EFFECTS OF PRENATAL STRESS AND/OR FOREBRAIN ATRX DEFICIENCY IN C57/B6 MALE MICE ON MATERNAL CARE AND EMOTIONAL, COGNITIVE AND SOCIAL DEVELOPMENT

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ABSTRACT

Early childhood experience and degree of parental-infant attachment influence the developing brain, notably in brain areas that support stress regulation, cognition and social behaviours. Animal models examining gestational stress suggest that sustained changes in gene expression in response to prenatal stress and/or natural variations in mother-pup interactions during the first week of postnatal life is mediated by changes in chromatin structure and DNA methylation. Although the exact mechanism is not completely understood, the chromatin remodeling factor *ATRX* is stably regulated by maternal care. We examined prenatal stress effects in *ATRX* deficient mice on the allocation of maternal behaviour and long-term behavioural phenotypes in the offspring. Our findings demonstrate an unexpected role for *ATRX* in prenatal and early postnatal mouse development involving experiences of social and affiliative interactions, such as parenting, with a persistent effect on DNA methylation, hippocampal development, cognition, and anxiety-related and social behaviours in the adult offspring.

LIST OF ABBRIVIATIONS AND SYMBOLS USED

α: alpha
β: beta
μ: Mean
γ: gamma
ACH: Acetylcholine
ALT: Alternative Lengthening of Telomeres
ANOVA: Analysis of variance
ATRX: X-linked ATP-dependent helicase
BP: Base pair
C: cysteine
CDNA: complementary DNA
CNS: Central nervous system
CPG: Cytosine–phosphodiester–guanine
CRE: P1 tyrosine recombinase enzyme
5-hmC: cytosine hydroxymethylated at the carbon-five position
°C: Degrees centigrade
DDH2O: double deionised water
DF: Degrees of freedom
DNA: Deoxyribonucleic acid
DNMT-1/3a/3b: DNA methyltransfer-ase-one, three A or three B
DNTP: Deoxynucleoside triphosphate
ED: Embryonic day
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent
FC: Free Choline
G: Grams
H: Hour
H: 1/2/3/4 – histone-one, two, three, four
H2O: Water
HAT: Histone acetylase transferase
HCl: Hydrochloric acid
HET: Heterozygote
HPA: hypothalamic-pituitary-adrenal
IGG: Immunoglobulin G
KB: Kilobase
L: Litre
LTP: Long term potentiation
LOXP: Locus of X-over P1 sequence

mRNA: Messenger ribonucleic acid
µg: Microgram
µl: Microlitre
µM: Micromolar
ml: Millilitre
mM: Millimolar
MIN: Minute
M: Molar
MWM: Morris water maze
N: Number of (e.g., subjects)
NAD: Nicotinamide adenine dinucleotide
NCRNA: small non-protein-coding ribo-nucleic acid
NMDA: n-methyl, d-aspartate
NO: Nitric oxide
OA: Open arm
OF: Open field
P: probability (statistics)
%: Percentage
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
PND: Post-natal-day
qPCR: Qualitative polymerase chain reaction
RIPA: Radio-immunoprecipitation assay
RPM: revolutions per min
RNA: Ribonucleic acid
SAM: S-adenosyl-methionine
SEC: Second
SAP: Stretch attend posture
SE: Standard error
SEM: Standard error of mean
SNS: Sympathetic nervous system
SPSS: Statistical package for the social sciences
UV: Ultraviolet
V: Volts
WT: Wild type
WHO: World Health Organization

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CHAPTER 1: INTRODUCTION

During early mammalian brain development, intrauterine and perinatal environments have persistent effects on gene expression programs in the offspring. Altered gene expression programs— in response to intrinsic (genomic) and extrinsic (environmental) cues—are transmitted in proliferating neural precursor cells through cell division from mother to daughter cells and contribute to changes in neurogenesis, synaptic overproduction and pruning of synapses and receptors (Teicher et al., 2006). Herein, the early nurturing environment has long-term influences on molecular pathways (in germline and somatic cells) that support fetal and postnatal brain growth and potentially contribute to neural and behavioural mechanisms associated with emotional, social and cognitive development in the offspring (Huizink et al., 2004).

Growing evidence suggests early life experiences are also linked with physical and mental health in the offspring. Common risk factors include maternal physical health during pregnancy (e.g. exposure to stressful life events, major episodes of mental disorder, exposure to toxins, infection) and maternal use of non-prescribed drugs during pregnancy (Brown and Kahn, 1997; Brown et al., 1998; Brown and Buddle, 1996). For example, pregnant women with increased anxiety and stress levels are subject to increased risk for spontaneous abortion and preterm labour, as well as for having a malformed or growth-retarded newborn (specifically with reduced head circumference) (Huizink et al., 2004). Infants of depressed mothers also express less positive affect, and flatter affect upon maternal interaction as early as 2 months old (Brown and Lumley, 2000; Oberlander et al., 2008). Animal research supports gestational stress affects pregnancy outcomes and results in early programming of brain functions with permanent changes in neuroendo-

crine regulation and behavioural responses to stress in the offspring (Francis et al., 1999; Liu et al., 2000; Weaver et al., 2004a). These findings have further prompted research in the field of gestational stress on offspring brain growth and behaviour.

The underlying pathway(s) through which an organism's intrauterine and perinatal environment can influence the manner in which the genome is expressed remains rudimentary. Identifying the key biological mechanism(s) that regulate the activation and suppression of genes is crucial, as they play a key role in determining the timing and location of gene expression. In the nucleus of eukaryotic cells, chromatin structure can be reorganized to facilitate dynamic expression of the genome throughout brain development, leading to the silencing of genes that facilitate proliferation and activation of genes involved in differentiation (Berube et al., 2005). For example, maternal exposure to environmental and social stressors during gestation can alter the function of several chromatin remodelling mechanisms (germline and somatic)— including, nucleosome positioning, non-coding RNA interference, post-translational histone modifications, and direct methylation of the DNA itself—producing abnormalities in somatic, cognitive and social development, as well as increased risk of physical and mental illness in offspring later in life (reviewed in (Weaver et al., 2017))

Chromatin remodelling complexes, which are subcategorized into the SF1 and SF2 superfamilies, regulate gene expression by nucleosome positioning. As a chromatin remodelling protein, *ATRX* (an ATP-dependent helicase of the SF2 superfamily) has been extensively studied in several forms of cancer (Watson, Goldberg & Bérubé, 2015). Cells in which the Alternative Lengthening of Telomeres (ALT) pathway has been activated often show significant loss of *ATRX*, and this loss has been shown to promote tumour growth, contribute to genetic instability, and impair DNA damage response (Koschmann

et al, 2016; Watson, Goldberg & Bérubé, 2015). While the specific role that ATRX plays in telomere maintenance and recombination is still not entirely clear, the removal of ATRX causes significant disruptions to these systems (Ramamoorthy & Smith, 2015).

ATRX also plays a key role in neuronal survival throughout early differentiation and development, especially in the emerging forebrain, hippocampus and cerebellum (Berube et al., 2005). Children born with mutations in the *Atrx* gene have cognitive defects and developmental abnormalities, including social avoidance behaviour. Whether the primary function of *ATRX* lies in the promotion or repression of genes appears to be context dependent (Berube et al., 2005). *Atrx-null* transgenic mice show decreased mass and significant hypocellularity in the cortex and the dentate gyrus, leading to extreme social, cognitive and somatic deficits (Berube et al., 2005). When the effects of the loss of *Atrx* are examined at the DNA level, these deficits are accompanied by significantly increased levels of methylation at repetitive elements (Kernohan et al., 2010). In addition to the influences exerted by prenatal maternal stress on the function of epigenetic mechanisms in offspring, preliminary research also shows that the quality of postnatal care that offspring receive influences the expression of *ATRX* in the forebrain in a manner that is stable into adulthood (Weaver et al., 2006). Variations in the degree and quality of postnatal care have been associated with stable, long-term differences in learning and memory, and the expression of anxiety-related behaviour (Weaver et al., 2006). Male offspring of low licking/grooming (low-LG) mothers showed reduced expression of *ATRX* in the hippocampus in adulthood - when compared their adult male counterparts of high licking/grooming (high-LG) mothers - at levels comparable to transgenic *ATRX* knockdown models (Weaver et al., 2006); (Kernohan et al., 2010). Taken together, these

findings suggest deficits in *Atrx* in the neonatal brain may influence how mothers socially interact with their own offspring, altering the degree and/or quality of care offspring receive. Here I review research on maternal care-induced changes in gene expression, brain function and behaviour, and potential role of *Atrx* in neural mechanisms underlying emotional-social-cognitive behaviours.

1.1. *Overview of Embryonic Development*

During the first half of prenatal development (approximately gestational weeks 5 - 22 in humans), the embryo undergoes rapid neurogenerative cell proliferation in the regions surrounding the telencephalic vesicles (Meyer, 2001). Neurogenesis creates an excess of immature progenitor cells, that migrate outward to form the cortical plate (Meyer, 2001). The generation of cortical and sub-cortical progenitor cells is regulated by the expression of the transcription factor FoxP2 (among others), and the migration of the newly formed cells to their respective destinations is regulated in part by reelin, an extracellular glycoprotein (Meyer, 2001; Tsui et al., 2013). The FoxP family of proteins help to regulate embryonic neural precursors of myocardial and spinal cord tissue, and are critical to neurogenesis, morphogenesis and differentiation of cells in these regions (Tsui et al., 2013). At the ventricular zone, FoxP2 is required for radial precursor cells to transition to intermediate progenitors and neurons in preparation for their migration to cortical regions of the developing brain (Tsui et al., 2013). Progenitor neurons are generated in the neuroepithelium around the telencephalic vesicle before migrating outwards via radial glia to create the cortical plate (Meyer, 2001)

Interneurons are produced in the ganglionic eminence, and migrate outward to their destinations without following radial glia (Meyer, 2001). Cajal-Retzius cells guide

the migration of neurons and interneurons up towards the marginal zone along (the inner surface of the pia mater) by secreting reelin, which interacts with the disabled-1 (Dab1) cytosolic adaptor protein expressed by the migrating neurons (Meyer, 2001). Once migrating neurons have arrived at the marginal zone, the reelin provides a 'stop' signal, which induces the neurons to settle at their current position, with newly generated neurons migrating past older neurons that have already settled into the layers of the cortical plate (Meyer, 2001). The interaction between Reelin and Dab1 plays a key role in ensuring that neurons are positioned appropriately in the neural plate, and that the six distinct layers of cortex are formed correctly (Meyer, 2001). (see Figure 1).

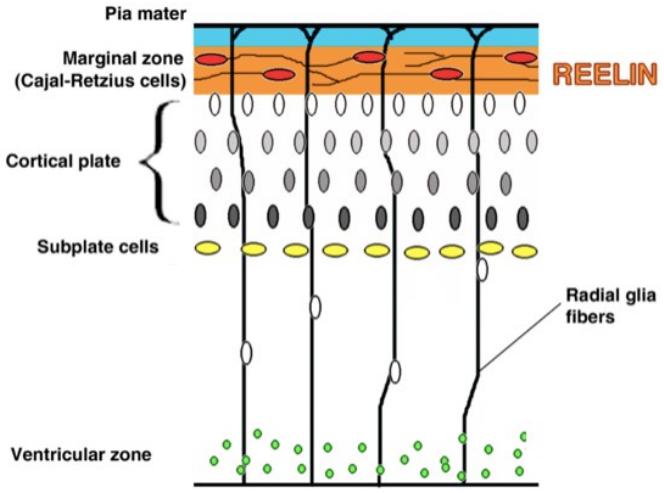


Figure 1: Murine corticogenesis: Progenitor neurons migrate from the ventricular zone of proliferation to the cortical plate. Reelin, secreted by Cajal-Retzius cells, attracts young neurons which migrate past older neurons to generate six layers of cortical tissue (Figure by CopperKettle, 2006, public domain).

Once cells have arrived in their respective cortical destinations, genomic expression switches from genes involved in proliferation and migration towards genes that facilitate differentiation (Berube et al., 2005; Meyer, 2001). As the cortical layers are highly diverse, a multitude of genes are required for the differentiation of developing neurons

and interneurons. As neurons mature and differentiate, they develop extrinsic and intrinsic axonal connections and expansive dendritic fields (Meyer, 2001). The overabundance of neurons generated during the initial half of gestational development then compete for target-derived neurotrophic factors, which guide these developing axonal and dendritic processes (Weaver et al., 2002). Once these networks of neural connections have been adequately formed and strengthened, programmed cell death is induced in extraneous or excess cells to hone neural connectivity in the mature brain (Weaver et al., 2002). Programmed cell death, or apoptosis, is presided over by the expression of a variety of tumor suppressor genes, including phosphoprotein 53 (p53), which cause the termination of cell division and essential cellular processes (Conte et al., 2012). Cells involved in facilitating neuronal migration to the cortex are removed after differentiation, potentially as a consequence of cytoplasmic degradation (Meyer, 2001).

1.2. *Overview of Embryonic Development of the Hippocampal Formation*

Development of the hippocampus (as well as several other subcortical regions) takes place simultaneously with the development of the cortex. The dentate gyrus (DG) region makes up the majority of the hippocampal formation, and plays a role in the formation of memory, spatial navigation, motivation and anxiety-like behaviours (Rolando and Taylor, 2014; Sigurdsson and Duvarci, 2015). The dentate gyrus begins to develop early in embryogenesis (on approximately embryonic day 13 in humans) and follows a pattern of neurogenesis, migration, differentiation and apoptosis similar to the cortex (Li and Pleasure, 2005). Early in its development, the hippocampal formation is composed of Cajal-retzius cells that have migrated from the telencephalic ventricular zone, and serve to draw later progenitor cells towards the outer edge to expand the hippocampal tissue

(Rolando & Taylor, 2014). DG progenitor cells migrate from the dentate neuroepithelium of the ventricular zone, and after reaching their destination differentiate into granular cells, which are a single cell type (Rolando and Taylor, 2014). This migration begins during embryogenesis, and continues on into perinatal development (Rolando and Taylor, 2014).

By the time an infant is born, the DG portion of the hippocampal fissure is fully populated with neural stem cells, which go on to migrate via primary radial glia to various destinations throughout the structure of the DG (Rolando and Taylor, 2014). Granular cells migrate through the dentate migratory stream in a set of distinct phases to create the CA1, CA2, CA3 and CA4 subsections of the hippocampal formation (Rolando and Taylor, 2014). From approximately postnatal day (PND) 3 and onwards, DG cells are then spatially reorganized to settle among various regions of the hippocampal formation (Rolando and Taylor, 2014). Stem cells (undifferentiated neural precursors) migrate to the fimbriodentate junction, a region situated where the supial fimbriae and the developing DG meet (Li et al., 2009). The fimbriodentate junction is responsible for the DGs ability to continually generate new neurons (neurogenesis) into and throughout adulthood (Rolando and Taylor, 2014). These undifferentiated neural stem cells may possibly emerge from the equi-longitudinal ventral hippocampus and migrate to the fimbriodentate junction close to the time of birth, but their specific origin has not yet been confirmed (Li, Fang, Fernandez, & Pleasure, 2013). As the hippocampal formation has a complex structure risen from an extended period of differentiation and migration and retains the ability to generate new neurons throughout adult life, proper regulation of the timing and spatial organization of the genome is critical to the development of the hippocampus.

1.3. *Mechanisms Regulating Gene Expression*

A genome represents the combined repository of genetic information of a given species, and contains all of the genes necessary to construct a complete, viable organism (Karp, 2013). A gene is a unit of deoxyribonucleic acid (DNA), which is a helical molecule composed of two nucleotide chains oriented in opposing directions with sugar-phosphate backbones facing outwards and the nitrogenous bases facing inward (Karp, 2013). In eukaryotic organisms, genes are encased into chromosomes, which are complexes of DNA and associated protein, or chromatin, which allow for the structure and organization of DNA (Karp, 2013) (Figure 2).

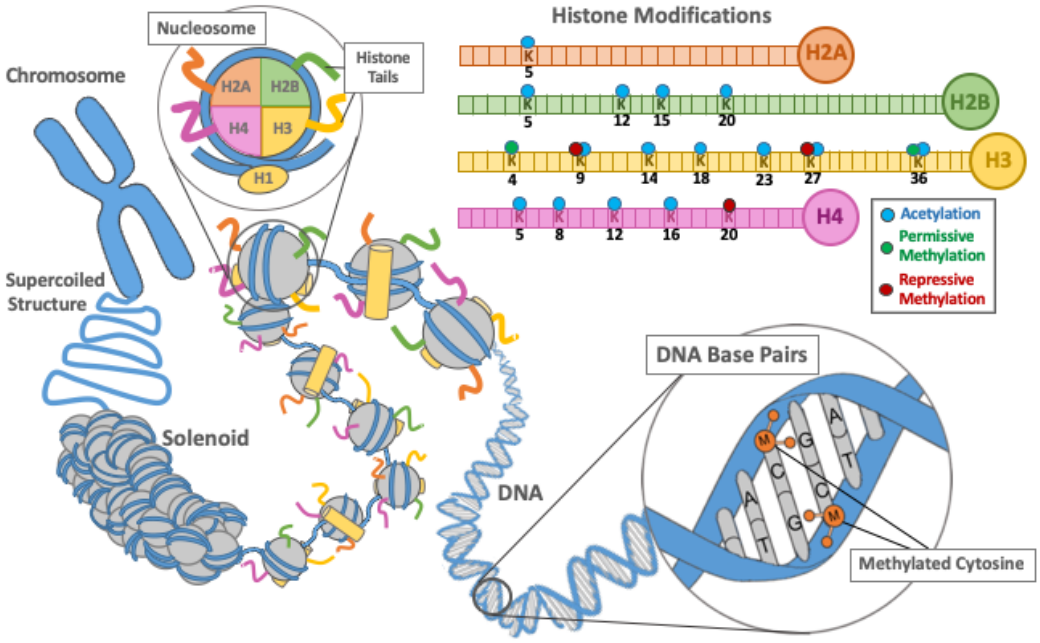


Figure 2: Chromatin structural organization (Figure taken from Weaver et al, 2017, Figure 1).

The DNA double helix strand is wrapped tightly around histone proteins, which are small, highly conserved proteins that possess a high density of basic amino acids, which serve to attract negatively-charged phosphate backbone of the DNA (Karp, 2013). Histone proteins and DNA are packaged into repeating nucleosomes units, which are

made up of a core nucleosome particle and a Histone 1 (H1) linker protein (Karp, 2013). Each core nucleosome particle consists of eight proteins in total - two H2A, two H2B, two H3, and two H4 proteins - along with any associated supercoiled DNA (Karp, 2013). Nucleosomes are compacted into a coil, which is arranged into looped domains; the exact positioning of nucleosomes is still unclear, and may change over the various stages of an organism's development (Karp, 2013).

Chromatin influences the expression of genes by organizing DNA into a tight, dense, and highly ordered structure, which limits the access of transcription factors and other molecular machinery required for gene transcription (Karp, 2013). When a given gene is expressed, or during certain phases of cell division, the structure of the relevant region of chromatin is loosened and uncoiled to allow transcriptional factors and molecular machinery to access the DNA (Karp, 2013). When chromatin is uncoiled in this accessible, transcriptionally active state, it is referred to as euchromatin; chromatin that is tightly packaged and therefore transcriptionally inactive, or silenced, is referred to as heterochromatin (Karp, 2013). Therefore, chromatin accessibility dynamics is an essential biological process across the lifespan, especially during early brain development (Figure 3).

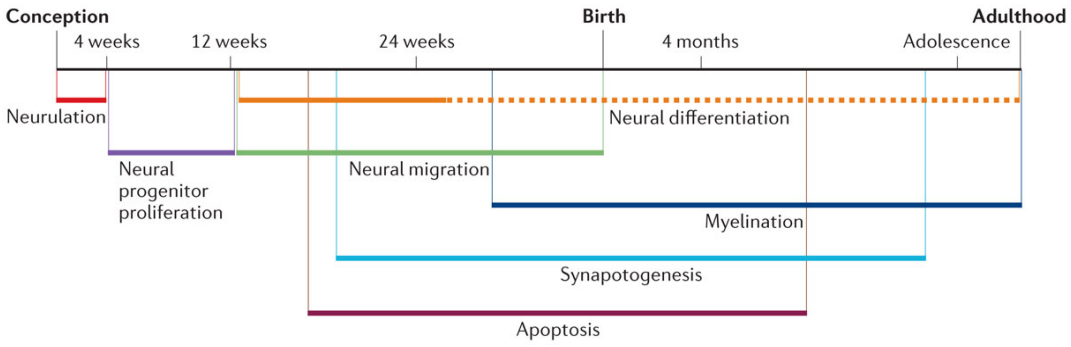


Figure 3: Processes regulated by chromatin across pre- and post-natal development (see text for details).

Epigenetics is a recent subfield of genetics research pertinent to investigating how external factors, such as the physical and social environment, can alter the structure of an organism's chromatin and consequently influence gene expression, resulting in a variety of cellular phenotypes (Bird, 2007). Although an organism's genome essentially does not vary, the possibility exists for several distinct epigenomes, which allow for the expression of different phenotypes from the same genome over time or among different populations of cells (Millan, 2013). Epigenetic mechanisms control which genes are available for expression during proliferation, migration, differentiation and apoptosis during early development, and produce stable gene expression throughout different cell populations into adulthood (Bird, 2007). Epigenetic mechanisms are highly sensitive to factors in an organism's environment, including toxin exposure, nutrient intake, infection and the experience of psychological stress (Kinsella and Monk, 2009). Consequently, these mechanisms act as a means for the environment to act on the genome of an organism (Bird, 2007).

1.4. *Maternal Stress and Embryonic development*

Maternal malnutrition, infection or exposure to toxins has been widely shown to affect the nature of neurogenesis, cellular migration, differentiation and synaptic refinement in the developing embryo (Kinsella and Monk, 2009). Additionally, mothers who experience elevated or prolonged psychological stress while they are pregnant produce offspring who show an increased prevalence of mental illness in the wake of toxin exposure, suggesting a possible increased vulnerability as a result of maternal gestational stress (Perera et al., 2006). A growing body of evidence indicates that the maternal psychological state can influence the in utero environment and have deleterious effects on embryonic development, which appears to affect brain regions implicated in mental illness and produce sta-

ble, lasting effects throughout the offspring's lifespan (Kinsella and Monk, 2009). In particular, chronic maternal exposure to common life stressors has been shown to affect fetal development of the central nervous system, and increase the likelihood that offspring may develop autism, mood disorders, or cognitive impairments later in life (Kinsella and Monk, 2009). Maternal stress has been theorized to act on embryonic development in two possible ways: by dysregulating the maternal-fetal hypothalamic-pituitary-adrenal (HPA) axis, or by disturbing the intrauterine environment by causing irregularities in blood flow in uterine arteries (Kinsella and Monk, 2009).

During gestation, in addition to the corticotropin-releasing hormone (CRH) generated from the maternal hypothalamus, the placenta also secretes CRH throughout pregnancy (Kinsella and Monk, 2009). These increased levels of CRH induce hyperactivity of the maternal HPA axis during mid- to late- pregnancy, leading to elevated maternal blood cortisol levels that resemble those of individuals with Cushing's disease (Kinsella and Monk, 2009). The developing fetus is shielded from this increase in cortisol by the placental 11B-HSD-2 enzyme, which converts cortisol to inactive cortisone; however, in high-stress mothers, 10-20% of maternal glucocorticoids appear to permeate the placental barrier (Kinsella and Monk, 2009). It appears that high maternal stress during gestation may inhibit the action of the 11B-HSD-2 enzyme, leading to increased exposure of the fetus to active cortisol (Glover et al., 2009). In the context of increased maternal HPA activation, the exposure of a fetus to active cortisol may have significant lasting effects on its development (Kinsella and Monk, 2009).

Maternal stress and/or anxiety may also act on the development of a fetus by altering the intrauterine environment: high-stress mothers also display increased blood flow

resistance in the uterine artery, which conveys nutrients to the fetus (Kinsella and Monk, 2009). A definitive link between uterine artery blood flow and the development of brain regions implicated in mental illnesses has not yet been established, but it appears that high resistance in the uterine artery has been correlated lower weight (for gestational age), indicating that a potential relationship with fetal development and function may exist (Harrington et al., 1996).

It is possible that changes in the fetal environment such as the ones described here may interfere with normal development by inducing atypical epigenetic patterns—and consequently, altered patterns of gene expression—during embryonic, fetal, and early childhood development (Monk, Spicer & Champagne, 2012). In particular, brain regions implicated in learning and memory, social behaviour, and stress reactivity are particularly susceptible to epigenetic dysregulation (Weaver et al., 2017).

1.5. *Epigenetic Mechanisms of Inheritance*

Irregularities in the fetal environment during prenatal development have been shown to alter the epigenome in the developing offspring; additionally, any altered epigenetic mechanisms present in parental germlines are inherited by offspring (Millan, 2013). Consequently, maternal gestational stress can produce stable alterations in the epigenetic profile and pattern of genome expression in offspring (Millan, 2013). Deviations from the typical epigenetic profile (or epimutations) in offspring are associated with increased risk for neurodevelopmental disorders and the emergence of mental or physical illnesses in adulthood (Millan, 2013).

During development, the HPA axis is especially susceptible to epigenetic repro-

gramming: individuals exposed to high maternal anxiety during gestation and low postnatal maternal care typically display elevated cortisol levels, increased stress reactivity and increased risk for mood disorders in adulthood (Weaver et al., 2017). Additionally, the epigenetic mechanisms that regulate cortical and sub-cortical neurogenesis and apoptosis are highly sensitive to the pre- and early postnatal environment, creating the potential for abnormal and/or highly stressful experiences to impede the development of a wide range of cortical functions by creating abnormal patterns of gene expression (Jensen, 2013).

1.6. *Classification of Chromatin Remodelling Protein Complexes*

There are four established types of epigenetic mechanisms: DNA methylation, post-translational histone modifications (see Figure 2), non-coding RNA interference, and chromatin remodeling (Bird, 2007). Chromatin remodeling enables the activation or silencing of distinct genotypes by either opening tightly-packaged heterochromatin to allow for access to the DNA, or by compressing sections of open euchromatin to render DNA inaccessible to transcriptional machinery (Bartholomew, 2014). Chromatin remodeling proteins act on the structure of chromatin by disassembling nucleosomes (see Figure 2), or by exchanging histone variants, which alters the positioning of affected nucleosomes on the DNA (Bartholomew, 2014).

Chromatin remodeling proteins are divided into two superfamilies: SF1 and SF2, which both contain a highly-conserved helicase core composed of two RecA-like protein domains (Fairman-Williams et al., 2010) (Figure 4). Each superfamily contains characteristic protein motifs with distinct sequential, structural and functional features, creating a variety of mechanisms of action and target substrates (Fairman-Williams et al., 2010).

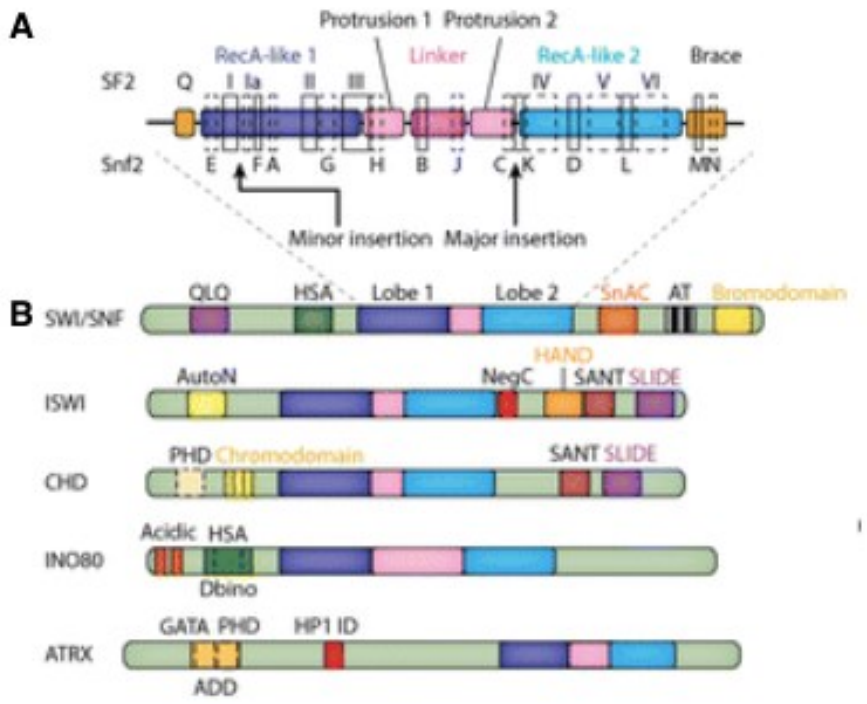


Figure 4: Classification of ATP dependent Chromatin Remodelling Complexes by properties and organization of domains. A) SF2 and snf2 motifs found in the helicase domain, along with insertion sites. Purple and blue sections correspond to the two RecA-like domains while pink sections correspond to the two protrusions and the linker DNA. B) Organization of the individualized domains for the five classes of snf2 chromatin remodelers. ((Figure taken from Fairman-Williams et al., 2010, Figure 1)

Under the SF2 superfamily is the Sucrose Non-Fermenting II (snf2) family, an assortment of ATP-dependent chromatin remodeling protein complexes distinguished by their SF2 RecA-like helicase core domains, a linker region, and two individualized protruding regions (DNA and/or Histone recognition elements) (Fairman-Williams et al., 2010). The snf2 family is further categorized into five subfamilies (Figure 4), based on the sequence of individualized motifs within their protruding regions, and the mechanisms by which they remodel and act on specific nucleosome sites (Bartholomew, 2014;

Fairman-Williams, Guenther & Jankowsky, 2010). The five subfamilies of the snf2 family are: SWI/SNF, ISWI, INO80 or SWR1 and α-thalassemia mental retardation syndrome X-linked (ATRX) (Bartholomew, 2014).

1.7. *ATRX Expression, Structure and Functional Role in Development*

The transcriptional regulator protein *ATRX* belongs to the SNF2 family of chromatin remodelers, and appears to be able to exert either a repressive or facilitative effect on chromatin structure (Bérubé et al, 2005). *ATRX* functions on the nucleosome level, and can work either independently or in conjunction with histone chaperone proteins to modify or create *de novo* nucleosome structures (Bartholomew, 2014). Expression of *ATRX* is localized to heterochromatin in the telomeric and pericentromeric regions of the chromosome, and appears to take part in preserving genomic integrity during transcription and replication in these regions (Bartholomew, 2014). *ATRX* also appears to participate in the repair of damaged DNA within its area of influence (Conté et al., 2012). The *ATRX* protein is composed of a snf2 helicase domain at its C-terminus, and an *ATRX-DNMT3-DNMT3L* (ADD) domain at its N-terminus (Figure 4) (Bartholomew, 2014). Between the two domains, there is a heterochromatin protein 1 (HD1 ID) motif, which facilitates interactions with various histone chaperones (Bartholomew, 2014).

The ADD domain of *ATRX* contains two functional pockets, which allow for the localization of *ATRX* by facilitating the recognition of the unmodified (H3K4me0) and as well as methylated (H3K9me2 and H3K9me3) lysine residues on H3 variants (Bartholomew, 2014). One ADD functional pocket appears to bind to unmodified H3K4me0 Lys residues in a manner that protects them from methylation (Otani et al., 2009). The other

ADD pocket acts to bind di- and tri-methylated lysine residues, and appears to interact with the HP1 ID domain in order to recruit HP1; *ATRX* must be able to interact with HP1 in order to assemble *de novo* nucleosomes in the pericentromeric and telomeric regions (Bartholomew, 2014). *ATRX* also associates with histone chaperones, such as death domain-associated protein (DAXX) in order to insert and remodel H3 variants (e.g., H3.1, H3.2, H3T) in the telomeric regions (Bartholomew, 2014).

In order to maintain heterochromatin and genomic integrity during transcription and replication, it is critical that *ATRX* be able to assemble and modify telomeric and pericentromic nucleosomes (Bartholomew, 2014). In addition to these regions, *ATRX* also appears to localize to cytosine-guanine (CG) rich regions of DNA—such as CpG islands (regions with > 50% GC dinucleotides, and an observed-to-expected CpG ratio > 60%) and interstitial telometric sequences (short telomere-like repeats located away from chromosomes ends)—to form segments of heterochromatin (Clynes & Gibbons, 2013). Individuals with *ATRX* syndrome show significant downregulation of genes situated closely to CG-rich regions; additionally this downregulation appears to increase in proportion to the proximity to and length of the CG-rich region (Clynes and Gibbons, 2013). This evidence indicates that the presence of *ATRX* may also act to prevent the silencing of genes that may typically be active in non-telemetric and pericentromeric regions (Clynes & Gibbons, 2013).

ATRX also appears to be able to enact nucleosome remodelling independently from replication processes: after exposure to replication inhibitors (e.g., Camptothecin, Aphidicolin) that triggers programmed cell death, *Atrx-null* cells demonstrate a higher rate of apoptosis, which indicates that the nucleosome remodeling functions of *ATRX* may

help to repair damage to DNA that may occur during replication (Clynes & Gibbons, 2013). When existing as single-strand DNA (ssDNA), telomeric, pericentromeric, and CG-rich regions of DNA create a G4 quadruplex structure, which can be observed during DNA replication in the lagging strand; this G4 structure appears to disrupt DNA replication processes, and interfere with genomic stability (Contè et al., 2012). As *ATRX* co-localizes with the enzyme DNA polymerase B at long tracks of ssDNA after replication (as opposed to localizing to the replication-fork), it has been theorized that *ATRX* may be able to bypass or repair the G4 structure in a replication-independent manner following replication or transcription (Clynes & Gibbons, 2013). With these findings in mind, it appears that *ATRX* may also operate to preserve heterochromatin integrity in telomeric, pericentromeric and CG-rich regions of terminally differentiated cells, which are typically unfavourable substrates for the formation of nucleosomes (Clynes & Gibbons, 2013).

In mammals, expression of *ATRX* is mainly confined to the hippocampal and cortical regions during development (Bérubé et al, 2005). Mutations of the *Atrx* gene lead to neurodevelopmental disorders, such as α-thalassemia X-linked mental retardation (Bérubé et al, 2005). α-thalassemia X-linked mental retardation produces extreme cortical hypocellularity, which leads to severe cognitive deficits, facial dysmorphism, microcephaly and skeletal and genital abnormalities in affected individuals (Bérubé et al, 2005). Overall, the ADD domain of *ATRX* appears to play a protective role by preventing the over-methylation of DNA in these regions, and it seem that *ATRX* plays a crucial role in ensuring the survival of newly generated cells in the hippocampus and cortex during embryonic development (Bérubé et al, 2005).

The hippocampus, in conjunction with other regions of the cortex, plays a key part

in a wide range of behavioural and cognitive functions (Sigurdsson and Duvarci, 2015). Reductions in the mass of the dorsal hippocampus have been shown to result in learning, memory, and spatial organization deficits (Sigurdsson & Duvarci, 2015). The ventral hippocampus appears to be involved in emotional and motivational processes, and reductions of mass in the hippocampus and disruptions of ventral hippocampal-cortical neural interactions have been associated with the emergence of psychological disorders, including anxiety and schizophrenia (Sigurdsson & Duvarci, 2015).

Expression of *ATRX* is highly sensitive to factors in the pre- and early postnatal environment (Weaver et al., 2012). Exposure to maternal gestational stress, and especially elevated maternal cortisol, has been shown to influence the expression of genes critical to normal development, including *Atrx* (Jensen, 2013). Low maternal care, which has been associated with maternal gestational stress, has also been shown to lead to lower rates of expression of *ATRX* in the forebrain (Weaver et al., 2006). Consequently, clarifying the relationship between the expression and functionality of *ATRX*, gestational stress, and maternal care is an important aim in the research of neurodevelopmental disorders in which there are reductions in cortical mass that lead to cognitive, social and emotional deficits.

1.8. *Modeling the developmental role of Atrx in mice*

Elucidating the exact role and function of *ATRX* is complex, as this protein is critical to early cortical and hippocampal development: *Atrx-null* mice are unable to survive past PND 2 (Conte et al., 2012). As *Atrx-null* animals are non-viable, an alternate transgenic model must be employed to model the decreased *ATRX* expression seen in offspring as a result of high maternal gestational stress and low postnatal maternal care. To this end,

researchers have implemented recombinase technologies to conditionally inactive the *Atrx* gene in developing offspring (Conte et al., 2012). The Cre-LoxP recombination system has been used to generate conditional knockouts: two LoxP sites are inserted into the wild-type *Atrx* allele to create a Floxed *Atrx* allele (Bérubé et al., 2005). Females homozygous for the Floxed *Atrx* allele can then be bred with Cre homozygous males, producing a conditional Knock-Out of *ATRX*, with heterozygous male offspring expressing the Floxed *Atrx* allele and Cre. Cre expression - and consequent *ATRX* knock-out - can be restricted to forebrain and CNS progenitor cells with the use of *Fork-head box G1* and *Nestin* promoters (Bérubé et al., 2005). Cre-LoxP recombinase *ATRX* knock-outs have been used to explore the impact of reduced ATRX expression in the hippocampus and forebrain during development on cognition and behaviour. These studies provide a groundwork for understanding how *ATRX* facilitates development by preserving chromatin integrity throughout cell replication and transcription, and by preventing the methylation of CG-rich regions of DNA (Bérubé et al., 2005; Bartholomew, 2014).

1.9. *Generation of Atrx Gene Targeted Mice*

ATRX conditional knockdown mice are created with the use of a Cre-LoxP recombination system augmented by a *Nestin* promoter, which restricts Cre expression to forebrain neuroprogenitor cells (Berube et al., 2005). A 6.2-kb *SstI* fragment of the *Atrx* gene was used to engineer a targeting construct containing a floxed *Neo*^{r} cassette within intron 17 and *loxP* sites flanking exon 18 (Figure 5). Two herpes simplex virus thymidine kinase (HSV TK) genes are included to allow for positive and negative selection of recombinant ES cell clones. Female mice homozygous for the floxed Atrx allele ($Atrx^{loxP}$) are bred to heterozygous Foxg1Cre male mice to generate knockout male mice ($Atrx^{Foxg1Cre}$).

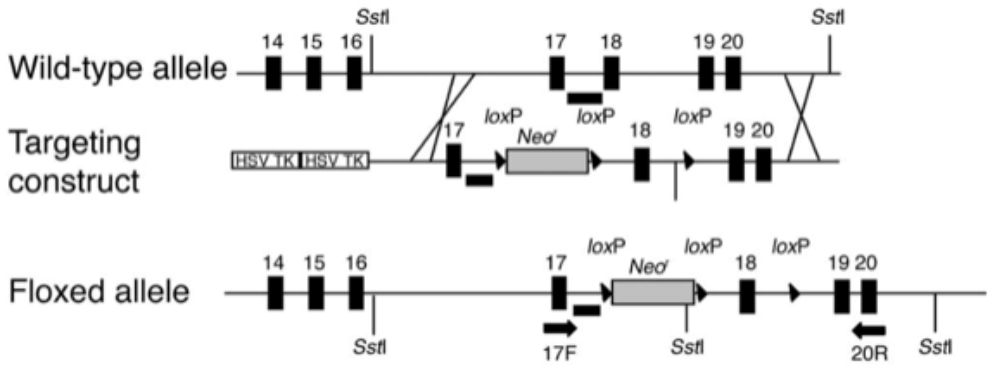


Figure 5: Generation of *Atrx* gene-targeted mice (taken from Berube et al., 2005, Figure 1)

Female dams homozygous for the floxed Atrx allele ($Atrx^{loxP}$) are then bred with Emx1Cre males (C57BL6 background) to create heterozygous conditional knockdown male offspring, referred to as $Atrx^{Emx1Cre}$. *Atrx* and *Cre* wild-type (WT) animals are bred as controls. *Cre* (i.e. *ATRX* knock-down) is active from E10-12 onward. Loss of *ATRX* results in widespread hypocellularity in the neocortex and hippocampus, and reduced size of the forebrain (Berube et al., 2005). Additionally, cortical progenitors isolated from *Atrx-null* mice appear to undergo enhanced apoptosis during differentiation (Berube et al., 2005). These findings demonstrate that *ATRX* plays a key role in cell survival during early neuronal differentiation, and the increased neuronal loss as a result of the absence of *ATRX* may contribute to the severe mental retardation seen in human patients with *Atrx* mutations.

1.10. *Research Question*

Deficits in forebrain *ATRX* expression are known to be associated with hippocampal neuron function and manifested as cognitive delay, altered stress reactivity and altered social interactions. The differential allocation hypothesis implies that animals can sense the health of their offspring through variations in offspring behaviour, and will adjust their

maternal behaviour accordingly; however, the specific impact that differing levels of parental investment has on the development of offspring remains unclear.

Firstly, this study aims to characterize and compare behavioural, social, and cognitive outcomes of Atrx homozygous (*Atrx*^{WT}) and Atrx heterozygous (*Atrx*^{HET}) male offspring, including the degree of maternal care received during the first week of postnatal life. As the females born in litters containing *Atrx*^{HET} males are genotypically *Atrx*^{WT}, they may receive the same level of maternal care as their *Atrx*^{HET} male littermates and will be treated as a type of control for the effects of early maternal care versus genotype. Secondly, this study aims to examine the effects of maternal gestational stress in the third trimester - from approximately gestational day (GD) 13-20 - to determine its differential impact on social, behavioural and cognitive outcomes in the presence of decreased *ATRX* expression and lower maternal care.

1.11. *Main Objectives:*

I. Characterize maternal behaviours of C57B (*Atrx*^{WT}) female mice toward their *Atrx*^{WT} and *Atrx*^{HET}male offspring.

II. Assess the impact of genotype and early maternal care on the long-term development of cognitive, emotional and social behaviour in *Atrx*^{WT} and *Atrx*^{HET} male offspring, and in female offspring from *Atrx*^{WT} and *Atrx*^{HET}-containing litters.

III. Compare the behaviours of gestationally stressed and non-stressed mothers, and assess the outcomes of offspring in adulthood to determine how the *Atrx*^{HET} and low-maternal care animals may be differentially affected by the effects of gestational stress.

CHAPTER 2: MATERIALS AND METHODS

2.1. *Animal Housing and Husbandry*

This experiment made use of wild-type (*Atrx* ^{WT}) and transgenic (*Atrx*^{HET}) C57BL mice. Wild-type *ATRX* ^{WT} C57BL mice were obtained from the Jackson Laboratory (Bar Harbor, ME); transgenic *Atrx*^{loxP} and Cre lox recombinase lines were donated by the Dr. David Picketts lab (University of Ottawa). These three lines were bred to establish a colony, and all mice used in the experiment were from of generation F4 or later.

Mice were housed in a colony room under a 12h:12h reversed light cycle (lights off at 0930h). Temperature in the colony room was maintained at 21 °C ± 2 °C. Mice were housed in polypropylene cages (42 cm x 21 cm x 20 cm; Allentown Caging Equipment Co.) with wire lids, containing corn cob and Enviro-Dri bedding, and a red polycarbonate mouse house (NICRAM animal supplies). Rodent chow (Purina Lab Chow) and acidified water were supplied ad libitum. For breeding, one male and one female were housed together for five to seven consecutive days, or until pregnancy was confirmed by the presence of a sperm plug. *ATRX*^{HET} male offspring were produced by mating *Atrx*^{loxP} females with Cre males (Figure 6). Female offspring reared in *ATRX*^{HET} litters were genotypically wild-type, but differed from females reared in litters among wild-type *ATRX* ^{WT} males in terms of perinatal environment. Pups remained with the dam until weaning (when pups weighed >10.0 g each, approximately PND 21 - 30), upon which the offspring were housed with same-sex littermates in groups up to five. All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

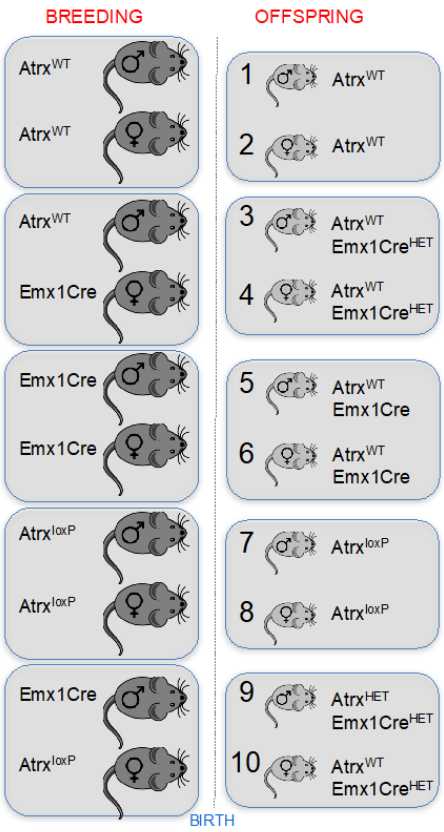


Figure 6: Breeding strategy to generate *ATRX*$^{+/-}$ transgenic mice (see text for details)

2.2. *Genotyping*

Mice were generated as described above and DNA from ear punch tissue of 21-day old pups was genotyped by PCR. Ear punches were heated (98 °C) in 75 ml digestion solution for 1 hour and vortexed for 30 minutes. 75 ml of neutralization solution was added to each sample and vortexed (See Appendix A for digestion and neutralization formulae). DNA was isolated by centrifuging at 4000 RPM for 3 min and removing supernatant. Isolated DNA in supernatant was stored at -25 °C until genotyping.

To prepare PCR samples, 10 µl extracted DNA supernatant was added to 20 µl master mix (See Appendix A for Master Mix formula) containing the *Cre* or *Atrx* primer sets (Table 1)

Target	Forward Primer	Reverse Primer	Product Length
Cre	ATGCTTCTCCGTTTGCCG	CCTGTTTTGCACGTTCACCG	200bp
Atrx	GGTTTTAGATGAAAATGAAGAG	TGAACCTGGGGACTTCTTG	1kb
AtrxloxP		CCACCATGATATTCGGCAAG	1.5kb

Table 1: Primers utilized to genotype animals (see text for details).

PCR amplifications were performed using BioRad 96-deep well C1000 Touch™ and S1000 Thermo Cycler Chassis (See Appendix A for PCR Conditions). Optimal conditions for *Atrx* PCR amplification were determined using an eight-temperature gradient at stage four of PCR (reannealing). Temperatures for reannealing were as follows: 65 °C, 64.1 °C, 62.1 °C, 59.3 °C, 55.9 °C, 53 °C, 51 °C, 50°C. Gene expression was assessed using gel electrophoresis. A 1.5% agarose (Argarose RA, Cat #: 232-731-8, Amresco) gel was prepared in 1X TBE buffer. 2 µl Biorad UView™ 6x ultraviolet Loading Dye was added to 10 µl of each PCR sample. Samples were loaded to each well. 8 µl of Biorad Amplisize 50-2000 bp Molecular Ruler (*Atrx* gels) or Biorad 100 bp molecular ruler was added to 2 µl Biorad UView™ 6x ultraviolet Loading Dye and loaded to the last gel well to allow band identification. The electrophoresis unit was run at 200V for 25 min (*Cre*) or 60 min (*Atrx*). Gels were imaged using a UV lightbox (Bio-Rad).

To confirm staining, the $Atrx^{loxp}$ bands gels were post-stained using gel red. Gels were incubated in a 1:10,000 dilution of gel red:dH_20 for 10 minutes then washed with dH20 for 2 minutes. Gels were imaged using a UV lightbox (Bio-Rad) (Figure 7). The optimal temperature for *ATRX* reannealing was found to be between 50-51 °C

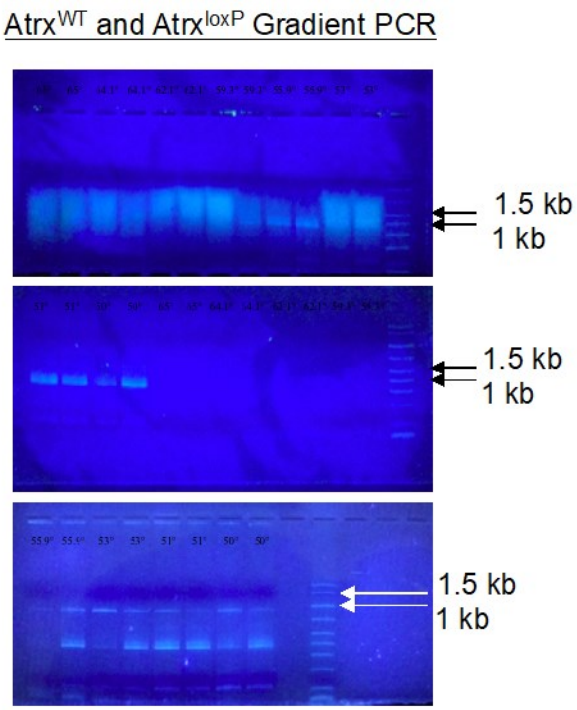


Figure 7: Gel electrophoresis of pooled samples of *Atrx*^{WT} and *Atrx*^{loxP} amplified using a gradient PCR. The optimal primer reannealing temperature for *Atrx*^{WT} (1 kb) and *Atrx*^{loxP} (1.5 kb) was 50-51°C.

2.3. *Prenatal Stressors*

During the last trimester of gestation (GD 12 - 19), pregnant females were either subjected to a seven-day course of restraint stress (RS), or left undisturbed in their home cages as non-stressed (N-S) controls. Fecal pellets were collected from all groups to assess maternal corticosterone (CORT) during this period. Pregnant females in the RS condition were removed from the colony room and individually placed in transparent plexiglas restrainers while being exposed to bright light (about 500lx) for 30 minute sessions, three times per day at approximately 9:00, 13:00 and 16:00 from GD 12 - 19. GD 0 was established by the presence of a sperm plug. After each trial, the restrainers were thoroughly cleaned with water containing detergents and dried with paper towel to avoid olfactory influences on subsequently tested animals.

Baseline CORT levels were established in the RS treatment group from fecal samples collected from the home cages before the onset of the first stressor on GD 12. Females' home cages were changed at the beginning of each day, and any fecal pellets produced over the course of the stress treatments were collected at the end of the day (at least six hours after the onset of the first stressor) to ensure that the samples reflected the stress-related increase in fecal CORT. Fecal samples were also taken from the N-S group over the GD 12 - 20 period. Samples were stored at -20°C after collection.

2.4. *Enzyme-linked immunosorbent assay*

In order to assess the concentration of CORT present in RS and N-S pregnant females, a protein isolation and Ab enzyme-linked immunosorbent assay (ELISA) was used to process the fecal samples collected from GD 12 - 20 (Enzo Life Sciences Corticosterone ELISA kit ADI-900-097). Protein isolation and Ab enzyme-linked immunosorbent assay (ELISA). Frozen faecal pellets were weighted and mechanically broken down in ice-cold TBS supplemented with protease inhibitors (P8340; Sigma) and 2 mM EDTA, and then ultracentrifuged (100,000g, 20 min, 4°C). The resultant Triton X-100 insoluble pellet was then dissolved to half of the initial homogenization volume in 70% FA and centrifuged (14,000g, 10 min, 4°C). The supernatant was evaporated off to 70% of the volume with a nitrogen stream and neutralized to pH 7 with 5M NaOH in 1M Tris. ELISAs were used to detect corticosterone (detection range (DR) 7.4 - 250 pg/mL; ENZO, Cat#: ADI-900-097). From pilot results for each assay, 10-40 µg of each sample was loaded in triplicate alongside an 8-point standard curve in duplicate (with controls) in the 96-well microtiter plates supplied by each manufacturer. Detection was performed with horseradish peroxidase (HRP)-labeled, Fc-specific IgG and read in the microplate

reader (Epoch, BioTek) at a wavelength of 450 nm. The blank corrected levels of each target protein are reported in picogram/mL.

2.5. *Maternal Care Observations*

A subset of dams from each experimental group were observed to determine if the demonstration of maternal behaviours were affected by the genotype of the offspring or by gestational stress treatments. Dams' maternal behaviour was observed and scored daily in real-time for 60 min at 0800h, 1100h, 1300h, 1500h and 2130h. During each observation period, the frequency of the following affiliative behaviours were scored every 3 min as in Champagne (2003a) and Popoola (Popoola et al., 2015): no contact with pups (NCP), which may or may not include self-grooming (SG), nest building (NB), or feeding behaviours (F), passive nursing (PN), arched-back-nursing (ABN) ranked as level 1 (low blanket posture) and 2-4 (high postures favorable for milk ejection), licking and grooming pups, separated pups (SP) and pup retrieval (PR). Non-affiliative maternal behaviours (or 'no contact' behaviours) consist of a dam making no contact with her pups and often being accompanied by self-grooming behaviour (licking, nibbling and combing-like actions of the fur), nest building (changing the positioning or location of the pine shavings around the nest), and feeding behaviour (nibbling at the feeder, consuming rat chow or drinking water). Passive nursing was scored when the dam was on her side to nurse her pups, or used the sides of the cage to support her while nursing. Blanket posture or ABN 1 was observed when the dam was flat over the pups. ABN consisted of graded degrees of arching, levels 2-4, based on kyphosis or the bend of the knees and steepness of back arching of the pups. Separated pups were recorded when a dam had pups away from the nest, isolated or in small groups. Pup retrieval was the transfer of pups back into the nest. Behav-

iours were scored on the MouseWatch application (I.C.G. Weaver; Novum Scientific, Halifax, NS., Canada).

2.6. *Open Field Test*

A variety of anxiety-like behaviours were assessed with the open field test (OFT), where animals were placed in a 70 x 70 cm box and observed for 10 minutes. Scored behaviours included rearing, rearing against a wall, stretched approaches, frequency of and time spent grooming, and the expulsion of urine or fecal boli. Stretched approaches were defined as instances in which the animal advanced on their forelegs and then retracted, while keeping their hind legs stationary. Time spent in the centre and periphery of the field, as well as overall locomotion were tracked in real-time with Noldus' Ethovision XT 9 software (see Appendix B for more details).

2.7. *Social Investigation Test*

Following OFT, animals' social propensity was evaluated with the social investigation task (SIT). In this experiment, a modified version of the SIT as outlined in *Long-Lasting Consequences of Neonatal Maternal Separation on Social Behaviours in Ovariectomized Female Mice* (Tsuda & Ogawa, 2012) was used. Animals went into preparation for the SIT a minimum of 24 hours after completion of the OFT.

In preparation for the SIT, animals were singly-housed in a larger cage (18 x 28 cm) for 24 hours. On the day of testing, cages were moved into a separate testing room, and at least 30 minutes prior to the test, an empty wire mesh container was introduced into the cage to allow the animal to habituate to its presence. At the beginning of each test, a naïve stimulus mouse of the opposite sex was introduced to the cage in the wire

mesh container, so that the experimental mouse would be able to investigate and interact with the stimulus mouse without being able to touch it.

Experimental animals were then observed for a period of 15 minutes. Recorded behaviours include time spent sniffing the stimulus mouse (social affinity), time spent sniffing towards the stimulus mouse from the corner of the cage (inconspicuous social investigation), and the number of stretched approaches. Time spent in in the cage periphery (avoidant behaviour) and overall locomotor activity were tracked in real-time using Noldus Ethovision XT 9 software (see Appendix B for more details).

2.8. *Morris water maze*

Animals' cognitive and spatial memory ability were assessed with the Morris water maze (MWM). The MWM was conducted in a 110 cm diameter plastic pool. Water was made opaque by the addition of approximately 150 ml of white, non-toxic, tempera-based paint to the water. The pool was filled one day prior to testing to allow the water to warm to room temperature. The platform was submerged approximately 1.5 cm under the surface, at a depth where the animals could easy climb onto the platform. Time spent in each quadrant, time spent in the periphery of the water maze (thigmotaxis), proximity to platform, distance traveled, and latency to reach the platform were tracked in real-time with Noldus Ethovision XT 9 software (see Appendix C for more details).

On each trial of the Morris water maze, the mouse would be introduced into the pool from either the north, east, south or west edge of the pool. A different order of entry directions was used for each day of testing and was consistent between mice in order to ensure that animals were required to use external spatial cues in order to navigate to the platform. If the mouse successfully found the platform within the allotted time (60 sec-

onds), then it would be allowed 10 seconds to view its surroundings before being removed from the pool. If the mouse was unable to find the platform within 60 seconds of being introduced to the pool, it would be guided to the platform before being retrieved from the water, in order to establish an association between the platform and escape from the pool. There was a five-minute delay between trials, during which animals were placed in holding cages under a heat lamp or on a heating pad.

MWM testing was divided into four phases: acquisition, reversal, a probe trial, and a visible platform phase. Animals underwent 4 trials per day in all phases except for the probe, which consisted of a single trial. During the acquisition phase, the platform was located in the southeast quadrant of the pool, and mice underwent 4 trials per day, over the course of three consecutive days. Following acquisition mice entered the reversal phase, in which the platform was moved to the opposite (northwest) quadrant of the pool, and mice again were tested for three days. After the acquisition and reversal phases, a single probe trial was conducted, in which there was no platform and the mouse was allowed to swim for 60 seconds so that its search patterns could be observed. Finally, mice underwent the visible platform phase as a control, in which the platform was moved to the northwest quadrant of the pool and was marked with a flag as a visual aid.

2.7 Euthanasia and Tissue Collection

After completing behavioural testing, animals were euthanized in a HiRoad Rodent Euthanasia System®, which employs an isoflurane/carbon dioxide combination to induce loss of anaesthesia followed by asphyxiation. After euthanasia, ear punches, tail snips, liver tissue, spleens, whole brains, and the intact GI tract were immediately collected and snap-frozen on dry ice. All tissue samples were stored at -80°C after collection.

2.8 Gene expression analysis by quantitative PCR

Total RNA was prepared from snap-frozen frontal cortex, hippocampal and cerebellum tissue using the RNeasy mini kit protocol (Qiagen, Valencia, CA, USA) and quantified with a Take3 Micro-volume Plate on an Epoch Spectrophotometer (BioTek). RNA integrity was confirmed using an Experion Automated Electrophoresis System and RNA StdSens chip (Bio-Rad). The RNA quality index value for all samples was >7.9 with low degradation. 2 µg of RNA was converted into cDNA using the iScript cDNA Synthesis Kit (BioRad) according to manufacturer's instruction. A pooled RNA sample from each set of extractions was used as a no-RT control, containing the nRT reagent in place of the RT, and underwent the same thermocycling as the RT samples. Reverse transcription-PCR was performed on the CFX96 Touch Real-Time Detection System (BioRad, Hercules, CA, USA) using primers as listed in Table 2. For each primer set, an annealing temperature gradient was conducted on a pooled 1:10 diluted cDNA sample and the optimal range was discovered to be 60-65ºC. Each 10µl reaction contained 2µl of template, 2.5µl of PCR primer, 2.5µl of PCR-grade H_2O water and 5µl of SsoFast EvaGreen Supermix (Bio-Rad). A no-template and no-RT control was used to control for contamination in the samples or reagents. Standard curves for each primer set were generated, and primer efficiencies were incorporated into the CFX Maestro software (version 1.1, Bio-Rad, Hercules, CA, USA). The thermocycling began with a 2min activation at 95ºC, followed by 40 cycles of 5s of denaturation at 95 ºC and annealing/extension at 60 ºC for 30s. This was finished with a melt curve from 65-95 ºC which increased by 0.5 ºC every 5sec. Each sample was run in triplicate and any replicate with deviation of greater than 0.2Cq from the mean was excluded from the analysis. Relative mRNA expression for each gene target

was calculated using the Livak and Schmittgen's 2^-ΔΔCT method and normalized to β-actin as a reference gene (Livak and Schmittgen, 2001) and standardized to the wildtype samples of the same age and the same run of the qPCR analysis (Livak and Schmittgen, 2001). When required, where gene-, treatment- and region-matched samples crossed over multiple plates, the average Cq for a pooled sample common to each plate served as an interplate calibrator and was adjusted to match across plates. Following this, using HPRT as the reference gene, the ΔCq was determined. The ΔCq, or expression, was then compared with a two-way ANOVA between sex and treatment for each region analyzed. Any values greater than two standard deviations from the mean were considered outliers and were removed from analysis.

Target	Gene	Forward Primer	Reverse Primer
β-actin	*Actb*	5'-agccttccttcttgggtatgg-3'	5'-acacagagtacttgcgctcag-3'
α-thalassemia mental retardation syndrome	*Atrx*	5'-agccttccttcttgggtatgg-3'	5'-acacagagtacttgcgctcag-3'

Table 2: Primers utilized for gene expression (see text for details).

2.9. *Bisulfite Pyrosequencing DNA methylation analysis*

Total genomic DNA was prepared from snap-frozen frontal cortex, hippocampal and cerebellum tissue using the DNeasy mini kit protocol (Qiagen, Valencia, CA, USA). DNA purity (A280/260) and concentration (ng/ul) was evaluated using 2µl nanodrop with the Take3 micro-volume plate on an Epoch Microplate Spectrophotometer (Biotek, Winooski, VT., USA). Based on previous published methods (Delaney et al., 2015; Tost and

Gut, 2007), 20 ng of template gDNA from each sample was bisulfite-converted using the EpiTect Fast DNA Bisulfite Kit (Cat#: 59824, Qiagen N.V, Venlo, The Netherlands), to convert unmethylated cytosine residues to uracil residues in single-stranded DNA while methylated cytosines remained unmodified. Custom assays covering the Atrx promoter were designed using the PyroMark Assay Design software (v2.0; Qiagen N.V, Venlo, The Netherlands) and validated to amplify single PCR products (Atrx = 400nt) using the PyroMark PCR Kit (Cat#: 978703, Qiagen N.V, Venlo, Netherlands) with the primer sequences listed in Table 3 (Integrated DNA Technologies, Inc. (IDT), Coralville, Iowa, USA). PCR conditions for each assay were as follows: 95°C, 15 minutes; (94°C, 30 sec; 56°C, 30 sec; 72°C, 30 sec) × 50 cycles; and 72°C, 10 min, during which the 5-methylcytosines are amplified as cytosines and the uracils are amplified as thymines. The PCR products were then purified using the QIAquick PCR Purification Kit (Cat#: 28106, Qiagen N.V, Venlo, Netherlands), to remove any residue of the PCR reaction that might interfere with the outcome of the sequencing results. Analysis of gene promoter-specific DNA-methylation was performed using the PyroMark Q24 Advanced pyrosequencer (Qiagen N.V, Venlo, Netherlands). Each kit used according to manufacture's guidelines.

Gene	Forward Primer (5' - 3')	Reverse Primer (3' - 5')	Sequencing Primers
Atrx	TTGGTGTTTTTTTTTTTGTTGATGAG	(Biotin)TCTCCTCCTCCCTAAACAAAAT	TTGTTGATGAGGTGG

Table 3: Primers utilized for bisulfite PCR and pyrosequencing (see text for details).

2.10. *Statistical Analysis*

Unless otherwise stated within the text, statistical analyses were performed using analysis

of variance (ANOVA) for repeated measures with Tukey's Honestly Significant Difference (HSD) *post-hoc* tests. The statistical package for the social sciences (SPSS Inc., USA) software was used for data analysis. In general, data are expressed as mean ± SEM (standard error of mean) within each figure.

CHAPTER 3: RESULTS

Previous literature indicates that maternal care and early life experiences can significantly alter gene expression and physiology in offspring (Weaver et al., 2017). Offspring exposed to high maternal anxiety and low maternal care show decreased expression of the Atrx gene, which regulates chromatin remodelling during neurogenesis and cell differentiation in the developing hippocampus (Berube et al., 2005). Proper development of the hippocampus is essential for the performance of tasks involving visual spatial organization, learning or memory and disruption of the ventral hippocampal–cortical connections has been shown to be a factor in the development of mental illnesses, specifically schizophrenia and anxiety (Sigurdsson et al., 2009). In this study, we have employed a Cre recombinase technology to conditionally delete the Atrx gene from developing mice pups, and then examine maternal behaviour of stressed and non-stressed mothers toward these offspring. In adulthood, these $Atrx^{HET}$ and $Atrx^{WT}$ animals were then assessed on their cognitive, social, and emotional behaviour, and tissue from the hippocampus, prefrontal cortex and cerebellum were assessed for *Atrx* mRNA transcript expression and methylation at *Atrx* promoter sites.

3.1. *Genotyping of Cre and Atrx transgenic offspring*

The specific genetic identification of genetically engineered animals in a litter is critical to research. We confirmed the genotype of offspring from all five breading paradigms (Figure 8). Importantly, gel electrophoresis of DNA isolated from heterozygous $Atrx^{Emx1Cre}$ males demonstrated that *Cre* recombinase under the control of *Forkhead Box G1* was effective in deleting exon 18 (compare lanes 7, 8 with lanes 9 , 10 in Figure 8).

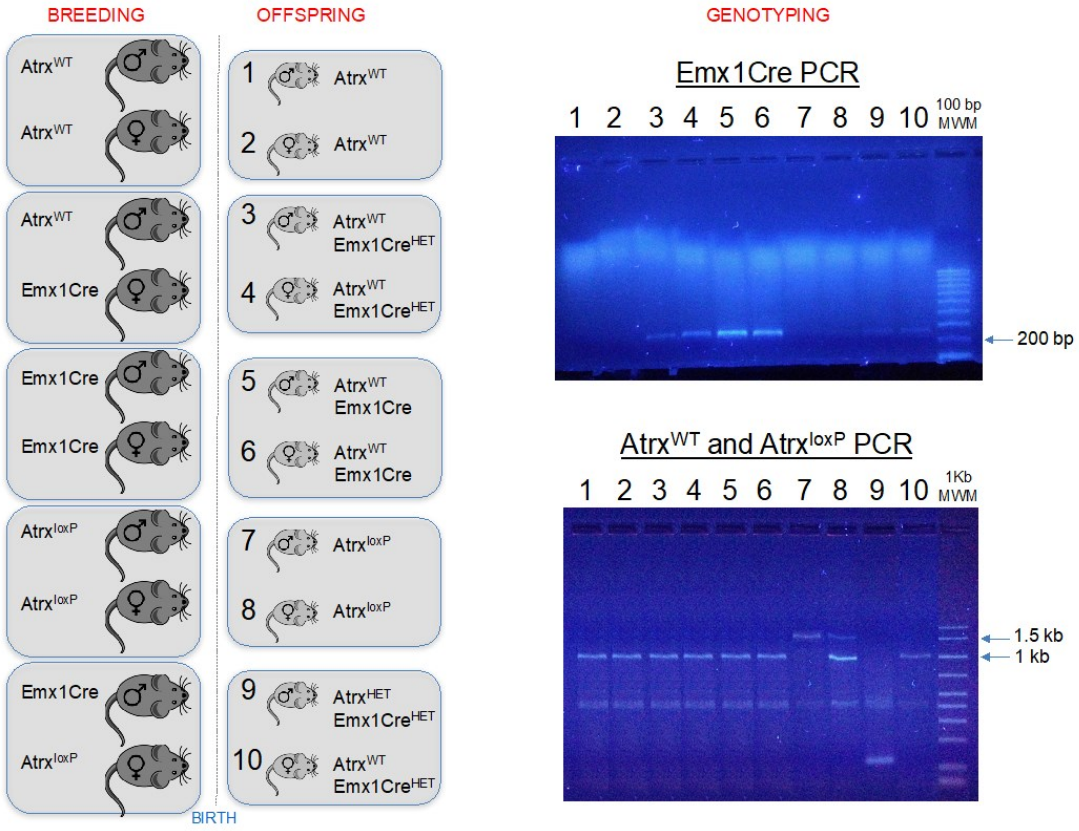


Figure 8: Breeding strategy to generate *Atrx* transgenic mice and gel electrophoresis of male and female *Emx1Cre* (*Emx1Cre*^{HET}) and *Atrx* alleles (*Atrx*^{WT}, *Atrx*^{loxP} and *Atrx*^{HET}) amplified by PCR.

3.2. *Effects of Atrx*^{HET} *Genotype in Offspring on Maternal Behaviour*

Maternal nurturing behaviour (Weaver et al., 2004b) and the context (Connors et al., 2015) of the early rearing environment have profound influences on postnatal development in the offspring. To examine these interactions, Emx1Cre males were mated with Atrx^{loxP} females and then raised their offspring in standard housing and the mother-infant interactions were monitored during the first week of postnatal life (see section 2.5 for details). Dams raising female *Atrx*^{WT} and male *Atrx*^{HET} pups behaved differently toward offspring relative to females raising male and female *Atrx*^{WT} pups (Figure 9).

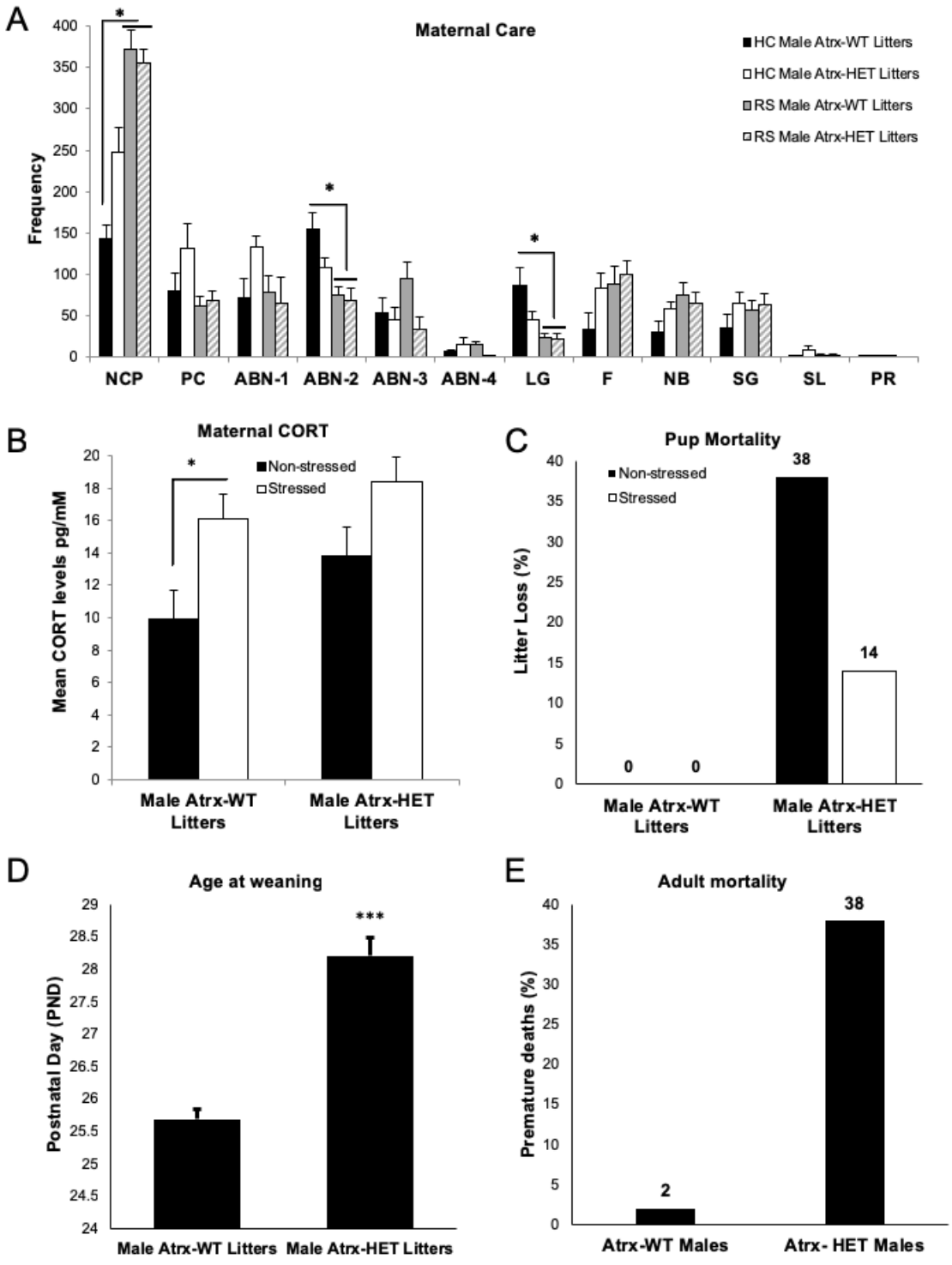


Figure 9: *Atrx* haploinsufficiency in pups and gestational stress treatment alters maternal behaviour towards offspring, maternal CORT levels and proportion of litters that did not survive to weaning age. Mean ± S.E.M. frequency: A) maternal behaviours (*including No Contact with Pups, Passive Contact, Arched-Back Nursing postures 1-4, Licking and Grooming, Feeding, Nest Building,*

Self-Grooming, Split Litter and Pup Retrieval), B) maternal fecal CORT (n=4/group; *p< 0.05). Genotype increases age at weaning and incidences of incidences of mortality in adulthood. Mean ± S.E.M. frequency: D) age at weaning.

3.2.1. Time spent not in contact with offspring

A three-way ANOVA revealed a statistically significant main effect of gestational stress treatment on time spent away from pups [F(1, 27) = 16.571, p = .0001]. Multiple comparisons revealed that non-stressed mothers of *Atrx*^{HET} litters did not differ significantly from mothers of *Atrx*^{WT} litters in terms of time away from pups; however, stressed mothers of both genotypes spent significantly more time away compared to non-stressed mothers of *Atrx*^{WT} pups ($p > 0.05$) (Figure 9A).

3.2.2. Maternal licking and grooming of offspring

A three-way ANOVA revealed a statistically significant main effect of treatment on time spent licking and grooming pups [F(1, 27) = 9.214 p = .005]. As with time away from pups, non-stressed mothers of *Atrx*^{HET} and *Atrx*^{WT} litters did not significantly differ in the amount of time spent engaging in licking and grooming behaviours, but stressed mothers of *Atrx*^{HET} and *Atrx*^{WT} pups both spent significantly less time grooming their litters than non-stressed mothers of *Atrx*^{WT} litters ($p < 0.05$) (Figure 9A).

3.2.3. Maternal low-level arched-back nursing of offspring

A three-way ANOVA revealed a statistically significant main effect of treatment on time spent in ABN-2 nursing posture [F(1, 27) =14.885, p = .001]. Non-stressed mothers of *Atrx*^{HET} and *Atrx*^{WT} litters did not differ in the amount of time spent ABN-2 nursing, but stressed mothers of *Atrx*^{HET} and *Atrx*^{WT} pups both spent significantly less time in the ABN-2 nursing posture non-stressed mothers of *Atrx*^{WT} litters ($p < 0.05$) (Figure 9A).

3.2.4. Effects of *Atrx*^{HET} genotype and stress treatments on maternal CORT

A three-way ANOVA revealed a statistically significant main effect of both pup genotype [$F(1, 18) = 5.365$, $p = .033$] and of treatment [$F(1, 18) = 16.210$, $p = .001$] on maternal fecal CORT levels during the third trimester (GD 13 - 20) (Figure 9B). Mothers pregnant with *Atrx*^{WT} litters showed a significant increase in maternal fecal CORT as a result of gestational stress ($p < 0.05$), while mothers pregnant with *Atrx*^{HET} litters did not. Additionally, mothers caring for *Atrx*^{HET} male litters (both stressed and non-stressed) showed a much higher rate of litter mortality (38% and 14% for *Atrx*^{HET} non-stressed and stressed litters, versus 0% litter loss in *Atrx*^{WT}-only litters) (Figure 9C). The Fisher's exact test, however, was not significant (2-tailed, $p > 0.05$). Litters containing *Atrx*^{HET} males also took significantly longer to reach a viable weight (> 10.0 g) at which they could be weaned and housed separately ($N=65$; $p < 0.0001$) (Figure 9D). In adulthood, *Atrx*^{HET} males experienced a much higher rate of adult mortality (Figure 9E) as a result of injuries sustained from fighting with male cage-mates, self-mutilation, and tissue necrosis (*Atrx*^{HET} male deaths were 38; *Atrx*^{WT} male deaths were 2). The Fisher's exact test, however, was not significant (2-tailed, $p > 0.05$).

3.3. *Effects of Atrx*^{HET} *Genotype and Gestational Stress on Emotional Behaviour*

During the open field test, time spent in the inner field was not significantly influenced by genotype, sex or prenatal stress treatment. A three-way ANOVA revealed a statistically significant main effect of genotype [$F(1,89) = 13.280$, $p = .0001$], and a two-way interaction between treatment and genotype [$F(1,89) = 9.524$, $p = .003$] on locomotor activity (Figure 10A).

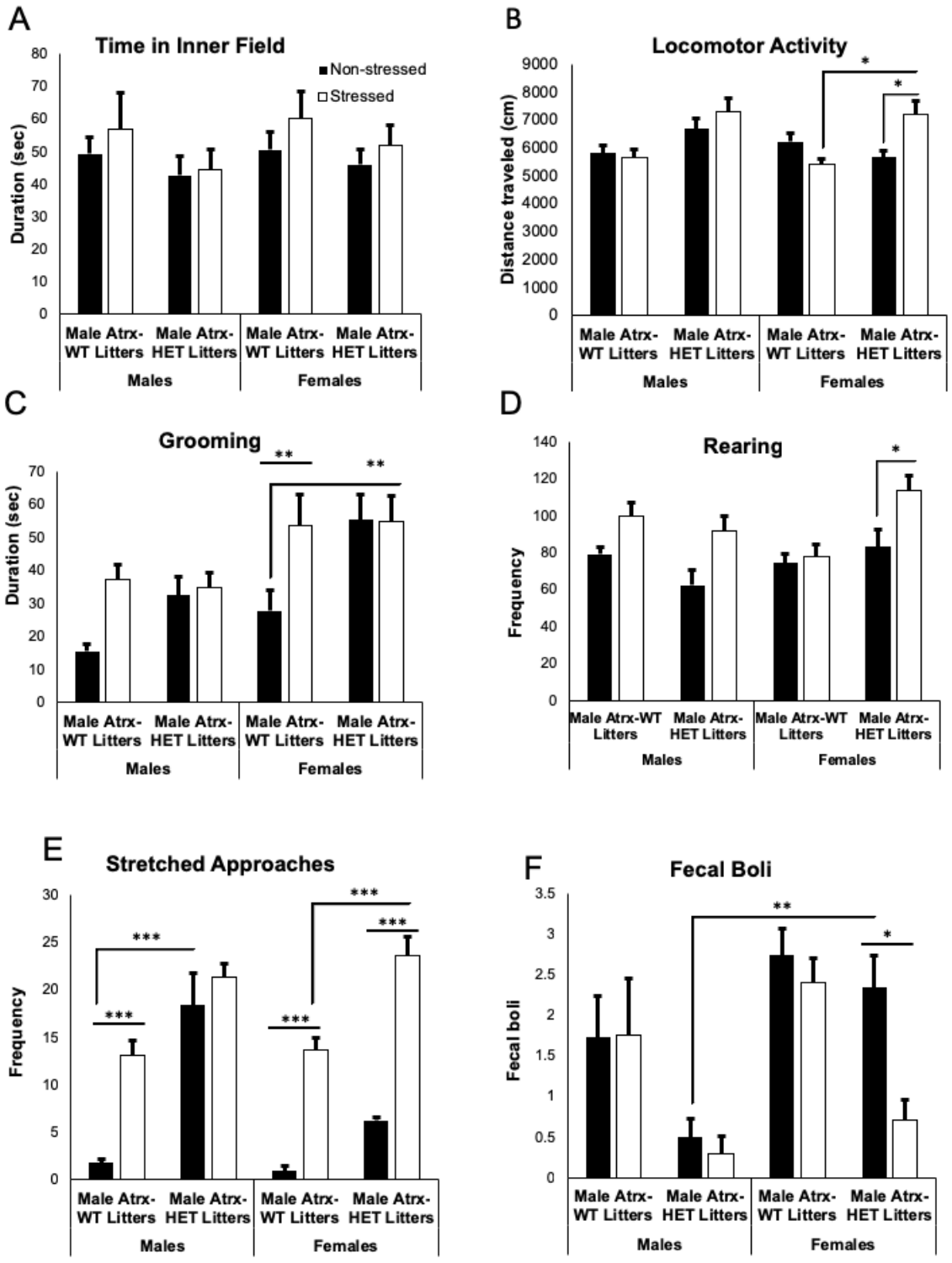


Figure 10: Genotype and stress treatment influenced the expression of anxiety-like behaviours. Mean ± S.E.M. frequency of A) time in inner field, B) locomotor activity, D) rearing, E) stretched approaches, and F) production of fecal boli during the Open Field Test. Mean ± S.E.M. duration of

C) grooming behaviours during Open Field testing. (N=8/group; *p< 0.05; **p< 0.001, ***p< 0.0001).

Females reared in litters containing *Atrx*^{HET} males showed significant increases in locomotor activity related to gestational stress: females from gestationally stressed *Atrx*^{HET}-containing litters were significantly more active in the open field than females from non-stressed *Atrx*^{HET} litters ($p = 0.043$), or than their counterparts from gestationally-stressed *Atrx*^{WT}-only litters ($p = 0.033$) (Figure 10B).

A three-way ANOVA revealed significant main effects of treatment [$F(1,89) = 13.549$, $p = .0001$], sex [$F(1,89) = 17.461$, $p = .0001$], and of genotype [$F(1,89) = 4.292$, $p = .041$] on the amount of time spent grooming (Figure 10C). Additionally, there was also a two-way interaction between treatment and genotype [$F(1,89) = 10.539$, $p = .002$]. Males of either genotype did not show differences in grooming behaviours as a result of gestational stress. Females from *Atrx*^{HET} and *Atrx*^{WT} litters, however, showed differential outcomes related to gestational stress. Gestationally stressed females from *Atrx*^{WT} litters, as well as both stressed and non-stressed females from *Atrx*^{HET} litters, exhibited significant increases in time spent self-grooming when compared to non-stressed females from *Atrx*^{WT} litters ($p = 0.002$, 0.007, and 0.004, respectively).

Females also showed unique outcomes related to litter genotype and gestational stress in terms of rearing behaviours: a three-way ANOVA revealed a statistically significant main effect of treatment [$F(1,89) = 24.152$, $p = .0001$] and a two-way interaction effect of sex and genotype [$F(1,89) = 6.426$, $p = .013$] on the frequency of rearing (Figure 10D). Gestationally stressed females from *Atrx*^{HET} litters showed significantly higher frequency of rearing behaviours than non-stressed females from *Atrx*^{HET} litters ($p = 0.01$).

A three-way ANOVA revealed a significant main effect of sex [$F(1, 89) = 6.115$, $p = .015$)], and genotype [$F(1,89) = 82.978$, $p = .0001$] on the number of stretched approach postures exhibited in the open field. Additionally, there was a significant two-way interaction between treatment and sex [$F(1, 89) = 13.976$, $p = .0001$], and sex and genotype [$F(1, 89) = 6.862$, $p = .010$], as well as a three-way interaction between treatment, sex and genotype, $F(1, 89) = 8.555$, $p = 0.004$]. Non-stressed *Atrx*^{HET} males a exhibited significantly increased number of stretched-approach postures compared to non-stressed *Atrx*^{WT} males ($p = 0.0001$), as did gestationally stressed *Atrx*^{WT} males ($p = 0.0001$). Stressed and non-stressed *Atrx*^{HET} males did not differ significantly in terms of stretched approaches (Figure 10E). Females from both *Atrx*^{HET} and *Atrx*^{WT} litter genotypes showed significant increases related to gestational stress ($p = 0.0001$, $p = 0.0001$). Stressed females from *Atrx*^{HET} litters also exhibited significantly higher frequency of stretched approaches than stressed females from *Atrx*^{WT} litters ($p = 0.001$).

The number of fecal boli produced over the course of the ten-minute open field test was also observed (Figure 10F). A three-way ANOVA revealed a significant main effect of sex [$F(1, 89) = 13.799$, $p = .0001$] and genotype [$F(1, 89) = 20.502$, $p = .0001$] on the number of fecal boli produced during the open field test. Non-stressed and stressed females from *Atrx*^{WT} litters, as well as non-stressed females from *Atrx*^{HET} litters, produced a significantly higher number of fecal boli than non-stressed *Atrx*^{HET}males ($p = 0.0001$, 0.005, and 0.001, respecitively). Stressed females from *Atrx*^{HET} litters also produced significantly fewer fecal boli than non-stressed *Atrx*^{HET} females ($p = 0.04$).

3.4. *Effects of Atrx^{HET} Genotype and Gestational Stress on Cognitive Behaviour*

When examining performance the Morris water maze, a three-way ANOVA revealed significant main effects of genotype [$F(1, 2159) = 214.116$, $p = .0001$] and sex [$F(1, 2159) = 5.138$, $p = .024$], as well as a two-way interactions between genotype and sex [$F(1, 2159) = 6.366$, $p = .012$] and genotype and treatment [$F(1, 2159) = 11.955$, $p = .001$] on animals' average latency to reach the platform (Figure 11A). Non-stressed males and females from *Atrx*^{HET}-containing litters both took significantly longer to reach the platform than their non-stressed *Atrx*^{WT} counterparts ($p < 0.0001$). Only females from *Atrx*^{HET}-containing litters appeared to show gestational stress-related increases in latency, with stressed females from *Atrx*^{HET} litters showing significantly longer latency to reach the platform than their non-stressed peers ($p = 0.05$).

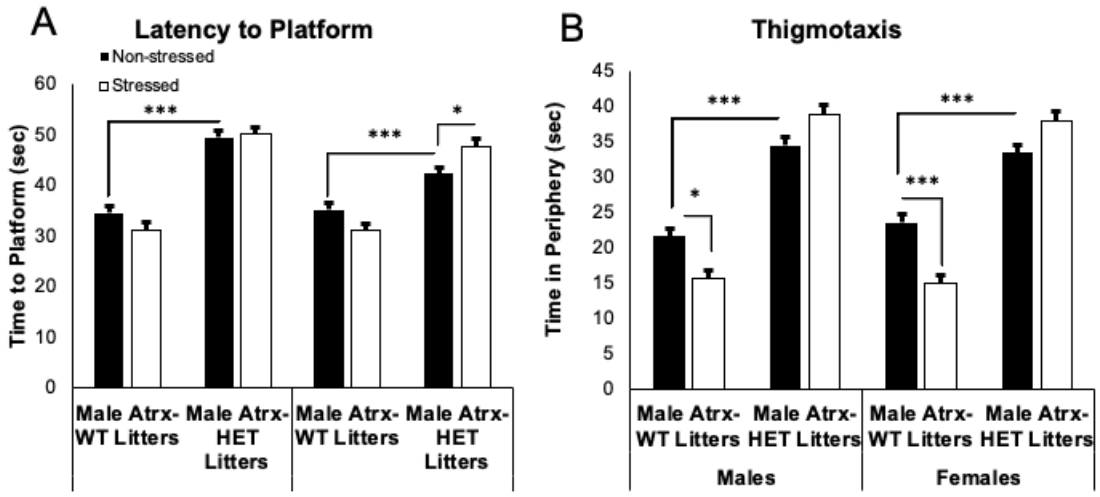


Figure 11: Genotype and stress treatment influenced cognitive behaviours. Mean ± S.E.M duration of A) time to reach reach platform and B) time spent in periphery during the Morris Water Maze task. (N=8/group; *p< 0.05; ***p< 0.0001).

When examining thigmotaxis (Figure 11B), or time spent swimming around the periphery of the pool (ie: not actively searching for the platform), a three-way ANOVA

revealed a significant main effect of genotype [$F(1, 2159) = 377.757$, $p = .0001$], and a two-way interaction between genotype and treatment [$F(1, 2159) = 44.373$, $p = .0001$]. Non-stressed males and females from *Atrx*^{HET} litters both showed significant increases in thigmotaxis when compared to their non-stressed counterparts from *Atrx*^{WT} litters ($p < 0.0001$). *Atrx*^{HET} litter animals did not appear to be sensitive to the effects of gestational stress in terms of thigmotaxis, as they did not show significant differences in time spent in the periphery of the arena. Gestationally stressed males and females from *Atrx*^{WT}-only litters, however, showed significantly *decreased* thigmotaxis compared to their non-stressed counterparts ($p = 0.039$ and 0.0001 respectively). See Figure 12A-C and Figure 13Figure 13A-C for male and female performance, respectively.

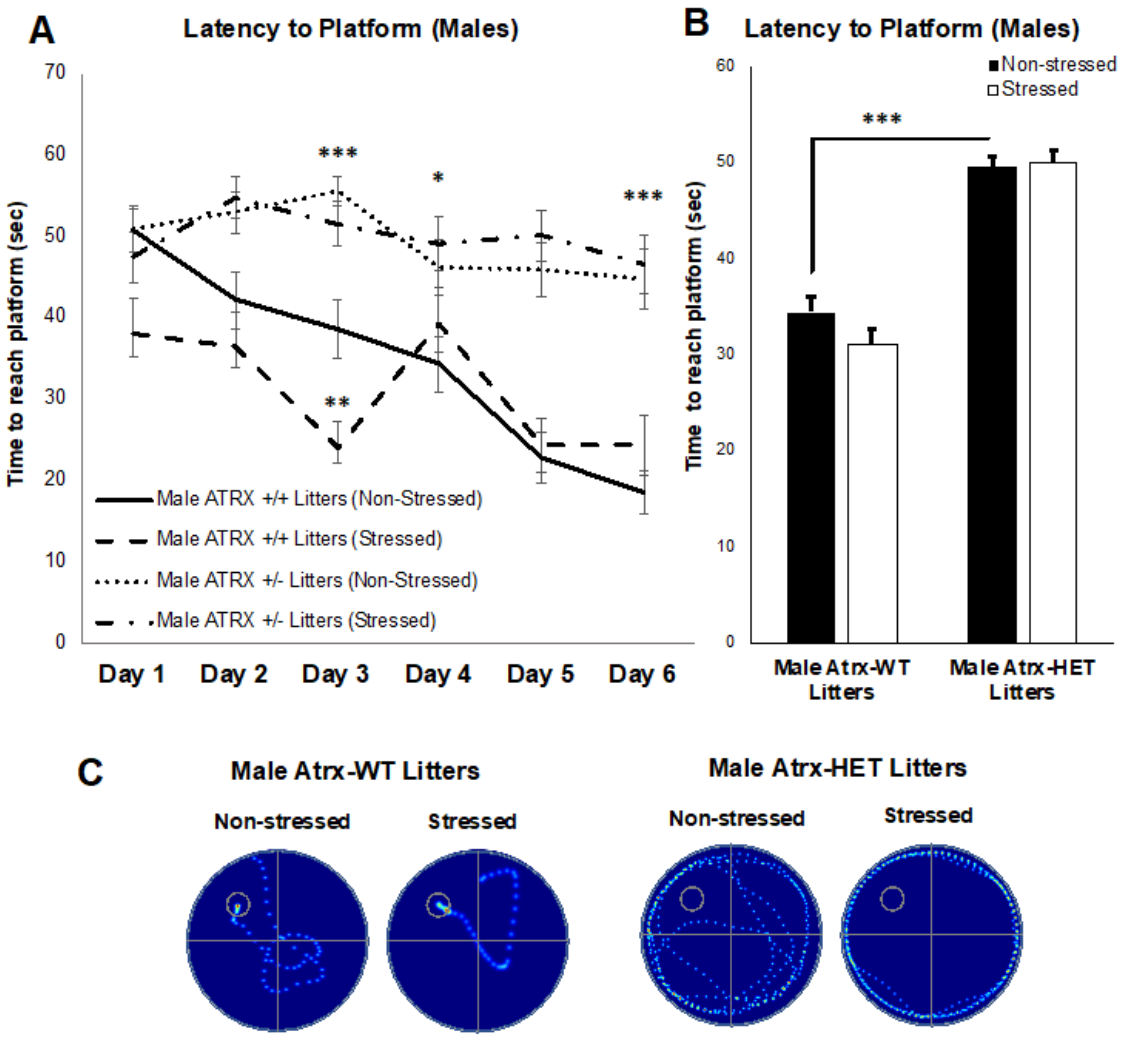


Figure 12: Genotype and stress treatment influenced cognitive behaviour in *Atrx*-WT and *Atrx*-HET males. Mean ± S.E.M duration of time to reach reach platform A) per day of testing and B) over all days of testing on the Morris Water Maze task. (N=8/group; *p< 0.05; ***p< 0.0001.) Raw heatmap data C) swim patterns from the final day of testing.

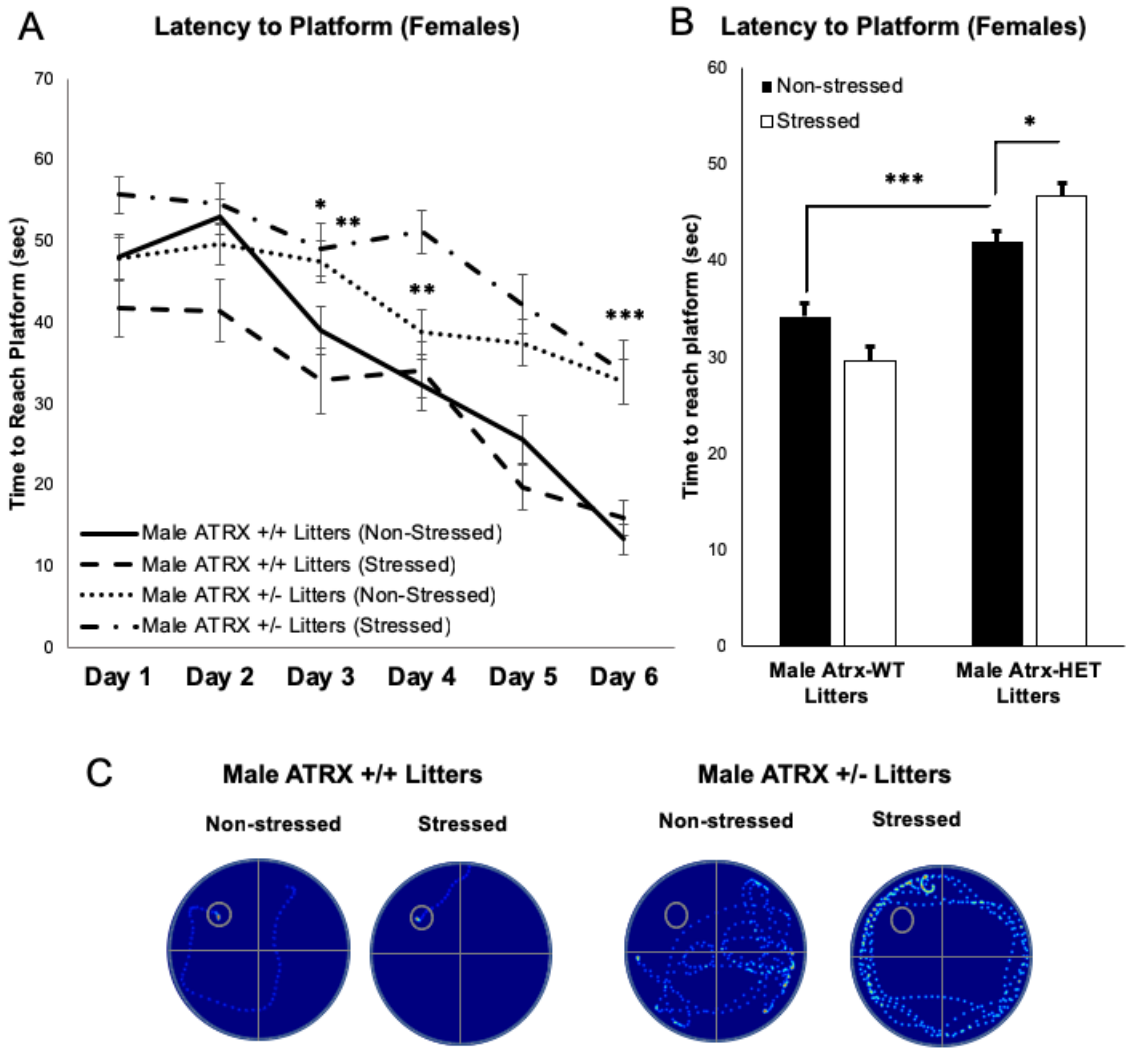


Figure 13: Genotype and stress treatment influenced cognitive behaviour in females raised with *Atrx*-WT and *Atrx*-HET males. Mean ± S.E.M duration of time to reach reach platform A) per day of testing and B) over all days of testing on the Morris Water Maze task. (N=10/group; *p< 0.05; ***p< 0.0001.) Raw heatmap data C) swim patterns from the final day of testing.

Finally, we examined time spent in the target quadrant during the probe trial of the Morris water maze, in which a three-way ANOVA revealed a significant main effect of genotype [$F(1, 81) = 11.669$, $p = .001$], and a two-way interaction between genotype and sex [$F(1, 81) = 18.119$, $p = .0001$] on time spent searching in the target quadrant. Both gestationally non-stressed and stressed $Atrx^{HET}$ males spent significantly less time in the target quadrant than their male $Atrx^{WT}$ counterparts ($p = 0.013$). Females did not show

significant differences in time spent in the target quadrant related to either litter genotype or gestational stress (Figure 14).

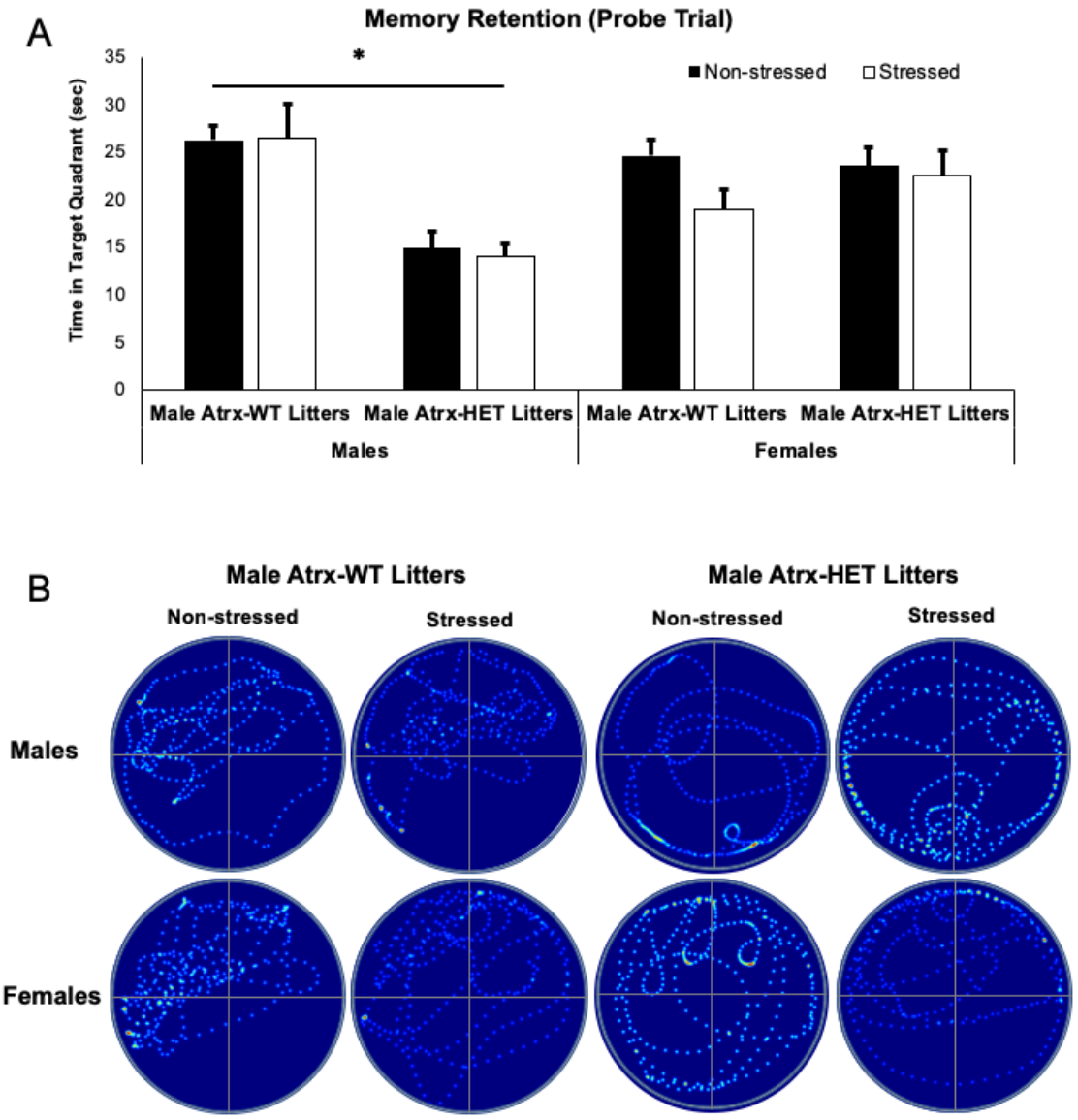


Figure 14: Genotype and gestational stress treatment influenced cognitive behaviour. Mean ± S.E.M duration of A) time spent in platform target quadrant during probe trial of Morris Water Maze task (N=8/group; *p< 0.05). Raw heatmap data C) swim patterns from probe trial (target quadrant in top left).

3.5. *Effects of Atrx^{HET} Genotype and Gestational Stress on Social Behaviour*

In the social investigation task, social affinity was quantified by the amount of time that a mouse spent sniffing the stimulus mouse (an *Atrx*^{WT} mouse of the opposite

sex). A three-way ANOVA revealed a significant main effect of genotype [F(1, 82) = 4.551, p = .036] and sex [F(1, 82) = 22.073, p = .0001], as well as a significant two-way interaction between genotype and sex [F(1, 82) = 21.327, p = .0001] on social affinity (Figure 15A).

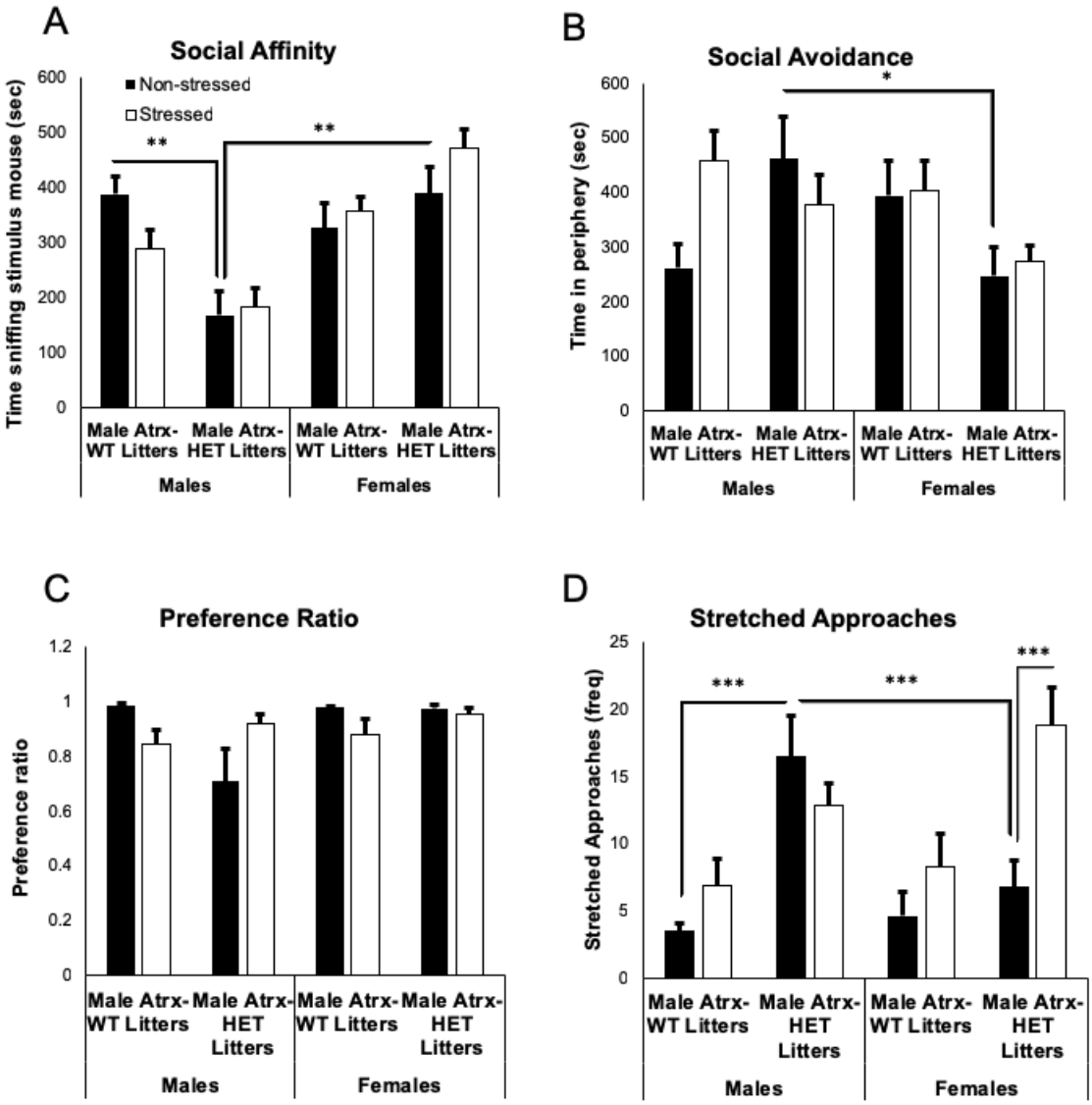


Figure 15: Genotype, sex and gestational stress treatment influenced frequency and duration of social behaviours. Mean ± S.E.M. duration of A) time spent sniffing social stimulus mouse, B) time spent in the periphery of the testing arena, and C) ratio of time spent sniffing spent sniffing social stimulus mouse over time spent sniffing from periphery during the Social Investigation Task. Mean ± S.E.M. frequency of D) stretched approaches during Social investigation task (N=8/group; *p< 0.05; **p< 0.001, ***p< 0.0001).

Non-stressed *Atrx*^{HET} male mice spent significantly less time investigating the stimulus mouse than non-stressed *Atrx*^{WT} males ($p = 0.003$), as well as significantly less time than non-stressed females from *Atrx*^{HET}-containing litters ($p = 0.001$). There were no gestational stress-related differences in social affinity.

Social avoidance (Figure 15B) was measured in terms of time spent in the periphery of the arena during the social investigation task (ie: as far away from the stimulus mouse as possible). A three-way ANOVA revealed a significant two-way interaction between genotype and sex [$F(1, 83) = 9.700$, $p = .003$] on social avoidance. Non-stressed *Atrx*^{HET} male mice spent significantly more time in the periphery of the arena than non-stressed females from *Atrx*^{HET}-containing litters ($p = 0.013$). Additionally, an animals' "preference ratio" (Figure 15C) was determined by calculating the amount of time the animal spent sniffing spent sniffing the stimulus mouse over time spent sniffing at a distance (from the periphery of the arena) during the Social Investigation Task. No significant differences in preference ratio were found related to sex, litter genotype, or gestational sex treatment.

The number of stretched approaches (Figure 15D) exhibited during the social investigation task was also examined, and a three-way ANOVA revealed significant main effects of genotype [$F(1, 83) = 52.936$, $p = .0001$] and treatment [$F(1, 83) = 12.462$, $p = .001$], as well as two-way interactions between sex and treatment [$F(1, 83) = 14.337$, .0001] and genotype and sex [$F(1, 83) = 5.735$, $p = .019$]. Additionally, a three-way interaction between genotype, sex and treatment [$F(1, 83) = 10.506$, $p = .002$] on the number of stretched approaches was found. Non-stressed *Atrx*^{HET} males showed a significantly higher frequency of stretched approach postures compared to non-stressed *Atrx*^{WT} males ($p < 0.0001$) and non-stressed females from *Atrx*^{HET} litters ($p < 0.0001$). Only females

from *Atrx*^{HET} litters showed gestational stress-related changes in the frequency of their stretched approach behaviour, with females from gestationally stressed *Atrx*^{HET} litters showing a significantly higher incidence of stretched approaches compared to females from non-stressed *Atrx*^{HET} litters ($p < 0.0001$)

3.6. *Effects of Atrx^{HET} Genotype on Brain Weight*

When examining the effects of genotype and gestational stress on whole brain weight, a three-way ANOVA revealed a significant main effect of genotype [$F(3, 35) = 36.477$, $p = 0.0001$] and a significant interaction effect of genotype and sex [$F(1, 35) = 49.107$, $p = 0.0001$] (Figure 16A). *Atrx*^{HET} males consistently exhibited significantly lower brain weights than all other genotypes ($p \leq 0.002$). When comparing weights of the frontal cortex, hippocampus, and cerebellum, no significant differences were found ($p \geq 0.51$) (compare Figure 16B-D).

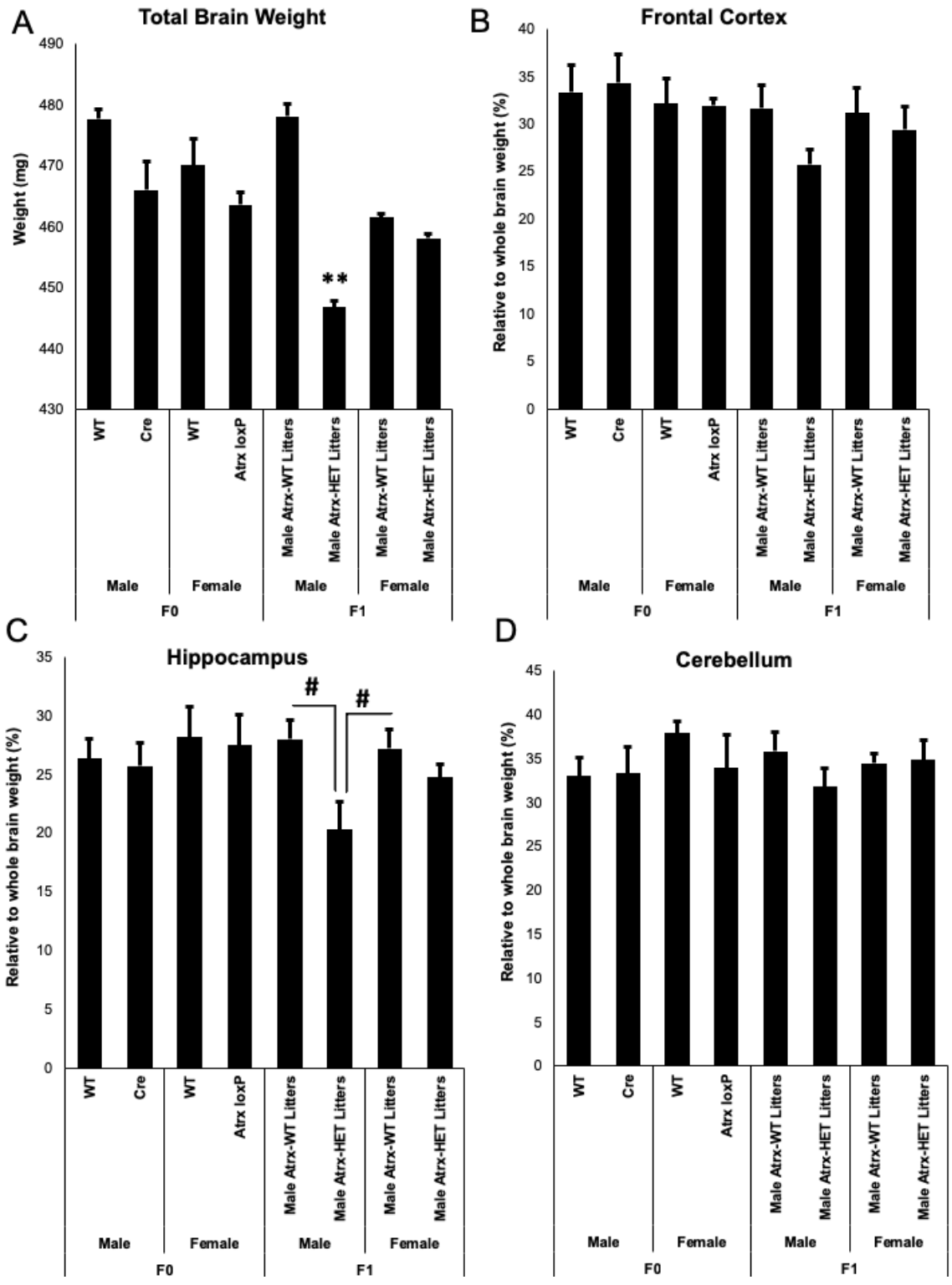
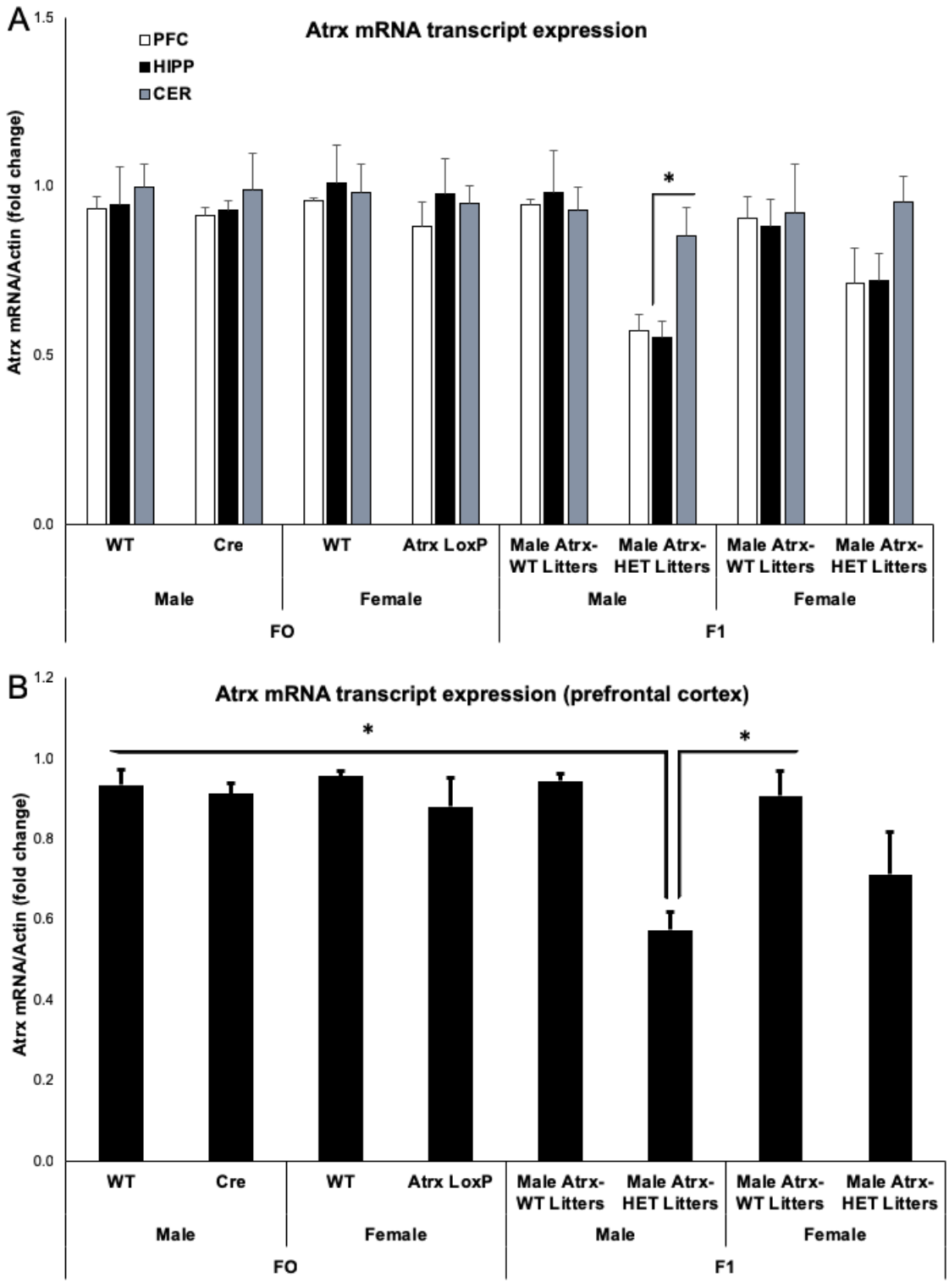


Figure 16: Genotype influenced overall brain weight, but not frontal cortex, hippocampal or cerebellar weights. Non-significant differences were observed in hippocampas weight ($p \leq 0.52$). Mean ± S.E.M. weight of A) whole brain, B) frontal cortex, C) hippocampus, D) cerebellum. (N=4/group; **$p < 0.001$; # $p \leq 0.52$).

3.7. *Effects of Atrx^{HET} Genotype on Atrx expression*

A three-way ANOVA revealed significant main effects of genotype on *Atrx* mRNA transcript expression in the prefrontal cortex [$F(1, 7) = 9.381$, $p = 0.0001$] and hippocampus [$F(1, 7) = 3.566$, $p = .029$], but not in the cerebellum. *Atrx*^{HET} males showed significantly decreased expression of *Atrx* mRNA transcripts compared to all other groups ($p \leq 0.011$) except for females from *Atrx*^{HET} litters. *Atrx*^{HET} males also showed significantly decreased *Atrx* mRNA transcript expression in the forebrain an and hippocampus when compared to the cerebellum, a difference which was not present in any other treatment groups (Figure 17A). In the hippocampus, *Atrx*^{HET} males also showed significantly decreased expression of *Atrx* mRNA transcripts compared to their *Atrx*^{WT} male counterparts ($p = 0.049$). No significant differences in *Atrx* mRNA transcript expression were found in the cerebellum (compare Figure 17B-D).


A
Atrx mRNA transcript expression
PFC
HIPP
CER
Atrx mRNA/Actin (fold change)
1.5
1.0
0.5
0.0
*
WT
Cre
WT
Atrx LoxP
Male Atrx-WT Litters
Male Atrx-HET Litters
Male Atrx-WT Litters
Male Atrx-HET Litters
Male
Female
Male
Female
FO
F1
B
Atrx mRNA transcript expression (prefrontal cortex)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
WT
Cre
WT
Atrx LoxP
Male Atrx-WT Litters
Male Atrx-HET Litters
Male Atrx-WT Litters
Male Atrx-HET Litters
Male
Female
Male
Female
FO
F1

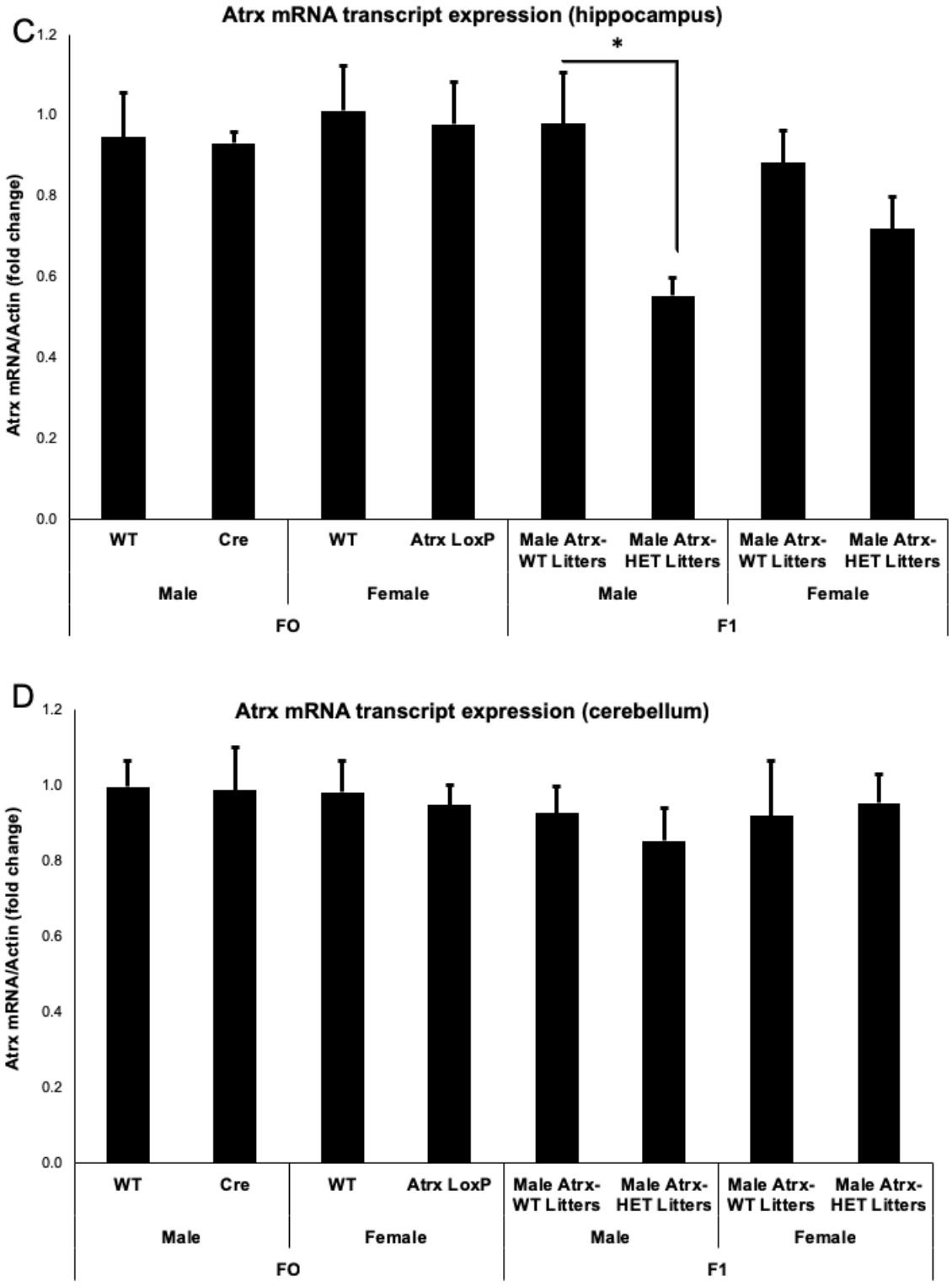
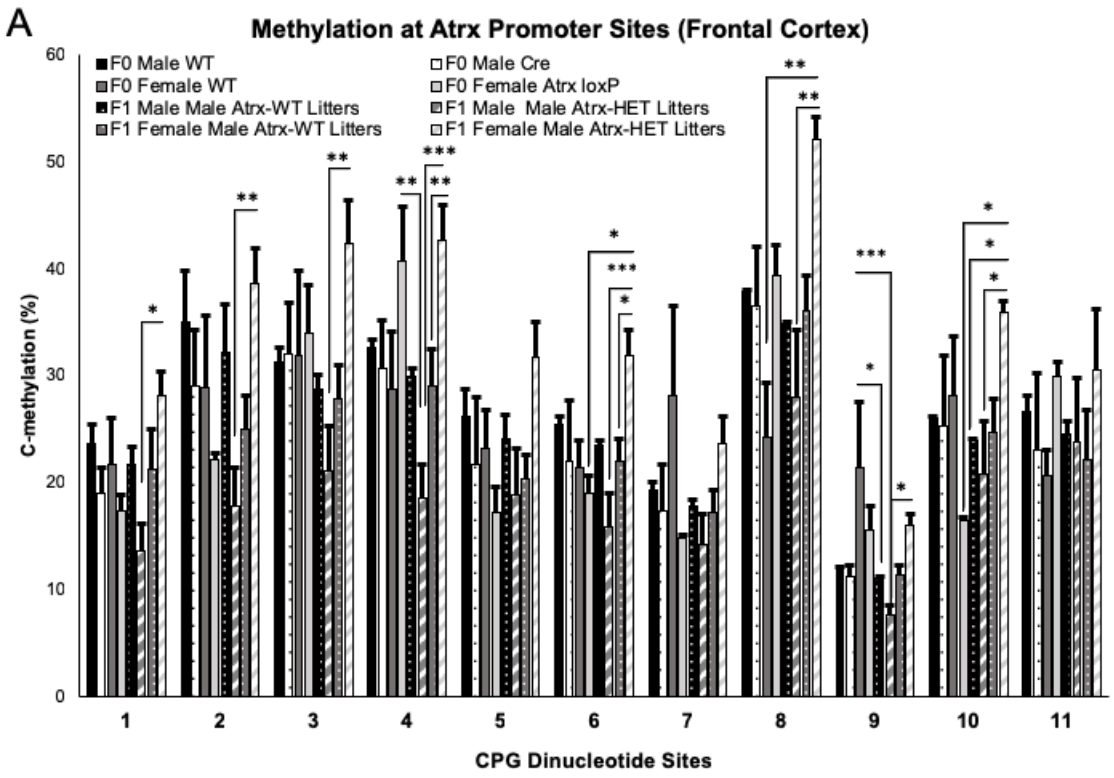
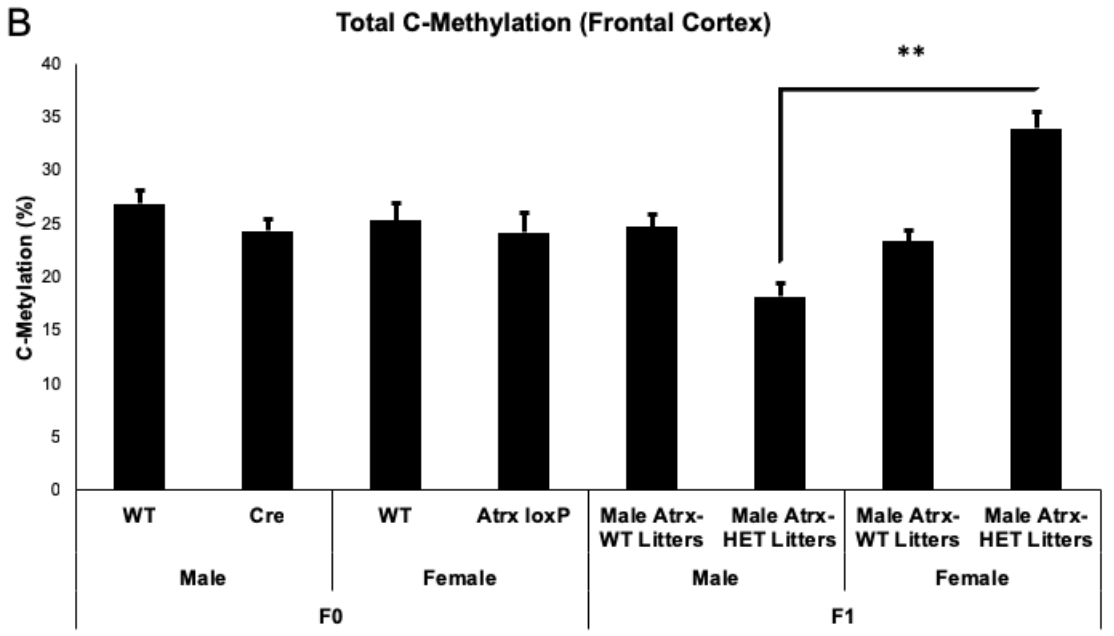


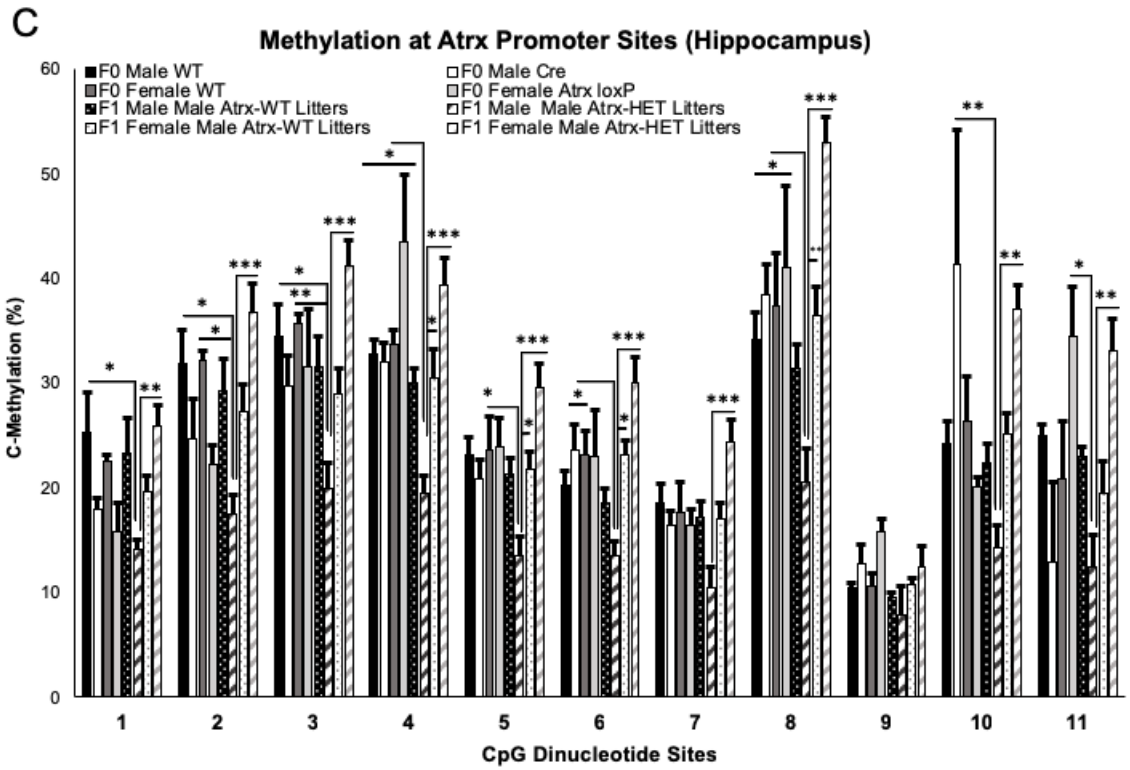
Figure 17: Genotype influenced levels of *Atrx* mRNA transcript expression in the hippocampus and prefrontal cortex. Mean ± S.E.M. Atrx mRNA transcript expression in A) prefrontal cortex, hippocampus and cerebellum, B) prefrontal cortex, C) hippocampus, D) cerebellum. (N=8/group; *p< 0.05).

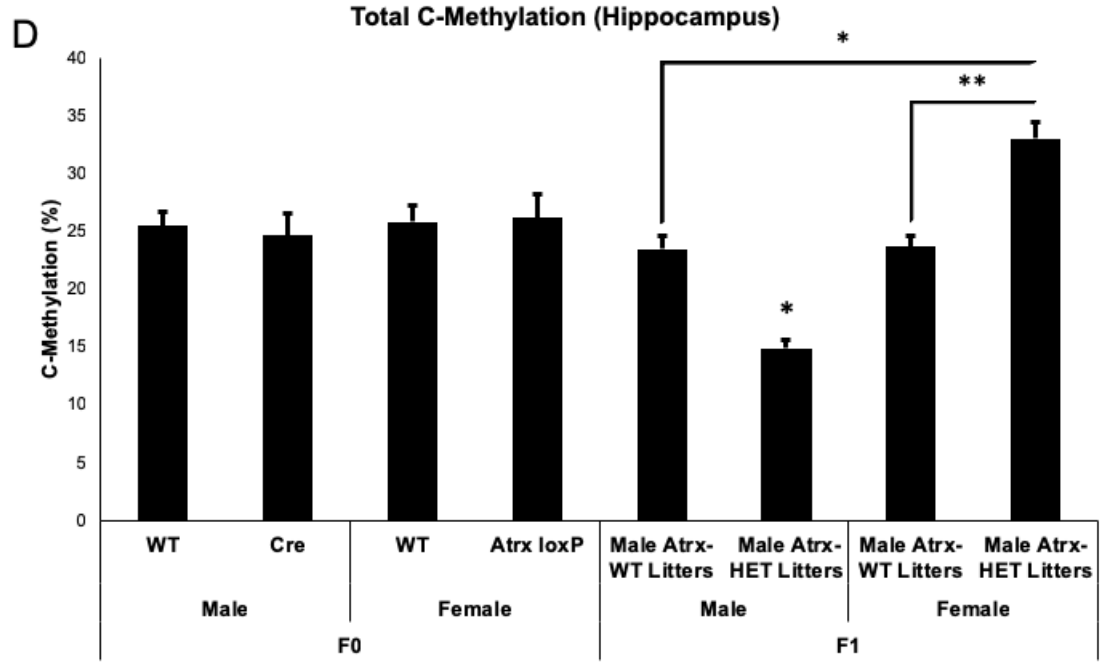
3.8. *Effects of Atrx^{HET} Genotype on Methylation at Atrx Promoter Sites*

A two-way ANOVA revealed a significant interaction between genotype and sex [$F(1, 7) = 5.914$, $p = 0.006$] on the overall level of C-methylation across thirteen *Atrx* promoter sites in the frontal cortex. *Atrx*^{HET} male animals showed significantly decreased levels of overall methylation when compared with females from *Atrx*^{HET} litters ($p = 0.006$) (Figure 18). In the prefrontal cortex and hippocampus (Figure 18A-D), a two-way ANOVA revealed a significant main effect of sex [$F(1, 7) = 15.099$, $p = 0.0001$] on overall C-methylation of *Atrx* promoter sites. *Atrx*^{HET} males showed significantly lower levels of overall methylation when compared with all other genotype groups ($p \leq 0.26$). Females from *Atrx*^{HET} litters also showed significantly higher methylation compared to both males and females from *Atrx*^{WT} litters ($p = 0.039$ and 0.008 respectively). No significant differences in overall C-methylation between groups were observed in the cerebellum (Figure 18E-F).


A
Methylation at Atrx Promoter Sites (Frontal Cortex)
F0 Male WT
F0 Female WT
F1 Male Male Atrx-WT Litters
F1 Female Male Atrx-WT Litters
F0 Male Cre
F0 Female Atrx loxP
F1 Male Male Atrx-HET Litters
F1 Female Male Atrx-HET Litters
C-methylation (%)
CPG Dinucleotide Sites


B
Total C-Methylation (Frontal Cortex)
C-Metylation (%)
WT
Cre
WT
Atrx loxP
Male Atrx-WT Litters
Male Atrx-HET Litters
Male Atrx-WT Litters
Male Atrx-HET Litters
Male
Female
Male
Female
F0
F1


C
Methylation at Atrx Promoter Sites (Hippocampus)
F0 Male WT
F0 Female WT
F1 Male Male Atrx-WT Litters
F1 Female Male Atrx-WT Litters
F0 Male Cre
F0 Female Atrx loxP
F1 Male Male Atrx-HET Litters
F1 Female Male Atrx-HET Litters
C-Methylation (%)
CpG Dinucleotide Sites


D
Total C-Methylation (Hippocampus)
C-Methylation (%)
WT
Cre
WT
Atrx loxP
Male Atrx-WT Litters
Male Atrx-HET Litters
Male Atrx-WT Litters
Male Atrx-HET Litters
Male
Female
Male
Female
F0
F1

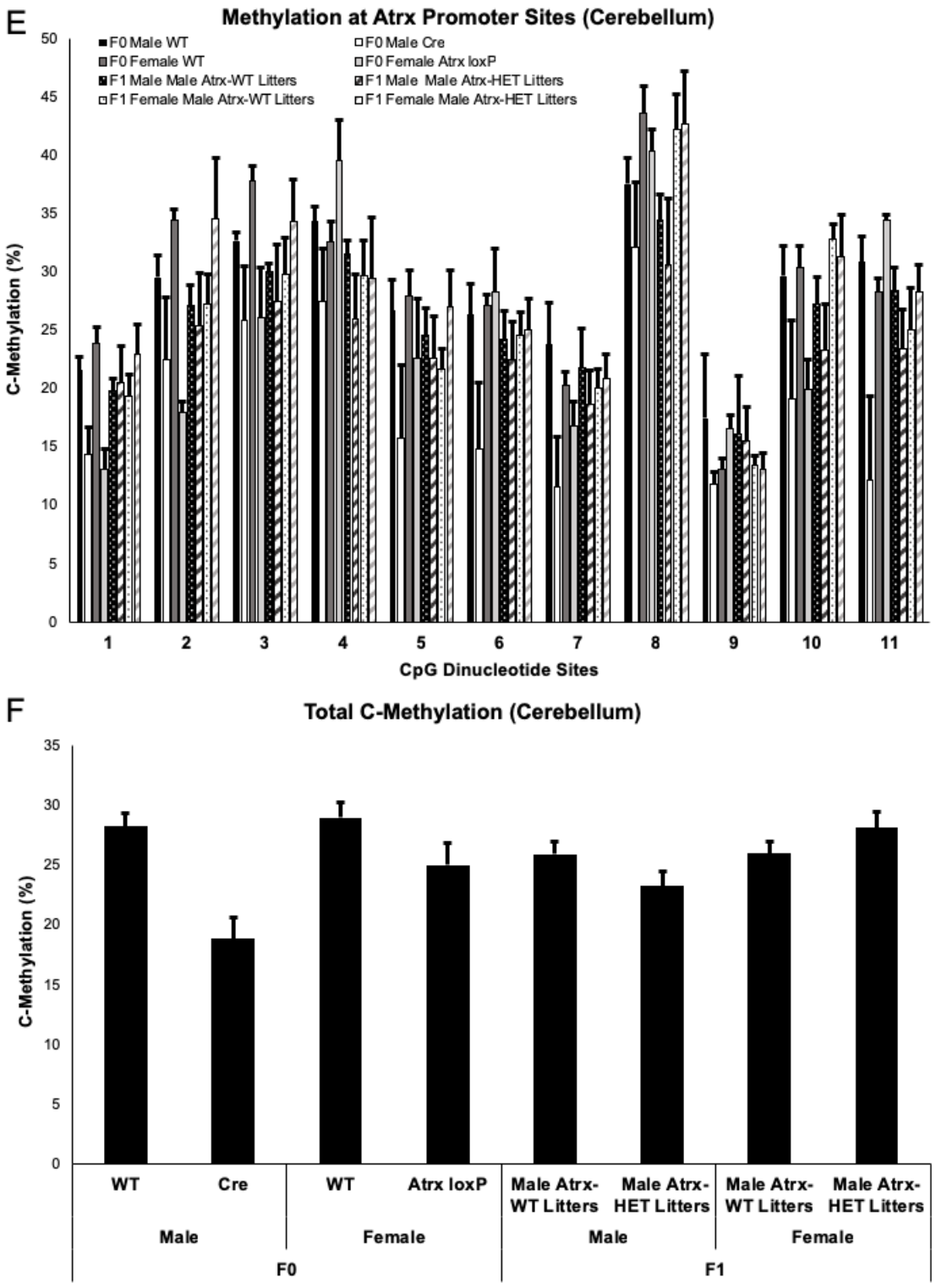


Figure 18: Genotype and sex influenced methylation levels at *Atrx* promoter sites in the frontal cortex and hippocampus. Mean ± S.E.M. methylation levels at 11 CPG dinucleotide sites in the A) frontal cortex, C) hippocampus, E) cerebellum. Mean ± S.E.M. overall methylation rates in the B) frontal cortex, D) hippocampus, F) cerebellum. (N=4/group; *p< 0.05; **p< 0.001, ***p< 0.0001).

CHAPTER 4: DISCUSSION

Early life experience and environment - including the pre- and post-natal environment and the quality of maternal care received - have been shown to have a significant impact on gene expression and physiology in offspring (Weaver et al., 2017). It has been previously shown that offspring who are exposed to high maternal anxiety and receive poor maternal care show significantly reduced expression of the gene *Atrx* in the hippocampal region (Weaver et al., 2017). The *Atrx* gene plays a role in the regulation of chromatin remodeling during early neurogenesis and cell differentiation in the developing embryo, and in the perinatal development of the hippocampal regions (Weaver; Bèrubè et al., 2005). Normal development of the hippocampus is critical for learning and memory, as well as any tasks requiring visuo-spatial organization; additionally, disruption of the ventral hippocampus to the cortex appears to play a role in the development of mental illnesses such as schizophrenia and anxiety, and various developmental delays (Sigurdsson & Duvarci, 2015).

In this study, Cre recombinase technology has been employed to conditionally knock-down the *ATRX* gene in developing mice pups in order to model the genotype of pups that receive low maternal care. The objective of this study was to determine whether the $Atrx^{HET}$ genotype gives rise to a distinctive phenotype in terms of the quality of early maternal care received; emotional, cognitive and social behaviour, both in the $Atrx^{HET}$ males themselves and in their female littermates. Additionally, as both *ATRX* and maternal stress/anxiety have been shown to significantly shape physiological and behavioural outcomes in offspring, our objective was also to investigate whether the $Atrx^{HET}$ pheno-

type was sensitive to maternal gestational stress in a manner different to that of animals from *Atrx*^{WT} litters.

It was found that the presence of the *Atrx*^{HET} genotype in a litter did not influence the quality of maternal care; however, maternal care was influenced by the experience of gestational stress. *Atrx*^{HET} offspring also went on to display a unique cognitive, behavioural and emotional profile in adulthood, as well as some differential outcomes as a result of gestational stress exposure as compared to their *Atrx*^{WT} counterparts. *Atrx*^{HET} males also showed decreased overall brain weights as well as region-specific decreases in the expression of *Atrx* mRNA transcripts and methylation of *Atrx* promoter sites. Given the observed increase in stress reactivity and anxiety-like behaviours in low maternal care offspring, this study has the implication of broadening our understanding of the physiological mediators of early life experiences and establishing a reliable genetic model and genotyping protocol to further investigate ATRX expression concurrently with social, emotional and cognitive behaviours in offspring.

4.1. *Effects of Reduced Atrx Expression and Gestational Stress on Maternal Care*

Female C57BL mice pregnant with *Atrx*^{WT} offspring showed elevated fecal cortisol levels during the third trimester of pregnancy (GD 13 - GD20) as a result of gestational restraint stress. Mice pregnant with *Atrx*^{HET} offspring, however, did not show a difference in fecal CORT expression as a result of gestational stress. Exposure to gestational stress resulted in females tending to *Atrx*^{WT} male-containing litters spending an increased amount of time away from their pups, as well as showing decreases in two key maternal affiliative behaviours: low-level arched-back nursing (ABN-2) and the licking and

grooming of pups. Mothers tending to litters containing *Atrx*^{HET} males did not show significant differences in maternal behaviour as a result of gestational stress.

Additionally, mothers caring for *Atrx*^{HET} male litters (both stressed and non-stressed) showed a much higher rate of litter mortality in terms of litters that did not survive to reach weaning age. Litters containing *Atrx*^{HET} males also took significantly longer to reach a viable weight at which they could be weaned and housed separately (> 10.0 g). In adulthood, *Atrx*^{HET} males experienced a much higher rate of adult mortality as a result of injuries sustained from fighting with male cage-mates, self-mutilation, and tissue necrosis.

These findings indicate that the *Atrx*^{HET} genotype shows a differential or decreased sensitivity to gestational stress in terms of the impact on maternal CORT expression and maternal care. Non-stressed mothers of *Atrx*^{HET} litters did not differ significantly terms of maternal care from non-stressed mothers of *Atrx*^{WT} litters or from stressed mothers of *Atrx*^{HET} litters, indicating that the presence of the *Atrx*^{HET} genotype may result in a maternal care phenotype in non-stressed mothers that is similar to the one seen in gestationally stressed mothers of *Atrx*^{WT} litters. As a result, the mothers of *Atrx*^{HET} litters may be less sensitive to the effects of gestational stress.

4.2. *Effects of Reduced Atrx Expression and Gestational Stress on Emotion*

When mice were evaluated on the Open Field test, genotype, sex, or gestational stress did not appear to have an effect on the time spent in either the inner field or the periphery of the arena, which both are traditionally regarded in behavioural studies as indices of anxiety-like behaviour. Additionally, males did not show genotype- or gestational stress-related

differences in terms of overall locomotor activity, or behaviours such as rearing or grooming. Females, however, appeared to be differentially affected by the effects of gestational stress as a result of being reared in litters containing *Atrx*^{HET} males. Gestationally stressed females from *Atrx*^{HET} male-containing litters showed significantly increased locomotor activity in the Open Field when compared to non-stressed females from *Atrx*^{HET} male litters, and were also more active than their stressed counterparts from *Atrx*^{WT} male litters. Females from *Atrx*^{WT} male litters did not show a corresponding gestational stress-related increase in activity.

Females from *Atrx*^{HET} male litters also showed significant gestational stress-related increases in rearing behaviours that were not present in females from *Atrx*^{WT} male litters. These appear to indicate that the presence of the *Atrx*^{HET} genotype within a litter may lead to increased sensitivity to factors in the pre- and post-natal environment, even in sibling animals that do not carry the *Atrx*^{HET} genotype themselves.

Alternately, females raised in *Atrx*^{WT} male litters showed significant increases in time spent grooming in the Open Field as a result of gestational stress. Females from *Atrx*^{HET} male litters did not show a similar stress-related increase in grooming, but both spent significantly more time performing grooming behaviours compared to non-stressed females from *Atrx*^{WT} male litters. A similar - but non-significant - trend was observed in *Atrx*^{WT} and *Atrx*^{HET} males as a result of gestational stress: stressed *Atrx*^{WT} males showed increased grooming at levels similar to those seen in non-stressed *Atrx*^{HET} males. As repetitive self-grooming is a behaviour typically seen as a result of stress and/or anxiety in mouse models of autism, the gestational stress may be creating a behavioural phenotype in Atrx^{WT} animals that is similar to that found in *Atrx*^{HET} animals (Dunn & Swiergiel, 1999; McFarlane et al, 2008). Taken together, these findings indicate that, despite the

same *Atrx*^{WT} genotype, the behavioural outcomes of an individual are still heavily influenced by the genotype of their littermates, potentially as a result of genotype-related differences in the quality of maternal care received.

During the Open Field test, non-stressed *Atrx*^{HET} males also exhibited a much higher frequency of the stretched approach posture - another behaviour traditionally associated with anxiety in animal models - than their non-stressed male *Atrx*^{WT} peers. Gestationally-stressed *Atrx*^{WT} males also showed a significantly higher frequency of stretched approaches than non-stressed *Atrx*^{WT} males, at levels similar to *Atrx*^{HET} males. Stressed and non-stressed *Atrx*^{HET} males did not differ significantly in terms of stretched approach behaviour.

A similar effect was seen in females, where gestational stress led to dramatically increased incidence of the stretched approach posture, although non-stressed females from *Atrx*^{WT} and *Atrx*^{HET} litters did not differ significantly. These findings indicate that gestational stress in *Atrx*^{WT} animals - both male and female - appears to lead to increases in anxiety-like behaviour while genotypically *Atrx*^{HET} males are not significantly affected, as even non-stressed *Atrx*^{HET} males already show heightened stretched approach behaviour when compared to non-stressed *Atrx*^{WT} males. Taken together, these findings indicate that the *Atrx*^{HET} genotype may potentially give rise to a behavioural phenotype that is similar to that produced by gestational stress.

Finally, it was shown that gestationally female mice raised in *Atrx*^{HET} litters showed a dramatic decrease in the number of fecal boli produced over the course of the ten-minute open field test when compared to females from all other groups. Male mice did not show a significant difference in the number of in fecal boli related to genotype or gestational stress. These findings indicate that sex, gestational stress, and family genotype

appear to interact and potentially act on gastrointestinal function, in agreement with previous literature stating that exposure to early life stress, such as maternal separation or abuse can disrupt gastrointestinal function, often via dysregulation of the HPA-axis (Bailey & Coe, 1999; Barreau et al, 2004; Drossman et al, 1995; O'Mahony et al, 2011).

In summary, the trends observed during the open field test indicate that the *Atrx*^{HET} genotype only appears to influence certain emotional behaviour, and is not sensitive to the effects of gestational stress. Additionally, the effects of gestational stress on emotional behaviour in *Atrx*^{WT} animals appear to mediated by sex and the early family environment, specifically the genotype of the siblings. These findings may have significant implications for human families, as it has been previously shown that the presence of autism or other pervasive developmental disorders in a family can have significant impact on social, cognitive and behavioural outcomes for non-autistic siblings, possibly by modulating the early life environment and the availability of parental care (Orsmond & Seltzer, 2009; Pilowsky et al, 2007; Toth et al, 2007).

4.3. *Effects of Reduced Atrx Expression and Gestational Stress on Cognition*

Performance on the Morris water maze indicated that both males and females from non-stressed *Atrx*^{HET} male litters consistently demonstrated significantly longer latency to reach the platform during the Morris Water Maze task than their counterparts from non-stressed *Atrx*^{WT} litters. Latencies in all groups were unaffected by gestational stress except for females from *Atrx*^{HET} male litters, which showed a significant stress-related increase in latency. As rodents are inherently motivated to escape the pool via the platform during this task, longer latencies to find the platform are typically interpreted as an indication of deficits in spatial navigation and problem-solving ability.

Consistent with results, both males and females from *Atrx*^{HET} litters also spent significantly more time in the periphery of the pool during the task than males and females from *Atrx*^{WT} litters. As the platform is never situated in the periphery of the pool in the Morris water maze, the mouse should quickly learn that there is no possibility of escape there; hence thigmotaxis is typically interpreted as a sign of increased anxiety and perseveration, indicating an absence of problem-solving ability (Vorhees & Williams, 2006). Animals from *Atrx*^{HET} litters were unaffected by gestational stress in terms of thigmotaxis; however, males from *Atrx*^{WT} litters showed *decreased* thigmotaxis related gestational stress. These results support previous literature stating that in genetically nominal individuals, gestational stress may potentially produce context-specific stress "inoculation" and may result in reduced sensitivity to stressful situations; however findings on this topic are mixed, and depend highly on the extent and nature of the gestational stress and on the context of stressors encountered in adulthood (Glover, O'Connor, & O'Donnell, 2010).

On the fourth day of the task, there was a reversal of the rules and the platform was moved to the from the southwest to the northeast quadrant (opposite) of the pool. Animals were tested for an additional three days with the platform in the new location, then on a single probe trial in which there was is no platform so that the animal's searching patterns can be observed. During this probe trial, animals would reasonably be expected to the majority of their time actively searching in the northeast quadrant, as the platform had most recently been located there during the reversal phase of testing. *Atrx*^{HET} males, however, spent a significantly lower amount of time in the target quadrant during the probe trial when compared to *Atrx*^{WT} males. Search patterns in either genotype-litter group were not affected by gestational stress.

These findings indicate that *Atrx*^{HET} males, and their female *Atrx*^{WT} littermates show increased anxiety-like behaviour and cognitive impairments in problem-solving and spatial navigation, demonstrating the importance of early life experience and parental care in shaping cognitive outcomes for offspring, and that the impact of the *Atrx*^{HET} genotype is not restricted to the individual. Human studies on autism have demonstrated similar findings, with non-autistic siblings of children with autism showing increased incidences of language and social deficits and pervasive developmental disorder, possibly as a result of changes in the early family environment (Pilowsky et al, 2007; Toth et al, 2007). It is worth noting that only *Atrx*^{HET} males showed specific deficits in working memory (ie: failure to search in the target quadrant during the probe trial of the maze), which highlights the need for further investigation to allow for differentiation between deficits related specifically to genotype over deficits related to shared early life environment and parental/maternal care.

4.4. *Effects of Reduced Atrx Expression and Gestational Stress on Social Behaviour*

During the social investigation task, *Atrx*^{HET} males demonstrated significantly decreased social affinity - as indicated by time spent investigating a stimulus mouse of the opposite sex - and increased anxiety-like behaviour when compared to *Atrx*^{WT} males and females from *Atrx*^{HET} litters. *Atrx*^{HET} males also showed increased social avoidance (time spent in the periphery of the testing arena, away from the stimulus mouse) compared to females from *Atrx*^{HET} litters. These findings are in line with human studies of ATRX syndrome, which typically produces significant social deficits and autistic-like behaviour (Gibbons, 2006).

Gestational stress appeared to only influence social behaviour of females raised in *Atrx*^{HET} litters, with gestational stress resulting in increased displays of anxiety-like behaviour during the social investigation task, while social affinity and avoidance were unaffected. Overall, females from *Atrx*^{HET} litters did not differ significantly in terms of social behaviour from females raised in *Atrx*^{WT} litters and showed decreased displays of anxiety-like behaviour compared to their *Atrx*^{HET} male littermates and gestationally stressed counterparts, in opposition to findings from human studies on the social behaviour of siblings of children with autism and other pervasive developmental disorders (Kaminsky & Dewey, 2001; Toth et al, 2007). These findings indicate that sex and early family environment appear to interact to moderate the effects of gestational stress on behaviour, and further research is required to elucidate the specific nature of these interactions.

4.5. *Effects of Reduced Atrx Expression on Brain Weight*

Atrx^{HET} males showed significantly decreased overall brain weight compared to all other genotype groups. However, when weights were compared across individual brain regions (the frontal cortex, hippocampus and cerebellum), no significant differences in brain weight were found. Non-significant decreases were found when hippocampal weights of *Atrx*^{HET} males were compared with that of males and females from *Atrx*^{WT} litters ($p = 0.051$ and 0.052 respectively). Although no differences were found in individual regions, these findings are in agreement with previous research indicating that the loss of ATRX results in hypocellularity and decreased hippocampal and cortical mass (Kernohan et al, 2010, Bérubé et al, 2005). The observed decreases in overall brain weights seen in

Atrx^{HET} individuals may be more evenly distributed throughout the cortex than previously expected, rather than localized in the forebrain, hippocampus.

4.6. *Effects of Reduced Atrx Expression on Atrx mRNA Transcript Expression*

Atrx^{HET} males showed significantly decreased *Atrx* mRNA transcript expression in the prefrontal cortex compared to all other groups except for females from *Atrx*^{HET} litters, and decreased transcript expression in the hippocampus compared to *Atrx*^{WT} males. Overall *Atrx* mRNA transcript expression in *Atrx*^{HET} brains was significantly lower in the prefrontal cortex and hippocampus when compared to the cerebellum, a difference not observed in the other genotypes. As the Cre-LoxP recombination system used to generate the *Atrx*^{HET} mice was augmented by a Nestin promoter that restricted Cre expression to forebrain neuroprogenitor cells, and ATRX is most extensively involved in neuronal survival during emergence of the forebrain and hippocampus, these results are in line with our expectations that reduced expression of *Atrx* promoter transcripts would be restricted to the prefrontal cortex and hippocampus (Berube et al., 2005).

4.7. *Effects of Reduced Atrx Expression on Methylation at Atrx promoter sites*

Atrx^{HET} males showed significantly decreased levels of methylation at *Atrx* promoter sites in the hippocampus, and significant decreases in methylation in the frontal cortex compared to their *Atrx*^{HET} littermates. No differences in methylation rate in the cerebellum were observed between groups. Interestingly, females from *Atrx*^{HET} litters showed significantly increased methylation in the hippocampus when compared to *Atrx*^{HET} males, as well as in the frontal cortex when compared to *Atrx*^{HET} males and *Atrx*^{WT} males and females. These findings indicate that females from *Atrx*^{HET} litters may be experiencing hy-

permethylation of *Atrx* promoter sites in the hippocampus as a result of some aspect of the early environment shared with their *Atrx*^{HET} littermates, and may explain some of the interactions between litter-genotype and sex demonstrated on social, cognitive and emotional behaviour.

4.8. *Concluding Remarks and Future Directions*

Our methods have helped to create a behavioural and epigenetic profile of the *Atrx*^{HET} genotype. Our findings suggest that *Atrx* haploinsufficiency greatly delays early development and hinders pup survival, increases social avoidance and some anxiety-like behaviours, and creates deficits in working memory and cognition. Additionally, the influences of *Atrx* haploinsufficiency are not restricted only to animals with the *Atrx*^{HET} genotype - *Atrx*^{WT} females reared in litters containing *Atrx*^{HET} males also show deficits in cognition, increases in some anxiety-like behaviours, and an altered profile of social behaviour unique from the behaviour of both *Atrx*^{HET} males and females from *Atrx*^{WT}-only litters. Analysis of collected brain tissue showed that, in line with expectations, *Atrx*^{HET} males showed decreased overall brain weight, localized decreases in *Atrx* mRNA transcript expression, and decreased methylation at *Atrx* promoter sites in the forebrain and hippocampus. It is still unclear whether deficits in ATRX expression solely effect behaviour indirectly, by disrupting formation of the parent-offspring bond, or have additional implications for stress reactivity and cognitive and social behaviours in offspring. These findings open the doors to investigating a multitude of factors that may influence ATRX gene expression during development or alternatively, that may be influenced by the degree of ATRX expression during development.

Using this protocol, future studies can measure the influence of varying degrees of maternal care on ATRX expression concurrently with the associations between reduced ATRX expression and social, emotional and cognitive behaviours of offspring. One promising avenue for future research may be to perform rescue experiments determine if the deficits shown by *Atrx*^{HET} animals could be alleviated by drug intervention. Currently, we are conducting experiments attempting to reduce the negative impact of the *Atrx*^{HET} genotype by infusing ubiquitin-specific protease 10 (USP10) or RING1b inhibitor directly into the dentate gyrus in order to stimulate ATRX overexpression, or H2AX ubiquitination/histone deacetylase inhibition to promote repair protein recruitment to DNA damage sites in vivo. Both drugs represent promising potential avenues for research that could be developed into treatments for human patients with ATRX syndrome and other ATRX-related disorders, and may help to further elucidate the role of ATRX in development of abnormal social and cognitive behaviours.

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Weaver, I.C.G., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., and Meaney, M.J. (2004b). Epigenetic programming by maternal behavior. Nature Neuroscience *7*, 847-854.

Weaver, I.C., Grant, R.J., and Meaney, M.J. (2002). Maternal behavior regulates long-term hippocampal expression of BAX and apoptosis in the offspring. J Neurochem *82*, 998-1002.

Williams, M. T., & Vorhees, C. V. (2006). Morris water maze: Procedures for assessing spatial and related forms of learning and memory. Nature Protocols*, 1*(2), 848-858.

APPENDIX A: Cre and Atrx Genotyping Protocol

1. Label PCR tubes, top and side, to match samples.
2. Vortex and spin the Thermopol Buffer, dNTP's and primer mix (not Taq Polymerase).
3. Prepare master mix for n+1 samples. Gently mix by hand and spin.

   Master Mix:

   2.5 µl 10X Thermopol buffer (Cat #: B9004S, BioLabs Inc.)
   0.5 µl 10 mM dNTP's (Cat #: N0447L, BioLabs Inc.)
   0.5 µl primer mix
   0.25 µl Taq DNA Polymerase (Cat #:M0267X, BioLabs Inc.)
   11.25 µl dH_2O

4. Aliquot 20 µl of master mix to each PCR tube.
5. Spin the DNA samples.
6. Transfer 10 µl of the extracted DNA supernatant to each sample tube containing master mix, according to the label.
7. Load the PCR tubes into the Thermocycler and run *ATRX* or *Cre* program.

ATRX: Total run time 2:04:47

1. 2.0 min 95 ℃
2. 30 sec 95℃
3. 30 sec 53℃
4. 1.30 min 72 ℃
5. Step 2 - 4 x34℃
6. 5.0 min 72 ℃
7. ∞4℃

Cre: Total run time 1:14:50

1. 2.0 min 95 ℃
2. 20 sec 95℃
3. 20 sec 60℃
4. 30 sec 72℃
5. Step 2 - 4 x34℃
6. 5.0 min 72 ℃
7. ∞4℃

Electrophoresis Protocol

1. Prepare a 1.5% agarose gel (Argarose RA, Cat #: 232-731-8, Amresco). Dissolve agarose in 1X TBE buffer. Microwave for 120 sec. (until clear) and pour into electrophoresis unit.

	Long gel	**Short gel**
Agarose	1.5 g	0.975 g
TBE	100 ml	65 ml

2. Label clean microtubes by sample and transfer 10 µl of each PCR sample to the corresponding microtube. Store the remaining PCR in the fridge.
3. Add 2 µl UView dye (Cat #: 166-5112, BioRad) to each PCR sample. Gently mix by hand.

4. Prepare DNA ladder. In a clean microtube, mix 8 µl ladder with 2 µl UView dye (Cat #: 166-5112, BioRad) for one lane.

	ATRX	Cre
Ladder	2000-50 bp Molecular Rular (Cat #:170-8200, BioRad)	100 bp Molecular Rukar (Cat#: 170-8202, BioRad)

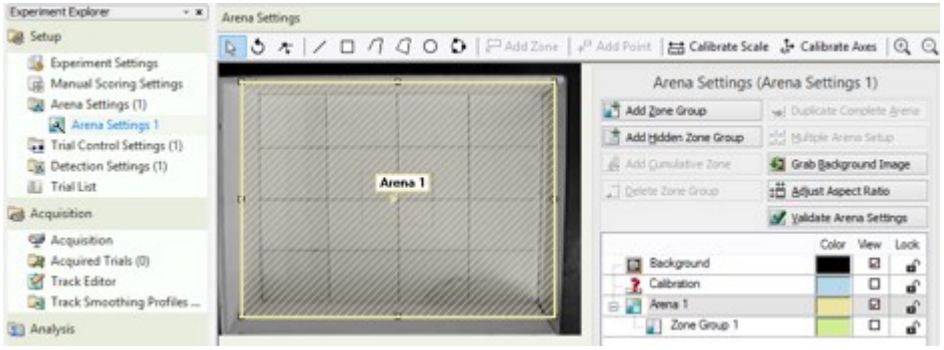
5. Load 15 µl of each sample into wells of the gel. Put 10 µl of ladder in the last well.
6. Run the gel at 200V for approximately 25 minutes (Cre) or 60 minutes (ATRX).
7. Image and print the gel.

APPENDIX B – OPEN FIELD TEST ANIMAL TRACKING PROTOCOL

Open Ethovision XT (version 9) and select **New default experiment**. A window prompt will appear; name your file and select where you'd like to save it with the Browse button.

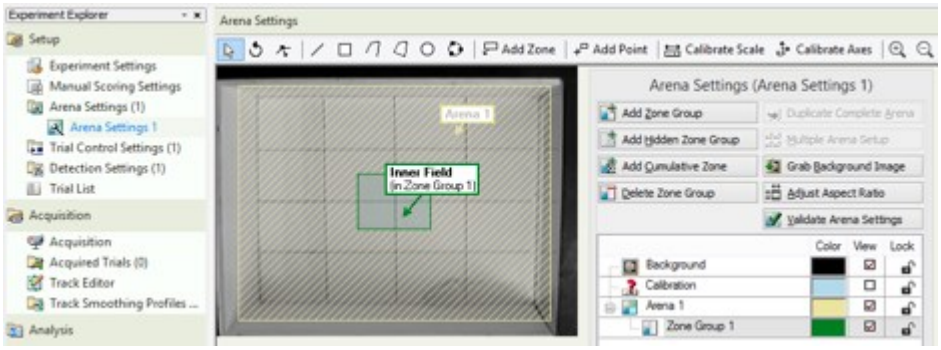
You can now use the **Experiment Explorer** menu on the left side of the screen to navigate. For the Open Field Test, the default Experiment and Manual Scoring settings are fine as they are, so expand the **Arena Settings** option and select **Arena Settings 1**. This will create a new arena settings profile.

Ethovision will prompt you for a video file to use as a background image. Click on **Browse** and select one of the videos you'll be using. The video will begin to play; click **Grab** to take a snapshot to use as a background for the arena. With **Arena 1** highlighted in the menu on the right, use the **square tool** to draw a box around the arena, which marks the boundary for the area in which you'd like to track the mouse. You'll want to include some of the walls, but not too much or the software may track the mouse's reflection. It should look like this:

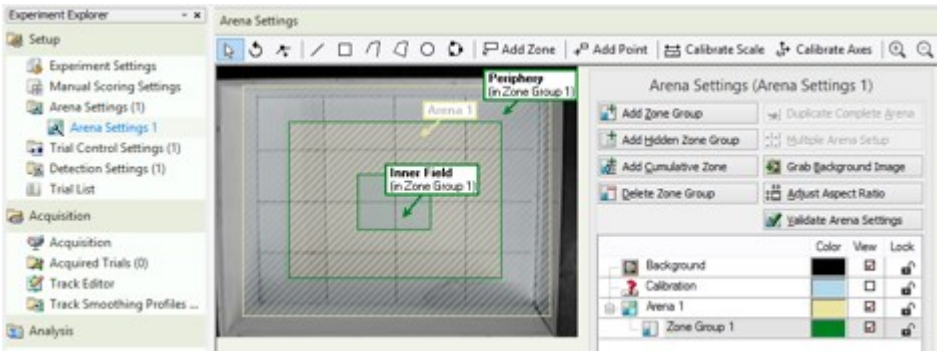


Next, click on **Zone Group 1** and then the **Add Zone** button. Click on the centre of the field and name the zone "Inner Field" when prompted. Use the **square tool** to draw

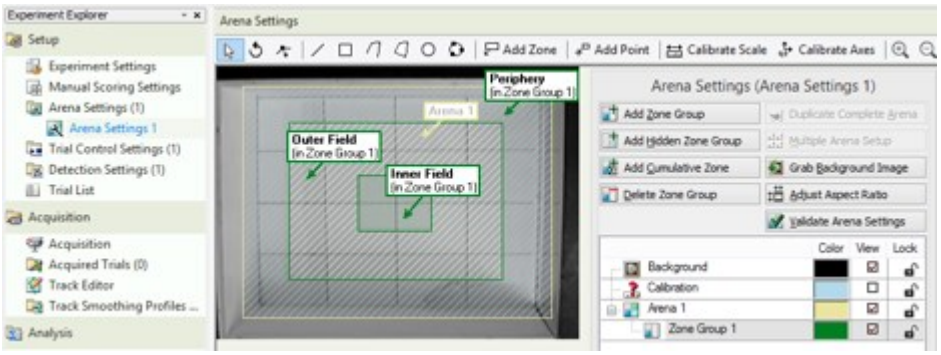
a box around the inner area in the middle of the field. You may want to change **color** of Zone 1 so that it stands out more, like this:



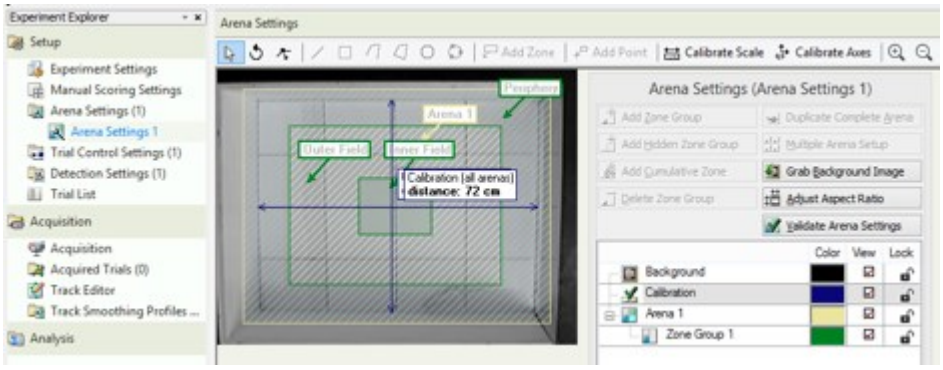
Click **Add Zone** again and click on the outer edge of the field. Name this zone "Periphery". Use the square tool to draw a box to define the inside edge of the periphery. The box should be halfway between the walls of the field and the first line of the grid pattern on the floor, like this:



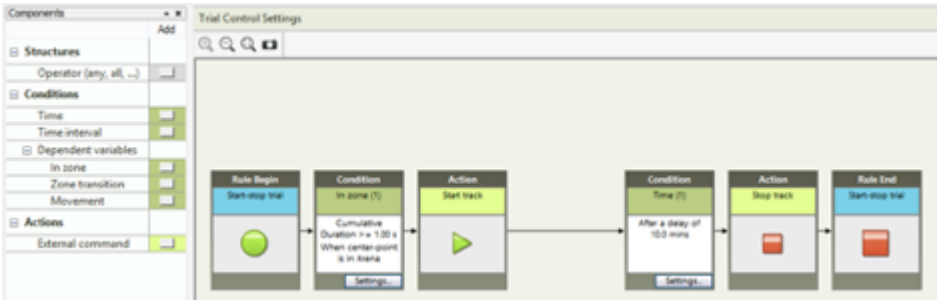
Select **Add Zone** again and click in the area between the Inner Zone and the Periphery. Name this area "Outer Field".



Finally, calibrate the arena by selecting **Calibrate Scale**. Click on one edge of the field where the floor meets the wall and, holding the mouse down, draw a straight horizontal line to the other edge. Enter “72 cm” when prompted. Select **Calibrate Scale** again, and this time draw a vertical line across the field.

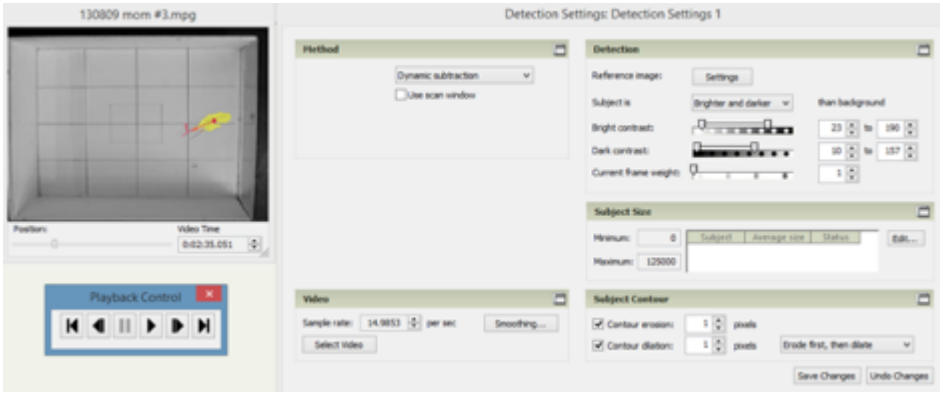


Expand the **Trial Control Settings** option in the left sidebar and select **Trial Control Settings 1**.



In the **Conditions (Time)** box (on the right), click on Settings. Select **Condition is met:** and **After a delay of:** and change the time to “10 min”. With these settings, Etho-vision will begin data acquisition once the animal has been detected in the arena for 1 second, and stop once 10 minutes have passed.

Expand **Detection Settings** and select **Detection Settings 1** to open the profile.

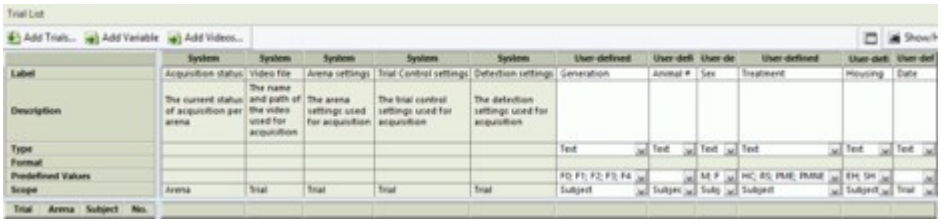


In the **Detection Settings** window under **Method**, select **Dynamic Subtraction** from the drop down menu. Under **Detection**, click **Reference Image: Settings** and another window will open. In this window, select **Start Learning (C)** and close the window once it's finished creating a clear background image.

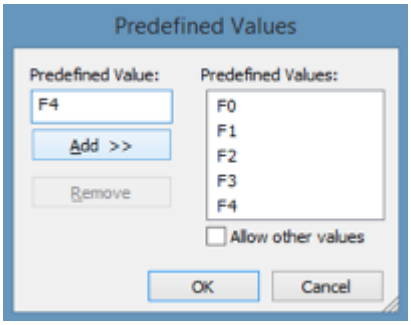
Next, click the drop down menu next to **Subject is** and select **Brighter and darker** (or whatever is appropriate for your footage). Adjust the sliders so that the mouse is detected properly. Only the mouse's body and should show up **yellow** on the footage, with a **red dot** indicating its centre point. You can use the **Playback Control** to play the video and ensure that the animal is being detected in all areas of the field, including the darker edges and corners.

Detection noise is represented in **orange**, and you should try to minimize it wherever possible. However, as long as the **red centre point** is consistently tracking the mouse's movement and not the environment, then the data should be fine. Lastly, under **Video**, turn the **Sample rate** down to "14.9853" per second (Noldus' recommended rate for normal behaviour in mice). Save your data settings.

Select **Trial List** from the left sidebar. Click on **Add Variable** and add 6 variables – **Generation**, **Animal #**, **Sex**, **Treatment**, **Housing**, and **Date**. For **Date** only, in the **Scope** setting to **Trial** instead of **Subject**.



You can add **Predefined Values** for each variable by clicking the drop-down menu. This will prompt a small window to open, where you can enter your desired values and add them to a list. When you add a set of predefined values to a variable, **untick** the **Allow other values** box.



For the following variables, add these predefined values:

Sex – M; F

Litter Genotype - WT; HET

Treatment – HC; RS

When you are done, your Trial List Screen should resemble the one shown above.

Under **Analysis**, expand **Analysis Profiles** and select **Analysis Profile 1**. It should already have 2 dependent variables set up: **Distance Moved** and **Velocity**.

In the **Dependent Variables** sidebar under the **Location** heading, click on **In Zone**. In the window that appears, deselect **Arena**, and select only **Inner Field**. Click **Add** to close the window. Repeat this 2 more times, with **Periphery** and then **Outer Field**. Make sure that only 1 zone is selected in each dependent variable, so that Ethovision will record the amount of time the mouse spends in each zone independently. When you are finished, your analysis profile will look like this:

Selected Dependent Variables	Description
Distance moved	Distance moved of the center-point
Velocity	Velocity for the center-point
In zone	When center-point is in Inner Field
In zone 2	When center-point is in Periphery
In zone 3	When center-point is in Outer Field

Return to the **Trial List** screen. Click on the **Add Trials** button in the top left corner of the screen. A window will open; enter the number of trials that you wish to add. It's a good idea to enter smaller batches of trials at first and then run them through acquisition before adding more to ensure that they are properly acquired.

When you have added trials, click on the small "**…**" button in the **Video file** column. Ethovision will open a window and prompt you to select a source video for the trial. Once the video has been added, go through the other columns and fill in the information about the animal. Note that the **date** must be entered year, month, day, without spaces (eg: August 9, 2013 = 130809). The **paste** (ctrl + v) and **multi-select** (shift + click) functions are useful here for filling in multiple cells with the same information.

Next, select the **Arena**, **Trial Control**, and **Detection settings** profiles that you'd like to use for each trial. It is important to note that once you have used a settings profile in **acquisition** mode, you cannot change make changes to it. You can, however, make a **duplicate** profile that can be altered.

Here are some other things to keep in mind when deciding which profiles to use:

Arena settings:

- You should be able to use the same **Arena settings profile** for each trial that takes place on the same day. When acquiring videos from a different day of testing, or when processing a large batch of trials from the same day, it is a good idea to check that the arena profile is still suitable.
- To check if an arena profile will work for a new trial, **right-click** on it and make a **duplicate**. In the duplicate profile, select **Grab Background Image** and take an image from the video you'll be using in your trial. If the arena matches the new image, then you may use the original profile.
- If the arena does not match the image, tweak the new duplicate profile to fit, and then select the arena profile in the **Trial list** screen to use it for acquisition.

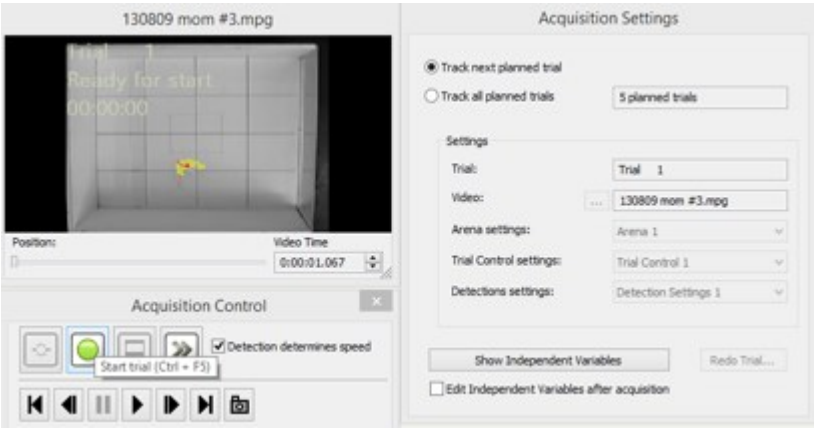
Trial control settings:

- You should be able to use the same **trial control settings** for almost all of the trials, unless for some reason you do not want to begin acquisition as soon as the video begins (ie: if an experimenter's hand is visible in frame).
- To create a new **trial control settings** profile, right-click on the existing one and select **duplicate**. Watch the video file for this trial and note the time as soon as the hand or obstruction is no longer in view. Then, in the profile, go to **Condition (In zone)** box and click on **Settings**. Set the acquisition to begin at the time you noted (when only the mouse is in view) and be sure to select this new profile for your trial in the **trial list** screen.

Detection Settings:

- As with the arena, you should be able to use the same **detection settings** for trials that take place on the same day.
- The easiest way to determine if a detection setting profile is suitable is to set up the trial in the trial list screen, then go to the **acquisition** screen and play the video (use the **play** button, not the green **start trial** button). If the mouse is being tracked consistently and there is little or no background noise, then the detection profile is suitable for this trial.
- Make sure to return playback to the beginning of the video before starting acquisition, or Ethovision will begin acquiring data part way through the video.
- If a mouse is not being detected properly, then go to **detection settings** and create a **duplicate** of one of your existing profiles. In the duplicate profile, click **Select Video** and pick the video you'd like to use for your trial. Under the **Detection – Reference image** heading, click on **Settings**, and then click **Start Learning (C)** to build a new dynamic background image. You can then use the contrast sliders to fine tune detection.

When all of your **trial list** settings are in order, go to the **acquisition** screen:



Before you begin **acquisition**, it's good practice to play each video briefly to make sure the mouse is being tracked properly. Use the **play** button in the **Acquisition Control** menu, not the green **Start trial** button (marked in the image above). Be sure to return playback to the beginning of the video before you start acquisition, or Ethovision will begin acquiring data wherever you left off in the footage.

You can click on the **Show Independent Variables** option to check that the information entered in the **trial list** screen is accurate. There is also a **Track all planned trials** option that will automatically run all of the trials you have planned, but it's best to track trials individually unless you are confident that there will be no issues with the footage or detection. Lastly, make sure the **Detection determines speed** option is ticked, or acquisition will take place in real time (each video will take 10 minutes to process).

When you are ready, click **Start trial**, and the Ethovision will begin acquisition from the video. Ethovision will notify you when it is finished, and you may then run the next trial you have planned.

If you notice an issue during acquisition, you can click the red **stop** button. This will halt acquisition and move on to the next trial you have planned out in the **trial list**

screen. You can then click on **Redo trial** to clear the recording and try again from the beginning. Alternately, you can also return to the **trial list** screen later and **right-click** on the trial and **clear** it, re-enter the settings, and then run it in acquisition again once you have solved the issue.

To export your data, under **Analysis** > **Results**, click on **Analysis Output**. In the top left corner of the screen, click on the **Calculate** button. The data should fill in the cells on screen. You can now export your data as an Excel spreadsheet (or the format of your choice) by clicking on the **Export** button.

APPENDIX C – MORRIS WATER MAZE ANIMAL TRACKING PROTOCOL

If the files are in a format other than .mpg, use an application such as Format Factory to convert them. Open Ethovision XT (version 9) and select **New template experiment**. A window prompt will appear; select **Apply a pre-defined template**. Choose the option **From video file**, and click **Browse** to select a video.

Select **Rodents > Mouse**, **Morris water maze** and **Platforms, quadrants** and **Animal is Brighter than the background** from the options in the following window prompts. Name your experiment and use **Browse** to choose where to save it.

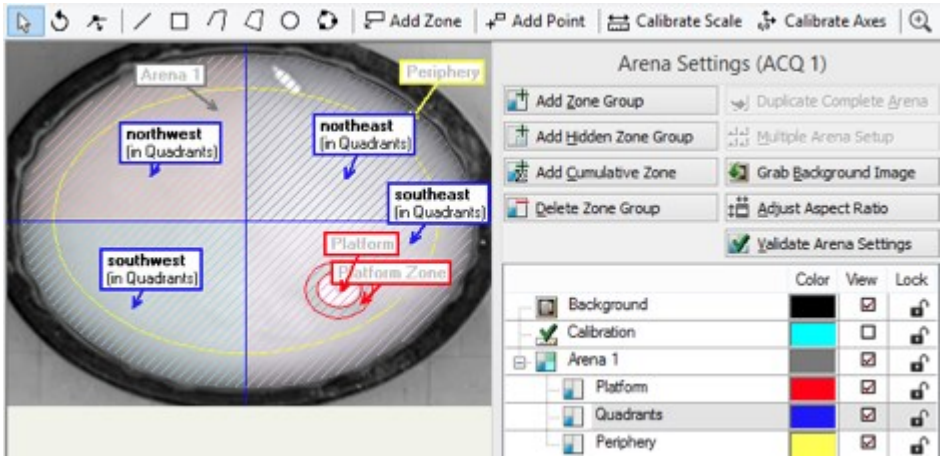
You can use the **Experiment Explorer** menu on the left of the screen to navigate. For the Morris Water Maze, the default Experiment and Manual Scoring settings are fine as they are, so expand the **Arena Settings** option and select **Platform and quadrants**. This will create a new arena settings profile.

Building the arena - **Acquisition Phase (Days 1-3/Trials 1-12)**

Once you open a new Arena Settings profile, Ethovision will automatically play the video file you selected earlier. If there is a mouse in the footage, wait until it isn't in the way of any of the arena features and then click on **Grab** to take a snapshot to use as a background for the arena.

Ethovision's default Morris water maze template will now appear over your snap-shot. You'll have to tweak the parameters to match your footage.

Ultimately, you want your arena setup to look something like this:



You may want to change the **Color** settings for each parameter in the **Arena Settings** menu to make them stand out more. You can also uncheck the **View** boxes next each layer/parameter of your arena in the Arena Settings menu to hide them, which can make tweaking the individual parameters easier.

Adjust the circle labeled **Arena 1** to encompass the area in which you'd like to track the mouse. You'll want to include all of the water's surface, but not much of the walls or Ethovision may track the mouse's reflection. Ensure that the **Arena 1** marker (the box with an arrow) is within the circle of the arena.

Next, in the **Arena Settings** menu, click on **Add Zone Group**. A new zone group will appear in the menu; right-click on it and rename it **"Periphery"**. Click on the **Create ellipse** (**circle**) tool in the upper menu bar and draw a circle within the arena, halfway between the edge of the arena and the platform. Make sure it is equidistant from the edge of the arena on all sides. In the upper menu, click **Add Zone**, then click on the space between the arena boundary and the circle you've just drawn. Name this zone "**Periphery**".

Next, adjust the 2 of the circles in the **Platform** parameter to match with the platform as shown in the snapshot. Have one circle surround the platform, and a second circle surround a small zone around it. There may be a leftover third circle; you can delete it.

Drag the **Platform** and **Platform Zone** markers within the appropriate circles. Under the **Quadrants** parameter, adjust the vertical and horizontal lines so that they divide the arena up into 4 equal quadrants. Drag the **northwest**, **northeast**, **southwest**, and **southeast** markers into the appropriate quadrants.

Highlight the **Calibrate** option in the **Arena settings** menu on the right. In the top menu, click on **Calibrate Scale**, and your cursor will turn into a plus sign. Click on one edge of the arena, and then draw a vertical line across the centre of the arena to the far edge. When prompted, enter "110 cm" as your measurement. Click on **Calibrate Scale** again and do this a second time, drawing a horizontal line across the arena. Again, enter "110 cm" as the measurement. Hide the calibration lines by un-ticking the **View** box under **Arena settings**.

Reversal Phase (Days 4-6/Trials 13-24)

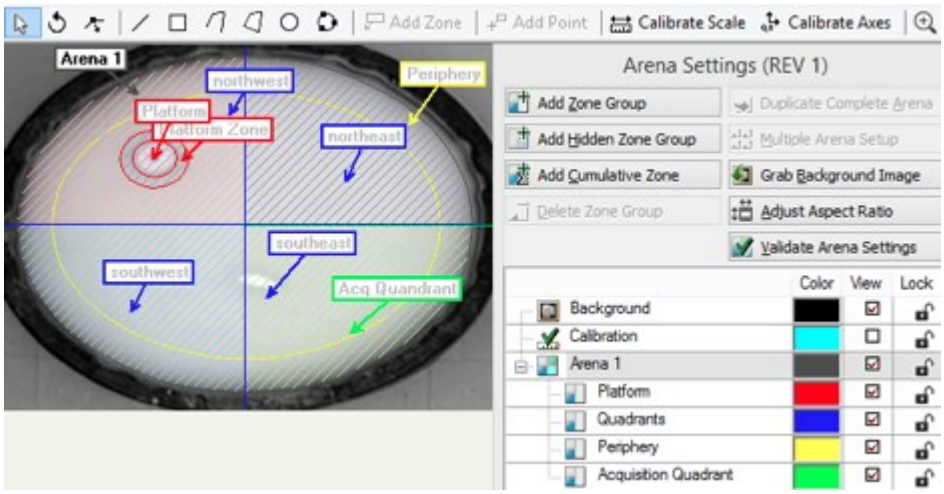
The arena for the reversal phase is similar to acquisition but with the addition of a new parameter, so you can simply alter an arena that you've already made. In the **Experiment Explorer** menu on the left of the screen, expand **Arena Settings** and right-click on the arena you previously made for Acquisition. Select **Duplicate**, and rename the new arena.

Click on **Grab Background Image** and then **Browse** to select your source video. Click on **Grab** to take a background snapshot, and tweak the arena parameters as necessary to suit the image. During Reversal trials, the platform will be up in the Northwest

quadrant instead of the Southeast, so move your **platform** and **platform zone** boundaries and markers accordingly.

Select **Add Zone Group**, and right click on the new zone group that appears to rename it "**Acquisition Quadrant**". With the **Acquisition Quadrant** zone group highlighted, use the **Create rectangle** (square) tool to draw a box around the southeast quadrant. Click on **Add zone**, and then click inside of the box you just drew around the southeast quadrant. Name the zone "**Acq Quadrant**".

Finally, unhide the **Calibration** layer to ensure that the measurements still match the dimensions of the arena. Adjust the lines as necessary, then hide the calibration layer again. When you are finished, your Reversal arena setup should look like this:

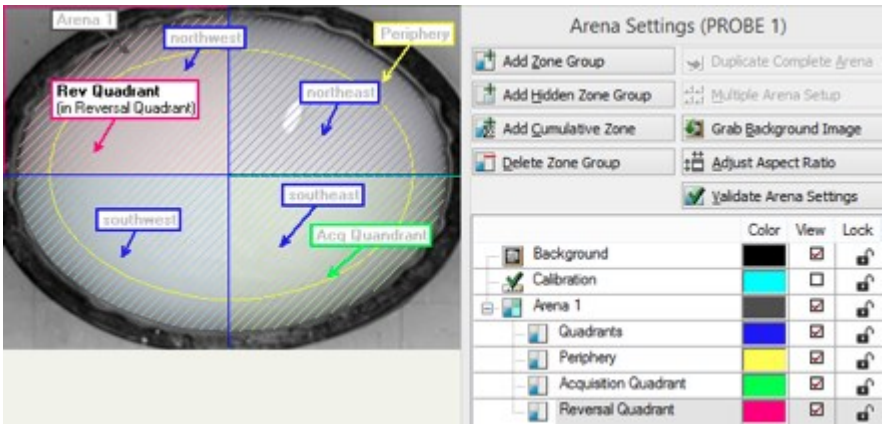


Probe Trial (Day 7/Trial 25)

Right-click on one your existing **Reversal** profiles to duplicate it, then rename it. Click **Grab Background Image**, select the video that you'd like to use, and click **Grab** to take a background. Tweak the **Arena**, **Periphery**, **Quadrants** and **Acquisition Quadrant** parameters to suit the new image as necessary.

Highlight the highlight the **Platform** layer under **Arena Settings**, and then click on **Delete Zone Group** to get of it. Then click **Add Zone Group**, then right-click on the new zone group to rename it "**Reversal Quadrant**". With **Reversal Quadrant** highlighted, click on the **Create rectangle** (square) tool and use it to draw a box around the **northwest quadrant**. Select **Add Zone** from the upper menu, and click inside of the box you just drew. Name the new zone "**Rev Quadrant**".

When you are finished, your arena setup should look something like this:

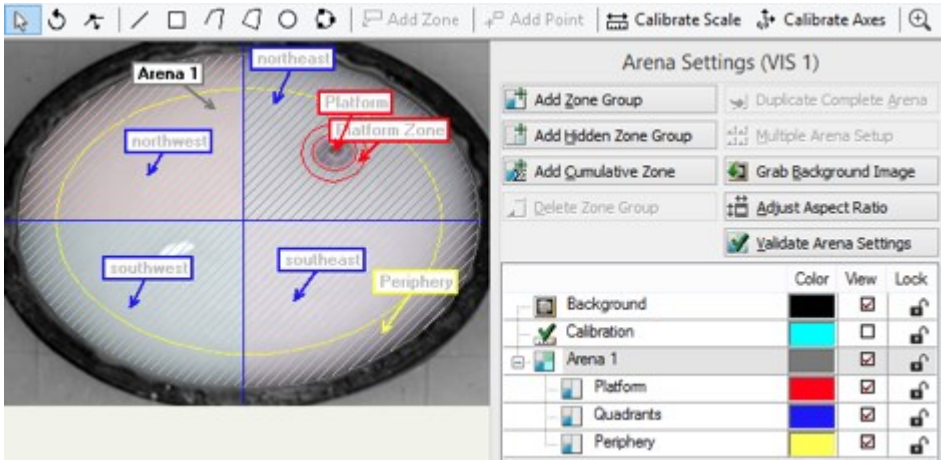


Visible Platform Trials (Day 8/Trials 26-29)

Right-click on one of your existing Acquisition profiles to duplicate it, then rename it. Click **Grab Background Image**, use **Browse** to select the video of your choice, and then grab a background snapshot.

Tweak the **Arena**, **Periphery**, and **Quadrants** parameters as necessary. The platform is now in the **northeast** quadrant of the maze, so move your **Platform** and **Platform Zone** boundaries and markers to suit. There are no Acquisition or Reversal quadrant zones in the Visible Platform arena.

When you are finished, your arena setup should look something like this:



Trial Control Settings

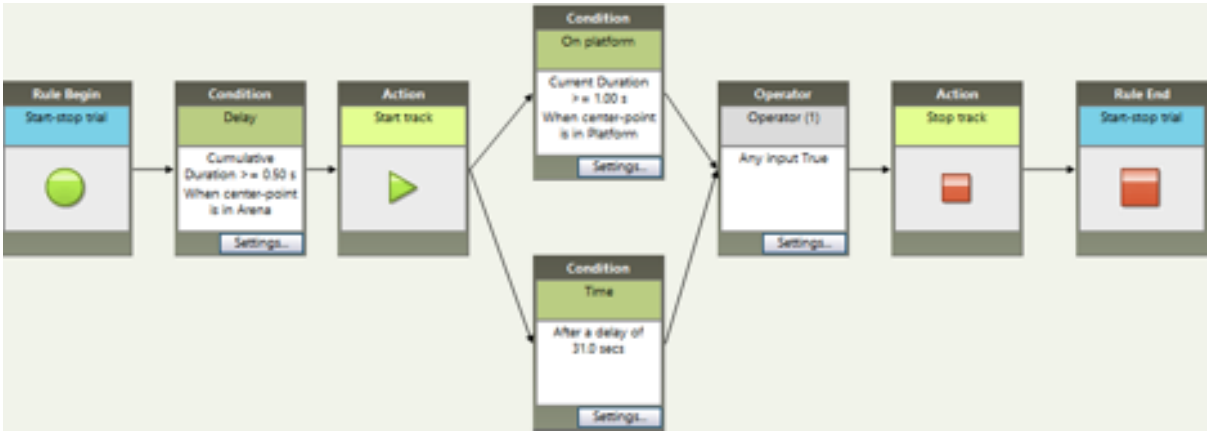
Expand the **Trial Control Settings** menu. Select **Max Track Duration 1 min** from the list of pre-set profiles. Delete the other 2 profiles; you will not be using them.

In the box labeled **Condition – Delay**, click on **Settings**. Set the parameters to **Cumulative Duration > When centre-point is in Arena > = 0.5 s**.

In the **Condition – On platform** box, click on **Settings**. Set the parameters to **Current Duration > When centre-point is in Platform > = 1.0 s**.

In the **Condition – Time** box. Click on **Settings.** Set the parameters to **After a delay of: 36.0 sec** (not 31 seconds as shown below).

When you are finished, your Trial control screen should look like this:



Detection Settings

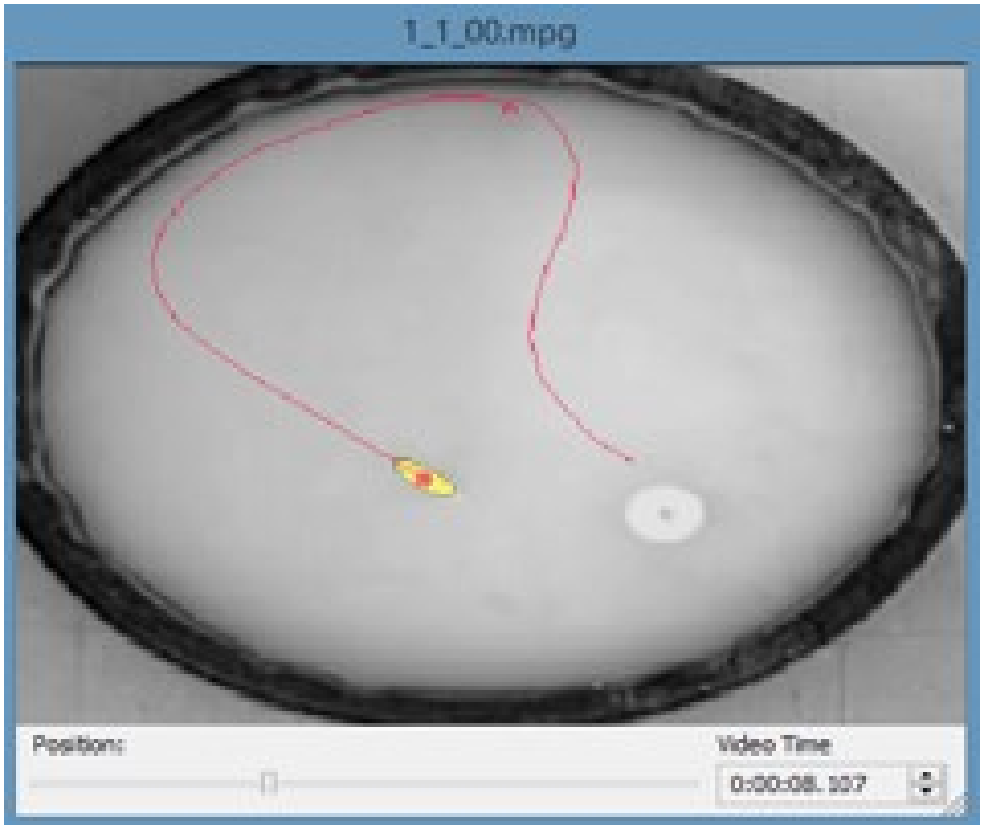
In the **Experiment Explorer** menu, expand **Detection Settings** and open a profile. Under **Video**, click on **Select Video**, and choose your video file. Next, click on **Reference Image > Settings**. In the window that pops up, click on **Start Learning (C)**. Ethovision will now make a dynamic background image by averaging all of the frames of the video. Once it's finished, click **< Grab Dynamic Image** and make sure that the **Use dynamic reference image** option is selected. Close the window.

Under **Video**, set the **sample rate** to **9.9902**. Then, under **Detection**, select **Subject is: Brighter than background** set the **Bright contrast** to a **minimum** of **10**, and a **maximum** of **200**. These parameters determine the range of contrast in which Ethovision will detect the subject animal, and the values given here are just a guideline to get you started.

Adjust the **Bright contrast** sliders until the mouse (and only the mouse) shows up as yellow and tracks smoothly when the video is played. If you find that Ethovision is picking up reflections or other visual noise instead of the mouse, try increasing the **mini-**

mum contrast. Too high of a minimum contrast, however, and Ethovision may not be able to detect the mouse.

When the mouse is being tracked properly, it will look something like this:



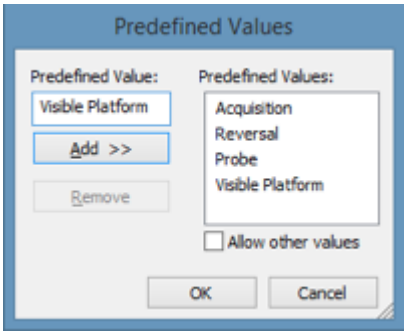
Under **Subject Size**, click **Edit**, and set the **minimum** to **30** pixels, and the **maximum** to **500** pixels. This setting puts a restriction on how large or small of an object that Ethovision can interpret to be the mouse. Again, these values are simply a guideline; if you are having issues with Ethovision picking up large shadows or ripples in the water, try reducing the maximum subject size. If Ethovision is tracking small spots or reflections in the water, try increasing the minimum subject size to filter them out.

You'll want to make a different detection settings profile for each phase of the Morris Water Maze (Acquisition, Reversal, Probe and Visible Platform) to ensure that the mouse is detected properly.

Trial List

Select **Trial List** from the left sidebar. Right click on the variable called "**Trial Name**" and select **Hide variable** to remove it from your trial list screen. Delete the 3 other **User-defined** variables: **Animal ID**, **Treatment**, and **Release Quadrant**.

Use the **Add Variable** button in the top menu bar to add the following 10 variables: **Phase**, **Day**, **Trial**, **Entry Direction**, **Generation**, **Animal**, **Sex**, **Treatment**, **Housing**, and **Found Platform**. For **Phase**, **Day**, **Trial**, **Entry Direction** and **Found Platform**, change the **Scope** to **Trial**. You can add **Predefined Values** for each variable by clicking the drop-down menu. This will prompt a small window to open, where you can enter your desired values and add them to a list. When you add a set of predefined values to a variable, **untick** the **Allow other values** box.



For the following variables, add these predefined values:

Day – 1; 2; 3; 4; 5; 6; 7; 8

Trial – 1; 2; 3; 4

Entry – N; E; S; W

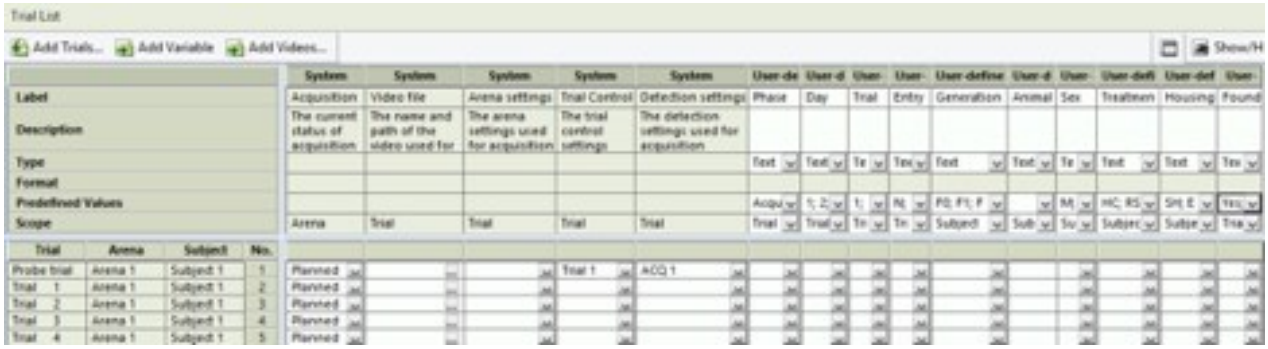
Sex – M; F

Treatment – HC; RS

Litter genotype – WT; HET

Found Platform – Yes; No

When you are done, your Trial List screen should look like this:



Analysis Profile

Under **Analysis**, expand **Analysis Profiles.** For the Morris Water Maze template, Ethovision generates 4 default templates: **Latency to reach platform**, **Path shape**, **Distance & Time** and **Heading**. From these, select **Latency to reach platform**.

The default *Latency to platform* is not a reliable measure in our case, as Ethovision often has difficulty detecting the white mouse on the white platform, and as a result may not register that the mouse has reached the platform.

To work around this, click on **In zone** (under **Location** in the **Dependent variables** menu on the left), and then select only **Arena** when prompted. This will track the total amount of time that the mouse spends in the water maze. We can then pair this with the variable **Found Platform: Yes/No**, or assume that if the mouse spent 35 seconds or more in the arena that it was unable to find the platform.

Next, click **In zone** again, and this time select **Acq Quadrant** and **Rev Quadrant**. Click **Add**. This will allow you to track how much time the mouse spends in those quadrants during the probe trial. If you are interested in tracking the mouse's activity (in terms of time) during other phases, click **In zone**, and select **Northwest quadrant** (Reversal), **Northeast quadrant** (Visible Platform), and **Southeast quadrant** (Acquisition)

from the list and **add** them as well. (The platform never appears in the southwest quadrant, so we typically don't bother to track it.)

When you are done, your analysis profile should look like this:

Selected Dependent Variables	Description
Latency to platform	When center-point is in Platform
Distance to zone	Distance from center-point to Platform
In zone 3	When center-point is in zone for the zones Acq Quandrant and Rev Quadrant
In zone	When center-point is in Arena
In quadrants	When center-point is in zone for the zones northwest, northeast and southwest

Preparing Trials

Return to the **Trial List** screen, and select **Add Trials…** and enter the number of trials you'd like to add.

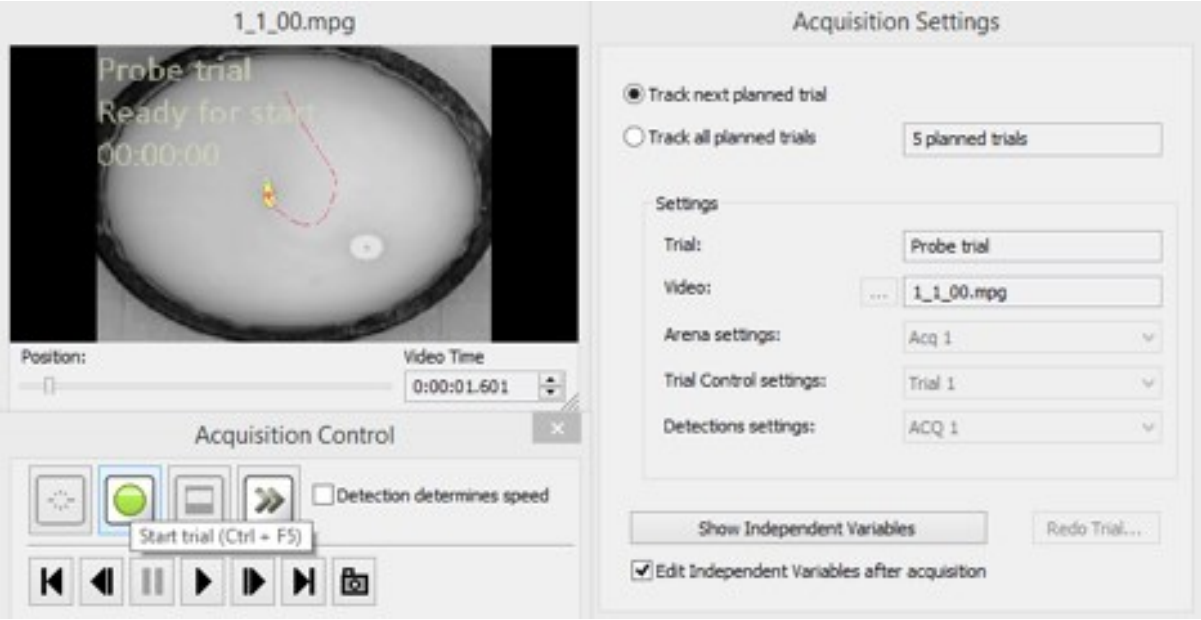
One of the most efficient ways to process analyze the videos is to group them by subject. In our study, there are 29 videos per animal (12 videos of Acquisition, 12 Reversal, 1 Probe, and 4 Visible Platform), so you can go ahead and add 29 trials.

Click on the "**…**" to select a source video file for each trial. Select which **Arena**, **Trial Control**, and **Detection Settings** profiles you'd like to use, and fill in the trial and animal information in the user-defined variables. You can use **Shift + Click** and **Control + V** to paste copied information into multiple cells.

System	System	System	System	System	User-defined	User-	User-	User-	User-define	User-d	User-	User-defi	User-def	User-de
Acquisition	Video file	Arena settings	Trial Control	Detection settings	Phase	Day	Trial	Entry	Generation	Animal	Sex	Treatment	Housing	Found
The current status of acquisition per arena	The name and path of the video used for acquisition	The arena settings used for acquisition	The trial control settings used for	The detection settings used for acquisition										
					Text	Text	Text	Tex	Text	Text	Text	Text	Text	Text
					Acquisition;	1;	1;	N;	F0; F1; F		M;	HC; RS;	SH; E	Yes;
Arena	Trial	Trial	Trial	Trial	Trial	Trial	Trial	Tri	Subject	Subjec	Subje	Subject	Subject	Trial
Planned	..._00.mpg	Acq 1	Trial 1	ACQ 1	Acquisition	1	1		F0	1	M	HC	SH	
Planned	..._01.mpg	Acq 1	Trial 1	ACQ 1	Acquisition	1	2		F0	1	M	HC	SH	
Planned	..._02.mpg	Acq 1	Trial 1	ACQ 1	Acquisition	1	3		F0	1	M	HC	SH	
Planned	..._03.mpg	Acq 1	Trial 1	ACQ 1	Acquisition	1	4		F0	1	M	HC	SH	
Planned	..._04.mpg	Acq 1	Trial 1	ACQ 1	Acquisition	2	1		F0	1	M	HC	SH	

Acquisition

When all of your **trial list** settings are in order, go to the **acquisition** screen:



Before you begin **acquisition**, it's good practice to play each video briefly to make sure the mouse is being tracked properly. Use the **play** button in the **Acquisition Control** menu, not the green **Start trial** button (marked in the image above). Be sure to return playback to the beginning of the video before you start acquisition, or Ethovision will begin acquiring data wherever you left off in the footage.

If you like, you can click on the **Show Independent Variables** option to check that the information entered in the **trial list** screen is accurate. There is also a **Track all planned trials** option that will automatically run all of the trials you have planned. However, unless you are extremely confident that there will be no issues with the footage or detection, it's best to track the trials individually. Lastly, make sure the **Detection determines speed** option is not ticked. (Unless you are processing especially long videos, it's best to analyze them in real time so that you can make sure they are being tracked properly.)

When you are ready, click **Start trial**, and the Ethovision will begin acquisition from the video. Watch the video carefully to make sure that the mouse is being tracked properly. Ethovision will notify you when it is finished, and you may then run the next trial you have planned.

If you notice an issue during acquisition, you can click the red **stop** button. This will halt acquisition and move on to the next trial you have planned out in the **trial list** screen. You can then click on **Redo trial** to clear the recording and try again from the beginning. Alternately, you can also return to the **trial list** screen later and **right-click** on the trial and **clear** it, re-enter the settings, and then run it in acquisition again once you have solved the issue.

Exporting Your Data:

When you are finished **acquiring** all of the trials that you need, select the **analysis profile** that you would like to export (**Latency to reach platform**, **Path shape**, **Distance & Time,** or **Heading**).

If you are only interested in a specific subset of the data, you can filter your results using the **Data Profiles** screen. **Right-click** on the "**All Data**" (default) profile and **duplicate** it, then **rename** it and adjust it to filter for the variables of your choice.

Under **Analysis** > **Results**, click on **Analysis Output**. In the top left corner of the screen, click on the **Calculate** button. The data should show up in the cells onscreen. You can now export your data as an Excel spreadsheet (or the format of your choice) by clicking on the **Export** button.