioural Tests

Observational data for the Open Field Task measured exploratory activity as an indicator for anxiety (See Table 3). A main effect of sex on rearing activity indicated males (43.77 ± 1.55 rears) were more active than females (33.19 ± 1.94 rears) with Welch's F (1, 57.969) = 18.22, $p < 0.0001$. No other behavioural measures were significant for this task and no effects of prenatal exposure to ionizing radiation were found.

Table 3. Observational Data on Open Field Task

Behaviour	**Sex**	**Sham**	**50 mGy**	**300 mGy**	**1000 mGy**	**Results**
Rears	M	44.83 ± 3.74	39.67 ± 3.06	50.00 ± 3.24	41.00 ± 3.47	Sex effect:
	F	*34.75 ± 3.24*	*29.25 ± 3.24*	*40.63 ± 3.24*	*28.86 ± 3.47*	$p < 0.0001$

Grid Crossings	M F	52.83 ± 6.07 *65.38 ± 5.26*	52.56 ± 4.96 *53.63 ± 5.26*	55.38 ± 5.26 *55.00 ± 5.26*	57.71 ± 5.62 *62.29 ± 5.62*	Not significant
Time in Center	M F	184.14 ± 14.1 *181.92 ± 12.2*	197.29 ± 11.5 *189.37 ± 12.2*	199.48 ± 12.2 *183.84 ± 12.2*	194.80 ± 13.01 *175.23 ± 12.2*	Not significant

The mean and S.E.M of each measured behaviour represented as occurrences of the behaviour, except for Time in Center, which is measured in seconds. For rearing behavior, males were significantly more active than females on average.

In the Social Anxiety Task, animals were observed for anxiety in relation to a stranger mouse (See Table 4). There was a main effect of prenatal exposure to ionizing radiation on rearing behaviour, $\lambda = 0.659$, F (12, 137.871) = 1.96, $p = 0.032$, $\eta^2 = 0.13$. In contrast, the 1000 mGy offspring (41.25 ± 2.57 rears) were more active than Sham offspring (28.67 ± 2.78 rears). There were no other measurable behavioural differences in either the Social Anxiety Task or the Porsolt Swim Task.

Table 4. Observational Data on Social Anxiety Task

Behaviour	**Sex**	**Sham**	**50 mGy**	**300 mGy**	**1000 mGy**	**Results**
Rears	M F	26.33 ± 4.20 *31.00 ± 3.63*	35.44 ± 3.43 *38.75 ± 3.63*	33.25 ± 3.63 *39.50 ± 3.63*	44.38 ± 3.63 *38.13 ± 3.63*	Dose effect: $p = 0.01$
# of Approaches	M F	28.50 ± 2.35 *23.75 ± 2.04*	25.56 ± 1.92 *25.50 ± 2.04*	23.00 ± 2.03 *28.13 ± 2.04*	30.50 ± 2.04 *27.50 ± 2.04*	Not significant
Total Time in Approach	M F	152.83 ± 17.09 *121.25 ± 14.80*	141.00 ± 13.95 *145.38 ± 14.80*	121.89 ± 14.80 *139.25 ± 14.80*	162.00 ± 14.80 *111.63 ± 14.80*	Not significant

The mean and S.E.M of each measured behaviour represented as occurrences or seconds. 1000 mGy animals were significantly more active in rearing than shams.

3.4.2. Genetic Analysis

Few dysregulated genes (see Figure 8) were discovered per brain region for males and females. Out of 31 genes analyzed, 1000 mGy males had 2 genes in the prefrontal cortex, 1 in the cerebral cortex, and 2 in the cerebellum dysregulated, whereas 1000 mGy females had 3 in the prefrontal cortex, 3 in the cerebral cortex, 4 in the hippocampus, and 2 in the cerebellum (see Table 5). In females, ROS regulator SOD1 was overexpressed relative to sham controls with a fold change of 1.23 ± 0.08 (2^ΔΔCQ + S.E.M.) in the cerebral cortex, as was GPX1 (1.15 ± 0.05). Synaptic activity gene PSD95 (1.99 ± 0.39) was the only other dysregulated gene in the cerebral cortex. In the prefrontal cortex, one ROS regulator gene, SOD3 (2.43 ± 1.02) was upregulated, as well as

two synaptic activity related genes, NOS3 (2.13 ± 0.93) and PSD95 (2.13 ± 0.69). In the female hippocampal region, there was a decrease in DNA damage regulator, GADD45 (0.84 ± 0.06), circadian rhythm gene, PER2 (0.88 ± 0.05), and synaptic activity gene, NeuN (0.72 ± 0.04). PSD95 (1.36 ± 0.22) was elevated, however. The cerebellum had two downregulated DNA methylation genes, DNMT3a (0.81 ± 0.06) and DNMT1 (0.71 ± 0.07).

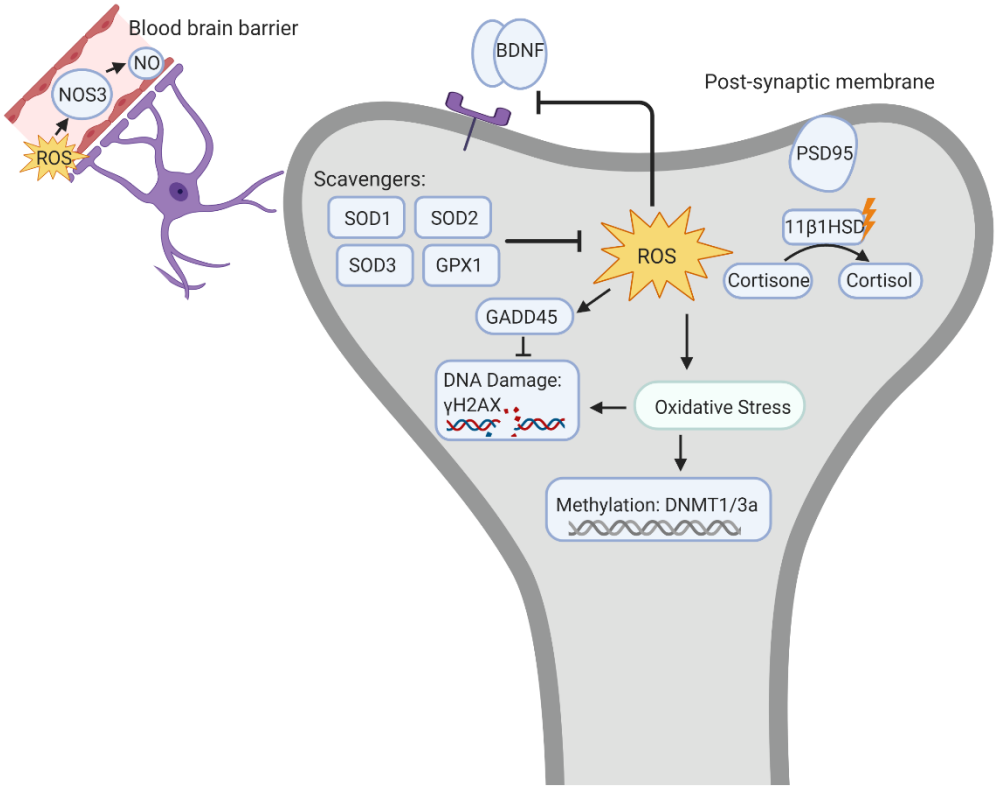

Figure 8. Related gene functions in relation to ROS. Dysregulated genes of C57Bl/6J brain regions. Each gene is differentially expressed depending upon region and animal sex. Endothelial nitric oxide (NOS3), superoxide dismutase 1, 2, 3 (SOD1, 2, 3), glutathione peroxidase 1 (GPX1), γH2AX, and post-synaptic density 95 (PSD95) were all upregulated. Growth arrest and DNA damage (GADD45), brain derived neurotrophic factor (BDNF), and DNA methyltransferase 1 and 3a (DNMT1/DNMT3a) were downregulated. Each gene is differentially expressed depending upon region and animal sex. Endothelial NOS leads to an increase in NO neurotransmission. ROS scavengers inhibit excess ROS. ROS inhibits BDNF and promotes oxidative stress, leading to DNA damage and methylation. Figure was created using biorender.com.

Table 5. Relative Gene Expression in 1000 mGy Offspring

Gene Expression in 1000mGy Offspring Relative to Sham (Fold Change ± SEM)					
ROS Regulation					
Gene	**Sex**	**Prefrontal Cortex**	**Cerebral Cortex**	**Hippocampus**	**Cerebellum**
SOD1	M F	1.79 ± 0.72 1.32 ± 0.52	1.08 ± 0.14 **1.23 ± 0.08**	1.09 ± 0.27 1.14 ± 0.09	1.00 ± 0.18 0.97 ± 0.13
SOD2	M F	**1.65 ± 0.29** 1.00 ± 0.35	1.12 ± 0.14 1.04 ± 0.04	1.29 ± 0.21 0.94 ± 0.07	**1.11 ± 0.06** 1.01 ± 0.08
SOD3	M F	1.42 ± 0.51 **2.43 ± 1.02**	1.08 ± 0.13 0.97 ± 0.20	*1.11 ± 0.27* *1.28 ± 0.27*	1.03 ± 0.55 0.94 ± 0.31
Catalase	M F	0.97 ± 0.55 0.33 ± 0.29	1.10 ± 0.09 0.94 ± 0.06	1.06 ± 0.15 1.32 ± 0.35	1.00 ± 0.06 1.04 ± 0.13
GPX1	M F	1.70 ± 0.82 1.62 ± 1.05	1.05 ± 0.06 **1.15 ± 0.05**	1.32 ± 0.18 1.01 ± 0.08	1.05 ± 0.49 0.80 ± 0.38
Cortisol Regulation					
11B1HSD	M F	1.40 ± 1.10 1.57 ± 0.93	nd	*0.75 ± 0.39* *1.19 ± 0.24*	1.12 ± 0.32 0.80 ± 0.11
11B2HSD	M F	1.17 ± 0.47 1.98 ± 0.89	nd	nd	0.96 ± 0.51 0.77 ± 0.37
GR	M F	1.04 ± 0.34 1.70 ± 0.95	0.98 ± 0.21 1.37 ± 0.24	1.15 ± 0.19 0.84 ± 0.09	0.78 ± 0.33 0.87 ± 0.42
Cell Cycle					
BTG2	M F	1.32 ± 0.54 1.32 ± 0.54	1.19 ± 0.25 0.77 ± 0.14	0.86 ± 0.26 1.41 ± 0.31	1.13 ± 0.12 1.04 ± 0.14
DNA Damage & Tumor Suppression					
yH2AX	M F	**3.38 ± 1.55** 0.94 ± 0.40	nd	nd	nd
p53	M F	1.58 ± 0.94 1.78 ± 1.05	0.97 ± 0.19 1.04 ± 0.01	1.83 ± 1.55 0.89 ± 0.63	1.05 ± 0.13 0.87 ± 0.06
GADD45	M F	2.28 ± 2.21 1.99 ± 1.30	nd	1.02 ± 0.25 **0.84 ± 0.06**	1.76 ± 0.21 0.86 ± 0.08
DDB2	M F	1.41 ± 0.99 0.36 ± 0.40	1.01 ± 0.04 0.95 ± 0.06	*0.88 ± 0.16* *1.31 ± 0.29*	0.74 ± 0.42 0.73 ± 0.27
MDM2	M F	1.72 ± 0.40 2.13 ± 0.94	nd	nd	nd
DNA Methylation					
DNMT3b	M F	nd	nd	*0.88 ± 0.21* *1.21 ± 0.36*	nd
DNMT3a	M F	nd	1.01 ± 0.08 0.76 ± 0.11	1.07 ± 0.22 0.97 ± 0.05	1.24 ± 0.17 **0.81 ± 0.06**
DNMT1	M	nd	0.90 ± 0.09	1.17 ± 0.20	1.07 ± 0.14

	F		1.00 ± 0.06	0.96 ± 0.07	**0.71 ± 0.07**
Circadian Rhythm					
PER2	M F	0.85 ± 0.57 0.26 ± 0.31	0.80 ± 0.24 0.85 ± 0.14	1.01 ± 0.23 **0.88 ± 0.05**	0.82 ± 0.19 0.79 ± 0.14
Synaptic Activity					
Synaptophysin	M F	nd	1.16 ± 0.18 0.76 ± 0.13	1.26 ± 0.14 0.82 ± 0.11	*1.09 ± 0.07* *0.94 ± 0.04*
BDNF	M F	nd	**0.57 ± 0.06** 0.86 ± 0.30	1.19 ± 0.33 0.74 ± 0.21	1.14 ± 0.16 1.06 ± 0.20
SLC6A5	M F	nd	nd	0.80 ± 0.22 0.91 ± 0.11	1.21 ± 0.16 1.13 ± 0.15
DCX	M F	nd	nd	1.81 ± 2.25 1.00 ± 0.49	nd
NOS3	M F	0.96 ± 0.37 **2.13 ± 0.93**	1.03 ± 0.31 1.40 ± 0.27	nd	nd
PSD95	M F	1.38 ± 0.47 **2.13 ± 0.69**	1.00 ± 0.32 **1.99 ± 0.39**	1.80 ± 0.54 **1.36 ± 0.22**	**1.55 ± 0.32** 0.79 ± 0.13
NeuN	M F	0.92 ± 0.09 2.11 ± 2.13	0.83 ± 0.15 1.46 ± 0.29	1.04 ± 0.33 **0.72 ± 0.04**	0.74 ± 0.29 0.73 ± 0.24
NeuroD	M F	0.52 ± 0.31 2.50 ± 2.30	1.00 ± 0.17 1.13 ± 0.30	1.80 ± 0.75 0.76 ± 0.28	0.82 ± 0.35 0.75 ± 0.24
NRF2	M F	0.24 ± 0.29 0.18 ± 0.33	nd	1.01 ± 0.12 0.88 ± 0.18	nd
Microglia					
TMEM119	M F	nd	1.29 ± 0.45 1.43 ± 0.22	1.18 ± 0.17 1.13 ± 0.26	1.13 ± 0.47 0.86 ± 0.36
Apoptosis					
CX3CR1	M F	nd	1.28 ± 0.13 1.26 ± 0.23	1.21 ± 0.17 1.11 ± 0.08	0.90 ± 0.59 0.93 ± 0.45
BAX	M F	nd	0.89 ± 0.21 1.36 ± 0.37	1.02 ± 0.28 0.82 ± 0.14	0.80 ± 0.28 1.23 ± 0.47
APAF1	M F	nd	1.49 ± 0.28 1.29 ± 0.39	1.20 ± 0.17 0.99 ± 0.14	0.82 ± 0.21 0.76 ± 0.23

Fold change of 1000 mGy males and females compared to sham controls (n = 3 to 8). Bolded numbers, highlight in red, are significant compared to controls. Sex differences are in italics and are shaded blue. Genes not detected due to multiple melt curve peaks were marked with nd. All significances are $p \leq 0.05$.

In the males, the cerebral cortex had one downregulated synaptic activity gene, BDNF (0.57 ± 0.06). The prefrontal cortex had an upregulation of ROS regulator SOD2 (1.65 ± 0.29), and a DNA damage gene γH2AX (3.38 ± 1.55). In the male hippocampi, ROS regulator SOD3 was upregulated in 1000 mGy males (1.11 ± 0.27) compared to 1000 mGy females (1.28 ± 0.27) and

11β1HSD (0.75 ± 0.39), DDB2 (0.88 ± 0.16), and DNMT3b (0.88 ± 0.21) were downregulated compared to 1000 mGy females. Synaptic activity gene and PSD95 (1.80 ± 0.54) was overexpressed in 1000 mGy males compared to sham controls. The cerebellum had an upregulation of ROS regulator SOD2 (1.11 ± 0.07) and synaptic protein PSD95 (1.55 ± 0.32). The cerebellum also had an upregulation of synaptophysin (1.09 ± 0.07) in 1000 mGy males compared to females (0.94 ± 0.04).

3.5. Discussion

During the late gestation developmental period, animals are susceptible to fetal programming under stressful or adverse in-utero conditions (30,154). Late gestation is a period of differentiation, synaptogenesis, and neuronal migration; while not a critical period for survival, it is a sensitive period that requires a healthy environment for proper neural development (26). Adverse neurological outcomes in both humans and rodents after prenatal exposure to various types of stress include decreased neurogenesis, impaired memory and learning, decreased synaptic plasticity, increased levels of depression, increased likelihood of attention deficit hyperactive disorder, and increased avoidance of novel situations (41,91,94,178,179). The current study examined the impact of prenatal exposures to low doses of ionizing radiation on the behavioural and genetic profiles in specific brain regions of adult offspring.

C57Bl/6J mice are a common rodent model for a broad spectrum of research; however, previous literature has implicated they are resistant to radiation exposures (180–182). In the present study, with doses of ionizing radiation of 1000 mGy and lower, we were unable to find adverse behavioural outcomes in correlation to prenatal exposures, aside from one main effect of treatment on rearing behaviour in the social anxiety task at the 1000 mGy dose, an indication of

hyperactivity. A main effect of sex on rearing in the open field task was also discovered—with males exhibiting higher activity than females—a departure from normal behaviour for this breed of mouse on this task (183,184). In isolation of other behavioural measures for each task, these are not indicative of any significant adverse behaviours. Specific to ionizing radiation, the period of organogenesis or embryonic development in C57Bl/6J mice has been shown to be neutrally sensitive to exposures less than 213 mGy, with cellular apoptosis and cognitive impairment as side effects (185,186). Considering the critical period, mid-gestational effects of ionizing radiation on C57Bl/6J animals, it is valuable to investigate late-gestation with this model; however, according to our lack of robust dose response and historical literature referring to C57Bl/6J mice as radiation-resistant, it is reasonable to expect that a behavioural response will only be measurable at higher doses of ionizing radiation (187).

There was a significant sex difference in gene dysregulation across the brain regions, which is a normal trend for brain region, stress type, and various mouse breeds (188,189). The number of dysregulated genes in each pathway and brain region, however, was limited, but indicate a trend of oxidative stress and damage. In the cerebral cortex of the female mice, two ROS regulatory genes, SOD1 and GPX1 were upregulated along with synaptic activity gene PSD95. SOD1, or superoxide dismutase 1, encodes for a cytoplasmic enzyme that will catalyze the dismutation of a free radical, such as superoxide, into hydrogen peroxide (190). Glutathione peroxidase, GPX1 encodes for a second enzyme that will reduce hydrogen peroxide into water. Both genes, in concert, work to reduce cellular stress due to ROS and act as an endogenous antioxidant system, indicating a level of stress-response in this region was active (see Figure 8). Post-synaptic density 95 is a synaptic anchoring protein in excitatory glutaminergic synapses, associated with synaptic plasticity. Disruption of the balance of excitatory versus inhibitory synapses, as indicated by the

upregulation in female cerebral cortices, can increase AMPA receptors, changing synaptic strength by inhibiting long-term potentiation and enhancing long-term depression (191,192). A link has been made between PSD95 overexpression and seizure activity, schizophrenia, and addictive behaviours (192). In the male cerebral cortices, males had one downregulated gene, the brain-derived neurotrophic factor or BDNF which is involved in synaptic formation and neurogenesis. Mouse knock-out models of BDNF show severe synaptic impairments and downregulation of the gene is linked to major depressive disorder and chronic stress states (193–195).

Prefrontal cortices of female mice had an upregulation of extracellular superoxide dismutase (SOD3), endothelial nitric oxide synthase (NOS3) and PSD95. As previously mentioned, SODs are markers for oxidative stress and will transform free radicals into hydrogen peroxide and oxygen. Endothelial NOS can be upregulated in the presence of ROS and promote nitric oxide neurotransmission and vascular changes (196). It has been reported that, SOD3 and NOS3 are both upregulated in the prefrontal cortices of schizophrenic brains; combined with the upregulation of PSD95, a pattern leading to potentially serious neural disruption can be appreciated (196). In a study looking at survivors of Chernobyl radiation exposure, there was an increase in schizophrenic-like disorders and frontotemporal limbic dysfunction, indicating this area is indeed susceptible to radiation-induce damage (197). In males, there was an increase in mitochondrial SOD2 and DNA damage marker, γH2AX. H2AX is a protein that will be phosphorylated after double-stranded breaks occur to become γH2AX; combined, the upregulation of these two genes indicate the presence of oxidative stress in the prefrontal cortex (198).

The hippocampi of females had the most dysregulation with a reduction in growth arrest and DNA damage (GADD45), period 2 (PER2), NeuN, and PSD95. GADD45, when overexpressed, can increase longevity and is associated with DNA repair (199). In response to cellular stress,

GADD45 may induce cell cycle arrest, DNA repair, or apoptosis, and if dysregulated, is correlated with the presence of tumors (200,201). PER2 is a circadian rhythm gene that, when disrupted, can lead to cognitive decline, impaired learning, and metabolic syndrome (202–205). NeuN is a biomarker for neurons; downregulation of this, along with PSD95 would indicate fewer cells in the region and a reduction of excitatory synaptic activity. In the males, there was an upregulation of SOD3, NeuN, and PSD95, an oppositional change in dysregulation of the hippocampi to the females. In a study investigating the effects of PSD95 knockout mice, it was discovered that male and female mice exhibit oppositional behavioural phenotypes in sociability and activity; exhibited in the present study, is a genetic expression and behavioural difference (206).

The final region analyzed was the cerebellum; in the females, there was a downregulation of two DNA methylation genes, DNMT3a and DNMT1, which are responsible for epigenetic modifications and transcriptional regulation. In the males, there was an increase in SOD2 and PSD95, again indicating oxidative stress and excitatory synapse activity.

Contrary to our behavioural results, the pattern of genetic disruption in both male and female offspring lead us to believe the threshold for fetal programming of adverse coping behaviour and anxious behaviour is just beyond the doses we have utilized here. These animals are displaying a neural response to prenatal stress exposures that either do not elicit behavioural changes at this level of disruption, or display changes that we have not investigated, such as late-cognitive decline or schizophrenic-behaviour as associated with the gene dysregulation patterns in other studies (181,185). Although changes in certain genes examined were observed with prenatal exposure to ionizing radiation (1000 mGy), these subtle changes in gene expression had no correlation or impact on behavioural outcome in the adult offspring.

Acknowledgements

This research was supported by NSERC-CRD, Bruce Power, and the Nuclear Innovation Institute.

Chapter 4 – Prenatal exposure to ionizing radiation induces adaptive behaviour and altered gene expressions in the brain of *Mus musculus* offspring

Christine Lalonde, Shayen Sreetharan, Alyssa Murray, Lisa Stoa, Mary Ellen Cybulski, Allison Kennedy, Sandhya Khurana, Sujeenthar Thamalingam, Joanna Wilson, Neelam Khaper, Simon Lees, Douglas Boreham, T.C. Tai

In preparation to submit to *Plos Bio*

4.1. Abstract

Environmental stress on a pregnant mother can produce either an adaptive or maladaptive phenotype in her offspring. When exposed to levels of ionizing radiation in early development, organogenesis may be disrupted, leading to loss of life or severe birth defects. During late gestation, most development is sensitive to exposures, such as the fetal brain, which is differentiating and forming connections. In order to investigate the phenotypical result of late-gestational exposures to radiation, pregnant *Mus musculus* (BALB/c) were randomly assigned into experimental groups: sham control, 50 mGy, 300 mGy, or 1000 mGy dosing condition on gestational day 15. Adult offspring were evaluated for behavioural abnormalities using the Porsolt swim task, open field task, and a social anxiety task. The prefrontal cortices, cerebral cortices, hippocampi, and cerebella were analyzed using quantitative real-time polymerase chain reaction for relative gene expression. Our results show that adult offspring prenatally exposed to 1000 mGy during late gestation, displayed hyperactive behaviour and active coping strategies with significant neural genetic dysregulation that is sexually dimorphic and regionally dependent. Exposure to doses below 1000 mGy had no effect on adult behavioural outcomes. These results suggest that in BALB/c mice, a threshold (1000 mGy) of ionizing radiation exists during pregnancy that is sufficient to induce a maladaptive response to novel environments.

4.2. Introduction

Fetal exposure to stressful environmental conditions has been shown to produce differences in adult predisposition to disease, such as the "Thrifty Phenotype," later flushed out as fetal programming (31,98). The founding researchers stipulated that early-life environmental deficiencies influenced the physiology and metabolism of the fetus, which would be considered an adaptive response, but is decidedly maladaptive when faced with an external environment in contrast to the predicted nature of deficient uterine conditions (32,161,162,207). The hypothesis was met with criticism, however, it withstood rigorous replication over the past two decades (29,30,154,155,208). Multiple studies have concluded that nutrient deprivation, hypoxia, and exposure to steroidal hormones lead to a maladaptive phenotype (208–211). Hypertension, obesity, diabetes, and other metabolic issues are hallmarks of this fetal programming phenotype.

The proposed mechanisms for fetal programming lie in gene expression modifications via high levels of glucocorticoids (GC) and reactive oxygen species (ROS; see Figure 9). Typically, active GC are prevented from crossing the placenta and interacting with the fetus via an enzymatic barrier, hydroxysteroid 11-beta dehydrogenase 2 (11β2HSD), which can convert cortisol into its inactive form, cortisone (103). When this barrier is overwhelmed by the mother's stress response, GC can bind to their receptors in the fetus. Mineralocorticoid receptors get occupied quickly because of a higher binding efficiency, and then glucocorticoid receptors begin to be occupied in higher numbers as well (212). The receptor will dimerize and translocate to the cellular nucleus, where it can modify gene expression directly by binding to the glucocorticoid response element (GRE), and by permitting methylation of CpG islands that are predominantly located near the GRE (90). Gene expression may be stimulated or repressed depending upon the methylation number and location (159,160).

The other mechanism, ROS, may be generated through ionizing radiation or through GC. Ionizing radiation can directly and indirectly disrupt electrons from their atoms, generating ROS, which may cause cellular damage via single-stranded and double-stranded DNA breaks. Multiple cellular mechanisms may be deployed to deal with the damage that include repair and apoptotic processes. Increased levels of ROS create an oxidative stress state, which may stimulate increased levels of glucocorticoids (90). Increased levels of glucocorticoids, in turn, generates increased levels of ROS (213). When a mother is exposed to high levels of a stressor, such as ionizing radiation (IR), higher concentrations of endogenous glucocorticoids (GC) will be produced in response to direct and indirect DNA damage in her body. Levels of ionizing radiation will also directly lead to the production of ROS within the fetus itself. Oxidative stress also influences gene regulation through a number of epigenetic mechanisms such as DNA methylation, histone modification, and non-coding RNA regulation (90).

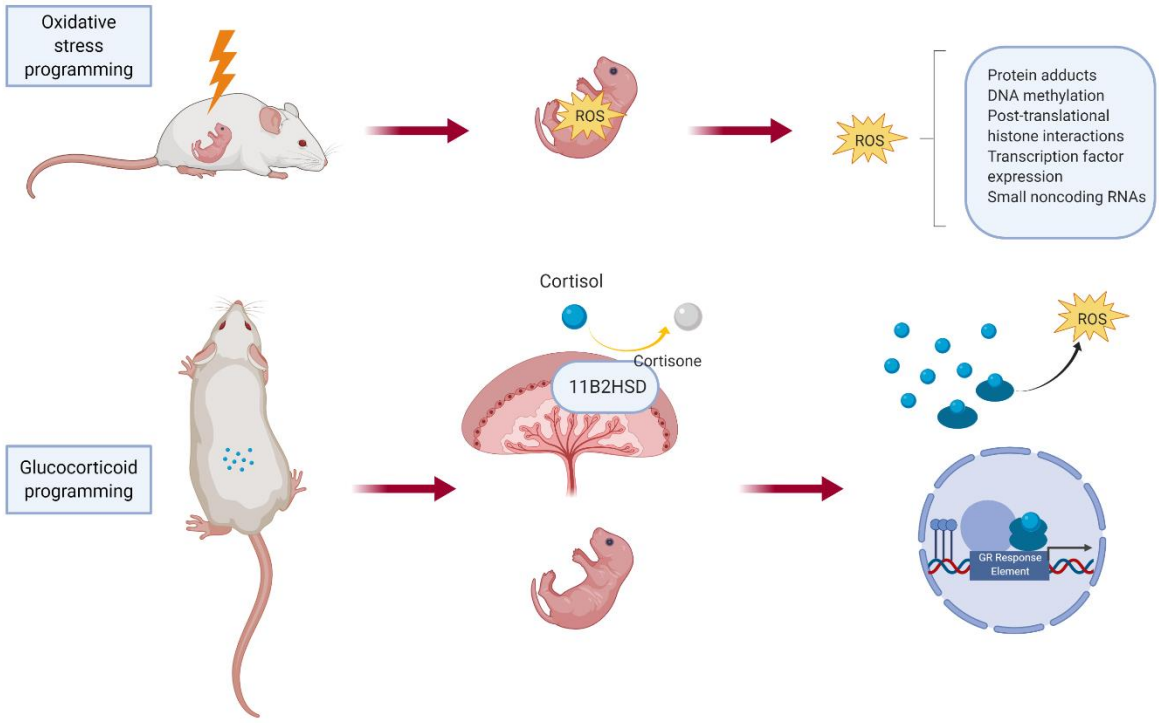

Figure 9. Epigenetic modification pathways. Intrauterine exposure to ionizing radiation leads to ROS induced epigenetic modifications via multiple mechanisms. In other stressors, active GC bind to their receptors in the fetus and translocate to the nucleus to bind to the GRE.

Various stressors during pregnancy can lead to genetic dysregulation in neural pathways that can be linked to problems with learning and memory, depression, attention, and inhibition (91,94,179,214,215). Early life exposures to radiation may lead to birth defects and loss of life due to organogenesis and critical development (91). Sensitive to radiation and other stressors, the late gestational period is neurodevelopmentally active, with neuronal migration, differentiation, and synaptogenesis occurring (26). Ionizing radiation exposures during pregnancy often come from medical diagnostics or airline travel; little is known about the adult behavioral effects of late-gestational exposures to low and sublethal doses (90,91). The hippocampus is a structure within the limbic system that is responsible for learning and memory based on reward and fear input from

other structures and is dense with GC receptors. The prefrontal cortex is linked to higher order cognition, inhibition, and social behaviours. The cerebellum, once known only for motor control, is also involved in cognitive processing and disruption can lead to addictions and depression. The cerebral cortex is responsible for sensory stimuli processing and perception. Each region is hypothetically a site for behavioural abnormalities. Similar to the adverse effect of the metabolic adaptive response to a uterine environment, abnormalities in behaviour could lead to socially damaging and dangerous practices in an appropriate context.

Our previous work indicated C57Bl/6J mice were not susceptible to doses of ionizing radiation 1000 mGy and under, with little behaviour and few genetic changes. BALB/c mice have been noted to be radiosensitive with an $LD_{50:30}$ more than 1000 mGy lower than C57Bl/6J mice (187,216). Here we have replicated the C57Bl/6J study to investigate fetal programming of brain and behaviour in a more susceptive mouse strain (187,216). In consideration of the pathways dysregulated in the C57Bl/6J mice in key brain areas related to inhibition, cognition, and other behaviour, we hypothesize a more robust behavioural and genetic response from the more vulnerable BALB/c mouse strain. Further, we hypothesize a similar maladaptive phenotype compared to the metabolic outcomes, whereby the adaptation is averse depending upon the environmental situation.

4.3. Results

4.3.1. Behavioural Tasks

Observational data for the Porsolt swim task measures escape behaviour as an indicator of coping skills (Figure 10. a). A main effect of treatment was observed F (3, 43) = 9.47, p = 0.001, η^2 = 0.398 (Figure 11). Adult animals prenatally exposed to 300 mGy during late gestation (138.067 ±

46.76, mean and S.E.M.), spent more time immobile than sham controls (96.73 ± 35.71); however, adult mice prenatally exposed to 1000 mGy (46.70 ± 50.37) spend far less time immobile compared to all other groups. There were no measurable differences in the open field task (Figure 10. b).

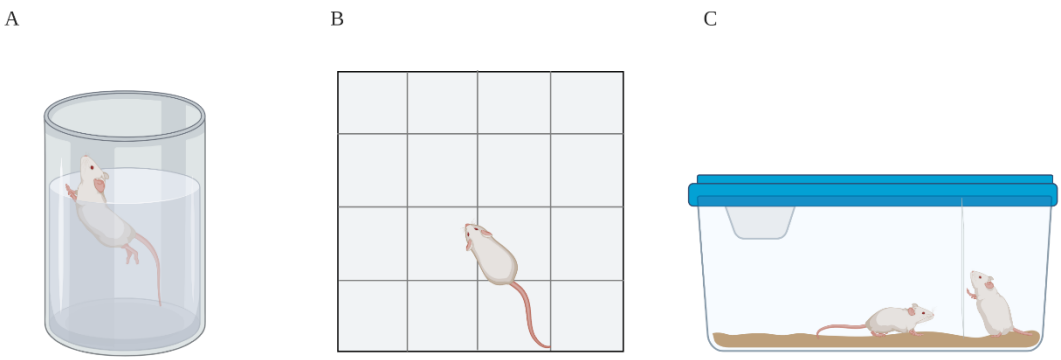

Figure 10. Behavioural tasks for *Mus musculus*. a) The Porsolt swim task: the mouse is placed in an inescapable container of water and is observed for latency-to-float and total immobility. b) Open field task: the mouse is placed in the center and is measured for grid crossings, rears, and time spent in center. c) Social anxiety task: the mouse is placed in a cage where it can see and smell a stranger. Approaches, total time in approach, and rears are recorded.

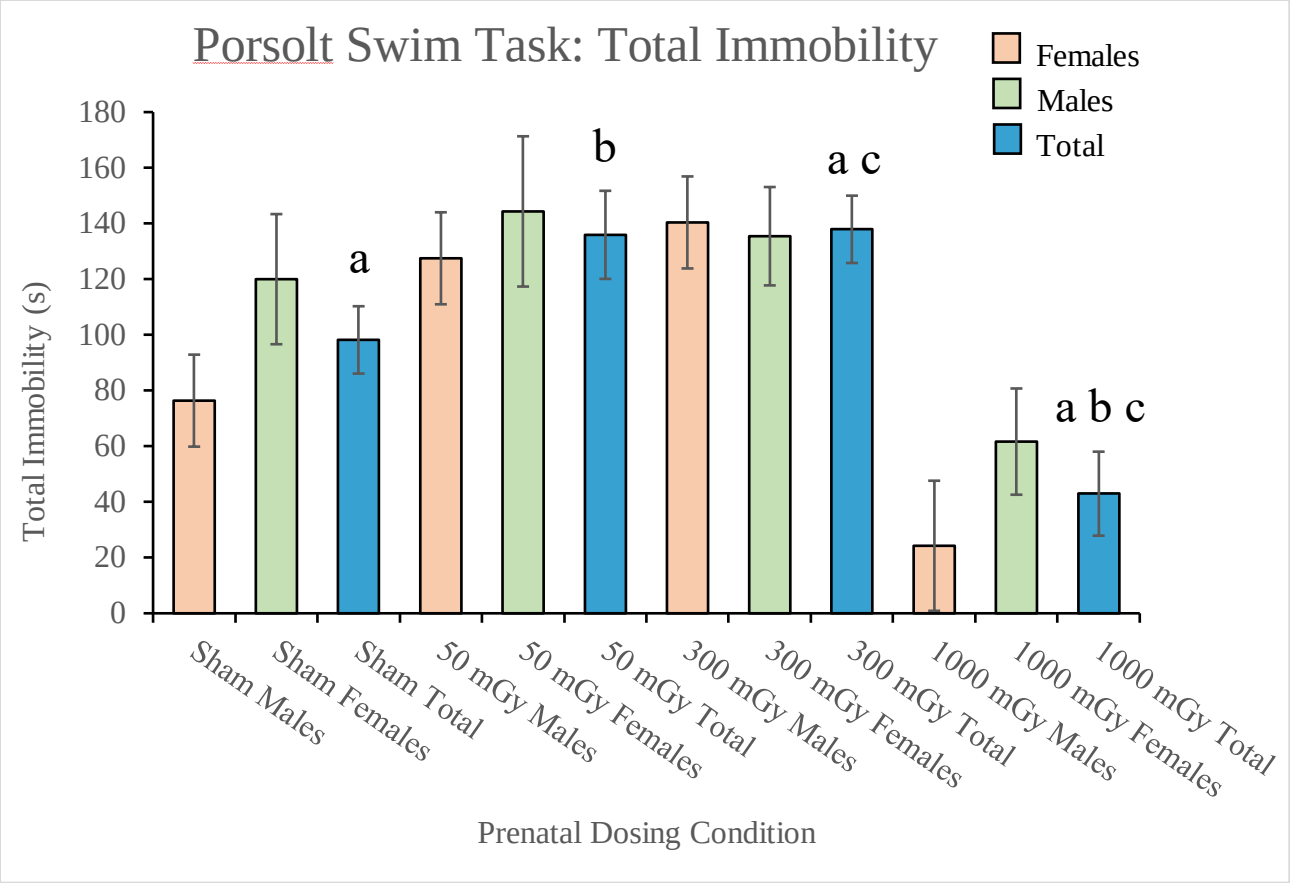

Figure 11. Total time (s) spent immobile in a Porsolt swim task. The prenatally exposed 1000 mGy dose condition spent far less time floating compared to sham, 50 mGy, and 300 mGy. The prenatally exposed 300 mGy condition spent significantly more time floating compared to sham controls.

In the social anxiety task, animals are measured on interactions with a stranger mouse (see Figure 10. c). There was a main effect of treatment, $\lambda = 0.517$, F (9, 119.404) = 4.14, $p = 0.001$, $\eta^2 = 0.198$ (Figure 12). For rearing activity, both 300 mGy (28.63 ± 2.64) and 1000 mGy (32.77 ± 3.086) were more active compared to shams (19.61 ± 2.402). For total number of approaches (Figure 13), 50 mGy (17.56 ± 1.16) and 1000 mGy (16.77 ± 1.36) were more active than shams (12.44 ± 1.06) and 300 mGy (13.15 ± 1.16). A main effect of sex was also observed, $\lambda = 0.853$, F (3, 49) = 2.807, $p = 0.049$, $\eta^2 = 0.147$. In the rearing condition, males (30.74 ± 1.91) were more active than females (22.80 ±1.91).

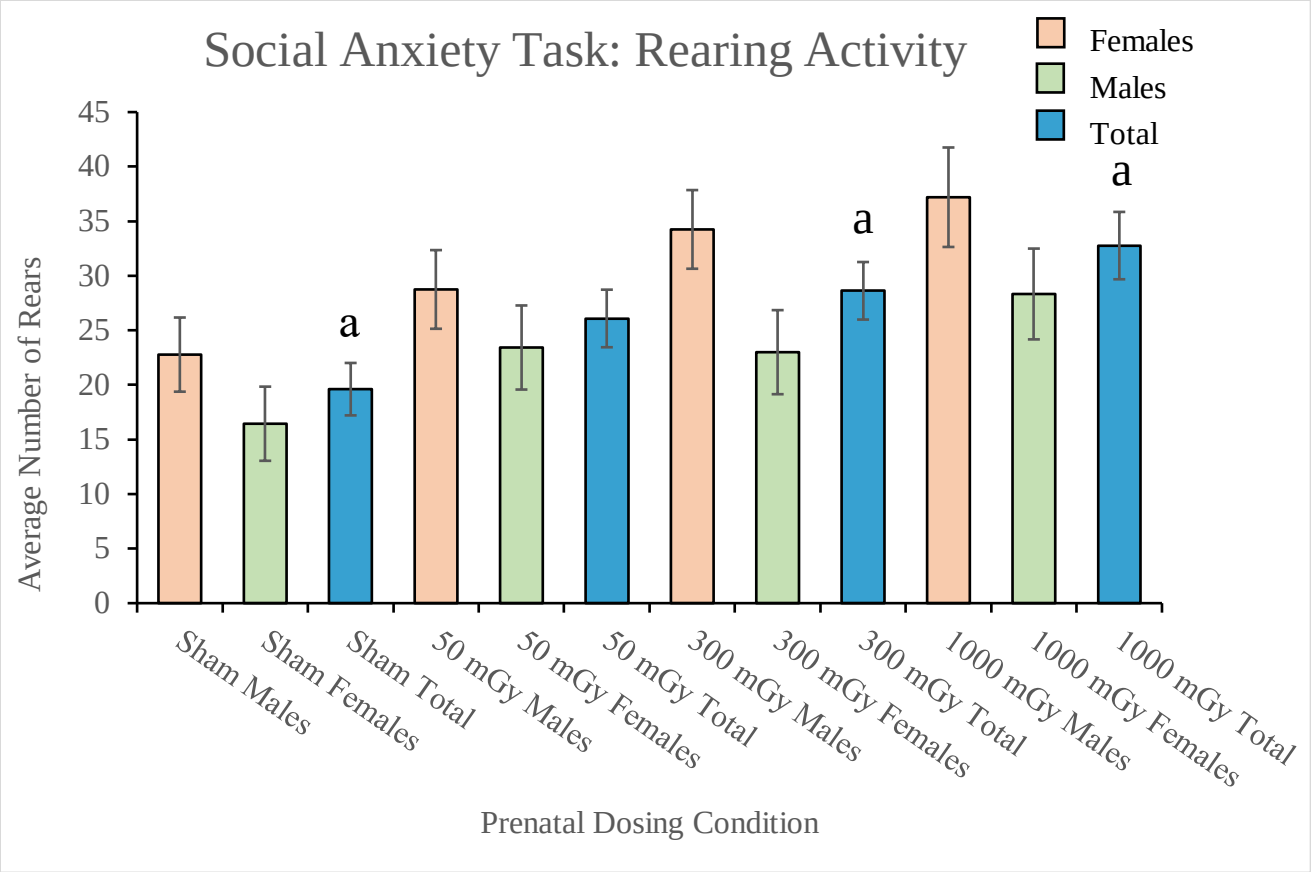

Figure 12. Average number of rears during the social anxiety task. Both the prenatally exposed 300 mGy and 1000 mGy conditions were more active compared to sham controls.

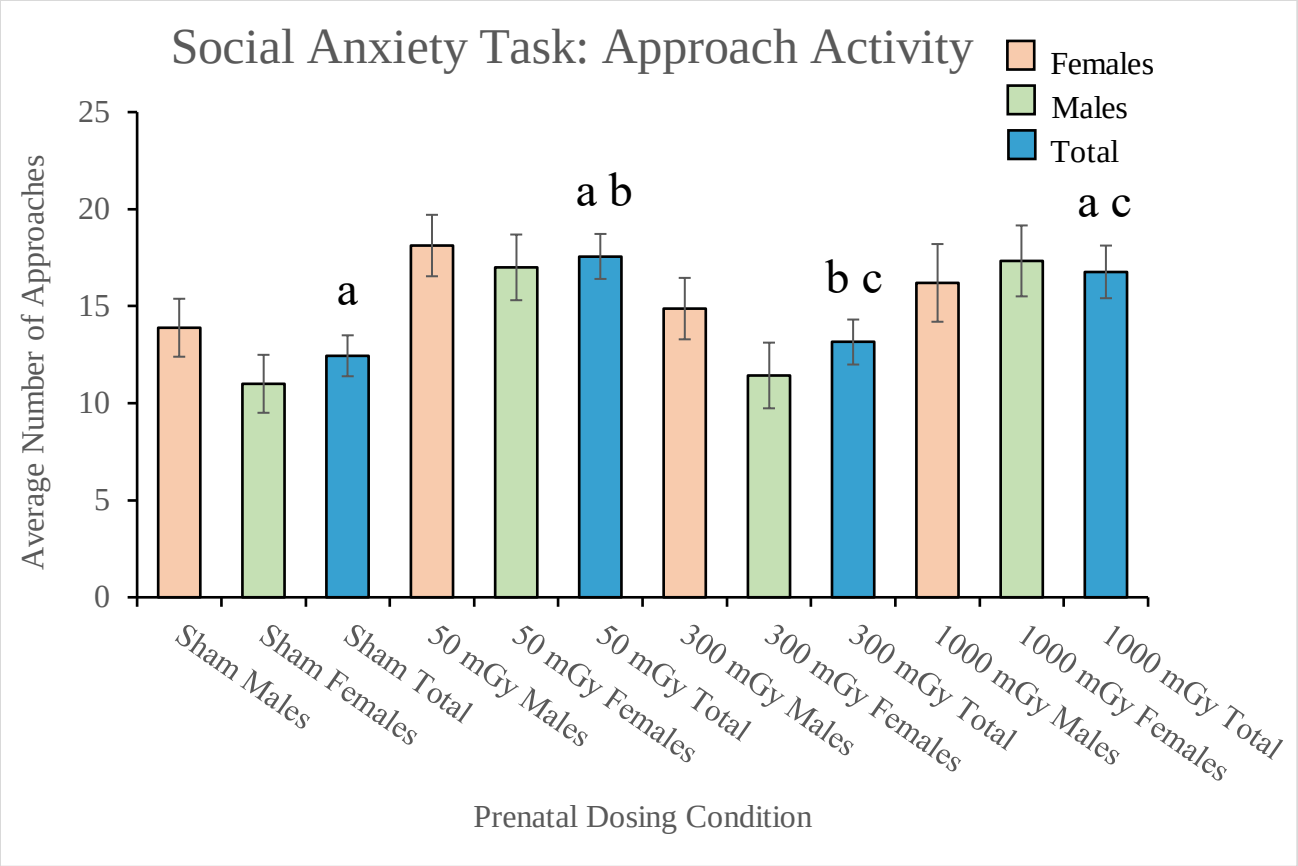

Figure 13. Average number of approaches to a stranger mouse during the social anxiety task. Both the prenatally exposed 50 mGy and 1000 mGy conditions were more active than sham controls and the 300 mGy condition.

4.3.2. Genetic Analysis

Out of 31 genes, 16 genes were dysregulated in the brains of 1000 mGy exposed males and 13 genes in females compared to their respective sham controls (Figure 14; Table 6). In the male PFC, ROS regulator SOD1 was overexpressed with a fold change of 1.66 ± 0.39 (2^ΔΔCQ ± S.E.M.), as were SOD3 (2.18 ± 0.74) and catalase (2.62 ± 0.87). Cell cycle gene, BTG2 (1.47 ± 0.22) was also upregulated, as was GADD45 (2.18 ± 0.74) and PER2 (2.66 ± 0.88). Genes with significant sex differences in the PFC were GR, γH2AX, TMEM119, APAF1, and BAX. In the cerebral cortices of the males, there were predominantly downregulated genes. NR3C1 (0.89 ± 0.11) and DDB2 (0.74 ± 0.06) were downregulated, as were PER2 (0.74 ± 0.07), NeuN (0.81 ±

0.09), and NeuroD (0.74 ± 0.07). The only gene upregulated in this region was APAF1 (1.22 ± 0.77). SOD1 and synaptophysin saw a main effect of sex in the cerebral cortices. In the cerebella, there were two dysregulated genes, synaptic activity gene BDNF (0.63 ± 0.05) and PSD95 (1.31 ± 0.16). NeuN and BAX were significantly different among 1000 mGy males and females in the cerebella.

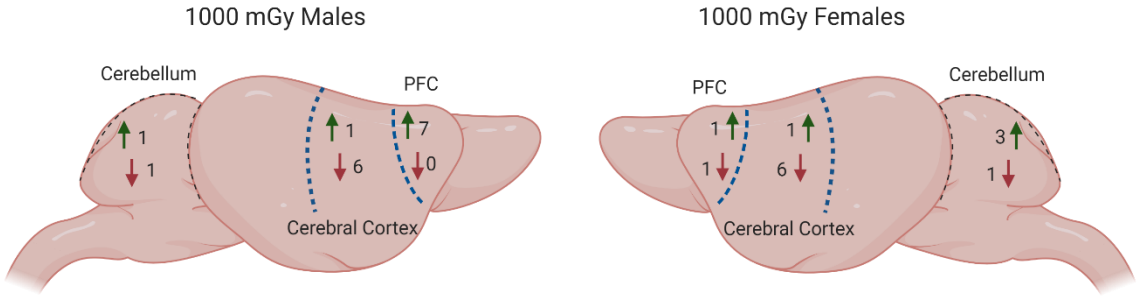

Figure 14. Differentially expressed genes in 1000 mGy male offspring versus 1000 mGy female offspring.

Table 6. Relative Gene Expression in BALB/c 1000 mGy Offspring

Gene Expression in 1000mGy Offspring Relative to Sham (Fold Change ± SEM)					
ROS Regulation					
Gene	**Sex**	**Prefrontal Cortex**	**Cerebral Cortex**	**Hippocampus**	**Cerebellum**
SOD1	M	**1.66 ± 0.39**	*0.86 ± 0.06*	1.36 ± 0.57	1.12 ± 0.54
	F	1.03 ± 0.21	*1.13 ± 0.07*	0.87 ± 0.23	1.00 ± 0.21
SOD2	M	1.59 ± 0.38	0.94 ± 0.05	1.39 ± 0.50	0.72 ± 0.15
	F	1.01 ± 0.10	0.90 ± 0.14	0.77 ± 0.22	0.82 ± 0.14
SOD3	M	**2.18 ± 0.74**	0.95 ± 0.91	1.60 ± 0.76	0.55 ± 0.16
	F	1.09 ± 0.46	0.77 ± 0.10	1.02 ± 0.48	1.23 ± 0.41
Catalase	M	**2.62 ± 0.87**	0.90 ± 0.11	0.82 ± 0.18	1.06 ± 0.11
	F	1.14 ± 0.52	**0.55 ± 0.44**	1.01 ± 0.14	1.01 ± 0.19

GPX1	M F	1.16 ± 0.30 1.09 ± 0.11	0.85 ± 0.07 0.98 ± 0.05	1.14 ± 0.22 1.16 ± 0.22	1.27 ± 0.14 0.92 ± 0.17
Cortisol Regulation					
11B1HSD	M F	nd	nd	0.78 ± 0.08 0.97 ± 0.11	0.97 ± 0.29 1.17 ± 0.27
11B2HSD	M F	1.06 ± 0.09 1.02 ± 0.08	nd	nd	0.87 ± 0.25 1.39 ± 0.18
GR	M F	*2.00 ± 1.50* *0.64 ± 0.38*	**0.89 ± 0.11** **0.64 ± 0.08**	nd	0.98 ± 0.13 0.70 ± 0.08
Cell Cycle					
BTG2	M F	**1.47 ± 0.22** 1.12 ± 0.20	0.86 ± 0.11 0.67 ± 0.22	1.17 ± 0.13 0.90 ± 0.14	0.60 ± 0.19 0.96 ± 0.27
DNA Damage & Tumor Suppression					
yH2AX	M F	*1.13 ± 0.51* *1.00 ± 0.76*	nd	nd	1.18 ± 0.46 1.36 ± 0.32
p53	M F	0.82 ± 1.19 0.94 ± 1.04	1.10 ± 0.11 0.93 ± 0.13	2.13 ± 1.20 0.87 ± 0.17	1.21 ± 0.43 0.86 ± 0.14
GADD45	M F	**2.18 ± 0.74** 1.09 ± 0.46	nd	1.12 ± 0.54 1.01 ± 0.23	0.90 ± 0.13 1.14 ± 0.24
DDB2	M F	1.21 ± 0.13 1.34 ± 0.16	**0.74 ± 0.06** 0.89 ± 0.05	1.38 ± 0.44 0.94 ± 0.18	1.02 ± 0.07 **1.37 ± 0.39**
MDM2	M F	1.22 ± 0.33 1.31 ± 0.18	nd	nd	1.02 ± 0.12 1.08 ± 0.14
DNA Methylation					
DNMT3b	M F	1.30 ± 0.27 **1.27 ± 0.09**	nd	1.28 ± 0.24 1.31 ± 0.14	0.59 ± 0.19 1.43 ± 0.41
DNMT3a	M F	nd	1.08 ± 0.26 0.69 ± 0.41	1.06 ± 0.09 1.08 ± 0.04	0.73 ± 0.21 1.19 ± 0.23
DNMT1	M F	nd	0.91 ± 0.06 0.94 ± 0.08	0.79 ± 0.16 0.95 ± 0.03	1.22 ± 1.27 1.48 ± 2.36
Circadian Rhythm					
PER2	M F	**2.66 ± 0.88** 0.88 ± 0.40	**0.74 ± 0.07** **0.80 ± 0.06**	1.15 ± 0.32 0.78 ± 0.13	nd
Synaptic Activity					
Synaptophysin	M F	nd	*1.01 ± 0.03* *1.01 ± 0.06*	1.06 ± 0.11 0.90 ± 0.15	1.14 ± 0.21 0.91 ± 0.12
BDNF	M F	nd	0.87 ± 0.36 **0.58 ± 0.11**	1.01 ± 0.22 0.89 ± 0.14	**0.63 ± 0.05** 0.85 ± 0.26
SLC6A5	M F	nd	0.90 ± 0.13 1.03 ± 0.12	nd	nd
DCX	M F	1.73 ± 0.75 1.04 ± 0.55	0.64 ± 0.29 **0.38 ± 0.08**	1.07 ± 0.07 0.81 ± 0.09	nd
NOS3	M F	1.16 ± 0.30 1.09 ± 0.11	0.95 ± 0.10 0.70 ± 0.33	nd	nd
PSD95	M F	1.27 ± 0.43 0.91 ± 0.12	0.90 ± 0.12 1.48 ± 0.49	1.46 ± 0.47 1.01 ± 0.18	**1.31 ± 0.16** **1.49 ± 0.32**

NeuN	M F	nd	**0.81 ± 0.09** **0.64 ± 0.08**	1.36 ± 0.64 0.64 ± 0.08	*0.81 ± 0.11* *0.72 ± 0.27*
NeuroD	M F	nd	**0.74 ± 0.09** 1.13 ± 0.35	2.15 ± 1.42 0.72 ± 0.52	0.42 ± 0.16 1.05 ± 1.07
NRF2	M F	1.19 ± 0.49 1.21 ± 0.42	nd	nd	nd
Microglia					
TMEM119	M F	*1.13 ± 0.74* *1.78 ± 1.35*	1.06 ± 0.15 1.07 ± 0.17	1.32 ± 0.74 0.93 ± 0.14	**2.30 ± 1.00** **1.81 ± 0.37**
Apoptosis					
CX3CR1	M F	nd	1.10 ± 0.06 0.87 ± 0.10	nd	nd
BAX	M F	*1.05 ± 0.44* *1.05 ± 0.75*	0.97 ± 0.08 0.68 ± 0.16	1.83 ± 1.14 0.84 ± 0.23	*0.81 ± 0.19* **0.77 ± 0.06**
APAF1	M F	*0.99 ± 0.44* *1.11 ± 1.10*	**1.22 ± 0.08** 0.92 ± 0.32	1.70 ± 1.32 0.86 ± 0.20	1.16 ± 0.46 1.58 ± 0.06

Fold change of 1000 mGy males compared to Sham controls. Bolded numbers, highlight in red, are significant compared to controls. Sex differences are in italics and are shaded blue. Genes not detected due to multiple melt curve peaks were marked with nd. All significances are $p \leq 0.05$.

Females displayed fewer dysregulated PFC genes than the males. DNA methylation gene DNMT3b (1.27 ± 0.09) was overexpressed. There was a significant disruption of the cerebral cortices, however, with a downregulation of catalase (0.55 ± 0.44), NR3C1 (0.64 ± 0.08), PER2 (0.80 ± 0.06), BDNF (0.58 ± 0.11), DCX (0.38 ± 0.08), and NeuN (0.64 ± 0.08). In the cerebella, there were a few dysregulated genes: DDB2 (1.37 ± 0.39), PSD95 (1.49 ± 0.32), TMEM119 (1.81 ± 0.37), and BAX (0.77 ± 0.06).

4.4. Discussion

Under normal conditions, during late gestation the fetal brain will continue to develop by neuronal migration, differentiation, cellular apoptosis, and synaptogenesis (217). Disruption of these processes can impair neural function, which can lead to changes in normative behaviour.

Rodent models are variable in radiation sensitivity; BALB/c mice are considered radiosensitive and are, therefore, suitable for fetal programming investigation (180–182). In the present study,

we have found BALB/c mice to be prenatally sensitive to doses 1000 mGy and lower. At the 300 mGy prenatal exposure, offspring display a trend towards behavioural disruption with increased immobility in the Porsolt swim task (PST) and increased rearing activity in the social anxiety task (SAT). Adult mice prenatally exposed to 1000 mGy display a robust behaviour profile, with increased activity in the PST and in two measures in the SAT. Previously utilized as a task to evaluate learned helplessness, the PST has recently been criticized for inaccurate evaluation of behavioural context. Still relevant to stress, immobility is indicative of a coping strategy, rather than despair (218). In a test-retest design, immobility increases with experience and antidepressants interfere with the learning-consolidation process and will and often produce a stimulation or sedative effect (219). The influx of GC during a stressful situation is key to contextual learning and if impaired inhibits the effect of immobility during the PST (220). In a study exploring the anti-depressant effects of sleep-deprivation, increased activity, as seen in the 1000 mGy condition is also induced with moderate levels of sleep-deprivation (221). In other words, immobility is a coping strategy and should not be considered a negative outcome of the task. The lack of immobility may indicate inhibition and can be considered a dangerous strategy when faced with an inescapable stressor. Similarly, the 1000 mGy offspring's increased activity in the SAT may be considered adversive. The increase in rearing activity and total number of approaches are indicative of hyperactive behaviour and decreased inhibition when faced with a novel situation and may be pathogenic (222).

In combination with the neural regions, the 1000 mGy offspring display a phenotype not characteristic of control animals. The prefrontal cortex (PFC) is responsible for higher order cognitive processing, which involves inhibition and modification of behaviour in stressful and novel situations (46,130,131). When faced with an unknown conspecific, such as in the SAT, it

may be prudent to consider the potential aggressiveness of the stranger; however, here we see increased exploration and approach. In the male animals prenatally exposed to 1000 mGy, eight differentially expressed genes were upregulated and one was downregulated in the PFC (see Figure 14). Three ROS regulator genes were upregulated: superoxide dismutase 1 (SOD1), which is cytoplasmic and will catalyze the dismutation of ROS into hydrogen peroxide (190). SOD3, an extracellular gene and catalase, a gene that encodes for the enzyme that catalyzes the hydrogen peroxide intermediates into water and oxygen, were also upregulated. In addition to these ROS relevant genes, GADD45, growth arrest and DNA damage gene, was overexpressed and is involved in a cellular response to stress, leading to arrest, repair, or apoptosis. Combined, these genes are indicative of oxidative stress within the PFC of the 1000 mGy prenatally exposed males. B-cell translocation gene 2 (BTG2) is radiation induced and is involved in anti-proliferation, neuronal apoptosis, and ROS inhibition (223). The other upregulated genes are the glucocorticoid receptor gene, NR3C1 and circadian rhythm gene, period 2 (PER2). Inhibition of the glucocorticoid receptor in the PFC has been shown to be associated with increased immobility on the PST (224). The females did not show as much of a robust dysregulation of genes in the PFC, with a downregulation of NR3C1 and an upregulation of DNA methylation gene DNMT3b.

The cerebral cortex is involved in sensory input and perception and both males and females prenatally exposed to 1000 mGy were significantly dysregulated in this region, with one upregulated gene each and six down. In the males, SOD1 was downregulated compared to the 1000 mGy females. Males also had a decrease in expression of damage specific DNA binding protein 2 (DDB2), which repairs ultra-violet damaged DNA. PER2 was downregulated, as were neuronal markers NeuN (neuronal nuclei antigen) and NeuroD (neuronal differentiation) which specifically mark new neurons. The one upregulated gene in the males was apoptosis protease

activating factor 1 (APAF1), which encodes for a protein that initiates apoptosis (225). In females, SOD1 was upregulated and catalase was downregulated. NR3C1 and PER2 were also downregulated. Similar to the males, synaptic activity and differentiation genes, BDNF (brain derived neurotropic factor), NeuN, and DCX (doublecortin) were also downregulated. Similar to the PFC, these genes indicate oxidative and cellular stress within the cerebral cortices.

The final region with differentially expressed genes is the cerebellum, which is also involved in cognition. The cerebella of both males and females had few dysregulated genes. In the males, BDNF was downregulated and transmembrane protein 119 was overexpressed, indicating microglia activity (226,227). In the females, cellular repair gene DDB2 was upregulated as was post-synaptic density 95, which is involved in excitatory synapses and is indicative of increased activity. Microglia marker, TMEM119 was also upregulated, but BAX (BCL-2 associated x protein), a cell apoptosis regulator was downregulated.

In comparison to the C57Bl/6J study, the BALB/c strain displayed not only a more robust behavioral phenotype, but significant genetic dysregulation. Both animal models displayed differentially expressed genes in ROS regulation, DNA damage, and synaptic activity. In addition to the other genetic changes seen across the neural regions in the BALB/c mice, it is evident that prenatal exposures to ionizing radiation may induce changes to behaviour and gene expression in response to damage. These changes in behaviour in response to novel situations may prove to be maladaptive, in consideration of the active coping strategy and inhibition towards anxiety, BALB/c mice may place themselves at risk for predation or injury. Upon exploration of our data, we believe morphological and regional volume analyses would be valuable in future research with this fetal programming model.

4.5. Materials and Methods

4.5.1. Ethics

All experimental protocols were approved by the Animal Care Committee of McMaster University (AUP-15-11-26) and are in accordance with the Canadian Council on Animal Care guidelines.

4.5.2. Animals and Housing

Male and female BALB/c mice (8-12 weeks) were obtained from Jackson Laboratories and were bred at McMaster University, Hamilton, ON. Two females were placed in a cage overnight with a single male. Pregnancies were confirmed with the presence of a vaginal plug in the morning, which was considered gestational day (GD) 1. Pregnant females were then singly housed for the duration of the gestational period. Standard rodent chow and water was provided ad-libitum (6.2% fat, 44.2% carbohydrate content, Teklad Diets Envigo, Madison, WI); and animals were maintained on a 12:12h light-dark cycle. Animals were housed in standard, non-vented cages.

4.5.3. Irradiation

Pregnant BALB/c mice were exposed to ionizing radiation with a 662 keV, ^{137}Cs γ-radiation Taylor Radiobiology source at McMaster University. Animals were transported in a temperature-controlled vehicle from the housing facility to the irradiation building in their home cages. Upon arrival, the cages were placed under shielding for acclimatization to the new location for 1 hour prior to exposures. Cages were place equidistant from the source and they received 10 mGy per minute dose on gestation day 15; fetal doses were measured at 8.9 mGy per minute. Dam and fetal dose rates were obtained using transplanted thermoluminescent dosimeters as previously described

(168). Animals were randomly assigned to either sham, 50, 300, or 1000 mGy exposure conditions.

4.5.4. Offspring

Two male and two female pups from each dam (n = 8) were utilized for behavioural and genetic testing. After weaning, pups were raised under normal conditions, 2-3 per cage, food and water *ad-libitum* until the age of 17-18 weeks. Pups then underwent behavioural testing and were euthanized by cervical dislocation within 24 hours of the last task. Relevant tissues were harvested, frozen immediately with liquid nitrogen, and then stored at -80°C until genetic analysis.

4.3.5. Behavioural Tests

All behaviour was recorded utilizing video-cameras. Experimenters and observers were blind to treatment groups. Behavioural tasks were conducted in random order to prevent ordering effects and were completed over the course of 1.5 days. Animals were brought into the testing rooms at least one hour prior to testing to acclimatize. All tasks were conducted during the light period and each task was washed between animal testing with 70% ethanol and water. All behaviour was scored using Behavioural Observation Research Initiative Software (BORIS) (172).

(1) Porsolt Swim Task

As a measure of stress coping strategies, animals were placed in buckets of water, at 32°C and were observed for a total of 5 minutes (125). Water depth was maintained at 9 inches in 11 x 11-inch plastic buckets (see Figure 10. a). Animals were placed in the center of the bucket and were dried off with paper towel and placed back into their home cages for at least an hour prior to participating in any other behavioural task. Animals were monitored by observers nearby, but out-

of-sight in case a rescue was required. Observed behaviour included latency-to-float and the total time spent immobile or floating. Immobility was defined as swimming cessation—movement involved in keeping the animal's head above water or to push itself from bumping into the wall were included in the immobility measure.

(2) Open Field Task

As a measure of general anxiety, four open field boxes (40 x 40 x 30 cm) were placed on the floor, see Figure 10. b, (173). Mice were placed in the center of the task and were allowed to explore the novel environment for 5 minutes. Behaviour measured by the observer included number of grid crossings to the center of the filed, total time spent within the center of the field, and the total number of rears. A grid was placed over the computer screen while utilizing BORIS software to create a central square measuring 20 x 20 cm. Grid crossings were defined as three or more paws across the line and rearing was defined as standing on two rear paws.

(3) Social Anxiety Task

To measure social anxiety, animals were placed in a clean, normal housing cage for a total of 5 minutes with a stranger mouse, see Figure 10. c, (174). The stranger mouse was matched for sex, but was not an experimental animal and was replaced with a new stranger after a few trials to prevent stress in the stranger mouse. The stranger was placed behind a plexiglass divider for protection and identification. The stranger mouse had reduced area (3 inches wide) to explore in order to prevent it from hiding from interactions with the experimental animal. Observed behaviour included rearing, as defined above, number of approaches, and total time in approach defined as being within 1.5 inches of the plexiglass, facing the stranger mouse.

4.3.6. Brain Dissections

Brain regions were determined utilizing Paxinos and Franklin's mouse brain atlas (175). The prefrontal cortex was delineated by a +2.2 mm anterior-posterior (AP) position from bregma. The cerebellum was removed using a scalpel, and the remaining cortex and hippocampus were removed using forceps and a scalpel (176). All dissections were conducted in sterile petri dishes on top of ice.

4.5.7. Primer Design

Primers were designed using Primer Bank, Primer3, Primer3Plus, and Gemi; sequences and accession numbers are listed in Supplementary Table 2, Appendix A. The primer for BDNF was designed based on previous literature (177). Primers were validated utilizing serial dilutions and specificity was analyzed using melt curves post amplification (127). Genes were chosen relevant to pathways in ROS regulation, cortisol regulation, cell cycling, DNA damage and tumor suppression, DNA methylation, circadian rhythm, synaptic activity, apoptosis, and microglia activity.

4.5.8. RNA Extraction and Complimentary DNA Synthesis

Neural tissue was extracted and complimentary DNA was synthesized according to our laboratory protocol http://dx.doi.org/10.17504/protocols.io.bez9jf96

4.5.9. RT-qPCR

Utilizing the QuantStudio5 Real-Time PCR System (Applied Biosystems), genes were analyzed comparing samples from the Sham and 1000 mGy animals (n = 4 to 8). A final concentration of

0.24 ng/µL of cDNA of each sample was added to 2.7 µL of DEPC water, 0.9 µL of both forward and reverse primers, and 7.5 µL of SYBR green master-mix (SensiFAST SYBR Lo-ROX, Bioline).

4.5.10. Statistical Analysis

All statistical analyses for behavioural and genetic comparisons were carried out using IBM SPSS 20.0 and jamovi version 1.2.12. Datasets were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene's test, $p > 0.05$. Inter-rater reliability was calculated using Pearson's Correlation, $r = 0.993$. General linear model analysis of variance (ANOVA) was conducted, otherwise Welch's test was utilized with alpha levels set to $p = 0.05$. All results are presented in mean ± standard error of the mean (S.E.M). Post-hoc analyses were conducted where appropriate, using Tukey's Honestly Significant Difference (HSD).

Acknowledgements

This research was supported by NSERC-CRD, Bruce Power, and the Nuclear Innovation Institute.

Chapter 5 – General Discussion, Conclusions and Future Directions

Fetal programming of adult disease has been extensively researched for the past few decades, linking poor nutrition, maternal stress, and exogenous administration of glucocorticoids to adult disease that may be heritable (29–33). GCs affect the physiological phenotype of offspring via gene expression directly and through DNA methylation (90,104). The impact of exposure of low dose ionizing radiation during late gestation of development is poorly understood.

Ionizing radiation exposures by accident, therapy, or through experimentation have highlighted critical periods of embryonic and fetal growth, whereby late gestation shows no or little mortality or birth defects at high doses (91). Early gestation exposures lead to appendage deformities, neurological disorders, and mortality (91). Typical prenatal exposures are through diagnostic procedures, airplane travel, or workplace accidents (90,91). Fear of toxicity to offspring is a reasonable response considering evidence during early gestation, but diagnostic and therapeutic ionizing radiation is often important, therefore flushing out the potential deleterious effects is important in understand the mechanisms of pathology and the foundation of prevention and amelioration.

This doctoral thesis sought to correlate neurological changes from different types of oxidative stress on rodent models. Defining a behavioural and genetic profile and understanding the adaptive responses to different prenatal stressors.

The first objective examined the effects of prenatal exposure to elevated levels of glucocorticoids by exposing pregnant Wistar-Kyoto rats to chronic dexamethasone (DEX) injections from gestational day 15 (equivalent of third trimester) until birth and behaviour was assessed in adult offspring. The elevated plus maze (EPM) was used to examine exploration and risk-taking

behaviour in contrast to the rodents' thigmotaxic predisposition and has been shown to be effective in illuminating anxiety traits with anxiolytic and anxiogenic drugs, where antidepressants and sedatives did not influence the behavioural effect (228). Within five minutes, rodents typically show avoidance of open arms, which are risky areas for the animal (124). The number of increased entries into the open arms of the prenatally DEX-exposed animals compared to naïve controls indicate an anxiolytic effect of DEX exposure, or, a reduction in inhibition. Similar to this effect, a study evaluating the possibility of hydrogen sulfide as an anxiolytic showed disinhibition in Wistar-Kyoto rats, with increased entries into the open arms (229). Another study used an ACE inhibitor on spontaneous hypertensive rats and also demonstrated that anxiety inhibition leads to increased open arm entries (230). Optogenetic manipulation of the prefrontal cortex-amygdala pathway changes the direction of behavioural responses in the EPM (231). Simulation of the pathway increases anxiety-like behaviour and reduced risk-taking behaviour and inhibition of the pathway increased behaviour. In consideration of the literature, this provides evidence that fetal programming of the prefrontal cortex leads to an adaptive phenotype with a loss of inhibition in anxiety-inducing situations.

In the Porsolt swim task (PST), animals were faced with an inescapable stressful situation which examines their coping strategies: passive or active based on their latency-to-float and total time spent immobile. Prenatally DEX-exposed adult offspring spent more time floating than the naïve controls, an energy-saving or passive coping strategy. Fetal programming models of maternal neglect on behaviour in the EPM and PST report behaviour similar to the findings here and also had dysregulated genes in the PFC (232). The combination of results from the first experiment defines an adaptive behavioural phenotype driven by modification of the PFC. The reduction of inhibition and the increase in a stress management strategy, along with neurotransmission and

methyltransferase gene overexpression in the PFC, indicates significant brain and behavioural changes occur in response to prenatal stress.

The second objective focused on the introduction of ionizing radiation as a maternal stressor. Doses of 50 mGy, 300 mGy and 1000 mGy were exposed to pregnant C57Bl/6J mice on gestational day 15 and behaviour was examined in adult offspring. These animals underwent behavioural testing that included open field for locomotor activity and generalized anxiety, the social anxiety task, and the Porsolt swim task. The C57Bl/6J mice, known for radiation resistance, only displayed changes in rearing activity during the social anxiety task. Across four brain regions, female and male 1000 mGy offspring had two-to-three dysregulated genes out of a panel of thirty that included DNA damage, synaptic activity, apoptosis, and ROS regulator genes. Given the few genes and single behavioural measure, it was concluded that C57Bl/6J animals were resistant to fetal programming of behavioural phenotypes to radiation doses 1000 mGy and under, and another mouse model was utilized in experiment three for further evaluation. The differentially expressed genes indicate, however, that there was some level of oxidative stress, but simply not enough for robust modification.

In a separate study, BALB/c mice were used to examine LDIR exposure with a similar experimental design for C57Bl/6J. No changes in dosing, housing, behavioural tasks, or genetic analysis were made and a striking difference in outcomes was observed. In these studies, the 300 mGy condition engaged in a passive coping in the PST, but the 1000 mGy animals employed an active strategy—continuing to swim throughout the task. Active strategies in this kind of situation is not necessarily a prudent choice, since energy wastage could be fatal. No differences in locomotive activity in the open field task were observed, but both the 50 mGy and 1000 mGy

animals were actively approaching the stranger mouse more often. The 300 mGy and 1000 mGy conditions also produced more rears in the SAT than the other groups.

In combination with the behavioural adaptation, there were sexually dimorphic gene changes in three regions of the brain. In the PFC, males had seven upregulated genes related to ROS, cell cycle, DNA damage, and GC receptors. In the male cerebral cortices, a ROS regulator was downregulated, as well as the GC receptor, DNA damage genes, and neuronal proliferation markers. An apoptosis factor gene was upregulated. In the cerebellum, two synaptic activity genes were dysregulated.

In contrast, the females showed only two dysregulated genes in the PFC: DNA methyltransferase and GC receptor genes. The cerebral cortex was modified in a similar pattern to the males, with ROS regulators, GC receptor, circadian rhythm, and neuronal proliferation markers differentially expressed. Female cerebella show microglia activation, synaptic activity, and a reduction in apoptosis genes.

The C57Bl/6J animals did not show as robust of a response compared to the BALB/c mice, presumably due to the noted radiation-resistance of this model (187). Indications of similar fetal programming of behaviour and genetic expression patterns did emerge; however, within the C57Bl/6J mice. The C57Bl/6J mice were extra active in the rearing conditions, whereas the BALB/c mice were extra active in two behavioural measures in the social anxiety task and hyperactive in the Porsolt swim task indicating disinhibition and active coping strategies. In comparison of genetic expression changes, the C57Bl/6J animals saw changes in ROS regulation, DNA damage, and synaptic activity pathways within the prefrontal cortex, as did the BALB/c's; however, the BALB/c mice had more pathways and genes modified. Within the cerebral cortex,

each animal model saw ROS regulation and synaptic activity changes. The BALB/c animals, again, had more changes in other regions and pathways. Overall, the C57Bl/6J animals produced a trend towards a similar pattern of behavioural and genetic phenotype modifications.

When comparing the BALB/c mice to the DEX treated offspring results, similarities in the behavioural phenotypes are clear with hyperactivity on anxiety tasks, but a different coping strategy on the Porsolt swim task. The Wistar-Kyoto rats had genetic expression changes for DNMT3b in the prefrontal cortex, as did the BALB/c animals. The rats also showed expression change in the neural transmission pathway, as did the BALB/c animals. Similarities in the behaviour and genetic expression patterns imply fetal programming through different forms of stress modulate similar regions, pathways, and behaviours.

Conclusions

Concluding points in support of fetal programming of adaptive mechanisms from each study are highlighted below.

Fetal programming of Wistar-Kyoto rats through synthetic glucocorticoid exposure leads to:

- Loss of inhibition in a novel situation (elevated plus maze)
- Passive coping strategies when faced with inescapable stress (Porsolt swim task)
- Gene expression changes associated with methylation, calcium signaling, neural transmission, neuronal growth and differentiation, and lysosomal homeostasis

Sublethal and low doses of ionizing radiation (< 1000 mGy) induces little change in C57Bl/6J mice:

- Hyperactivity in one measure, rearing, within the 1000 mGy condition (social anxiety task)
- Sexually dimorphic changes in brain regions, with more genetic expression changes in the female offspring
- Behavioural and genetic phenotype patterns similar to that of stress-induced phenotypes of BALB/c offspring
- Radiation resistant during fetal development

Sublethal dose of ionizing radiation (1000 mGy) induces adaptive behavioural phenotypes and genetic modification in BALB/c mice:

- Active coping strategy when faced with inescapable stress (Porsolt swim task)
- Loss of inhibition with hyperactivity in rearing and approach-to-stranger behaviours (social anxiety task)
- Significant sexually dimorphic gene changes in multiple brain regions associated with ROS regulation, cortisol regulation, DNA damage and tumor suppression, DNA methylation, synaptic activity, circadian rhythm, apoptosis, microglia, and cell cycling pathways
- Radiation sensitive during fetal development

The results from the experimental objectives support the hypothesis that fetal programming of behavioural and genetic phenotypes may come from alternate sources of stress and produce similar patterns of modification. The behavioural phenotypes are arguably adaptive depending up on the situation, similar to the thrifty phenotype, where when presented in the wrong environmental conditions, may be adverse to the offspring. Here, hyperactivity in either an exploration model or social model may place the animal in danger of predation or conspecific-aggression; however, it may also lead to escape or social engagement. Passive coping strategies, such as those seen in the

Porsolt swim task of the DEX-exposed offspring, can preserve energy which may be crucial to survival; however, an active coping strategy may prevent hypothermia and lead to escape. Unfortunately, the first mouse model did not have a significant behavioural phenotype change; however, previous work has shown the $LD_{50/30}$ for C57Bl/6J mouse model is 6300 ± 40 mGy whereas the BALB/c model $LD_{50/30}$ is 5000 ± 60 mGy which is a greater difference than the maximum dose utilized here for fetal programming and can explain fetal resistance to stress-induced modifications (187,216). And, finally, transcriptional changes leading to differentially expressed genes may occur through high concentrations of circulating GC. Direct expression changes occur when the GC receptors bind to the GRE promotor region, or indirectly through CpG methylation of this site and through the production of ROS (44,104,105,164). ROS, either produced via GC or ionizing radiation will cause oxidative stress and methylation changes (89,90). Through these mechanisms, sensitive neural regions are target sites for modification during development that will affect behavioural phenotypes.

Future Directions

In consideration of the results of the experimental objectives in this thesis and the literature, future analysis should include cognitive behavioural tasks that incorporate short and long-term memory, fear-conditioning, and a novel depression task that depicts a more comprehensive portrait of the behavioural phenotype of the offspring. Earlier and later ages should be considered as extra endpoints to see if there is a recovery or significant decline in behaviour. Dexamethasone exposures should be investigated on the BALB/c model to ensure a similar pattern of modifications. As well, methylation status of the genes in pathways with the most robust changes in expression should be conducted to elucidate mechanisms of action. Amelioration of phenotypes with environmental enrichment to investigate the plasticity of the phenotypes and potential

interventional methods should be explored, along with transgenerational effects and interventions. Each of these proposals would require significant time and expenditure; however, would provide insightful additions to the conclusions of this doctoral thesis.

References

1. Sun T, Sawyer RD. Art of War. Basic Books; 1994. 376 p.
2. Ruben A. Surviving your stupid, stupid decision to go to grad school. Broadway Books; 2010. 168 p.
3. Adams JJ. The mad scientist's guide to world domination : original short fiction for the modern evil genius. 363 p.
4. MacLeod H. Evil plans : having fun on the road to world domination. Portfolio/Penguin; 2011. 179 p.
5. Powell RA, Honey PL, Symbaluk DG. Introduction to learning and behavior. 608 p.
6. Hughes RN, Kaiser MJ, Mackney PA, Warburton K. Optimizing foraging behaviour through learning. J Fish Biol [Internet]. 1992 Dec [cited 2020 Apr 21];41(sb):77–91. Available from: http://doi.wiley.com/10.1111/j.1095-8649.1992.tb03870.x
7. Thornton A, Clutton-Brock T. Social learning and the development of individual and group behaviour in mammal societies. Philos Trans R Soc B Biol Sci [Internet]. 2011 Apr 12 [cited 2020 Apr 21];366(1567):978–87. Available from: https://royalsocietypublishing.org/doi/10.1098/rstb.2010.0312
8. Morand-Ferron J. Why learn? The adaptive value of associative learning in wild populations. Vol. 16, Current Opinion in Behavioral Sciences. Elsevier Ltd; 2017. p. 73–9.
9. Plath M, Liu K, Umutoni D, Gomes-Silva G, Wei J-F, Cyubahiro E, et al. Predator-induced changes of male and female mating preferences: innate and learned components. Curr Zool. 2019 Jun 1;65(3):305–16.
10. Black AH, Nadel L, O'Keefe J. Hippocampal function in avoidance learning and

punishment. Psychol Bull. 1977 Nov;84(6):1107–29.

11. Kumar RS, Narayanan SN, Kumar N, Nayak S. Exposure to Enriched Environment Restores Altered Passive Avoidance Learning and Ameliorates Hippocampal Injury in Male Albino Wistar Rats Subjected to Chronic Restraint Stress. Int J Appl basic Med Res. 2018;8(4):231–6.
12. Judah G, Gardner B, Kenward MG, DeStavola B, Aunger R. Exploratory study of the impact of perceived reward on habit formation. BMC Psychol. 2018 Dec 20;6(1):1–12.
13. Brust V, Schindler PM, Lewejohann L. Lifetime development of behavioural phenotype in the house mouse (Mus musculus) [Internet]. Vol. 12, Frontiers in Zoology. BioMed Central Ltd.; 2015 [cited 2020 Apr 14]. p. S17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26816516
14. Everitt BJ, Robbins TW. Neural systems of reinforcement for drug addiction: From actions to habits to compulsion. Vol. 8, Nature Neuroscience. Nature Publishing Group; 2005. p. 1481–9.
15. Miller RR, Polack CW. Sources of maladaptive behavior in 'normal' organisms. Vol. 154, Behavioural Processes. Elsevier B.V.; 2018. p. 4–12.
16. Ersche KD, Gillan CM, Simon Jones P, Williams GB, Ward LHE, Luijten M, et al. Carrots and sticks fail to change behavior in cocaine addiction. Science (80-). 2016 Jun 17;352(6292):1468–71.
17. Myers CE, Sheynin J, Balsdon T, Luzardo A, Beck KD, Hogarth L, et al. Probabilistic reward- and punishment-based learning in opioid addiction: Experimental and computational data. Behav Brain Res. 2016 Jan 1;296:240–8.
18. Hair P, Hampson SE. The role of impulsivity in predicting maladaptive behaviour among

female students. Pers Individ Dif. 2006 Apr 1;40(5):943–52.

19. Loxton NJ, Nguyen D, Casey L, Dawe S. Reward drive, rash impulsivity and punishment sensitivity in problem gamblers. Pers Individ Dif. 2008 Jul 1;45(2):167–73.

20. Barch DM, Ceaser A. Cognition in schizophrenia: Core psychological and neural mechanisms. Vol. 16, Trends in Cognitive Sciences. 2012. p. 27–34.

21. Diehl MM, Lempert KM, Parr AC, Ballard I, Steele VR, Smith D V. Toward an integrative perspective on the neural mechanisms underlying persistent maladaptive behaviors. Vol. 48, European Journal of Neuroscience. Blackwell Publishing Ltd; 2018. p. 1870–83.

22. Deisseroth K. Circuit dynamics of adaptive and maladaptive behaviour. Vol. 505, Nature. Nature Publishing Group; 2014. p. 309–17.

23. DeFelipe J. Sesquicentenary of the birthday of Santiago Ramón y Cajal, the father of modern neuroscience. Vol. 25, Trends in neurosciences. Elsevier Current Trends; 2002. p. 481–4.

24. DeFelipe J. Brain plasticity and mental processes: Cajal again. Vol. 7, Nature Reviews Neuroscience. Nature Publishing Group; 2006. p. 811–7.

25. Phenotypic Plasticity: Functional and Conceptual Approaches - Google Books [Internet]. [cited 2020 Apr 14]. Available from: https://books.google.ca/books?hl=en&lr=&id=7A3AdwmEi-UC&oi=fnd&pg=PA1&dq=plasticity+biology&ots=5kH-wBb3JZ&sig=nUqObPEHWWZqbBPqwHAnQ7wFV0A#v=onepage&q=plasticity biology&f=false

26. Verreet T, Verslegers M, Quintens R, Baatout S, Benotmane MA. Current Evidence for

Developmental , Structural , and Functional Brain Defects following Prenatal Radiation Exposure. 2016;2016.

27. Zucchi FCR, Yao Y, Ward ID, Ilnytskyy Y, Olson DM, Benzies K, et al. Maternal Stress Induces Epigenetic Signatures of Psychiatric and Neurological Diseases in the Offspring. 2013;8(2).

28. Korosi A, Naninck EFG, Oomen CA, Schouten M, Krugers H, Fitzsimons C, et al. Early-life stress mediated modulation of adult neurogenesis and behavior. Behav Brain Res [Internet]. 2012;227(2):400–9. Available from: http://dx.doi.org/10.1016/j.bbr.2011.07.037

29. Godfrey KM, Barker DJ. Fetal programming and adult health. Public Health Nutr. 2001 Apr;4(2b):611–24.

30. Sallout B, Walker M. The fetal origin of adult diseases. In: Journal of Obstetrics and Gynaecology. 2003. p. 555–60.

31. Hales CN, Barker DJP. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Vol. 35, Diabetologia. Springer-Verlag; 1992. p. 595–601.

32. Vaag AA, Grunnet LG, Arora GP, Brøns C. The thrifty phenotype hypothesis revisited. Diabetologia. 2012 Aug;55(8):2085–8.

33. McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: Prediction, plasticity, and programming. Vol. 85, Physiological Reviews. American Physiological Society; 2005. p. 571–633.

34. Chivers EK, Wyrwoll CS. Maternal Malnutrition, Glucocorticoids, and Fetal Programming: A Role for Placental 11β-Hydroxysteroid Dehydrogenase Type 2. In: Diet, Nutrition, and Fetal Programming. Springer International Publishing; 2017. p. 543–55.

35. Lindsay KL, Buss C, Wadhwa PD, Entringer S. The Interplay Between Nutrition and Stress in Pregnancy: Implications for Fetal Programming of Brain Development. Vol. 85, Biological Psychiatry. Elsevier USA; 2019. p. 135–49.

36. Lesage J, Blondeau B, Grino M, Bréant B, Dupouy JP. Maternal Undernutrition during Late Gestation Induces Fetal Overexposure to Glucocorticoids and Intrauterine Growth Retardation, and Disturbs the Hypothalamo-Pituitary Adrenal Axis in the Newborn Rat [1]. Endocrinology [Internet]. 2001 May 1 [cited 2020 May 15];142(5):1692–702. Available from: https://academic.oup.com/endo/article-lookup/doi/10.1210/endo.142.5.8139

37. Correia-Branco A, Keating E, Martel F. MaternaluUndernutrition and fetal developmental programming of obesity. Reprod Sci [Internet]. 2015 Feb 6 [cited 2020 May 15];22(2):138–45. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25001018

38. Seckl JR. Glucocorticoid programming of the fetus; adult phenotypes and molecular mechanisms. In: Molecular and Cellular Endocrinology. Elsevier; 2001. p. 61–71.

39. SECKL JR, MEANEY MJ. Glucocorticoid Programming. Ann N Y Acad Sci [Internet]. 2004 Dec 1 [cited 2020 Apr 14];1032(1):63–84. Available from: http://doi.wiley.com/10.1196/annals.1314.006

40. Matthews SG, Owen D, Kalabis G, Banjanin S, Setiawan EB, Dunn EA, et al. Fetal glucocorticoid exposure and hypothalamo-pituitary-adrenal (HPA) function after birth. In: Endocrine Research. Taylor & Francis; 2004. p. 827–36.

41. McGowan PO, Matthews SG. Prenatal stress, glucocorticoids, and developmental programming of the stress response. Vol. 159, Endocrinology. Oxford University Press; 2018. p. 69–82.

42. Kapoor A, Petropoulos S, Matthews SG. Fetal programming of hypothalamic-pituitary-

adrenal (HPA) axis function and behavior by synthetic glucocorticoids. Vol. 57, Brain Research Reviews. Elsevier; 2008. p. 586–95.

43. Huang Y, Cai G quan, Peng JP, Shen C. Glucocorticoids induce apoptosis and matrix metalloproteinase-13 expression in chondrocytes through the NOX4/ROS/p38 MAPK pathway. J Steroid Biochem Mol Biol. 2018 Jul 1;181:52–62.

44. Flaherty RL, Owen M, Fagan-Murphy A, Intabli H, Healy D, Patel A, et al. Glucocorticoids induce production of reactive oxygen species/reactive nitrogen species and DNA damage through an iNOS mediated pathway in breast cancer. Breast Cancer Res [Internet]. 2017 Mar 24 [cited 2020 Apr 14];19(1):35. Available from: http://breast-cancer-research.biomedcentral.com/articles/10.1186/s13058-017-0823-8

45. Carlén M. What constitutes the prefrontal cortex? Vol. 358, Science. American Association for the Advancement of Science; 2017. p. 478–82.

46. Miller EK, Cohen JD. An Integrative Theory of Prefrontal Cortex Function. Annu Rev Neurosci. 2001 Mar;24(1):167–202.

47. Gee DG, Humphreys KL, Flannery J, Goff B, Telzer EH, Shapiro M, et al. A developmental shift from positive to negative connectivity in human amygdala-prefrontal circuitry. J Neurosci. 2013 Mar 6;33(10):4584–93.

48. McEwen BS, Nasca C, Gray JD. Stress Effects on Neuronal Structure: Hippocampus, Amygdala and Prefrontal Cortex. Neuropsychopharmacology [Internet]. 2016 Jan 16 [cited 2018 Feb 23];41(1):3–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26076834

49. Klavir O, Prigge M, Sarel A, Paz R, Yizhar O. Manipulating fear associations via optogenetic modulation of amygdala inputs to prefrontal cortex. Nat Neurosci. 2017 Jun

1;20(6):836–44.

50. Wei J, Zhong P, Qin L, Tan T, Yan Z. Chemicogenetic Restoration of the Prefrontal Cortex to Amygdala Pathway Ameliorates Stress-Induced Deficits. Cereb Cortex [Internet]. 2018 [cited 2020 Apr 14];28:1980–90. Available from: https://academic.oup.com/cercor/article-abstract/28/6/1980/3813666

51. Eichenbaum H. Prefrontal-hippocampal interactions in episodic memory. Vol. 18, Nature Reviews Neuroscience. Nature Publishing Group; 2017. p. 547–58.

52. Faa G, Manchia M, Pintus R, Gerosa C, Marcialis MA, Fanos V. Fetal programming of neuropsychiatric disorders. Birth Defects Res Part C Embryo Today Rev [Internet]. 2016 Sep 1 [cited 2020 Apr 14];108(3):207–23. Available from: http://doi.wiley.com/10.1002/bdrc.21139

53. Goyal N, Siddiqui S, Chatterjee U, Kumar D, Siddiqui A. Neuropsychology of prefrontal cortex. Indian J Psychiatry. 2008;50(3):202.

54. Laubach M, Amarante LM, Swanson K, White SR. Cognition and Behavior What, If Anything, Is Rodent Prefrontal Cortex? Review [Internet]. [cited 2020 May 6];5(5):315–33. Available from: https://doi.org/10.1523/ENEURO.0315-18.2018

55. Muir JL, Everitt BJ, Robbins TW. The Cerebral Cortex of the Rat and Visual Attentional Function: Dissociable Effects of Mediofrontal, Cingulate, Anterior Dorsolateral, and Parietal Cortex Lesions on a Five-Choice Serial Reaction Time Task [Internet]. 1992 [cited 2020 May 6]. Available from: https://academic.oup.com/cercor/article-abstract/6/3/470/385516

56. Narducci R, Baroncelli L, Sansevero G, Begenisic T, Prontera C, Sale A, et al. Early impoverished environment delays the maturation of cerebral cortex. Sci Rep. 2018 Dec

1;8(1):1–15.

57. Burgos H, Hernández A, Constandil L, Ríos M, Flores O, Puentes G, et al. Early postnatal environmental enrichment restores neurochemical and functional plasticities of the cerebral cortex and improves learning performance in hidden-prenatally-malnourished young-adult rats. Behav Brain Res. 2019 May 2;363:182–90.

58. Andrews MH, Kostaki A, Setiawan E, McCabe L, Owen D, Banjanin S, et al. Developmental regulation of the 5-HT7 serotonin receptor and transcription factor NGFI-A in the fetal guinea-pig limbic system: Influence of GCs. J Physiol. 2004 Mar 16;555(3):659–70.

59. Moisiadis VG, Matthews SG. Glucocorticoids and fetal programming part 2: Mechanisms. Vol. 10, Nature Reviews Endocrinology. Nature Publishing Group; 2014. p. 403–11.

60. Kempadoo KA, Mosharov E V., Choi SJ, Sulzer D, Kandel ER. Dopamine release from the locus coeruleus to the dorsal hippocampus promotes spatial learning and memory. Proc Natl Acad Sci U S A. 2016 Dec 20;113(51):14835–40.

61. Schmidt SD, Furini CRG, Zinn CG, Cavalcante LE, Ferreira FF, Behling JAK, et al. Modulation of the consolidation and reconsolidation of fear memory by three different serotonin receptors in hippocampus. Neurobiol Learn Mem. 2017 Jul 1;142:48–54.

62. Anacker C, Hen R. Adult hippocampal neurogenesis and cognitive flexibility-linking memory and mood. Vol. 18, Nature Reviews Neuroscience. Nature Publishing Group; 2017. p. 335–46.

63. Yavas E, Gonzalez S, Fanselow MS. Interactions between the hippocampus, prefrontal cortex, and amygdala support complex learning and memory [version 1; peer review: 3 approved]. Vol. 8, F1000Research. F1000 Research Ltd; 2019.

64. Liao L, Yao X, Huang J, Bai S. Prenatal Stress Up-Regulated Hippocampal Glucocorticoid Receptor Expression in Female Adult Rat Offspring Estrés Prenatal Expresión del Receptor de Glucocorticoides del Hipocampo Regulado por Aumento en Crías de Ratas Hembras Adultas. Vol. 38, Int. J. Morphol. 2020.

65. Constantinof A, Moisiadis VG, Kostaki A, Szyf M, Matthews SG. Antenatal Glucocorticoid Exposure Results in Sex-Specific and Transgenerational Changes in Prefrontal Cortex Gene Transcription that Relate to Behavioural Outcomes. Sci Rep [Internet]. [cited 2020 May 5]; Available from: www.nature.com/scientificreports/

66. Mychasiuk R, Gibb R, Kolb B. Prenatal Stress Produces Sexually Dimorphic and Regionally Specific Changes in Gene Expression in Hippocampus and Frontal Cortex of Developing Rat Offspring. Dev Neurosci [Internet]. 2011 Mar [cited 2020 Mar 29];33(6):531–8. Available from: https://www.karger.com/Article/FullText/335524

67. Zhang TY, Hellstrom IC, Bagot RC, Wen X, Diorio J, Meaney MJ. Maternal care and DNA methylation of a glutamic acid decarboxylase 1 promoter in rat hippocampus. J Neurosci. 2010 Sep 29;30(39):13130–7.

68. Bagot R, Tse Y, Nguyen H, … AW-B, 2012 undefined. Maternal care influences hippocampal N-methyl-D-aspartate receptor function and dynamic regulation by corticosterone in adulthood. Elsevier [Internet]. [cited 2020 May 7]; Available from: https://www.sciencedirect.com/science/article/pii/S0006322312002648?casa_token=SZ7GIRdc9Y8AAAAA:FJkeIejB4lrQRODjiO5tPSSaev0_yld57RlRTzWPpQ053AjfnbnSnUQkHloJ4QeUg_1pEUNWnuM

69. Bagot R, Zhang T, … XW-P of the, 2012 undefined. Variations in postnatal maternal care and the epigenetic regulation of metabotropic glutamate receptor 1 expression and

hippocampal function in the rat. Natl Acad Sci [Internet]. [cited 2020 May 7]; Available from: https://www.pnas.org/content/109/Supplement_2/17200.short

70. Champagne DL, Bagot RC, van Hasselt F, Ramakers G, Meaney MJ, de Kloet ER, et al. Maternal Care and Hippocampal Plasticity: Evidence for Experience-Dependent Structural Plasticity, Altered Synaptic Functioning, and Differential Responsiveness to Glucocorticoids and Stress. J Neurosci [Internet]. 2008 Jun 4 [cited 2020 May 7];28(23):6037–45. Available from: http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.0526-08.2008

71. Nguyen H, Bagot R, Diorio J, … TW-, 2015 undefined. Maternal care differentially affects neuronal excitability and synaptic plasticity in the dorsal and ventral hippocampus. nature.com [Internet]. [cited 2020 May 7]; Available from: https://www.nature.com/articles/npp201519

72. van Hasselt FN, Cornelisse S, Yuan Zhang T, Meaney MJ, Velzing EH, Krugers HJ, et al. Adult hippocampal glucocorticoid receptor expression and dentate synaptic plasticity correlate with maternal care received by individuals early in life. Hippocampus. 2012 Feb;22(2):255–66.

73. Buckner RL. The cerebellum and cognitive function: 25 years of insight from anatomy and neuroimaging. Vol. 80, Neuron. Cell Press; 2013. p. 807–15.

74. Barua S, Kuizon S, Chadman K, Brown W, Junaid M. Microarray Analysis Reveals Higher Gestational Folic Acid Alters Expression of Genes in the Cerebellum of Mice Offspring—A Pilot Study. Brain Sci [Internet]. 2015 Jan 26 [cited 2020 Apr 14];5(1):14–31. Available from: http://www.mdpi.com/2076-3425/5/1/14

75. Pourié G, Martin N, Bossenmeyer-Pourié C, Akchiche N, Guéant-Rodriguez RM,

Geoffroy A, et al. Folate- and vitamin B $_{12}$ –deficient diet during gestation and lactation alters cerebellar synapsin expression *via* impaired influence of estrogen nuclear receptor α. FASEB J [Internet]. 2015 Sep 1 [cited 2020 Apr 14];29(9):3713–25. Available from: http://www.fasebj.org/doi/10.1096/fj.14-264267

76. Buss C, Davis EP, Muftuler LT, Head K, Sandman CA. High pregnancy anxiety during mid-gestation is associated with decreased gray matter density in 6–9-year-old children. Psychoneuroendocrinology [Internet]. 2010 Jan 1 [cited 2020 May 7];35(1):141–53. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0306453009002388

77. Sandman CA, Glynn LM, Davis EP. Is there a viability–vulnerability tradeoff? Sex differences in fetal programming. J Psychosom Res [Internet]. 2013 Oct 1 [cited 2020 May 7];75(4):327–35. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0022399913002754

78. Pascual R, Valencia M, Larrea S, Bustaman C. Single course of antenatal betamethasone produces delayed changes in morphology and calbindin-D28k expression in a rat's cerebellar Purkinje cells. Acta Neurobiol Exp (Wars). 2014;74:415–23.

79. McCormick CM, Mathews IZ. HPA function in adolescence: Role of sex hormones in its regulation and the enduring consequences of exposure to stressors. Vol. 86, Pharmacology Biochemistry and Behavior. Elsevier; 2007. p. 220–33.

80. Watson S, Mackin P. HPA axis function in mood disorders. Psychiatry. 2006 May 1;5(5):166–70.

81. Rentesi G, Antoniou K, Marselos M, Fotopoulos A, Alboycharali J, Konstandi M. Long-term consequences of early maternal deprivation in serotonergic activity and HPA function in adult rat. Neurosci Lett. 2010 Aug 9;480(1):7–11.

82. Vries A de, Holmes M, … AH-TJ of, 2007 undefined. Prenatal dexamethasone exposure induces changes in nonhuman primate offspring cardiometabolic and hypothalamic-pituitary-adrenal axis function. Am Soc Clin Investig [Internet]. [cited 2020 May 7]; Available from: https://www.jci.org/articles/view/30982

83. Bratt AM, Kelley SP, Knowles JP, Barrett J, Davis K, Davis M, et al. Long term modulation of the HPA axis by the hippocampus: Behavioral, biochemical and immunological endpoints in rats exposed to chronic mild stress. Psychoneuroendocrinology. 2001 Feb 1;26(2):121–45.

84. Kern S, Oakes TR, Stone CK, McAuliff EM, Kirschbaum C, Davidson RJ. Glucose metabolic changes in the prefrontal cortex are associated with HPA axis response to a psychosocial stressor. Psychoneuroendocrinology. 2008 May 1;33(4):517–29.

85. Swaab DF, Fliers E, Hoogendijk WJG, Veltman DJ, Zhou JN. Interaction of prefrontal cortical and hypothalamic systems in the pathogenesis of depression. In: Progress in Brain Research. Elsevier; 2000. p. 369–96.

86. Schutter DJLG. The cerebello-hypothalamic-pituitary-adrenal axis dysregulation hypothesis in depressive disorder. Med Hypotheses. 2012 Dec 1;79(6):779–83.

87. Stress Science: Neuroendocrinology - Google Books [Internet]. [cited 2020 Apr 14]. Available from: https://books.google.ca/books?hl=en&lr=&id=HJwqWQhQELMC&oi=fnd&pg=PA3&dq=stress+definition&ots=ooqPf-2b_-&sig=R_3UlYwxjQM-33ayUW_CmLRogJU#v=onepage&q=stress definition&f=false

88. Mora F, Segovia G, Del Arco A, De Blas M, Garrido P. Stress, neurotransmitters, corticosterone and body-brain integration. Brain Res. 2012 Oct 2;1476:71–85.

89. Hall EJ, Giaccia AJ. Radiobiology for the radiologist. 546 p.

90. Tharmalingam S, Sreetharan S, Kulesza A V., Boreham DR, Tai TC. Low-Dose Ionizing Radiation Exposure, Oxidative Stress and Epigenetic Programing of Health and Disease. Radiat Res. 2017 Oct;188(4.2):525–38.

91. Sreetharan S, Thome C, Tharmalingam S, Jones DE, Kulesza A V., Khaper N, et al. Ionizing Radiation Exposure During Pregnancy: Effects on Postnatal Development and Life. Radiat Res [Internet]. 2017 Jun 18 [cited 2019 Sep 27];187(6):647–58. Available from: http://www.bioone.org/doi/10.1667/RR14657.1

92. Verreet T, Quintens R, Dam D Van, Verslegers M, Tanori M, Casciati A, et al. A multidisciplinary approach unravels early and persistent effects of X-ray exposure at the onset of prenatal neurogenesis. 2015;1–21.

93. Kempf SJ, Toerne C Von, Hauck SM, Atkinson MJ, Benotmane MA, Tapio S. Long-term consequences of in utero irradiated mice indicate proteomic changes in synaptic plasticity related signalling. Proteome Sci [Internet]. 2015;10:1–7. Available from: http://dx.doi.org/10.1186/s12953-015-0083-4

94. Baskar R, Devi PU. Influence of gestational age to low-level gamma irradiation on postnatal behavior in mice. 2000;22:593–602.

95. Effects of Radiation on the Embryo and Fetus | Radiology Key [Internet]. [cited 2020 Jul 28]. Available from: https://radiologykey.com/effects-of-radiation-on-the-embryo-and-fetus/

96. Tai TC, Tai IT. Impact of Fetal Glucocorticoid Exposure on Gene Expression Profiles in the Adult Adrenal Gland | The FASEB Journal [Internet]. The FASEB Journal. 2006 [cited 2020 Mar 26]. Available from:

https://www.fasebj.org/doi/abs/10.1096/fasebj.20.5.lb38

97. Marciniak A, Patro-Małysza J, Kimber-Trojnar Ż, Marciniak B, Oleszczuk J, Leszczyńska-Gorzelak B. Fetal programming of the metabolic syndrome. Vol. 56, Taiwanese Journal of Obstetrics and Gynecology. Elsevier Ltd; 2017. p. 133–8.

98. Lalonde C. Hypertension. In: Encyclopedia of Evolutionary Psychological Science [Internet]. Cham: Springer International Publishing; 2018 [cited 2020 Mar 26]. p. 1–3. Available from: http://link.springer.com/10.1007/978-3-319-16999-6_731-1

99. Del Giudice M. Fetal programming by maternal stress: Insights from a conflict perspective. Vol. 37, Psychoneuroendocrinology. Pergamon; 2012. p. 1614–29.

100. Khurana S, Grandbois J, Tharmalingam S, Murray A, Graff K, Nguyen P, et al. Fetal programming of adrenal PNMT and hypertension by glucocorticoids in WKY rats is dose and sex-dependent. Bader M, editor. PLoS One [Internet]. 2019 Sep 4 [cited 2020 Jan 20];14(9):e0221719. Available from: http://dx.plos.org/10.1371/journal.pone.0221719

101. Fajersztajn L, Veras MM. Hypoxia: From Placental Development to Fetal Programming. Birth Defects Res [Internet]. 2017 Oct 16 [cited 2020 Mar 26];109(17):1377–85. Available from: http://doi.wiley.com/10.1002/bdr2.1142

102. Thompson LP, Al-Hasan Y. Impact of oxidative stress in fetal programming. J Pregnancy. 2012;2012.

103. Wyrwoll C, Keith M, Noble J, Stevenson PL, Bombail V, Crombie S, et al. ScienceDirect Fetal brain 11□-hydroxysteroid dehydrogenase type 2 selectively determines programming of adult depressive-like behaviors and cognitive function, but not anxiety behaviors in male mice. Psychoneuroendocrinology [Internet]. 2015 [cited 2018 May 4];59:59–70. Available from: http://dx.doi.org/10.1016/j.psyneuen.2015.05.003

104. Provençal N, Arloth J, Cattaneo A, Anacker C, Cattane N, Wiechmann T, et al. Glucocorticoid exposure during hippocampal neurogenesis primes future stress response by inducing changes in DNA methylation. Proc Natl Acad Sci. 2019 Aug 9;201820842.

105. Thomassin H, Flavin M, Espinas M-L, Grange T. Histone methylation: A dynamic mark in health, disease and inheritance. EMBO J. 2001;20(8):1974–83.

106. Clayton DF, Anreiter I, Aristizabal M, Frankland PW, Binder EB, Citri A. The role of the genome in experience-dependent plasticity: Extending the analogy of the genomic action potential. Proc Natl Acad Sci. 2019 May 24;201820837.

107. Yang X, Ewald ER, Huo Y, Tamashiro KL, Salvatori R, Sawa A, et al. Glucocorticoid-induced loss of DNA methylation in non-neuronal cells and potential involvement of DNMT1 in epigenetic regulation of Fkbp5. Biochem Biophys Res Commun. 2012 Apr 13;420(3):570–5.

108. Zannas AS, Chrousos GP. Epigenetic programming by stress and glucocorticoids along the human lifespan. Vol. 22, Molecular Psychiatry. Nature Publishing Group; 2017. p. 640–6.

109. Moore LD, Le T, Fan G. DNA Methylation and Its Basic Function. Neuropsychopharmacology [Internet]. 2012 [cited 2020 Mar 27];38. Available from: www.neuropsychopharmacology.org

110. Werner CT, Gancarz AM, Dietz DM. Mechanisms Regulating Compulsive Drug Behaviors. In: Neural Mechanisms of Addiction. Elsevier; 2019. p. 137–55.

111. Grgić G, Fatusić Z, Bogdanović G. Stimulation of fetal lung maturation with dexamethasone in unexpected premature labor. Med Arh. 2003 Jan 1;57(5–6):291–4.

112. Seckl JR. Glucocorticoid "Programming" and PTSD Risk. Ann N Y Acad Sci [Internet].

2006 Jul 1 [cited 2020 May 3];1071(1):351–78. Available from: http://doi.wiley.com/10.1196/annals.1364.027

113. Moisiadis VG, Matthews SG. Glucocorticoids and fetal programming part 1: Outcomes. Vol. 10, Nature Reviews Endocrinology. Nature Publishing Group; 2014. p. 391–402.

114. Weaver I, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, et al. Epigenetic programming by maternal behavior. nature.com [Internet]. 2004 [cited 2020 May 4]; Available from: https://www.nature.com/articles/nn1276

115. Turner JD, Alt SR, Cao L, Vernocchi S, Trifonova S, Battello N, et al. Transcriptional control of the glucocorticoid receptor: CpG islands, epigenetics and more. Biochem Pharmacol [Internet]. 2010 [cited 2020 May 4];(12):1860. Available from: https://hal.archives-ouvertes.fr/hal-00637161

116. Cottrell EC, Seckl JR. Prenatal stress, glucocorticoids and the programming of adult disease. Vol. 3, Frontiers in Behavioral Neuroscience. Frontiers Media SA; 2009.

117. Kapoor A, Dunn E, Kostaki A, Andrews MH, Matthews SG. Fetal programming of hypothalamo-pituitary-adrenal function: prenatal stress and glucocorticoids. J Physiol [Internet]. 2006 Apr 1 [cited 2020 Mar 27];572(1):31–44. Available from: http://doi.wiley.com/10.1113/jphysiol.2006.105254

118. George MS, Ketter TA, Post RM. Prefrontal cortex dysfunction in clinical depression. Depression [Internet]. 1994 Jan 1 [cited 2020 Mar 28];2(2):59–72. Available from: http://doi.wiley.com/10.1002/depr.3050020202

119. Barch DM, Sheline YI, Csernansky JG, Snyder AZ. Working memory and prefrontal cortex dysfunction: Specificity to schizophrenia compared with major depression. Biol Psychiatry. 2003 Mar 1;53(5):376–84.

120. Lemogne C, Delaveau P, Freton M, Guionnet S, Fossati P. Medial prefrontal cortex and the self in major depression. Vol. 136, Journal of Affective Disorders. Elsevier; 2012. p. e1–11.

121. Drevets WC. Functional anatomical abnormalities in limbic and prefrontal cortical structures in major depression. In: Progress in Brain Research. Elsevier; 2000. p. 413–31.

122. George MS, Nahas Z, Molloy M, Speer AM, Oliver NC, Li XB, et al. A controlled trial of daily left prefrontal cortex TMS for treating depression. Biol Psychiatry. 2000 Nov 15;48(10):962–70.

123. Arnsten AFT. Stress signalling pathways that impair prefrontal cortex structure and function. Vol. 10, Nature Reviews Neuroscience. Nature Publishing Group; 2009. p. 410–22.

124. Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat Protoc. 2007 Mar;2(2):322–8.

125. Can A, Dao DT, Arad M, Terrillion CE, Piantadosi SC, Gould TD. The mouse forced swim test. J Vis Exp. 2011;(58).

126. Paxinos G, Watson C, Diego S, Boston L, York N. The Rat Brain in Stereotaxic Coordinates Academic Press [Internet]. 1997 [cited 2020 Mar 29]. Available from: http://www.apnet.com

127. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 2001;25(4):402–8.

128. Nguyen P, Khurana S, Peltsch H, Grandbois J, Eibl J, Crispo J, et al. Prenatal glucocorticoid exposure programs adrenal PNMT expression and adult hypertension. J Endocrinol. 2015;227(2):117–27.

129. Grandbois J, Khurana S, Graff K, Nguyen P, Meltz L, Tai TC. Phenylethanolamine N-methyltransferase gene expression in adrenergic neurons of spontaneously hypertensive rats. Neurosci Lett. 2016 Dec 2;635:103–10.

130. Ridderinkhof KR, Van Den Wildenberg WPM, Segalowitz SJ, Carter CS. Neurocognitive mechanisms of cognitive control: The role of prefrontal cortex in action selection, response inhibition, performance monitoring, and reward-based learning. Brain Cogn. 2004 Nov 1;56(2 SPEC. ISS.):129–40.

131. Levy BJ, Wagner AD. Cognitive control and right ventrolateral prefrontal cortex: Reflexive reorienting, motor inhibition, and action updating. Vol. 1224, Annals of the New York Academy of Sciences. Blackwell Publishing Inc.; 2011. p. 40–62.

132. Kraeuter AK, Guest PC, Sarnyai Z. The Forced Swim Test for Depression-Like Behavior in Rodents. In: Methods in Molecular Biology. Humana Press Inc.; 2019. p. 75–80.

133. Molendijk ML, de Kloet ER. Coping with the forced swim stressor: Current state-of-the-art. Vol. 364, Behavioural Brain Research. Elsevier B.V.; 2019. p. 1–10.

134. Treit D, Fundytus M. Thigmotaxis as a test for anxiolytic activity in rats. Pharmacol Biochem Behav. 1988 Dec 1;31(4):959–62.

135. Xu Y, Sheng H, Wu T, Bao Q, Zheng Y, Zhang Y, et al. CRH/CRHR1 mediates prenatal synthetic glucocorticoid programming of depression-like behavior across 2 generations. FASEB J [Internet]. 2018 Aug 15 [cited 2020 May 5];32(8):4258–69. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1096/fj.201700948RR

136. Hiroi R, Carbone DL, Zuloaga DG, Bimonte-Nelson HA, Handa RJ. Sex-dependent programming effects of prenatal glucocorticoid treatment on the developing serotonin system and stress-related behaviors in adulthood. Neuroscience. 2016 Apr 21;320:43–56.

137. Zeng Y, Brydges NM, Wood ER, Drake AJ, Hall J. Prenatal glucocorticoid exposure in rats: programming effects on stress reactivity and cognition in adult offspring. Stress [Internet]. 2015 May 4 [cited 2020 May 5];18(3):353–61. Available from: http://www.tandfonline.com/doi/full/10.3109/10253890.2015.1055725

138. Cartier J, Zeng Y, Drake AJ. Glucocorticoids and the prenatal programming of neurodevelopmental disorders. Vol. 7, Current Opinion in Behavioral Sciences. Elsevier Ltd; 2016. p. 1–7.

139. Bayraktar G, Kreutz MR. Neuronal DNA Methyltransferases: Epigenetic Mediators between Synaptic Activity and Gene Expression? Vol. 24, Neuroscientist. SAGE Publications Inc.; 2018. p. 171–85.

140. Catania EH, Pimenta A, Levitt P. Genetic deletion of Lsamp causes exaggerated behavioral activation in novel environments. Behav Brain Res. 2008 Apr 9;188(2):380–90.

141. Del Prete D, Checler F, Chami M. Ryanodine receptors: Physiological function and deregulation in Alzheimer disease. Vol. 9, Molecular Neurodegeneration. BioMed Central Ltd.; 2014. p. 21.

142. Peng S, Li W, Lv L, Zhang Z, medicine XZ-D, 2018 undefined. BDNF as a biomarker in diagnosis and evaluation of treatment for schizophrenia and depression. discoverymedicine.com [Internet]. [cited 2020 Mar 31]; Available from: http://www.discoverymedicine.com/Shiyong-Peng/2018/10/bdnf-as-biomarker-in-diagnosis-and-treatment-for-schizophrenia-and-depression/

143. Feigen M. Should cancer survivors fear radiation-induced sarcomas? Vol. 1, Sarcoma. Hindawi Limited; 1997. p. 5–15.

144. Groen RS, Bae JY, Lim KJ. Fear of the unknown: Ionizing radiation exposure during pregnancy. Vol. 206, American Journal of Obstetrics and Gynecology. 2012. p. 456–62.

145. Normile D. Epidemic of fear. Vol. 351, Science. American Association for the Advancement of Science; 2016. p. 1022–3.

146. Shakerian R, Thomson BN, Judson R, Skandarajah AR. Radiation fear. J Trauma Acute Care Surg [Internet]. 2015 Jan [cited 2019 Dec 12];78(1):88–93. Available from: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=01586154-201501000-00011

147. Giarola RS, De Almeida GHO, Hungaro TH, De Paula IR, Godinho AF, Acencio ML, et al. Low-dose ionizing radiation exposure during pregnancy induces behavioral impairment and lower weight gain in adult rats. 2019 May 24 [cited 2019 Dec 12]; Available from: http://arxiv.org/abs/1905.10445

148. Tsou M, Liu J, Hammitt JK, Lu C, Kao SZ. Effect of Prenatal Exposure to Radiation on Birth Outcomes: Exploiting a Natural Experiment in Taiwan. Japanese Econ Rev [Internet]. 2019 May 28 [cited 2019 Dec 12];jere.12228. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/jere.12228

149. Bock J, Wainstock T, Braun K, Segal M. Stress In Utero: Prenatal Programming of Brain Plasticity and Cognition. Vol. 78, Biological Psychiatry. Elsevier USA; 2015. p. 315–26.

150. Ortinau C, Neil J. The neuroanatomy of prematurity: Normal brain development and the impact of preterm birth. Clin Anat [Internet]. 2015 Mar [cited 2019 Dec 14];28(2):168–83. Available from: http://doi.wiley.com/10.1002/ca.22430

151. Needleman S, Powell M. Radiation hazards in pregnancy and methods of prevention. Best Pract Res Clin Obstet Gynaecol. 2016 May 1;33:108–16.

152. Meijer HJM, Essers M, Struikmans H, Venselaar JLM, Poortmans P. Contributions and risks of radiation therapy in managing cancer during pregnancy. In: Managing Cancer During Pregnancy. Springer International Publishing; 2016. p. 41–54.

153. Godfrey KM, Barker DJ. Fetal programming and adult health. Public Health Nutr [Internet]. 2001;4(2B):611–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11683554

154. Lau C, Rogers JM, Desai M, Ross MG. Fetal Programming of Adult Disease. Obstet Gynecol [Internet]. 2011 Apr [cited 2019 Dec 14];117(4):978–85. Available from: http://insights.ovid.com/crossref?an=00006250-201104000-00030

155. Barker DJP. In utero programming of chronic disease. Vol. 95, Clinical Science. Portland Press Ltd; 1998. p. 115–28.

156. Khurana S, Grandbois J, Tharmalingam S, Murray A, Graff K, Nguyen P, et al. Fetal programming of adrenal PNMT and hypertension by glucocorticoids in WKY rats is dose and sex-dependent. Bader M, editor. PLoS One [Internet]. 2019 Sep 4 [cited 2019 Dec 14];14(9):e0221719. Available from: http://dx.plos.org/10.1371/journal.pone.0221719

157. Pascual R, Valencia M, Bustamante C. Antenatal betamethasone produces protracted changes in anxiety-like behaviors and in the expression of microtubule-associated protein 2, brain-derived neurotrophic factor and the tyrosine kinase B receptor in the rat cerebellar cortex. Int J Dev Neurosci. 2015 Jun 1;43:78–85.

158. Giarola RS, De Almeida GHO, Hungaro TH, De Paula IR, Godinho AF, Acencio ML, et al. Low-dose ionizing radiation exposure during pregnancy induces behavioral impairment and lower weight gain in adult rats. 2019 May 24 [cited 2020 Apr 29];1–15. Available from: http://arxiv.org/abs/1905.10445

159. Gupta S, Kim SY, Artis S, Molfese DL, Schumacher A, Sweatt JD, et al. Histone methylation regulates memory formation. J Neurosci. 2010 Mar 10;30(10):3589–99.

160. Greer EL, Shi Y. Histone methylation: A dynamic mark in health, disease and inheritance. Vol. 13, Nature Reviews Genetics. 2012. p. 343–57.

161. Hales CN, Barker DJP. The thrifty phenotype hypothesis. Br Med Bull [Internet]. 2001 Nov 1 [cited 2019 Dec 16];60(1):5–20. Available from: https://academic.oup.com/bmb/article/60/1/5/322752

162. Wells JCK. The thrifty phenotype as an adaptive maternal effect. Biol Rev. 2007;82(143):143–72.

163. Hales C, Barker D. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Int J Epidemiol [Internet]. 2013 Oct 1 [cited 2020 Jan 5];42(5):1215–22. Available from: https://academic.oup.com/ije/article-lookup/doi/10.1093/ije/dyt133

164. Raciti M, Ong J, Weis L, Edoff K, Battagli C, Falk A, et al. Glucocorticoids alter neuronal differentiation of human neuroepithelial-like cells by inducing long-lasting changes in the reactive oxygen species balance. Neuropharmacology. 2016 Aug 1;107:422–31.

165. Ganapathi R, Manda K. Later Life Changes in Hippocampal Neurogenesis and Behavioral Functions After Low-Dose Prenatal Irradiation at Early Organogenesis Stage. Int J Radiat Oncol Biol Phys. 2017 May 1;98(1):63–74.

166. Norton S, Donoso JA. Forebrain damage following prenatal exposure to low-dose X-irradiation. Exp Neurol. 1985 Feb 1;87(2):185–97.

167. Darmanto W, Inouye M, Murata Y. Proseding Seminar Naional Unirow Tuban. 2017.

168. Sreetharan S, Stoa L, Cybulski ME, Jones DE, Lee AH, Kulesza A V., et al.

Cardiovascular and growth outcomes of C57Bl/6J mice offspring exposed to maternal stress and ionizing radiation during pregnancy. Int J Radiat Biol [Internet]. 2019 Aug 3 [cited 2020 Jan 6];95(8):1085–93. Available from: https://www.tandfonline.com/doi/full/10.1080/09553002.2019.1589025

169. DePuy V, Berger VW. Counterbalancing. In: Wiley StatsRef: Statistics Reference Online [Internet]. Chichester, UK: John Wiley & Sons, Ltd; 2014 [cited 2020 Apr 17]. Available from: http://doi.wiley.com/10.1002/9781118445112.stat06195

170. Brooks JL. Counterbalancing for serial order carryover effects in experimental condition orders. Psychol Methods. 2012 Dec;17(4):600–14.

171. Rustay N, Browman K, Curzon P. Cued and Contextual Fear Conditioning for Rodents. In CRC Press/Taylor & Francis; 2008. p. 19–37.

172. Friard O, Gamba M. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Fitzjohn R, editor. Methods Ecol Evol [Internet]. 2016 Nov [cited 2020 Jan 6];7(11):1325–30. Available from: http://doi.wiley.com/10.1111/2041-210X.12584

173. Seibenhener ML, Wooten MC. Use of the open field maze to measure locomotor and anxiety-like behavior in mice. J Vis Exp. 2015 Feb 6;(96).

174. Crawley J, Bailey K. Anxiety-Related Behaviors in Mice. In 2008. p. 77–101.

175. Paxinos G, Franklin KBJ. Paxinos and Franklin's The mouse brain in stereotaxic coordinates.

176. Sultan F. Dissection of Different Areas from Mouse Hippocampus. BIO-PROTOCOL. 2013;3(21).

177. Elfving B, Plougmann PH, Müller HK, Mathé AA, Rosenberg R, Wegener G. Inverse

correlation of brain and blood BDNF levels in a genetic rat model of depression. Int J Neuropsychopharmacol [Internet]. 2010 Jun 2 [cited 2020 Mar 15];13(05):563–72. Available from: https://academic.oup.com/ijnp/article-lookup/doi/10.1017/S1461145709990721

178. Modgil S, Lahiri DK, Sharma VL, Anand A. Role of early life exposure and environment on neurodegeneration : implications on brain disorders. 2014;1–14.

179. Huizink AC, Dick DM, Sihvola E, Pulkkinen L, Rose RJ, Kaprio J. Chernobyl exposure as stressor during pregnancy and behaviour in adolescent offspring. Acta Psychiatr Scand [Internet]. 2007 Dec 1 [cited 2020 Mar 9];116(6):438–46. Available from: http://doi.wiley.com/10.1111/j.1600-0447.2007.01050.x

180. Jackson IL, Vujaskovic Z, Down JD. Revisiting Strain-Related Differences in Radiation Sensitivity of the Mouse Lung: Recognizing and Avoiding the Confounding Effects of Pleural Effusions. Radiat Res. 2010 Jan;173(1):10–20.

181. Ponnaiya B, Cornforth MN, Ullrich RL. Radiation-Induced Chromosomal Instability in BALB/c and C57BL/6 Mice: The Difference Is as Clear as Black and White. Radiat Res. 1997 Feb 7;147(2):121.

182. Roderick TH. The Response of Twenty-Seven Inbred Strains of Mice to Daily Doses of Whole-Body X. Vol. 20, Source: Radiation Research. 1963.

183. An X-L, Zou J-X, Wu R-Y, Yang Y, Tai F-D, Zeng S-Y, et al. Strain and Sex Differences in Anxiety-Like and Social Behaviors in C57BL/6J and BALB/cJ Mice. Vol. 60, Exp. Anim. 2011.

184. Bolivar VJ, Caldarone BJ, Reilly AA, Flaherty L. Habituation of Activity in an Open Field: A Survey of Inbred Strains and F 1 Hybrids. Vol. 30, Behavior Genetics.

Gershenfeld and Paul; 2000.

185. Gao WM, Lu HM, Dong JC, Zhang W, Zhou XY, Jenkins LW, et al. Postnatal growth, neurobehavioral and neurophysiologic changes of prenatal low-dose β-radiation from tritiated water in mice. Neurotoxicol Teratol. 2002 Mar 1;24(2):247–54.

186. Kitamura M, Itoh K, Matsumoto A, Hayashi Y, Sasaki * Ryohei, Imai Y, et al. PRENATAL IONIZING RADIATION-INDUCED APOPTOSIS OF THE DEVELOPING MURINE BRAIN WITH SPECIAL REFERENCES TO THE EXPRESSION OF SOME PROTEINS. Vol. 47, Kobe J. Med. Sci. 2001.

187. Grahn D, Hamilton KF. GENETIC VARIATION I N THE ACUTE LETHAL RESPONSE OF FOUR INBRED MOUSE STRAINS TO WHOLE BODY X-IRRADIATION' [Internet]. ncbi.nlm.nih.gov. [cited 2020 May 8]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1209824/

188. Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L, et al. Tissue-specific expression and regulation of sexually dimorphic genes in mice. [cited 2020 Mar 10]; Available from: www.genome.org/cgi/doi/10.1101/gr.5217506.

189. Nadler JJ, Zou F, Huang H, Moy SS, Lauder J, Crawley JN, et al. Large-Scale Gene Expression Differences Across Brain Regions and Inbred Strains Correlate With a Behavioral Phenotype. [cited 2020 Mar 10]; Available from: http://www.genetics.org/

190. de Haan JB, Newman JD, Kola I. Cu/Zn superoxide dismutase mRNA and enzyme activity, and susceptibility to lipid peroxidation, increases with aging in murine brains. Mol Brain Res. 1992 Apr 1;13(3):179–87.

191. El-Husseini AE, Schnell E, Chetkovich DM, Nicoll RA, Bredt DS. PSD-95 involvement in maturation of excitatory synapses. Science [Internet]. 2000 Nov 17 [cited 2020 Mar

14];290(5495):1364–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11082065

192. Keith D, El-Husseini A. Excitation control: Balancing PSD-95 function at the synapse. Front Mol Neurosci. 2008 Mar 28;1(MAR).

193. Vásquez CE, Riener R, Reynolds E, Britton GB. NMDA receptor dysregulation in chronic state: A possible mechanism underlying depression with BDNF downregulation. Vol. 79, Neurochemistry International. Elsevier Ltd; 2014. p. 88–97.

194. Guilloux JP, Douillard-Guilloux G, Kota R, Wang X, Gardier AM, Martinowich K, et al. Molecular evidence for BDNF-and GABA-related dysfunctions in the amygdala of female subjects with major depression. Mol Psychiatry. 2012 Nov 13;17(11):1130–42.

195. Pozzo-Miller LD, Gottschalk W, Zhang L, Mcdermott K, Du J, Gopalakrishnan R, et al. Impairments in High-Frequency Transmission, Synaptic Vesicle Docking, and Synaptic Protein Distribution in the Hippocampus of BDNF Knockout Mice. 1999.

196. Prabakaran S, Swatton JE, Ryan MM, Huffaker SJ, Huang JTJ, Griffin JL, et al. Mitochondrial dysfunction in schizophrenia: Evidence for compromised brain metabolism and oxidative stress. Mol Psychiatry. 2004 Apr 20;9(7):684–97.

197. Loganovsky KN, Loganovskaja TK. Schizophrenia Spectrum Disorders in Persons Exposed to Ionizing Radiation as a Result of the Chernobyl Accident. Schizophr Bull [Internet]. 2000 Jan 1 [cited 2020 Apr 20];26(4):751–73. Available from: https://academic.oup.com/schizophreniabulletin/article-lookup/doi/10.1093/oxfordjournals.schbul.a033492

198. Kuo LJ, Yang LX. γ-H2AX- A novel biomaker for DNA double-strand breaks. Vol. 22, In Vivo. 2008. p. 305–10.

199. Plyusnina EN, Shaposhnikov M V., Moskalev AA. Increase of Drosophila melanogaster

lifespan due to D-GADD45 overexpression in the nervous system. Biogerontology [Internet]. 2011 Jun [cited 2020 Mar 14];12(3):211–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21153055

200. Cretu A, Sha X, Tront J, Hoffman B, Liebermann DA. Stress sensor Gadd45 genes as therapeutic targets in cancer. Vol. 7, Cancer Therapy. Cancer Therapy; 2009. p. 268–76.

201. Salvador JM, Brown-Clay JD, Fornace AJ. Gadd45 in stress signaling, cell cycle control, and apoptosis. Adv Exp Med Biol. 2013;793:1–19.

202. Wang X, Wang L, Yu Q, Xu Y, Zhang L, Zhao X, et al. Alterations in the expression of Per1 and Per2 induced by Aβ31-35 in the suprachiasmatic nucleus, hippocampus, and heart of C57BL/6 mouse. Brain Res. 2016 Jul 1;1642:51–8.

203. Grimaldi B, Bellet MM, Katada S, Astarita G, Hirayama J, Amin RH, et al. PER2 controls lipid metabolism by direct regulation of PPARγ. Cell Metab. 2010 Nov 3;12(5):509–20.

204. Wang LM-C, Dragich JM, Kudo T, Odom IH, Welsh DK, O'Dell TJ, et al. Expression of the Circadian Clock Gene *Period2* in the Hippocampus: Possible Implications for Synaptic Plasticity and Learned Behaviour. ASN Neuro [Internet]. 2009 May 6 [cited 2020 Mar 14];1(3):AN20090020. Available from: http://journals.sagepub.com/doi/10.1042/AN20090020

205. Englund A, Kovanen L, Saarikoski ST, Haukka J, Reunanen A, Aromaa A, et al. NPAS2 and PER2 are linked to risk factors of the metabolic syndrome. J Circadian Rhythms [Internet]. 2009 May 26 [cited 2020 Mar 14];7(0):5. Available from: https://www.jcircadianrhythms.com/article/10.1186/1740-3391-7-5/

206. Winkler D, Daher F, Wüstefeld L, Hammerschmidt K, Poggi G, Seelbach A, et al. Hypersocial behavior and biological redundancy in mice with reduced expression of

PSD95 or PSD93. Behav Brain Res. 2018 Oct 15;352:35–45.

207. Hales CN, Barker DJP. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Int J Epidemiol. 2013 Oct;42(5):1215–22.

208. Khurana S, Grandbois J, Tharmalingam S, Murray A, Graff K, Nguyen P, et al. Fetal programming of adrenal PNMT and hypertension by glucocorticoids in WKY rats is dose and sex-dependent. PLoS One. 2019;14(9).

209. Burdge GC, Hanson MA, Slater-Jefferies JL, Lillycrop KA. Epigenetic regulation of transcription: A mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life? Br J Nutr. 2007 Jun;97(6):1036–46.

210. Chmurzynska A. Fetal programming: link between early nutrition, DNA methylation, and complex diseases. Nutr Rev [Internet]. 2010 Feb 1 [cited 2020 Apr 12];68(2):87–98. Available from: https://academic.oup.com/nutritionreviews/article-lookup/doi/10.1111/j.1753-4887.2009.00265.x

211. Nguyen P, Khurana S, Peltsch H, Grandbois J, Eibl J, Crispo J, et al. Prenatal glucocorticoid exposure programs adrenal PNMT expression and adult hypertension. J Endocrinol [Internet]. 2015 Nov 1 [cited 2020 Apr 12];227(2):117–27. Available from: https://joe.bioscientifica.com/view/journals/joe/227/2/117.xml

212. Vyas S, Rodrigues AJ, Silva JM, Tronche F, Almeida OFX, Sousa N, et al. Chronic Stress and Glucocorticoids: From Neuronal Plasticity to Neurodegeneration. 2016 [cited 2020 Apr 12]; Available from: http://dx.doi.org/10.1155/2016/6391686

213. Sato H, Takahashi T, Sumitani K, Takatsu H, Urano S. Glucocorticoid generates ROS to induce oxidative injury in the hippocampus, leading to impairment of cognitive function of rats. J Clin Biochem Nutr [Internet]. 2010 Nov [cited 2020 Apr 12];47(3):224–32.

Available from: http://www.ncbi.nlm.nih.gov/pubmed/21103031

214. Mcgowan PO, Matthews SG. Prenatal Stress, Glucocorticoids, and Developmental Programming of the Stress Response. [cited 2018 May 4]; Available from: https://watermark.silverchair.com/en.2017-00896.pdf?token=AQECAHi208BE49Ooan9kkhW_Ercy7Dm3ZL_9Cf3qfKAc485ysgAAAdYwggHSBgkqhkiG9w0BBwagggHDMIIBvwIBADCCAbgGCSqGSIb3DQEHATAeBglghkgBZQMEAS4wEQQMV1xbVyojpOwiEk1RAgEQgIIBiZtFpEBYIcqdSG22ittlBCFm2dkAv9tVnoBqvbZVTl1_Zr5Gbj3gak2_QJxn4S-v_00guyhvC-ogDASgiyeGpldTRAVjZI42vbaRvuNY_kD1omZBnPxijzla_t3Uz0XPnrPAwGVougaYeAJwKROIlmn1gRA2y_el9JsH3kSH-ONPmaCIlvQTFIs3QDzbzzOZZjgcVH5htG7pdRX-VbcN9VwXc6znLVVQxS9OydxJEXDRVw44y-WaeO-88Z_xXit5du8Y5vjbaJFfGG1hPn6cJuJ4f80lAi7aPwnmHMtmtQNGRvXtHUxatB0-6utrv8banb4lu65D8AwpQFkUcRKLddNmt18t-iUOrMzcQfA5QLFKCOYo6QiRhGk670hQbU82aDOUSAszGn6wyh2P0mx_8J-1C9x9WpoG1z7q4uxphtV8_4HkMHCDcTAbyt1oh4OQ3KP6wRGzhKa2ZOa90dQT7By-vC5TXShYsYljCLYGjBd0COKhVYxZdXYkjYzKRqRg-fnr7fPEr0VFqg

215. Modgil S, Lahiri DK, Sharma VL, Anand A. Role of early life exposure and environment on neurodegeneration: Implications on brain disorders. Vol. 3, Translational Neurodegeneration. BioMed Central Ltd.; 2014. p. 1–14.

216. Davidson CQ, Tharmalingam S, Niccoli S, Nemec-Bakk A, Khurana S, Murray A, et al. Dose threshold for radiation induced fetal programming in a mouse model at 4 months of age: Hepatic expression of genes and proteins involved in glucose metabolism and glucose uptake in brown adipose tissue. Torrens C, editor. PLoS One [Internet]. 2020 Apr 21

[cited 2020 May 8];15(4):e0231650. Available from: https://dx.plos.org/10.1371/journal.pone.0231650

217. Verreet T, Verslegers M, Quintens R, Baatout S, Benotmane MA. Current evidence for developmental, structural, and functional brain defects following prenatal radiation exposure. Vol. 2016, Neural Plasticity. Hindawi Limited; 2016.

218. De Kloet ER, Molendijk ML. Coping with the Forced Swim Stressor: Towards Understanding an Adaptive Mechanism. 2016 [cited 2020 Apr 13]; Available from: http://dx.doi.org/10.1155/2016/6503162

219. Commons KG, Cholanians AB, Babb JA, Ehlinger DG. The Rodent Forced Swim Test Measures Stress-Coping Strategy, Not Depression-like Behavior. Vol. 8, ACS Chemical Neuroscience. American Chemical Society; 2017. p. 955–60.

220. Reul JMHM, Collins A, Saliba RS, Mifsud KR, Carter SD, Gutierrez-Mecinas M, et al. Glucocorticoids, epigenetic control and stress resilience. Vol. 1, Neurobiology of Stress. Elsevier Inc; 2015. p. 44–59.

221. Hines DJ, Schmitt LI, Hines RM, Moss SJ, Haydon PG. Antidepressant effects of sleep deprivation require astrocyte-dependent adenosine mediated signaling. Transl Psychiatry. 2013 Jan 15;3(1):e212–e212.

222. Tanda K, Nishi A, Matsuo N, Nakanishi K, Yamasaki N, Sugimoto T, et al. Abnormal social behavior, hyperactivity, impaired remote spatial memory, and increased D1-mediated dopaminergic signaling in neuronal nitric oxide synthase knockout mice. Mol Brain [Internet]. 2009 Jun 18 [cited 2020 Apr 13];2(1):19. Available from: http://molecularbrain.biomedcentral.com/articles/10.1186/1756-6606-2-19

223. Mao B, Zhang Z, Wang G. BTG2: A rising star of tumor suppressors (Review) [Internet].

Vol. 46, International Journal of Oncology. Spandidos Publications; 2015 [cited 2020 Apr 13]. p. 459–64. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25405282

224. McKlveen JM, Myers B, Flak JN, Bundzikova J, Solomon MB, Seroogy KB, et al. Role of prefrontal cortex glucocorticoid receptors in stress and emotion. Biol Psychiatry. 2013 Nov 1;74(9):672–9.

225. Shakeri R, Kheirollahi A, Davoodi J. Apaf-1: Regulation and function in cell death. Vol. 135, Biochimie. Elsevier B.V.; 2017. p. 111–25.

226. Van Wageningen TA, Vlaar E, Kooij G, Jongenelen CAM, Geurts JJG, Van Dam AM. Regulation of microglial TMEM119 and P2RY12 immunoreactivity in multiple sclerosis white and grey matter lesions is dependent on their inflammatory environment. Acta Neuropathol Commun [Internet]. 2019 Dec 11 [cited 2020 Apr 13];7(1):206. Available from: https://actaneurocomms.biomedcentral.com/articles/10.1186/s40478-019-0850-z

227. Bennett ML, Bennett FC, Liddelow SA, Ajami B, Zamanian JL, Fernhoff NB, et al. New tools for studying microglia in the mouse and human CNS. Proc Natl Acad Sci U S A. 2016 Mar 22;113(12):E1738–46.

228. Pellow S, Chopin P, File SE, Briley M. Validation of open" closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. Vol. 14, Journal of Neuroscience Methods. 1985.

229. Donatti AF, Soriano RN, Leite-Panissi CRA, Branco LGS, de Souza AS. Anxiolytic-like effect of hydrogen sulfide (H2S) in rats exposed and re-exposed to the elevated plus-maze and open field tests. Neurosci Lett. 2017 Mar 6;642:77–85.

230. Yu Z, Zhao W, Ding L, Yu Y, Liu J. Anxiolytic effects of ACE inhibitory peptides on the behavior of rats in an elevated plus-maze. Food Funct. 2016 Jan 1;7(1):491–7.

231. Felix-Ortiz AC, Burgos-Robles A, Bhagat ND, Leppla CA, Tye KM. Bidirectional modulation of anxiety-related and social behaviors by amygdala projections to the medial prefrontal cortex. Neuroscience. 2016 May 3;321:197–209.

232. Montalvo-Ortiz JL, Bordner KA, Carlyle BC, Gelernter J, Simen AA, Kaufman J. The role of genes involved in stress, neural plasticity, and brain circuitry in depressive phenotypes: Convergent findings in a mouse model of neglect. Behav Brain Res. 2016 Dec 15;315:71–4.

Appendices

Appendix A – Supplementary Information: Primer Design for Dexamethasone Study

Pathway	Gene	Accession #	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
Methylation	COMT	NM_012531.2	TTCAAGCGTCGGGATCGG	TGCAGCACGTACTCAAACCA
	DNMT3b	NM_001003959.1	GATGAGGAGAGCCGAGAACG	CAGAGCCCACCCTCAAAGAG
Glutamate signaling	GRM4	NM_022666.1	CAGTGCGAGCAGCTAAGGG	GAGAATGGCTCCGGTCACTC
	SLC1A2	NM_001035233.1	GTCAATGCCGCACACAACTC	GAATGGATGCAGGGGATGGT
	GRIA2	NM_001083811.1	GCATCGCCACACCTAAAGGA	TCCTTGGAATCACCTCCCCC
	GRM2	NM_001105711.1	TTCCCTCCTCAGTCTTCCACT	CCTCCATAGGAAGCAGCGTT
Calcium signaling	RYR2	NM_001191043.1	CACTAAGCAGCATCCTGTGC	CTTCGGTCTTGGCTTCTCAGT
	CACNB2	NM_053851.1	CAGCTGCACTGTCGGAATCT	AGTCATTCCATTTTTGCCCTGA
	CACNA1B	NM_001195199.1	GGGCTAATCTGCCCCAGAAG	GAGAGCCGCATAGACCTTCC
	PLCH2	XM_017593885.1	AAATCCCAGAGTGCCCATCC	ATCGTTCCACCACAGGCAAA
	RYR1	XM_017590442.1	GGGCCATAACAACGGTGAGA	TCAGACGACATCGCAGTCAC
Glucocorticoid receptors	NR3C1	NM_012576.2	TGCTGGAGGTGATTGAACCC	TCACTTGACGCCCACCTAAC
	NR3C2	NM_013131.1	CAGTGCACAGTCCCATCACT	GGACTTGAAAGAGGGGAGCC
Neural transmission	SNAP25	NM_001270575.1	ATGTTGGATGAGCAAGGCGA	TCGGCCTCCTTCATGTCTTG
Neuronal differentiation, growth	MYT1L	NM_053888.1	TGTGGAGCCAGCCATACAAG	TGGGGCTGTTTATCTTGCGT
	LSAMP	NM_017242.1	CACTGAGGAACACTACGGCA	ACCCGGGTCTGAAAAGGACT
Lysosomal homeostasis	MBTPS1	NM_053569.1	GCGAGTAAACATCCCCCGAA	CCCAAATCTAGCAGGAGCCC
Reference genes	Aanat	XM_006247792.2	GACAAGACGTCTCCCTCTGG	GGTGGATGCTCAACATGGGT
	CycA	NM_017101.1	CAGACGCCGCTGTCTCTTTTC	CGTGATGTCGAAGAACACGGT
	Ywhaz	NM_013011.3	GGCAGAGCGATACGATGACA	AAGATGACCTACGGGCTCCT

All primers listed were designed using Primer3 or BLAST. Annealing temperatures was 58 degrees Celsius.

Appendix B – Supplementary Information 2: Primer Design for Radiation Study

Pathway	Gene	Accession #	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
ROS Regulation	SOD1	NM_011434	CGGATGAAGAGAGGCATGTTGGA	CAATGATGGAATGCTCTCCTGAG
	SOD2	NM_013671	TTACGACTATGGCGCGCTGGA	TCGTGGTACTTCTCCTCGGTG
	SOD3	NM_011435	GGCCTGTGGCTCTGTCACC	CCTATCTTCTCAACCAGGTCAAG
	Catalase	NM_009804	CCTGACATGGTCTGGGAC	CCATAGCCATTCATGTGCCG
	GPX1	NM_008160	GGTGCTGCTCATTGAGAATGTCG	GGGAAACCGAGCACCACCAG
Cortisol Regulation	11β1HSD	NM_001044751	GAAGAGTTCAGACCAGAAATGCT	CAATACCACATGGGCTCCCATTT
	11β2HSD	NM_008289	TCTTTGGTGCACTTGAGCTGACC	AGGCTGCCAAGCAGGGGTATG
	GR	NM_001361209	AGGCCGCTCAGTGTTTTCTA	TACAGCTTCCACACGTCAGC
Cell Cycle	BTG2	NM_007393.3	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
DNA Damage & Tumor Suppression	γH2AX	NM_010436	GCCTCTCAGGAGTACTGAGGG	CCCGAAGTGGCTCAGCTCTTT
	p53	AB020317	GGAAGACTCCAGTGGGAACC	TCTTCTGTACGGCGGTCTCT
	GADD45A	NM_007836	GAAAGGATGGACACGGTGGG	GGGTCTACGTTGAGCAGC
	DDB2	NM_028119.5	ACCGAGTACGTCATGGCTCCC	CTTGGCTTCGGGCTCCAGCT
	MDM2	NM_001288586	CCTGGATCAGGATTCAGTTTCTG	TCATCATCCTCATCTGAGAGCTC
DNA Methylation	DNMT3A	NM_007872	GAGGGAACTGAGACCCCAC	CTGGAAGGTGAGTCTTGGCA
	DNMT3B	NM_001122997	AGCGGGTATGAGGAGTGCAT	GGGAGCATCCTTCGTGTCTG
	DNMT1	NM_001199433	AAGCCTGGTGTTGTCTACCGAC	CATCCAGGTTGCTCCCCTTG
Circadian Rhythm	Per2	NM_011066	ACAAGAAGGCCAAGGGGAAGG	GGCTCTCACTGGACATTAGCAG
Synaptic Activity	Doublecortin	NM_001110222	ATGTCAACCGGGAAAGCACA	TGGTGGAACCACAGCAACTT
	NeuN	NM_001039168	GGCAAATGTTCGGGCAATTCG	TCAATTTTCCGTCCCTCTACGAT
	NeuroD	NM_010894.2	ATGACCAAATCATACAGCGAGAG	TCTGCCTCGTGTTCCTCGT
	PSD-95	NM_007864.3	GGCGGAGAGGAACTTGTCC	AGAATTGGCCTTGAGGGAGGA
	Synaptophysin	NM_009305.2	CAGTTCCGGGTGGTCAAGG	ACTCTCCGTCTTGTTGGCAC
	NOS3	NM_008713	TTTGCTGCCCTTGGCCTGCG	CTCTGAACTCATGTACCAGCCG
	NRF2	NM_010902	CAGCACATCCAGACAGACACCA	TGGGAATGTCTCTGCCAAAAGCT
Microglia	TMEM119	NM_146162	TTCACCCAGAGCTGGTTCCATA	GAGTGACACAGAGTAGGCCA
Apoptosis	CX3CR1	NM_009987.4	TCACCGTCATCAGCATCGAC	CGCCCAGACTAATGGTGACA
	BAX	NM_007527	CTGGATCCAAGACCAGGGTG	GTGAGGACTCCAGCCACAAA
	BCL2	NM_009741	GCGTCAACAGGGAGATGTCA	GCATGCTGGGGCCATATAGT
	APAF1	NM_001282947.1	CGTCTTCCAGTGTAAGGACAGT	CCATAGATGGTGACCCACCC
Reference Genes	Beta Actin	NM_007393.3	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
	RPL29	NM_009082.2	ACATGGCCAAGTCCAAGAAC	TGCATCTTCTTCAGGCCTTT

All primers listed were designed using Primer Bank, Primer3, Primer3Plus, or Gemi. Annealing temperature was 58 degrees Celsius.