EFFECTS OF PRENATAL STRESS AND/OR FOREBRAIN ATRX DEFICIENCY IN C57BL/6 MICE ON REGULATION AND EXPRESSION OF AUTISM RISK GENES

By

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ABSTRACT

Early life experiences, such as stress *in utero* and early mother-offspring interactions, have been shown to influence brain development and consequently, behaviour into adulthood. In humans, maternal stress during the third trimester has been associated with children developing Autism Spectrum Disorder (ASD), a neurodevelopmental disorder characterized by deficits in social behaviour. The molecular mechanisms underlying the development of idiopathic ASD remain rudimentary, particularly as idiopathic cases lack a known genetic cause. Despite this, several genes have been shown to be dysregulated in humans with idiopathic ASD. *Atrx* is one such gene that encodes a chromatin remodeling protein responsible for widespread regulation of gene expression. Animal models with *Atrx* deficiencies show abnormal brain development and ASD-like behaviour into adulthood, that may be due to reduced *ATRX* function in regulating gene expression. Furthermore, *Atrx* expression is influenced by early maternal care and exposure to drugs that modify the epigenome. In this study, we investigated the effect of reduced *Atrx*, through genetic manipulation or potentially induced by prenatal stress exposure, on expression and promoter regulation of genes implicated in ASD. We found that mice exposed to stress *in utero* and that received low quality post-natal maternal care, had reduced *ATRX* and *mTOR* expression in the adult brain, and reduced methylation at both the *Atrx* and *Mtor* promoters. These finding suggest that early life experiences are capable of influencing expression and regulation of ASD risk genes. Additionally, we found that *Mtor* promoter regulation was not affected in animals with reduced *ATRX* expression, indicating that *ATRX* does not directly regulate the *Mtor* promoter. This study expands on our understanding of epigenetic mechanisms involved in the development of behavioural phenotypes seen in idiopathic ASD.

LIST OF ABBREVIATIONS AND SYMBOLS USED

α: alpha
Δ: delta
%: Percentage
5mc: 5-methylcytosine
AIC: Akaike information criterion
ASD: Autism Spectrum Disorder
ATP: Adenosine triphosphate
ATRX: α-thalassemia/mental retardation, X-linked
BDNF: Brain derived neurotrophic factor
bp: Base pair
BMP: Bone morphogenic protein
cDNA: Complementary DNA
CER: Cerebellum
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
°C: Degrees centigrade
DAXX: Death domain-associated protein
ddH_2O: Double deionised water
DF: Degrees of freedom
DNA: Deoxyribonucleic acid
DNMT: DNA methyltransferase
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
F0: First generation
F1: Second generation
FASD: Fetal Alcohol Spectrum Disorders
FC: Frontal cortex
gDNA: Genomic DNA
H 1/2/3/4: Histone one, two, three, four
HAT: Histone acetylase transferase
HC: Home cage
HDAC: Histone deacetylase
HEM: Hemizygotic
HIPP: Hippocampus
IPC: Interplate calibrator
kDa: Kilodaltons
LG: Licking and grooming
LR: Likelihood ratio
LTP: Long term potentiation
LoxP: Locus of X-over P1 sequence
MBP: Methyl-CpG binding proteins
MeCP2: Methyl CpG-binding protein 2
mRNA: Messenger ribonucleic acid
mTOR: Mammalian target of rapamycin
mTORC 1/2: mTOR complex one, two
MWM: Morris water maze
µg: Microgram
µL: Microlitre
µM: Micromolar
ml: Millilitre
N: Number of subjects
nm: Nanometer
NTC: No template control
NRT: No reverse transcriptase
NPC: Neural precursor cells
NSC: Neural stem cells
NTD: Neural tube defects
p: Probability (statistics)
p53: Phosphoprotein p53
PCR: Polymerase chain reaction
PHD: Plant homeodomain
PI3K-Akt: Phosphoinositide-3-kinase – protein kinase B
p-mTOR: Phosphorylated mTOR
qPCR: Quantitative PCR
RIPA: Radio-immunoprecipitation assay
RNA: Ribonucleic acid
RPM: Revolutions per min
RQI: RNA quality indicator

RS: Restraint stressed
RT: Reverse transcriptase
RT-qPCR: Real-time qPCR
S6K: Ribosomal protein S6 kinase
SEM: Standard error of mean
Ser: Serine
SF: Superfamily
SFARI: Simons Foundation Autism Research Initiative
Swi/Snf: SWItch/Sucrose Non-Fermentable
TBS: Tris-buffered Saline
UV: Ultraviolet
WT: Wild-type

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CHAPTER 1: INTRODUCTION

Organisms of the same species are highly diverse and vary physically, physiologically, and behaviourally. Yet, genetic diversity within a species is relatively small. All humans, for example, are estimated to share 99.4% of the total 3.2 billion base pairs (bp) in their genetic sequence (Genomes Project et al., 2015), leaving only 0.6% variability in genotype to account for the vast phenotypic variability between individuals. Even when individual humans share 100% of their genetic sequence, which is often the case with monozygotic twins, each individual develops their own unique phenotypes (A. H. Wong, Gottesman, & Petronis, 2005). Further, an individual's phenotypes vary as they develop and mature while each cell within the individual contains the same DNA sequence through their entire lifespan (Ralston & Shaw, 2008). The diversity that emerges within individuals throughout their life does not always result from genetic variation. In 1997, Wilmut, Campbell and colleagues showed that an organism's DNA sequence does not undergo irreversible changes as the organism matures when they cloned a lamb from adult lamb cells (Wilmut, Schnieke, McWhir, Kind, & Campbell, 1997). Rather, it is the manner in which an individual's genes are regulated and expressed, through epigenetic modifications, that results in the variable phenotypes within and between individuals. Epigenetics was coined by Conrad Waddington to describe the study of how diverse phenotypes arise through variable gene expression (Bird, 2007; Millan, 2013). More recent interpretations of the term epigenetics refer to studying specific molecular mechanisms by which gene regulation, without altering DNA sequences, is related to phenotypic outcomes (Bird, 2007; Day & Sweatt, 2012; Slatkin, 2009).

The epigenome is comprised of chemical modifications that interact with the genome to regulate transcription (Day & Sweatt, 2011). Some epigenetic modifications, such as DNA methylation, often repress the expression of genes (Siegfried et al., 1999). Others, such as histone acetylation, promote upregulation of gene transcription (Struhl, 1998). Though the molecular mechanisms by which epigenetic modifications influence gene transcription differ, none affect the sequence of DNA (Urnov & Wolffe, 2001). Instead, these chemical modifications are made 'above' the genome, as the prefix 'epi' suggests (Urnov & Wolffe, 2001).

Studying the epigenome allows for a more complete understanding of how phenotypic differences arise. This is of particular interest to scientists aiming to understanding the etiology of idiopathic Autism Spectrum Disorder (ASD), which unlike syndromic ASD, cannot be explained by known genetic mutations. Though these two subtypes of ASD can be differentiated by etiology, the core symptoms of both idiopathic and syndromic ASD are deficits in social behaviour and repetitive/restrictive behaviour (American Psychiatric Association. & American Psychiatric Association. DSM-5 Task Force, 2013). Despite not being characterized by a specific genetic mutation, people with idiopathic ASD have dysregulated expression in 161 genes associated with ASD (Liu et al., 2017) suggesting that aberrant epigenetic regulation of gene expression may underlie the development of the behavioural deficits seen in ASD. However, dysregulation of a single gene is typically insufficient to result in ASD (Xiong et al., 2019). These findings imply that development of ASD is polygenic, involving dysregulated expression of several genes. In idiopathic cases, this means there may be widespread epigenetic dysregulation (Berko & Greally, 2015).

The Simons Foundation Autism Research Initiative (SFARI) has compiled studies identifying 913 ASD-associated genes. *Atrx* is a gene listed in the SFARI database that is associated with ASD (Brett et al., 2014; Deneault et al., 2018) and codes for a protein, *ATRX*, known to regulate the expression of several other genes throughout the genome (Kernohan et al., 2010). Mutations to *Atrx* cause ATR-X syndrome, characterized by severe cognitive deficits (Gibbons, Suthers, Wilkie, Buckle, & Higgs, 1992) and dysregulated gene expression (Law et al., 2010). Reduced expression of *Atrx* in mouse models results in impaired performance in cognitive tasks, reduced long-term potentiation (LTP) in the hippocampus, and increased cell death (Gugustea, Tamming, Martin-Kenny, Berube, & Leung, 2019; Tamming et al., 2019; Tamming et al., 2017). It could be that reductions in *Atrx* are leading to these behavioural outcomes through an increase in cell death. However, the relationship between reduced *Atrx* expression on the regulation of other genes that may be underlying this increase in cell death remains rudimentary.

Several studies have shown evidence that early life experiences during intrauterine and peri-natal development have lasting impacts on an individual's developmental trajectory. For instance, maternal stress during gestation has been associated with increased

incidences of ASD in children (Beversdorf, Stevens, & Jones, 2018; Walder et al., 2014). In particular, there was a positive association between mothers who were pregnant during the Quebec Ice Storm reporting higher stress levels, and their children scoring higher on the Autism Spectrum Screening Questionnaire (Walder et al., 2014). As the etiology of ASD remains poorly understood, it is unclear as to how many cases of idiopathic ASD are associated with prenatal stress. However, it has been shown that the severity of ASD symptoms increases in children who experienced prenatal stress (Varcin, Alvares, Uljarevic, & Whitehouse, 2017).

Furthermore, it has previously been shown that expression of *Atrx* is influenced by early life environment and can be manipulated with chemicals that alter epigenetic modifications (Weaver, Meaney, & Szyf, 2006). As previously mentioned, idiopathic ASD arises without a known genetic mutation, yet there is widespread gene dysregulation. *Atrx* is implicated in ASD, encodes a protein involved in regulating gene expression, and is influenced by early life experiences. For these reasons, we are interested in studying the relationship between early life experiences, such as stress *in utero* and variable maternal care, and decreased *Atrx* expression. More specifically, how reductions in *Atrx* expression might affect the regulation of other genes implicated in ASD.

In this introduction, I will review literature on the role of the chromatin remodeling protein, *ATRX*, in gene regulation and shaping brain development, the influence of stress on maternal behaviour and offspring gene expression, and the way in which dysregulated expression of ASD risk genes may result in ASD-like phenotypes.

1.1. *Gene Regulation in Early Brain Development*

Neuronal development is a complex process that is guided by spatiotemporally precise gene expression (Waterston, Lander, & Sulston, 2002). During prenatal and perinatal development, cell division and differentiation is guided by intrinsic (differential gene expression) and extrinsic (intercellular signaling) molecular cues in a precise spatiotemporal manner (Jiang & Nardelli, 2016) that are, in part, regulated by epigenetic modifications (Mager & Bartolomei, 2005).

The first steps begin around the fifth week of embryonic development in humans during neurulation, which is triggered by the expression of neural inducers in the ectoderm germ layer (Wilson & Hemmati-Brivanlou, 1997). The expression of the neural

inducers ‘noggin’, ‘follistatin’, and ‘chordin’ by cells in the ectoderm have all been demonstrated to induce the development of neuronal tissue (Hemmati-Brivanlou, Kelly, & Melton, 1994; Lamb et al., 1993; Sasai, Lu, Steinbeisser, & De Robertis, 1995). Expression of these genes inhibits the bone morphogenic protein (BMP) pathway, permitting cells to differentiate into neurons (Bond, Bhalala, & Kessler, 2012). In this early phase of development, regulation of gene expression is critical to coordinate the cellular and molecular signaling underlying the complex morphological changes in the neural plate to form the neural tube (Figure 1), which is the precursor for the central nervous system (CNS) (Squire, 2013).

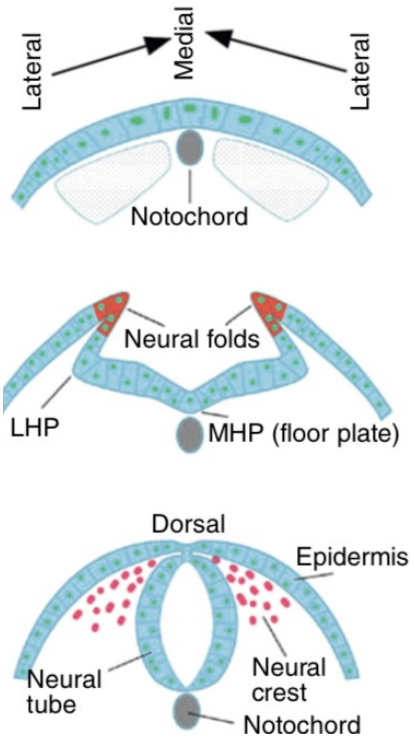

Figure 1. Illustration of transverse section of neural plate undergoing morphological changes of neurulation to form neural tube. Adapted from Squire (2013).

The importance of gene regulation is emphasized by the fact that dysregulation of genes involved in neural tube formation can lead to improper closure resulting in neural tube defects (NTD) (Figure 2) (Botto, Moore, Khoury, & Erickson, 1999). During this period in embryonic development, mutations to gene expression, lack of substrates like folic acid, and exposures to toxins such as ethanol, can all disrupt the chemical signaling involved in proper neural tube closure (A. J. Copp & Greene, 2010; Imbard, Benoist, & Blom, 2013; Zhou, Sari, Powrozek, Goodlett, & Li, 2003). The developmental

consequences vary in severity depending on which region of the tube fails to close (Figure 2). Although the precise molecular mechanisms underlying neural tube closure have yet to be elucidated, the fact that this process can be severely impacted by changes to gene expression and environmental cues suggest that precise regulation and coordinated expression of several genes is required for typical CNS development.

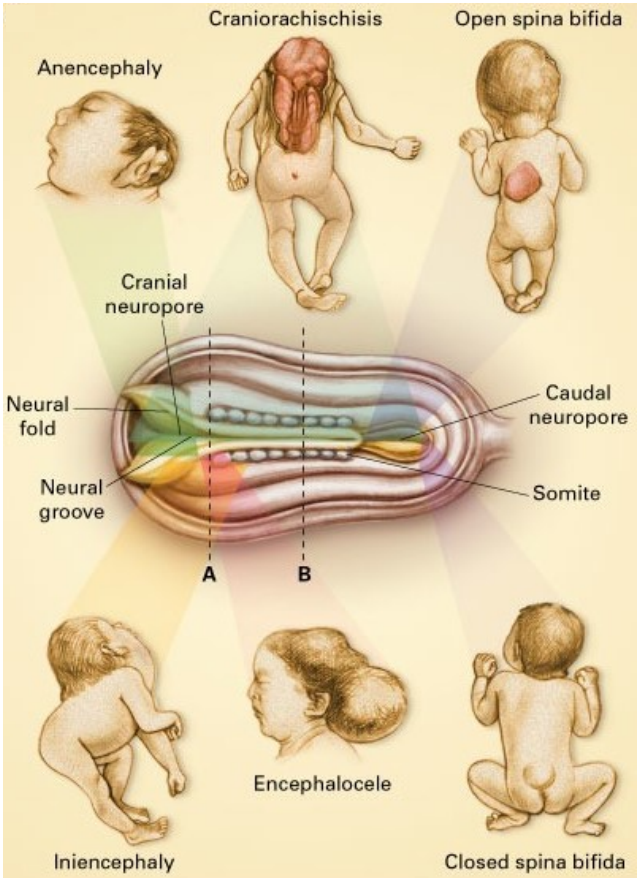

Figure 2. Developmental consequences of neural tube defects. The point at which closure fails will determine the developmental consequence. Adapted from Botto et al. (1999).

At the end of the first trimester and spanning into the second trimester of embryonic brain development, the majority of neural stem cells (NSC) that will form the adult cerebral cortex are generated (Bystron, Blakemore, & Rakic, 2008). There is an upregulation in genes encoding neuronal proliferation, migration, differentiation, cell adhesion, branching and myelination (Iruretagoyena et al., 2014). The burst in neurogenesis during this period causes the embryonic brain to be particularly susceptible to changes to the environment that can alter epigenetics influencing cortical development, and ultimately

leading to different developmental outcomes. For instance, exposure to ethanol during this period leads to significantly reduced cortical thickness due to reductions in neurogenesis (Miller, 1989), and can result in Fetal Alcohol Spectrum Disorders (FASD) (May et al., 2013). Exposure to ethanol *in utero* has been shown to alter DNA methylation in progenitor cells, which influences the offspring's developmental trajectory often resulting in the development of FASD (Zhou et al., 2011). Environmental influences on gene expression have pronounced effects on cognitive outcomes at this point in development as cells rely heavily on genetic cues to develop the cortex.

The third trimester of human brain development is characterized by significant brain growth. In humans, the average gestational term is approximately 38 weeks (Jukic, Baird, Weinberg, McConnaughey, & Wilcox, 2013), however, the World Health Organization estimates that more than 1 in 10 infants are born prematurely. Infants born during the third trimester often have reduced brain volume and slower developmental trajectories (Bouyssi-Kobar et al., 2016), altered neuronal circuitry and diminished performance in socio-cognitive tasks (Fischi-Gomez et al., 2015), and emotional and behavioural dysregulation as children (Clark, Woodward, Horwood, & Moor, 2008) compared to babies that develop *in utero* to term. These findings suggest that although infants can survive when born preterm, remaining in the intrauterine environment through the third trimester is optimal for brain development.

In the perinatal period, an infant's experiences and external environment continue influencing development of brain regions and by consequence, behavioural phenotypes (Fox, Levitt, & Nelson, 2010). The frontal cortex, for instance, is responsible for regulating several behaviours such as social behaviour, emotional regulation, and decision making (Rudebeck, Bannerman, & Rushworth, 2008). Disruptions to early cortical development is associated with reduced cortical volume (Kapellou et al., 2006), impaired social behaviour (Talamini, Koch, Luiten, Koolhaas, & Korf, 1999), and diagnosis of neurodevelopmental disorders, such as ASD (Chomiak, Karnik, Block, & Hu, 2010). Early life experiences that alter the trajectory of frontal cortex development lead to varying behavioural outcomes (Berger-Sweeney & Hohmann, 1997). The hippocampus is another brain region that profoundly affects behaviour when development is dysregulated (Lipska & Weinberger, 2002). While the hippocampus is primarily responsible for memory

formation and spatial memory (Eichenbaum, 1999), atypical development of this region can result in disordered social behaviour and emotional regulation (Uchida et al., 2011). Children with ASD, who have disordered social behaviour, have irregularly shaped hippocampi compared to neurotypical controls (Dager et al., 2007). The developmental trajectories of both the frontal cortex and the hippocampus can have profound impacts on behaviour. Furthermore, these two regions are highly interconnected and various behaviours are regulated by the functional coordination of these two brain regions (Sigurdsson & Duvarci, 2015).

In summary, individuals are particularly vulnerable to the effects of altered gene expression during early brain development as this is a period when cells depend heavily on genetic cues for typical cell division and differentiation. When gene expression is dysregulated and cells do not receive typical guidance from their environment, the neuronal pathways that are formed can be defective and will negatively impact brain development and organization. These changes to neuronal pathways in the frontal cortex and hippocampus lay the foundation for behaviour into adulthood. When the environment influences changes to gene regulation during embryonic development, early life experiences have the capacity to alter an organism's developmental trajectory and shape behaviour into adulthood.

1.2. *Epigenetic Mechanisms*

Epigenetic modifications are chemical markings that occur either directly on DNA or indirectly on the proteins associated with DNA to alter the accessibility of DNA to transcription factors that regulate gene expression (Sweatt, 2009). Epigenetic marks regulate various aspects of gene expression in neural and non-neural tissues (e.g. timing, levels, silencing, de-repression, poised for expression, etc.) (Weinhold, 2006), which are crucial for programming physical, cognitive, social and emotional development, accounting for a large portion of phenotypic variability between individuals (Haque, Gottesman, & Wong, 2009; Shah et al., 2015). These modifications are affected by environmental factors and contribute to the development of the organism's phenotypes. In early-life, external factors of both a physical nature, such as exposure to toxins, and social nature, such as reduced maternal interaction, alter epigenetic modifications on genes and persistently affect gene expression into adulthood (Champagne, 2008; Perera & Herbstman, 2011). The

most well studied epigenetic modifications are DNA methylation and histone post-translational modifications, though others such as RNA-interference exist (Sweatt, 2009).

1.3. *Chromatin*

DNA is capable of compaction into structures called chromatin so that the 3.2 billion base pairs in an individual human's genome condense into the nucleus of each cell (Alberts et al., 2002). The basic subunit of chromatin is the nucleosome (Figure 3), which is a section of double-stranded DNA that is approximately 146 base pairs long wrapped around a histone octamer consisting of duplicates of H2A, H2B, H3 and H4 variants (Khorasanizadeh, 2004). Histone proteins have a high density of positively charged, basic amino acids which attract the negatively charged phosphate groups in DNA. Histone tails can be covalently modified which will alter the affinity of the DNA-histone bond and can lead to either increases or decreases in chromatin compaction (Bannister & Kouzarides, 2011). Nucleosomes can be brought into closer proximity when H1 variants bind DNA, condensing the structure further into solenoids (Allan, Hartman, Crane-Robinson, & Aviles, 1980). Inhibiting H1 from binding DNA makes chromatin more accessible for transcriptional activation (Allan et al., 1980). Solenoids can then supercoil into the most transcriptionally repressed conformation of chromatin.

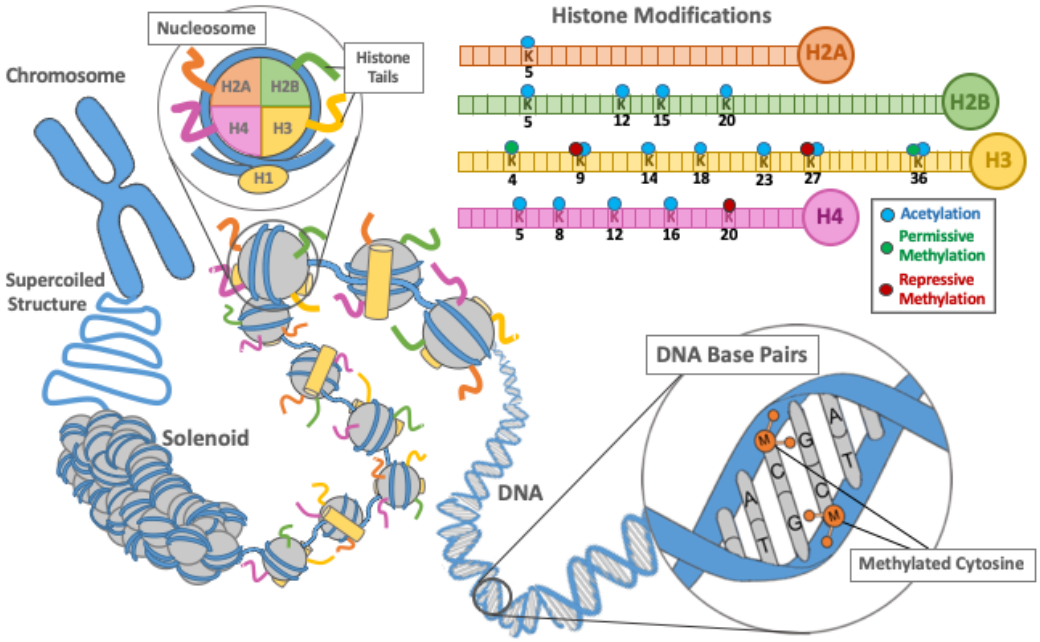

Figure 3. Chromatin structural organization. Adapted from Weaver et al. (2017).

The conformation of chromatin is inherently involved in gene regulation by determining which genes are readily transcribed and which are transcriptionally silent. Open conformations of chromatin allow DNA replication machinery to access genes, while genes tightly packed into a closed conformation will be transcribed less (Jantzen, Fritton, & Igo-Kemenes, 1986). Nucleosome spacing determines whether chromatin is in a closed (heterochromatin) or open (euchromatin) conformation (Fedorova & Zink, 2008).

The elements that control chromosome conformation are epigenetic modifications, such as histone post-translational modifications (Mellor, Dudek, & Clynes, 2008). Post-translational acetylation and/or methylation of histone proteins alters the affinity of the DNA-histone bond, consequently affecting the accessibility of DNA by transcription factors and replication machinery (Struhl, 1998). For instance, acetylation at specific sites on histone tails decreases the affinity of the bonds between DNA and histone proteins and increases transcription, while repressive methylation at other sites strengthens DNA-histone bond affinity and downregulates transcription (Figure 3) (Weaver et al., 2017).

While histone acetylation and methylation regulate gene transcription by altering DNA-histone interactions, DNA methylation regulates gene expression through direct chemical modifications to nucleotides (Keshet, Yisraeli, & Cedar, 1985). Cytosine bases can be methylated and converted to 5-methylcytosine (5mC) by DNA methyltransferases (DNMTs) (Rountree, Bachman, Herman, & Baylin, 2001). DNA methylation, primarily at regulatory gene promoter regions, has been shown to downregulate transcription of the modified sequence (Moore, Le, & Fan, 2013). The conversion of cytosine to 5mC recruits protein complexes, such as methyl-CpG binding proteins (MBP) (Free et al., 2001), that downregulate gene expression by occupying the promoter region and limiting transcription factor binding (De Smet et al., 1995). Upon binding with 5mC, MBPs recruit other proteins that can alter transcriptional regulation through chromatin restructuring, such as histone deacetylases (Nguyen, Gonzales, & Jones, 2001).

Post-translational modifications to histones and DNA methylation can be modified throughout the lifespan (Xiao, Wang, & Kong, 2019; Zentner & Henikoff, 2013), resulting in changes to chromatin conformation. When chromatin structure is dysregulated and elements that are typically in the closed conformation become accessible, the

consequence is aberrant gene expression that can be pathological (Ando et al., 2019). For example, changes to chromatin accessibility can lead to binding of different transcription factors to promoters and also different histone variants to increase transcription of oncogenes (Saeed et al., 2012).

1.4. *Chromatin Remodeling Proteins*

Chromatin remodeling proteins alter the accessibility of DNA by modifying chromatin structure (Fairman-Williams, Guenther, & Jankowsky, 2010). They promote or repress transcription by exchanging histone variants or fully disassembling nucleosomes (Fairman-Williams et al., 2010), which can reversibly restructure chromatin from heterochromatin to euchromatin (Sokpor, Xie, Rosenbusch, & Tuoc, 2017). One important functional distinction is made between ATP-dependent complexes and modifiers of acetylation of histones: histone acetyl transferases (HATs) or histone deacetylases (HDACs) (Vignali, Hassan, Neely, & Workman, 2000). ATP-dependent complexes directly alter DNA-histone interactions while HATs and HDACs alter the degree of acetylation of histones, which in turn alters DNA-histone bonding (Vignali et al., 2000). These proteins are essential for various chromatin remodeling processes, such as restructuring and stabilizing chromatin, that occur during transcription and DNA repair (Pyle, 2008). As such, there are a large number of chromatin remodeling proteins that are classified into superfamilies (SF) (Figure 4). Proteins within SF1 and SF2 have almost identical catalytic cores that are structurally similar to the recombination protein, RecA (Singleton, Dillingham, & Wigley, 2007). Distinctions between families within SF1 and SF2 are drawn by genetic, structural and functional differences (Fairman-Williams et al., 2010).

Chromatin remodeling proteins play a critically important role in regulating gene expression, particularly in development, as mutations to these proteins often results in dysregulated gene expression and altered developmental trajectories (Ronan, Wu, & Crabtree, 2013). In addition, several forms of cancer are associated with mutations in genes encoding chromatin remodeling proteins (Nair & Kumar, 2012).

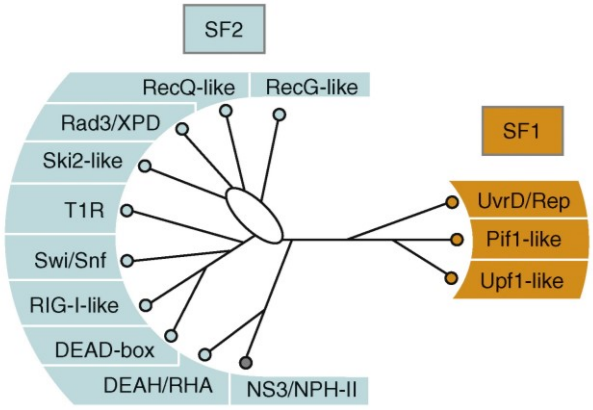

Figure 4. Classification of chromatin remodeling proteins by superfamilies (SF1 and SF2) and families. Adapted from Fairman-Williams et al., 2010.

1.4.1. ATRX

The main isoform of *ATRX* is a large protein (~280 kDa) categorized in SF2 as a member of the SWItch/Sucrose Non-Fermentable (Swi/Snf) family, and more specifically as an Snf2 protein (Ryan & Owen-Hughes, 2011). Members of the Snf2 family, such as *ATRX*, are involved in transcriptional regulation, recombination, replication and DNA repair (Carlson & Laurent, 1994). *ATRX,* specifically, is important in sliding, remodeling or removing histone variants to alter chromatin structure (Picketts et al., 1996). *ATRX* contains a plant homeodomain (PHD) type zinc finger motif and ATP-ase/helicase domain (Dhayalan et al., 2011), suggesting it is involved in transcriptionally regulating other genes through restructuring chromatin (Aasland, Gibson, & Stewart, 1995). The PHD-type zinc finger acts as a reader of epigenetic modifications by recognizing modifications on H3 (Sanchez & Zhou, 2011), allowing it to deposit H3.3 histone variants upon forming a larger complex involving other proteins such as the death domain-associated protein (DAXX) (Figure 5) (Clynes, Higgs, & Gibbons, 2013; Drane, Ouararhni, Depaux, Shuaib, & Hamiche, 2010). The ATP-ase/helicase domain uses energy from ATP to restructure chromatin (Ryan & Owen-Hughes, 2011).

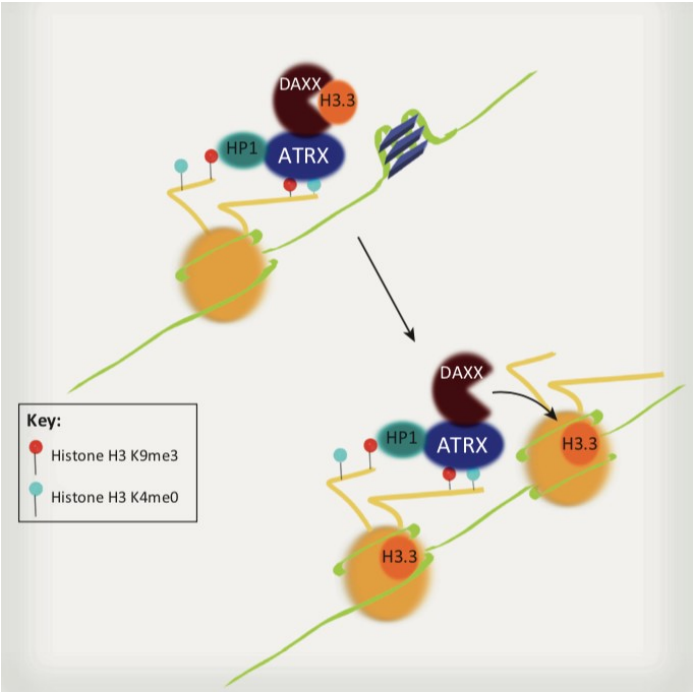

Figure 5. Illustration of the role of *ATRX* in H3.3 deposition. Figure from Clynes et al. (2013).

Several roles for *ATRX* in gene regulation have been proposed. *ATRX* is found at heterochromatin regions, such as telomeres, suggesting it is important for transcriptional repression (Clynes et al., 2013). Lack of *ATRX* expression results in telomere instability (L. H. Wong et al., 2010) and aberrant gene expression. Mutations to the *ATRX* protein have been associated with changes in methylation patterns (Gibbons et al., 2000). Further, it has been observed that mutations to *ATRX* downregulates the expression of other genes, *HBA1* and *HBA2* (Wilkie et al., 1990), suggesting functional *ATRX* is required for transcription of these genes. It has also been found that *ATRX* is important for resolving G-quadruplexes, that impede gene transcription, indicating that *ATRX* is found at intragenic regions and facilitates transcription of specific genes (Levy, Kernohan, Jiang, & Berube, 2015). Together, these studies demonstrate the functional diversity of *ATRX* in gene regulation.

1.5. *Functional Role of ATRX in the brain*

ATRX plays a critical role in the earliest stages of development. Gastrulas that are *ATRX*-null in embryonic stem cells are incapable of surviving beyond gestational day 9.5 (Garrick et al., 2006) demonstrating an essential role for *ATRX* at the beginning of

embryonic development. Furthermore, when *ATRX* is ablated in mice during embryonic development in neural precursor cells (NPC) specifically, the mice survive long enough to be born but die soon after birth (Watson et al., 2013). In the aforementioned mouse model, *ATRX* levels are ablated around embryonic day 8.5 through Cre-recombination to excise exon 18 of *Atrx* at the onset of *Foxg1* driven Cre expression (Berube et al., 2005; Watson et al., 2013). These mice have incomplete cortical development, as well as under-developed dentate gyri of the hippocampus due to increased apoptosis (Berube et al., 2005), which suggests that *ATRX* is critical for cell survival during the earliest stages of brain development. As these mice that lack *ATRX* in NPCs do not survive into adulthood, alternative models of *ATRX* deficient mice had to be developed in order to understand how reduced *ATRX* levels in the brain impact behaviour. In a conditional knockout (cKO) model where *Atrx* expression is reduced in NPCs during embryonic development rather than ablated, mice are capable of surviving into adulthood (Tamming et al., 2017). Due to *Atrx* being X-linked, this model reduces *Atrx* expression in females in a mosaic pattern where only some NPCs are *Atrx*-null. The resulting females mice have reduced, not ablated, *Atrx* expression, disrupted endocrine signaling, deficits in object recognition, contextual fear, and spatial memory, but normal working memory in adulthood (Tamming et al., 2017). The findings from these studies demonstrate that the timing and the degree to which *ATRX* is reduced during embryonic development will influence the extent to which development is negatively impacted.

While *ATRX* expression is critical during embryonic development, it continues to play an important role in early postnatal brain development. In mouse models where *Atrx* expression is knocked out in excitatory neurons of the forebrain at the onset of CamKII expression, mice have structural changes to synapses in adulthood, along with impairments in long-term memory formation in males (Tamming et al., 2019) and reduced LTP in the hippocampus (Gugustea et al., 2019). Although these mice survive into adulthood, unlike mice that are *Atrx*-null in NPCs during prenatal development, they have anatomical and physiological deficits in the forebrain that are likely underlying the observed cognitive deficits. The mice in (Gugustea et al., 2019) and (Tamming et al., 2019) show more severe cognitive deficits in adulthood compared to the female mice described in (Tamming et al., 2017), likely due to the fact that the former have ablated *Atrx* expression

in the forebrain, while the latter have reductions in *Atrx* expression throughout the brain. Thus, the degree to which *Atrx* expression is reduced in the forebrain has differential impacts on behaviour in adulthood.

Several studies have reported impairments resulting from *ATRX* deficiencies; however, it remains unclear how a reduction in *Atrx* expression in the brain leads to the observed behavioural deficits. *ATRX*, being a chromatin remodeling protein, has transcriptional control over large portions of the genome. Knowing that *ATRX* deficiency leads to an increase in apoptosis (Berube et al., 2005) and that *ATRX* partners with *MeCP2* to transcriptionally repress genes implicated in ASD (Kernohan et al., 2010), we expect that reduced *ATRX* expression in the forebrain is associated with widespread dysregulation of other genes important for cell survival and synaptic plasticity.

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

An organism's early life experiences are capable of influencing gene expression by altering epigenetic modifications, resulting in altered developmental outcomes (Roth & Sweatt, 2011). Stress exposure during intrauterine development has been associated with dysregulated gene expression (Mueller & Bale, 2008). Also, maternal care during early postnatal life has been shown to regulate epigenetic programming of gene expression into adulthood (Weaver et al., 2004). Regulation of *Atrx* expression is affected by early mother-pup interactions (Weaver et al., 2006), which may affect widespread gene regulation as *ATRX* is a chromatin remodeling protein. There is a gap in knowledge about the impact of adverse early life experiences on *Atrx* expression and the way in which the regulation and expression of other genes may be impacted.

1.6.1. Gestational Stress

Previous studies have shown that maternal stress during pregnancy can have an impact on offspring development and behaviour into adulthood. Males exposed to stress *in utero* have increased anxiety-like behaviour, poorer learning outcomes later in life, altered gene expression in adulthood (Liao et al., 2019). Growing evidence suggests that prenatal exposure to stress can impact neurodevelopment and increase an individual's susceptibility to major neurodevelopmental disorders (Beversdorf et al., 2018). Previous work in the Weaver lab has shown that exposure to the effects of physical/psychological

restraint stress during the third trimester of intrauterine development is associated with altered social, cognitive and emotional behaviour in adulthood, similar to the behaviour seen in mice that have reduced *Atrx* expression. Changes to *Atrx* expression in these mice exposed to gestational stress have yet to be assessed.

1.6.2. Postnatal Mother-Pup Interactions

Maternal behaviour towards offspring during the postnatal period prior to weaning influences epigenetic programming. Mice born to mothers who are highly nurturing toward their offspring in early life are less susceptible to stress in adulthood when compared to mice whose mothers are less nurturing (Caldji, Hellstrom, Zhang, Diorio, & Meaney, 2011). Maternal stress during pregnancy affects the mother's behaviour towards offspring in the early postnatal period. In rodent models, variations in maternal licking and grooming (LG) of pups during early life is epigenetically programmed through changes in chromatin structure and DNA methylation, resulting in permanent changes in gene expression in the hippocampus of offspring, including *Atrx* expression (Weaver et al., 2006). Restraint stress exposure during pregnancy can cause dams to show increased depressive-like symptoms postpartum (Haim, Sherer, & Leuner, 2014). Pregnant rats exposed to a long-term restraint stress during the third trimester of pregnancy were observed to show reduced active maternal behaviours towards their pups (Smith, Seckl, Evans, Costall, & Smythe, 2004) indicating that gestational stress is capable of influencing maternal behaviour towards offspring. Recent work in the Weaver lab shows that pregnant mice exposed to chronic restraint stress during the third trimester showed reduced active and increased passive care towards their pups. In the present study, we will investigate whether this change in maternal behaviour toward the offspring is associated with changes to *Atrx* promoter regulation and expression in offspring.

1.7. *Generation of Atrx Gene Targeted Mice*

As previously described, mice with *ATRX* deficiencies onset in the earliest stages of development are not viable (Berube et al., 2005; Garrick et al., 2006). In order to assess the effects of reduced *ATRX* expression in early life and the behaviour of these animals in adulthood, we used a conditional knockout that silences *Atrx* expression around embryonic day 10-12 in the C57BL/6 background strain. The transgenic mice were

generated using a Cre/LoxP recombination system. Two LoxP sites were inserted into the *Atrx* sequence flanking exon 18, that encodes the ATP-ase domain (Berube et al., 2005) to generate *Atrx* floxed female mice. These mice were then bred with male mice expressing Cre recombinase. Cre expression in the offspring of this breeding pair is driven by *Emx1* expression to target cells in the forebrain. *Emx1* is expressed around embryonic day 10-12 in precursor cells of excitatory neurons and glia (Gorski et al., 2002), therefore the *Atrx*-cKO will be limited to these types of cells. The resulting transgenic mouse, which will be referred to as $Atrx^{HEM}$, is a Cre-positive hemizygous male that is *Atrx*-null due to the floxed *Atrx* gene being X-linked. The cKO of exon 18 is equivalent to *Atrx*-null, as transcription results in a non-functional, truncated *ATRX* protein (Berube et al., 2005). Furthermore, the cKO does not lead to the generation of new *ATRX* isoforms that rescue the diminished function (Berube et al., 2005). From this breeding scheme, all females will express wild-type *Atrx* ($Atrx^{WT}$).

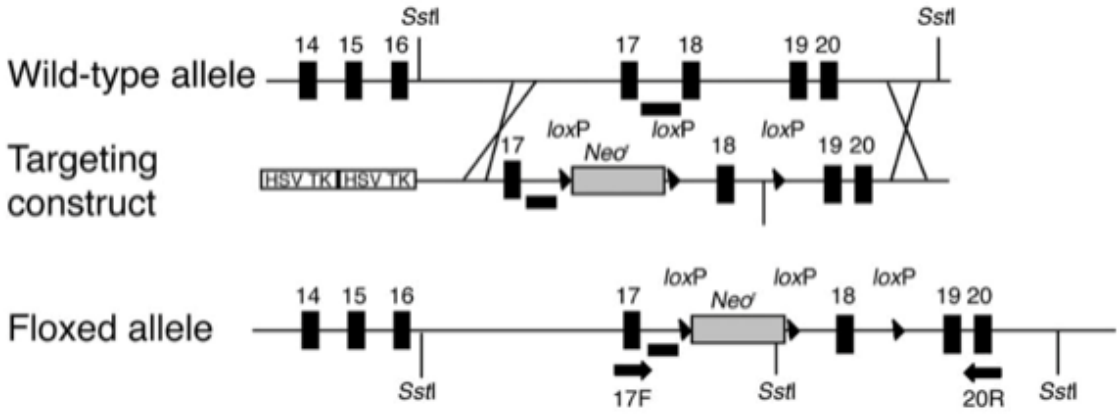

Figure 6. Generation of *Atrx* floxed gene construct. Figure from Berube et al. (2005).

1.8. *mTOR, an ASD Risk Gene*

Mammalian target of rapamycin (*mTOR*) is an atypical serine/threonine kinase that regulates cellular growth and proliferation, protein synthesis, and neuronal plasticity (Asnaghi, Bruno, Priulla, & Nicolin, 2004). Together with several other proteins, *mTOR* forms two structurally and functionally distinct complexes: *mTORC1* and *mTORC2* (Figure 6) (Gilbert & Man, 2017). The complexes vary in their molecular composition and are involved in distinct signaling cascades that vary both upstream and downstream of *mTOR* (Figure 7). Both *mTORC1* and *mTORC2* are sensitive to different factors in the cell that

can alter the activity of the pathways (Wullschleger, Loewith, & Hall, 2006). *mTORC1* can sense factors such as growth factors, available nutrients, energy, and stress, while *mTORC2* activity has only been shown to be affected by growth factors (Gilbert & Man, 2017). The role of *mTORC1* is currently shown to be more functionally broad compared to that of *mTORC2*.

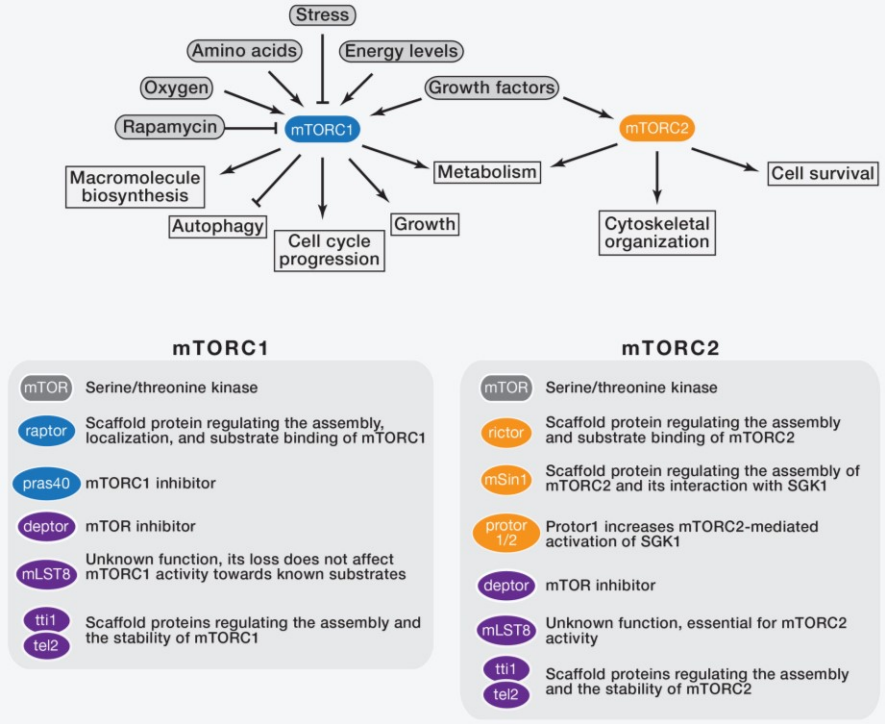

Figure 7. Proteins involved in forming *mTORC1* and *mTORC2*. Both complexes are sensitive to environmental factors through upstream signaling and affect changes to downstream signaling cascades of various functions. Adapted from Gilbert & Man (2017).

As *mTOR* is involved in many intracellular signaling cascades, mutations or dysregulated expression can have dire consequences on the individual. For instance, mutations to *Mtor* are associated with cancers (Grabiner et al., 2014), abnormal brain growth and cognitive dysfunction (Baynam et al., 2015). Furthermore, *Mtor* is listed in the SFARI database as an ASD risk gene. ASD is characterized by changes to brain growth and synaptic regulation (Bourgeron, 2015; Fombonne, Roge, Claverie, Courty, & Fremolle, 1999). The PI3K-Akt pathway involving *mTORC1* is involved in several important processes in neurons, such as forming long-term memories through LTP (Opazo, Watabe, Grant, & O'Dell, 2003), that are dysfunctional in ASD. Several studies have reported dysregulation of the PI3K-Akt pathway is associated with ASD (Cusco et al.,

2009; Kelleher & Bear, 2008; Onore, Yang, Van de Water, & Ashwood, 2017). In the brains of humans with idiopathic ASD, expression of proteins in the PI3K-Akt pathway involving *mTORC1* are downregulated (Nicolini, Ahn, Michalski, Rho, & Fahnestock, 2015) and synaptic pruning involving *mTORC1* has been shown to be impaired (Tang et al., 2014).

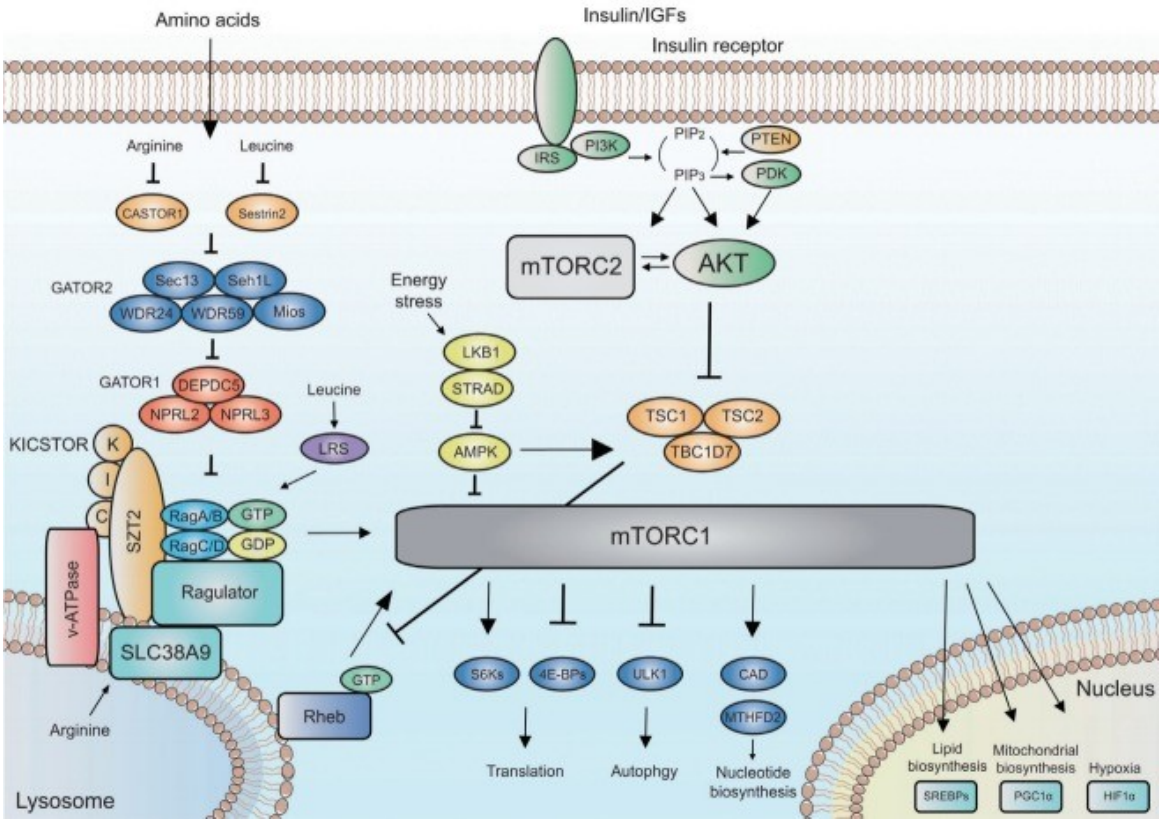

Figure 8. Upstream regulators and downstream functional outcomes of pathways involving *mTORC1* and *mTORC2*. Figure from Kim & Lee (2019).

As previously mentioned, various factors can influence the activity of *mTOR* complexes. First, *mTOR* activity is regulated through phosphorylation at two main sites. Ser2448 is phosphorylated by both upstream and downstream factors in the PI3K-Akt pathway (Chiang & Abraham, 2005; Sekulic et al., 2000). *mTOR* is also capable of autophosphorylation at Ser2481 (Soliman et al., 2010). Phosphorylation of *mTOR* results in activation of *mTORC1* and *mTORC2,* which will alter the activity of several intracellular pathways. The activity of pathways involving *mTOR* can also be influenced by intercellular signaling and environmental factors. For instance, gestational stress in the form of maternal immune activation leads to a down regulation in *mTOR* expression as well as

expression of other proteins in the PI3K-Akt pathway (Nicolini et al., 2015). *mTORC1* activity is also inhibited by DNA damage through *p53* signaling (Budanov & Karin, 2008; Feng, Zhang, Levine, & Jin, 2005). Modifications to *mTOR* pathway activity can result from various factors and will impact neurodevelopment and neuronal function in adulthood.

The relationship between *ATRX* and *mTOR* has yet to be investigated despite the fact that both genes are implicated in idiopathic ASD, the proteins they encode are involved in regulating cell death/survival, and the activity of both can be influenced by stress and DNA damage. Previous work in the Weaver lab has shown that the *Mtor* promoter region in neurons of *Atrx*^{HEM} mice is occupied by *MeCP2*, indicating it is likely transcriptionally repressed. However, no studies have investigated changes to *mTOR* expression related to *ATRX* deficiencies. Further, few studies have investigated the role of gestational stress and altered maternal care or *Mtor* regulation and expression.

1.9. *MeCP2*

Methyl CpG binding protein 2 (*MeCP2*) is an MBP that is involved in transcriptional regulation through locating and binding to 5-methylcytosine regions in CpGs (Mellen, Ayata, Dewell, Kriaucionis, & Heintz, 2012; Nan, Campoy, & Bird, 1997). At promoter regions, *MeCP2* occupancy represses gene expression (Nan et al., 1997) through recruiting histone deacetylase complexes to modify DNA-histone bonds (Jones et al., 1998). In this way, and through association with chromatin remodelling proteins, *MeCP2* is capable of inducing changes to chromatin regulation (Kernohan, Vernimmen, Gloor, & Berube, 2014). In particular, *MeCP2* recruits *ATRX* to promoter regions and it has been observed that mice that are *MeCP2* deficient have disrupted *ATRX* localization and by consequence, transcriptional regulation (Nan et al., 2007). These studies suggest a critical role for *MeCP2* in transcriptional silencing and that dysregulated expression of *MeCP2* can lead to widespread dysregulation of gene expression throughout the genome.

MeCP2 is listed by SFARI as an ASD risk gene as mutations to *Mecp2* are associated with Rett syndrome and syndromic ASD (Shibayama et al., 2004). Although *Mecp2* mutations account for small portion of ASD cases, dysregulated expression of *MeCP2* is commonly observed in the forebrains of people with ASD (Nagarajan, Hogart, Gwye, Martin, & LaSalle, 2006). Furthermore, in the brains of people with ASD, *Mecp2*

promoter is hypermethylated and X-chromosome inactivation is altered (Nagarajan et al., 2008). The imprinted brain theory posits that people with ASD have altered expression of imprinted genes, in favour of increased paternal expression (Badcock, 2011). As *ATRX* associates with *MeCP2* to transcriptionally silence genes at imprinting control regions (Kernohan et al., 2010), we are interested in investigating the effects of *ATRX* deficiency on *MeCP2* expression in the brain to gain an understanding of mechanisms of gene dysregulation that may be potentially underlying idiopathic ASD.

1.10. *Present Research*

There is a knowledge gap in our current understanding of the molecular mechanisms and signaling pathways underlying the development of idiopathic ASD. Studying the influence of early life experiences on the expression of genes that are important for neurodevelopment, such as *Atrx* and *Mtor*, may lead to a more complete understanding of their function.

In this study, we will use banked tissue from second-generation (F1) adult male *Atrx*^{WT} and *Atrx*^{HEM} mice, as well as female *Atrx*^{WT} mice, to investigate the relationship between *Atrx* and *Mtor*, two candidate genes in idiopathic ASD. We will also look at the impact of early life experiences on the regulation and expression of these genes. While all females were *Atrx*^{WT}, some were raised with male *Atrx*^{HEM} siblings, which has been shown to reduce quality of maternal care towards offspring. This will allow us to measure the impact of reduced quality maternal care on *Atrx* expression and regulation. In addition, a group of first-generation (F0) female mice were exposed to chronic physical and physiological restraint stress during the third trimester of pregnancy. Using tissue from the F1 mice exposed to gestational stress will allow us to study how early life stress impacts forebrain gene expression and regulation into adulthood.

First, we will measure *ATRX*, *mTOR* and phosphorylated-*mTOR* (*p-mTOR*) protein expression levels in the forebrains of adult *Atrx*^{WT} and *Atrx*^{HEM} mice using enzyme-linked immunosorbent assay (ELISA). Some mice will have experienced chronic stress exposure in the third trimester of gestation, while others did not. Offspring also received variable levels of maternal care in the early postnatal period. This will provide insight as to how protein expression changes in adulthood as *Atrx* is reduced transgenically, or through

early life exposures. Next, we will quantify mRNA expression levels using real-time quantitative polymerase chain reaction (RT-qPCR) in forebrain samples from these same animals. This will allow us to understand if differences to protein expression are due to post-transcriptional modifications or differences in DNA transcription. Finally, we will investigate changes to DNA methylation of the *Atrx* and *Mtor* promoters using sodium bi-sulfite pyrosequencing, providing insight as to how epigenetic regulation of these genes change under various early life conditions.

The main goal of this study is to better understand the relationship between early life experiences and gene expression and regulation in the forebrain, which may also help to understand how ASD-like phenotypes emerge with no genetic mutation.

1.11. *Main Objectives*

I. Characterize *ATRX*, *mTOR* and *p-mTOR* expression in the forebrains of $Atrx^{WT}$ and $Atrx^{HEM}$ mice that are stress naïve or exposed to stress *in utero*.

II. Investigate the effects of early life stress exposure and reduced quality of maternal care on *Atrx, Mtor,* and *Mecp2* expression in the forebrain.

III. Assess changes to epigenetic regulation of *Atrx* and *Mtor* promoter in response to modified *ATRX* expression, and early life experiences.

IV. Investigate the role of *ATRX* in regulating the expression of ASD risk gene expression in the forebrain by drawing correlations between *ATRX* expression and the expression and regulation of ASD risk genes.

CHAPTER 2: MATERIALS AND METHODS

2.1. *Tissue Collection and Dissection*

Animals were euthanized by asphyxiation using an isofluorane and carbon dioxide gas combination in the HiRoad Rodent Euthanasia System® (Lab Etc. Incorporated). Whole brains, as well as, ear punches, tail snips, livers, spleens, and intact gastrointestinal tracts were collected from each animal and immediately snap-frozen on dry ice. The samples were subsequently stored at -80°C.

Dissections of the frozen whole brain were performed on a chilled metal surface placed on ice. Using a scalpel and spatula, the brains were split along the interhemispheric fissure and the cerebellum, hippocampi and frontal cortex were isolated. While freeing the cerebellum, the colliculus inferior and remaining area of the pons were removed. The olfactory bulbs were removed, and the prefrontal cortex was isolated. Each hemisphere was rotated such that the medial surface was exposed. Using the spatula, the medial surface was removed to separate the fornix and roll the hippocampus out of the cortex. Each region was divided into three equal parts and placed into separate 1.5mL microcentrifuge tubes. Brain regions were weighed and stored at -80°C.

2.2. *DNA Extraction and Bisulfite Pyrosequencing*

Genomic DNA (gDNA) was extracted from one of the three previously divided sections of the frontal cortex, hippocampus and cerebellum of each mouse using the DNeasy® Blood & Tissue Kit (Cat. #: 69506, Qiagen, Valencia, CA, USA) following the protocol in the July 2006 Handbook. The purity and concentration of DNA was assessed using 2µL of sample on the Take3 micro-volume plate on an Epoch Microplate Spectrophotometer (Biotek, Winooski, VT, USA). Any samples with a 260/280nm ratio less than 1.8 were excluded as this measure would indicate protein contamination. The measured concentrations (ng/µL) were then used to determine the volume of template gDNA required for 20 ng, which is the optimal amount of template required for pyrosequencing (Cummings et al., 2013).

The template gDNA underwent a bisulfite conversion reaction using the EpiTect® Bisulfite Kit (Cat. #: 59104, Qiagen, Valencia, CA, USA) to convert cytosine residues to uracil residues through deamination without affecting 5-methylcytosine residues

(Delaney, Garg, & Yung, 2015). The reaction occurred in 200µL PCR strip tubes in the C1000 Touch^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as per the temperature cycling protocol shown in Table 1. The bisulfite converted DNA was then purified using buffers and purification columns in the aforementioned kit to terminate the conversion reaction and remove contaminants from the product prior to continuing the protocol.

Table 1. 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

Table 2. 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

The bisulfite treated DNA was used as a template to generate amplicons through PCR using EpiMark® Hot Start *Taq* DNA polymerase (Cat. #: M0490L, New England

BioLabs® Inc., Ipswich, MA, USA) in the C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA.) following the cycling protocol in Table 2. The primers were designed to specifically assess DNA methylation at the promoter regions of the *Atrx, Mtor* and *Line1* genes (see Table 3.). The PCR products were then run on a 2% agarose gel and visualized with UView™ 6x Loading Dye (Cat. #: 166-5112, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and compared to a 50-2000bp ladder AmpliSize® Molecular Ruler (Cat. #: 170-8200, Bio-Rad Laboratories, Inc., Hercules, CA, USA) to verify that there was a single band produced for each primer set, indicating specificity of the primer. The PCR products for each primer were excised using a razor blade and extracted from the gel using the QIAquick® Gel Extraction Kit (Cat. #: 28706, Qiagen, Valencia, CA, USA) following the Quick-Start protocol from July 2015.

Table 3. Sequence for primers used in PCR reaction and for pyrosequencing.

Gene	F primer (5'-3')	R primer (5'-3')	Sequencing Primers (5'-3')
Atrx	TTGGTGTTTTTT TTTTTTGTTGAT GAG	TAAAACAAATC CCTCCTCCTCT (Biotin)	TTGTTGATGAGGTGG
Mtor	GTGGAGGGGAT TTGTAGTAG	(Biotin) ACTCCA AACCCCAAACT CAC	GTTTTTAGTTTTTTTT TGTTTGTTA
Line1	AGGGAGAGTTAGA TAGTG	(Biotin) AACTAT AATAAACTCCA CCC	GTGCATTGAACCCTCAA AGG

Not all PCR products were run on the 2% agarose gel. After confirming that the PCR product generated by the primers listed in Table 3 were of the same length of base pairs as expected to be produced, the subsequent PCR products were purified to remove contaminants using the QIAquick® PCR Purification Kit (Cat. #: 69506, Qiagen, Valencia, CA, USA) rather than by gel extraction.

The purified PCR product of the bisulfite treated samples were used for pyrosequencing in the PyroMark® Q24 Advanced pyrosequencer (Qiagen, Valencia, CA, USA) using the PyroMark® Q24 Advanced CpG Reagents (Cat. #: 970922, Qiagen, Valencia, CA, USA) and following the Quick-Start protocol from December 2012.

2.3. *RNA Extraction and Quantitative PCR*

RNA was extracted from one of the three previously divided sections of the frontal cortex, hippocampus and cerebellum of each mouse using the RNeasy® Plus Mini Kit (Cat. #s: 74134 and 74136, Qiagen, Valencia, CA, USA) following the protocol in the kit. On the day of extraction, RNA concentration was measured with a 2µL sample on the Take3 micro-volume plate on an Epoch Microplate Spectrophotometer (Biotek, Winooski, VT, USA). Purity of RNA was assessed using the 260/280 nm ratio. Samples with a ratio less than 1.8 were excluded. Integrity of 12 RNA samples was assessed on the day of extraction using Experion (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The RNA Quality Indicator (RQI) values of the samples were above 9, which indicates acceptable integrity for RT-qPCR (Taylor, Wakem, Dijkman, Alsarraj, & Nguyen, 2010).

Table 4. 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 (°C)
Priming	5	25
Reverse transcription	20	46
Reverse transcriptase inactivation	1	95

RNA was then reverse transcribed into single-stranded complimentary DNA (cDNA) in the C1000 Touch^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in a 20µL reaction in 200µL PCR strip tubes following the cycling protocol in Table 4. The reverse transcriptase (RT) used was iScript Reverse Transcription Supermix for RT-qPCR (Cat. #: 1708841, Bio-Rad Laboratories, Inc., Hercules, CA, USA). See Table 5 for primer sequences. A control with no template (NTC) and another with no RT (NRT) were generated following the same cycling protocol in order to assess contamination of the reagents or across wells. Following the conversion reaction, 2µL from each sample of cDNA was pooled in a 1.5 mL microcentrifuge tube. The pooled cDNA was used to generate a concentration gradient by diluting samples to 1 x10^{n} (n = number in dilution series) for five dilutions.

Table 5. Sequences of primers used in RT-qPCR reactions.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Gapdh	GTTGTCTCCTGCGACTTCA	GGTGGTCCAGGGTTTCTTA
Atrx	GTCCGAGCCAAAAACATGAC	GTCATGAAGCTGCACCA
Mecp2	CCCAAGGAGCCAGCTAAGAC	GCTTTGCAATCCGCTCTATG
mTOR	AGCTTCGTGGCCTATCAG	ATGAGCAGCGTCAGTGATGT
HPRT	AAGCTTGCTGGTGAAAAGGA	TTGCGCTCATCTTAGGCTT
b-actin	TTGCTGACAGGATGCAGAAG	ACATCTGCTGGAAGGTGGA

To optimize the concentration and temperature conditions for each RT-qPCR primer set, five dilutions of pooled cDNA were run in triplicate technical replicates in the CFX96 qPCR Instrument (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with an annealing temperature gradient. Plates were set up such that the concentration gradient varied by columns and the annealing temperature varied by row (refer to Table 6 for temperature cycling protocol). When optimizing primers, the temperature cycling protocol concluded with a melt curve analysis that ranged from 65-95 ºC and increased by 0.5 ºC every 5 seconds.

Table 6. 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	60 for *HPRT* and *b-actin* 62.7 for *Mtor* 63 for *Atrx* 65 for *Mecp2*
Melt-curve (only for primer temp optimization)	0.5s/each increase in 1 ºC	65-95

The optimal dilutions were determined to be 1 x10^1 for all primer sets. These dilutions were chosen as these were the conditions that lead to the quantitative cycle (Cq) to be between 20 and 30 for the pooled cDNA samples. The optimal annealing temperatures were determined to be 60 ºC for *HPRT* and *b-actin,* 63 ºC for *Atrx*, 62.7 ºC for *Mtor*, and 65 ºC for *Mecp2* using the data produced from the melt curve. At these optimal annealing temperatures and dilution concentrations, a melt curve with a single peak, indicating annealing specificity and lack of primer dimerization (Taylor et al., 2010), was produced for each primer set. To confirm that the primers were effectively amplifying the intended target sequence, samples from each primer set were run on a 2% agarose gel with UView™ 6x Loading Dye (#166-5112, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and compared to a 50-2000bp ladder AmpliSize® Molecular Ruler (Cat. #: 170-8200, Bio-Rad Laboratories, Inc., Hercules, CA, USA) to verify that the amplicon band was of the expected length.

Master mix was generated by adding 5µL of Sso Advanced Universal SYBR® Green Supermix (Cat. #: 1725274, Bio-Rad Laboratories, Inc., Hercules, CA, USA), 0.5µL of 10µM forward primer, 0.5µL of 10µM reverse primer, and 2µL or PCR-grade water per sample. Within each 10µL reaction in the 96 well plate, 8µL of master mix and 2µL of template cDNA of the previously determined optimal dilution was used.

There were eight biological replicates per group and each sample was run in two technical replicates. When technical replicates deviated more than 0.5 Cq, they were repeated in triplicate and the average of the two technical replicates within 0.2 Cq was used in the analysis. Samples were only excluded once replicated in triplicate and no replicate was within 0.2 Cq of another. To make valid comparisons between samples that spanned over multiple plates, an inter plate calibrator (IPC) was used. The IPC consisted of the pooled cDNA sample diluted to 1 x10^1 with *Gapdh* primers in the master mix. The threshold was then set on each data file according to the Cq for the IPC at the temperature at which the plate was run.

Using the CFX Maestro software (Bio-Rad Laboratories, Inc., Hercules, CA, USA), the average Cq for each sample was used to calculate the normalized expression (ΔCq) with *HPRT* and *b-actin* as the reference genes. Reference gene stability was assessed using the geNorm function in the software.

2.4. *Protein Extraction and Enzyme Linked Immunosorbent Assay*

Protein was extracted from one of the three previously divided sections of the frontal cortex, hippocampus and cerebellum of each mouse. Ice cold Tris-buffered saline (TBS) with protease inhibitors (P8340; Sigma) and 2 mM ethylenediaminetetraacetic acid (EDTA) was added to each tube. The tissue was homogenized and centrifuged for 20 minutes at 100 000g at 4 ºC. The resulting Triton X-100 insoluble pellet was dissolved to half the initial homogenization volume in 70% formic acid and centrifuged for 10 minutes at 14 000g at 4 ºC. Next, the supernatant was evaporated using a nitrogen stream to 70% of the original volume. The solution was subsequently neutralized to pH 7 with 5M sodium hydroxide in 1M TBS and used in the ELISA assays.

ELISA was performed to measure *ATRX* (Cat. #: LS-F3104, LifeSpan BioSciences, Inc.), *mTOR* (Cat. #: MBS762851, MyBioSource.com) and *p-mTOR* (Cat. #: PEL-mTOR-S2448, Ray Biotech) protein expression levels following the respective protocols provided in each kit.

2.5. *Statistical Analyses*

Animals were earmarked and given a unique number. During analysis, the researchers were blinded to genotype and experimental group. Akaike information criterion (AIC) scores likelihood ratios (LR) and p-values were reported (Wagenmakers & Farrell, 2004). Due to the R package used (lme4), 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). For the group comparisons, linear mixed-effects models were used with genotype ($Atrx^{WT}$ or $Atrx^{HEM}$) and stress condition (home cage or restraint stress) as the between factors, and brain region [frontal cortex (FC), hippocampus (HIPP), cerebellum (CER)] 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. *ATRX, mTOR and p-mTOR Protein Expression*

We quantified *ATRX, mTOR,* and *p-mTOR* expression in the frontal cortex, hippocampus, and cerebellum of F0 and F1 female and male mice. F0 female groups consisted of two genotypes ($Atrx^{WT}$ and floxed *Atrx)* and two stress conditions (stress naïve and gestational stress). F0 male groups consisted of two genotypes ($Atrx^{WT}$ and Cre) and none were exposed to 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 groups consisted of two genotypes ($Atrx^{WT}$ and $Atrx^{HEM}$) and two stress conditions (stress naïve and exposed to gestational stress). There were four mice per group. We report *ATRX, mTOR* and *p-mTOR* expression, as well as the ratio of *p-mTOR* to total *mTOR.* Models were compared using AIC and LR.

3.1.1. ATRX

There were no significant differences in *ATRX* protein levels in the frontal cortex, hippocampus or cerebellum of F0 male mice (all $p > 0.05$; Figure 9A) or F0 female mice (all $p > 0.05$; Figure 9B) based on genotype or stress condition. F1 male $Atrx^{HEM}$ mice had reduced expression of *ATRX* compared to male $Atrx^{WT}$ mice (AIC = -63.173, LR = 10.57, $p = 0.001$; Figure 9C). F1 males exposed to gestational stress had reduced *ATRX* expression compared to male $Atrx^{WT}$ mice (AIC = -64.353, LR = 9.39, $p = 0.002$). When comparing expression levels across brain regions, the most *ATRX* expression was measured in the cerebellum, followed by the hippocampus and the least in the frontal cortex (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 not in the hippocampus (CI 95% = -0.009, 0.255) or 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 reduced *ATRX* expression compared to F1 females raised with male $Atrx^{WT}$ siblings (AIC = -68.365, LR = 7.95, $p = 0.005$; Figure 9D). In addition,

female mice exposed to gestational stress had reduced *ATRX* compared to stress naïve F1 females (AIC = -62.444, LR = 13.88, p < 0.001).

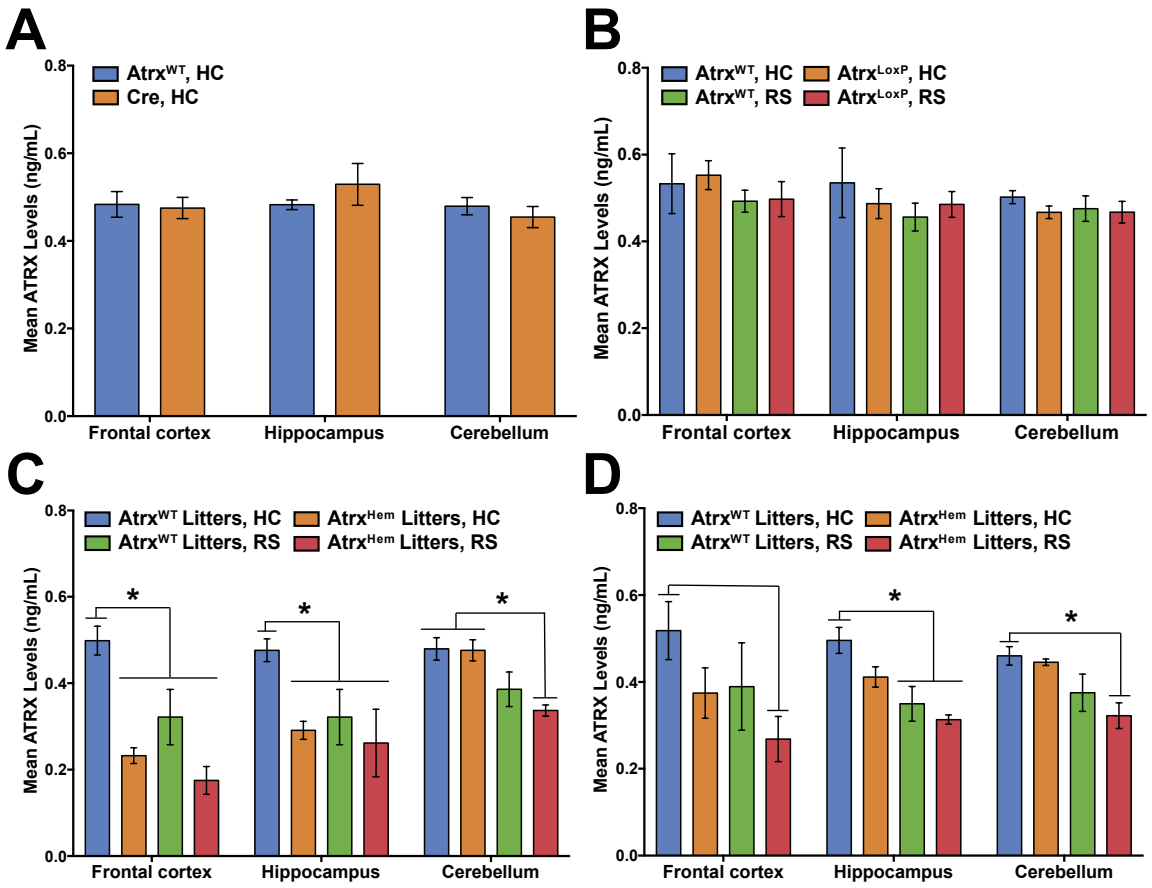

Figure 9. Effect of prenatal stress exposure and/or *Atrx* deficiency on *ATRX* protein levels in adult male and female offspring. *ATRX* expression in (A) WT or Cre fathers housed in homecage (HC), (B) *Atrx*^{WT} or *Atrx*^{LoxP} mothers housed in HC or restraint stressed (RS) during pregnancy, (C) *Atrx*^{WT} or *Atrx*^{HEM} male offspring from HC or RS mothers, (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).

3.1.2. mTOR

In F0 males, *mTOR* expression levels did not differ by genotype in the hippocampus (CI 95% = -0.117,0.051) or the cerebellum (CI 95% = -0.024, 0.029). However, in the frontal cortex, F1 male *Atrx*^{WT} mice had higher *mTOR* expression compared to male Cre expressing mice (CI 95% = 0.009, 0.069), which caused a genotype by brain region interaction (AIC = -70.702, LR = 8.472, p = 0.014; Figure 10A).

In F0 females, there were no main effects of genotype or stress on *mTOR* expression (both $p > 0.05$). However, when comparing *mTOR* levels between brain regions, the cerebellum had higher expression compared to the frontal cortex and hippocampus (AIC = -59.445, LR = 91.08, $p < 0.001$; Figure 10B). There was also an interaction between genotype and region (AIC = -146.21, LR = 6.02, $p = 0.049$). The 95% CIs revealed no significant effect of genotype within each brain region (CI 95% FC = -0.024, 0.076, HIPP = -0.050, 0.015, CER = -0.024, 0.007). There was a trend for genotype by stress condition by region interaction (AIC = -148.23, LR = 5.65, $p = 0.059$). In the frontal cortex of the F0 female stressed *Atrx*^{WT} mice, there was a significant negative correlation between *ATRX* and *mTOR* expression ($r = -0.999$, $p = 0.01$).

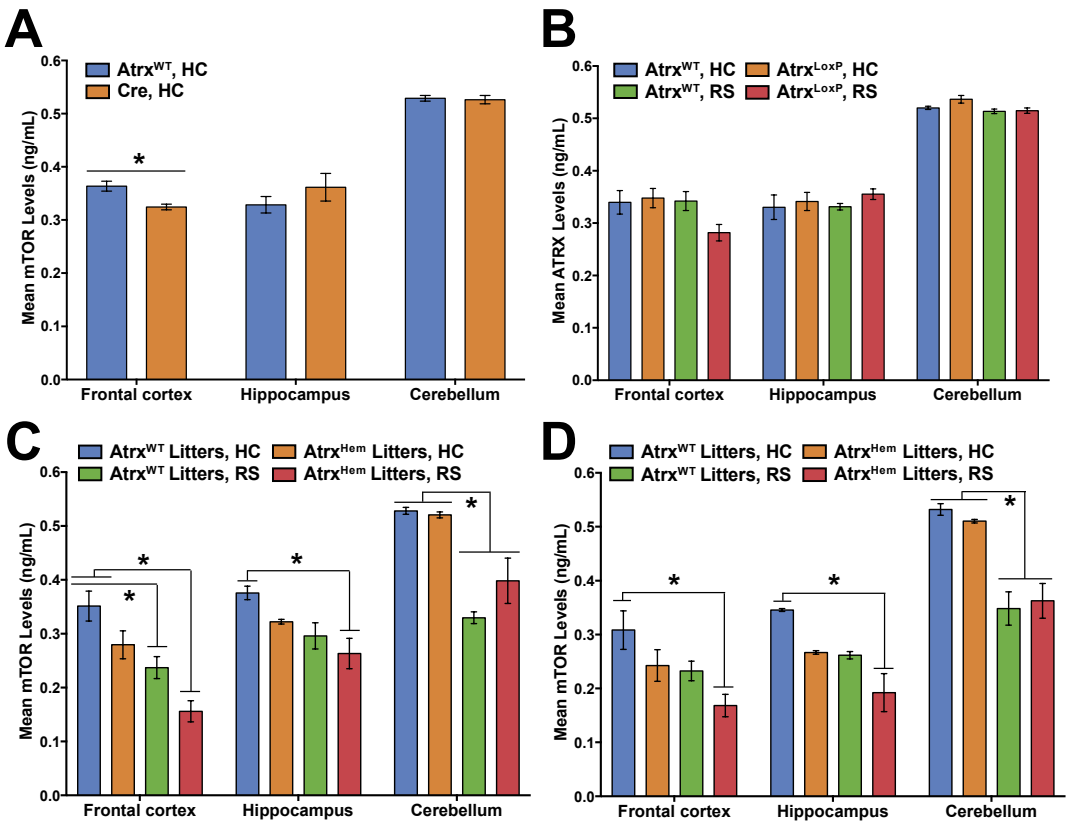

Figure 10. Effect of prenatal stress exposure and/or *Atrx* deficiency on *mTOR* protein levels in adult male and female offspring. *mTOR* expression in (A) WT or Cre fathers housed in homecage (HC), (B) *Atrx*^{WT} or *Atrx*^{LoxP} mothers housed in HC or restraint stressed (RS) during pregnancy, (C) *Atrx*^{WT} or *Atrx*^{HEM} male offspring from HC or RS mothers, (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).

F1 males exposed to gestational stress had decreased *mTOR* expression (AIC = -86.836, LR = 23.81, $p < 0.001$; Figure 10C). The effect of stress was most pronounced in the frontal cortex (CI 95% FC = 0.047, 0.191), followed by the cerebellum (CI 95% CER = 0.104, 0.216) and the hippocampus (CI 95% HIPP = 0.019, 0.120), which lead to an interaction between gestational stress condition and brain region (AIC = -114.71, LR = 11.29, $p = 0.004$). When comparing between brain regions, *mTOR* expression was greatest in the cerebellum, followed by the hippocampus, and least in the frontal cortex (AIC = -61.095, LR = 51.55, $p < 0.001$). In addition, male *Atrx*^{HEM} mice showed a trend toward reduced *mTOR* expression compared to male *Atrx*^{WT} mice (AIC = -106.815, LR = 3.83, $p = 0.050$), and this difference was reversed in the cerebellum leading to a genotype by region interaction (AIC = -110.93, LR = 15.07, $p < 0.001$). There was also a significant negative correlation between *ATRX* and *mTOR* expression in the cerebellum of F1 male *Atrx*^{HEM} mice who were exposed to gestational stress ($r = -0.99$, $p = 0.03$).

F1 female mice raised with male *Atrx*^{HEM} siblings had reduced *mTOR* expression compared to females raised with male *Atrx*^{WT} siblings (AIC = -104.322, LR = 9.75, $p = 0.002$; Figure 10D). There was a main effect of stress on *mTOR* expression in F1 females: F1 females that experienced gestational stress had reduced *mTOR* compared to females that were stress naïve (AIC = -89.279, LR = 24.79, $p < 0.001$). This effect of stress was most pronounced in the frontal cortex compared to the hippocampus and cerebellum (AIC = -111.81, LR = 12.31, $p = 0.002$). Across brain regions, *mTOR* expression was greatest in the cerebellum (AIC = -55.106, LR = 60.97, $p < 0.001$). Within the frontal cortex and cerebellum, there were no differences in *mTOR* expression between females raised with male *Atrx*^{WT} or *Atrx*^{HEM} siblings (CI 95% FC = -0.012, 0.140, CER = -0.122, 0.130). However, females raised with male *Atrx*^{HEM} siblings had reduced *mTOR* expression in the hippocampus compared to females raised with male *Atrx*^{WT} siblings (CI 95% HIPP = 0.008, 0.141), which created a sibling genotype by brain region interaction (AIC = -116.71, LR = 7.41, $p = 0.025$). Further, there was a negative correlation between *ATRX* and *mTOR* expression in the cerebellum of F1 female mice that experienced gestational stress and were raised with male *Atrx*^{HEM} siblings.

3.1.3. p-mTOR

In F0 males, *p-mTOR* expression was highest in the cerebellum compared to other regions (AIC = -40.928, LR = 16.72, $p < 0.001$; Figure 11A). Similarly, in F0 females, *p-mTOR* expression varied by brain region and was highest in the cerebellum (AIC = -102.11, LR = 24.39, $p < 0.001$; Figure 11B).

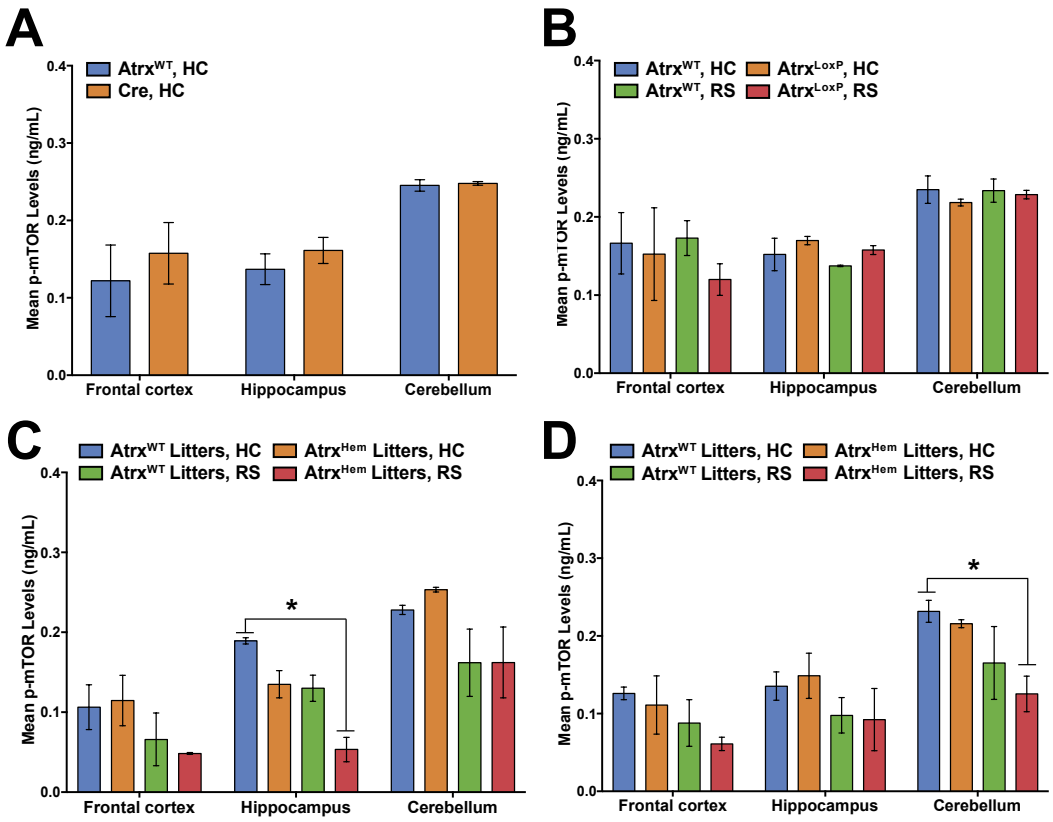

Figure 11. Effect of prenatal stress exposure on *p-mTOR* expression in adult male and female offspring. *P-mTOR* expression in (A) WT or Cre fathers housed in homecage (HC), (B) *Atrx*^{WT} or *Atrx*^{LoxP} mothers housed in HC or restraint stressed (RS) during pregnancy, (C) *Atrx*^{WT} or *Atrx*^{HEM} male offspring from HC or RS mothers, (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).

There was a main effect of stress on *p-mTOR* expression in the brains of F1 male mice: F1 male mice exposed to gestational stress had reduced *p-mTOR* expression compared to male mice who were stress naïve (AIC = -103.07, LR =14.68, $p < 0.001$; Figure 11C). The cerebellum had the highest levels of *p-mTOR* expression, followed by the hippocampus, and the lowest levels were in the frontal cortex (AIC = -87.04, LR = 32.71, $p < 0.001$). There was no significant effect of genotype in the frontal cortex or the

cerebellum (CI 95% FC = -0.061, 0.071, CER = -0.096, 0.070). In the hippocampus, however, male *Atrx*^{HEM} mice had decreased expression of *p-mTOR* compared to male *Atrx*^{WT} mice (CI 95% HIPP = 0.008, 0.123), which lead to an interaction between brain region and genotype (AIC = -111.25, LR = 7.35, p = 0.025).

In F1 females, there was a main effect of stress: females exposed to gestational stress had reduced *p-mTOR* expression compared to females that were stress naïve (AIC = -109.251, LR = 9.46, p = 0.002; Figure 11D). Across brain regions, the cerebellum had the highest levels of *p-mTOR* expression, followed by the hippocampus, and the frontal cortex (AIC = -97.269, LR = 23.44, p < 0.001).

3.1.4. p-mTOR to total mTOR Ratio

The ratio of *p-mTOR* to *mTOR* did not differ by genotype, stress condition or brain region for F0 males (all p > 0.05; Figure 12A**)** or F0 females (all p > 0.05; Figure 12B**)**.

F1 male *Atrx*^{HEM} mice had reduced *p-mTOR/mTOR* expression in the hippocampus (CI 95% HIPP = 0.017, 0.317; Figure 12C) compared to male *Atrx*^{WT} mice. There was an interaction between genotype and brain region (AIC = -27.431, LR = 7.1554, p = 0.028), as there was no effect of genotype in the frontal cortex or the cerebellum (CI 95% FC = -0.321, 0.137, CER = -0.130, 0.159). There was a significant positive correlation between *p-mTOR/mTOR* ratio and *ATRX* expression in the cerebellum of male *Atrx*^{HEM} mice who were stress naïve (r = 0.99, p = 0.002).

In F1 females, the ratio of *p-mTOR* to *mTOR* did not differ by sibling genotype, gestational stress exposure or brain region (all p > 0.05; Figure 12D). There were trends toward a positive correlation between *ATRX* and *p-mTOR/mTOR* expression in the cerebellum (r = 0.99, p = 0.07) and frontal cortex (r = 0.99, p = 0.9) of females that did not experience gestational stress but were raised with male *Atrx*^{HEM} mice. There was also a trend for a negative correlation between *ATRX* and *p-mTOR/mTOR* expression in the cerebellum (r = -0.99, p = 0.06) of females that were stress naïve and were raised with male *Atrx*^{WT} mice.

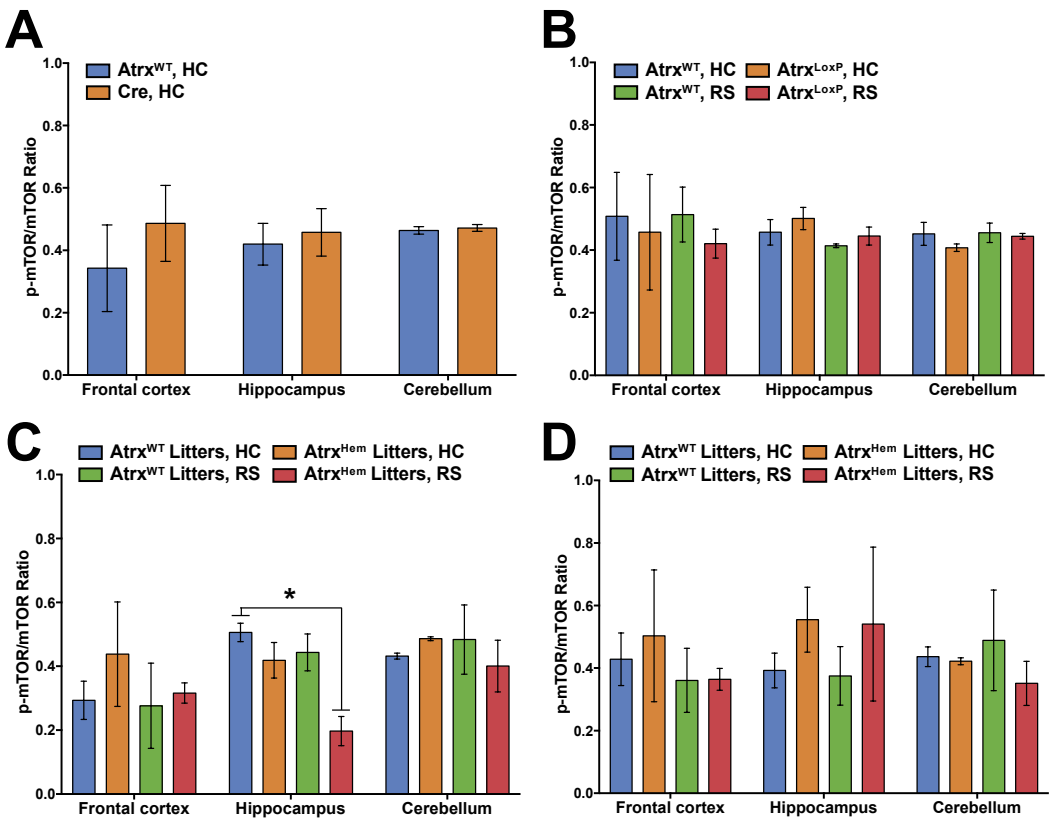

Figure 12. Effect of prenatal stress exposure and/or *Atrx* deficiency on *p-mTOR/mTOR* ratio in brains of adult male and female offspring. *p-mTOR/mTOR* ratio in (A) WT or Cre fathers housed in homecage (HC), (B) $Atrx^{WT}$ or $Atrx^{LoxP}$ mothers housed in HC or restraint stressed (RS) during pregnancy, (C) $Atrx^{WT}$ or $Atrx^{HEM}$ male offspring from HC or RS mothers, (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).

3.2. *Atrx, Mtor, and Mecp2 mRNA expression*

We quantified *Atrx, Mtor,* and *Mecp2* expression in the frontal cortex, hippocampus, and cerebellum of F1 female and male mice. 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 groups consisted of two genotypes ($Atrx^{WT}$ and $Atrx^{HEM}$) and two stress conditions (stress naïve and exposed to gestational stress). There were eight mice per group. Models were compared using AIC and LR.

3.2.1. Atrx

In F1 male mice, models including the main effects of genotype (AIC = 11.47, LH = 4.68, p = 0.031; Figure 13A), brain region (AIC = 13.36, LH = 8.57, p = 0.014), and the interaction between genotype, gestational stress exposure, and brain region (AIC = 9.82, LH = 8.25, p = 0.016) differed significantly from the null model (AIC = 8.80). Male mice that were stress naïve had reduced *Atrx* expression in the frontal cortex (CI 95% = 0.219, 0.763), but no significant difference in the hippocampus (CI 95% = -0.053, 0.522) or cerebellum (CI 95% = -0.053, 0.522). There was no significant effect of genotype for the males that were exposed to gestational stress.

In F1 female mice, the model including a main effect of brain region (AIC = 29.57, LH = 21.44, p < 0.001; Figure 13B) on *Atrx* mRNA expression differed significantly from the null model (AIC = 12.134). *Atrx* mRNA levels were lower in the cerebellum and did not differ between the frontal cortex and hippocampus.

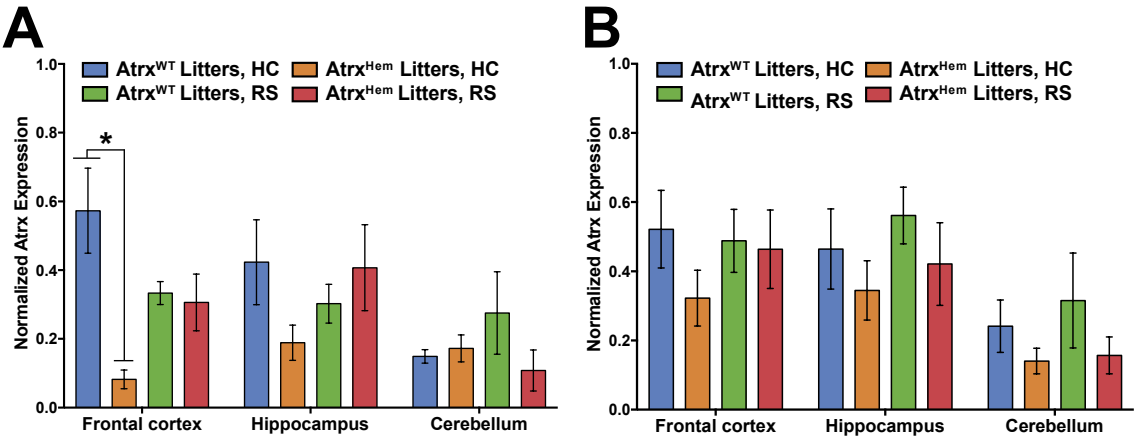

Figure 13. Effect of prenatal stress exposure and/or *Atrx* deficiency on *Atrx* mRNA transcript levels in adult male and female offspring. *Atrx* expression in (A) $Atrx^{WT}$ or $Atrx^{HEM}$ male offspring from HC or RS mothers, (B) $Atrx^{WT}$ female offspring from HC or RS mothers and raised with $Atrx^{WT}$ or $Atrx^{HEM}$ males. Mean ± SEM. (N=8/group; *p<0.05).

3.2.2. Mtor

In F1 male mice, *Mtor* mRNA levels did not differ between the genotypes, maternal stress conditions or the brain regions for the male mice (all p > 0.05; Figure 14A). In F1 female mice, the model including the main effect of brain region (AIC = -2.73, LH = 16.33, p < 0.001; Figure 14B) differed significantly from the null model (AIC = -15.06).

The highest levels of *Atrx* mRNA were measured in the hippocampus, followed by the frontal cortex, and least in the cerebellum.

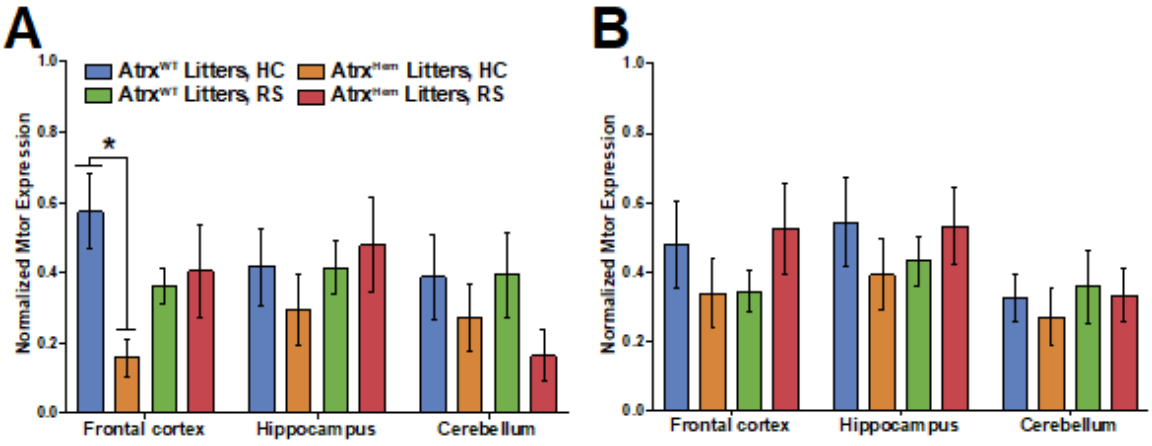

Figure 14. Effect of prenatal stress exposure and/or *Atrx* deficiency on *Mtor* mRNA transcript levels in adult male and female offspring. *Mtor* expression in (A) $Atrx^{WT}$ or $Atrx^{HEM}$ male offspring from HC or RS mothers, (B) $Atrx^{WT}$ female offspring from HC or RS mothers and raised with $Atrx^{WT}$ or $Atrx^{HEM}$ males. Mean ± SEM. (N=8/group; *p<0.05).

3.2.3. Mecp2

In F1 male mice, *Mecp2* expression levels differed between the brain regions (AIC = 38.97, LH = 28.04, $p < 0.001$; Figure 15A). Highest levels were measured in the frontal cortex, followed by the hippocampus and the lowest levels were in the cerebellum. In F1 female mice, the model including the main effect of brain region (AIC = 24.95, LH = 33.95, $p < 0.001$; Figure 15B) differed significantly from the null model (AIC = -5.00). The highest levels of *Mecp2* expression were measured in the hippocampus, followed by the frontal cortex, and least in the cerebellum.

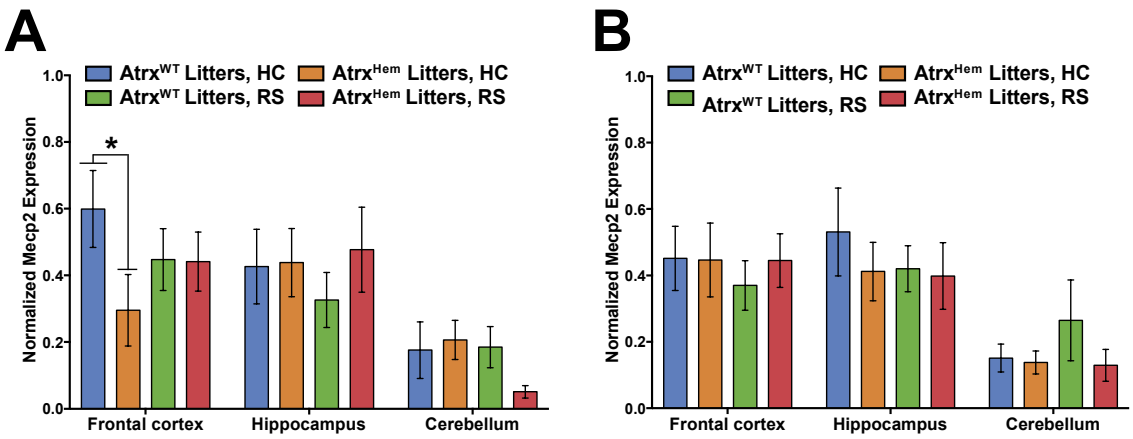

Figure 15. Effect of prenatal stress exposure and/or *Atrx* deficiency on *Mecp2* mRNA transcript levels in adult male and female offspring. *Mecp2* expression in (A) *Atrx*^{WT} or *Atrx*^{HEM} male offspring from HC or RS mothers, (B) *Atrx*^{WT} female offspring from HC or RS mothers and raised with *Atrx*^{WT} or *Atrx*^{HEM} males. Mean ± SEM. (N=8/group; *p<0.05).

3.3. *Regulation of Atrx, Mtor and Line1 promoter*

We quantified promoter methylation at various sites on the *Atrx, Mtor,* and *p-Line1* promoters in the frontal cortex, hippocampus, and cerebellum of F0 and F1 female and male mice. F0 female groups consisted of two genotypes (*Atrx*^{WT} and floxed *Atrx)* and two stress conditions (stress naïve and gestational stress). F0 male groups consisted of two genotypes (*Atrx*^{WT} and Cre) and none were exposed to 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 groups consisted of two genotypes (*Atrx*^{WT} and *Atrx*^{HEM}) and two stress conditions (stress naïve and exposed to gestational stress). There were four mice per group. Models were compared using AIC and LR.

3.3.1. Atrx

In F0 males, the model including the main effect of brain region (AIC = 139.61, LR = 6.13, p = 0.047; Figure 16) differed significantly from the null model (AIC = 137.48) as methylation was higher in the cerebellum compared to the frontal cortex and hippocampus. In F0 females, there were no significant differences in *Atrx* promoter methylation across groups (all p>0.05; Figure 17). In F1 males, the model including the main effect of exposure to gestational stress (AIC = 345.33, LR = 5.35, p = 0.021; Figure 18)

differed significantly from the null model (AIC = 314.98), as F1 males who were exposed to gestational stress had increased methylation at the *Atrx* promoter. In F1 females, the model including the main effect of sibling genotype (AIC = 324.19, LR = 13.79, $p < 0.001$; Figure 19) and exposure to gestational stress (AIC = 320.54, LR = 10.13, $p = 0.002$) differed significantly from the null model (AIC = 312.41). Females raised with male $Atrx^{HEM}$ mice had increased *Atrx* promoter methylation compared to females raised with male $Atrx^{WT}$ mice. Additionally, females exposed to gestational stress had increased *Atrx* promoter methylation compared to females who were stress naïve.

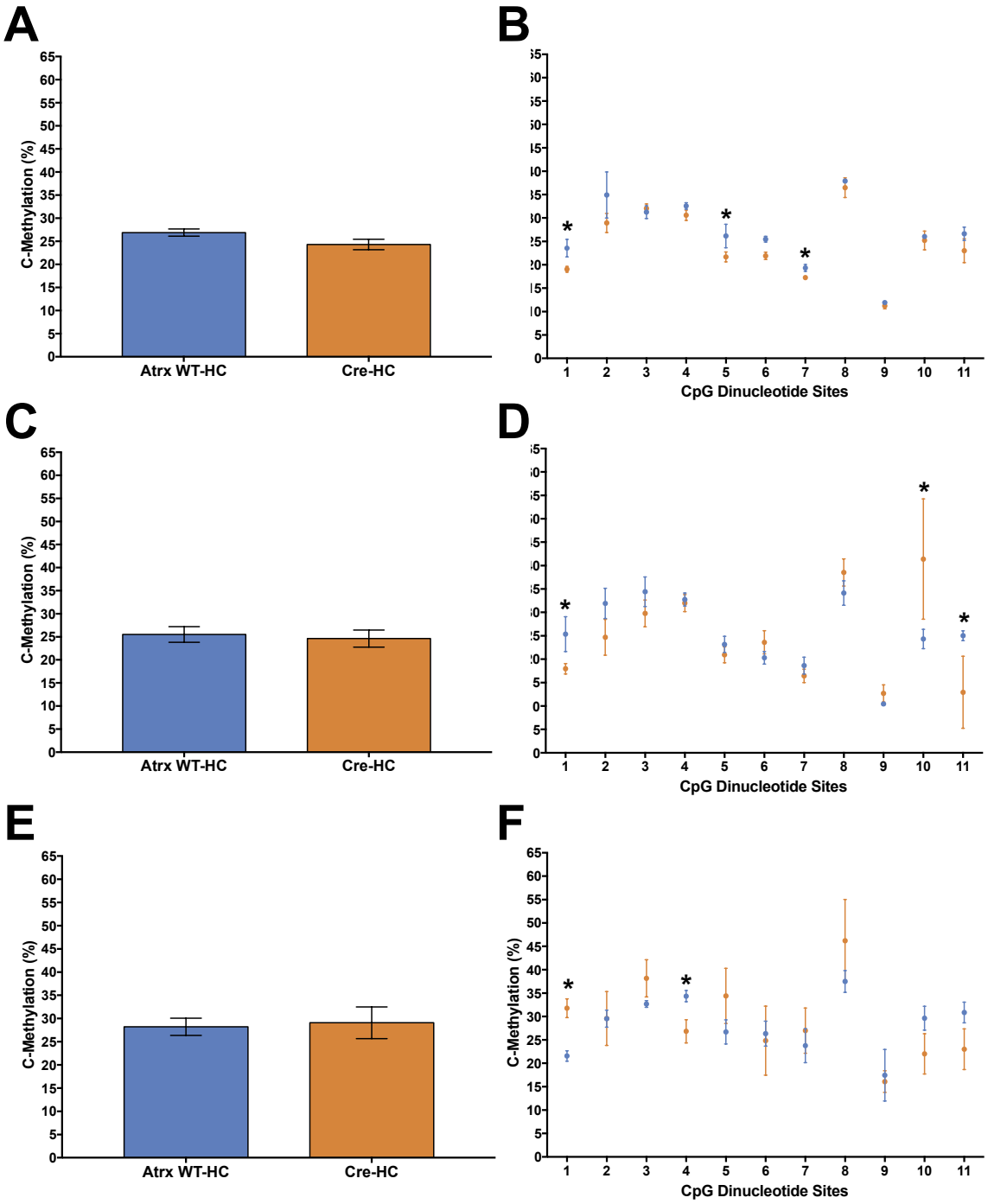

Figure 16. *Atrx* gene promoter methylation in F0 males housed in home cage (HC) and WT or Cre genotype. Overall and site specific DNA methylation levels at 11 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

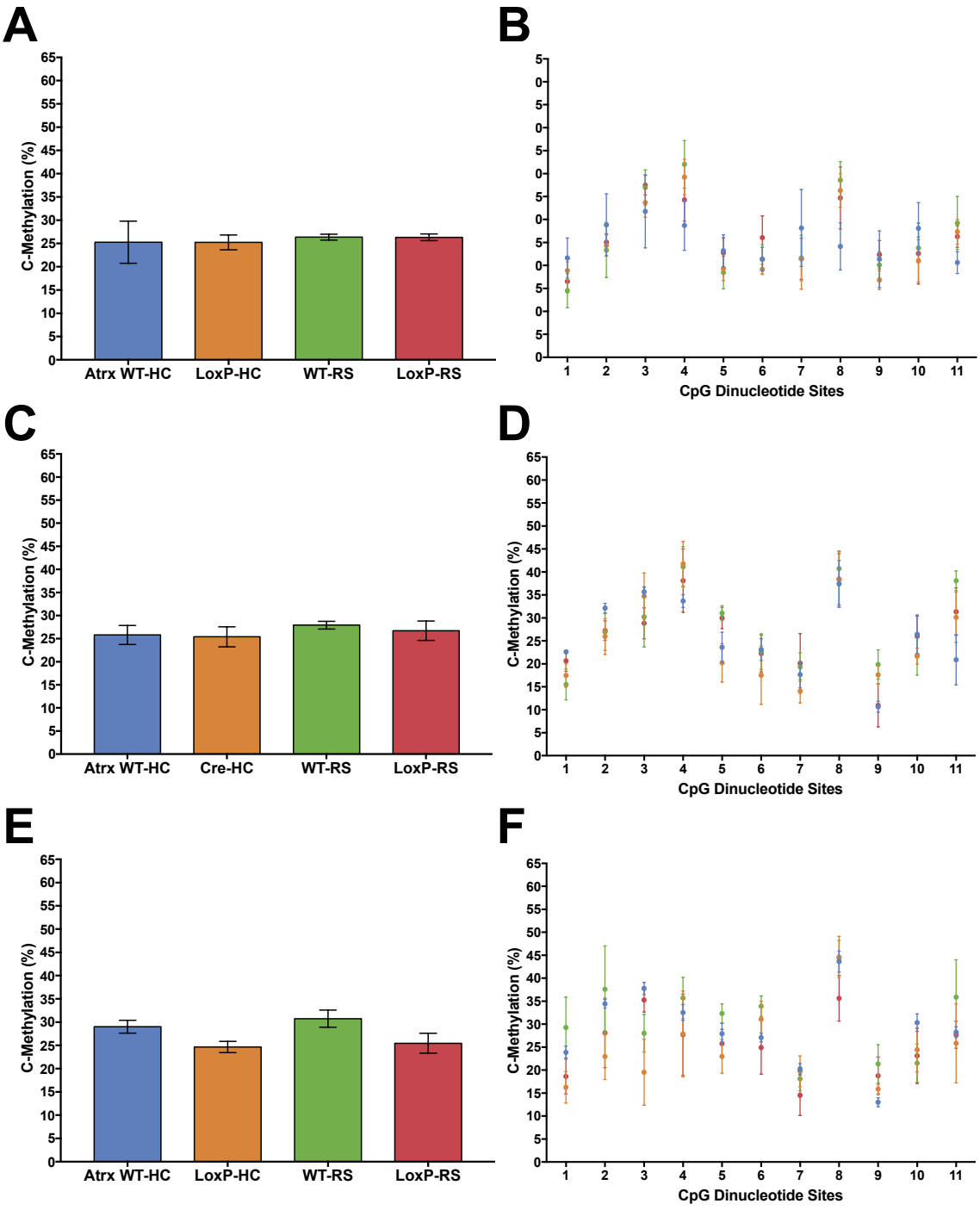

Figure 17. *Atrx* gene promoter methylation in F0 females housed in home cage (HC) or restraint stressed (RS) and WT or *Atrx*^{LoxP} genotype. Overall and site specific DNA methylation levels at 11 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

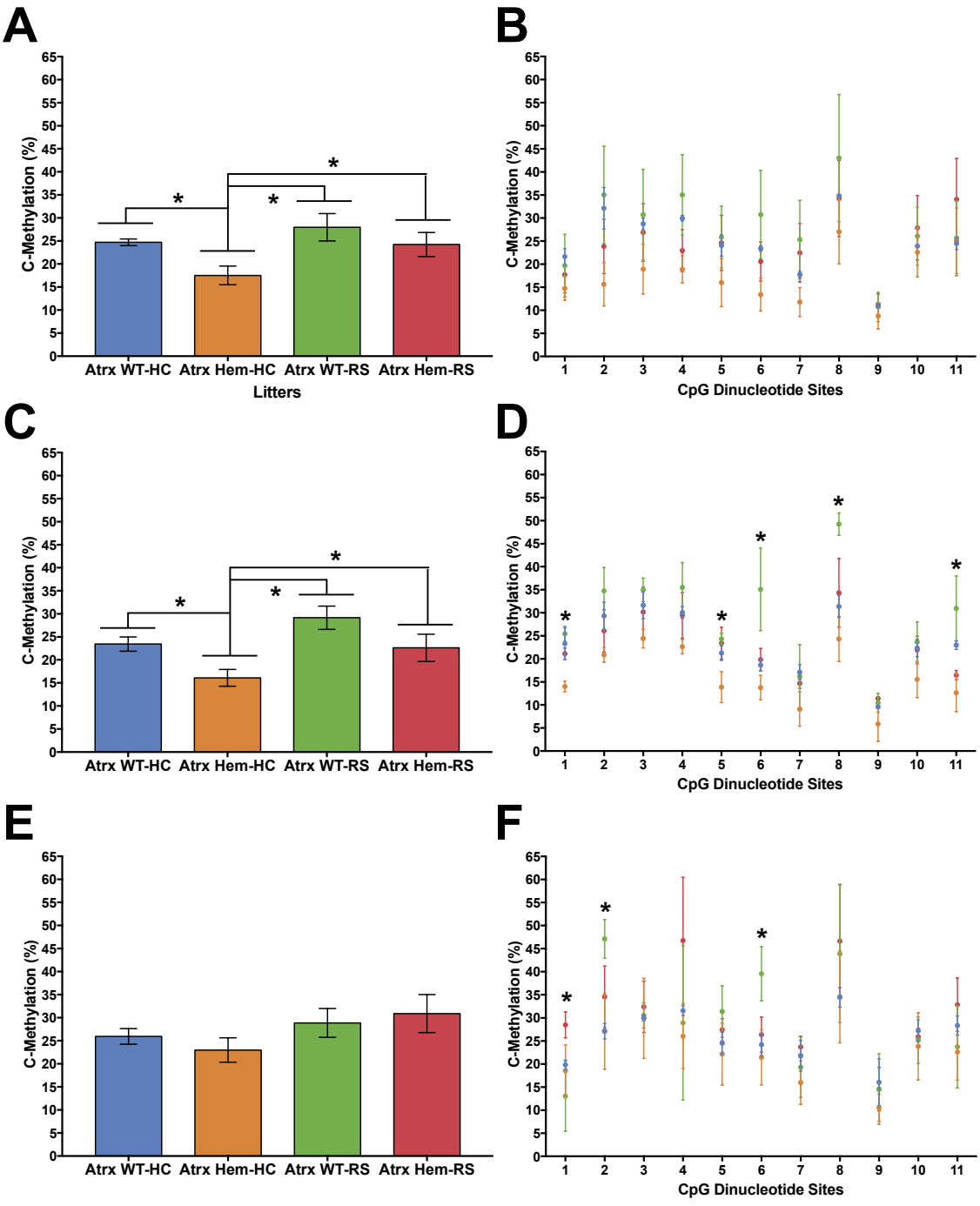

Figure 18. *Atrx* gene promoter methylation in F1 males with *Atrx*^{WT} or *Atrx*^{HEM} genotype and mothers housed in home cage (HC) or restraint stressed (RS). Overall and site specific DNA methylation levels at 11 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

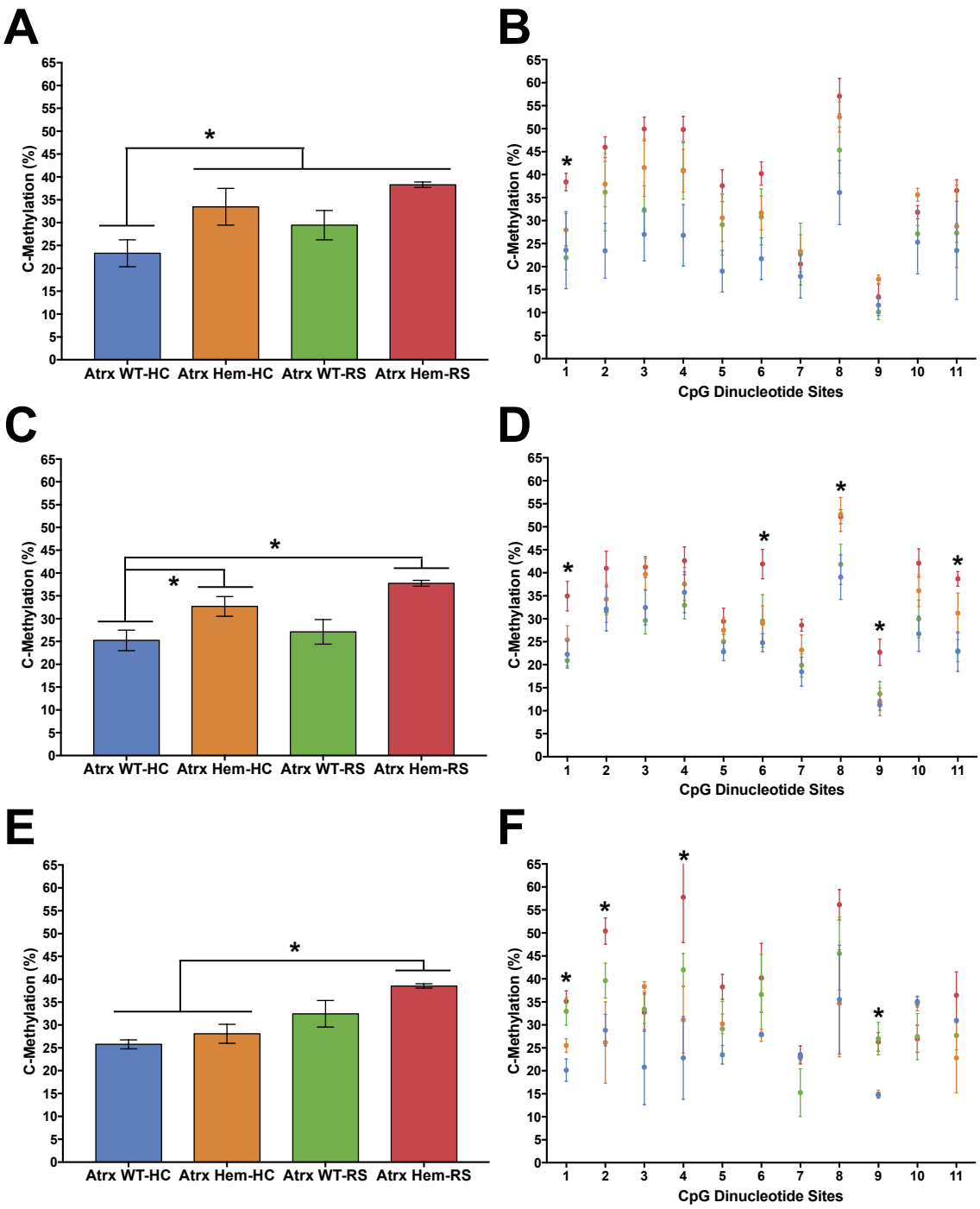

Figure 19. *Atrx* gene promoter methylation in F1 females reared with *Atrx*^{WT} or *Atrx*^{HEM} males and mothers housed in home cage (HC) or restraint stressed (RS). Overall and site specific DNA methylation levels at 11 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

3.3.2. Mtor

There were no differences in overall *Mtor* promoter methylation F0 male mice. There was no model that differed significantly from the null model (all $p > 0.05$, AIC = 172.15; Figure 20). Compared to the $Atrx^{WT}$ males, the *Cre* males showed a lower percent DNA methylation in CpG dinucleotide position 1 in the frontal cortex and position 2 and 9 in the hippocampus ($p<0.05$; Figure 20B, D). The *Cre* males displayed, however, significantly higher DNA methylation in position 17 in the frontal cortex and position 9 in the cerebellum ($p<0.05$; Figure 20B, F).

There were no differences between *Mtor* promoter methylation across brain regions, genotypes, or stress conditions in F0 female mice. No model differed significantly from the null model (all $p > 0.05$, AIC = 342.09; Figure 21). Within the F0 females, although there was no significant genotype difference in overall *Mtor* DNA percent methylation between the frontal cortex and the hippocampus, there were a few differences in specific CpG site methylation (Figure 21B, D). Compared to their non-stressed counterparts, stressed females displayed lower DNA methylation in positions 3, 5, 7 and 9 in the frontal cortex and position 1 in the hippocampus ($p<0.05$; Figure 21B, D). The *Atrx* floxed females displayed significantly lower overall *Mtor* DNA methylation in the cerebellum ($p<0.001$). This pattern was relatively consistent across all CpG dinucleotide positions in the cerebellum ($p<0.05$; Figure 21F).

The F1 males showed a main effect of genotype (AIC = 421.19, LR = 12.78, $p<0.001$) and exposure to gestational stress (AIC = 345.62, LR = 11.12, $p = 0.001$) differed significantly from the null model (AIC = 312.41; Figure 22). $Atrx^{HEM}$ males displayed lower overall *Mtor* DNA percent methylation in their frontal cortex and hippocampus compared to the $Atrx^{WT}$ males ($p<0.05$, $p<0.001$; Figure 22A,C). This pattern was relatively consistent across the CpG dinucleotide positions in the frontal cortex and the hippocampus ($p<0.05$; Figure 22B,D). The prenatally stressed males displayed higher overall *Mtor* DNA percent methylation in their cerebellum when compared to the non-stressed groups ($p<0.001$; Figure 22E). This pattern of high DNA methylation in the cerebellum was significant in CpG dinucleotide positions 1, 14, 18, and 19 ($p<0.05$, $p<0.001$; Figure 22F).

In F1 females, models including the main effect of sibling genotype (AIC = 318.49, LH = 12.80, $p < 0.001$; Figure 23), gestational stress (AIC = 313.50, LH = 7.82, $p = 0.005$), and brain region (AIC = 311.27, LH = 7.59, $p = 0.023$) differed from the null model significantly (AIC = 307.68). F1 females raised with male *Atrx*^{HEM} mice, and those that experienced gestational stress, had increased *Mtor* promoter methylation. Between brain regions, the frontal cortex and hippocampus had increased methylation compared to the cerebellum. This pattern was relatively consistent across the CpG dinucleotide positions in the frontal cortex (Figure 23B). In the hippocampus, the females raised with *Atrx*^{WT} males displayed higher *Mtor* DNA methylation in the CpG dinucleotide position 16 (Figure 23D). In the cerebellum of stressed females raised with *Atrx*^{HEM} brothers, most CpG dinucleotide positions displayed higher percent methylation than the non-stressed females raised with *Atrx*^{WT} (Figure 23F). This pattern was significant in positions 1, 3, 9, 10, 11, 14, 15, 17, 19 and 24 ($p<0.05$; Figure 23B, D, F).

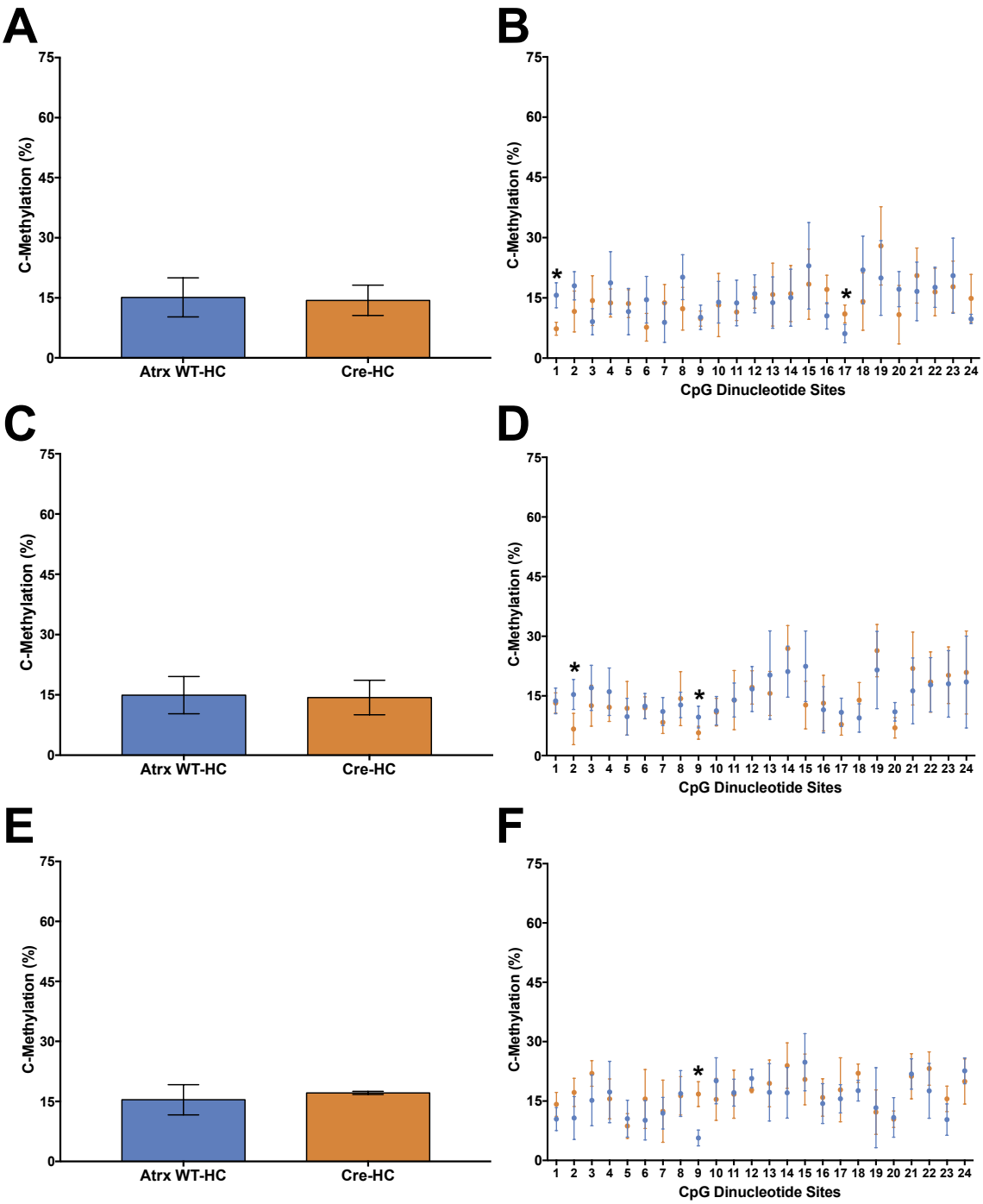

Figure 20. *Mtor* gene promoter methylation in F0 males housed in home cage (HC) and WT or Cre genotype. Overall and site specific DNA methylation levels at 24 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

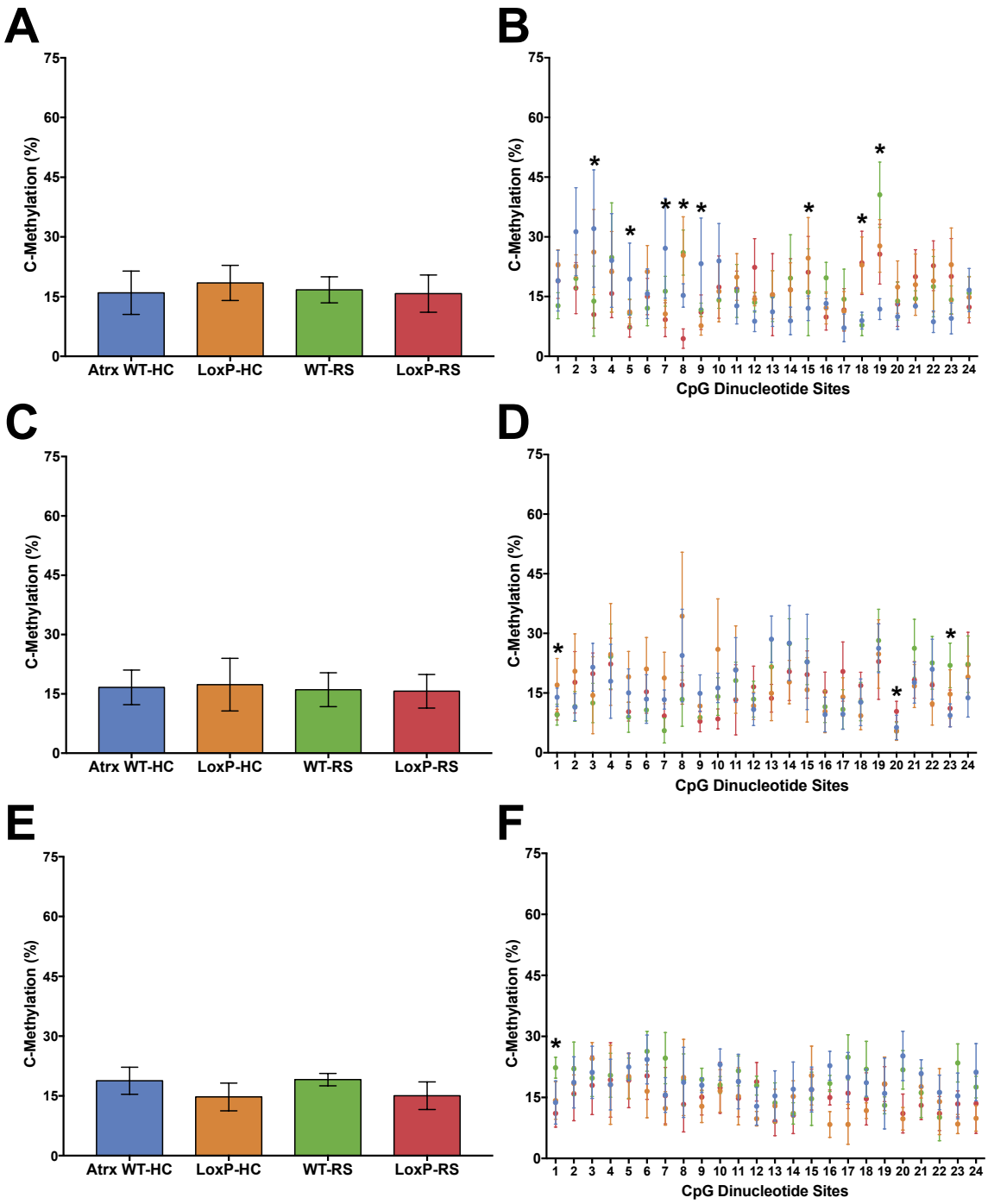

Figure 21. *Mtor* gene promoter methylation in F0 females housed in home cage (HC) or restraint stressed (RS) and WT or *Atrx*^{LoxP} genotype. Overall and site specific DNA methylation levels at 24 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

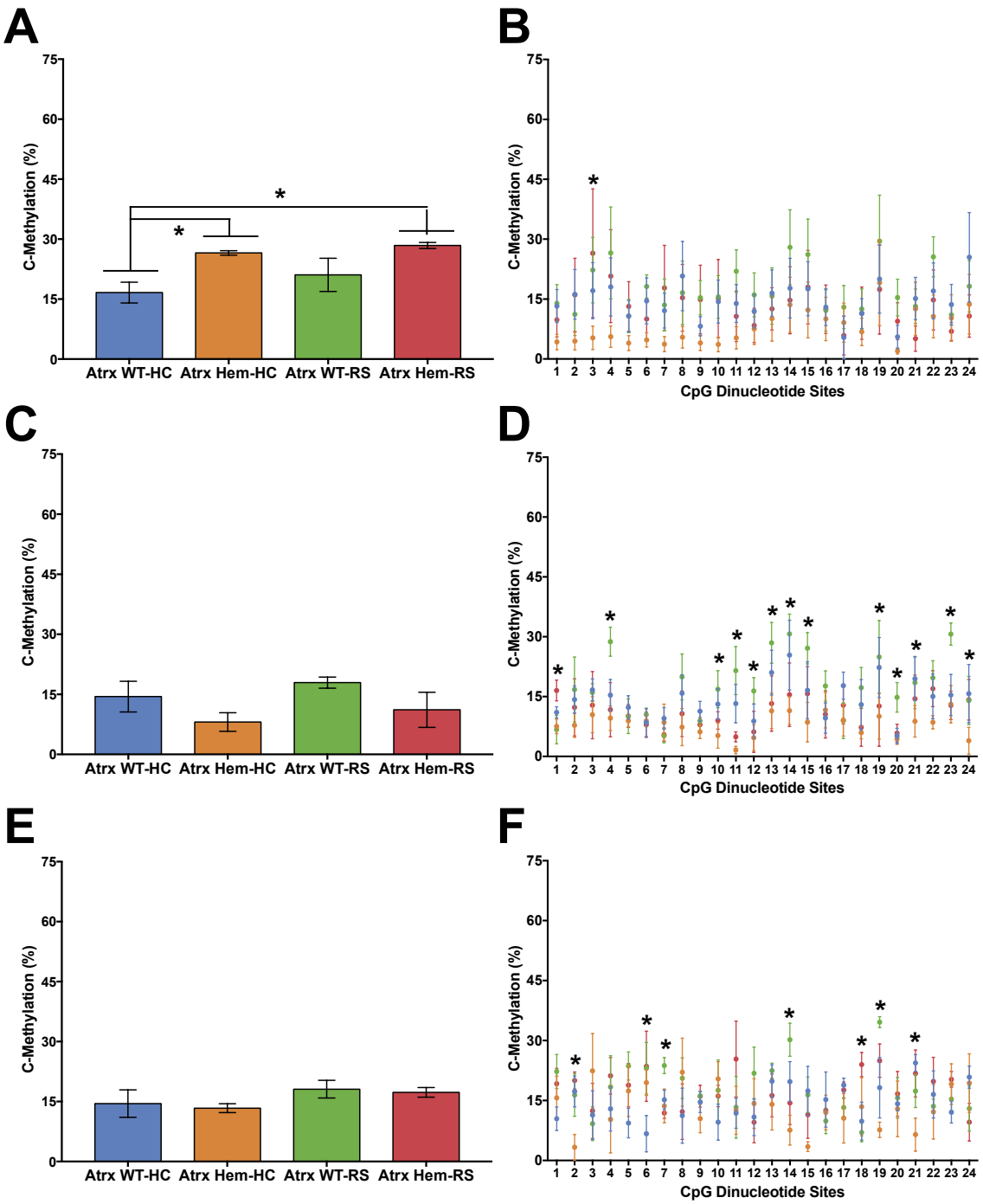

Figure 22. *Mtor* gene promoter methylation in F1 males with *Atrx*^{WT} or *Atrx*^{HEM} genotype and mothers housed in home cage (HC) or restraint stressed (RS). Overall and site specific DNA methylation levels at 24 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

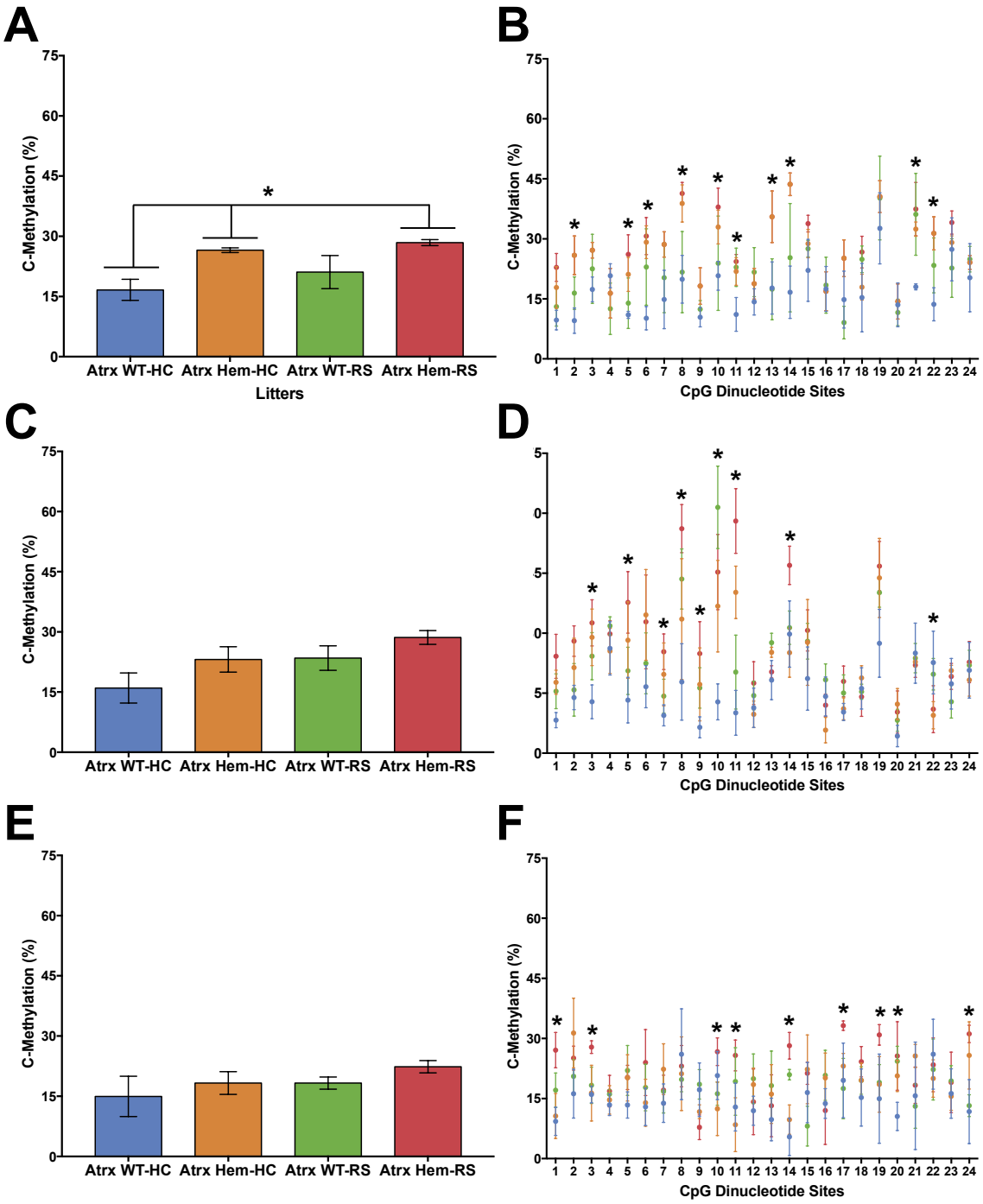

Figure 23. *Mtor* gene promoter methylation in F1 females reared with *Atrx*^{WT} or *Atrx*^{HEM} males and mothers housed in home cage (HC) or restraint stressed (RS). Overall and site specific DNA methylation levels at 24 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

3.3.3. Line1

In the F0 male mice, there were no models including main effects or interactions that differed from the null model (all $p > 0.05$; Figure 24). Further, there were no significant correlations between *Line1* promoter methylation and protein expression in any brain regions in F0 male mice.

In F0 female mice, models including the main effect of genotype (AIC = 241.80, LR = 7.45, $p = 0.006$) as well as the models including the interaction between genotype and brain region (AIC = 237.01, LR = 12.47, $p = 0.002$), and genotype, stress condition and brain region (AIC = 228.54, LR = 25.87, $p < 0.001$) differed significantly from the null model (AIC = 236.35; Figure 25). Within the frontal cortex, there was no effect of genotype on *Line1* promoter methylation in stress naïve females (CI 95% = -6.620, 1.418; Figure 25B). Methylation levels in the stressed F0 females, however, did vary by genotype: *Atrx* floxed females had higher levels of *Line1* promoter methylation compared to the WT females (CI 95% = 5.632, 7.858). The findings were similar in the hippocampus (CI 95% no-stress = -2.259, 3.920; stress = 2.324, 7.805; Figure 25C, D). In the cerebellum, WT F0 females that were stressed had increased *Line1* promoter methylation compared to the stressed *Atrx* floxed females (CI 95% = 0.380, 7.168). There were no effects of genotype in the stress naïve F0 females (CI 95% = -5.730, 1.226). There was a significant positive correlation between *Line1* promoter methylation and *ATRX* expression ($r = 0.99$, $p = 0.004$) in the cerebellum of wild-type, stress naïve F0 female mice. There was a significant negative correlation between *Line1* promoter methylation and *mTOR* expression ($r = -0.99$, $p = 0.006$) in the hippocampus of wild-type, stressed F0 female mice. There was a significant positive correlation between *Line1* promoter methylation and *p-mTOR/mTOR* ratio ($r = 0.99$, $p = 0.029$) in the hippocampus of wild-type stressed F0 female mice.

In F1 male mice, there was a main effect of brain region (AIC = 203.07, LR = 7.71, $p = 0.021$), and interactions between genotype and brain region (AIC = 198.35, LR = 7.62, $p = 0.022$), exposure to gestational stress and brain region (AIC = 197.63, LR = 6.90, $p = 0.032$), and genotype, exposure to gestational stress, and brain region (AIC = 194.73, LR = 7.98, $p = 0.019$) that differed significantly from the null model (AIC = 199.37; Figure 26). Within the frontal cortex, there was no difference in *Line1* promoter

methylation between *Atrx*^{WT} and *Atrx*^{HEM} stress naïve F1 male mice (CI 95% = -0.892, 5.442), but the *Atrx*^{WT} males exposed to gestational stress had increased methylation compared to the *Atrx*^{HEM} counterparts (CI 95% = 0.254, 5.236). Within the hippocampus, there was no difference in *Line1* promoter methylation between *Atrx*^{WT} and *Atrx*^{HEM} in either stress condition. Within the cerebellum, there was no effect of genotype in F1 males exposed to gestational stress (CI 95% = -5.086, 0.921), however, in stress naïve males, *Atrx*^{WT} had increased *Line1* promoter methylation compared to male *Atrx*^{HEM} mice (CI 95% = 0.108, 4.052). There was a significant negative correlation between *Line1* methylation and *ATRX* expression in the cerebellum of stress naïve F1 male *Atrx*^{WT} mice ($r = -0.99$, $p = 0.011$). There was a significant positive correlation between *Line1* methylation and *mTOR* expression in the frontal cortex of stress naïve F1 *Atrx*^{HEM} mice ($r = 0.99$, $p = 0.049$).

In F1 female mice, there was a main effect of exposure to gestational stress (AIC = 221.30, LR = 8.64, $p = 0.003$; Figure 27) and an interaction between gestational stress and brain region (AIC = 218.86, LR = 8.51, $p = 0.014$) that differed significantly from the null model (AIC = 214.66). F1 females exposed to gestational stress had increased *Line1* promoter methylation in the cerebellum (CI 95% = 1.749, 5.227) compared to stress naïve F1 females. However, there were no differences in methylation driven by stress in the frontal cortex or hippocampus of F1 females. There was a significant negative correlation between *Line1* promoter methylation and *mTOR* expression ($r = -1$, $p < 0.001$) in the hippocampus of stress naïve F1 female mice raised with male *Atrx*^{HEM} mice. However, in the hippocampus of F1 females exposed to gestational stress raised with male *Atrx*^{HEM} mice, there was a significant positive correlation between *Line1* promoter methylation and *mTOR* expression ($r = 0.99$, $p = 0.047$). In the cerebellum of stress naïve F1 female mice raised with male *Atrx*^{WT} mice, there was a negative correlation between *Line1* promoter methylation and *mTOR* expression ($r = -0.99$, $p = 0.021$). While those females that were exposed to gestational stress showed a positive correlation between *Line1* promoter methylation and *mTOR* expression ($r = 0.99$, $p = 0.023$).

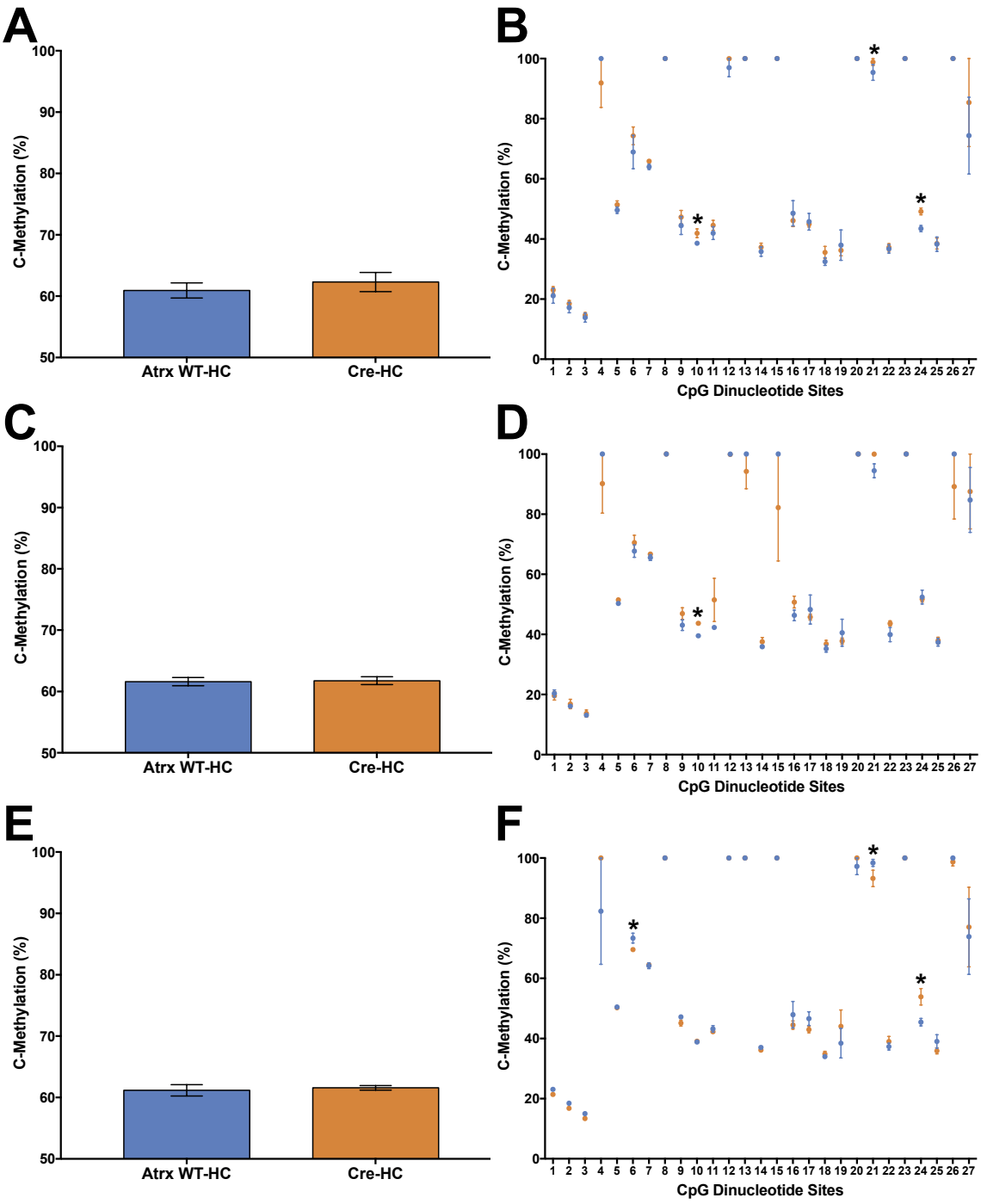

Figure 24. *Line1* gene promoter methylation in F0 males housed in home cage (HC) and WT or Cre genotype. Overall and site specific DNA methylation levels at 27 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

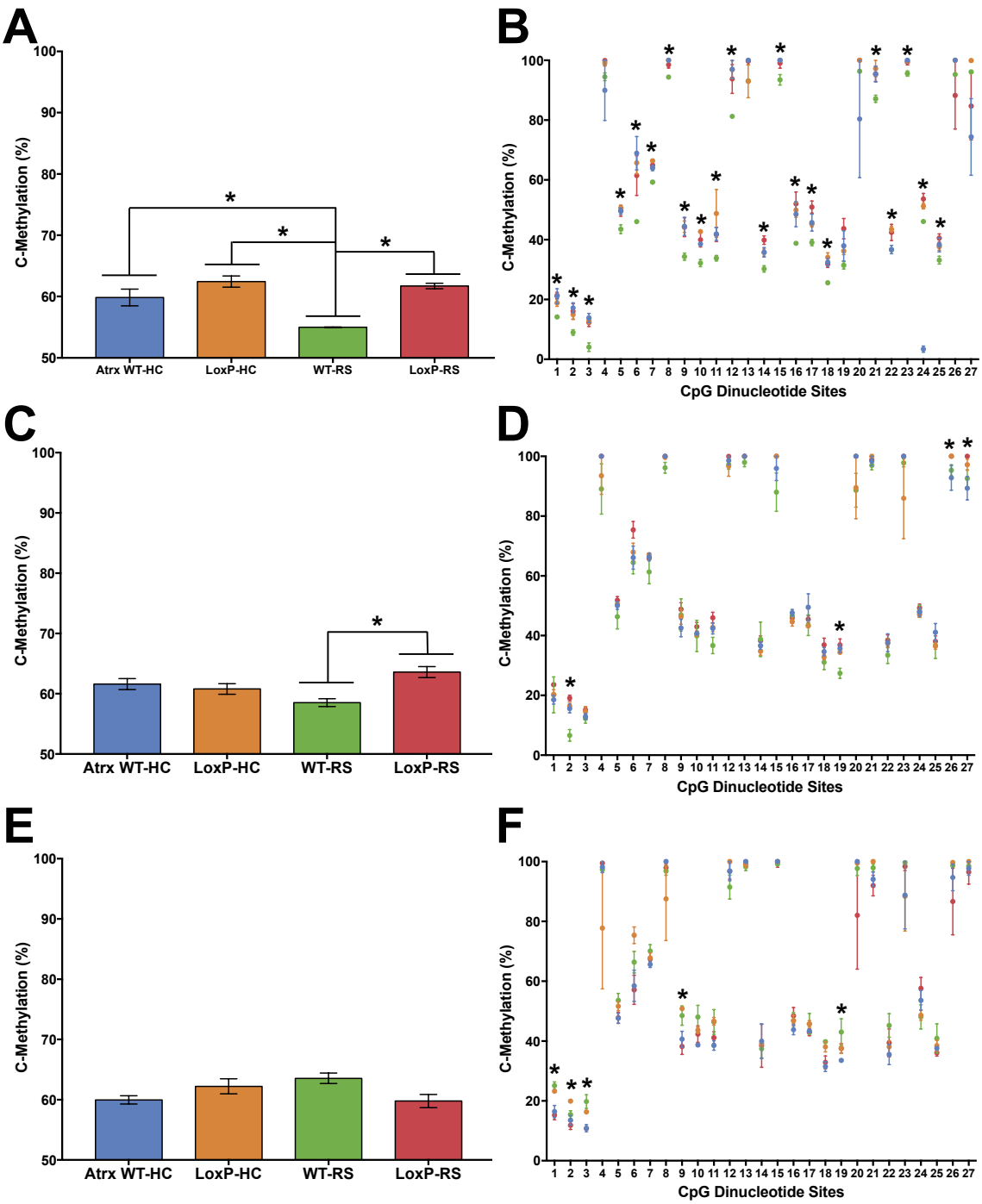

Figure 25. *Line1* gene promoter methylation in F0 females housed in home cage (HC) or restraint stressed (RS) and WT or *Atrx*^{LoxP} genotype. Overall and site specific DNA methylation levels at 27 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

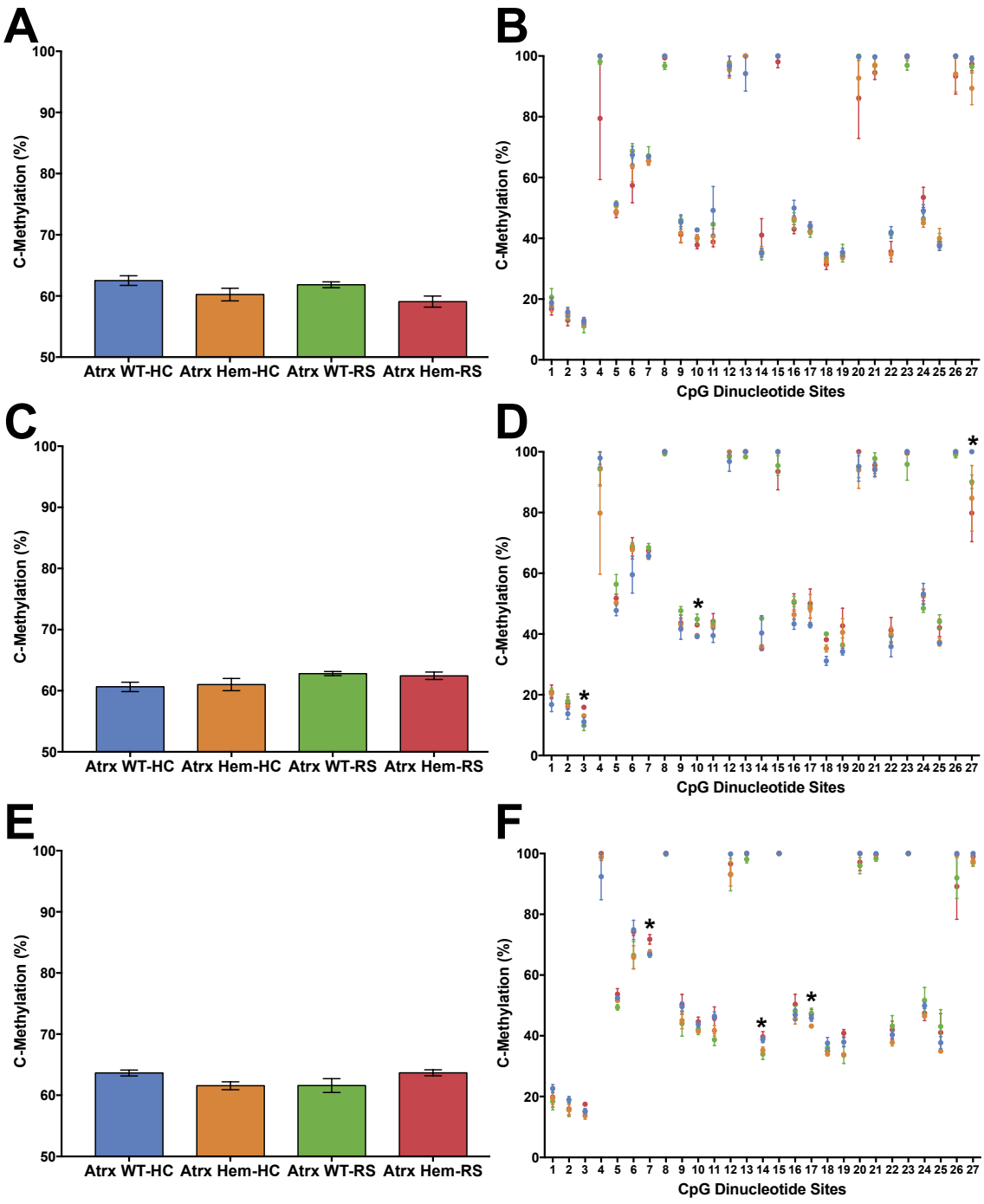

Figure 26. *Line1* gene promoter methylation in F1 males with *Atrx*^{WT} or *Atrx*^{HEM} genotype and mothers housed in home cage (HC) or restraint stressed (RS). Overall and site specific DNA methylation levels at 27 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

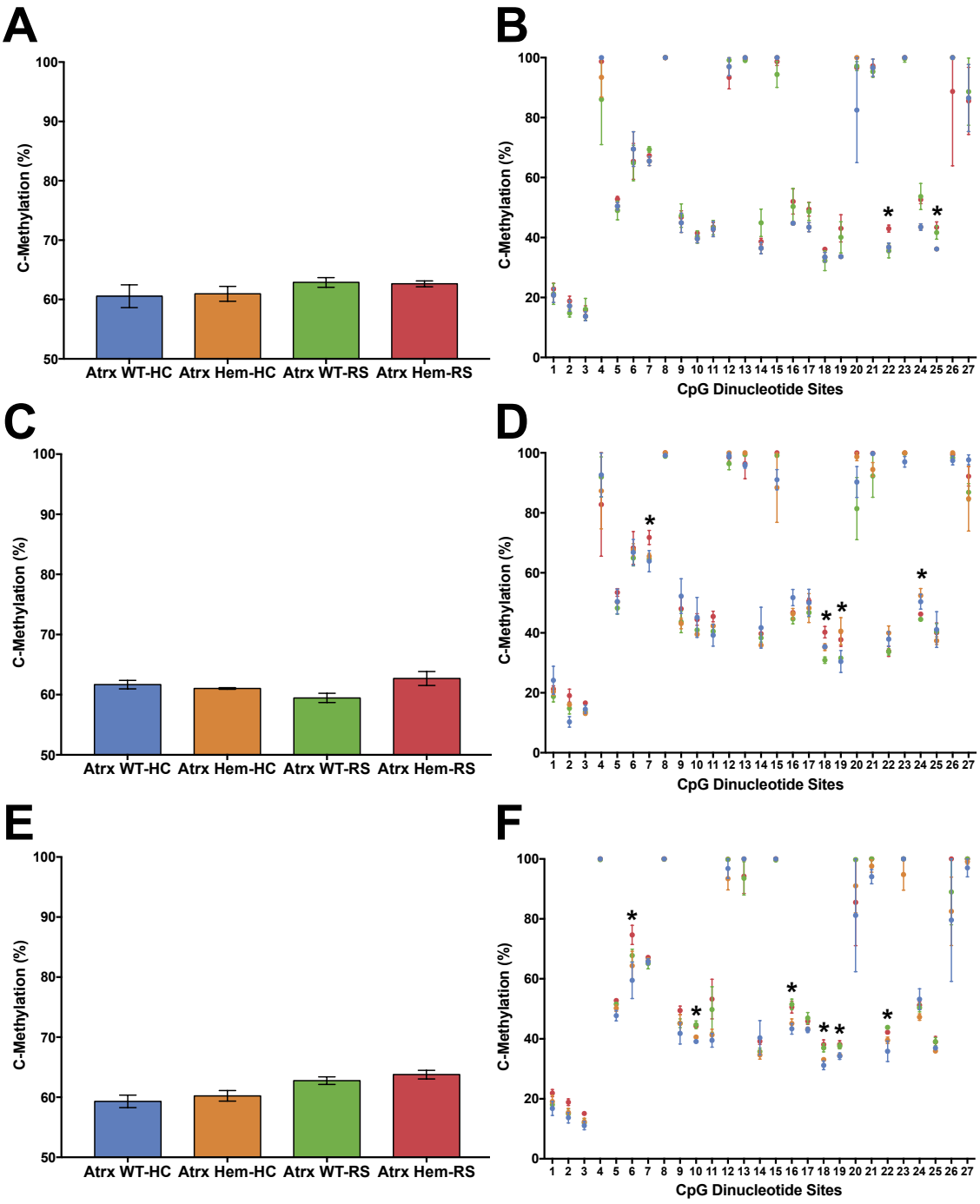

Figure 27. *Line1* gene promoter methylation in F1 females reared with *Atrx*^{WT} or *Atrx*^{HEM} males and mothers housed in home cage (HC) or restraint stressed (RS). Overall and site specific DNA methylation levels at 27 CpG sites in (A-B) frontal cortex, (C-D) hippocampus, (E-F) cerebellum. Mean ± SEM. (N=4/group; *p<0.05).

3.4. *Relationship between Atrx, Mtor, Line1 and Cognitive Behaviour in F1 mice*

Before sacrifice, each offspring had been assessed for cognitive impairments in problem-solving and spatial navigation in adulthood. A Morris water maze (MWM) was used to investigate learning and memory retention by measuring the time spent in the correct quadrant during a probe trial. Our previous findings showed that *Atrx*^{HEM} males and their female *Atrx*^{WT} littermates show decreased time spent in the correct quadrant compared to the *Atrx*^{WT} males and their female *Atrx*^{WT} littermates. We therefore examined the relationship between brain region-specific *Atrx*, *Mtor* and *Line*1 regulation in the offspring from each treatment groups with their probe trial performance.

There was a significant negative correlation ($p = 0.02$) between MWM probe trial performance and overall *Atrx* gene promoter methylation in the cerebellum of the *Atrx*^{HEM} male offspring of non-stressed mothers (Figure 28A). There was a significant negative correlation between MWM probe trial performance and *mTOR* protein levels in the cerebellum of the *Atrx*^{WT} male offspring exposed to stress during gestation ($p = 0.045$; Figure 28B) and in the hippocampi of *Atrx*^{HEM} males exposed to gestational stress ($p = 0.045$; Figure 28C). There was also a significant positive correlation between MWM probe trial performance and *mTOR* protein levels in the hippocampi of the *Atrx*^{HEM} males with non-stressed mothers ($p = 0.01$; Figure 28D). There was a significant negative correlation between MWM probe trial performance and *p-mTOR* protein levels in the cerebellum of *Atrx*^{WT} males exposed to gestational stress ($p = 0.046$; Figure 28E). There was a significant positive correlation between MWM probe trial performance and *Line1* methylation in the hippocampi of the *Atrx*^{HEM} males exposed to gestational stress ($p = 0.04$; Figure 28F).

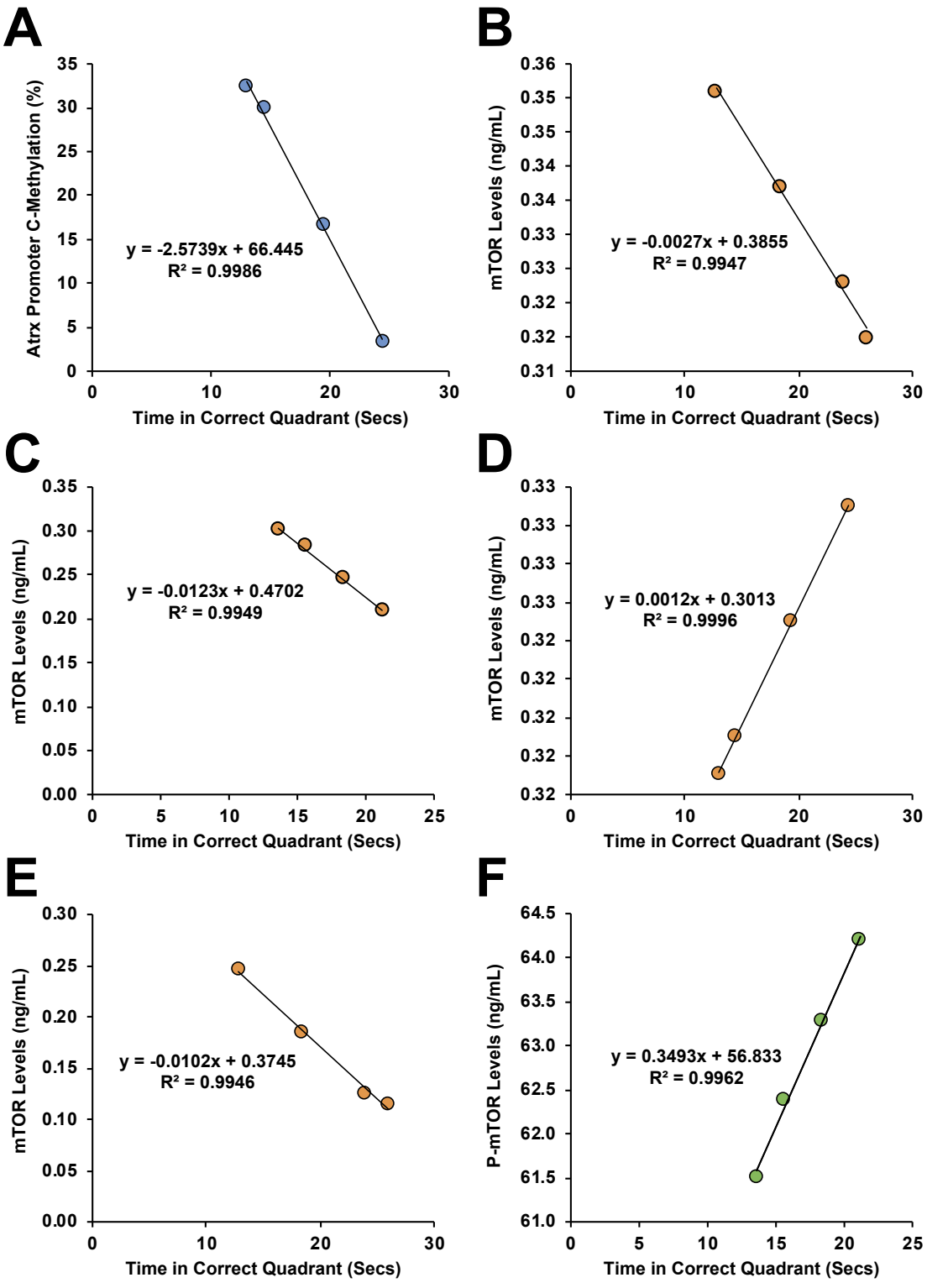

Figure 28. Relationship between Morris water maze probe trial performance and specific molecular markers in F1 males of *Atrx*^{WT} and *Atrx*^{HEM} genotype and mothers housed in home cage (HC) or restraint stressed (RS). Correlation between time spent in the correct quadrant and: (A) overall *Atrx* gene promoter methylation

in the cerebellum of *Atrx*^{HEM} offspring of HC mothers; *mTOR* protein levels in the (B) cerebellum of *Atrx*^{WT} offspring of RS mothers, (C) hippocampus of *Atrx*^{HEM} offspring of RS mothers, and (D) hippocampus of *Atrx*^{HEM} offspring of HC mothers; (E) *p-mTOR* protein levels in the cerebellum of *Atrx*^{HEM} offspring of RS mothers; and (F) overall *Line1* promoter methylation in the hippocampus of *Atrx*^{HEM} offspring of RS mothers. (N=4/group).

There was a significant positive correlation between MWM probe trial performance and *ATRX* protein levels in the cerebellum of *Atrx*^{WT} female offspring exposed to gestational stress and reared with *Atrx*^{HEM} brothers ($p = 0.034$; Figure 29A). There was also a significant negative correlation between MWM probe trial performance and *mTOR* protein levels in the cerebellum of the *Atrx*^{WT} female offspring exposed to gestational stress and reared with *Atrx*^{HEM} brothers ($p = 0.04$; Figure 29B), and a significant positive correlation in the cerebellum of the *Atrx*^{WT} females with *Atrx*^{HEM} brothers and non-stressed mothers ($p = 0.03$; Figure 29C). There was a negative correlation between MWM probe trial performance and *p-mTOR* protein levels in the cerebellum of the *Atrx*^{WT} female offspring exposed to gestational stress and reared with *Atrx*^{WT} brothers ($p = 0.02$; Figure 29D). There was a significant negative correlation between MWM probe trial performance and overall *Line1* promoter methylation in the cerebellum of *Atrx*^{WT} females with *Atrx*^{WT} brothers and non-stressed mothers ($p = 0.04$; Figure 29E).

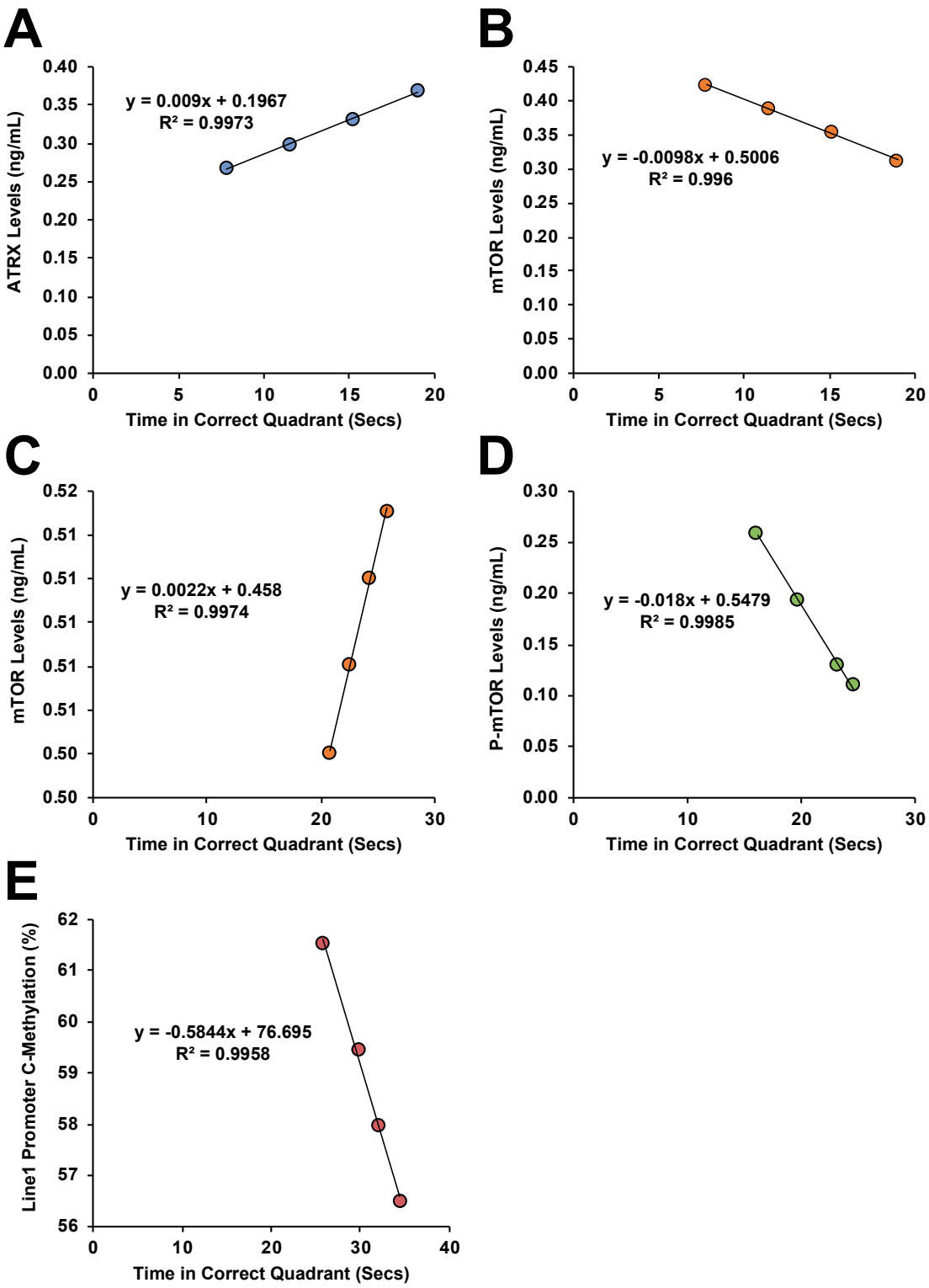

Figure 29. Relationship between Morris water maze probe trial performance and specific molecular markers in F1 females reared with *Atrx*^{WT} and *Atrx*^{HEM} males and mothers housed in home cage (HC) or restraint stressed (RS). Correlation between time spent in the correct quadrant and: (A) *ATRX* protein levels in the cerebellum of

Atrx^{WT} offspring of RS mothers and reared with *Atrx*^{HEM} males; *mTOR* protein levels in the (B) cerebellum of *Atrx*^{WT} offspring of RS mothers and reared with *Atrx*^{HEM} males, and (C) cerebellum of *Atrx*^{WT} offspring of HC mothers and reared with *Atrx*^{HEM} males; (D) *p-mTOR* protein levels in the crebellum of *Atrx*^{WT} offspring of RS mothers and reared with *Atrx*^{WT} males; (E) overall *Line1* promoter methylation in the cerebellum of *Atrx*^{WT} offspring of HC mothers and reared with *Atrx*^{WT} males. (N=4/group).

CHAPTER 4: DISCUSSION

The purpose of this study was to examine the relationship between early life experiences and gene expression and regulation in the forebrain of mice to gain insight into the mechanisms involved in the development of idiopathic ASD. In this study, we showed that early life experiences, in particular exposure to stress *in utero* and early rearing interactions, have an effect on the expression and regulation of the ASD risk genes *Atrx* and *Mtor*. Both *ATRX* and *mTOR* expression was decreased in the brains of mice exposed to stress during prenatal development. While mRNA expression was not significantly impacted by early life experiences, we observed altered *Atrx* and *Mtor* promoter methylation in mice that received lower quality maternal care during early postnatal development, as well as in mice exposed to stress *in utero*. We also determined that decreased *ATRX* expression in both $Atrx^{HEM}$ and $Atrx^{WT}$ mice that received reduced quality maternal care and were exposed to stress *in utero*, was associated with decreased *mTOR* expression. These findings extend our understanding of the effects of adverse early life experiences on brain development, particularly in regard to the expression and regulation of ASD risk genes.

4.1. *Effects of Atrx-cKO and Early Life Experiences on Protein Expression*

Overall, we showed that exposure to stress *in utero*, reduced maternal care, and genetic manipulation of the *Atrx* gene is associated with reduced *ATRX* and *mTOR,* and *p-mTOR* throughout the brain. The ratio of *p-mTOR* to *mTOR* expression was not affected.

4.1.1. ATRX

The F1 male $Atrx^{HEM}$ mice expressed significantly less *ATRX* than their $Atrx^{WT}$ counterparts, as we had predicted. The cKO transgenic model used in this study was designed to excise exon 18 of the *Atrx* gene around embryonic day 10 in cells that express *Emx-1*, and simultaneously Cre-recombinase. We had expected that all cells in the forebrain would have *Atrx* exon 18 excised as *Emx-1* is expressed throughout the developing forebrain (Gulisano, Broccoli, Pardini, & Boncinelli, 1996), and thus *ATRX* expression would be significantly reduced in both the frontal cortex and hippocampus. However, the effect of the cKO on reduced *ATRX* expression was only significant within the frontal cortex and not in the hippocampus. It could be that we did not find a significant reduction in *ATRX* in the hippocampus of our transgenic mice as *Emx-1* is expressed to varying

degrees within the hippocampus. When *Emx-1* is homozygously knocked out, cerebral cortex development is consistently impaired, while hippocampal development is differentially impaired across regions (Yoshida et al., 1997). Further, *Emx-1* mutants develop smaller dentate gyri of the hippocampus, but do not show a change to cell proliferation in the subventricular zone when visualized using immunocytochemistry (Hong et al., 2007), indicating that *Emx-1* is not uniformly expressed in the hippocampus. Though *Emx-1* is expressed in the hippocampus, the lack of uniform expression may not be sufficient to drive the excision of *Atrx* exon 18 in the entire hippocampus of the transgenic model used in this study, which can explain the lack of reduction of *ATRX* expression in the hippocampi of $Atrx^{HEM}$ mice compared to $Atrx^{WT}$ mice.

By measuring *ATRX* protein expression in the brain of mice exposed to stress during different points in the lifespan, we found that the timing of stress exposure lead to varying consequences on *ATRX* expression in the brain. While the F0 female mice that were subject to restraint stress during the third trimester of pregnancy did not show any differences in *ATRX* expression in the brain due to stress, the F1 female mice had decreased *ATRX* expression when exposed to stress during early brain development compared to the F1 females that were stress naïve. Previous studies have shown that prenatal stress during brain development is capable of affecting protein expression into adulthood (Welberg & Seckl, 2008). However, to our knowledge, this is the first study to characterize the effects of stress on *ATRX* expression in the brain. These findings contribute to existing literature by showing that stress exposure during critical periods in brain development can have a lasting impact on expression of the chromatin remodelling protein, *ATRX,* in the brain that are not observed when the stressor is presented during adulthood.

Furthermore, these findings expand on our understanding of how the timing of stress exposure may lead to variable effects in the brain, and consequently to behaviour, through altered cellular processes. Previous work has shown that intrauterine stress exposure is associated with social behaviour deficits into adulthood (de Souza et al., 2013), while the effects of restraint stress during adulthood on mouse behaviour are minimal (Sadler & Bailey, 2016). Although behavioural differences have been characterized, there is a gap in understanding of the cellular and molecular mechanisms driving the effects of stress on the development of these behavioural differences. One theory suggests that

glucocorticoid exposure impacts behaviour differently in adulthood compared to during early development due to differences in neurogenesis (Odaka, Adachi, & Numakawa, 2017). Prenatal brain development is characterized by rapid neurogenesis, while in comparison, the rate of neurogenesis is negligible in adulthood (Sorrells et al., 2018). Additionally, neurogenesis occurs throughout the developing brain, including in the frontal cortex and hippocampus, while adult neurogenesis is limited to the hippocampus (Odaka et al., 2017). As such, the impact of glucocorticoids on neurogenesis will have different behavioural consequences depending on the timing of exposure (Figure 28) (Odaka et al., 2017).

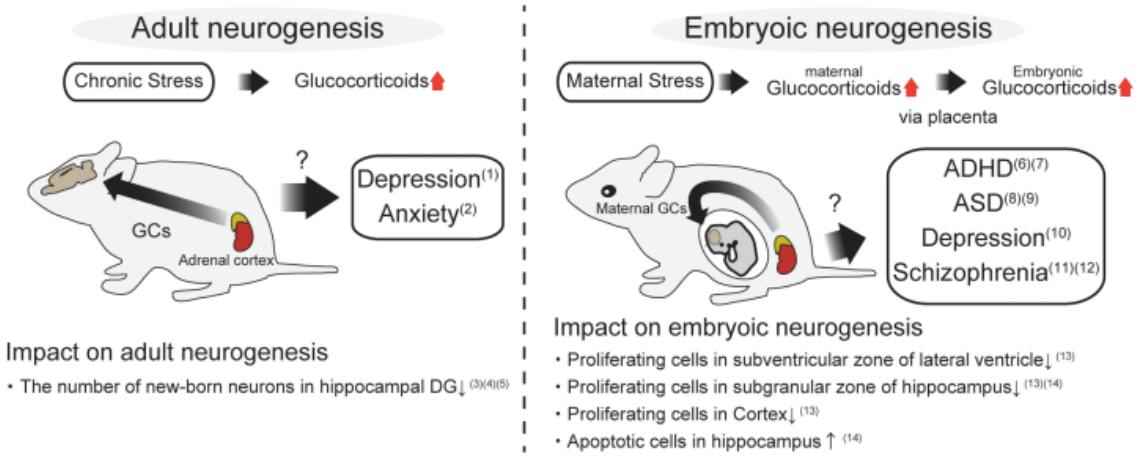

Figure 30. Impact of glucocorticoid exposure in adulthood and during embryonic development on neurogenesis and development of behavioural disorders. Figure from Odaka et al. (2017).

Though the relationship between glucocorticoid exposure and *ATRX* expression and function is not well characterized, it is established that reduced *ATRX* expression impairs neurogenesis (Ritchie, Watson, Davidson, Jiang, & Berube, 2014; Seah et al., 2008). As *ATRX* is important in stabilizing chromatin and DNA damage repair (Clynes et al., 2013), reduced *ATRX* expression is associated with increased sensitivity to factors that induce DNA damage and apoptosis through *p53*-dependent pathways (Conte et al., 2012; Seah et al., 2008). In *ATRX*-null mice, there is a reduction in neural progenitor cells in the cortex and hippocampus resulting from increased *p53*-mediated apoptosis (Seah et al., 2008). Reduced neurogenesis, and increased apoptosis in the frontal cortex and hippocampus is associated with altered brain development and behavioural deficits (Ernst,

2016; Guidi et al., 2008). The mice that showed decreased *ATRX* in the brain in the present study, have been observed to have impaired cognitive and social behaviour in work previously done in the Weaver lab. Thus, our finding that prenatal stress, but not stress in adulthood, leads to decreased *ATRX* expression throughout the brain, suggests that further investigation is warranted into the relationship between stress exposure *in utero*, reduced *ATRX* expression in the brain, and altered neurogenesis and apoptosis.

In addition to early life stress, we found that early rearing environment also had a significant effect on *ATRX* expression into adulthood when we compared *ATRX* expression in the brains of F1 females raised with either $Atrx^{WT}$ or $Atrx^{HEM}$ siblings. In the Weaver lab, it has previously been observed that mothers with $Atrx^{HEM}$ males in their litter show reduced quality maternal care toward their offspring. Knowing that reduced quality of postnatal maternal care is associated with decreased *ATRX* expression (Weaver et al., 2006), our finding that F1 females raised with male $Atrx^{HEM}$ mice had reduced *ATRX* expression compared to females raised with $Atrx^{WT}$ mice was in line with our expectations.

The significant reductions in *ATRX* expression that we observed in F1 male $Atrx^{HEM}$ mice, F1 females exposed to stress, and F1 females raised with male $Atrx^{HEM}$ mice contribute to our understanding of how brain development may be affected by early life experiences through changes to *ATRX* expression. The association between reduced *ATRX* expression and cognitive function has been well studied through mutations to *Atrx*, however, few studies have investigated how stress exposure impacts *ATRX* expression and function. The present study shows evidence that prenatal stress has an effect on expression of the ASD-related protein *ATRX*, suggesting the importance of further studies investigating the role of *ATRX* in the mechanism relating third trimester stress exposure to the development of idiopathic ASD.

4.1.2. mTOR

Similar to the trends observed in *ATRX* expression, *mTOR* expression in the brain was differentially affected by the timing of stress exposure. F0 female mice did not show a change in *mTOR* expression in the brain in response to restraint stress during gestation, but *mTOR* expression was decreased in the brains of F1 female mice exposed to stress *in*

utero when compared to stress naïve F1 females. Previous studies have examined the relationship between prenatal stress and *mTOR* signalling in non-neuronal tissue. For instance, it has been shown in non-neuronal cell cultures that hypoxia during prenatal development inhibits *mTORC1* signalling through increased DNA damage response signalling (Brugarolas et al., 2004). In the present study, we showed that prenatal stress exposure is associated with decreased *mTOR* expression in neuronal tissue specifically. While it has previously been shown that glucocorticoid exposure influences *BDNF* and PI3K-Akt signalling *in vitro* (Kumamaru, Numakawa, Adachi, & Kunugi, 2011), and that deficits in PI3K-Akt/*mTOR* signalling are associated with neurodevelopmental disorders (Wang et al., 2017), the findings in the current study begin to bridge the gap in knowledge between prenatal stress exposure, reduced *mTOR* expression in the brain, and the development of idiopathic neurodevelopmental disorders.

Like F1 females, F1 males exposed to stress *in utero* also had decreased *mTOR* expression in the brain compared to stress naïve males. In F1 males, this effect was most pronounced in the frontal cortex, the area that is, in part, responsible for emotional regulation and social behaviour (Bicks, Koike, Akbarian, & Morishita, 2015). Frontal cortex development is impaired in humans with ASD (Ha, Sohn, Kim, Sim, & Cheon, 2015), and dysregulation of the *mTOR* pathway in murine models results in human ASD-like cortical changes and behaviour (Huang, Chen, & Page, 2016; Ryskalin, Limanaqi, Frati, Busceti, & Fornai, 2018). The finding that prenatal stress is associated with a greater reduction in *mTOR* expression in the frontal cortex than in the hippocampus, was limited to males only. Interestingly, males are significantly more susceptible to developing ASD than females, though the mechanisms behind this sex difference are poorly understood (Werling & Geschwind, 2014).

In addition, we found that early rearing conditions had an effect on *mTOR* expression in the brain. F1 females raised with male $Atrx^{HEM}$ mice had reduced *mTOR* expression compared to females raised with $Atrx^{WT}$ mice. This finding indicates that the pup's early environment has an impact on *mTOR* expression in the brain into adulthood. There are various ways in which the early environment of F1 females reared with male $Atrx^{HEM}$ mice may vary from that of females reared with $Atrx^{WT}$ mice. First, mother-offspring interactions can influence offspring gene expression in adulthood. As previously

mentioned, quality of maternal care towards offspring in litters containing male *Atrx*^{HEM} mice is reduced compared to litters containing only *Atrx*^{WT} mice. Early mother-offspring interactions play an important role in shaping gene expression in the offspring into adulthood (Weaver et al., 2006). Second, offspring genotype can affect interactions between siblings (Ashbrook, Sharmin, & Hager, 2017). We did not however characterize interactions between siblings in early life. This limits our ability to determine the precise early life interaction that is associated with decreased *mTOR* expression. However, isolating the effect of inter-sibling interactions on gene expression into adulthood would inherently introduce other confounding variables. By removing mother-offspring interactions immediately following birth, offspring gene expression would likely be impacted due to early life neglect (Montalvo-Ortiz et al., 2016). Thus, while we did not distinguish between the exact early interactions that are associated with reduced *mTOR* expression, we determined that early rearing environment does have an impact on *mTOR* expression in the brain.

We found that *mTOR* expression was not affected by the *Atrx*^{HEM} transgenic model as there was no significant decrease in *mTOR* expression in the brains of F1 male *Atrx*^{HEM} mice compared to male *Atrx*^{WT} mice. This finding suggests that *ATRX* does not directly regulate *mTOR* expression. However, knowing that *mTOR* expression is not directly affected by *ATRX*, and that both *ATRX* and *mTOR* expression in the brain are impacted by prenatal stress exposure, the effects of decreased protein expression related to ASD-like phenotypes may involve different molecular pathways that converge on common cellular processes. Neurogenesis is known to be affected in ASD (Packer, 2016) and apoptosis contributes to the development of ASD (Dong, Zielke, Yeh, & Yang, 2018). Prenatal stress affects neurogenesis in the hippocampus (Lemaire, Koehl, Le Moal, & Abrous, 2000; Weinstock, 2011) and increases expression of proapoptotic proteins in the frontal cortex and hippocampus (Glombik et al., 2015). Both *ATRX* and *mTOR* are involved in cell survival during neurogenesis, as well as apoptosis through *p53*. Reduced *ATRX* is associated with increased *p53*-mediated apoptosis (Seah et al., 2008). Additionally, *p53* senses stressors in the cellular environment and as a critical regulator of *mTORC1,* influences the activity of *mTOR*-mediated pathways involved in cell growth, division and apoptosis (Feng et al., 2005). Reduced *mTOR* expression is associated with dysregulated cell survival mechanisms (Hung, Garcia-Haro, Sparks, & Guertin, 2012). In

this study, we showed that prenatal stress is associated with decreased *ATRX* and *mTOR* expression in the brain. Given that *p53* is sensitive to stress and is closely related to both *ATRX* and *mTOR*, future studies may aim to investigate *p53*-mediated apoptosis as a mechanism behind the association between prenatal stress and idiopathic ASD.

4.1.3. p-mTOR

In this study, we showed that early life stress has an effect on *p-mTOR* expression in the brain. We found that F1 males and females exposed to stress *in utero* had reduced *p-mTOR* expression throughout the brain compared to their stress naïve counterparts. While we reported similar trends in overall *mTOR* expression, measuring the phosphorylation state of *mTOR* is important as it is indicative of pathway activity (Altomare et al., 2004). Seeing as the PI3K-Akt/*mTOR* pathway is involved in cell survival, we chose to focus on the expression of *p-mTOR* that is phosphorylated at Ser 2448 specifically. This site is associated with upstream activation of *mTOR* via the PI3K-Akt pathway (Chiang & Abraham, 2005; Sekulic et al., 2000). Furthermore, *mTORC1* is only phosphorylated at Ser2448 when the protein complex is intact (J. Copp, Manning, & Hunter, 2009), indicating it is activated and functional. Seeing as prenatal stress exposure is associated with reduced *p-mTOR* expression and *p-mTOR* expression is associated with cell proliferation (Lee, 2017), this finding would suggest an association between prenatal stress and reduced cell proliferation, potentially via reduced *p-mTOR*.

Similar to *mTOR*, there was no significant effect of transgenic manipulation of *Atrx* expression on *p-mTOR* expression levels in the brain. This finding suggests that the reductions in *p-mTOR* are not driven by direct regulation of *Mtor* by *ATRX.*

4.1.4. Ratio of p-mTOR to mTOR

In this study, we found that there was no significant effect of prenatal stress, early rearing conditions on the ratio of *p-mTOR* to total *mTOR* expression. There were no significant differences in the ratio of expression within F0 male and female, and F1 female mice. The F1 male mice showed a genotype by region interaction as in the hippocampus specifically, the $Atrx^{HEM}$ mice had reduced ratio of *p-mTOR* to total *mTOR* compared to $Atrx^{WT}$ mice.

Quantifying the ratio of *p-mTOR* to total *mTOR* provides information about the

overall activation of pathways involving *mTOR*. A decrease in the ratio of *p-mTOR* to total *mTOR* is indicative of a decrease in *mTORC1* dependent translation from mRNA to protein, which can affect cellular processes like cell survival (Schmitz, Chao, & Wyse, 2019). Drugs that increase the ratio of *p-mTOR* to total *mTOR* restore the impairments to cellular survival seen when the ratio is decreased (Schmitz et al., 2019). Decreases in the expression of both *p-mTOR* and total *mTOR* will not lead to a significant decrease the ratio of *p-mTOR* to *mTOR*. However, the effect of a decrease in the absolute expression of *mTOR* and *p-mTOR* without a change in the ratio on physiological changes in the cell has yet to be explored. While the ratio of *p-mTOR* to total *mTOR* provides an estimate of the activity of *mTOR* pathways, measuring the binding of *mTORC1* to downstream targets involved in cellular survival, such as *S6K*, through immunoprecipitation would provide a more accurate understanding of relative levels of activity in the *mTOR* pathway of interest.

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

In this study, we did not observe changes to mRNA expression driven by early life experiences. The $Atrx^{HEM}$ mice showed decreased *Atrx* expression in the frontal cortex, indicating an effect of genotype on *Atrx* mRNA expression. While we expected to observe reduced *Atrx* throughout the entire forebrain, the reduction in *Atrx* transcript levels was limited to the frontal cortex as the $Atrx^{HEM}$ mice did not show a significant decrease in transcript levels in the hippocampus. Furthermore, there was no effect of genotype on *Mtor* or *Mecp2* expression, as the $Atrx^{HEM}$ mice did not have significantly altered levels of these mRNA transcripts in any brain region. These findings suggest that the differences observed in protein expression were not likely to be driven by differences in transcription of genes. Rather, transcription of these genes was unaffected by early life experience and the differences observed in protein expression associated with prenatal stress are likely due to other mechanisms.

Quantifying mRNA expression with RT-qPCR is indicative of transcription levels of the genes targeted with specific primers. mRNA levels are not directly proportional to protein expression levels, which indicates that post-transcriptional modifications affect protein expression (Csardi, Franks, Choi, Airoldi, & Drummond, 2015). Thus, the lack of

correlation between the changes to protein and mRNA expression that were observed in this study may be due to differences in transcription and to post-transcriptional modifications.

4.2.1. Atrx

As we expected, we observed decreased *Atrx* mRNA transcript expression in the frontal cortex of male *Atrx*^{HEM} mice relative to male *Atrx*^{WT} mice. This allows us to conclude that our genetic mouse model was effective in reducing *Atrx* expression in the frontal cortex. We expected that expression in the hippocampus in the male *Atrx*^{HEM} mice would mirror the effect seen in the frontal cortex. However, we did not observe a significant reduction in *Atrx* in the hippocampus. This may be due to the same reason outlined in the discussion about *ATRX* protein levels in the frontal cortex and hippocampus, involving non-uniform *Emx-1* expression in the hippocampus.

Additionally, there was no effect of prenatal stress or rearing condition on *Atrx* expression. We did not see differences in forebrain *Atrx* expression that followed the trends we observed in *ATRX* expression in F1 females and males exposed to stress, and F1 females reared with male *Atrx*^{HEM} mice. As there were no differences to *Atrx* transcription resulting from prenatal stress exposure, it is likely that the reduction in protein expression is due to post-transcriptional modifications that influence protein translation.

4.2.2. Mtor

We observed a significant decrease in *Mtor* mRNA expression in the frontal cortex of F1 male *Atrx*^{HEM} mice. *Atrx*-null mice have decreased *Mtor* mRNA expression in the frontal cortex. This finding suggests that a lack of *Atrx* mRNA influences a reduction in *Mtor* mRNA expression. Interestingly, there were no differences in *mTOR* protein expression in the F1 male *Atrx*^{HEM} and *Atrx*^{WT} mice, indicating that while this reduction in *Mtor* mRNA may be statistically significant, it may not be biologically significant.

In F1 female and male mice, there was no effect of prenatal stress exposure on *Mtor* mRNA transcript levels in the brain. While we did not see an effect of stress on *Mtor* mRNA in the brain, we may have seen changes to *mTOR* protein levels as *mTOR* protein function is cyclical and varies with circadian rhythm (Cao, 2018).

4.2.3. Mecp2

As *MeCP2* is known to recruit *ATRX* and *Mecp2* deficient mice have disrupted *ATRX* localization at chromatin (Nan et al., 2007), we were interested in measuring the influence of reduced *Atrx* expression on *Mecp2* mRNA transcript levels. There was no significant change to *Mecp2* expression in the brains of F1 male $Atrx^{HEM}$ mice compared to $Atrx^{WT}$ males. Though *ATRX* interacts with *MeCP2* to silence gene transcription, the lack of reduction of *Mecp2* in $Atrx^{HEM}$ mice is indicative that *ATRX* expression does not directly affect *Mecp2* expression.

Additionally, there were no effects of prenatal stress or early rearing on *Mecp2* mRNA transcript levels in F1 males or females. While we did not see differences in *Mecp2* mRNA expression due to stress, further investigation into the mechanism by which stress reduces expression of *ATRX* and *mTOR* may be concerned with *MeCP2* activity.

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

To better understand the epigenetic mechanisms by which prenatal stress and early life interactions alter *ATRX* and *mTOR* expression, we measured methylation at various sites along the *Atrx, Mtor* and *Line1* promoters. In general, enriched methylation at these promoters indicates silencing of gene expression. In F1 mice, we found that the *Atrx* promoter was differentially methylated in response to prenatal stress exposure, early rearing, and male genotype. We also found that the *Mtor* promoter in female mice was differentially methylated in response to stress. Interestingly, we observed differences in *Line1* promoter methylation in F0 females in response to stress and genotype.

4.3.1. Atrx

F1 female mice exposed to prenatal stress showed an overall increase in promoter methylation compared to stress naïve females indicating that stress has a silencing effect on the *Atrx* promoter. F0 female mice exposed to stress in adulthood, however, did not show any changes to promoter methylation. These findings indicate that early life stress, specifically, is capable of influencing *Atrx* gene regulation into adulthood.

4.3.2. Mtor

F1 male *Atrx*^{HEM} mice did not show any significant changes to *Mtor* methylation compared to *Atrx*^{WT} males. The reduction of *ATRX* in *Atrx*^{HEM} mice is not sufficient to drive changes in *Mtor* promoter methylation, suggesting that *ATRX* likely does not directly regulate *Mtor* expression.

However, we found that prenatal stress affects *Mtor* methylation in females. There was an increase in *Mtor* promoter methylation in the brains of F1 females exposed to prenatal stress compared to stress naïve F1 females. Furthermore, this finding is specific to prenatal stress, as methylation of *Mtor* was unaffected in F0 females exposed to stress. These findings indicate that prenatal stress impacts methylation status of *Mtor*.

In addition, early rearing conditions was associated with differences in *Mtor* promoter methylation. F1 females reared with *Atrx*^{HEM} mice had increased *Mtor* promoter methylation compared to those reared with *Atrx*^{WT} males, suggesting that early interactions play an important role in programming *Mtor* methylation status.

4.3.3. Line1

In the frontal cortex and hippocampus, there were significant differences between *Line1* methylation by genotype of F0 females exposed to gestational stress. In F1 females exposed to prenatal stress, there was increased *Line1* methylation compared to stress naïve females. *Line1* promoter methylation is an indication of global DNA methylation (Ohka et al., 2011). As such, our findings suggest that prenatal stress exposure, as well as the interaction between stress exposure and floxed *Atrx*, are capable of influencing global DNA methylation.

Interestingly, *Atrx*^{HEM} mice that had reduced *ATRX* expression in the brain, did not show differences in global methylation compared to *Atrx*^{WT} mice. Though these mice have reduced *ATRX*, the finding that global methylation is not altered indicates that there may not be a complete dysregulation of chromatin structure. As *ATRX* is known to be located primarily at heterochromatin during cell division (McDowell et al., 1999), it may be useful to measure changes to *ATRX* function using chromatin immunoprecipitation (ChIP) assays. This would show how *ATRX* binding at heterochromatin is affected by prenatal stress.

4.4. *Concluding Remarks and Future Directions*

Overall, we showed that prenatal stress exposure and early rearing conditions influence the expression and regulation of genes implicated in ASD pathogenesis. Additionally, our findings suggest that *ATRX* does not directly regulate the *Mtor* promoter. However, *ATRX* and *mTOR* expression are both decreased in brains of mice exposed to prenatal stress. Furthermore, *Atrx* and *Mtor* promoter regions have enriched methylation in mice that experienced prenatal stress compared to stress naïve mice. We saw similar changes to protein expression and promoter regulation in the brains of females reared with $Atrx^{HEM}$ males, suggesting that early life interactions can also influence the expression of these genes. Changes to *Line1* methylation resulting from prenatal stress exposure and early rearing conditions also indicate that these early life experiences influence widespread genome methylation. These findings extend the understanding that pre- and perinatal experiences are formative in shaping gene expression throughout the lifespan. Through long-term alterations to epigenetic programming, early life experiences influence gene expression and developmental trajectories.

Knowing that *ATRX* and *mTOR* expression, and *Atrx* and *Mtor* regulation, are affected by stress, future studies may assess binding of glucocorticoid receptors with the promoter region of *Atrx* and *Mtor.* This would clarify whether there is a direct relationship between prenatal stress and regulation of these ASD risk genes and would further solidify the understanding that prenatal stress exposures are associated with idiopathic ASD.

While our findings addressed changes to gene regulation and protein expression, we did not assess how the function of *ATRX* and *mTOR* were affected by our manipulations. Measuring changes to chromatin accessibility through assay for transposase-accessible chromatin with high throughput sequencing (ATAC-seq) would provide insight to modifications to *ATRX* function associated with prenatal stress or early rearing conditions. Also, measuring *mTOR* binding with downstream targets in the *mTOR* pathway would assess changes in *mTOR* function.

Additional future studies may aim to delineate the relationship between stress, apoptosis, and idiopathic ASD. The effects of stress on pathways involving *ATRX* and *mTOR* may converge through aberrant *p53* function. Assessing the role of *p53*-mediated

apoptosis in brains of mice exposed to prenatal stress may help elucidate the molecular pathways connecting prenatal stress exposures and idiopathic ASD pathogenesis.

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