EFFECTS OF PRENATAL STRESS AND/OR FOREBRAIN *ATRX* DEFICIENCY IN C57BL/6 MICE ON CELLULAR METABOLOSIM, DNA DAMAGE, *ATM* PROMOTER METHTLATION AND GENE EXPRESSION

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ABSTRACT

Early life experiences and maternal-infant attachment influence brain development, notably areas that support stress regulation, cognition, and social behaviour. Animal models examining gestational stress suggest that sustained changes in gene expression in response to prenatal stress and/or altered rearing conditions during the first week of postnatal development is mediated by changes in chromatin structure, DNA methylation and DNA damage pathways. Although the exact mechanism is not completely understood, the chromatin remodelling protein *ATRX* is regulated by maternal care and works to mediate *Atm*, a gene responsible for DNA damage detection. *ATRX* and *ATM* play a key role in gene expression regulation, which is in part regulated by mitochondrial dynamics and mitigation of metabolic pathways. *ATRX* exerts its effects by conformational changes to chromatin resulting in epigenetic modification, such as DNA methylation, responsible for downstream gene activation. *ATM* works to detect double stranded breaks by phosphorylating downstream proteins. Both *ATRX* and *ATM* are highly regulated; changes in expression can cause synaptic deficits contributing to neurodevelopmental disorders, such as idiopathic autism.

We examined mitochondrial function, reactive oxygen species generation, oxidative DNA damage, *Atm* gene expression and *Atm* promoter methylation in brain regions relevant to autistic-like behaviours using a mouse model with reduced *Atrx* expression. Males exposed to *Atrx* deficiency and/or prenatal stress exhibited an impaired mitochondrial metabolic potential, increased H_2O_2 production, increased *Atm* expression, and reduced *Atm* promoter methylation in the murine forebrain. Females reared with *Atrx* deficient males showed increased *Atm* promoter methylation, suggesting rescue mechanism(s) or hormonal sex differences are involved in stress regulation of our *Atrx* deficient models.

Our findings demonstrate that early life environment, particularly stress, plays a role in mechanisms underlying metabolism, gene expression, DNA damage detection and ultimately neurodevelopment. This study expands the current knowledge surrounding epigenetic mechanisms and early life experience, while providing a potential mechanism for interactions between *ATRX* and *ATM*, which is linked with sex differences in neural cell metabolism and DNA damage pathways in adult offspring of gestationally stressed mothers.

LIST OF ABBREVIATIONS AND SYMBOLS USED

α: alpha
Acetyl-CoA: Acetyl coenzyme A
AD: Alzheimer's disease
ADD: ATRX-DNMT3-DNMT3L
AIC: Akaike information criterion
AMA: Antimycin A
ASD: Autism spectrum disorder
A-T: Ataxia-telangiectasia
ATM: Ataxia telangiectasia mutated
ATP: Adenosine triphosphate
ATL: Alternate lengthening of telomeres
ATRX: α-thalassemia/mental retardation, X-linked
BER: DNA base excision repair
bp: Base pair
cDNA: Complementary DNA
CER: Cerebellum
CHD: Chromodomain-Helicase-DNA binding
ChIP: Chromatin immunoprecipitation
CI: Confidence interval
cKO: Conditional knockout
CNS: Central nervous system
CpG: Cytosine–phosphodiester–guanine
Cq: Quantitative cycle
Cre: P1 tyrosine recombinase enzyme
DAXX: Death domain-associated protein
Δ**:** delta
°C: Degrees centigrade
ddH_2O: Double deionised water
DDR: DNA damage response
DF: Degrees of freedom
DNA: Deoxyribonucleic acid
DNMT: DNA methyltransferase
DSB: Double-stranded breaks
DSM: Diagnostic and Statistical Manual of Mental Disorders
E2F: E2 transcription factor family
ECAR: extracellular acidification rate
EDTA: Ethylenediaminetetraacetic acid
ETC: Electron transport Chain
F0: First generation
F1: Second generation
FACS: Fluorescence-activated cell sorting
FC: Frontal cortex
FCCP: carbonyl cyanide 4-(trifluoromethoxy)phenyhydrazone
gDNA: Genomic DNA
H 1/2/3/4: Histone one, two, three, four
8-OHdG: 8- hydroxy-2'-deoxyguanosine
HEM: Hemizygotic
HIPP: Hippocampus
H_2O_2: Hydrogen peroxide
HPA: Hypothalamic-Pituitary-Adrenal Axis
INO80: INOsitol requiring 80
IPC: Interplate calibrator
ISWI: Imitation SWItch
kDa: Kilodaltons
LG: Licking and grooming
LR: Likelihood ratio
LTP: Long term potentiation
LoxP: Locus of X-over P1 sequence
MBD: Methyl-CpG binding domain
MeCP2: methyl-CpG binding protein 2
µg: Microgram
µL: Microlitre
µM: Micromolar
MFI: Mean fluorescence intensity

MRI: Magnetic resonance imaging
MRN: Mre11-Rad50-Nbs1
mRNA: Messenger ribonucleic acid
ml: Millilitre
N: Number of subjects
NAD+: nicotinamide adenine dinucleotide
nm: Nanometer
NTC: No template control
NRT: No reverse transcriptase
OCR: oxygen consumption rate (OCR)
%: Percentage
p: Probability (statistics)
p53: Phosphoprotein p53
PCR: Polymerase chain reaction
PI3K-Akt: Phosphoinositide-3-kinase – protein kinase B
PIKKs: PI3K-like Protein Kinases'
qPCR: Quantitative PCR
RPM: Revolutions per min
RNA: Ribonucleic acid
ROS: Reactive oxygen species
RQI: RNA quality indicator
RT: Reverse transcriptase
RT-qPCR: Real-time qPCR
SAM: S-adenosylmethionine
SEM: Standard error of mean
Ser: Serine
SF: Superfamily
SFARI: Simons Foundation Autism Research Initiative
SWI/SNF2: SWItch/Sucrose Non-Fermentable-2 protein
TBS: Tris-buffered Saline
TDG: Thymine-DNA-glycosylase
UV: Ultraviolet
WT: Wild-type

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CHAPTER 1: INTRODUCTION

Neurodevelopmental disorders are characterized by brain dysfunction (such as sensory motor and speech problems) and cognitive impairments (such as learning and organizational skills) typically manifesting during developmental years of early childhood (Aspromonte et al., 2019; van Loo & Martens, 2007). Complex neurodevelopmental disorders, such as autism spectrum disorder (ASD), are said to develop via complex interactions between gene expression and the environment (van Loo & Martens, 2007). Similarly, phenotypic determinants develop through interplay of genes and the environment, originating during the pre-natal or post-natal periods (Paus, 2013). Twin studies have demonstrated the significance of genetic influence on neurodevelopmental disorders, such as autism. Studies suggest neurodevelopmental phenotypes and genotypes are highly heritable in twins (Taniai et al., 2008). When studying developmental gene expression, it is important on consider not only the genetic make-up but also the environment in which the individual is developing (pre- and post-natal).

Gene expression is controlled and regulated at different levels; with epigenetic modifications ultimately influencing the production of proteins encoded by the DNA (Jiang et al., 2019). Epigenetic modifications impact multiple nuclear processes, such as chromatin remodeling, DNA packaging and ultimately gene expression (Jiang et al., 2019; McKittrick et al., 2004). Epigenetic mechanisms have been highlighted in the role of neurodevelopmental disorders by characterizing genes involved in neurodevelopment. The Simons Foundation Autism Research Initiative (SFARI) has compiled studies identifying 913 autism spectrum disorder-associated genes. Autism is one of the most common neurological disorders seen in children, characterized by disturbances in social interactions and communication as well as exhibiting repetitive behaviours (American Psychiatric Association; DSM-5, 2013). Studying the epigenome is an important tool to understanding idiopathic ASD. Although idiopathic autism is defined as having no known genetic mutations, dysregulated expression of specific genes associated with ASD is seen in individuals with idiopathic ASD (Liu et al., 2017). Current research suggests both genetics and environmental factors play a role in the etiology of ASD, suggesting that epigenetic mechanisms as well as early life experiences may influence the dysregulation of neurodevelopment (Grafodatskya et al., 2010). Animal models examining gestational

stress suggest that sustained changes in gene expression in response to prenatal stress and/or natural variations in mother-pup interactions during the first week of postnatal life is mediated by changes in chromatin structure and DNA methylation (Bérubé et al., 2005; Weaver et al., 2017).

Chromatin remodeling factor *ATRX* is listed in the SFARI database as an ASD associated gene (Brett et al., 2014; Deneault et al., 2018). Chromatin remodelling is the dynamic modification to the structure of chromatin structure (condensed vs. unpacked), to allow regulatory transcription machinery proteins access to the underlying DNA sequence. *ATRX* protein is essential for brain development and plays a key role in gene expression regulation. It exerts its effects by binding to heterochromatin resulting in epigenetic modifications responsible for downstream gene activation (De La Fuente et al., 2011). Mutations in the *Atrx* gene can cause synaptic deficits contributing to neurodevelopmental disorders, such as *ATR-X* syndrome, characterized by developmental deficits and cognitive abnormalities (Gibson 2006). Although the exact mechanism is not completely understood, studies have shown that *Atrx* is, in part, regulated by early life environment and maternal care (Murgatroyd & Spengler., 2011; Weaver et al., 2006).

Intrauterine and postnatal development involves interactions between genes and the environment, through early life experiences and epigenetic modifications. In mammals, early life experience and the degree of parental-infant attachment influence brain development, notably areas that support stress regulation, cognition and social behaviours (Weaver et al., 2006; Kundakovic & Champagne, 2014). It's suggested that the most sensitive period to environmental stressors is during early embryonic development, though the effects of epigenetic changes caused by gene-environment interactions is dependent on the genes that are implicated (Fang et al., 2014). Studies have shown that maternal stress during gestation has been associated with increased incidences of ASD in children (Beversdorf, Stevens, & Jones, 2018; Walder et al., 2014). As previously mentioned, *Atrx* expression is influenced by the pre- and early postnatal environment. It is known as an ASD associated gene and encodes for a protein involved in regulating gene expression. This raises the question of how early life experiences and maternal care effect *Atrx* expression and the relationship between *Atrx* expression regulation and ASD risk genes,

such as the ataxia telangiectasia mutated (*Atm*) gene, which is involved in DNA damage recognition and cell cycle progression of neuronal cells.

This introduction will review previous literature surrounding the role of chromatin remodeling protein, *ATRX*, in genetic regulation and brain development. It will also focus on the effect of early life experiences and the impact on offspring gene expression, particularly when there is dysregulation in ASD risk genes, such as *Atm*.

1.1. *Gene Regulation in Early Brain Development*

Brain development is the complex, adaptive process involving macroanatomic, cellular and molecular mechanisms working synchronously within a highly constrained context (Stiles & Jernigan, 2010). During early brain development, neural progenitor cells differentiate in accordance with a cascade of molecular signalling to form different populations of embryonic cells (Stiles & Jernigan, 2010). Embryonic structures are then specified by inductive interactions and morphogenetic processes (Lumsden & Kiecker, 2013). Thickening of dorsal ectodermal progenitor cells forms the neural plate (Lumsden & Kiecker, 2013). Once the neural plate is formed, the expression of neural inducers triggers neurulation; which forms the neural tube (Lumsden & Kiecker, 2013; Wilson & Hemmati-Brivanlou, 1997). Inductive interactions via neural inducers play an important role in permitting neurogenesis and ultimately gene expression (Bier, 1997). The neural tube, along with migrating neural crest cells help to form the central nervous system and the peripheral nervous system, respectively (Lumsden & Kiecker, 2013).

Molecular gene expression and environmental input influence brain development; beginning as early as the third gestational week during embryonic development and extends throughout one's lifespan (Stiles & Jernigan, 2010). As progenitor cells begin to differentiate, their gene expression programs become more definitive as a result of gene regulatory networks, namely transcription factors (Reik, 2007; Sonawane et al., 2017). Cell differentiation is a tissue specific process dependent on tissue environment and aforementioned transcription factors and their protein products (Sonawane et al., 2017). Protein synthesis is essential for brain development. Dysregulation of typical protein synthesis during mRNA translation and associated mechanisms for protein synthesis often contribute to an increase the risk factor for neurodevelopmental, neurodegenerative and neuropsychiatric disorders (Wiebe et al., 2020). Together these regulatory networks

composed of genes, transcription factors and their gene products work in synchronous, requiring high specificity to ensure proper brain development and functioning.

Neuron production occurs shortly after conception, with many neurons migrating to different brain regions, destined to become elaborate neural connections. Although many neurons are produced before birth, much of the synaptic connections that these neurons form occur during the postnatal period and continue into adulthood (Stiles and Jernigan, 2010). Studies using magnetic resonance imaging (MRI) have demonstrated the continuation of neuronal development into early adulthood (ages 4-21) and provide evidence that neural structures mature according to specific trajectories and timelines (Gogtay et al., 2004; Jernigan et al., 2011). Neuronal development can be categorized in laymen's terms as 'growth spurts,' which indicate changes in brain chemistry and morphology (Jernigan et al., 2011). Although it is unclear whether changes to the adult brain are attributed to the continual growth in white matter or the shaping of neuronal processes, it is evident that changes are in fact occurring during this period (Jernigan et al., 2011). What is more evident, however, are the structural changes in the brain during childhood and adolescence (Jernigan and Gamst, 2005). Changes in the volume of brain structures, such as the frontal cortex, thalamus, nucleus accumbens and cerebral white matter, rapidly change during childhood and throughout adolescence (**Figure 1**) (Jernigan and Gamst, 2005).

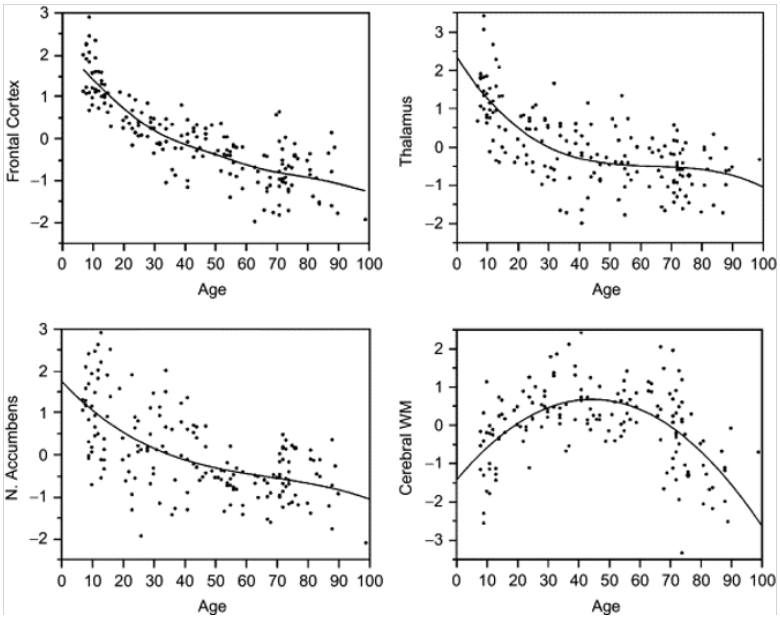

Figure 1. Estimated volumes of brain structures plotted against age using standard residuals. From Left, volumes of frontal cortex, thalamus, nucleus accumbens, and cerebral white matter. Adapted from Jernigan and Gamst (2005).

In summary, brain development is a highly regulated process that begins during the per-natal period and continues throughout the lifespan. The use of MRI has allowed for expansive research into the structural development of the brain in terms of neuronal trajectories and developmental timelines. Structural development is highly regulated by regulatory networks consisting of genes, transcription factors and gene products, which if disrupted, can increase the risk of neurodevelopmental, neurodegenerative and neuropsychiatric disorders.

1.2. *Mechanisms of Epigenetic Inheritance*

During periods of extensive growth, maternal and environmental factors (e.g., maternal care, nutrition, infection) can influence perinatal brain development (Stiles and Jernigan, 2010). For example, variation in mother-neonate interactions induce long-term epigenetic alterations, with implications for the development of neural circuits and the function of these circuits in the adult offspring (Weaver et al., 2004). The adverse effects of maternal and environmental stressors will be discussed more extensively later but contribute greatly to perinatal development. Along with mother-neonate interactions, stress may also influence brain development and gene expression (Weaver et al., 2004). These maternal and environmental factors also influence embryonic development in addition to perinatal development. Embryonic development is highly susceptible to change in the intrauterine environment. Factors such as maternal nutrition influence fetal development, leading to changes in fetal survival strategies that will impact future health of the offspring (Reusens et al., 2008). For example, a low-protein maternal diets alter gene expression in fetal islet cells which modify fetal beta cell phenotype (Reusens et al., 2008). Additionally, adverse maternal hormonal environments have been shown to influence fetal brain development. Changes in maternal hormone levels (e.g., thyroid hormones & glucocorticoids) can affect gene expression during fetal development (Miranda & Sousa, 2018).

In rodent studies, evidence suggests that the maternal environment can influence the phenotype of the offspring, which is mediated by changes in gene expression (Champagne and Curley, 2009). Mitotically inherited epigenetic changes can occur within germ cells due to improper assembly of DNA, and thus have the potential to be passed from one generation to the next (from parent to offspring) (Fang et al., 2014). Though the

effects of these epigenetic changes on a developing embryo depends on the genes implicated, it is clear that the most sensitive period to environmental stressors is during early embryonic development (Fang et al., 2014). The effects of epigenetic changes on offspring, however severe, can be lasting and detrimental to the health of the developing young (Millan, 2013). In many instances, altering the epigenetic code of the offspring can increase the likelihood of many neurodevelopmental disorders, such as autism and schizophrenia (Millan, 2013). These neurodevelopmental disorders are typically accompanied by epigenetic regulation processes that have been activated as a result of epigenetic changes, in turn allowing the disorder to persist (Millan, 2013). The hypothalamic-pituitary-adrenal (HPA-) axis is particularly susceptible to epigenetic alterations, as offspring subject to prenatal stress and low maternal care typically have elevated cortisol levels, leading to increased amounts of stress and a greater chance of developing stress related disorders later in life (Weaver et al., 2017). The HPA-axis is responsible for the neuroendocrine stress response, by initiation the release of stress specific hormones. Persistent exposure to stressors, facilitates stress adaptivity within individuals, which can result in epigenetic changes (Fang et al., 2014).

Molecular mechanisms that are involved in epigenetic changes and regulations of gene expression include chromatin remodelling, histone modification and DNA methylation (Champagne and Curley, 2009). These epigenetic mechanisms guide brain development. The epigenetic machinery used in these processes are vulnerable to changing environmental factors which can lead to long-term changes in gene expression. In the brain DNA methylation, acts to regulate the gene expression by the covalent addition of a methyl group (Hwang et al., 2017). This process is catalyzed by DNA methyltransferases (DMTs), a family of enzymes that play a key role in modification of brain function in adulthood (Simmons et al., 2013; Hwang et al 2017). Epigenetic changes via DNA methylation and DMTs are variable depending on brain regions but are highly expressed during early life, suggesting an important role in brain development and function (Simmons et al., 2013). Epigenetic modifications via chromatin remodelling and histone modifications will be discussed in the following section.

1.3. *Chromatin*

Chromatin is composed of condensed DNA and histone proteins that are organized in a repeating pattern across the genome within the cell nucleus (Lee & Orr-Weaver, 2001). The main role of chromatin is to aid in the structural organization of the genome, which regulates gene expression (Tyagi et al., 2016).

There are five families of histone proteins, known as the core canonical histones and are as follows: H1, H2A, H2B, H3, H4 (Lee & Orr-Weaver, 2001). The most basic structural element of chromatin is the nucleosome, which is comprised of the repeating pattern of DNA and linker histone H1, wrapped around a core octamer of histone proteins H2A, H2B, H3, and H4 (**Figure 2**) (Pisetsky, 2017; Lee & Orr-Weaver, 2001; Tyagi et al., 2016). The core octamer of the nucleosome can then be split into four histone dimer pairs, forming a helicase-like structure to which then binds DNA (Clapier & Cairns, 2014). Each family of histones contains variants that substitute the core canonical histones within the nucleosome, altering the structure and function of the nucleosome (Tyagi et al., 2016). Histones are positively charged conserved proteins which face outwards along the helicase like structure and can easily bind to the negatively charged phosphate backbone of DNA and facilitate the movement of the core octamer histone proteins along the DNA backbone (Lee & Orr-Weaver, 2001; Clapier & Cairns, 2014). The extensive structure of chromatin provides genetic information that influences many biological processes, such as DNA replication and gene transcription (Pisetsky, 2017; Fang et al., 2014).

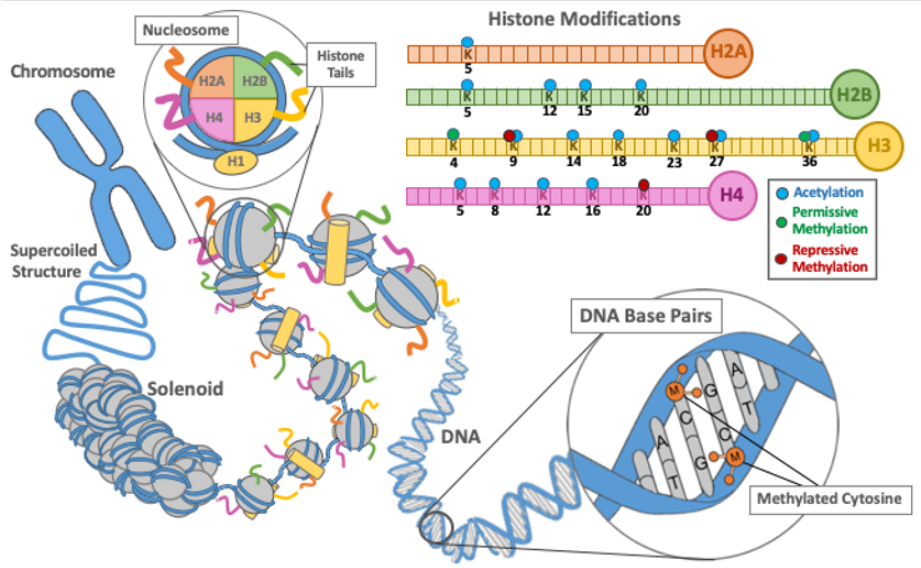

Figure 2. Structural organization of chromatin demonstrating histone H1 tightly wrapped around a core octamer of histone proteins (H2A, H2B, H3, H4) that form the helicase structure of DNA. Adapted from Weaver et al. (2017).

The structure of chromatin provides plasticity as it is subject to proofreading and extensive remodelling (Pisetsky, 2017; Fang et al., 2014). This is an important type of repair mechanisms in response to environmental and physical stressors in developing organisms (Pisetsky, 2017; Fang et al., 2014). To repair errors in DNA sequences, chromatin must constantly switch between DNA packing and unpacking states (Zhang et al., 2016). In the DNA packing state, the nucleosomes are tightly condensed. Whereas in the unpacked state, the genetic material loosens to allow for the chromatin remodelers to access the DNA to alter chromatin architecture and allow DNA replication machinery to access specific genetic sequences (Zhang et al., 2016).

1.4. *Linking Cell Metabolism, Chromatin and DNA Damage Pathways*

Dynamic chromatin organization and gene regulation underlies brain development and function. Homeostasis of cellular metabolism is an essential process for regulating intracellular activities, including signalling to chromatin, in response to changes in the cellular microenvironment (**Figure 3**). Digested nutrients (e.g., glucose, amino acids, vitamins, minerals, and fatty acids) are absorbed into the bloodstream through capillaries in the villi that line the small intestine. These metabolites passively and actively diffuse through the plasma and nuclear membrane of cells. The ability to alter gene expression in

response to changing metabolite availability is governed by 1) metabolic enzymes; 2) histone-modifying complexes; and 3) chromatin remodeling complexes, and require the coordination of metabolic state and gene expression programs (Dai et al., 2020). Metabolic enzymes convert metabolites into the substrates—e.g., acetyl-CoA (acetyl-CoA), S-adenosylmethionine (SAM)—that mediate the transfer of chemical groups by covalent histone and DNA-modifying complexes (epigenetic writers) such as histone acetyltransferases/deacetylases and DNA methylases/demethylases, respectively (Dai et al., 2020). Metabolic enzymes also convert metabolites into the co-factors—e.g., adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NAD^+)—that mediate the transfer of chemical groups by ATP-dependent chromatin-remodeling complexes (Dai et al., 2020). Examples of such complexes are SWItch/Sucrose Non-Fermentable (SWI2/SNF2) and the imitation SWI (ISWI) group, which can open chromatin. The open form of chromatin allows for selective binding of transcription factors that, in turn, recruit RNA polymerase II to transcribe nuclear genes. Effector proteins (epigenetic readers) detect and bind to methylated DNA, resulting in changes in selective binding of transcription factors that, in turn, alter gene expression (Dai et al., 2020). Examples of epigenetic readers are methyl-CpG binding domain family proteins (e.g., MBD1-4, MeCP2), thymine-DNA-glycosylase and DNA base excision repair (TDG/BER) machinery (Dai et al., 2020).

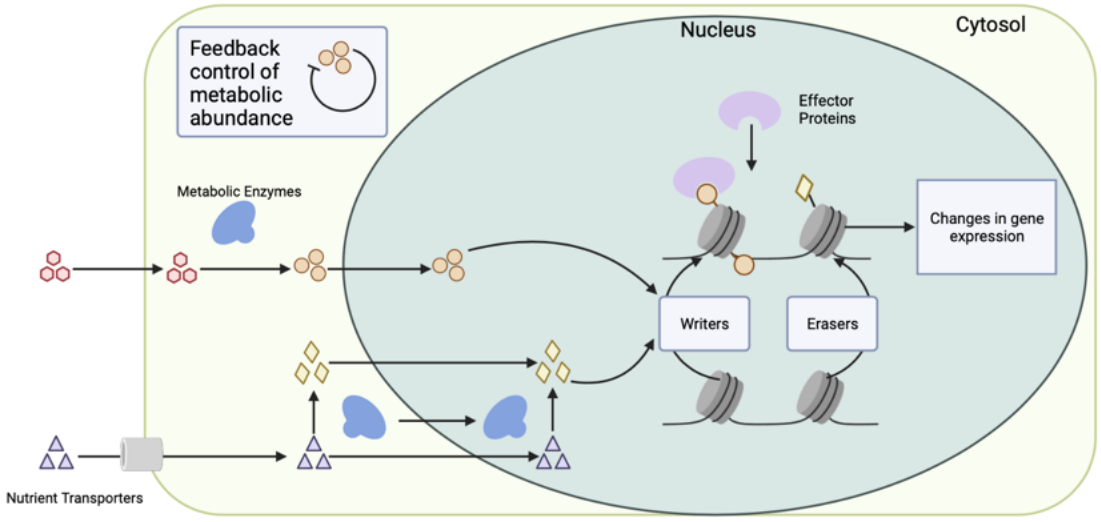

Figure 3. Interplay between cellular metabolism, chromatin-modifying metabolites and epigenetic machinery. Cytoplasmic-nuclear partitioning is used as a regulatory mechanism to link metabolic state to transcriptional outcomes. Transcription factors that are activated by extracellular signals, such as hormones, direct a transcriptional response to changes in metabolic state. In addition, key components of intermediary metabolism are cofactors or co-substrates of chromatin-modifying enzymes. Metabolic enzyme levels and substrate availability regulate chromatin-modifying enzymes, chromatin structure and gene expression. Adapted from Dai et al. (2020).

Metabolism can be divided into two classes: catabolic processes (the breakdown of molecules, which releases energy) and anabolic processes (the synthesis of proteins, lipids, and nucleic acids, which requires energy). Metabolic pathways such as methionine metabolism and redox balance, work to maintain the pool of several of these metabolites and thus help regulate the epigenomic landscape, in concert with chromatin modifiers, remodellers and transcription factors (**Figure 4**). Herein, although the underlying pathways linking metabolism with transcriptional regulation have not yet been fully elucidated, changes in metabolite availability are associated with specific molecular mechanisms that promote canonical and emerging DNA and histone modifications at targeted gene regions have been mechanistically linked to cellular signalling in health and disease (Dai et al., 2020; Melnyk et al., 2012; Zawia et al., 2009).

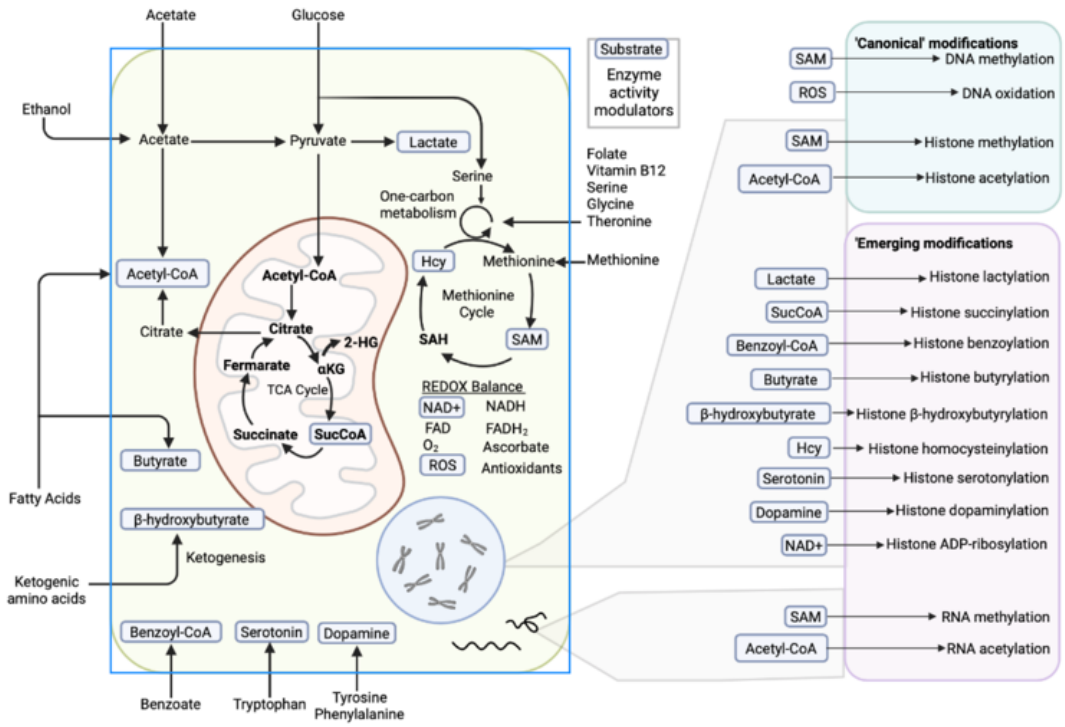

Figure 4. Metabolic pathways and chromatin-modifying metabolite production. Pathways related to central-carbon, one-carbon and methionine metabolism, acetate metabolism, ketogenesis, and redox balance feed the pools of several of these metabolites and thus help regulate the epigenomic landscape, in concert with chromatin modifiers, remodellers and transcription factors. 2-HG, 2-hydroxyglutarate; αKG, α-ketoglutarate; FAD, flavin adenine dinucleotide; Hcy, homocysteine (hcy as a histone modification); ROS, reactive oxygen species; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SucCoA, succinyl-CoA; TCA, tricarboxylic acid. Adapted from Dai et al. (2020).

A critical function of metabolism is oxidative phosphorylation, a process that uses oxygen to produce energy for the cell in the form of ATP as well as the regulation of mitochondrial reactive oxygen species (ROS) production from the mitochondrial respiratory chain, to maintain optimal cell function (Piedrafita et al., 2015; see **Figure 4**). Under conditions of excess nutrient supply and low ATP demand, increased ROS act as a feedback signal to slow substrate oxidation and to divert carbohydrates to storage as fat. This helps to decrease overall ROS production and facilitate more efficient energy usage. During an organism's lifespan environmental changes (i.e., osmotic imbalance, chemical, temperature, or biotic interactions with other species) can affect cellular functioning (Piedrafita et al., 2015). These stress-induced changes can then persist, affecting cellular metabolite levels as well as mitochondria biosynthesis and function (Piedrafita et al., 2015). Mitochondrial dysfunction or fluctuations in cellular oxygenation have been implicated in

different disease states. For example, a combination of excess nutrition and physical inactivity, an underlying driver for the metabolic syndrome, chronically over activates these redox signaling pathways and may contribute to pathologies associated with neurological disorders, such as Alzheimer's disease (AD) and autism (Melnyk et al., 2012; Zawia et al., 2009).

Epigenetic mechanisms controlling gene expression and oxidative damage pathways are in part mediated by alterations in DNA methylation at CpG dinucleotides (Zawia et al., 2009). CpG sites consist of cytosine-guanine repeats along a DNA sequencing in the 5'-3' direction. Methylation of the cytosine nucleotides in these regions are important for gene regulation. Tissue-specific gene expression is dependent on promoter methylation patterns, which are established *in utero* (Melnyk et al., 2012). DNA methylation is usually associated with gene silencing due to 1) the blocking of DNA binding proteins that act as or recruit transcriptional activators or 2) the recruitment of MBD proteins, which recruit transcriptional corepressor complexes (Valinluck et al., 2004). DNA methylation is regulated by metabolism as it is driven by the diet acquired essential amino acid methionine (Dai et al., 2020). Methionine undergoes a conversion to a methyl-donor metabolite, SAM, which is used as a substrate for DNA and histone methyltransferase (Dai et al., 2020). Disruption to this metabolic pathway, such as decreased metabolite or substrate availability, or altered metabolic states that protect cells under stressed conditions, have been shown to alter levels of DNA and histone methylation in both mice and humans (Dai et al., 2020; Piedrafita et al., 2015).

Oxidative stress and/or damage occurs when antioxidant defense mechanisms fail to control the amount of reactive oxygen species generated from metabolic pathways or environmental exposures to oxidative agents (Melnyk et al., 2012). Alterations in antioxidant systems, such as the initiation of the DNA damage pathway, has been associated with free radical damage seen in some idiopathic cases of AD and autism (Melnyk et al., 2012; Zawia et al., 2009). There is some evidence to suggest that the oxidation of guanine bases may impact specific aspects of epigenetic signalling, such as the binding of transcription factors (Ghosh & Mitchell, 1999; Valinluck et al., 2004). Specifically, 8-hydroxydeoxyguanosine (*8-OHdG*) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (*8-oxodG*) can be used as biomarkers of oxidative stress (Long et al., 2012; Ghosh & Mitchell, 1999;

Valinluck et al., 2004). *8-OHdG* is formed through the hydroxylation of guanine by reactive oxygen species and excreted via bodily fluids (Long et al., 2012). Thus, *8-OHdG* level found in the blood and urine can be used as an indicator to correlate the degree of internal DNA damage (Long et al., 2012). Although the interactions between DNA methylation and oxidative damage is still unclear, there is some evidence to suggest that oxidation of guanine in CpG dinucleotides reduce MBP binding (Valinluck et al., 2004). Monitoring cellular metabolism is therefore a central part of understanding disease states and developing effective interventions.

In summary, changes in chromatin not only contributes to DNA repair mechanisms, but changes in the state of chromatin (condensed vs. unpacked) is important in gene regulation during periods of cellular plasticity, such as development (Harabula &Pombo, 2021). Together the organization of chromatin, DNA and chromatin remodelling proteins allow for control over gene expression to maintain normal cell specific functions, which is of particular importance in the brain (Lamonica & Zhou, 2019). Detrimental changes to chromatin and chromatin remodelling proteins can negatively impact the proper functioning of important genes and therefore gene expression, causing gene mutations and brain disorders like Fragile X Syndrome and ASD, respectively. (Lamonica & Zhou, 2019; Zhao et al., 2018). Chromatin regulators, such as chromatin remodelling complexes, are involved in the formation, modification and maintenance of the epigenetic information critical for development (Lamonica & Zhou, 2019; Allis & Jenuwein, 2016).

1.5. *Chromatin Remodeling Proteins*

Chromatin remodelling complexes modify chromatin to allow for transcriptional signalling and ultimately proper gene expression. Chromatin remodeling proteins are often classified by the way in which the complexes interact with specific biological processes involving chromatin, such as chromatin assembly and editing (Clapier & Cairns, 2014). Remodeling complexes can be categorized as such that mediate post-translational modifications on the histone, or remodelers which alter the histone-DNA contact using ATP hydrolysis (Tyagi et al., 2016; Clapier & Cairns, 2009). Remodelers can also include enzymes that allow for proper spacing of the nucleosomes that controls gene repression by maintaining the tightly condensed packing state of chromatin (Clapier et al., 2017).

Dysregulation of these enzymes can cause ATP-dependent remodeling complexes to facilitate histone sliding, ejection and incorporations of histone variants (Clapier et al., 2017). Chromatin remodelers that utilize ATP can be classified into four family's knowns as, SWI/SNF2 (SWItch/Sucrose Non-Fermentable-2), ISWI (Imitation SWItch), CHD (Chromodomain-Helicase-DNA binding) and INO80 (INOsitol requiring 80) (Tyagi et al., 2016; Clapier &Cairns, 2014). Each of these subfamilies elicit different effects on the nucleosome and utilize different mechanisms for chromatin remodelling (Clapier et al., 2017). In this study, we explore a chromatin remodeler belonging to the SWI/SNF2 family, known as *ATRX* (Picketts et al., 1996; De La Fuente et al., 2011).

1.5.1. *ATRX*

ATRX is a chromatin remodelling protein that is essential for brain development and plays a key role in gene expression regulation (Weaver et al., 2017). It exerts its effects by binding to heterochromatin resulting in epigenetic modifications, such DNA demethylation, responsible for downstream gene activation. *ATRX* is regulated and finely tuned; small changes in expression can cause synaptic deficits contributing to neurodevelopmental disorders, such as autism.

As previously mentioned, *ATRX* belongs to the switch/sucrose non-fermenting family (SWI/SNF2), which utilizes ATP to facilitate chromatin remodeling (Picketts et al., 1996; De La Fuente et al., 2011). This family of remodelling complexes works to eject, insert or slide histones to disrupt the interactions between histone domains and the genomic DNA (Zhang et al., 2016; Becker & Workman, 2013). The approximate loci of *ATRX* on the X chromosome lies between xq12-q21.3, containing 36 exons which span approximately 300 kb pairs long (Gibbons et al., 1995; Picketts et al., 1996). The structure of *ATRX* can be divided into three main sections: A hydrophilic N-terminal, a central portion containing alternating hydrophilic and hydrophobic section, and finally a C-terminal domain (Picketts et al., 1996). Further investigations revealed that *ATRX* is comprised of an *ATRX-DNMT3-DNMT3L* (*ADD*) domain at the N-terminus and a *SNF2* helicase domain located at the C-terminus (Bartholomew, 2014). The central portion of the protein contains a heterochromatin protein 1 DNA-binding motif that allows for histone proteins to interact with the chromatin remodeler (Bartholemew, 2014). An important feature of *ATRX* is the *ADD* domain at the N-terminus. The *ADD* domain is a zinc finger domain,

which acts similarly to DNA methyltransferase 3 (DNMT3) (Gibbons, 2006; Xie et al., 1999). Zinc fingers are 100 bp nucleotide domains of one or more zinc ions, that ensure that the folds of the implicated protein are stabilized (Vandevenne et al., 2013). This *ATRX-ADD* domain interaction is the site of mutations associated with *ATRX* deficiencies (Gibbons et al., 2008).

ATRX functions as chromatin remodelling protein involved in the formation of heterochromatin in mammalian telomeres (De La Fuente et al., 2011). Typically, in embryonic stem cells of mice, *ATRX* is recruited to telomeres during the S phase of mitosis and plays an important role in telomere stability during the telomere replication process (Clynes & Gibbons, 2013). Telomers consist of tandem repeats which contribute to folding conformations that allow for protection against DNA damage (Sfeir et al., 2009). Telomere with G rich tandem repeats can form G quadruplex structures, which have been shown to be the preferential for binding *ATRX* (Clynes & Gibbons, 2013). G quadruplex structures slows or halts replication and transcription by forming bulking DNA or blocking these processes (Amorim et al., 2016). It's suggested that the binding of *ATRX* to these complexes works to combat the formation of bulky DNA by favoring histone deposition H3.3 that maintains the preferential conformation of DNA (Amorim et al., 2016; Clynes et al., 2015). Knowing this, *ATRX* deficits may result in an increased amount of bulky or damaged DNA, along with impeded replication and transcription (Amorim et al., 2016). Mutated or damaged cells avoid telomere shortening by the addition of tandem repeats by the use of telomerase, or via a telomerase-independent mechanism known as the Alternative Lengthening of Telomeres (ATL) pathway (Clynes et al., 2015). Mutations in the *ATRX*/*DAXX* chromatin remodelling complexes have been associated with increased activity of the ATL pathway, often utilized by certain subsets of human cancers and immortalized cell lines (Lovejoy et al., 2012). It has been shown that the loss of *ATRX* protein and mutations in the *ATRX* gene are hallmarks for ATL-immortalized cell lines, or the origin of ATL proliferating cells (Lovejoy et al., 2012). Additionally, loss of *ATRX* protein and mutations in the *ATRX* gene may work to impair the DNA damage response; an important DNA repair mechanism (George et al., 2020; Lovejoy et al., 2012). In summary, *Atrx* gene encodes for a *SNF2*-type chromatin remodeling protein which regulates gene expression by modifying the structure of chromatin, and mutations in *ATRX* of loss

of *ATRX* protein results in DNA replication stress and telomere instability in developing brains and gene specific tissues (Gugustea et al., 2019).

1.6. *Functional Importance of ATRX in Brain Development*

In mammals, the expression of *ATRX* is typically confined to cortical and hippocampal regions of the brain during development (Bérubé et al., 2005). These regions play an important role in a number of behavioural and cognitive functions. The hippocampus is especially important in spatial navigation as well as learning and memory, while cortical regions are associated with cognitive processes and emotional regulation (Sigurdsson & Duvarci, 2015). Loss of *ATRX* has been associated with differences in synaptic plasticity as well as memory deficits in mice (Gugustea et al., 2019). Alternatively, human mutations in *ATRX* are associated with genetic neurodevelopmental disorders such as ATR-X syndrome and α-thalassemia (Bérubé et al., 2005). ATR-X syndrome can be characterized by a variety of physiological and behavioural deficits, such as cognitive impairments, microcephaly, seizures, skeletal and genital abnormalities and developmental delays (Gibbons, 2006). ATR-X syndrome is often accompanied by α-thalassemia, which is associated with the downregulation of α-globin genes, producing a detectable hematological marker (Mitson et al., 2011; Clynes et al., 2013; Gibbons, 2006). As *ATRX* interacts with various binding partners it is considered an X-chromosome *trans* acting protein that facilitates proper functioning of many different genes during replication (Gugustea et al., 2019; Clynes et al., 2013).

Gain-loss studies have shown the functional importance of *ATRX* during development. Inactivation of *ATRX* results in reduced growth, increased telomeric transcription and telomere instability; early inactivation can result in lethality and reduced brain sizes (Bérubé, 2011; Garrick et al., 2006; Goldberg et al., 2010; Drané et al., 2010). Overexpression of *ATRX* results in more variable phenotypes, but still leads to negative effects on the developing embryo in both pre-and early post-natal periods. For example, *ATRX* overexpression can result in neurodevelopmental delays, craniofacial anomalies, increase incidence of embryonic death, neural tube defects and abnormal behaviours (Bérubé, 2011; Bérubé, 2002). Behavioural changes in offspring due to mutated *ATRX* can be regulated by mother-neonate interactions and early life experiences. Variations in mother-

neonate interactions induce long term epigenetic alterations, implicating development and proper functioning of neural circuits in adult offspring (Weaver et al., 2004).

1.7. *Effects of Early Life Experiences on Gene Expression*

Prenatal and postnatal environment plays a critical role during developmental periods and have an important impact on disease and health trajectories later in life (Szyf, M, 2009). Examples of this has been seen in birth weight correlations with the likelihood of developing obesity or heart disease (Szyf, M, 2009; Ozanne & Constancia, 2007). Additionally, maternal nutrition studies have shown that maternal environment (i.e., nutritional restrictions, gestational diabetes, maternal stress, drug exposure) impact intrauterine growth, physiology, metabolism and health of offspring (Weaver, 2014; Mousseau & Fox, 1998). Early life nutrition has been implicated in altering the bioavailability of enzymes essential for DNA synthesis and post-translational modifications, meaning that early life nutrition may impact epigenetic programming that exerts lifelong changes (Weaver, 2014). Exposure to toxins, such as cocaine and teratogens, during the prenatal period can contribute to impaired cognitive abilities, improper brain development and alters behaviour of the offspring (Singer et al., 2004; Kyle, 2006).

The effects of maternal prenatal environment have been widely studied in understanding the developmental impact of an altered prenatal environment, however postnatal environment should also be seen as a critical period that can influence development (Champagne, 2010). Longitudinal studies of abuse and neglect demonstrated the increased risk of cognitive impairment, social/emotional difficulties and risk of mental and physical disease (Champagne, 2010; Trickett & McBride-Chang, 1995). As previously mentioned, an area of the brain that is particularly susceptible to epigenetic changes caused by early life experiences is the HPA axis. Adults who experience childhood abuse or neglect are associated with increased HPA and immune system activation (Champagne, 2010; De Bellis et al., 1999). It has been well documented that maternal behaviour and mother-offspring relationship can influence an offspring's social and genetic outcome (Champagne & Curley, 2005; Francis et al., 1999; Lui et al., 1997; Champagne et al., 2003; Meaney, 2001). Reduced maternal care, as well as high environmental pre- and post-natal stress, have the capacity to alter genetic expression and induce lifelong

detrimental changes in the brain of developing offspring (Champagne et al., 2003; Meaney, 2001; Weaver et al., 2004).

1.7.1. *Gestational Stress*

Prenatal maternal stress or excess glucocorticoids has the potential to alter the expression levels of many genes that are essential for normal brain development, such as *ATRX*. During pregnancy, glucocorticoids are involved in shaping offspring health and behaviour outcomes which can produce lifelong effects. This means that the environment in utero may cause health and behavioural issues during post-natal development, which may persist into adulthood (Glover et al., 2018). Research suggests that stress during early pregnancy may hinder normal embryonic development (Grace et al., 2015). Subsequent research findings suggests that stress in the later stages of pregnancy may have detrimental effects on mental, emotional, and behavioural development in infancy and early childhood (Grace et al., 2015; Huizink et al., 2003; O'Connor et al., 2003). However, it is also important to note that severity of effects resulting from stress varies between offspring, with some experiencing little to no biological consequences (Glover et al., 2018; Glover, 2015). There is still a need for more research when determining if the type of stress (e.g., anxiety, depression, life event stress, etc.) influences outcomes for the offspring (Glover et al., 2018).

There are many different protocols used to simulate stress when working with animal models. To replicate the effects of gestational stress, pregnant rodent models can be exposed to a psychological/physical stress and effects on the brain and/or behaviour of parents and offspring can be quantified (Weinstock, 2017). Types of stressors employed can range from suspension, crowding, restraint, unpredictable noise and flashing lights (Baier et al., 2012). Although these protocols have a common goal to induce stress, the stressors may not be comparable and the stress response may vary in gestating mothers (Baier et al., 2012). Nonetheless, during pregnancy genetic changes as a result of maternal and environmental stress are passed on to the offspring, be it adaptive or detrimental (Glover et al., 2018). Stress during pregnancy is likely to influence postnatal relationships with offspring. In rodent studies, maternal stress can lead to a decrease in maternal care given to offspring during the postnatal period, which has been shown to reduce genome-wide gene expression (including *Atrx)* in certain brain regions (Weaver et al., 2006).

1.7.2. *Postnatal Mother-Offspring Interactions*

During the postnatal period, parental investment and nutrition provided determines gene expression profiles which contribute to the organization and function of neural circuits and molecular pathways that support growth, survival and behaviour (Weaver, 2014). Quality of postnatal environment has been associated with long-term changes in gene expression (Champagne, 2010). Prolonged periods of decreased maternal care results in increased HPA response to stress and impaired cognitive ability (Champagne, 2010; Lippman et al., 2007; Weaver, 2006). Studies focusing on the neurobiological and behavioural consequences of decreased maternal care during infancy have emphasized the role of early life experiences in the development of stress responsivity and certain social behaviours (Champagne & Curley, 2005; Harlow et al., 1965). In both rodent and primate models, adults who were raised in environments with decreased maternal care or artificial maternal care showed deficits in play behaviour, cognitive impairments and increased stress responsivity (Champagne & Curley, 2005; Lehmann et al., 1999).

In mice, maternal care is shown by the frequency of maternal licking and grooming (LG) of the offspring following the first few days of birth (Weaver, 2014). As previously mentioned, maternal care (i.e., LG) has lasting impacts on the HPA axis reactivity and anxiety-like behaviours (Weaver et al., 2006). High LG within the first few days of life can lead to better stress adaptivity and reduce anxiety-like behaviours (Weaver et al., 2006). An example of this can be seen in previous work done in the Weaver Lab, where both male and female mice who were born to a prenatally stressed mother; via social, cognitive and behavioural manipulations, exhibited a greater level of social avoidance, anxiety-like behaviour and cognitive delays. Similar studies have shown that offspring who receive increased LG will be better adapted to unfamiliar social interactions and less likely to exhibit anxiety-like behaviour (Liu et al., 1997). In many species, including mice, adulthood peer interactions can be influenced by early social experiences (Champagne and Curley, 2009). This is demonstrated by studies looking at female offspring and quantity of maternal care given (High vs Low LG) during the initial postnatal period (Champagne & Curley, 2005). Female offspring raised in low LG environments provided low LG to their offspring, while females raised with high LG provided high LG (Champagne & Curley, 2005; Champagne et al., 2003; Francis et al., 1999).

In summary, when offspring are subject to differential maternal care in the form of LG within the first few days of life, the maternal care ensued upon them actively aids in normal development of the neural systems, with variations in maternal grooming directly influencing hippocampal development (Weaver et al., 2006; Liu et al., 2000). Early social interactions can be transmitted across generations via maternal behaviour and are mediated by alterations within the HPA axis (Champagne & Curley, 2005; Champagne et al., 2003; Francis et al., 1999; Weaver et al., 2006).

1.8. *Generation of ATRX Gene Targeted Mice*

A gene targeted approach can be used to create *ATRX* deficient animal models. This is particularly useful when studying the physiological and behaviours effects caused by reduced or mutated *ATRX*, much like those that were previously mentioned (Bérubé et al., 2006; Gibson, 2006). In mice, *ATRX* expression has been detected as early as day 11 in embryonic development (Bérubé et al., 2005). Because of this, conditional knockout (cKO) models are used to silence *ATRX* expression around embryonic day 10-12 in the C57BL/6 background strain. To do this, mice were generated using a Cre-LoxP recombination system. Cre recombinase is a site-specific recombinase enzyme that recognizes and binds LoxP, a sequence of palindromic repeats (Szeberényi, 2013). When two LoxP sequences flank the target sequence it leads to deletion of the sequence between the LoxP sites (**Figure 5**) (Szeberényi, 2013). Since LoxP sequences don't naturally exist in mammalian DNA, in order to generate *ATRX* floxed female mice, two LoxP sites are inserted into the *Atrx* sequence which flanks exon 18, encoding the ATP-ase domain (Szeberényi, 2013; Bérubé et al., 2005). The female mice are then bred with male mice expressing Cre recombinase to produce the sequence deletion via Cre/LoxP recombination (Szeberényi, 2013). Cre expression of offspring resulting from Cre/LoxP recombination is driven by the *Emx-1* gene (Gorski et al., 2002). *Emx-1* is simultaneously expressed with Cre-recombinase in the developing cerebrum around embryonic day 11-12 and plays a role in the differentiation and proliferation of neural stem cells (Gorski et al., 2002). Mutations in the *Atrx* gene are located on the X chromosome, which means that using this model, male mice will be Cre-positive hemizygous and express un unstable form of *Atrx*. These male mice will be referred to as $Atrx^{HEM}$. Additionally, female mice will express wild-type *Atrx* ($Atrx^{WT}$) as a result of the aforementioned Cre/LoxP system.

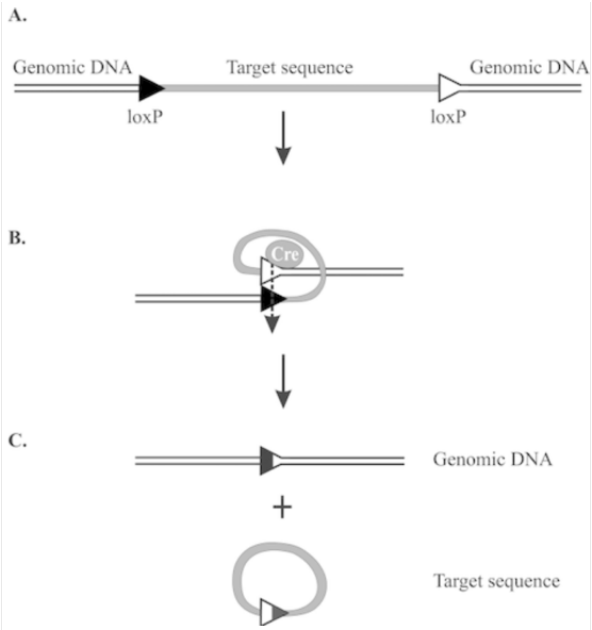

Figure 5. Gene deletion by Cre/LoxP system. Where (A) represents LoxP sites flanking a target sequence, which is able to complementary base-pair while Cre protein binds (B) resulting in target sequence deletion from the genomic DNA (C).

1.9. *ATM*

Ataxia Telangiectasia mutated (*ATM*) protein is essential in detecting double-stranded DNA breaks and activating molecular pathways during the DNA damage response (DDR) (Cara et al., 2016; Shiloh, 2006). *ATM* belongs to a family of signalling proteins called 'PI3K-like protein kinases' (PIKKs), which contain serine/threonine kinase activity that phosphorylates various substrates during DDR (Shiloh, 2006; Abraham, 2004). Signalling proteins in the PIKKs family, such as *ATM*, play a central role in cell growth, gene expression and genome surveillance in response to replication stress (Abraham, 2004). Much like *ATRX, ATM* activation is regulated by H3.3; with loss of H3.3 causing *ATM* deficiency (Han et al., 2018; Zhang et al., 2016). Since *ATRX* is mediated by the ADD domain to facilitate H3.3 binding, studies suggest that *ATRX* might play a role in mediating DNA damage by regulating the activity of *ATM* (Han et al., 2018). Genomic integrity is maintained by a number of biological mechanisms, such as replication, cell check points and DNA damage repair (Czornak et al., 2008). Exogenous or endogenous DNA-damaging agents may cause double-stranded breaks, resulting in DNA legions which can have various detrimental effects (Czornak et al., 2008). For example, *ATM* activation due to double-stranded breaks promotes cellular senescence or apoptosis

as a form of DNA repair (Vačevska et al., 2020; Denchi & de Lange, 2007; Karlseder et al., 1999). DNA repair machinery works to maintain genomic integrity and is composed of a network of over 700 proteins, all of which are phosphorylated by *ATM* and other PIKKs (Czornak et al., 2008; matsuoka et al., 2007). When double-stranded breaks occur, repair machinery works to activate *ATM* and other PIKKs (Czornak et al., 2008). Specifically, *Mre11-Rad50-Nbs1* (*MRN*; also known as *Nibrin* or *p95*) protein complexes work to increase DNA-tethering which induces conformational changes in *ATM* from monomeric form into the active form (Czornak et al., 2008; Shiloh, 2006). *ATM* then has the ability to recruit more *ATM* to the damaged DNA sites and additional downstream targets, by amplifying DNA damage signals in a cyclical process (
Figure 6) (Shiloh, 2006; Shiloh & Lehmann, 2004).

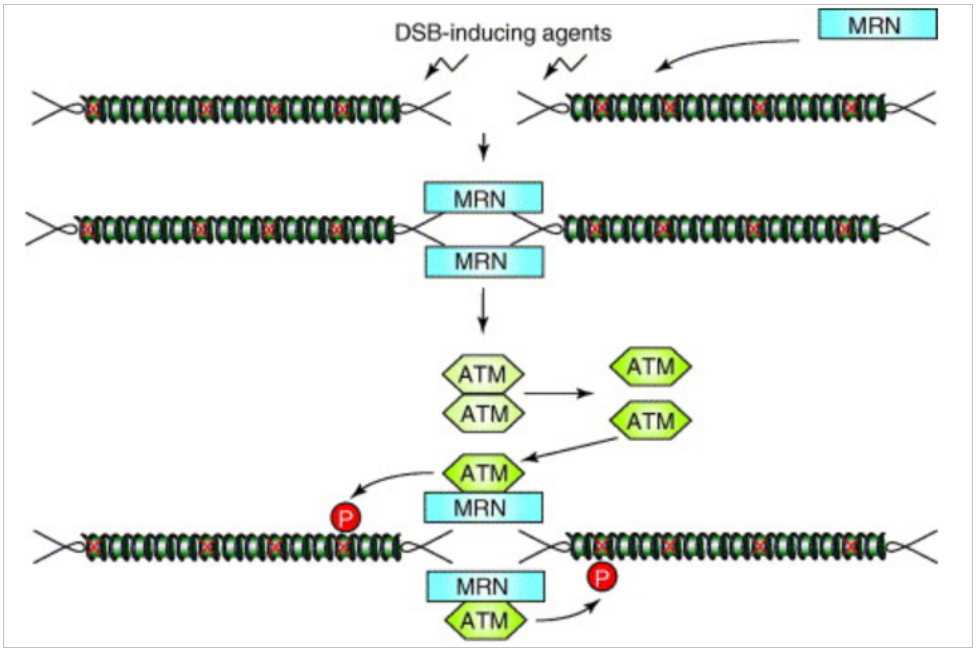

Figure 6. Early events in the DNA-Damage Response to double-stranded DNA breaks, where *MRN* complexes are recruited to the damage site and activates *ATM* to respond in a cyclical manner. Adapted from Shiloh (2006).

Loss of *ATM* results in clinical diagnosis of ataxia telangiectasia (A-T), a genomic instability syndrome (Shiloh, 2006; Han et al, 2018). A-T is characterized by early childhood onset of neuronal degeneration, resulting in neuromotor dysfunction, immunodeficiency, chromosomal fragility, predisposition to certain cancers and extreme sensitivity to double-strand DNA breaks (Shiloh, 2006; Chun & Gatti, 2004). This genetic disorder has

an autosomal recessive pattern and can be diagnosed in the individuals presenting symptoms, with subsequent testing performed on siblings, after symptoms become most apparent around age ten (Perlman et al., 2003). Genetic testing can be used as a conformation of disease by measuring *ATM* protein levels, *ATM* kinase activity and *ATM* mutations (Chun & Gatti, 2004; Perlman et al., 2003). Studies have shown that A-T presents with the absence or dysfunction of the *ATM* protein and mutations in the *Atm* gene (Chun & Gatti, 2004). Treatment for A-T is based on symptom management and tailored to the individual needs of the patient. Often neurorehabilitation is used to combat the rapid neurodegeneration (Perlman et al., 2003). Additionally, drug treatments may be useful in easing the severity of symptoms, as well as surgical corrections and genetic counselling to increase disease understanding (Perlman et al., 2003).

Proper functioning of *ATM* relies heavily on environmental conditions during a narrow range of time during development (Cara et al., 2016). Interestingly, in addition to inducing senescence or apoptosis in response to DNA damage, *ATM* also play a key role in cells of the central nervous system (CNS) and works to signal pathways used for mediating oxidative stress, insulin signalling, synaptic vesicle behaviour and GABAergic development and excitatory/inhibitory equilibrium in hippocampal neurons (Pizzamiglio et al., 2020). Knowing this, *ATM* is said to play an essential role in neuronal homeostasis and involved in additional neurological conditions beyond A-T (Pizzamiglio et al., 2020). Studies using Drosophila models have shown that even partial reduction in *ATM* activity causes neurodegeneration similar to AD and Parkinson's disease (Pizzamiglio et al., 2020; Petersen et al., 2012). The involvement of *ATM* and cognition has been widely described. In A-T, cognitive processes like attention, memory, decision making, and learning have been found to be significantly impaired, with involvement of both the cerebellum and the hippocampus (Pizzamiglio et al., 2020; Hoche et al., 2014; Volkow et al., 2014).

Together, these finding highlight how *ATM* is fundamental in DNA damage repair and cognition. Although the relationship between *ATRX* and *ATM* has only begun to be investigated, there is reason to suggest the activity of these genes and their protein products can be influenced by stress and DNA damage, in turn regulating cell survival and/or cell death. Additionally, the mechanism in which *ATRX* acts upon *ATM* has yet to be

discovered but may play an important role in understanding the complex process of early neuronal development.

1.10. *Present Research*

Although there is an abundance of research pertaining to maternal care and development, there is a lack of understanding in the molecular mechanisms underlying neurodevelopmental disorder acquisition, such as idiopathic autism. By studying the influence of early life experiences on the expression of gene that are essential for neurodevelopment, such as *ATRX* and *ATM*, a more complete understanding of gene-environment mechanisms may be obtained.

The aims of this study surround two hypotheses. First, that maternal stress during pregnancy and low levels of maternal LG during the first week of postnatal life is associated with decreased *Atrx* gene expression in the frontal cortex, hippocampus and cerebellum of adult offspring. Secondly, that reduced *Atrx* gene expression and reduced function inhibits the expression of *Atm*, that controls neuronal cell metabolism, growth and survival in the offspring. In attempts to determine the relationship between prenatal stress, frequency of maternal LG behaviour and changes in neuronal gene expression (*Atrx, Atm*), banked whole brain mouse tissue will be used from a colony that has been genetically altered to have lower levels of *ATRX* protein and wildtype control mice (*C57/BL6J* background). Seahorse analysis, metabolic staining and enzyme-linked immunosorbent assays (ELISAs) will allow for the analysis of cellular metabolism and DNA oxidative stress biomarkers in transgenic and wildtype mice. Real-Time Quantitative Polymerase Chain Reactions (RT-qPCR) will allow for the analysis and quantification of *Atrx* expression in the frontal cortex, hippocampus and cerebellum of adult male and female, prenatal-stressed and non-stressed tissue. Additionally, bisulfite pyrosequencing will be used to determine the pattern of DNA methylation on the *Atrx* and *Atm* promoters.

This study will build on our understanding of the molecular mechanisms involved in epigenetic programming in offspring by maternal behaviour and how the effect of adverse early-life experiences is rendered permanent. From a biological perspective, this work will provide novel insights into brain development and the mechanisms underpinning complex genetic disorders.

1.11. *Main Objectives*

I. Characterize mitochondria respiration and DNA oxidative stress in the forebrains of *Atrx*^{WT} and *Atrx*^{HEM} mice that are stress naïve or exposed to stress *in utero*.

II. Determine *Atm* expression in the forebrains of *Atrx*^{WT} and *Atrx*^{HEM} mice that are stress naïve or exposed to stress *in utero*.

III. Explore the effects of early life stress exposure and reduced quality of maternal care on *Atm* expression in the forebrain.

IV. Compare changes to epigenetic regulation of the *Atm* promoter in response to modified *Atrx* expression, and early life stress.

CHAPTER 2: MATERIALS AND METHODS

2.1. *Tissue Collection and Dissection*

Prior to dissection, records were obtained for each frozen post-mortem brain pertaining to gender, generation and allelic frequency. Dissections were performed on a chilled metal plate to obtain sections of the frontal cortex, hippocampi and cerebellum. Placed ventral side down, whole brains were split along the interhemispheric fissure using a scalpel and spatula. From each hemisphere, the cerebellum was isolated. From the remaining hemispheric tissue, the olfactory bulbs were removed, and the prefrontal cortex was isolated. The section was rotated so that the medial surface was exposed. Using a scalpel, the medial surface was removed, and the hippocampus was separated from the cortex. Each desired brain region was divided into equal sections and placed into 1.5mL microcentrifuge tubes. Each section was weighed and stored at -80ºC.

2.2. *Extracellular Flux Analysis*

The effects of *Atrx* deficiency and/or gestational stress on mitochondrial function in frontal cortex, hippocampus and cerebellum was assessed using a XF24 extracellular flux analyzer following the manufacturer's instructions (Seahorse Bioscience, Billerica, MA, USA). The XF24 creates a transient, 7 µl chamber in specialized microplates enabling oxygen concentrations associated with respiring neurons to be determined in real time. The assay uses the built-in injection ports on XF sensor cartridges to add modulators of cell respiration into each well during the assay to reveal the key parameters of mitochondrial function (**Figure 7**). The metabolic rate of the same cell population is measured repeatedly. To maintain normal cell physiology, a temperature control system maintains the cellular environment at 37°C.

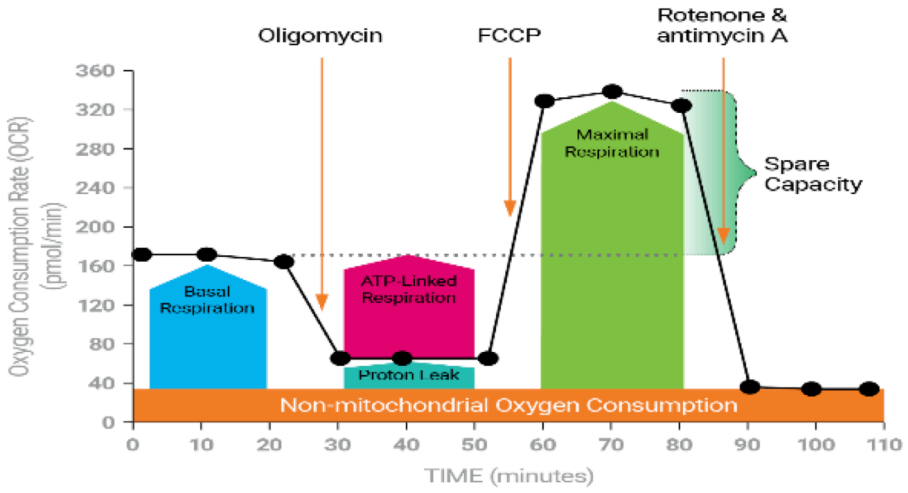

Figure 7. Agilent Seahorse XF Cell Mito Stress Test profile, showing the key parameters of mitochondrial function. Adapted from Manual Part Number 103016-400 (2006).

In brief, 2 x10^6 frontal cortex, hippocampal, and cerebellum cells dissociated in XF assay media (unbuffered DMEM containing 10 mM glucose, 2 mM glutamine and 1 mM pyruvate) were adhered on XF24 plates (using Cell TAK) and assessed under basal conditions and in response to the following modulators of the electron transport chain (ETC) activity within the mitochondrial inner membrane (**Figure 8**). The first injection was 1 µM oligomycin (O4876; Sigma), which inhibits ATP synthase (complex V). It decreases electron flow through the ETC, resulting in a reduction in mitochondrial respiration (i.e., a reduction in the oxygen consumption rate, OCR). This decrease in OCR is linked to cellular ATP production. The second injection was 2.5 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (C2920; Sigma), which is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential. As a result, electron flow through the ETC is uninhibited, and oxygen consumption by complex IV reaches the maximum. The FCCP-stimulated OCR is then be used to calculate spare respiratory capacity, defined as the difference between maximal respiration and basal respiration. Spare respiratory capacity is a measure of the ability of the cell to respond to increased energy demand or under stress (Xun, et al., 2012). The third injection was a mixture of 0.1 µM rotenone (R8875; Sigma), a complex I inhibitor, and 1 µM antimycin A (AMA) (A8674; Sigma), a complex III inhibitor. This combination shuts down mitochondrial respiration and enables the calculation of non-mitochondrial respiration driven by processes outside the mitochondria. Finally, the Seahorse software

generates a 'Cell Energy Phenotype Test' to evaluate the metabolic potentials for OCR and extracellular acidification rate (ECAR) by calculating changes after stress conditions relative to baseline. OCR is reported in units of pmol/minute and ECAR in mpH/minute.

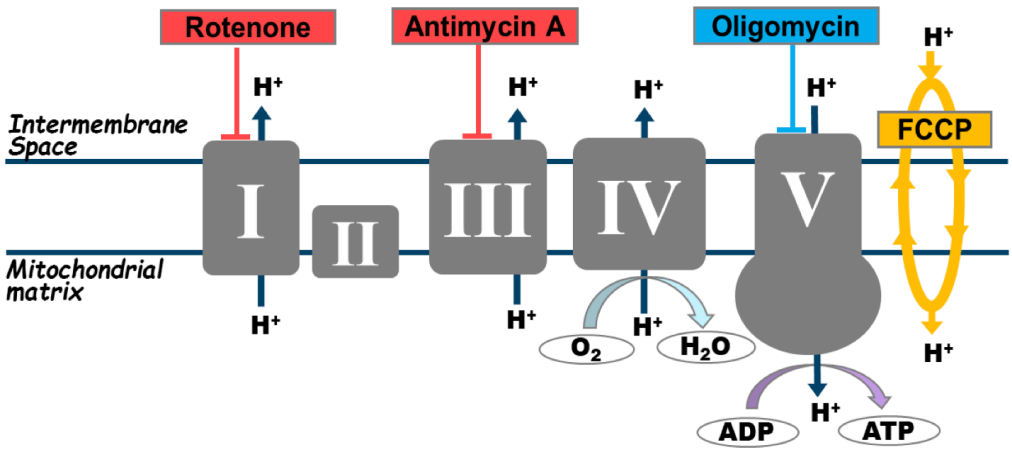

Figure 8. Agilent Seahorse XF Cell Mito Stress Test modulators of the electron transport chain (ETC). Adapted from Manual Part Number 103016-400 (2006).

2.3. *Metabolic stain*

The effects of *Atrx* deficiency and/or gestational stress on reactive oxygen species (ROS) H_2O_2 levels in frontal cortex, hippocampus and cerebellum cells was assessed using a BD FACSCanto™ II Flow Cytometer System following the manufacturer's instructions (Becton, Dickinson and Company Bioscience, San Jose, USA). Frontal cortex, hippocampal, and cerebellum cells were incubated (30 min, 37°C) with 2.5 µM of the general oxidative stress indicator CM-H2DCFDA (Molecular Probes #C6827) in fluorescence-activated cell sorting (FACS) buffer (PBS pH 7.4 [Gibco #10010], 1% FBS, and 5 mM EDTA). Excess stain was washed off and the mean fluorescence intensity (MFI) was measured by flow cytometry and analysed with BD FACSDiva software (provided with the instrument).

2.4. *Enzyme Linked Immunosorbent Assay*

One of the three sets previously divided tissue samples of the frontal cortex, hippocampus and cerebellum of each mouse was thawed and placed in 5 ml of homogenization buffer (0.1M phosphate buffer, pH 7.4, containing 1mM EDTA). The tissue was homogenized by sonication (40 %, 6x10 sec pulses) and centrifuged for 10 minutes at

1,000xg. The supernatant was purified using the reagents and protocol from the DNeasy® DNA Kit (Qiagen, Valencia, CA, USA). The extracted DNA was digested using nuclease P1 (Sigma N8630) following the manufacturer's instructions. Tris (M) was used to adjust the pH to 7.5-8.5. One unit of alkaline phosphatase was added per 100µg of DNA and incubated for 30 minutes at 37°C, before boiling for 10 minutes and placing on ice. ELISA were used to detect 8-Hydroxy-2'-deoxyguanosine (*8-OHdG*) levels according to the protocol provided with the kit (detection range, 0.94 to 60ng/ml; Cat. #: SKT-120-96S, StressMarq Biosciences Inc.). From the pilot results for each assay, 10 to 40 ng of each sample was loaded in triplicate alongside an eight-point standard curve in duplicate (with controls) in the 96-well microtiter plates supplied by each manufacturer. Detection was performed using horseradish peroxidase-labeled, Fc-specific IgG and read in the microplate reader at a wavelength of 450nm. The blank corrected *8-OHdG* levels for each sample are reported in ng/ml.

2.5. *RNA Extraction Procedure and Integrity Confirmation Assay*

RNA extraction was performed on one of the three sets of tissue samples using the reagents and protocol from the RNeasy® Plus Mini Kit (Qiagen, Valencia, CA, USA). Immediately following the extraction, RNA concentration was measured with 2µL sample on the Take3 micro-volume plate specified for the Epoch Microplate Spectrophotometer (Biotek, Winooski, VT, USA). Parameters were set using 260/280nm ratio with a 320nm reference. Any samples with a ratio smaller than 1.8 were excluded as this would indicate protein contamination. Additionally, the integrity of 12 RNA samples were assessed by loading 100ng/uL of each RNA sample onto an RNA StdSens chip, which was processed on an Experion Automated Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). RNA Quality Indicator (RQI) values above 7.8 indicate acceptable integrity for RT-qPCR (Taylor et al., 2010). The RQI value for all samples tested was >8 with low degradation (**Figure 9**). All RNA samples remained stored at -80°C until used.

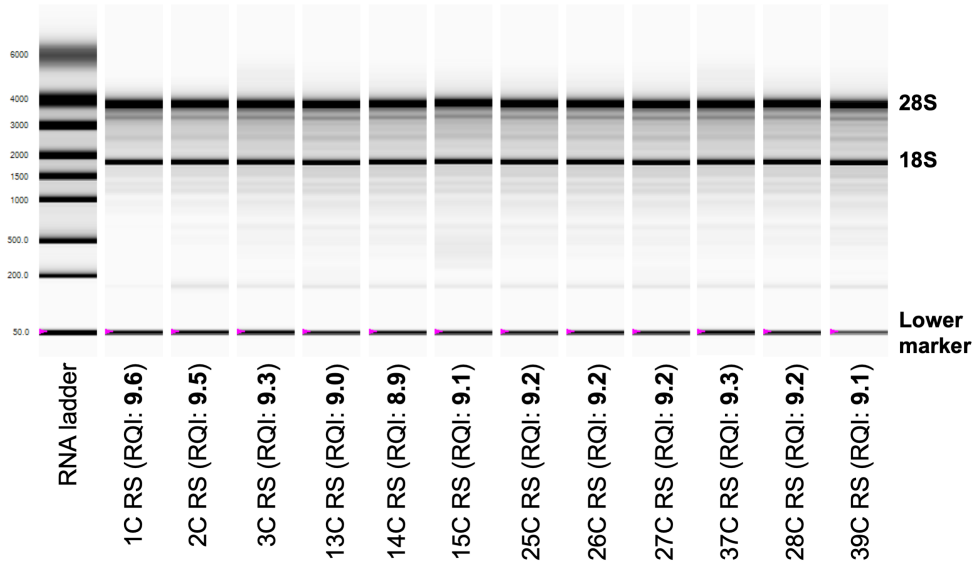

Figure 9. Virtual gel generated by Experion automated electrophoresis system. RNA ladder values are fluorescence units. The gel shows successful separation of the samples with a lower marker peak that is at least 5 fluorescence units above the baseline and well-resolved 18S and 28S ribosomal RNA (rRNA peaks). Lane loading and RQI for each sample is listed.

2.6. *Reverse Transcription and Quantative PCR*

Extracted RNA was used to make complimentary DNA (cDNA) via reverse transcription by generating 20µL reaction in 200µL PCR strip tubes in the C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and following the thermal cycling protocol (**Table 1**). This reaction requires the use of a reverse transcriptase (RT) known as iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Two controls were used to assess contamination across the samples. The first was a control without any RNA sample, known as no-template control (NTC). For this control sample volume was substituted with RNA-free water. The second control doesn't contain RT (NRT), by using No-RT Control Supermix instead of the iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Once the reaction was complete, 2µL of each sample of cDNA was pooled into a 1.5mL microcentrifuge tube and used to generate a concentration gradient by diluting samples to $1x10^n$ (n=number in dilution series) for five dilutions.

Table 1. Temperature cycling protocol for reverse transcription to generate cDNA in the C1000 TouchTM Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Step	Time (minutes)	Temperature (ºC)
Priming	5	25
Reverse transcription	20	46
Reverse transcriptase inactivation	1	95

The five-dilution series of pooled cDNA was used to optimize the concentration and temperature conditions for each primer used in RT-qPCR (**Table 3**). See **Table 2** for primer sequences. The dilutions were run in triplicate technical replicates in the CFX96 qPCR Instrument (Bio-Rad Laboratories, Inc., Hercules, CA, USA). During the primer optimization, the thermal cycling protocol finished with a melt curve analysis, which ranged from 65-96ºC and increased by 0.5ºC every second (**Table 3**).

Table 2. Primer sequences used in RT-qPCR reactions.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Atm	CAGCAGCACCTCTGATTCTTACAAC	ACCTTAGCCTTAGGACCTGACTGG
β-actin	TTGCTGACAGGATGCAGAAG	ACATCTGCTGGAAGGTGGA
CycA	CATCCTAAAGCATACAGGTCCTG	TCCATGGCTTCCACAATGTT
HPRT	AAGCTTGCTGGTGAAAAGGA	TTGCGCTCATCTTAGGCTT

Table 3. Temperature cycling protocol for RT-qPCR in the CFX 96 qPCR Instrument (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Step	Time	Temperature (ºC)
Activation	2 min	95
Denaturation	5s	95
Annealing/ Extension	30s	62.7 for *Atm* 60 (*β-actin, CycA, HPRT)*
Melt-curve (only for primer temp optimization)	0.5s/each increase in 1 ºC	65-95

For *Atm, β-actin* and *HPRT*, the optimal dilutions were $1x10^1$. The optimal dilution for *CycA* was $1x10^2$.These dilutions led to quantitative cycle (Cq) values between 20-30 for pooled cDNA samples. For *Atm* the optimal annealing temperature was determined to be 64ºC, while for *CycA* it was determined to be 63.4ºC, while *β-actin* and *HPRT* was 60ºC. For RT-qPCR, a master mix was generated by using 5µL of Sso Advanced Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Additionally, 0.5µL of 10µM forward primer and 5µL of 10µM reverse primer, and 2µL of PCR-grade water was added to the master mix. These measurements were used for each sample. Using a 96 well plate, the 10µL reaction for each well was made up of 8µL of master mix and 2µL of template cDNA, using the optimal dilution.

There were eight biological replicates per group and each sample was run in three technical replicates. When technical replicates deviated more than 0.2Cq, they were repeated and the average of the triplicates within 0.2Cq was used. An inter-plate calibrator (IPC) was used in order to compare samples that spanned over multiple plates. The IPC was generated using the previously pooled cDNA diluted to $1x10^1$, using *ATM* primers in the master mix. For each data set, the threshold was set according to the Cq value for the IPC at the temperature at which the plate was run.

The average Cq for each sample was used to calculate the normalized expression (ΔCq) with *β-actin, CycA,* and *HPRT* as reference genes, using CFX Maestro Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.7. *DNA Extraction and Bisulfite Pyrosequencing*

Genomic DNA (gDNA) extraction from one of the three sets of tissue samples was performed using DNeasy® Blood and Tissue Kit and protocol in the July 2006 handbook (Qiagen, Valencia, CA, USA). Concentration and purity of the samples were quantified by placing 2µL of sample on the Take3 micro-volume plate specified for the Epoch Microplate Spectrophotometer (Biotek, Winooski, VT, USA). Parameters were set using 260/280nm ratio with a 320nm reference. Any samples with a ratio smaller than 1.8 were excluded as this would indicate protein contamination. Measured concentration outputs (ng/µL) were used to determine the volume of template gDNA required to achieve 20ng; the optimal amount of template gDNA used for pyrosequencing (Cummings et al., 2013).

Once the determined volumes for each sample was generated, the template gDNA underwent a bisulfite conversion reaction using protocol and reagents from the Epitect® Bisulfite Kit (Qiagen, Valencia, CA, USA). This process is used to convert cytosine residues to uracil residues through deamination without affecting the 5-methlycytosine residues (Delaney et al., 2015). The reactions were prepared in 200µL PCR strip tubes and cycled through the C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the temperature cycling protocol (**Table 4**). Once the bisulfite conversion was complete, the DNA was purified using reagents and specialty spin columns provided in the Epitect® Bisulfite Kit to terminate the conversion reaction and remove contaminants.

Table 4. Temperature cycling protocol for bisulfite conversion reaction in the C1000 TouchTM Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Step	Time (minutes)	Temperature (ºC)
Denaturation	5	95
Incubation	25	60
Denaturation	5	95
Incubation	85	60
Denaturation	5	95
Incubation	175	60
Hold	Indefinite	20

ATM gene promoter primers were generated for PCR amplification on bisulfite treated DNA using EpiMark® Hot Start *Taq* DNA polymerase (New England BioLabs® Inc., Ipswich, MA, USA) in the C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the optimized temperature cycling protocol (**Table 5**). To verify the PCR product created the desired amplicons, some samples were run on a 2% agarose gel and visualized with UView™ 6x Loading Dye (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The samples were compared to a 50-2000bp AmpliSize® Molecular Ruler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR products were excised and extracted from the gel using the protocol from July 2015 and reagents from the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA, USA).

Table 5. Temperature cycling protocol for PCR amplification reaction in the C1000 TouchTM Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Step	Time	Temperature (ºC)
1	30 seconds	95
2	30 seconds	95
3	60 seconds	56
4	30 seconds	68
5	Repeat steps 2-4, 50 times	
6	5 minutes	68
7	Indefinite	4

After checking the PCR products produced the desired base pair length using the primers listed in **Table 6,** all PCR products which weren't run on a gel were purified using the QIAquick® Purification Kit (Qiagen, Valencia, CA, USA). The purified PCR products were used for pyrosequencing using PyroMark® Q24 Advanced software and pyrosequencer (Qiagen, Valencia, CA, USA) The December 2012 protocol was used along with PyroMark® Advanced CpG Reagents (Qiagen, Valencia, CA, USA).

Table 6. Primer sequences used in PCR reaction and for pyrosequencing.

Gene	F primer (5'-3')	R primer (5'-3')	Sequencing Primers (5'-3')
Atm	(Biotin) GTTTTATTGGAGGT GTTGAATT	CTCCCCTCAAAA CAATCT	CCCCTCAAAACAATCTC

2.8. *Statistical Analyses*

Animals were earmarked and given a unique number. Akaike information criterion (AIC) scores, likelihood ratios (LR) and p-values were reported (Wagenmakers & Farrell, 2004). Samples were analysed using the R package lme4, which states that a higher AIC score indicates a better model as the output score is for the model lacking that factor (i.e., the AIC score for genotype is for the statistical model lacking genotype as a factor). Linear mixed-effects models were used for the group comparisons, where genotype ($Atrx^{WT}$ or $Atrx^{HEM}$) and stress condition (home cage or restraint stress) were used as

the between factors, and brain region [frontal cortex (FC), hippocampus (HIPP), cerebellum (CER)] was used as the within factor, independently for the male and female mice from F0 and F1 generations. Interactions were analysed using 95% confidence intervals (CIs). The R stats version 3.6.2 – "Dark and Stormy Night" (https://www.r- project.org; R Foundation for Statistical Computing, Vienna, Austria) was used for all of the analyses and the graphs were generated in Graph Pad Prism VII® using group means and standard errors.

CHAPTER 3: RESULTS

3.1. *Mitochondrial Respiration and ROS Production*

We quantified the oxygen consumption rate (OCR), an indicator of mitochondrial respiration, and extracellular acidification rate (ECAR), an indicator of glycolysis, in the frontal cortex, hippocampus, and cerebellum of F1 male and female mice (Dranka, et al., 2010). Genotypes of the F0 male groups were *Atrx*^{WT} and *Cre*, where none of the mice were exposed to stress. F1 male groups consisted of two genotypes (*Atrx*^{WT} and *Atrx*^{HEM}) and two stress conditions (stress naïve and exposed to gestational stress). F0 female groups were *Atrx*^{WT} and floxed *Atrx,* with two stress conditions (stress naïve and gestational stress). F1 female groups consisted of one genotype (*Atrx*^{WT}), two rearing conditions (raised with male *Atrx*^{WT} mice and raised with male *Atrx*^{HEM} mice), and two stress conditions (stress naïve and exposed to gestational stress). F1 male *Atrx*^{HEM} mice had decreased levels of *ECAR* compared to male *Atrx*^{WT} mice (AIC = -54.121, LR = 13.42, p = 0.05; **Figure 10A**).

F1 males exposed to gestational stress had decreased levels of *ECAR* compared to male *Atrx*^{WT} mice (AIC = -52.231, LR = 8.22, p = 0.05). There was also an interaction between genotype and brain region (AIC = -67.411, LR = 14.20, p = 0.001). According to the 95% CIs, the effect of genotype was only significant in the frontal cortex (CI 95% = 0.071, 0.425) and the hippocampus (CI 95% = -0.05, 0.232), but not the cerebellum (CI 95% = -0.066, 0.115). Finally, there was an interaction between genotype, gestational stress condition, and brain region (AIC = -67.812, LR = 7.74, 0.050). F1 female (all *Atrx*^{WT}) mice raised with male *Atrx*^{HEM} siblings had decreased levels of *ECAR* compared to F1 females raised with male *Atrx*^{WT} siblings (AIC = -73.643, LR = 11.01, p = 0.05; **Figure 10B**). In addition, female mice exposed to gestational stress had decreased levels of *ECAR* compared to stress naïve F1 females (AIC = -45.643, LR = 12.64, $p < 0.05$).

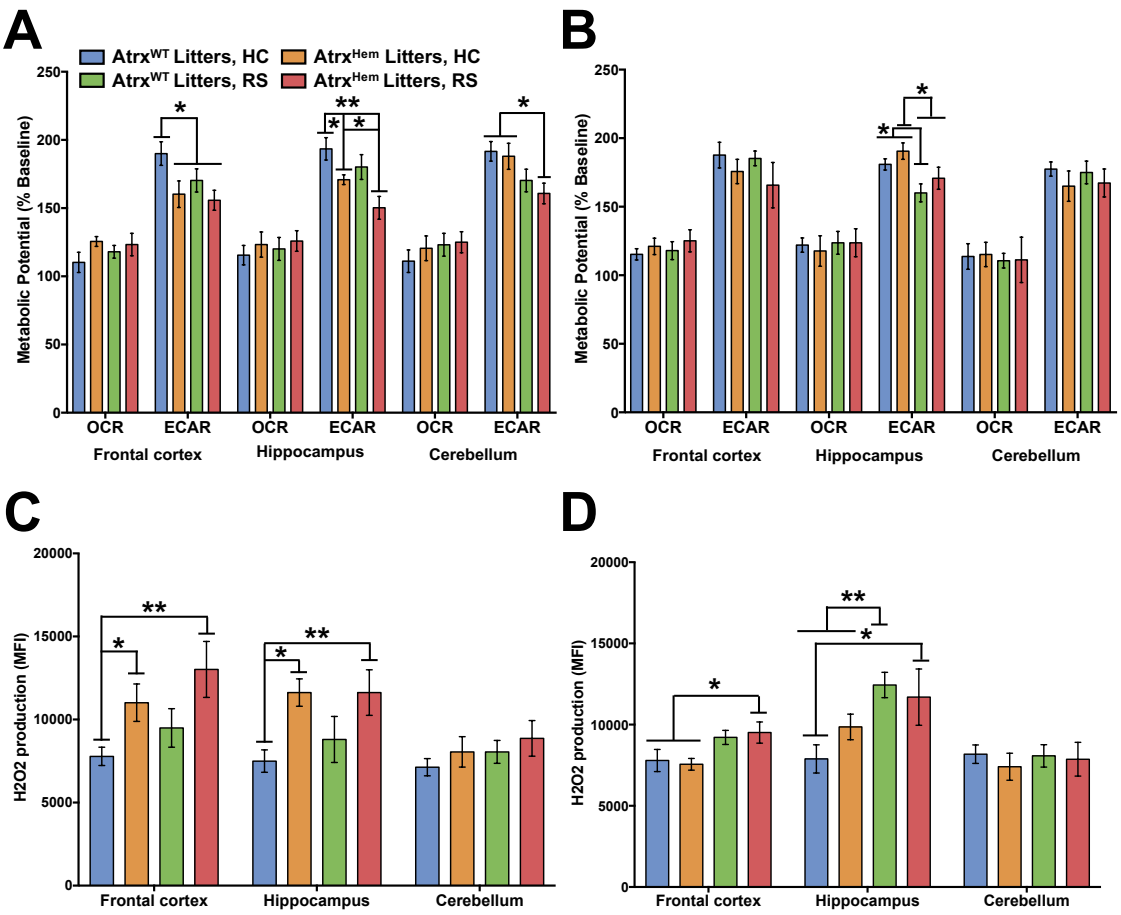

Figure 10. Effect of prenatal stress exposure and/or *Atrx* deficiency on mitochondrial respiration (A,B) and ROS production (C,D) in adult male and female offspring. OCR/ECAR and H_2O_2 levels in (A,C) *Atrx*^{WT} or *Atrx*^{HEM} male offspring from HC or RS mothers, (B,D) *Atrx*^{WT} female offspring from HC or RS mothers and raised with *Atrx*^{WT} or *Atrx*^{HEM} males. Mean ± SEM. (N=4/group; *p<0.05, **p<0.001).

We quantified hydrogen peroxide (H_2O_2) levels in the frontal cortex, hippocampus, and cerebellum of F1 male and female mice (**Figure 10C, D**). F1 male *Atrx*^{HEM} mice had increased levels of H_2O_2 compared to male *Atrx*^{WT} mice (AIC = -73.645, LR = 10.56, p = 0.05; **Figure 10A**). F1 males exposed to gestational stress had increased levels of H_2O_2 compared to male *Atrx*^{WT} mice (AIC = -46.342, LR = 10.64, p = 0.005). There was also an interaction between genotype and brain region (AIC = -76.634, LR = 11.68, p = 0.001). According to the 95% CIs, the effect of genotype was only significant in the frontal cortex (CI 95% = 0.083, 0.654) and the hippocampus (CI 95% = -0.04, 0.4325), but not the cerebellum (CI 95% = -0.078, 0.110). Finally, there was an interaction between genotype, gestational stress condition, and brain region (AIC = -67.812, LR = 7.74,

0.050).

F1 female (all *Atrx*^{WT}) mice raised with male *Atrx*^{HEM} siblings had increased levels of H_2O_2 compared to F1 females raised with male *Atrx*^{WT} siblings (AIC = -81.564, LR = 12.05, p = 0.05; **Figure 10B**). In addition, female mice exposed to gestational stress had increased levels of H_2O_2 compared to stress naïve F1 females (AIC = -43.6435, LR = 13.72, $p < 0.05$), suggesting persistent effects of prenatal stress on mitochondria respiration.

3.2. *8-OHdG Levels*

At high levels, ROS can lead to impaired physiological function through cellular damage of DNA. We quantified levels of *8-OHdG*, which is a biomarker for DNA oxidative stress (Valavanidis et al., 2009), in the frontal cortex, hippocampus, and cerebellum of F1 male and female mice. Four mice were used per group. Models were compared using AIC and LR.

F1 male *Atrx*^{HEM} mice had increased levels of *8-OHdG* compared to male *Atrx*^{WT} mice (AIC = -63.173, LR = 10.57, p = 0.001; **Figure 11A**). F1 males exposed to gestational stress had increased levels of *8-OHG* compared to male *Atrx*^{WT} mice (AIC = -64.353, LR = 9.39, p = 0.002). When comparing expression levels across brain regions, the highest levels of *8-OHdG* was measured in the frontal cortex, followed by the hippocampus and the least in the cerebellum (AIC = -62.484 LR = 13.26, p = 0.001). There was also an interaction between genotype and brain region (AIC = -67.411, LR = 14.20, p = 0.001). According to the 95% CIs, the effect of genotype was only significant in the frontal cortex (CI 95% = 0.084, 0.330) and the hippocampus (CI 95% = -0.009, 0.255), but not the cerebellum (CI 95% = -0.074, 0.126). Finally, there was an interaction between genotype, gestational stress condition, and brain region (AIC = -77.613, LR = 6.45, 0.040).

F1 female (all *Atrx*^{WT}) mice raised with male *Atrx*^{HEM} siblings had increased levels of *8-OHdG* compared to F1 females raised with male *Atrx*^{WT} siblings (AIC = -68.365, LR = 7.95, p = 0.005; **Figure 11B**). In addition, female mice exposed to gestational stress had

increased levels of *8-OHdG* compared to stress naïve F1 females (AIC = -62.444, LR = 13.88, $p < 0.001$).

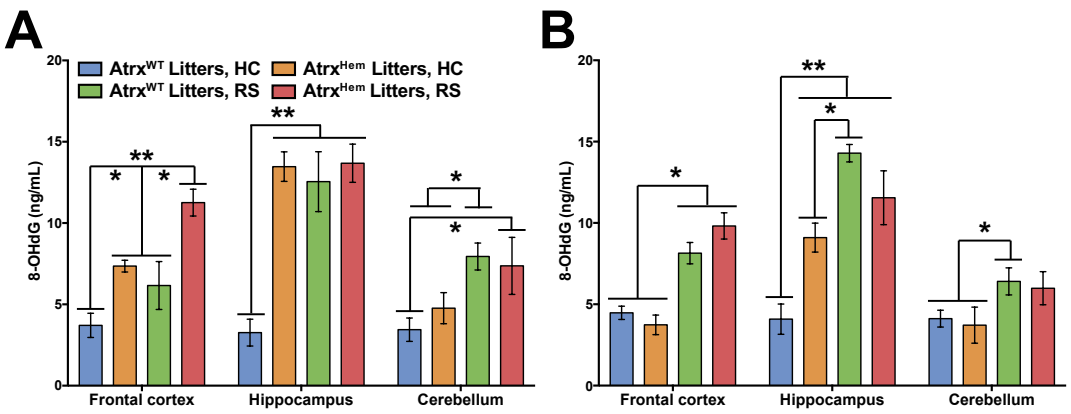

Figure 11. Effect of prenatal stress exposure and/or *Atrx* deficiency on *8-OHdG* levels in adult male and female offspring. *ATRX* expression in (A) *Atrx*^{WT} or *Atrx*^{HEM} male offspring from HC or RS mothers, (B) *Atrx*^{WT} female offspring from HC or RS mothers and raised with *Atrx*^{WT} or *Atrx*^{HEM} males. Mean ± SEM. (N=4/group; *p<0.05, **p<0.001).

3.3. *Regulation of Atm promoter*

Promoter methylation was quantified at various sites on the *Atm* promoter in the frontal cortex, hippocampus, and cerebellum of F0 and F1 male and female mice. Eight mice were used per group. Models were compared using AIC and LR.

3.3.1. Atm mRNA expression

Atm expression was quantified in the frontal cortex, hippocampus, and cerebellum of F1 male and female mice. Eight mice were used per group. Models were compared using AIC and LR. In F1 male mice, models including the main effects of genotype (AIC = 10.35, LH = 5.47, p = 0.041; **Figure 12A**), brain region (AIC = 12.26, LH = 7.43, p = 0.012), and the interaction between genotype, gestational stress exposure, and brain region (AIC = 9.53, LH = 7.32, p = 0.015) differed significantly from the null model (AIC = 7.91). stress naïve HC male mice had reduced *Atm* expression in the frontal cortex (CI 95% = 0.220, 0.872), hippocampus (CI 95% = 0.218, 0.631) and cerebellum (CI 95% = -0.221, 0.522). *Atm* mRNA levels were lower in the cerebellum and did not differ between the frontal cortex and hippocampus.

In F1 female mice, the model including a main effect of genotype region (AIC = 30.43, LH = 18.32, $p < 0.001$; **Figure 12B**), and the interaction between genotype, gestational stress exposure, and brain region (AIC = 8.72, LH = 7.38, $p = 0.017$) differed significantly from the null model (AIC = 13.125). The highest levels of *Atm* expression were measured in the hippocampus, followed by the frontal cortex, and least in the cerebellum.

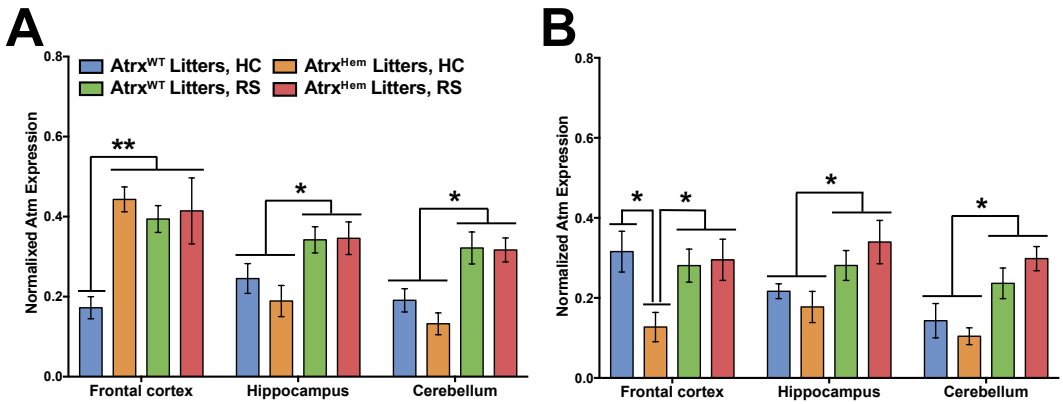

Figure 12. Effect of prenatal stress exposure and/or *Atrx* deficiency on *Atm* mRNA transcript levels in adult male and female offspring. *Atm* expression in (A) *Atrx*^{WT} or *Atrx*^{HEM} male offspring from HC or RS mothers, (B) *Atrx*^{WT} female offspring from HC or RS mothers and raised with *Atrx*^{WT} or *Atrx*^{HEM} males. Mean ± SEM. (N=8/group; *p<0.05; **p<0.001).

3.3.2. *Atm Gene Promoter Methylation*

In F1 males, offspring of the HC mothers had higher levels of *Atm* promoter methylation compared to the offspring of RS mothers AIC = 261.37, LR = 25.33, $p < 0.001$). While the male *Atrx*^{WT} mice had higher levels of *Atm* promoter methylation in their hippocampi and cerebellum compared to the frontal cortex, male *Atrx*^{HEM} mice had the highest levels of methylation in their frontal cortex, causing a genotype by region interaction (AIC = 243.83, LR = 9.93, $p = 0.007$). Finally, there was a genotype by maternal stress condition by brain region interaction (AIC = 237.91, LR = 6.19, $p = 0.045$; **Figure 13**). The difference based on maternal stress was present in all 24 individual methylation sites except for sites 14, 17, 20, 21, 22 and 24. Yet the difference was significant only sites 20 (AIC = 304.86, LR = 16.44, $p < 0.001$), and 24 (AIC = 348.10, LR = 17.04, $p < 0.001$; **Figure 13**).In F1 females, the offspring of RS mothers had lower levels of *Atm* promoter methylation than the offspring of HC mothers (AIC = 300.34, LR =18.34, $p < 0.001$) and the difference was greater in the cerebellum compared to other regions (AIC = 286.94, LR = 7.13, $p = 0.028$; **Figure 14**). This trend was present in all 24 methylation sites

except for sites 17, 20, and 23, in which *Atm* promoter methylation was higher in the offspring of the RS mothers. However, the difference was significant only in site 20 (AIC = 290.87, LR = 6.76, p = 0.034). On site 23 females raised with male *Atrx*^{WT} siblings had higher levels of *Atm* promoter methylation than females raised with male *Atrx*^{HEM} siblings (AIC = 257.67, LR = 11.54, p = 0.001; **Figure 14**).

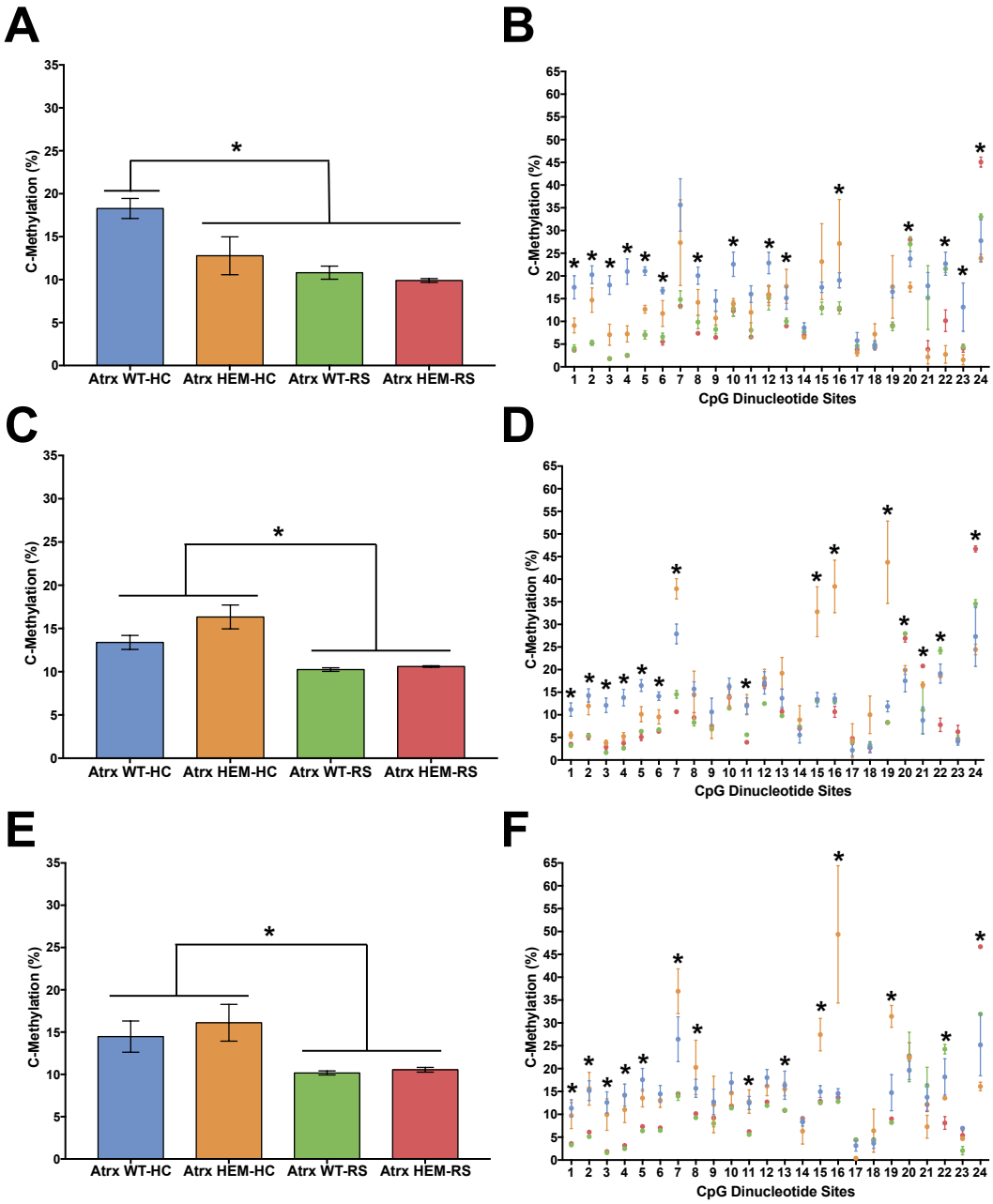

Figure 13. *Atm* gene promoter methylation in F1 males with *Atrx*^{WT} or *Atrx*^{HEM} genotype and mothers housed in home cage (HC) or restraint stressed (RS). Overall and site-specific DNA methylation levels at 11 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

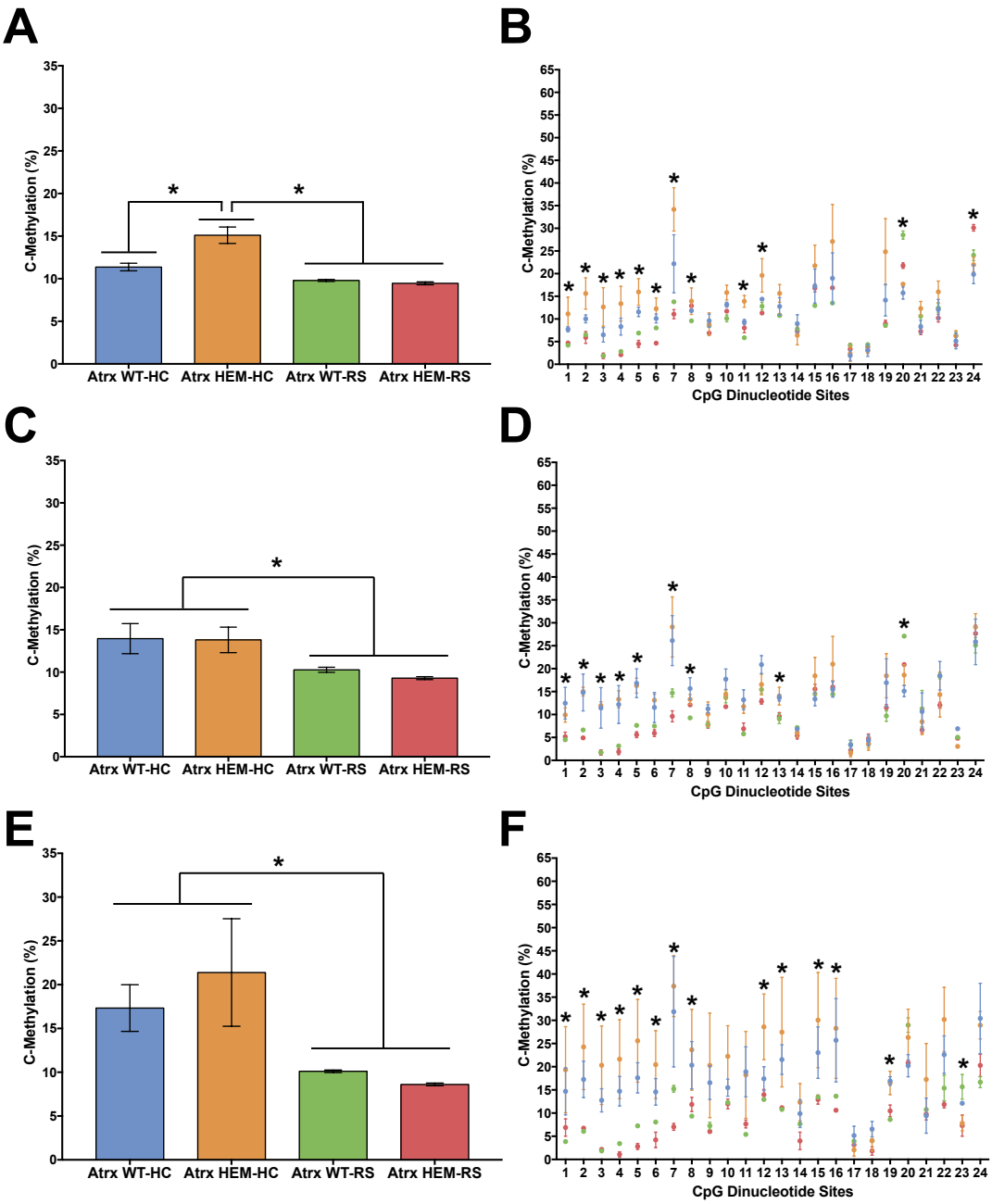

Figure 14. *Atm* gene promoter methylation in F1 females reared with *Atrx*^{WT} or *Atrx*^{HEM} males and mothers housed in home cage (HC) or restraint stressed (RS). Overall and site-specific DNA methylation levels at 11 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

CHAPTER 4: DISCUSSION

The purpose of this study was to examine interactions between early life experiences, metabolism and gene regulation and/or gene expression in the forebrain of mice, in the hopes to gain more insight into the mechanisms involved in neurodevelopmental disorders, such as idiopathic autism. This study shows that early life experiences, particularly exposure to stress un utero and early mother-pup interactions, may influence metabolism as well as the expression and regulation of DNA damage recognition and cell cycle progression gene*, Atm.* The expression of *Atm* was found to be increased in the brains of mice exposed to prenatal stress during development. Additionally, we observed altered *Atm* promoter methylation in mice that received lower quality maternal care during early postnatal development, as well as in mice exposed to stress *in utero*. Findings suggest that decreased *ATRX* expression mice ($Atrx^{HEM}$) which were exposed to stress *in utero* and received reduced quality of maternal care, was associated with altered *Atm* expression. This study demonstrates only a small portion of the research required to understand the effects of early life stress on gene expression. However, the result from this study allows for a better understanding of how adverse early life experiences effect neurodevelopment, in particular, the expression and regulation of DNA damage and cell cycle progression genes.

4.1. *Effects of Atrx-cKO and Mitochondrial Respiration and ROS Production*

Quantifying the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), gives insight into the indicators of mitochondrial respiration and glycolysis, respectively. High rates of ECAR are said to indicate a reduction in the extracellular pH, meaning there may be an increase in cellular glycolysis. However, extracellular pH is influenced by multiple biologic process and molecules, so more testing would need to be performed to definitively mark high ECAR scores with increased glycolysis. Overall, there were no significant variations in the oxygen consumption rate, which implies that mitochondrial respiration was unaffected by the *Atrx*-cKo models. When looking at ECAR, the rate of extracellular acidification decreased when exposed to gestational stress, which is true for both males and females. Stress naïve $Atrx^{HEM}$ males showed decreased ECAR compared to their wildtype counterpart. Additionally, stress naïve females

raised with *Atrx* deficient males showed increased ECAR compared to their stressed counterpart. These findings may indicate that frontal cortex and hippocampus cells of male *Atrx*^{HEM} mice well as prenatally stressed mice exhibit decreased glycolysis. These findings are consistent with the results seen in cells of the frontal cortex for female siblings of male *Atrx*^{HEM} mice, whereas in the hippocampus of these female siblings may be exhibiting an increase in glycolysis. Together, the suspected variation in glycolysis would be a feature of heightened mitochondrial activity, where overall metabolic potential is impaired. Studies have shown that glycolytically supressed cells upregulate mitochondrial function and rely on oxidative phosphorylation to obtain ATP required for survival (Shiratori et al., 2019). The potential decrease of glycolytic capacities in male *Atrx*^{HEM} mice and gestationally stressed animals could suggests that cells within the frontal cortex and hippocampal cells may be diverging from anaerobic glycolysis and could be using a new substrate for glycolysis to gain the required ATP. The cells within this region may have converted part of their energy production from oxidative phosphorylation to aerobic glycolysis, which is a phenomenon known as the Warburg effect (Liberti & Locasale, 2016). Anaerobic respiration is often seen in neurodegenerative disorders and cancer pathology (Pathak et al., 2013; Liberti & Locasale, 2016). The most common theory surrounding the Warburg effect in dysregulated cell types is that certain intermediates of aerobic glycolysis are important in maintaining cellular dysfunction and proliferation. The increase in ECAR seen in the hippocampus of females reared with *Artx*^{HEM} males suggests that there is an increase in glycolysis. This could indicate that the metabolic potentials of females reared with *Atrx* deficient males is less suspectable to changes under stressed conditions. This will be discussed further in the sections in surrounding *Emx-1* expression.

Reactive oxygen species (ROS) are common by-products of normal aerobic cellular metabolism and play important physiological roles in intracellular cell signalling and homeostasis. Mitochondria are considered a primary intracellular site of H_2O_2 (ROS) production. Thus, quantifying the levels of H_2O_2 can help to elucidate the metabolic activity of *Atrx* deficient models and stress. Results showed increased H_2O_2 levels for both males and females exposed to prenatal stress. *Atrx*^{HEM} males had increased H_2O_2 levels compared to their wildtype counterparts. Additionally, stress naïve females raised with *Atrx*

deficient males showed decreased H_2O_2 levels compared to their stressed counterpart. These findings suggest a persistent effects of prenatal stress on mitochondria respiration, further testing is required to conclude the results of these preliminary findings. Generating cultured cell lines to be used for immunohistochemistry could help to elucidate the effects of maternal stress on mitochondrial cells and ROS production. Mitochondrial ROS produced from aerobic respiration work to regulate oxidative phosphorylation and ATP production. Increased ROS can act as feedback to slow substrate oxidation, which in turn facilitates more efficient energy usage. Although low levels ROS may help to facilitate adaptation to stress via cellular signalling, high levels of ROS are deleterious and trigger oxidative stress (Scialò et al., 2017). Oxidative stress resulting from increased ROS has been associated with free radical damage seen in some idiopathic cases of AD and autism (Melnyk et al., 2012; Zawia et al., 2009).

4.2. *Effects of Atrx-cKO and Early Life Experience on DNA Damage*

Quantifying the *8-OHdG* levels within samples indicate levels of oxidative damage of DNA by reactive oxygen and nitrogen species. This test was performed to determine if *Atm* would be an appropriate target gene for this study. *8-OHdG* was used as a marker for oxidative stress and given that *Atm* is involved in DNA damage detection, the results from this assay would indicate the potential presence of *Atm* expression. Overall, there was an increase in *8-OHdG* levels for both male and female mice that experienced stress. This occurred regardless of whether the stress experienced was via gestational stress or altered rearing interactions. Male mice with reduced *Atrx* expression ($Atrx^{HEM}$) showed increased *8-OHdG* levels compared to their $Atrx^{WT}$ counterpart. This trend persisted in the hippocampus, where there was the largest difference in *8-OHdG* levels.

These results were as expected, since stress and ROS can induce oxidative changes, which recruits DNA repair mechanisms. Reduced *ATRX* has been associated with DNA damage and oxidative stress (Nandakumar et al, 2017; Leung et al., 2013). Under both conditions in this study (gestational vs. stress naïve), there may be significant changes in the availability of metabolic enzymes used as substrates and cofactors, which are then used to regulate the epigenomic landscape with chromatin modifiers and remodellers, and transcription factors used for epigenome-modifying enzymes (Dai et al., 2020; Melnyk et al., 2012; Zawia et al., 2009). Since we know that *ATRX* plays an important

role in chromatin remodelling and that metabolite availability plays a role in chromatin remodelling, there is a potential link between metabolism, oxidative stress, and proper functioning of *ATRX*. Significant changes to metabolite availability may then cause changes to stress protection, such as the activation of DNA repair mechanisms. One such repair mechanism may be the recruitment of *ATM,* which detects double stranded breaks and activates DNA damage pathways. Although the link between *ATRX* and *ATM* is not yet completely understood, there is some evidence to suggest that *ATRX* mediates DNA damage by regulating the activity of *ATM* (Han et al., 2018). Studies in cancer suggests that using *ATRX*-mutated/loss models cause increased DNA damage and hyperactivation of ATM (Dyer et al., 2017). This would again give rationale to the use of *Atm* as a potential target of interest in our hemizygous *Atrx* models.

4.3. *Effects of Atrx-cKO and Early Life Experience on mRNA Expression*

The quantification of mRNA expression with RT-qPCR indicates transcription levels of the genes targeted with specific primers. There was an overall effect on mRNA expression and exposure to prenatal stress, as Atm expression increase with gestational stress for both males and females. There was a varying effect on mRNA expression and early life experiences. Stress naïve *Atrx* deficient males ($Atrx^{HEM}$) showed increased *Atm* expression, which subsequently decrease when comparing brain regions. Pups born with an *Atrx* deficiency would have received reduced levels of maternal care during the postnatal period (Weaver et al., 2004). In this study, reduced maternal care is categorized as a stressor, as maternal care is crucial for typical brain development (Weaver et al., 2004). A stress response to reduced maternal care or gestational stress may activate the *Atm* pathways, thus leading to increased expression of *Atm* within the frontal cortex of this group. For the hippocampus and cerebellum of the stress naïve $Atrx^{HEM}$ male mice, the mRNA expression levels were not significantly increased compared to the $Atrx^{WT}$ counterparts.

We expected Male $Atrx^{HEM}$ mice to express significantly more *Atm* than their $Atrx^{WT}$ counterparts, however, we expected to see this trend in the hippocampus as well. The cKO transgenic model used in this study targeted excision of exon 18 of the *Atrx* gene around day 11-12 in cells that simultaneously express Cre-recombinase and *Emx-1*. *Emx-1* is expressed in the forebrain, thus, we expected that all cells within the forebrain that express *Emx-1* to have exon 18 of the *Atrx* gene excised (Gorski et al., 2002). This

would include all cells within the frontal cortex and hippocampus. When looking at *Atm* expression, we expected that the cKO model would express less *Atrx* within these regions, which in turn, would lead to increased expression of *Atm*. Increased *Atm* expression was only seen within the frontal cortex and not the hippocampus. *Emx-1* is expressed in a graded fashion within the forebrain as early as embryonic day 10.5, with varying levels of expression during embryonic development (Nakagawa et al., 1999). Specifically, expression levels within the hippocampus of *Emx-1* varies. So, although *Emx-1* is expressed throughout the hippocampus, the variations in expression patterns may mean that there wasn't total excision of exon 18 of the *Atrx* gene in cells expressing *Emx-1*. Thus, with the graded variations in expression patterns of *Emx-1*, our models may not have had a significant reduction in *Atrx* within the hippocampus, leading to the decreased expression seen in *Atm.* This may also be the case with the metabolic potentials of females reared with *Atrx* deficient mice. Cells within the hippocampus of these mice may experience relatively normal levels of glycolysis, as they are less affected by the cKO model. Meaning that although the ECAR appears to be increased compared to other groups within this region, these rates may be indicative of relatively normal cellular function.

Females reared with *Atrx* deficient males ($Atrx^{HEM}$) under stress naïve conditions showed reduced expression in all brain regions. Within the frontal cortex, stress naïve $Atrx^{WT}$ females showed increased expression compared to stress naïve females reared with *Atrx* deficient males ($Atrx^{HEM}$). These results were not necessarily as expected. Previous studies have shown that mothers with $Atrx^{HEM}$ males in their litters show decreased maternal care towards all pups within the litter, including females. As previously stated, *ATRX* may help to mediate *Atm* pathways associated with stress and altered rearing conditions may elicit a stress response within these mice. Knowing this, it was expected that decreased *Atrx* expression within our cKO models would also result in increased *Atm* expression for females raised with $Atrx^{HEM}$ males. Although females who are reared with $Atrx^{HEM}$ males experience altered rearing conditions, they are not *Atrx* deficient. As a result of the Cre-LoxP breeding system used, females express wildtype *Atrx*. This could mean that the expression of wildtype *Atrx* mediates the extent to which *Atm* is expressed. Meaning, that although these animals are experiencing altered rearing conditions, which may elicit a stress response, the response isn't as significant for the $Atrx^{HEM}$ female

varieties. Additionally, there are significant sex differences in brain activity in response to stress circuity. One study suggests that group housed stressed females have less adrenal hypertrophy compared to those solitary-housed females (Westenbroek et al., 2005; Beery & Kaufer, 2015). Evidently, sex differences may also be related to neurochemical variations between males and females. Hormones such as oxytocin and vasopressin are important for regulating stress response to social events, which have sex differences in both their production, release location, density of their receptors and their roles in social behaviour (Bales & Carter, 2003; Carter, 2007; Beery & Kaufer, 2015).

Lastly, mRNA expression levels are not directly proportional to the *8-OHdG* expression levels. Although the results from both tests are similar, there is some indication that there may be other reasons for cellular oxidative stress. Specifically, there is increased levels of *8-OHdG Atrx*^{HEM} males in both the hippocampus and cerebellum. For this group there is decreased *Atm* expression. *Atm* expression controls cell growth and genome surveillance due to replication stress. It likely that other genes activated in response to stress alongside *Atm*, which may explain the discrepancies between the oxidative stress levels and *Atm* expression.

4.4. *Effects of Atrx-cKO and Early Life Experience on Promoter Regulation*

Measuring methylation at various sites along the *Atm* promoters gave insight into the epigenetic mechanisms by which prenatal stress and early life interactions alter *Atm* gene regulation. Typically, methylation at these promoters indicates silencing or reduced gene expression. We found that the *Atm* promoter showed reduced methylation in response to prenatal stress exposure in both male and female mice. *Atrx* deficient mice which experienced altered rearing interactions showed varying methylation levels across brain regions and between sexes. There were also varying levels of methylation along specific sites of the *Atm* promoter. However, there were specific sites along the *Atm* promoter that showed a particularly interesting methylation pattern, which will be discussed more in depth within this section.

4.4.1. *Atm Promoter Methylation*

Quantifying the methylation using bisulfite pyrosequencing allows for more insight into potential methylation patterns in response to stress and early life experiences.

When looking at overall methylation, both males and female showed reduced methylation when exposed to prenatal stress. As expected, when there is increased expression there is typically reduced methylation. Epigenetic mechanisms, such as methylation act to regulate gene expression by changing the state of chromatin (Harabula & Pombo, 2021). The open or unpacked form of chromatin allows gene transcription, while the closed or condensed form prevents gene activity. Chromatin remodelling complexes are essential in the formation, modification, and maintenance of epigenetic information, such as methylation. Changes to chromatin remodelling proteins (such as *Atrx* cKO models) can impact proper functioning of important genes and thus ultimately changes gene expression and methylation patterns. Hence the results from RT-qPCR showing increased *Atm* expression within prenatally stressed mice and reduced methylation.

Stress naïve *Atrx* deficient HC males (*Atrx*^{HEM}) showed a decrease in methylation within the frontal cortex, whereas in the hippocampus and cerebellum there was increased methylation. These results are consistent with those from the RT-qPCR experiments examining *Atm* expression, although they are not necessarily as we expected. We expected that methylation could be decreased for all brain sections for stress naïve *Atrx*^{HEM} male mice. The same reasoning behind the *Atm* expression difference can be applied for this group. The graded variations in *Emx-1* expression within the hippocampus may have resulted in decreased expression of *Atm* and ultimately increased methylation within the hippocampus. *Emx-1* expression is restricted to the forebrain, which means that our cKO models do not have reduced *Atrx* expression within the cerebellum. For this study the cerebellum was used as a control region. Herein, *Atrx* expression should be unaffected in *Atrx*^{HEM} groups within the cerebellum. This would explain why there was increased *Atm* expression and decreased methylation within this region. Having typical *Atrx* expression may mediate the expression and methylation of *Atm* within the cerebellum, although the mechanism of action is still unknown. Methylation was increased for stress naïve females who grew up with *Atrx* deficient males (*Atrx*^{HEM}) in both the frontal cortex. These results are also consistent with those from the RT-qPCR experiments examining *Atm* expression. These results can be explained by reasoning previously mentioned surrounding the breading system and *Atrx* expression. As a result of the Cre-LoxP breeding system, females will express wildtype *Atrx,* which may mediate the extent to which *Atm* is expressed. The

increased methylation acts to repress or silence the expression of *Atm* for stress naïve females reared with *Atrx* deficient males.

Methylation at individual CpG dinucleotide sites varied by sex and brain region, but sites 15-18 showed increased variation, which was particularly interesting. Investigating further into CpG site specific transcription binding factors may give insight into the regula-tion of both Atm and Atrx. Looking specifically at the Atm promoter allowed for visualiza-tion of transcription factors that may be implicated at those specific CpG binding sites. Two transcription binding factors were of interest: p53 and E2F varieties. Along the Atm pro-moter, p53 is located at sites 15, 16, and 18, while E2F is located at sight 17 (**Figure 15**). Looking at how these transcription factors interact with both Atrx and Atm may help to de-termine a potential pathway involved in neurodevelopment.

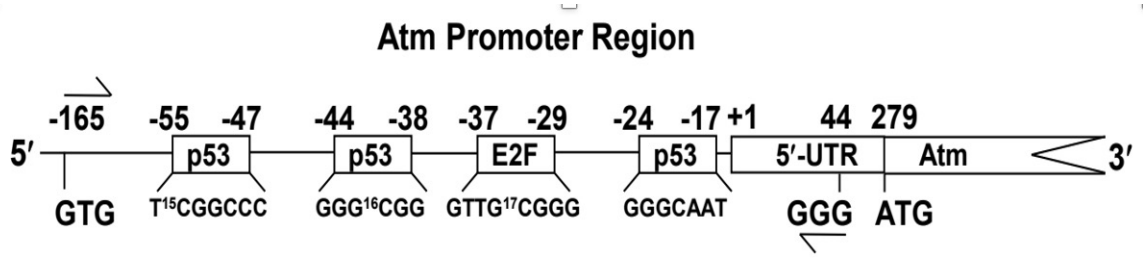

Figure 15. *Atm* gene promoter region depicting CpG dinucleotide sites of transcription binding factors, *p53* and *E2F*. Where *p53* is located at CpG sites 15, 16 and 18, while *E2F* is located at GpG site 17.

ATRX-null models have been associated with DNA damage and replicative stress, through interactions between both *Atm* and *p53* (Nandakumar et al, 2017; Leung et al., 2013). Some studies suggest that *ATRX* inhibits *p53*-mediated apoptosis (Conte et al., 2012). Meaning our cKO *ATRX* models could have increased *p53*-mediated apoptosis in response to DNA damage. *In vitro* studies have revealed that *ATRX* aggregates at DNA damage sites and interacts with the *Mre11-Rad50-Nbs1* protein complexes involved in double-strand breaks and stalled replication forks (Nandakumar et al, 2017; Leung et al., 2013). Interestingly, when double-stranded breaks occur, repair machinery works to activate *ATM* and other PIKKs (Czornak et al., 2008). Activation of *Mre11-Rad50-Nbs1* protein complexes induce conformational changes in *ATM* from monomeric form into the active form (Czornak et al., 2008; Shiloh, 2006). This conformational change in *ATM* allows for the recruitment of additional *ATM* to the damaged DNA sites, along with

downstream targets that amplifies the DNA damage signal (Shiloh, 2006; Shiloh & Lehmann, 2004). Potentially *ATRX* inhibits *p53*-mediated apoptosis to allow for the activation of *ATM* and associated repair machinery.

ATM phosphorylates many downstream targets, which initiates apoptosis and promotes DNA repair (Chen et al., 2011; Lee & Paull, 2007). As previously stated, *Mre11-Rad50-Nbs1* protein complexes stimulate the kinase activity of *ATM* to increase substrate recruitment by *ATM* and induce conformational changes that increase *ATM* substrate affinity (Lee & Paull, 2007). *ATM* kinase activity works towards the phosphorylation of *p35* and *E2F*, as well as a network of over 700 proteins. Apoptosis and DNA damage detection is, in part, mediated by *p53*. While E2F works to regulate the expression of genes important for cell proliferation. Although the exact pathway has not yet been discovered, our findings may give insight into how ATRX and ATM interactions work to mediate DNA damage. A potential mechanism of action is that, in our cKO *ATRX* models, reduced *ATRX* expression increases *ATM* expression, which then initiates phosphorylation of downstream targets which play a crucial role in cellular integrity. Additionally, reduced *ATRX* expression may allow for increased *p53* expression, which could then act as a potential feedback loop for *ATM* expression and the phosphorylation of both *p53* and *E2F*. **Figure 16** shows the potential interactions between *ATRX* and *ATM*.

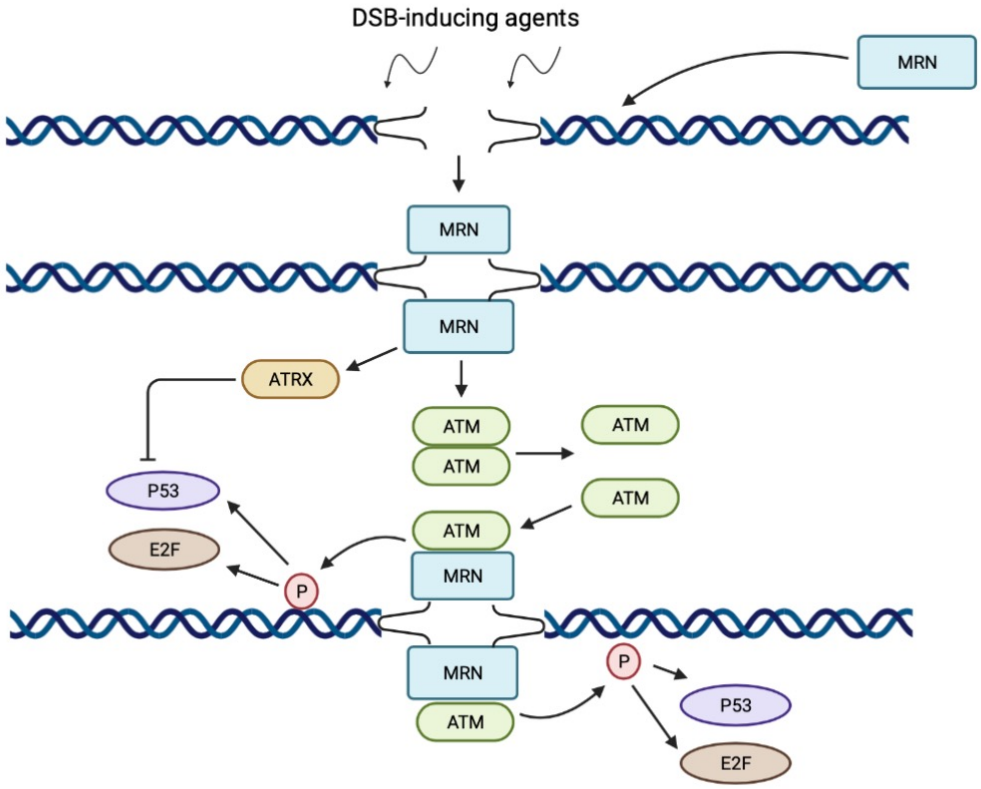

Figure 16. Proposed mechanism action between *ATRX*, *ATM a*nd transcription binding factors (*p53* & *E2F*) to early events in the DNA-Damage Response to double-stranded breaks. *MRN* complexes are recruited to the damage site and activates *ATM* to respond in a cyclical manner and causes recruitment of *ATRX*. Activation of ATM leads to phosphorylation of *p53* and *E2F*, while *ATRX* inhibits the activity of *p53*. Adapted from Shiloh (2006).

4.5. *Concluding Remarks and Future Directions*

Our findings suggest that prenatal stress exposure influence cellular metabolism and the expression and regulation of genes implicated in neurodevelopment, such as ASD pathogenesis. Our studies also suggest that there are persistent effects of prenatal stress on mitochondria respiration, however, oxidative stress mediated by ROS may not be indicative of *Atm* gene expression. Additionally, our findings suggest that the *ATRX* protein does not directly regulate the *Atm* gene promoter, but may play a role in regulating downstream targets initiated by the activation of *Atm.*

The *Atm* gene promoter region was found to have decreased methylation in mice experience prenatal stress compared to stress naïve mice. *Atm* gene promoter methylation was found to be counterbalanced with *Atm* gene expression levels, indicating that

methylation may act to repress or silence *Atm* gene expression. Expression in the brains of gestationally stressed groups were increased, suggesting that early life interactions can influence the expression of the *Atm* gene. Together, these findings help to increase the understanding that prenatal experiences are involved in shaping gene expression. Epigenetic programing, such as chromatin remodelling and metabolite-genome interactions, as well as early life experiences work to influence neurodevelopment throughout one's lifespan.

Future studies may aim to accurately describe the interactions between the stress, apoptosis, and neurodevelopment. The effects of stress on pathways involving *ATRX* and *ATM* may converge through transcription factors, such as *p53* and *E2F*. Assessing the role of *p53*-mediated apoptosis and E2F-mediated proliferation via assays such as ChIP (chromatin immunoprecipitation) analysis may give insight into the activity of *ATM.* Additionally*,* this study explores only one of the possible pathways between *ATRX*-*ATM* and early life interactions. Using alternative knockout animal models or targeting alternative biomarkers seen in both stress and metabolism may help to better understand the molecular pathways involved in prenatal stress exposure and neurodevelopment. It is important to note that although these findings point towards an interaction between stress and epigenetic regulation of *Atm* and *Atrx*, more robust testing is required to draw definitive conclusions. Additionally, using a larger sample size would help to increase the significance of the results found in future studies.

While our findings addressed changes to gene regulation and methylation, we did not assess how the protein expression of *Atm* was affected by our manipulations. Nor did we compare the results from previous studies of *ATRX* expression with our current results. Measuring changes to protein using tailored ELISA assays would allow for a more complete picture of how changes in early life experiences influence DNA damage detection gene, *Atm*. A comparative study would help to elucidate the relationship between *ATRX*, *ATM and* prenatal stress. Our findings do however propose a potential mechanism of action for *ATRX* and *ATM* in response to DNA damage, which can be used in future studies.

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