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Assessment of Autistic-like Behaviour of Mice Lacking ATRX in Forebrain Glutamatergic Neurons

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Abstract

Mutations in *ATRX*, which encodes a chromatin remodeling protein, have been identified in patients with autism and are associated with ATR-X syndrome, a genetic disorder characterized by cognitive deficits, developmental delays, and autistic-like behaviour. Despite the relationship between *ATRX* and autism, it is not yet known how mutations lead to autistic behaviour. To investigate this relationship, we generated two mouse models displaying distinct timing of *Atrx* inactivation in forebrain excitatory neurons. With these models, we performed behavioural paradigms to assess behaviour related to autism. Behavioural testing revealed that the conditional deletion of *Atrx* in differentiated forebrain neurons during embryogenesis leads to aggressive social behaviour, overgrooming stereotypies, and an exaggerated startle response in male mice. However, these autistic-like traits were not observed after a postnatal deletion of *Atrx* in forebrain neurons. We conclude that neuronal ATRX is required at early stages of forebrain development to suppress autistic-like behaviour in male mice.

Keywords

ATRX, autism spectrum disorder, social behaviour, aggression, stereotypies, overgrooming, startle response, pre-pulse inhibition, ATR-X syndrome

Summary for Lay Audience

ATRX is a protein that alters the way DNA is packaged in the cell during various cellular processes that are important for proper development. Mutations that reduce ATRX activity cause ATR-X syndrome, a genetic disorder that presents with cognitive impairments, various developmental delays, and autistic-like behaviour. Additionally, mutations in the *ATRX* gene have been identified in patients with autism spectrum disorder. Despite this relationship between *ATRX* and autism, it is not yet known how mutations in *ATRX* ultimately result in autistic behaviour. To begin to address this question, we generated two mouse models in which *Atrx* was deleted in the cells of the brain that transmit information, called neurons, at distinct developmental time points - either in the embryo or several weeks after birth. We then investigated if either of these mouse models display behaviour related to autism by performing behavioural tests that assess sociability, repetitive and stereotyped behaviour, and startle response to sound. Although our group previously identified learning and memory impairments in mice with *Atrx* inactivation in neurons beginning ~20 days after birth, we did not detect any autistic-like behaviour. However, early inactivation of *Atrx* in neurons in the embryo resulted in behaviour that is considered to be "autistic-like" in mice. Specifically, male mice were aggressive toward other mice and handlers, exhibited excessive self-grooming, and an exaggerated response to sound. Overall, we conclude that only an early disruption in *Atrx* gene function in neurons can cause autistic-like behaviour.

Co-Authorship Statement

I designed, conducted, analyzed, and interpreted all experiments presented in this thesis with the following exceptions:

In Chapter 3, tail rattling episodes were scored from videos of the three-chamber social test by Katherine Quesnel in Figure 3-2.

In Appendix B, Katherine Quesnel performed the brain dissections, cryosectioning, immunofluorescence staining, and imaging to validate the absence of ATRX protein in the AtrxNexCre mice.

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Abbreviations

Abbreviation	Meaning
5-ALA	5-aminolevulinic acid
αCaMKII	alpha-Ca^{2+}/calmodulin-dependent protein kinase II
µl	Microliter
ACTL6B	Actin-like protein 6B
ADD	ATRX-DNTM3-DNTM3L
ADNP	Activity-dependent neuroprotector homeobox
ANOVA	Analysis of variance
aPAR	Ancestral pseudoautosomal
ARID1B	AT-rich interactive domain-containing protein 1B
ARRIVE	Animal Research: Reporting of In Vivo Experiments
ASD	Autism Spectrum Disorder
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate synthase
ATRX	ATRX chromatin remodeler
ATR-X	Alpha-thalassemia X-Linked Intellectual Disability Syndrome
ATRXt	Truncated form of ATRX
BAF	BRG1- or BRM-associated factors
BCL11A	B-cell CLL/lymphoma 11A protein
CAD	Canadian dollars
CHD	Chromodomain-helicase-DNA-binding protein
CHD7	Chromodomain-helicase-DNA-binding protein 7
CHD8	Chromodomain-helicase-DNA-binding protein 8
CHIP-seq	Chromatin immunoprecipitation sequencing
cKO	Conditional knockout
cm	Centimeter
CNV	Copy number variation
CTCF	CCCTC-binding factor
DAXX	Death domain associated protein 6
Db	Decibel
DID	DAXX interacting domain
DNA	Deoxyribonucleic acid
DNMT3	DNA methyltransferase 3
DSB	Double-strand break
DSM	Diagnostic and Statistical Manual and Mental Disorders
EZH2	Enhancer of zeste homolog 2
F	F-statistic value
FOXP1	Forkhead box protein P1
G	Guanine
GABA	Gamma-aminobutyric acid
$GABA_AR$	GABA type A receptor
Gabrβ3	$GABA_AR$ subunit beta3
GATA	GATA binding protein 1

GC	Guanine-cytosine
H3	Histone variant H3
H3.3	Histone variant H3.3
H3K4	Histone 3 lysine 4
H3K4me0	Histone 3 lysine 4 unmethylated
H3K4me3	Histone 3 lysine 4 trimethylation
H3K9	Histone 3 lysine 9
H3K9me3	Histone 3 lysine 9 trimethylation
H3K27me3	Histone 3 lysine 27 trimethylation
HeLa	Henrietta Lacks (cervical cancer cells)
hNP	Human neuronal progenitor cells
HP1α	Heterochromatin protein 1, alpha
HR	Homologous recombination
HU	Hydroxyurea
ID	Intellectual disability
Igf2	Insulin-like growth factor 2
iPSC	Induced pluripotent stem cells
IQ	Intelligence quotient
JMJD1C	Jumonji domain containing 1C protein
kb	Kilobase
kDa	Kilodalton
KDM5C	Lysine demethylase 5C protein
LTP	Long-term potentiation
m	Minute
mc	Multiple comparison
MeCP2	Methyl-CpG-binding protein
MEF2C	Myocyte enhancer factor 2C protein
miR-137	microRNA-137
MRN	Mre11-Rad50-Nbs1
mRNA	Messenger RNA
ms	Millisecond
nBAF	Neural BAF complex
Nex	Neuronal differentiation 6
Nlgn3	Neuroligin 3
Nlgn4	Neuroligin 4
Nrxn1α	Neurexin 1 alpha
OMIM	Online Mendelian Inheritance in Man
p	Probability value
p53	Tumour suppressor protein 53
PBRM1	Protein polybromo-1
PML	Promyelocytic leukemia
PolII	RNA polymerase II
PPI	Pre-pulse inhibition
PWS-IC	Prader-Willi Syndrome imprinting center
Rad54	DNA repair and recombination protein RAD54
RNA	Ribonucleic acid
s	Second

SEM	Standard error of the mean
SFARI	Simons Foundation Autism Research Initiative
Shank	SH3 and multiple ankyrin repeat domains protein family
Shank2	SH3 and multiple ankyrin repeat domains 2 protein
Shank3	SH3 and multiple ankyrin repeat domains 3 protein
SMARCA4	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A, Member 4 protein
SMARCC1	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin Subfamily C Member 1 protein
SMARCC2	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin Subfamily C Member 2 protein
SMC1A	Structural maintenance of chromosomes 1A protein
SMC3	Structural maintenance of chromosomes 3 protein
SNV	Single-nucleotide variant
SRY	Sex-determining region Y
SWI/SNF	Switch/sucrose non-fermenting
TBR1	T-box, Brain 1 transcription factor
VNTR	Variable number tandem repeat
XCI	X chromosome inactivation
Xlr3b	X-linked lymphocyte-regulated protein 3b

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Chapter 1

1 Introduction

1.1 Autism spectrum disorder (ASD)

1.1.1 Prevalence and societal burden

Autism spectrum disorder (ASD) is a group of heterogeneous neurodevelopmental conditions characterized by difficulties in social communication and the presence of stereotyped and repetitive behaviour (Abrahams & Geschwind, 2008; Chen, Peñagarikano, Belgard, Swarup, & Geschwind, 2015; Lai, Lombardo, & Baron-Cohen, 2014). ASD is a major healthcare issue, with a population prevalence estimated to be approximately 1-2% (Rogge & Janssen, 2019). Specifically in Canada, it is estimated that among children and youth 5-17 years old, 1 in 66 have been diagnosed with ASD (Public Health Agency of Canada, 2018). Reports suggest an increase in the number of diagnosed cases of ASD, however it is unclear if this is due to a true increase of cases or improved diagnostic techniques accompanied by heightened public and medical awareness (Rogge & Janssen, 2019). The rising number of people being diagnosed with ASD, in combination with the life-long care required, makes autism a major societal concern. It is challenging to encompass all the costs related to ASD, and it is difficult to compare costs between countries due to varying health care systems and reporting methods. However, it is clear that significant costs are incurred to the individuals diagnosed with ASD, their family, and health systems (Rogge & Janssen, 2019). A Canadian public policy study estimated the annual cost of supporting an individual with ASD to be between $26,639 - $130,000 CAD, depending on the severity of symptoms (Dudley & Emery, 2014). The severity of ASD and the presence of comorbidities including intellectual disabilities (ID) contribute to increased costs. Individuals with more severe ASD and co-occurring ID require more medical care, intensive early behavioural therapy, and special education during childhood (Rogge & Janssen, 2019). Altogether, the total lifetime cost of supporting an individual with ASD in Canada is estimated to be between $1.2 million - $4.7 million CAD (Dudley & Emery, 2014).

1.1.2 Genetic etiology of ASDs

The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) defines ASD without subtypes, and places emphasis on two groups of core features: (1) difficulties in social communication and social interaction and (2) restricted and repetitive behaviour, interests, or activities (Lai et al., 2014). The latter core feature encompasses alterations in sensory processing, including hypo- or hyper-reactivity to sensory input, as well as unusual interest in sensory aspects of the environment (Lai et al., 2014). In addition to these core features of ASD, more than 70% of individuals with ASD have other co-occurring conditions. In the DSM-4, language delay was considered to be a defining feature of ASD, but is now classified as a co-occurring condition, along with intellectual disability (approximately 45%). Other common co-occurrences include tic disorders (14-38%), motor abnormalities (79%), gastrointestinal issues (9-70%), and epilepsy (8-30%). Additionally, many ASD patients experience anxiety (42-56%) and depression (12-70%), and 68% of patients exhibit aggressive behaviour, often directed towards caregivers (Lai et al., 2014). A small proportion of ASD patients (5%) have "*syndromic autism*", in which autistic traits are observed within a genetic syndrome such as Rett syndrome, Fragile X syndrome, or ATR-X syndrome (Gibbons, 2006; Lai et al., 2014). It is now well established that males are affected more frequently than females, at a 4:1 ratio (Lai et al., 2014). This sex-difference in prevalence suggests the potential for female-specific protective effects, such that females require a greater load to reach diagnostic threshold. Alternatively, there may be male-specific risks that heighten their susceptibility (Krishnan, 2018; Robinson, Lichtenstein, Anckarsäter, Happé, & Ronald, 2013). Although the etiological factors that contribute to ASD are still being explored, the majority of cases are thought to be the result of complex interactions between genetic and non-genetic (environmental) risk factors (Lai et al., 2014).

Although diagnosis is currently limited to behavioural traits, ASDs display high heritability, indicating a strong genetic cause (Hammer et al., 2015). Twin studies have demonstrated that monozygotic twins have higher concordance rates of ASD (60-90%) than dizygotic twins (0-30%; Bourgeron, 2015). Twin studies have also revealed that a large number of the genetic contributions to ASD are shared with other neurodevelopmental disorders, such as ID (>40%; Bourgeron, 2015). Moreover, recent

work has highlighted the important contribution of *de novo* variants and inherited copy number variants (CNVs) in ASD, confirming a strong genetic component (Abrahams & Geschwind, 2008; Chen et al., 2015; Sanders et al., 2013). In 10-30% of individuals with ASD, a single genetic change, including a chromosomal rearrangement, CNV, insertion and deletion, or single nucleotide variant (SNV), is penetrant enough to cause ASD (Pinto et al., 2014). Additionally, monogenic forms of the disorder, in which changes to a single gene are responsible for the ASD phenotype, are estimated to account for 10-20% of all ASD cases (Bourgeron, 2015). Recent work has focused on identifying genes and gene networks that are associated with ASD susceptibility. To date, hundreds of ASD susceptibility genes have been identified and shown to share commonalities in synaptic, transcriptional, and epigenetic mechanisms (Geschwind, 2011; Loke, Hannan, & Craig, 2015; Voineagu et al., 2011; Yang, Sau, Lai, Cichon, & Li, 2015).

1.1.3 Epigenetic contribution to ASD

Genome-wide association studies of ASD have identified mutations in numerous genes encoding proteins with epigenetic functions. These include epigenetic factors that bind directly to DNA to regulate gene expression, those involved in the exchange of methyl groups from histones or DNA, and those involved in chromatin organization and remodeling (Geschwind, 2011; Loke et al., 2015; Voineagu et al., 2011; Yang et al., 2015). Mutations in transcription factors that bind to DNA and regulate expression of genes in the developing brain, including *MEF2C*, *FOXP1*, and *TBR1,* have been implicated in ASD (Hamdan et al., 2010; Le Meur et al., 2010; McDermott, Study, Clayton-Smith, & Briggs, 2018; O ' Roak et al., 2012). Alternatively, altered expression levels of these transcription factors has also been detected in ASD patients. For example, Chien et al. (2013) performed gene expression profiling of lymphoblastoid cell lines from male patients with ASD and noted increased expression in *FOXP1* compared to the control subjects. Mutations in genes encoding histone demethylation proteins have also been associated with ASD, including *KDM5C*, a demethylase of histone 3 lysine 4 (H3K4; Adegbola, Gao, Sommer, & Browning, 2008; Tahiliani et al., 2007), and *JMJD1C*, a demethylase for histone 3 lysine 9 (H3K9; Castermans et al., 2007; Neale et al., 2012). Notably, alterations in the histone methylation landscape have been reported in human brain postmortem studies of ASD.

Disruption of the H3K4me3 landscape for several genes with known neurodevelopmental functions was observed in frontal cortex samples from ASD patients compared to controls (Shulha et al., 2012).

Proteins involved in chromatin organization and remodeling have important roles in the developing brain. As such, mutations in these proteins frequently result in neurodevelopmental disorders and ASDs (Gabriele, Lopez Tobon, D'Agostino, & Testa, 2018). For example, MeCP2 exerts a transcriptional regulatory role through global binding of methylated cytosines and regulating chromatin organization (Karaca & Brito, 2019), as well as directly interacting with histones by binding to trimethylated histone 3 lysine 27 (H3K27me3; Lee, Kim, Yun, Ohn, & Gong, 2020). Females with a deletion of *MECP2* display Rett syndrome, whereas individuals with a duplication of the *MECP2* gene develop ASD and ID (Amir et al., 1999; Ramocki et al., 2009). The MeCP2 protein partners with the X-linked alpha-thalassemia/intellectual disability protein, ATRX, and the cohesin complex to modulate higher-order chromatin structure, specifically, long-range chromatin looping (Kernohan et al., 2010). Mutations in the cohesin complex members *SMC3* and *SMC1A* cause Cornelia de Lange syndrome as well as ASD phenotypes (Deardorff et al., 2007; Iossifov et al., 2014). Similarly, mutations in the chromatin remodeling protein ATRX are associated with ATR-X syndrome as well as autism (Gibbons et al., 1995; Gibbons, Picketts, Villard, & Higgs, 1995).

Chromatin remodeling factors, such as *ATRX*, use the energy from ATP hydrolysis to move and exchange nucleosomes, allowing the alteration of DNA-nucleosome interactions. Mutations in genes encoding chromatin remodeling complex subunits appear to be a recurrent theme in neurodevelopmental disorders and ASDs (Iakoucheva, Muotri, & Sebat, 2019). Several components of the Switch/Sucrose non-fermenting (SWI/SNF) chromatin remodeling neuronal-specific BAF (nBAF) complex are encoded by genes in which rare mutations are linked to Coffin-Siris syndrome and ASD, including *ACTL6B* (BAF53b), *SMARCC1* (BAF155), *SMARCC2* (BAF170), *ARID1B* (BAF250b), *PBRM* (BAF180), *BCL11A* (BAF100A), and *SMARCA4* (BRG1; Basak et al., 2015; Halgren et al., 2012; Devlin et al., 2012; Nord et al., 2011; O ' Roak et al., 2012; Tsurusaki et al., 2012; Wenderski et al., 2020). Other SWI/SNF chromatin remodeling proteins have been

implicated in ASD, notably individuals with mutations in the *ADNP* gene are reported to display intellectual disabilities as well as autism (Helsmoortel, 2014; Vandeweyer, 2014). Finally, mutations in the CHD family of chromatin remodeling factors including *CHD8* and *CHD7* have been observed in ASD patients (Gabriele et al., 2018). Specifically, CHD8 regulates the transcription of diverse genes in the developing brain, and mutations in *CHD8* are among the highest confidence risk factors for ASD, and account for approximately 0.5% of all cases (Barnard, Pomaville, & O'Roak, 2015; Suetterlin et al., 2018). These discoveries underscore the importance of epigenetic factors in ASD, many of which influence the expression of other ASD-associated genes in the developing brain (Bourgeron, 2015; Iakoucheva et al., 2019).

A single mutation in an ASD-susceptibility gene encoding an epigenetic factor could conceivably have broad effects by directly and indirectly altering the expression of other ASD genes (Iakoucheva et al., 2019). Numerous studies have provided examples in which mutations in ASD-associated transcription factors and chromatin remodeling proteins result in broad transcriptional changes. Notwell et al. (2016) performed chromatin immunoprecipitation sequencing (ChIP-seq) for the transcription factor TBR1 during mouse cortical neurogenesis, and showed that TBR1-bound regions are enriched adjacent to ASD genes. In *Tbr1* knockout mice, several of these ASD genes were misregulated in the developing neocortex (Notwell et al., 2016). Similarly, transcriptomic analysis of the developing brain in a transgenic mouse model of *Foxp1* suggests that mutations lead to the dysregulation of ASD genes (Araujo et al., 2015). A human neuronal progenitor (hNP) cell model of *FOXP1* knockdown demonstrated that many dysregulated genes are indirectly influenced by reduction of this transcription factor (Araujo et al., 2015). This indirect regulation of ASD genes was also observed in an iPSC (induced pluripotent stem cell) model with a knockdown of *CHD8* (Sugathan et al., 2014). CHD8 has also been shown to directly bind to and regulate the expression ASD-associated genes in iPSC neuroprogenitor cells and in *Chd8* mutant mouse models (Cotney et al., 2015; Platt et al., 2017; Suetterlin et al., 2018; Sugathan et al., 2014). Interestingly, sexually dimorphic changes in ASD gene expression were observed in *Chd8* mutant mice which correlated with sexually dimorphic behavioural changes (Jung et al., 2018). The majority of misregulated ASD-associated genes identified in these studies encode proteins involved in synapse formation and

maintenance (Bourgeron, 2015; Iakoucheva et al., 2019). Therefore, the emergence of genes encoding chromatin remodeling proteins and transcription factors as prominent ASD-susceptibility genes is in part due to the ability of these proteins to influence neuronal development and synaptic plasticity (Bourgeron, 2015).

1.1.4 Investigating autistic-like behaviour in genetically modified mice

Despite the identification of numerous ASD-associated genes, the mechanisms by which impaired gene function leads to behavioural changes is unclear (Chen et al., 2015). One method to test the implications of candidate gene mutations for ASD is to generate mice with gene mutations or loss of function. Neuroanatomical and genetic similarities between mice and humans support the use of mouse models to further clarify the biological mechanisms underlying ASD (Silverman, Yang, Lord, & Crawley, 2010). As the diagnostic criteria for ASD are mainly behavioural, behavioural assays are used to phenotype ASD mouse models and identify analogs of behavioural changes associated with ASDs (Crawley, 2007; Silverman et al., 2010; Möhrle et al., 2020). Comprehensive assays have been developed to assess the core symptoms of ASD-like traits in rodents including social, and stereotyped or repetitive behaviour. Other assays were developed to investigate co-occurring symptoms such as deficits in communication, impaired learning and memory, aggressive behaviour, and anxiety (Silverman et al., 2010).

Mice are highly social animals that nest in groups, communicate with scent marks and vocalizations, and engage in other social interactions (Chen et al., 2015). Impairments in reciprocal social interactions can be measured by quantifying parameters such as sniffing, following, and other physical contact (Crawley, 2008; Silverman et al., 2010). Similarly, sociability and preference for social novelty can also be assessed by scoring the amount of time a subject mouse spends in contact with a novel mouse versus and non-social object, or a novel mouse versus and familiar mouse (Crawley, 2008; Silverman et al., 2010). During social interactions, mice are able to communicate in part through olfactory cues and vocalizations. Several behavioural tasks can be used to evaluate the olfactory and auditory cues emitted by mice (Crawley, 2007). Olfactory habituation and dishabituation tests assess the ability of mice to discriminate between the same and different odors

(Crawley, 2007; Silverman et al., 2010). Additionally, to investigate auditory cues, ultrasonic vocalizations emitted by mice in social situations can be recorded and quantified (Crawley, 2007). Repetitive behaviour is defined as normal behaviour that persist for unusually long periods of time, such as long periods of self-grooming and digging behaviour. Mice also exhibit spontaneous stereotypies including circling and jumping (Silverman et al., 2010). Finally, behavioural assays have been established to assess anxiety, aggression, learning and memory, and hyper- or hypo-reactivity to sensory stimuli (Silverman et al., 2010). Anxiety-related behaviour can be evaluated using paradigms such as open-field activity and light-dark exploration (Crawley, 2008; Crawley & Paylor, 1997). Aggressivity can be assessed in the resident-intruder test and observing aggressive tendencies of interacting males. Learning and memory are measured using object recognition tasks, spatial learning and memory tasks, contextual and cued fear learning, and working memory tasks (Crawley, 2008; Crawley & Paylor, 1997). Finally, sensitivity to auditory stimuli is assessed using acoustic startle paradigms (Valsamis & Schmid, 2011).

Mice with genetic disruptions of ASD-associated genes encoding synaptic proteins have been generated and assessed for the presence of ASD-like behaviour. Many of these models display multiple behavioural alterations that are associated with ASD (Möhrle et al., 2020). For example, mice with a complete knockout of *Nlgn4* display reduced reciprocal social interactions, low sociability, lack of social novelty, reduced aggression, and impaired ultrasonic communication (El-Kordi et al., 2013; Jamain et al., 2008). Similarly, *Nlgn3* knockout mice demonstrate reduced preference for social novelty and ultrasonic vocalizations (Radyushkin et al., 2009). Although *Nrnx1* knockout mice do not display prevalent alterations in social behaviour, they have been observed to engage in repetitive self-grooming (Etherton, Blaiss, Powell, & Sudhof, 2009). Finally, mice with a knockout of the $GABA_AR$ β3 subunit (*Gabrβ3)* display several behavioural changes associated with ASD, including low sociability, lack of social novelty, and repetitive and stereotyped behaviour (DeLorey, Sahbaie, Hashemi, Homanics, & Clark, 2008). Additionally, mice in which candidate genes encoding proteins with epigenetic functions have been disrupted also display ASD-like behaviour. For example, *Mecp2* heterozygous mice display prominent impairments in social behaviour including social avoidance, impaired social recognition, and reduced social interest (Gemelli et al., 2006; Moretti, Bouwknecht,

Teague, Paylor, & Zoghbi, 2005). Although there is high phenotypic variability between the different mouse models, this variability resembles the heterogeneity observed in individuals with ASD (Huguet, Ey, & Bourgeron, 2013). Findings of behavioural traits relevant to the symptoms of ASD in candidate gene mutant mice reinforces the interest in analyzing these genes to further understand how these genetic disruptions contribute to ASD symptoms.

1.2 The ATRX chromatin remodeling protein

1.2.1 The *ATRX* gene and protein

Mutations in the X-linked alpha-thalassemia/intellectual disability (*ATRX)* gene have been identified in individuals with autism (Aspromonte et al., 2019; Brett et al., 2014; Gong et al., 2008; Li et al., 2017; Munnich et al., 2019; Yu et al., 2013), and *ATRX* has been classified as an ASD-associated gene by the Simons Foundation Autism Research Initiative (SFARI) Gene database (https://gene.sfari.org/). The *ATRX* gene is located on the long arm of the X-chromosome (Xq21.1) and contains 36 exons distributed along approximately 300 kb of genomic DNA (Picketts, 1996). The gene was originally identified in children who develop severe cognitive disability, various developmental defects, and mild α-thalassemia (ATR-X syndrome; OMIM: 301040; Gibbons et al., 1995). The gene encodes a transcript that extends 10,488 nucleotides in length, and at least two alternatively spliced transcripts exist (Picketts, 1996). The full-length transcript encodes a large protein of 280 kDa. Alternative splicing of exon 6 results in a 265 kDa protein product (Picketts, 1996). An additional transcript results from a premature stop codon, resulting in the failure to remove intron 11, producing a truncated protein isoform of 180 kDa, referred to as ATRXt (Garrick et al., 2004).

ATRX is an ATP-dependent chromatin remodeling protein that belongs to the SWI/SNF superfamily of chromatin remodeling factors (Gibbons et al., 1995, 2008). ATRX contains two highly conserved functional domains. The first is the N-terminal ATRX-Dnmt3-Dnmt3L (ADD) domain which contains a C2-C2 GATA-like zinc finger, a plant homeodomain, followed by an alpha-helical region (Argentaro et al., 2007; Figure 1-1).

This ADD domain displays high homology to the zinc finger domain of the DNMT3 family of DNA methyltransferases, which have been shown to bind to N-terminal tails of histone H3 (Argentaro et al., 2007). Similarly, the ADD domain of ATRX targets the protein to chromatin through recognition of epigenetic marks on histone H3 tails. Specifically, the ADD domain interacts with histone H3 tails that are trimethylated at lysine 9 (H3K9me3) and unmethylated at lysine 4 (H3K4me0; Dhayalan et al., 2011). The C-terminal domain of ATRX contains a SWI/SNF domain comprised of seven highly conserved colinear helicase motifs and confers ATPase function (Gibbons, 2006; Gibbons et al., 1995; Figure 1-1). This ATPase motif of ATRX is most similar to that in Rad54, a translocase involved in transcription initiation and nucleotide excision repair (Picketts, Tastan, Higgs, & Gibbons, 1998).

ATRX is exclusively nuclear and localizes to the nuclear matrix during interphase (Bérubé, Smeenk, & Picketts, 2000). As the cell enters mitosis, ATRX is phosphorylated and localizes to pericentromeric heterochromatin through association with HP1α via a variant PxVxL motif (Bérubé et al., 2000; McDowell et al., 1999; Figure 1-1). In postmitotic neurons, ATRX is recruited to pericentromeric heterochromatin by MeCP2 via a MeCP2 binding site located within the ATPase domain (Nan et al., 2007; Figure 1-1). ATRX also localizes to the heterochromatic inactive X chromosome, as well as the pericentric heterochromatin of the Y chromosome in neonatal spermatogonia (Baumann & De La Fuente, 2009; Baumann, Schmidtmann, Muegge, & De La Fuente, 2008). ATRX is enriched at other heterochromatic repetitive sequences including ribosomal DNA (rDNA), telomeres, and pericentromeric major satellite repeats through its association with Fas death domain-associated protein (DAXX; Gibbons et al., 2000; Law et al., 2010; McDowell et al., 1999). Through a DAXX interacting domain (DID) near the center of the protein, ATRX forms a complex with DAXX (Tang et al., 2004; Figure 1-1). Together, ATRX and DAXX deposit histone variant H3.3 at these heterochromatic regions (Drané, Ouararhni, Depaux, Shuaib, & Hamiche, 2010; Lewis, Elsaesser, Noh, Stadler, & Allis, 2010). DAXX is also responsible for targeting ATRX to promyelocytic leukemia (PML) nuclear bodies; however, ATRX also contains a domain at its C-terminus that targets the protein to PML bodies independent of DAXX (Xue et al., 2003; Figure 1-1). Additionally, ATRX has been shown to specifically localize at G-rich sequences that have the propensity

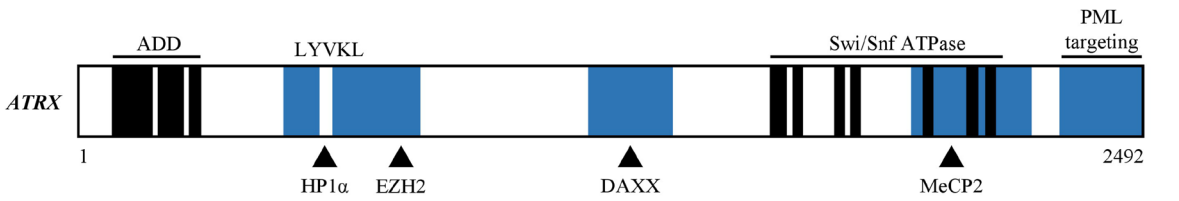

Figure 1-1 Schematic of the structure of the full length ATRX protein.

The conserved ADD and Swi/Snf ATPase domains are indicated by the black regions. Protein interaction sites, including HP1α, EZH2, DAXX, and MeCP2 interacting sites, are indicated in blue. The PML targeting site necessary for targeting ATRX to PML nuclear bodies is also indicated in blue. Modified from review article by Nathalie Bérubé (Bérubé, 2011).

to form secondary structures called G- quadruplexes, as well as RNA-DNA hybrids known as R-loops (Law et al., 2010; Nguyen et al., 2017). Finally, ATRX has been shown to interact with enhancer of zeste homolog 2 (EZH2), however, this interaction could not be confirmed in a recent study (Cardoso et al., 1998; Qadeer et al., 2019; Figure 1-1).

1.2.2 Established cellular functions of ATRX

As a chromatin remodeling protein, ATRX utilizes the energy from ATP hydrolysis to alter the position of nucleosomes and subsequently change the association between DNA and histones. Chromatin remodelers are involved in numerous cellular processes, including regulation of transcription, DNA recombination and repair, and mitotic chromosome segregation (Gibbons et al., 1995), and ATRX is no exception.

1.2.2.1 ATRX in gene regulation

ATRX has the ability to act both as an activator and a repressor of gene expression using different mechanisms. ATRX was first suspected to regulate the expression of other genes when it was observed that patients with *ATRX* mutations often present with α-thalassemia, a mild form of anemia with reduced ability to produce hemoglobin (Gibbons et al., 1995). It was eventually discovered that ATRX regulates the expression of the α-globin gene by binding to the secondary structures formed by the GC-rich variable number tandem repeat (VNTR) near the α-globin locus (Law et al., 2010). Mutations in *ATRX* result in decreased transcription of the α-globin gene, resulting in α-thalassemia in some patients (Gibbons et al., 1995).

ATRX has also been shown to localize to G-rich regions within specific gene bodies. ATRX specifically localizes at G-rich sequences that have the propensity to form bulky secondary structures called G-quadruplexes, as well as RNA-DNA hybrids known as R-loops (Law et al., 2010; Nguyen et al., 2017). In the newborn mouse brain, ATRX binds G-rich gene bodies with high predicted propensity to form G-quadruplexes and promotes H3.3 incorporation, potentially preventing the formation of these structures to promote transcriptional elongation (Levy, Kernohan, Jiang, & Bérubé, 2014). The absence of ATRX results in decreased H3.3 incorporation and increased RNA polymerase II (PolII)

occupancy, suggesting stalling of transcriptional machinery during transcriptional elongation, resulting in decreased expression of these genes (Levy et al., 2014). In the mouse brain, ATRX and H3.3 are enriched at G-rich gene regions of several pseudoautosomal (aPAR) genes, including *Nlgn4,* resulting in reduced transcript levels, pointing to ATRX transcriptional regulation of these aPAR genes through this mechanism (Levy et al., 2014).

ATRX also regulates gene transcription by acting as a co-factor for nuclear hormone receptors. In the mouse testes, ATRX is a co-factor for the nuclear androgen receptor (Bagheri-fam et al., 2011). When *Atrx* is deleted in Sertoli cells, expression of the androgen receptor-dependent gene *Rhox5* is decreased (Bagheri-fam et al., 2011). Conversely, ATRX enhances *Rhox5* promoter activity in the presence of the androgen receptor (Bagheri-fam et al., 2011). There is other evidence that ATRX may play a role in mediating nuclear hormone receptor gene activity in the brain. Ablation of ATRX in the mouse forebrain and anterior pituitary early in development results in a decrease in thyroid hormone responsive gene expression (Watson et al., 2013).

Another mechanism by which ATRX regulates gene expression is by regulating long-range chromatin interactions. ATRX was shown to interact with the cohesin complex and MeCP2 to suppress the expression of an imprinted gene network in the neonatal mouse brain (Kernohan et al., 2010; Kernohan, Vernimmen, Gloor, & Bérubé, 2014). ATRX and MeCP2 maintain a nucleosome configuration conducive to CCCTC-binding factor (CTCF) binding at the *H19* imprinting control region, thus enabling long-range chromatin looping configurations across the *H19-Igf2* imprinted locus that are required for expression control (Kernohan et al., 2014). Finally, ATRX can influence gene expression by directly regulating the expression of a microRNA in postmitotic hippocampal neurons in mice. ATRX directly binds to the promotor region of *microRNA-137* (*miR-137*; Tamming et al., 2020), a known regulator of pre- and post-synaptic processes as well as memory (Mahmoudi & Cairns, 2017). The regions of ATRX binding correspond to H3.3 occupancy and the repressive mark, H3K27me3. In the absence of ATRX, there is reduced H3K27me3 occupancy at the *miR-137* gene locus and a subsequent increase of *miR-137* expression. RNA sequencing analysis of hippocampi from mice with postnatal loss of neuronal *Atrx*

revealed changes in expression of numerous synaptic genes, some of which are directly regulated by ATRX (i.e. *Nlgn4*) and others that are downstream targets of miR-137 (i.e. *Shank2*; Tamming et al., 2020). Many synaptic genes were affected only in mutant male hippocampi and were associated with distinct hippocampal structural and synaptic defects along with impaired long-term contextual memory (Tamming et al., 2020), and reduced long-term potentiation in the Schaffer collateral and the medial perforant synaptic pathways (Gugustea, Tamming, Martin-Kenny, Bérubé, & Leung, 2019).

1.2.2.2 ATRX in DNA replication, DNA repair, and mitosis

ATRX is also implicated in DNA replication and repair. During S-phase, when DNA replication occurs, ATRX is recruited to heterochromatin and telomeres (Huh et al., 2016; Watson et al., 2013; Wog et al., 2010). ATRX loss of function results in prolonged S-phase progression and hypersensitivity to drug treatments that inhibit DNA synthesis and causes replication fork stalling (Huh et al., 2016; Leung et al., 2013; Watson et al., 2013). Specifically, ATRX-null cells exhibit defects in checkpoint activation and in replication restart. Treatment of these cells with hydroxyurea (HU) lead to increases in stalled replication forks and slow replication forks, indicating that ATRX is involved in the restart of stalled replication forks and required for DNA replication (Clynes et al., 2014; Leung et al., 2013). ATRX binds to the Mre11-Rad50-Nbs1 (MRN) complex that is essential for the restart of stalled replication forks, as well as double stranded break (DSB) repair (Clynes et al., 2014; Robison, Elliott, Dixon, & Oakley, 2004). Depletion of ATRX also results in the accumulation of DSBs at telomeres and telomere dysfunction (Huh et al., 2016; Watson et al., 2013; Wong et al., 2010).

Telomeres have large G-rich repetitive sequences that have the propensity to form secondary structures, such as G-quadruplexes (Rhodes & Lipps, 2015). Treatment of ATRX-null neuroprogenitor cells with a G-quadruplex stabilizing ligand increased levels of DNA damage, indicating that ATRX aids in the replication of telomeric G-quadruplex structures (Watson et al., 2013). It was proposed that these DNA secondary structures at telomeres persist in the absence of ATRX and form a barrier to DNA replication, leading to increased replication fork stalling (Clynes et al., 2015). Finally, ATRX functions during

homologous recombination (HR)-mediated repair of DSBs (Juhász, Elbakry, Mathes, & Löbrich, 2018). ATRX and DAXX function together to deposit histone H3.3 during extended DNA repair synthesis (Juhász et al., 2018), when sequence information is copied from the undamaged sister chromatid during HR (Wright, Shah, & Heyer, 2018). ATRX-deficient cells exhibit defects in sister chromatid exchange during HR at DSBs, leading to impaired DNA repair (Juhász et al., 2018). Therefore, it is proposed that ATRX facilitates the chromatin remodeling required for sister chromatid exchange during extended DNA repair synthesis during HR (Juhász et al., 2018).

ATRX is also required for mitotic progression in human cultured cells and in neuroprogenitors (Ritchie et al., 2008). ATRX-depleted human HeLa cells displayed aberrant cell division including a prolonged transition from prometaphase to metaphase, abnormal chromosome congression at the metaphase plate, defective sister chromatid cohesion, and more decondensed chromosomes (Ritchie et al., 2008). Not surprisingly, the loss of ATRX in mouse embryonic stem cells reduces their growth capacity, precluding the generation of a conventional knockout model (Garrick et al., 2006). Conditional early inactivation of *Atrx* at the morula stage resulted in early embryonic lethality due to placental defects (Garrick et al., 2006). A forebrain specific loss of *Atrx* resulted in an increase in cortical progenitor cell mitotic defects, replication stress, and p53-dependent apoptosis leading to a reduction of the caudal-medial cortex, loss of the dentate gyrus, and hypocellularity of several cortical layers of the neonatal forebrain (Bérubé et al., 2005, 2000; Seah et al., 2008). These results demonstrate the severe outcomes of ATRX inactivation in proliferating cells.

1.2.3 Mutations in *ATRX* cause ATR-X syndrome

The *ATRX* gene was originally identified because mutations of the gene were detected in a rare X-linked disorder that is characterized by intellectual disability, various developmental abnormalities, and α-thalassemia (ATR-X syndrome; OMIM 301040; Gibbons et al., 1995). The prevalence of ATR-X syndrome in the general population is currently unknown; however, a report from 2008 identified over 200 cases from 182 families, with 113 different associated mutations (Gibbons et al., 2008). A wide variety of

mutations occur but a large majority of them are missense hypomorphic mutations that result in a partial loss of gene function with a small proportion of remaining normal *ATRX* mRNA (<1-30%; Gibbons et al., 2008; Picketts, 1996). It is theorized that this occurs because a complete loss of ATRX may be lethal (Gibbons et al., 2008). This is further supported by the occurrence of mutation skipping in the case of nonsense mutations, allowing for phenotypic rescue (Gibbons et al., 2008). The majority of *ATRX* mutations cluster in the highly conserved ADD domain (50%) and the ATPase domain (30%; Gibbons et al., 2008), suggesting that mutations in these domains lead to reduced or impaired protein function (Picketts, 1996).

ATR-X syndrome almost exclusively affects males. Female carriers experience skewed X-inactivation and as a result are asymptomatic except for very mild hematologic changes reported in some cases (Gibbons et al., 1995). For ATR-X syndrome cases, pregnancy usually proceeds to term and birth weight is normal in 90% of cases (Gibbons & Higgs, 2000). Neonates present with microcephaly, hypotonia and associated feeding difficulties, as well as abnormal movements and seizures (Gibbons & Higgs, 2000). Throughout childhood, patients are delayed in all milestones including walking and speaking; most have no speech or are limited to few words or signs (Gibbons & Higgs, 2000). Intellectual disability is the most prevalent symptom in ATR-X syndrome patients and occurs in 95% of cases (Gibbons & Higgs, 2000). In addition to profound intellectual disabilities, there are several other common symptoms reported. In an analysis of 145 cases, it was reported that the majority experience characteristic facial (94%), skeletal (91%), and genital (80%) abnormalities. Other symptoms include gut dysmotility (75%), microcephaly (76%), short stature (65%), and seizures (35%). Less frequently reported symptoms include cardiac defects (18%) and renal or urinary abnormalities (14%; Gibbons & Higgs, 2000). Finally, 87% of cases exhibit α-thalassemia, although there is considerable variation in the severity of these hematological effects (Gibbons & Higgs, 2000). Anecdotally, it has also been reported that some patients exhibit autistic-like behaviour (Gibbons, 2006). These patients are described to show little interest or recognition of those around them and avoid eye contact (Gibbons, 2006). Some patients also exhibit repetitive stereotypic movements, such as pill-rolling or hand flapping (Gibbons, 2006). At this time, there is no cure or treatment

for ATR-X syndrome, however, management of the disorder is multidisciplinary and genetic counselling can be offered to patients and families (Gibbons, 2006).

1.2.4 *ATRX* is an autism-associated gene

A subset of ATR-X syndrome patients exhibit autistic-like behaviour such as reduced interest in those around them and avoiding eye contact (Gibbons, 2006). The recent discovery of autistic patients harboring mutations in *ATRX* have strengthened the relationship between *ATRX* and ASD (Table 1-1). Male carriers of a rare mutation in *ATRX* have been reported, and missense variants of the gene were detected in male ASD patients. Gong et al. (2008) reported two affected brothers who inherited a causative variant in *ATRX* from their unaffected mother. The latter experienced skewed X-inactivation and did not show any symptoms of ID or ASD. A similar method of inheritance was reported by Munnich et al. (2019), in which a male patient with ASD also presented with ID and hypotonia. The variant identified in this case was classified as likely pathogenic. Fitzgerald et al. (2015), as well as Yu et al. (2013), reported male autistic patients that experience co-occurring developmental disabilities associated with hemizygous missense mutations in *ATRX.* Finally, Li et al. (2017) reported another missense variant in a patient that presented with ASD and macrocephaly. These reports describe the handful of identified male patients that present with ASDs and have a causative or likely pathogenic mutation in *ATRX.* Interestingly, for studies in which female family members were included in the analysis, the female carriers of these *ATRX* mutations were asymptomatic due to skewed X-inactivation (Aspromonte et al., 2019; Gong et al., 2008; Li et al., 2017; Munnich et al., 2019; Yu et al., 2013). Notably, Brett et al. (2014) reported a female patient with ASD who also presented with developmental delays, facial abnormalities, but displayed a moderate IQ (Brett et al., 2014). A mutation in *ATRX* was identified in this case along with other inherited mutations in ASD-associated genes. The identified *ATRX* mutation is novel and was inherited from an unaffected mother. In that particular case, the phenotypic consequences of this mutation are currently unknown (Brett et al., 2014).

Table 1 ASD patients with reported mutations in *ATRX*.

Patients	Reported	Mutation	Clinical Phenotype	Additional Information
Patients #1 and #2 (brothers)	Gong et al., 2008	p.G1676A	- ASD	- Mother transmitted mutation to 2 affected sons - Mother did not have symptoms and showed XCI skewing
Patient #3	Yu et al., 2013	Missense variant 76918960T>C c.A4031G p.K1344R	- ASD - Developmental regression at 2 years - Seizures	- No additional information
Patient #4 (female)*	Brett et al., 2014	Novel variant p.P609A	- ASD - Developmental delays - Facial abnormalities - Moderate IQ	- Inherited other mutations in ASD-associated genes - Mutation is novel so the significance is unknown - Inherited mutation from mother
Patients #5 and #6 (brothers)	Fitzgerald et al., 2015	Missense variant 76939069G>C	- ASD - Learning disabilities - Delayed speech & language development - Macrocephaly - Genital deformities	- Inherited mutation from mother
Patient #7	Li et al., 2017	Missense variant c.6280G>A p.V20941	- ASD - Macrocephaly	- In this study, *ATRX* was classified as an ASD-related microcephaly-macrocephaly risk gene
Patient #8	Munnich et al., 2019	c.6740A>C p.H2247P	- ASD - ID - Hypotonia	- Mutation is classified as “likely pathogenic” - Inherited mutation from mother

*Variant is novel and phenotypic consequences are currently unknown. Variant must be studied further to determine the contribution to the clinical phenotype. ASD = autism spectrum disorder; XCI = X-chromosome inactivation; IQ = intelligence quotient; ID = intellectual disability

The identification of ASD patients harboring *ATRX* mutations strengthens the relationship between ATRX and ASD and support *ATRX* as an ASD-susceptibility gene. The SFARI Gene database provides information on genes that are associated with autism risk. Each gene in the database is assigned a score that reflects the strength of evidence linking it to the development of autism. Based on the scientific discoveries that have linked *ATRX* and ASD, *ATRX* has been classified as a Category 1 (High Confidence) gene indicating there is strong evidence that *ATRX* is implicated in autism (https://gene.sfari.org/).

1.3 Thesis Summary

Hypomorphic mutations in *ATRX* are associated with an intellectual disability syndrome known as ATR-X syndrome (Gibbons et al., 1995). Furthermore, mutations in *ATRX* have been identified in autism patients, distinguishing *ATRX* as an ASD-associated gene (Aspromonte et al., 2019; Brett et al., 2014; Gong et al., 2008; Li et al., 2017; Munnich et al., 2019; Yu et al., 2013). The Bérubé laboratory has previously demonstrated that the loss of *Atrx* in mouse forebrain alters gene expression (Levy et al., 2014; Tamming et al., 2020). Specifically, transcription of ASD-susceptibility genes, including *Nlgn4*, *Shank2*, and others encoding synaptic proteins, are altered upon the loss of *Atrx* (Levy et al., 2014; Tamming et al., 2020). In male mice with a postnatal loss of *Atrx* in excitatory forebrain neurons (AtrxCaMKIICre model), these transcriptional changes are associated with synaptic ultrastructural defects and impaired LTP (Gugustea et al., 2019; Tamming et al., 2020). Additionally, these mice display hippocampal-dependent long-term memory deficits (Tamming et al., 2020). However, it is unclear whether these mice exhibit any ASD-like traits. I hypothesized that the loss of *Atrx* in forebrain excitatory neurons would result in the development of autistic-like behaviour.

In order to address this hypothesis, we characterized the impact of *Atrx* loss in forebrain excitatory neurons on ASD-like traits in AtrxCaMKIICre mice. The results indicate that neither male or female mutant mice exhibit alterations in social behaviour, startle responses, or the presence of stereotypies. To address whether an earlier inactivation of *Atrx* would have an alternate outcome, we also investigated the presence of ASD-like behaviour in the AtrxNexCre model, in which *Atrx* is deleted post-mitotically in dorsal

telencephalic neurons that develop into cortical and hippocampal excitatory neurons. Indeed, this earlier inactivation of *Atrx* in non-proliferating cells led to behaviour associated with ASD in male mice, including self-grooming stereotypies, an exaggerated startle response, and aggressive social behaviour. These findings suggest that *Atrx* inactivation in neurons must occur early during brain development to instigate behavioural changes related to ASD in adulthood. Overall, this study reveals how the timing of *ATRX* dysfunction dictates the behavioural outcomes related to ASD.

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Chapter 2

2 Effects of a postnatal *Atrx* conditional knockout in neurons on autism-like behaviours in male and female mice

Based on emerging evidence of *ATRX* as an autism-associated gene, we sought to determine if a conditional loss of *Atrx* in forebrain excitatory neurons in mice would result in autistic-like behaviour. Despite sexually dimorphic changes observed in olfaction, we did not observe alterations in social behaviour. Additionally, we did not detect the presence of digging, grooming, or vertical episode stereotypes. Finally, we did not identify changes in startle responses or pre-pulse inhibition to an acoustic stimulus in either male or female mice. Overall, the postnatal knockout of *Atrx* in forebrain excitatory neurons did not lead to autism-related behaviour in male or female adult mice.

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2.1 Introduction

Autism spectrum disorder (ASD) is a behaviourally defined condition characterized by deficits in social and communicative abilities, impaired sensory gating, as well as the presence of stereotyped behaviours (Abrahams & Geschwind, 2008; Chen et al., 2015). Recent work has highlighted the important contribution of *de novo* variants and inherited copy number variants in ASD, confirming a strong genetic component of this disease (Abrahams & Geschwind, 2008; Chen et al., 2015; Sanders et al., 2013). Numerous autism susceptibility genes have been identified and shown to share commonalities in synaptic, transcriptional, and epigenetic mechanisms (Geschwind, 2011; Loke et al., 2015; Yang et al., 2015). Mouse models have typically been used to investigate the behavioural implications of genetic mutations associated with ASD (Crawley, 2008; Silverman et al., 2010). However, these studies often omit the investigation of the sex-specific effects of these genetic mutations, limiting the potential translational applications. In the general population, ASD occurs at a 4:1 male:female ratio, highlighting the need to study the

outcome of genetic mutations in both male and female model systems (Abrahams & Geschwind, 2008; Chen et al., 2015).

In this study, we describe the impact of targeted inactivation of *Atrx* in glutamatergic neurons on behaviours related to autism in male and female mice. ATRX belongs to the SWI/SNF family of chromatin remodeling factors (Gibbons, Picketts, Villard, & Higgs, 1995; Gibbons et al., 2008). Mutations in the *ATRX* gene are associated with an intellectual disability syndrome referred to as ATR-X syndrome, characterized by autistic-like behaviours in addition to cognitive deficits, intellectual disabilities, and developmental delays (Gibbons, 2006). Furthermore, autistic carriers of rare mutations in *ATRX* have been discovered and missense variants in *ATRX* have been identified in male ASD patients. Interestingly, female carriers of *ATRX* mutations experience skewed X-inactivation, and as a result, are asymptomatic (Aspromonte et al., 2019; Brett et al., 2014; Gong et al., 2008; Li et al., 2017; Munnich et al., 2019; Yu et al., 2013).

Previous studies have demonstrated that the loss of *Atrx* in mouse forebrain causes changes in gene expression (Kernohan et al., 2010; Levy et al., 2014). Specifically, transcription of autism susceptibility genes, including the monogenic *Neuroligin-4* (*Nlgn4)*, are altered upon loss of *Atrx* (Levy et al., 2014). Additionally, sexually dimorphic transcript changes have been revealed in the adult mouse hippocampus upon the loss of *Atrx* in excitatory neurons (Tamming et al., 2020). However, autistic behaviours were not evaluated in that report, and should be addressed given the link between *ATRX* mutations and ASD.

In this study, we characterize the impact of *Atrx* loss in neurons on autistic-like behaviours in male and female mice. We used the AtrxCamKIICre model (Tamming et al., 2020) where *Atrx* is ablated in forebrain excitatory neurons postnatally, thus bypassing deleterious effects of ATRX loss-of-function previously observed in neural progenitors during brain development (Bérubé et al., 2005; Watson et al., 2013). An array of behavioural assays was performed to investigate the presence of autistic-like behaviours, including deficits in sociability, altered sensory gating, and the presence of repetitive or stereotyped behaviours. These investigations revealed minimal behavioural deficits related to autism in both male and female mice. Interestingly, we identified changes in olfaction,

particularly odor discrimination, in both male and female mice upon the conditional loss of *Atrx* in neurons. Overall, this study demonstrates that a conditional loss of *Atrx* in forebrain excitatory neurons postnatally does not result in typical autistic-like traits in male or female mice.

2.2 Materials and Methods

2.2.1 Animal Care and Husbandry

Mice were exposed to a 12-hour-light/12-hour-dark cycle and with water and chow ad libitum. The *Atrx*^{loxP} females (129/Sv background) have been described previously (Bérubé et al., 2005). *Atrx*^{loxP} mice were mated with C57BL/6 mice expressing Cre recombinase under the control of the *αCaMKII* gene promoter (Tsien et al., 1996). The progeny includes hemizygous male mice that produce no ATRX protein in forebrain excitatory neurons (*Atrx*-cKO^{MALE}). The *Atrx*-cKO males were mated to *Atrx*^{loxP} females to yield homozygous deletion of *Atrx* in female mice (*Atrx*-cKO^{FEMALE}). Male and female littermate floxed mice lacking the Cre allele were used as controls (Ctrl^{MALE}; Ctrl^{FEMALE}). Consequently, male and female mice are from different hybrid generations. Control littermates from the same hybrid generation as the corresponding conditional knockout mice were used for behavioural assays. Genotyping of tail biopsies for the presence of the floxed and Cre alleles was performed as described previously (Bérubé et al., 2005). Conditional loss of the ATRX protein in postnatal neurons was previously verified in adult *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} forebrain (Tamming et al., 2020). All procedures involving animals were conducted in accordance with the regulations of the Animals for Research Act of the province of Ontario and approved by the University of Western Ontario Animal Care and Use Committee (2017-048). Behavioural assessments started with less demanding tasks and moved to more demanding tasks in the following order: open field test, marble-burying assay, induced self-grooming, pre-pulse inhibition (PPI) and startle response, social approach, and 3-chamber social tests. ARRIVE guidelines were followed: mouse groups were randomized, experimenters were blind to the genotypes, and software-based analysis was used to score mouse performance in all the tasks. All behavioural tasks were performed between 9:00 AM and 4:00 PM. All behavioural assays were performed when mice were

between 3-7 months of age. 3 cohorts of male and female mice were used to reach the final sample size ($Ctrl^{MALE}$: 17; *Atrx*-cKO^{MALE}: 10; $Ctrl^{FEMALE}$:13; *Atrx*-cKO^{FEMALE}: 13). Statistics were calculated by two-way repeated measures ANOVA with Sidak's multiple comparison test or unpaired Student's T-tests, as indicated in the figure legends.

2.2.2 Odor Habituation and Discrimination

The odor habituation and discrimination assay was performed as previously described (Arbuckle, Smith, Gomez, & Lugo, 2015) to assess olfaction. Individual mice were placed into a clean cage with a wire lid and allowed to habituate to the testing room for 30 minutes. The mice were then presented with an odor on a cotton swab (either almond, banana, or water as a control) for a 2-minute trial. For each trial, 50µl of water, almond extract, or banana extract (Club House) was pipetted onto the tip of a cotton swab and the swab was then secured to the wire cage top through the water bottle opening. The mice were presented with the same odor three times before being presented with a new odor, for a total of nine trials. During the 2-minute trials, the amount of time that the mouse spent sniffing the odor was recorded by an investigator blind to the genotype. Sniffing was defined as the animal's nose being in proximity to the cotton swab (2cm or closer), and oriented toward the swab.

2.2.3 Social Approach

This test was performed as previously described (Jamain et al., 2008) to assess for sociability with conspecific mice. For two consecutive days prior to the test day, individual mice were habituated to the open area for 10 minutes. On the test day, pairs of unfamiliar, same-sex conspecific mice were placed into the cage. Behaviour of the mice was recorded by the AnyMaze software and video-tracking system. The time spent in social interaction, defined as the experimental mouse sniffing the stranger mouse, was manually scored by investigators unaware of the genotype.

2.2.4 3-Chamber Social Tests (Social Preference and Novelty)

The social preference and social novelty assessments were performed as described (El-Kordi et al., 2013; Jamain et al., 2008) with minor modifications. Individual mice were placed in the 3-chambered box and allowed to freely explore the arena during a 10-minute habituation period. After the habituation period, an unfamiliar, same-sex mouse of a different genotype (stranger 1) was placed in one of the side chambers under a wire cage. An identical wire cage containing an inanimate object was placed in the opposite chamber. The test mouse was then allowed to explore the entire 3-chambered arena for 10 minutes. The amount of time spent in each chamber was recorded by the AnyMaze video-tracking system. Following this period, a second unfamiliar, same-sex mouse of a different genotype (stranger 2) was placed into the wire cage previously containing the inanimate object. The test mouse was then allowed to explore the 3-chambered arena for 10 minutes. The amount of time spent in each chamber was recorded by the AnyMaze video-tracking system. Based on the amount of time spent in each chamber, a 'sociability index' and a 'social novelty index' was calculated as previously described (El-Kordi et al., 2013). The sociability index was calculated as: $time_{stranger}/(time_{stranger}+time_{object})$ x 100. The social novelty index was calculated as: $time_{novel}/(time_{novel}+time_{familiar})$ x 100.

2.2.5 Marble Burying

The test was performed as previously described (Deacon, 2006) with modifications to evaluate repetitive digging behaviour. Mice were brought into the test room to habituate in their home cages for approximately 30 minutes prior to the test. The test cages were filled with 4 cm of wood-chip bedding, with 12 evenly spaced glass marbles placed on the surface. Individual mice were then placed in the test cage and permitted to explore for 30 minutes. Following the test, the number of marbles buried (>3/4 surface covered) was counted and recorded by investigators blind to the genotype.

2.2.6 Induced Self-Grooming

The test was performed as previously described (El-Kordi et al., 2013; Kalueff, Wayne Aldridge, Laporte, Murphy, & Tuohimaa, 2007) to evaluate repetitive grooming

tendencies. Mice were individually habituated in an empty test cage for 30 minutes prior to the test. To amplify natural grooming tendencies, mice were misted with water 3 times at 10 cm distance of the upper-back. Following this misting, the grooming behaviour of each mouse was recorded by the Anymaze video-tracking system for 30 minutes. The time that each individual mouse spent grooming during this 30-minute trial was manually scored by the rater, unaware of the genotype.

2.2.7 Open Field Test

Mice were brought into the testing room to habituate in their home cages approximately 30 minutes prior to the test. Mice were placed in a 20 cm x 20 cm arena with 30 cm high walls. Locomotor activity was automatically recorded in 5-minute intervals over 2 hours (AccuScanInstrument)(Tamming et al., 2017). For each mouse the number of vertical episodes was assessed.

2.2.8 Pre-Pulse Inhibition and Startle Response

The pre-pulse inhibition and startle response tests were performed as previously described (Valsamis & Schmid, 2011) to assess sensory gating. Mice underwent two days of habituation prior to the testing day, to acclimate the mice to the apparatus. During this habituation, mice were individually placed in the chamber apparatus and exposed to background noise (65 db) for 5 minutes (SR-LAB, San Diego Instruments). On the test day, individual mice were placed in the chamber and acclimated for 10 minutes with background noise. The mice then underwent a habituation block, consisting of fifty acoustic startle trials, with 20 ms stimulus of 115 db, and intertrial interval of 20 seconds. After the habituation block, mice underwent a pre-pulse inhibition block consisting of ten sets of five types of trials randomly ordered with variable intertrial intervals of 10, 15, or 20 seconds. Four of the five trial types consisted of pre-pulses (intensity of 75 or 80 db, length of 20 ms), separated from the startle stimulus (intensity of 115 db, length of 40 ms) by an interstimulus interval of either 30 ms or 100 ms. The fifth trial type was a startle pulse alone. The startle response was measured by the movement of the mouse on the platform, which generates a transient force analyzed by the software. The startle magnitude

recorded was an average for the ten trials of each trial type and startle magnitudes of pre-pulse trials were normalized to the pulse-only trial.

2.3 Results

2.3.1 Sexually dimorphic olfaction differences in *Atrx*-cKO mice

As olfactory impairments can confound the interpretation of other tests, especially social behaviour assays, we first wanted to address whether the loss of *Atrx* in excitatory neurons of the forebrain alters olfaction in male and female mice. To do this, we performed the odor discrimination and habituation assay (Arbuckle et al., 2015). In this test, mice were presented with multiple odors for 2-minute trials, during which the amount of time spent sniffing the odor was recorded. During this test, *Atrx*-cKO^{MALE} mice spent significantly less time sniffing the odors throughout the nine trials compared to Ctrl^{MALE} mice (ANOVA, **p=0.004; Fig.2-1A). In particular, *Atrx*-cKO^{MALE} mice spent significantly less time sniffing the cotton swab when first presented with the banana odor (multiple comparisons, ***p<0.001). There was no significant difference in the overall amount of time spent sniffing the odors throughout the test between *Atrx*-cKO^{FEMALE} and Ctrl^{FEMALE} mice. However, *Atrx*-cKO^{FEMALE} mice did spend significantly more time sniffing the cotton swab when first presented with the banana odor (multiple comparison, ****p<0.0001; Fig. 2-1B). Overall, the results of this test suggest that the loss of *Atrx* in forebrain excitatory neurons results in sexually dimorphic changes in olfaction that must be considered in subsequent behaviour testing of these mice.

2.3.2 Social assays reveal sex-differences, but not genotypic-differences, in *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice

Given that *ATRX* mutations are associated with autistic traits in humans, we next sought to investigate if the loss of *Atrx* in forebrain excitatory neurons has an effect on social behaviour. Changes in sociability and social preference are some of the most common deficits observed in mouse models with autism-associated genetic mutations (Courchet et al., 2018; El-Kordi et al., 2013; Jamain et al., 2008; Nuytens et al., 2013; Tong et al., 2019).

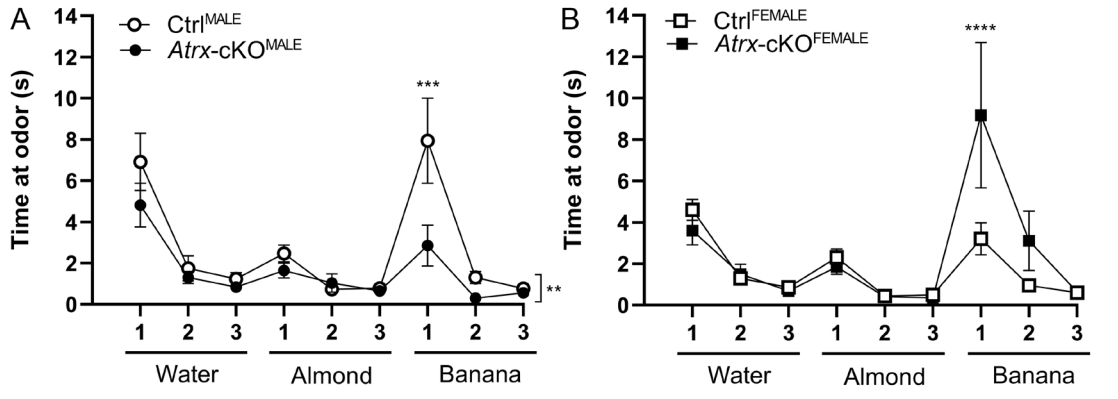

Figure 2-1 *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice exhibit differences in olfaction during the odor habituation and discrimination assay.

A) The amount of time spent sniffing a cotton swab saturated with an odor during nine, 2-minute trials. *Atrx*-cKO^{MALE} (n=24) mice spend less time sniffing cotton swabs with corresponding odors compared to Ctrl^{MALE} (n=17) (twANOVA, $F_{(1,351)}$=8.203, **p=0.004; mc, ***p<0.001). B) *Atrx*-cKO^{FEMALE} (n=19) mice spend more time sniffing the cotton swab with the banana odor when first exposed to the scent compared to Ctrl^{FEMALE} (n=15) (twANOVA, $F_{(1,288)}$=3.188, p=0.075; mc, ****p<0.0001). Error bars: ± SEM.

As such, we first investigated sociability of *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice by means of the social approach assay, as described previously (Jamain et al., 2008). There was no significant difference in the total amount of time that *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice spent interacting with a stranger mouse compared to controls (Fig. 2-2A). However, when these results were grouped and analyzed by sex, male mice (*Atrx*-cKO^{MALE} and Ctrl^{MALE}) spent more time socially interacting with the stranger mouse compared to female mice (*Atrx*-cKO^{FEMALE} and Ctrl^{FEMALE}) (ANOVA, *p=0.048; Fig 2-2A). When social interaction was analyzed over one-minute intervals during the 10-minute test, there were no genotypic or sex-differences (Fig. 2-2B).

We also investigated social preference and social novelty in the three-chambered paradigm (El-Kordi et al., 2013). During the first part of the paradigm, social preference was assessed as mice were placed into a three-chambered apparatus and were free to explore between the chambers. The outer two chambers contained either a novel object or a stranger mouse, while the centre chamber remained empty. Both Ctrl^{MALE} and *Atrx*-cKO^{MALE} mice demonstrated a preference for the chamber containing a stranger mouse compared to the object and the empty chamber (multiple comparisons, ****p<0.0001; Fig. 2-2C). Similarly, both Ctrl^{FEMALE} and *Atrx*-cKO^{FEMALE} mice preferred exploration of the stranger mouse (multiple comparisons, *p=0.030, ****p<0.0001; Fig. 2-2D). No genotypic differences were observed in social preference between groups (Fig. 2-2C-D). Social novelty was investigated during the second part of the paradigm in which the outer chambers contained either the stranger mouse from the first part of the test (familiar mouse) or a novel mouse. Ctrl^{MALE} and *Atrx*-cKO^{MALE} mice both spent more time in the chamber containing the novel mouse compared to the familiar mouse and the empty chamber (multiple comparisons, ***p<0.001, ****p<0.0001; Fig. 2-2E). Interestingly, although Ctrl^{FEMALE} and *Atrx*-cKO^{FEMALE} mice both spent significantly less time in the empty chamber, neither demonstrated a preference for the novel mouse over the familiar mouse (multiple comparisons, ***p<0.001; Fig. 2-2F). Sociability and social memory indexes were calculated based on the social preference and social novelty results. *Atrx*-cKO and Ctrl mice did not display genotypic or sex-differences in their sociability indexes (Fig. 2-2G). Similarly, there was no genotypic difference in social memory indexes, however, male mice (Ctrl^{MALE} and *Atrx*-cKO^{MALE}) displayed a greater social memory index than female

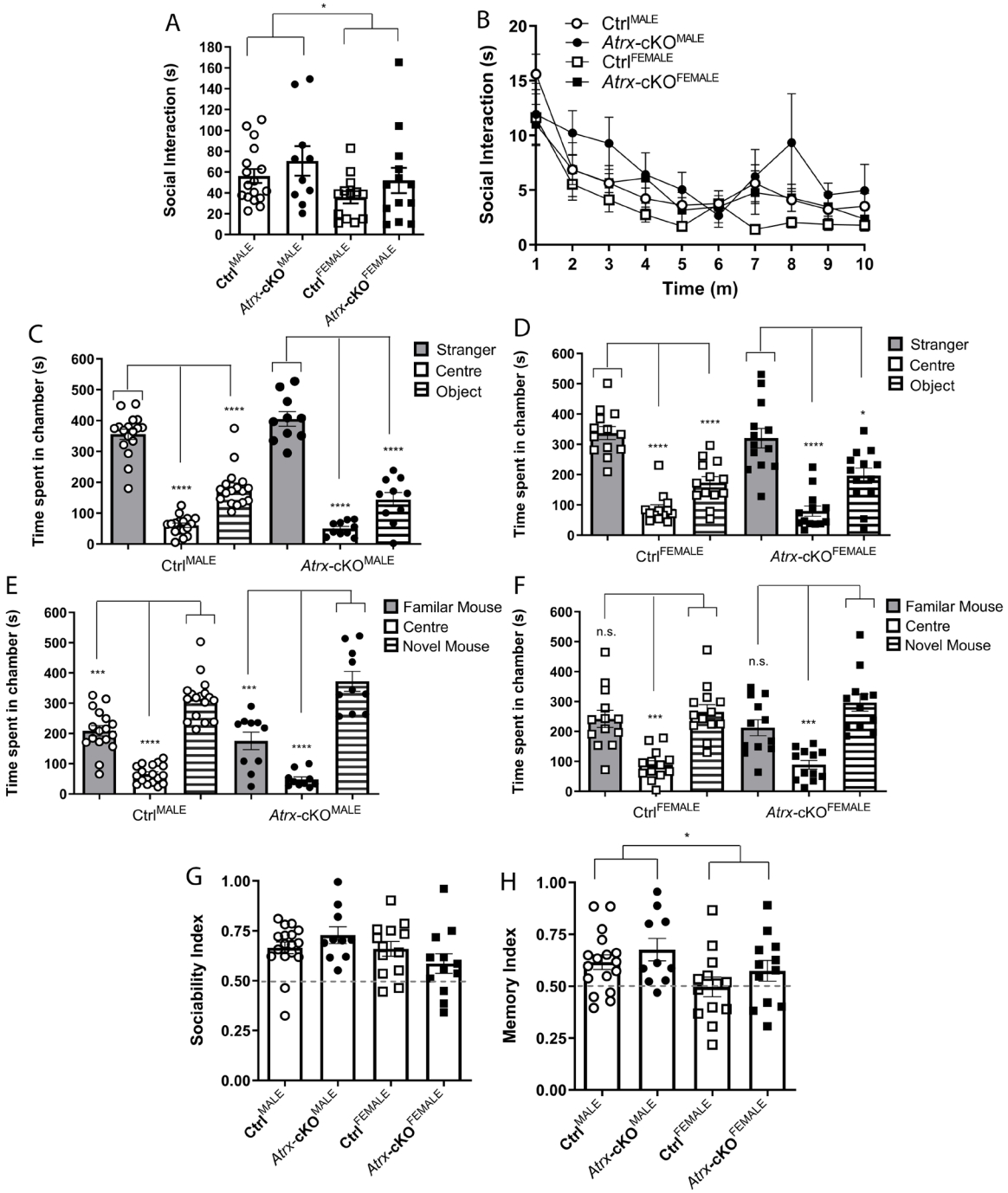
A
Social Interaction (s)
CtrlMALE
Atrx-cKOMALE
CtrlFEMALE
Atrx-cKOFEMALE
B
CtrlMALE
Atrx-cKOMALE
CtrlFEMALE
Atrx-cKOFEMALE
Social Interaction (s)
Time (m)
C
Time spent in chamber (s)
Stranger
Centre
Object
CtrlMALE
Atrx-cKOMALE
D
Time spent in chamber (s)
Stranger
Centre
Object
CtrlFEMALE
Atrx-cKOFEMALE
E
Time spent in chamber (s)
Familar Mouse
Centre
Novel Mouse
CtrlMALE
Atrx-cKOMALE
F
Time spent in chamber (s)
Familar Mouse
Centre
Novel Mouse
n.s.
CtrlFEMALE
Atrx-cKOFEMALE
G
Sociability Index
CtrlMALE
Atrx-cKOMALE
CtrlFEMALE
Atrx-cKOFEMALE
H
Memory Index
CtrlMALE
Atrx-cKOMALE
CtrlFEMALE
Atrx-cKOFEMALE

Figure 2-2 Social behaviour assays reveal lack of genotypic difference between *Atrx*-cKO and control mice.

A) Total amount of social interaction with a mouse conspecific for genotype and sex during the 10-minute social approach assay (twANOVA, genotype, $F_{(1,49)}$=2.496, p=0.121; twANOVA, sex, $F_{(1,49)}$=4.086, *p=0.048). B) Social interaction over 1-minute intervals during the social approach assay (twANOVA, genotype, $F_{(1,51)}$=1.489, p=0.228; twANOVA, sex, $F_{(1,51)}$=3.425, p=0.070). C) Amount of time $Ctrl^{MALE}$ (twANOVA, $F_{(2,32)}$=78.39, ****p<0.0001; mc, ****p<0.0001) and *Atrx*-cKO^{MALE} (twANOVA, $F_{(2,18)}$=58.26, ****p<0.0001; mc, ****p<0.0001) spent in the empty centre chamber and chambers containing either a stranger mouse or novel object in the social preference assay. D) Amount of time $Ctrl^{FEMALE}$ (twANOVA, $F_{(2,24)}$=31.39, ****p<0.0001; mc, ****p<0.0001) and *Atrx*-cKO^{FEMALE} (twANOVA, $F_{(2,24)}$=14.88, ****p<0.0001; mc, *p<0.05, ****p<0.0001) spent in chambers containing either a stranger mouse or novel object, or the empty centre chamber E) Time spent in the empty centre chamber and chambers containing a familiar mouse or a novel mouse in the social novelty assay for E) $Ctrl^{MALE}$ (twANOVA, $F_{(2,32)}$=51.38, ****p<0.0001; mc, ***p<0.001, ****p<0.0001) and *Atrx*-cKO^{MALE} mice (twANOVA, $F_{(2,18)}$=27.26, ****p<0.0001; mc, ***p<0.001, ****p<0.0001), and F) $Ctrl^{FEMALE}$ (twANOVA, $F_{(2,24)}$=11.77, ***p<0.001; mc, ***p<0.001) and *Atrx*-cKO^{FEMALE} mice (twANOVA, $F_{(2,22)}$=12.50, ***p<0.001; mc, ***p<0.001). G) The sociability index for each mouse was calculated as the time spent in the stranger mouse chamber, divided by the total time in the stranger mouse and novel object chambers (twANOVA, genotype, $F_{(1,28)}$=0.019, p=0.890; twANOVA, sex, $F_{(1,48)}$=3.574, p=0.065). H) The social memory index for each mouse was calculated as the time spent in the novel mouse chamber, divided by the total time in the familiar mouse and novel mouse chambers (twANOVA, genotype, $F_{(1,48)}$=2.217, p=0.143; twANOVA, sex, $F_{(1,48)}$=5.811, *p=0.019). *Atrx*-cKO^{MALE}: n=10, $Ctrl^{MALE}$: n=17, *Atrx*-cKO^{FEMALE}: n=13, $Ctrl^{FEMALE}$: n=13. Error bars: ± SEM.

mice ($Ctrl^{FEMALE}$ and *Atrx*-cKO^{FEMALE}) (ANOVA, *p=0.019; Fig. 2-2H). Altogether, these results demonstrate that the loss of *Atrx* in forebrain excitatory neurons postnatally does not result in social deficits in male and female mice.

2.3.3 Repetitive behaviours are not altered in *Atrx*-cKO^{MALE} and *Atrx* cKO^{FEMALE} mice

Previous studies using autism mouse models have demonstrated that the mutant mice often present with repetitive and stereotyped behaviours (Burrows et al., 2015; Cheng et al., 2018; El-Kordi et al., 2013; Jung et al., 2018; Nuytens et al., 2013). We tested for the presence of these repetitive and stereotyped behaviours in both *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice using various tests. The marble-burying assay was used to assess repetitive burying and digging by placing mice in a cage with 12 marbles and recording how many marbles were buried following a 30-minute period. Percentage of marbles buried during the marble-burying assay was not significantly different when comparing *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice to their respective controls. There also was no difference when comparing between genotypes or sexes. However, the interaction was significantly different between groups, suggesting the loss of *Atrx* in forebrain excitatory neurons has opposing effects on marble burying when comparing male and female mice (ANOVA, *p=0.047; Fig. 2-3A).

We also investigated the presence of repetitive grooming tendencies by misting mice with water to induce grooming behaviours. The total amount of time spent grooming during the 30-minute induced self-grooming assay was not significantly different between *Atrx*-cKO mice and controls, or between sexes (Fig. 2-3B). When the results of this test were analyzed over 5-minute intervals, similarly, there was no difference in the amount of time spent grooming between *Atrx*-cKO^{MALE} and $Ctrl^{MALE}$ mice (Fig. 2-3C) or *Atrx*-cKO^{FEMALE} and $Ctrl^{FEMALE}$ mice (Fig. 2-3D). Interestingly, results from the open field test show a significant increase in the number of vertical episodes (including rearing and jumping) of female mice (*Atrx*-cKO^{FEMALE} and $Ctrl^{FEMALE}$ mice) compared to male mice (*Atrx*-cKO^{MALE} and $Ctrl^{MALE}$) (ANOVA, ****p<0.0001; Fig. 2-3E). When these results were analyzed in 10-minute intervals, it is apparent that these sex-differences in vertical episodes

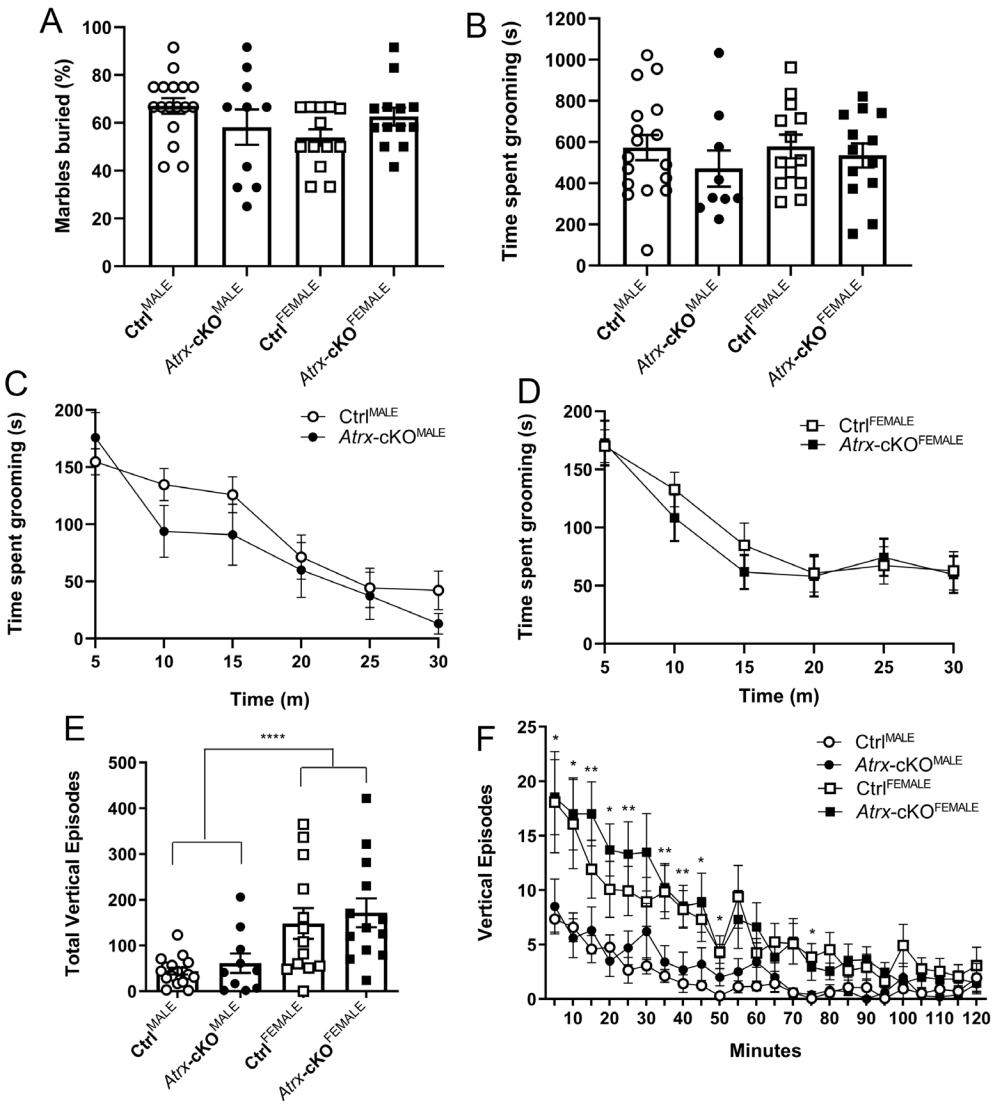
A
Marbles buried (%)
100
80
60
40
20
0
Ctrl MALE
Atrx-cKO MALE
Ctrl FEMALE
Atrx-cKO FEMALE
B
Time spent grooming (s)
1200
1000
800
600
400
200
0
Ctrl MALE
Atrx-cKO MALE
Ctrl FEMALE
Atrx-cKO FEMALE
C
Time spent grooming (s)
200
150
100
50
0
Ctrl MALE
Atrx-cKO MALE
5
10
15
20
25
30
Time (m)
D
Time spent grooming (s)
200
150
100
50
0
Ctrl FEMALE
Atrx-cKO FEMALE
5
10
15
20
25
30
Time (m)
E
Total Vertical Episodes
****
500
400
300
200
100
0
Ctrl MALE
Atrx-cKO MALE
Ctrl FEMALE
Atrx-cKO FEMALE
F
Vertical Episodes
25
20
15
10
5
0
Ctrl MALE
Atrx-cKO MALE
Ctrl FEMALE
Atrx-cKO FEMALE
10
20
30
40
50
60
70
80
90
100
110
120
Minutes

Figure 2-3 *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice do not display stereotyped behaviours.

A) Percentage of marbles buried during 30-minute marble burying task (twANOVA, genotype, $F_{(1,49)}$=0.0003, p=0.985; twANOVA, sex, $F_{(1,49)}$=1.038, p=0.313; twANOVA, interaction, $F_{(1,49)}$=4.132, *p=0.047). B) Total time grooming during 30-minute water-induced grooming task (twANOVA, genotype, $F_{(1,48)}$=1.200, p=0.279; twANOVA, sex, $F_{(1,48)}$=0.268, p=0.607). C-D) Amount of time spent grooming over 5-mintue intervals during water-induced grooming task (twANOVA, males, $F_{(1,44)}$=2.386, p=0.125; twANOVA, females, $F_{(1,44)}$=0.562, p=0.455). E) Total number of vertical episodes during 120-minute open field test (twANOVA, genotype, $F_{(1,49)}$=0.673, p=0.416; twANOVA, sex, $F_{(1,49)}$=18.71, ****p<0.0001). F) Number of vertical episodes over 10-minute intervals during the 120-minute open field test (twANOVA, genotype, $F_{(1,51)}$=0.866, p=0.356; twANOVA, sex, $F_{(1,51)}$=19.19, ****p<0.0001; mc, *p<0.05, **p<0.01). *Atrx*-cKO^{MALE}: n=10, Ctrl^{MALE}: n=17, *Atrx*-cKO^{FEMALE}: n=13, Ctrl^{FEMALE}: n=13. Error bars: ± SEM.

occurred primarily within the first 60 minutes of the open-field test (multiple comparisons; *p<0.05, **p<0.01; Fig. 2-3F). In addition to these sex-differences, there was no genotypic difference between the total vertical episodes or the vertical episodes over time. Similarly, there were no significant differences between *Atrx*-cKO mice and controls when analyzing vertical episodes. These behavioural analyses suggest that the loss of *Atrx* in forebrain excitatory neurons postnatally does not result in repetitive or stereotyped behaviours typically associated with autism.

2.3.4 *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice display typical startle responses to acoustic stimuli

Previous studies have reported that rodent models with autism-associated genetic mutations can display an exaggerated startle response, or impaired pre-pulse inhibition, to an acoustic stimulus (Crawley, 2007; Jiang et al., 2018; Luo, Norris, Gordon, & Nithianantharajah, 2018; Scott et al., 2018). These impairments are associated with deficits in sensory gating and auditory processing often reported in ASD patients (Ebishima et al., 2019; Takahashi & Kamio, 2018). As such, we wanted to investigate if *Atrx*-cKO mice display hypersensitivity to an acoustic startle stimulus. The pre-pulse inhibition and startle response assay using an acoustic stimulus was performed, as described previously (Valsamis & Schmid, 2011). Mice were placed in a chamber and exposed to fifty trials of an acoustic stimulus (20 ms, 115 db). *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice demonstrated a similar startle response to the acoustic stimuli compared to their respective controls (Fig. 4-2 A-B). Additionally, there was no significant difference in startle responses when comparing genotypes (*Atrx*-cKO vs. Ctrl). Notably, there was a significant increase in the startle response of male mice (*Atrx*-cKO^{MALE} and Ctrl^{MALE}) compared to female mice (*Atrx*-cKO^{FEMALE} and Ctrl^{FEMALE}) (ANOVA, *p<0.023).

We also performed a set of trials that investigated pre-pulse inhibition to the acoustic stimulus by first exposing mice to a pre-pulse that preceded the acoustic stimulus. These trials varied in the intensity of the pre-pulse (75 db or 80 db), and the amount of time between the pre-pulse and the acoustic “pulse” stimulus (30 ms or 100 ms). Additionally, there was one trial that only involved a “pulse” without a pre-pulse. Startle responses to

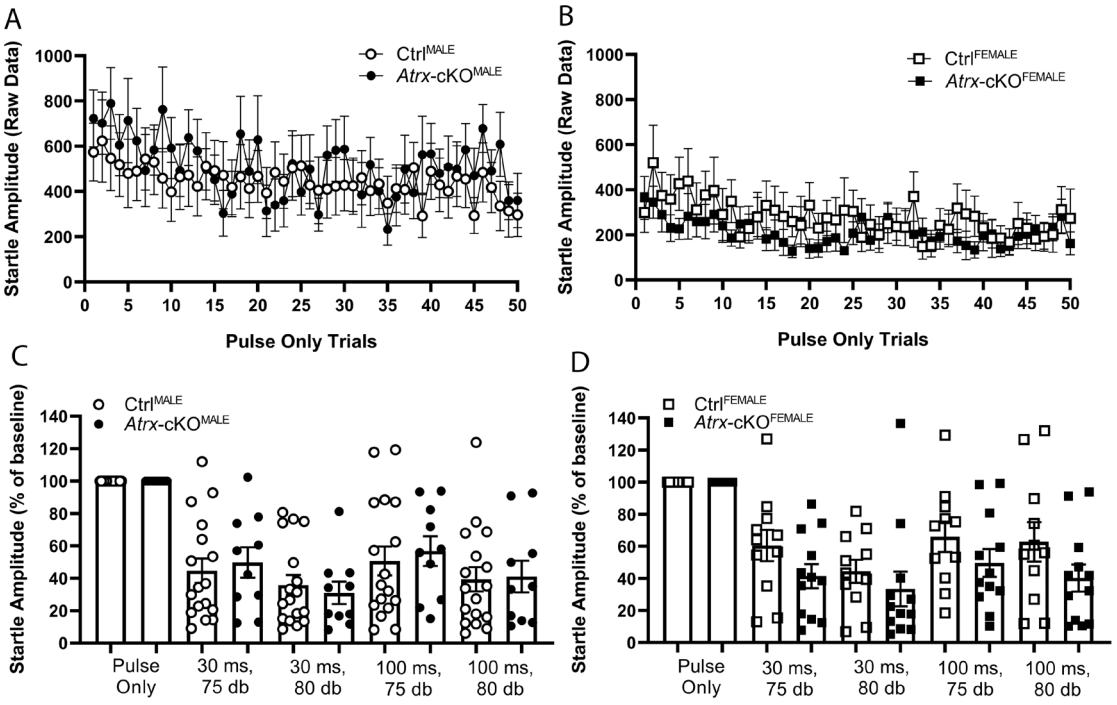

Figure 2-4 Acoustic startle response of *Atrx*-cKO mice is typical.

A-B) Quantification of startle responses (recorded in millivolts) to fifty "pulse only" trials (twANOVA, genotype, $F_{(1,48)}$=0.084, p=0.773; twANOVA, sex, $F_{(1,48)}$=5.555, *p=0.023). C-D) Averaged pre-pulse inhibition for four trial types, varying in interstimulus intervals (30 ms or 100 ms) and pre-pulse intensity (75 db or 80 db). Startle responses for these trials are expressed as a percentage of the normalized "pulse only" trial (baseline) (Student's t-test, *Atrx*-cKO^{MALE} and Ctrl^{MALE} for each trial type, p>0.05; Student's t-test, *Atrx*-cKO^{FEMALE} and Ctrl^{FEMALE} for each trial type, p>0.05). *Atrx*-cKO^{MALE}: n=10, Ctrl^{MALE}: n=17, *Atrx*-cKO^{FEMALE}: n=11, Ctrl^{FEMALE}: n=12. Error bars: ± SEM.

this “pulse-only” trial were used as a baseline, and results from all other trials were expressed as a percentage of this baseline. For all pre-pulse trials, both *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice did not demonstrate significant difference in their startle responses to the acoustic stimulus compared to the controls (Fig. 4-2 C-D). Overall, these results suggest that the postnatal loss of *Atrx* in neurons does not result in an exaggerated startle response or impaired pre-pulse inhibition in male or in female mice.

2.4 Discussion

In this study we present an assessment of the effects of a postnatal conditional knockout of the autism susceptibility gene *Atrx* on autistic-like behaviours in male and female mice. We provide evidence that the postnatal loss of *Atrx* in forebrain excitatory neurons does not result in social deficits, stereotypies and repetitive behaviours, or sensory gating deficits. We identified differences in olfaction for both male and female mice upon the postnatal conditional loss of *Atrx* in neurons, however, these differences in olfaction did not impair social behaviours.

Prior to investigating ASD-related behaviours, we first sought to determine whether *Atrx*-cKO adult mice present with any olfactory differences compared to controls. We performed the odor discrimination and habituation assay to assess if *Atrx*-cKO mice showed habituation to a repeatedly presented odor and were able to discriminate between a novel odor (Arbuckle et al., 2015). By establishing that experimental mice are able to detect and discriminate between odors, results of subsequent social behaviour assays can be more accurately interpreted. Results of the odor habituation and discrimination assay suggest that *Atrx*-cKO^{MALE} mice have deficits in olfaction, as they spent overall less time smelling multiple odors compared to controls. In particular, odor discrimination may be affected in *Atrx*-cKO^{MALE} mice as demonstrated by decreased time smelling a novel odor following repeated presentation of another. These differences in olfaction displayed by *Atrx*-cKO^{MALE} mice are important to consider when interpreting results from social behaviour assays that require odor discrimination. It is interesting to note that olfaction deficits have been reported in both ASD patients as well as models with autism-associated genetic mutations. In a recent clinical study, nine children with ASD demonstrated impaired

olfactory adaption compared to a control group (Kumazaki et al., 2018). Additionally, another study reported that mice with haploinsufficiency of the autism-associated gene, T-box, Brain 1 (*Tbr1*), displayed impairments in olfactory discrimination (Huang et al., 2019). Therefore, these impairments in olfaction, particularly odor discrimination, may be an indication of autistic-like features.

While there was no overall difference in olfaction when we compared *Atrx*-cKO^{FEMALE} mice to controls, *Atrx*-cKO^{FEMALE} mice demonstrated increased time spent sniffing the cotton swab when presented with the banana odor for the first trial. These results suggest that their odor discrimination may be heightened compared to that of Ctrl^{FEMALE}. Altogether, although *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice display differences in olfaction compared to controls, we did not identify any genotypic-differences in social behaviours either in the social approach test or the 3-chamber paradigm. Therefore, any differences in olfaction of *Atrx*-cKO mice did not result in impairments in social recognition or discrimination based on olfactory cues.

Although there were no identified genotypic effects on the behaviours analyzed here, analysis of the data revealed potential sex-differences in sociability, social novelty, startle response and anxiety levels. Sex differences in startle amplitude and marble burying have not yet been reported in the literature for the hybrid strain that was used in this study and should be repeated using a replication cohort. However, it is important to note that our experimental approach was not designed to detect differences based on sex, as male and female mice were obtained from a different generations of strain hybrids.

The *Atrx*-cKO^{FEMALE} mice used in our study experience a complete loss of ATRX expression in forebrain excitatory neurons postnatally. However, in humans, females harbouring *ATRX* mutations are typically carriers and are asymptomatic due to skewed X-inactivation (Aspromonte et al., 2019; Brett et al., 2014; Gong et al., 2008; Li et al., 2017; Munnich et al., 2019; Yu et al., 2013). As such, the clinical relevance of any observed differences we observed in this model are limited. Nevertheless, our model allows the exploration of the basic biology of ATRX function in neurons and the potential effects on the behavioural outcomes. We theorize that the absence of autistic-like phenotypes

observed in *Atrx*-cKO mice is due to the timing at which *Atrx* is deleted in forebrain excitatory neurons, which starts at 2-3 weeks of age. ASD is clinically defined as a developmental disorder due to the majority of symptoms becoming apparent in the first few years of life. Therefore, genetic mutations that contribute to autistic phenotypes may need to occur during embryogenesis or be inherited (Abrahams & Geschwind, 2008; Chen et al., 2015; Geschwind, 2011). Future studies should utilize additional Cre/loxP systems to investigate if the loss of *Atrx* in differentiated forebrain excitatory neurons during embryogenesis leads to autistic-like behaviours in male and female mice.

In conclusion, a postnatal conditional knockout of the autism susceptibility gene *Atrx* did not result in autistic-like behaviours in either male or female mice. Although changes in olfaction were observed in both male and female *Atrx*-cKO mice, these differences did not result in impaired social recognition or discrimination. These findings suggest that the postnatal loss of ATRX is insufficient to cause the subset of autistic behaviours tested here and support the idea that ASD is a developmental disorder where disruptions occur at early stages of brain development**.**

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Chapter 3

3 Deletion of *Atrx* in embryonic forebrain neurons results in autistic-like behaviour in male mice

Mutations in *ATRX* are associated with an X-linked intellectual disability and have also been identified in patients with autism. Despite growing evidence of *ATRX* as an autism-associated gene, we previously failed to identify autistic-like behaviour in male and female mice after targeted ablation of ATRX in excitatory neurons in the postnatal forebrain. Here, we investigated whether targeting ATRX earlier during embryonic brain development would result in autistic-like behaviour. Using the Cre/loxP system, we deleted *Atrx* in postmitotic neurons of the dorsal forebrain and evaluated the behaviour of adult male mice. Our findings revealed that this conditional loss of *Atrx* leads to overgrooming stereotypies, an exaggerated startle response, as well as aggressive social interactions with other mice. We conclude that the conditional loss of neuronal *Atrx* must occur early during embryonic development to cause select autistic-like behaviour in male mice.

3.1 Introduction

ATR-X syndrome is a neurodevelopmental disorder that presents with mild to severe cognitive deficits, microcephaly, hypomyelination, seizures, and developmental delays (Gibbons, 2006). ATR-X syndrome results from hypomorphic mutations in the *ATRX* gene. ATRX is an ATP-dependent chromatin remodeling protein required to maintain chromatin structural integrity and to regulate gene expression (Gibbons, Picketts, Villard, & Higgs, 1995; Gibbons et al., 2008; Kernohan, Vernimmen, Gloor, & Bérubé, 2014; Levy, Kernohan, Jiang, & Bérubé, 2014; Tamming et al., 2020). In addition to cognitive deficits and delays, some patients with ATR-X syndrome exhibit autistic-like features, including showing little interest in those around them and avoiding eye contact (Gibbons, 2006). Autism spectrum disorder (ASD) is a group of neurodevelopmental conditions characterized by difficulties in social communication and the presence of stereotyped and repetitive behaviour (Abrahams & Geschwind, 2008; Chen et al., 2015; Lai et al., 2014).

Although diagnosis is currently limited to behavioural traits, ASDs display high heritability indicating a strong genetic cause (Hammer et al., 2015). Recently, several missense variants in *ATRX* have been identified in male ASD patients (Aspromonte et al., 2019; Brett et al., 2014; Gong et al., 2008; Li et al., 2017; Munnich et al., 2019; Yu et al., 2013), strengthening the relationship between ATRX and autism and supporting *ATRX* as an autism susceptibility gene. However, the mechanisms by which *ATRX* mutations result in autism and autistic-like behaviour is not currently understood.

Mouse models in which *Atrx* is lost early in embryogenesis highlight the importance of ATRX during brain development, and how *ATRX* loss-of-function may result in neuronal defects. The germline knockout of *Atrx* is lethal early in embryogenesis, while the conditional deletion of *Atrx* in neuroprogenitors results in DNA replication stress, excessive DNA damage, and apoptosis of cortical progenitor cells (Bérubé et al., 2005, 2000; Ritchie et al., 2008; Watson et al., 2013). Additionally, mice lacking exon 2 of the gene were generated that result in reduced ATRX protein levels. These mice were reported to display deficits in learning and memory including novel object recognition, spatial memory, and contextual fear memory (Shioda et al., 2011, 2018). Similar behavioural impairments were observed in female mice with mosaic expression of ATRX in the central nervous system (Tamming et al., 2017).

To investigate how cell type specific deletions of *Atrx* influences behaviour of male and female mice, Tamming et al. (2020) previously deleted *Atrx* in postnatal excitatory neurons of the forebrain using the Cre/loxP system under the control of the *αCaMKII* gene promoter. Behavioural analysis demonstrated that this conditional loss of *Atrx* resulted in impaired long-term spatial learning and memory in male mice (*Atrx*-cKO^{MALE}), but not female mice (Tamming et al., 2020). Additionally, *Atrx*-cKO^{MALE} mice experienced transcriptional changes associated with synaptic ultrastructural defects and had impaired long-term potentiation (LTP; Gugustea, Tamming, Martin-Kenny, Bérubé, & Leung, 2019; Tamming et al., 2020). However, despite these neuronal changes, the mice did not develop autistic-like behaviour, including altered social behaviour, repetitive or stereotyped behaviour, or changes in sensory gating to an acoustic stimulus. We theorize that the

absence of autistic-like phenotypes observed in *Atrx*-cKO^{MALE} mice was due to the timing at which *Atrx* was deleted in forebrain excitatory neurons, starting at 2-3 weeks of age.

Given that autism is a neurodevelopmental disorder, it is possible that dysregulation of causative genes must occur during embryogenesis or be inherited (Abrahams & Geschwind, 2008; Chen et al., 2015; Geschwind, 2011). We thus sought to investigate if *Atrx* inactivation during early stages of brain development would result in autistic-like behaviour. We characterized the AtrxNexCre model in which *Atrx* was deleted post-mitotically in dorsal telencephalic neurons starting during embryogenesis at ~E11.5 (Goebbels et al., 2006). Using an array of behavioural tests, we investigated if these mice displayed changes in social interactions, repetitive or stereotyped behaviour, and altered sensory gating. In male mice (*Nex*-cKO^{MALE}) we identified autistic-like behaviour including aggressive social behaviour, an overgrooming stereotype, and an exaggerated startle response. Overall, these results demonstrate that the conditional loss of neuronal *Atrx* during embryogenesis is sufficient to result in autistic-like behaviour in adult male mice.

3.2 Materials and Methods

3.2.1 Animal Care and Husbandry

Mice were exposed to a 12-hour-light/12-hour-dark cycle and with water and chow ad libitum. The *Atrx*^{loxP} females (129/Sv background) have been described previously (Bérubé et al., 2005). *Atrx*^{loxP} mice were mated with C57BL/6 mice expressing Cre recombinase under the control of the *NEX* gene promoter (Goebbels et al., 2006). The progeny includes hemizygous male mice that produce no ATRX protein in neurons of the dorsal telencephalon starting at ~E11.5 (*Nex*-cKO^{MALE}). Wildtype littermate male mice that express the Cre allele were used as controls (*Nex*-Ctrl^{MALE}). Absence of the ATRX protein in postmitotic neurons has been verified in the dorsal forebrain of *Nex*-cKO^{MALE} embryos at E.13.5 and at P20 in the hippocampus and cortex (Katherine Quesnel, unpublished; Appendix B). Genotyping of tail biopsies for the presence of the floxed and Cre alleles was performed as described previously (Bérubé et al., 2005). Primer sequences were as follows:

Primer	Sequence (5' - 3')
ATRX – F	AGAAATTGAGGATGCTTCACC
ATRX – R (wildtype)	TGAACCTGGGGACTTCTTTG
ATRX – R (loxP)	CCACCATGATATTCGGCAAG
CRE - 3b	TGACCAGAGTCATCCTTAGCG
CRE - 5b	AATGCTTCTGTCCGTTTGCC
SRY – F	GCAGGTGGAAAAGCCTTACA
SRY - R	AAGCTTTGCTGGTTTTTGGA

All procedures involving animals were conducted in accordance with the regulations of the Animals for Research Act of the province of Ontario and approved by the University of Western Ontario Animal Care and Use Committee (2017-048). Behavioural assessments started with less demanding tasks and moved to more demanding tasks in the following order: odor habituation and discrimination task, self-grooming assay, pre-pulse inhibition (PPI) and startle response, and 3-chamber social tests. ARRIVE guidelines were followed: mouse groups were randomized, experimenters were blind to the genotypes, and software-based analysis was used to score mouse performance in all the tasks. All behavioural tasks were performed between 9:00 AM and 4:00 PM with mice between the age of 3 and 7 months. Behavioural tasks were performed with 2 cohorts of *Nex*-Ctrl^{MALE} and *Nex*-cKO^{MALE} mice to reach the final sample size, which is indicated in the figure legends. Statistics were calculated by one-way repeated measures ANOVA with Tukey's multiple comparison test, two-way repeated measures ANOVA with Sidak's multiple comparison test, or unpaired Student's T-tests, as indicated in the figure legends.

3.2.2 Odor Habituation and Discrimination

The odor habituation and discrimination assay was performed as previously described (Arbuckle et al., 2015) to assess olfaction. Individual mice were placed into a clean cage with a wire lid and allowed to habituate to the testing room for 30 minutes. The mice were then presented with an odor on a cotton swab (either almond, banana, or water as a control) for a 2-minute trial. For each trial, 50µl of water, almond extract, or banana extract (Club House) was pipetted onto the tip of a cotton swab and the swab was then secured to the wire cage top through the water bottle opening. The mice were presented with the same odor three times before being presented with a new odor, for a total of nine trials. During the 2-minute trials, the amount of time that the mouse spent sniffing the odor was recorded

by an investigator blind to the genotype. Sniffing was defined as the animal's nose being in proximity to the cotton swab (2cm or closer), and oriented toward the swab.

3.2.3 3-Chamber Social Tests (Social Preference and Novelty)

The social preference and social novelty assessments were performed as described (El-Kordi et al., 2013; Jamain et al., 2008) with minor modifications. Individual mice were placed in the 3-chambered box and allowed to freely explore the arena during a 10-minute habituation period. After the habituation period, an unfamiliar, same-sex mouse of a different genotype (stranger 1) was placed in one of the side chambers under a wire cage. An identical wire cage containing an inanimate object was placed in the opposite chamber. The test mouse was then allowed to explore the entire 3-chambered arena for 10 minutes. The amount of time spent in each chamber was recorded by the ANY-maze video-tracking system (Stoelting Co). Following this period, a second unfamiliar, same-sex mouse of a different genotype (stranger 2) was placed into the wire cage previously containing the inanimate object. The test mouse was then allowed to explore the 3-chambered arena for 10 minutes. The amount of time spent in each chamber was recorded by the ANY-maze video-tracking system. Based on the amount of time spent in each chamber, a 'sociability index' and a 'social novelty index' was calculated as previously described (El-Kordi et al., 2013). The sociability index was calculated as: $time_{stranger}/(time_{stranger}+time_{object})$ x 100. The social novelty index was calculated as: $time_{novel}/(time_{novel}+time_{familiar})$ x 100. Videos recorded during these trials were further analyzed to determine the number of tail rattling episodes that occurred during the trail, a sign of active aggression (Miczek & O'Donnell, 1978). Each tail rattling event was scored, and it was noted which chamber the test mouse was in when displaying this sign of aggression. Videos were manually scored by an investigator, blinded to the genotype.

3.2.4 Self-Grooming Assay

The test was performed as previously described (Mehta, Gandal, & Siegel, 2011) to evaluate spontaneous grooming as a measure of repetitive behaviour. Mice were individually acclimated in an empty test cage for 15 minutes prior to the test. Following

acclimation, the grooming behaviour of each mouse was recorded by the ANY-maze video-tracking system for 15 minutes. The time that each individual mouse spent grooming during this 15-minute trial was manually scored by an investigator blinded to the genotype of the animals.

3.2.5 Pre-Pulse Inhibition and Startle Response

The pre-pulse inhibition and startle response tests were performed as previously described (Valsamis & Schmid, 2011) to assess sensory gating. Mice underwent two days of habituation prior to the testing day, to acclimate the mice to the apparatus. During this habituation, mice were individually placed in the chamber apparatus and exposed to background noise (65 db) for 5 minutes (SR-LAB, San Diego Instruments). On the test day, individual mice were placed in the chamber and acclimated for 10 minutes with background noise. The mice then underwent a habituation block, consisting of fifty acoustic startle trials, with 20 ms stimulus of 115 db, and intertrial intervals of 20 seconds. After the habituation block, mice underwent a prepulse-inhibition block consisting of ten sets of five types of trials randomly ordered with variable intertrial intervals of 10, 15, or 20 seconds. Four of the five trial types consisted of prepulses (intensity of 75 or 80 db, length of 20 ms), separated from the startle stimulus (intensity of 115 db, length of 40 ms) by an interstimulus interval of either 30 ms or 100 ms. The fifth trial type was a startle pulse alone. The startle response was measured by the movement of the mouse on the platform, which generates a transient force analyzed by the software. The startle magnitude recorded was an average for the ten trials of each trial type and startle magnitudes of pre-pulse trials were normalized to the average of the pulse-only trials.

3.3 Results

3.3.1 Differences in olfaction upon the embryonic neuronal loss of *Atrx*

Changes in olfaction can confound results of social behaviour assays that require test mice to discriminate between two distinct odors, such as the 3-chamber social assay. Therefore, we first addressed if the embryonic loss of *Atrx* in neurons alters olfaction in mice through the odor discrimination and habituation assay (Arbuckle et al., 2015). Mice were presented

with multiple odors for 2-minute trials, during which the amount of time spent sniffing the odor was recorded. During this test, *Nex*-cKO^{MALE} mice spent significantly less time sniffing the odors throughout the nine trials compared to *Nex*-Ctrl^{MALE} mice (ANOVA, ***p=0.0006; Fig.3-1A). In particular, *Nex*-cKO^{MALE} mice spent significantly less time sniffing the cotton swab when first presented with the banana odor (multiple comparisons, ****p<0.0001). There was no difference in the amount of time *Nex*-cKO^{MALE} mice spent sniffing the control cotton swabs saturated with water compared to *Nex*-Ctrl^{MALE} mice (Fig.3-1A). This suggests that the decreased time *Nex*-cKO^{MALE} mice spent sniffing the odors is due to changes in olfaction rather than differences in exploration of the swabs. These results demonstrate that the loss of *Atrx* in embryonic neurons results in altered olfaction that must be considered in subsequent behaviour testing of these mice.

3.3.2 *Nex*-cKO^{MALE} mice exhibit aggressive behaviour

We next sought to investigate the social behaviour of *Nex*-cKO^{MALE} mice. To do this, we examined social preference and social novelty in the 3-chambered social test (El-Kordi et al., 2013). Social preference was assessed in the first part of the paradigm, in which test mice were placed into a 3-chambered apparatus with the outer two chambers containing either a novel object or a stranger mouse, while the centre chamber remained empty. The test mice were free to explore the chambers during a 10-minute trial, and the amount of time spent in each chamber was recorded. Interestingly, although *Nex*-cKO^{MALE} mice showed a strong preference for spending time in the chamber containing the stranger mouse (multiple comparison, ****p<0.0001), the control mice did not show the expected preference for the stranger mouse compared to the object (Fig.3-2A). The sociability index was then calculated as the ratio of the difference to the sum of time spent exploring the novel mouse and time spent exploring the novel object (El-Kordi et al., 2013). A sociability index above 0.50 indicates that the test mouse demonstrated a preference towards the novel mouse over the object during the social preference test. Five *Nex*-Ctrl^{MALE} mice had a sociability index below 0.50, indicating these mice did not display a typical preference towards the novel mouse (Fig.3-2B). *Nex*-cKO^{MALE} mice did not display differences in their sociability indexes compared to *Nex*-Ctrl^{MALE} (Fig.3-2B);

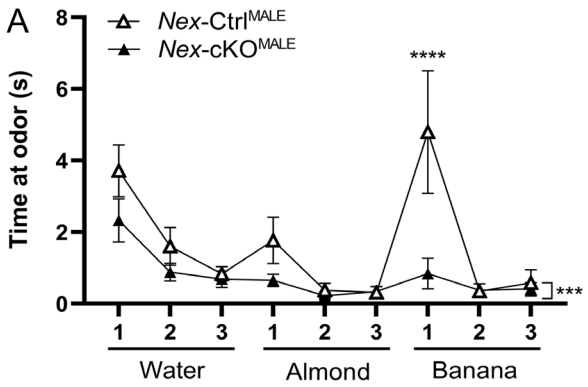

Figure 3-1 The conditional embryonic loss of *Atrx* leads to changes in olfaction.

The amount of time spent sniffing a cotton swab saturated with an odor during nine, 2-minute trials. *Nex*-cKO^{MALE} (n=15) mice spend less time sniffing cotton swabs with corresponding odors compared to *Nex*-Ctrl^{MALE} (n=13; twANOVA, $F_{(1,234)}$=12.20, ***p=0.0006; mc, ****p<0.0001).

however, we cannot draw conclusions regarding the sociability of *Nex*-cKO^{MALE} mice due to the poor performance of the control group.

Social novelty was then investigated during the second part of the paradigm in which the outer chambers contained either the stranger mouse from the first part of the test (familiar mouse) or a novel mouse. Again, the control mice did not demonstrate the expected preference for the novel mouse over the familiar mouse. However, *Nex*-cKO^{MALE} mice did demonstrate a preference for the chamber containing the novel mouse compared to that with the familiar mouse (multiple comparisons, *p=0.031; Fig.3-2C). Social memory indexes were then calculated as the ratio of the difference to the sum of time spent exploring the novel mouse and time spent exploring the familiar mouse (El-Kordi et al., 2013). The index provides a measurement of the ability of the test mouse to distinguish between the familiar and novel mouse and demonstrate an expected preference towards the novel mouse. *Nex*-cKO^{MALE} mice did not display differences in their social memory indexes compared to *Nex*-Ctrl^{MALE} (Fig.3-2D). However, the majority of *Nex*-Ctrl^{MALE} mice failed the social novelty assay, based on a social memory index below 0.50 (Fig.3-2D). Although we are not able to draw conclusions regarding the social behaviour of *Nex*-cKO^{MALE} relative to a typically behaving control group, these results demonstrate that the *Nex*-cKO^{MALE} exhibit increased sociability to a mouse vs an object, and to a novel mouse vs a previously encountered mouse.

It was unexpected that the control groups performed so poorly in the above assays. Throughout the care and husbandry of *Nex*-cKO^{MALE} mice, we noted that *Nex*-cKO^{MALE} mice are highly aggressive towards both handlers and littermates, often attacking and wounding the control mice they are caged with. We thus re-analyzed the videos of the 3-chamber social assay to investigate if *Nex*-cKO^{MALE} mice display aggression towards stranger mice in this social paradigm. A common sign of aggression in mice is tail rattling (Miczek & O'Donnell, 1978). Tail rattling has been proposed to function as a threat signal towards other mice (Scott & Fredericson, 1951), or as a signal that elicits fighting in mice (St. John, 1973). To assess if *Nex*-cKO^{MALE} mice display this aggressive signal towards stranger mice more than *Nex*-Ctrl^{MALE} mice, each tail rattling episode during the 3-chamber social assay was scored. During the social preference trial, *Nex*-cKO^{MALE} mice displayed

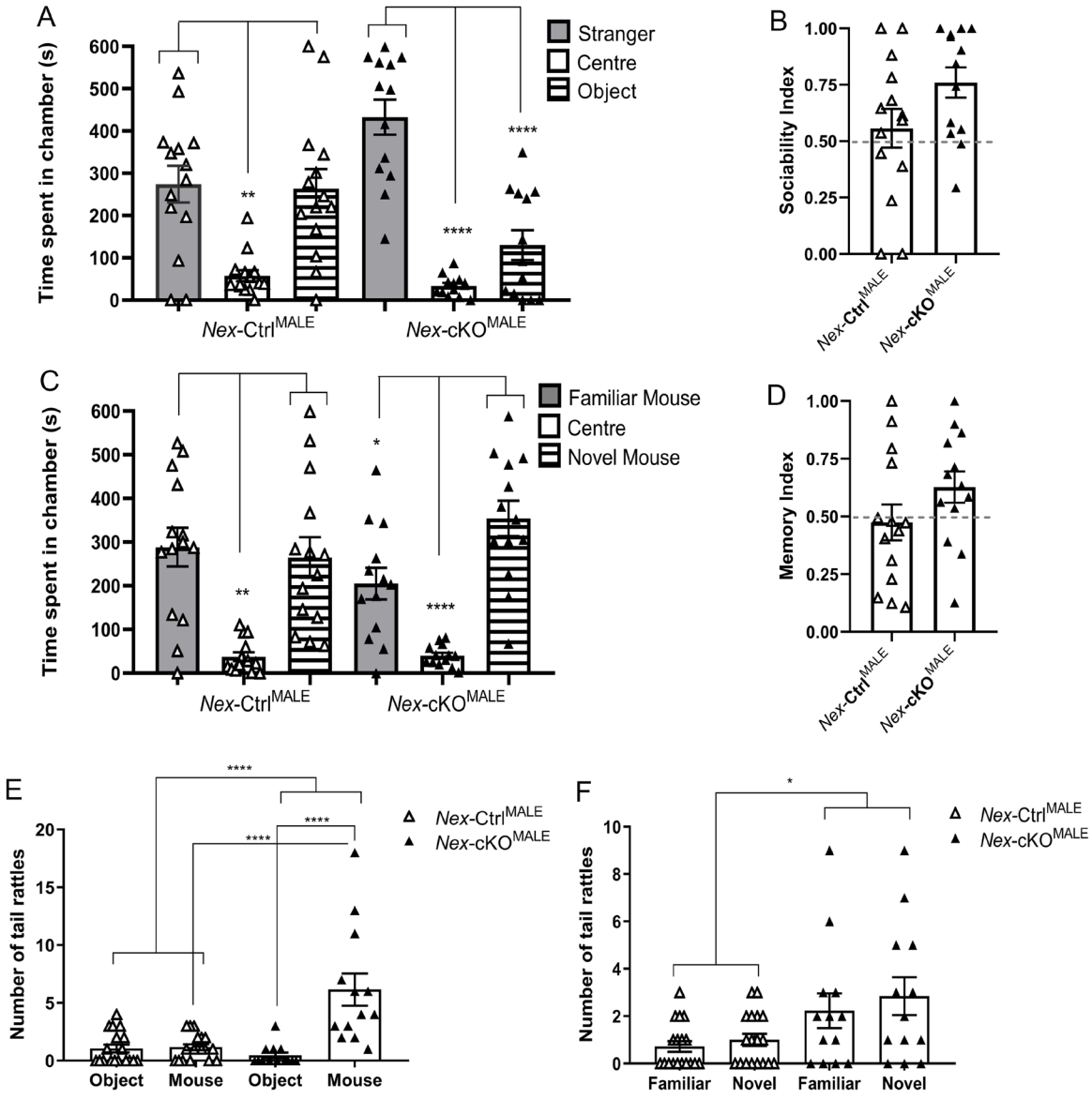
A
Time spent in chamber (s)
600
500
400
300
200
100
0
Stranger
Centre
Object
**
****
****
Nex-Ctrl MALE
Nex-cKO MALE
B
Sociability Index
1.00
0.75
0.50
0.25
0.00
Nex-Ctrl MALE
Nex-cKO MALE
C
Time spent in chamber (s)
600
500
400
300
200
100
0
Familiar Mouse
Centre
Novel Mouse
**
*
****
Nex-Ctrl MALE
Nex-cKO MALE
D
Memory Index
1.00
0.75
0.50
0.25
0.00
Nex-Ctrl MALE
Nex-cKO MALE
E
Number of tail rattles
20
15
10
5
0
****
****
****
Nex-Ctrl MALE
Nex-cKO MALE
Object
Mouse
Object
Mouse
F
Number of tail rattles
10
8
6
4
2
0
*
Nex-Ctrl MALE
Nex-cKO MALE
Familiar
Novel
Familiar
Novel

Figure 3-2 *Nex*-cKO^{MALE} mice display aggressive social behaviour.

A) Amount of time *Nex*-Ctrl^{MALE} (n=14; twANOVA, $F_{(2,26)}$=7.19, **p=0.003; mc, **p<0.01) and *Nex*-cKO^{MALE} (n=13; twANOVA, $F_{(2,24)}$=28.92, ****p<0.0001; mc, ****p<0.0001) spent in chambers containing either a stranger mouse or novel object, or the empty centre chamber. B) The sociability index for each mouse was calculated as the time spent in the stranger mouse chamber, divided by the total time in the stranger mouse and novel object chambers (T-test, p=0.078). C) Time spent in the empty centre chamber and chambers containing a familiar mouse or a novel mouse in the social novelty assay for *Nex*-Ctrl^{MALE} (n=14; twANOVA, $F_{(2,26)}$=9.203, ***p<0.001; mc, **p<0.01) and *Nex*-cKO^{MALE} mice (n=13; twANOVA, $F_{(2,24)}$=16.45, *p=0.031; mc, ****p<0.0001). D) The social memory index for each mouse was calculated as the time spent in the novel mouse chamber, divided by the total time in the familiar mouse and novel mouse (T-test, p=0.154). E) The number of tail rattling episodes while in chambers containing either a novel object or stranger mouse during the 3-chamber social assay. *Nex*-cKO^{MALE} mice display more tail rattling episodes towards the stranger mouse than the novel object (mc, ****p<0.0001), and towards the stranger mouse compared to *Nex*-Ctrl^{MALE} mice (owANOVA, ****p<0.0001; mc, ****p<0.0001). F) The number of tail rattling episodes while in chambers containing either a familiar mouse or novel mouse during the 3-chamber social assay. *Nex*-cKO^{MALE} mice display more tail rattling episodes towards stranger mice compared to *Nex*-Ctrl^{MALE} mice (owANOVA, *p=0.012). Error bars: ± SEM.

more tail rattling episodes in the chamber containing the stranger mouse, compared to *Nex*-Ctrl^{MALE} mice (multiple comparison, ****p<0.0001; Fig3-2E). This aggressive behaviour appeared to be socially specific, as *Nex*-cKO^{MALE} mice only displayed these tail rattling episodes in the chamber containing the stranger mouse, not the novel object (multiple comparison, ****p<0.0001). Overall, throughout the social preference trial of the 3-chamber social test, *Nex*-cKO^{MALE} mice had more tail rattling episodes than *Nex*-Ctrl^{MALE} mice (ANOVA, ****p<0.0001; Fig3-2E). Similarly, during the social novelty trial, *Nex*-cKO^{MALE} mice displayed more tail rattling episodes in chambers containing both familiar and novel mice, compared to *Nex*-Ctrl^{MALE} mice (ANOVA, *p=0.012; Fig3-2F). Altogether, the loss of *Atrx* in forebrain excitatory neurons during embryogenesis does not alter the ability of adult male mice to socially interact with stranger mice; however, they display signs of aggression when interacting with other mice. Moreover, this aggressive behaviour may have affected their control littermates, perhaps providing an explanation for their poor performance of *Nex*-Ctrl^{MALE} mice in these tests.

3.3.3 Overgrooming stereotypies and an exaggerated startle response in *Nex*-cKO^{MALE} mice

Studies using mouse models with ASD-associated genetic alterations often report the presence of repetitive and stereotyped behaviour (Burrows et al., 2015; Cheng et al., 2018; El-Kordi et al., 2013; Jung et al., 2018; Nuytens et al., 2013). We therefore wanted to investigate if *Nex*-cKO^{MALE} mice display repetitive or stereotyped behaviour. Throughout handling and husbandry, we noted large patches of hair loss on several *Nex*-cKO^{MALE} mice but not control mice, potentially due to over-grooming tendencies (Fig.3-3A). These observations prompted us to assess the natural grooming tendencies of *Nex*-cKO^{MALE} mice relative to their controls. *Nex*-cKO^{MALE} mice spent significantly more time grooming themselves compared to *Nex*-Ctrl^{MALE} mice over 15 minutes (T-test, **p=0.002; Fig.3-3B). When the results were analyzed over 5-minute intervals, *Nex*-cKO^{MALE} mice displayed increased time grooming during the first 10 minutes of the test (multiple comparisons, **p<0.01; Fig.3-3C).

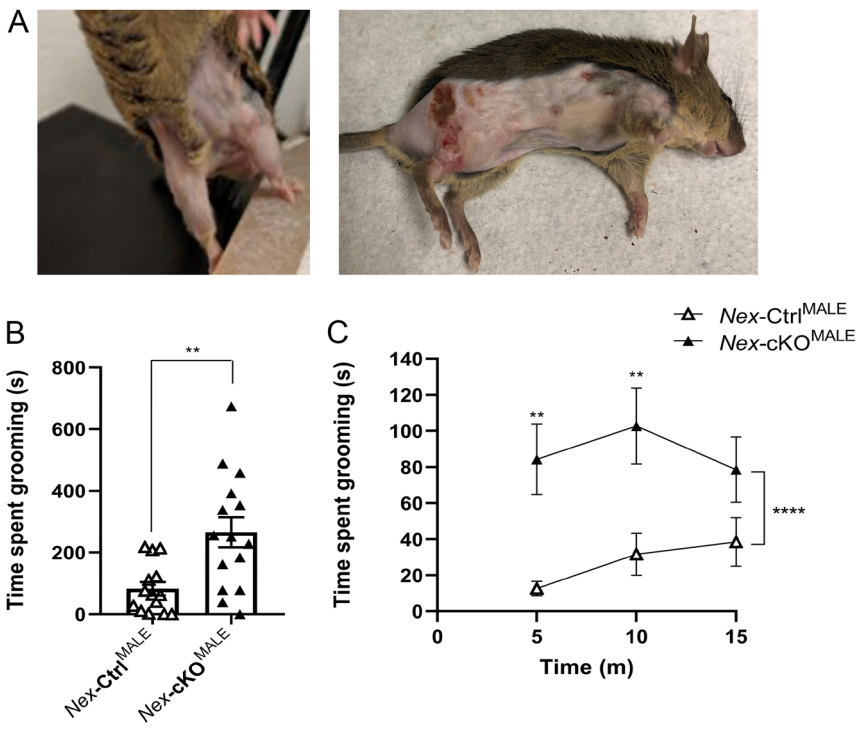

Figure 3-3 The embryonic conditional loss of neuronal *Atrx* results in an overgrooming stereotype.

A) Images of 4-month-old *Nex*-cKO^{MALE} mice with patches of hair loss and self-inflicted lesions due to overgrooming. B) Total time grooming during 15-minute grooming task for *Nex*-Ctrl^{MALE} (n=14) and *Nex*-cKO^{MALE} mice (n=15; T-test, **p=0.002). C) Amount of time spent grooming over 5-mintue intervals during grooming task (twANOVA, $F_{(1,81)}$=21.50, ****p<0.0001; mc, **p<0.01).

Another common trait observed in models with ASD-associated genetic alterations is an exaggerated startle response, or impaired pre-pulse inhibition, to an acoustic stimulus (Crawley, 2007; Jiang et al., 2018; Luo et al., 2018; Scott et al., 2018). These differences are associated with changes in sensory gating and auditory processing that are often reported in ASD patients (Ebishima et al., 2019; Takahashi & Kamio, 2018). We thus investigated if the embryonic conditional loss of neuronal *Atrx* would result in hypersensitivity to an acoustic startle stimulus. The pre-pulse inhibition and startle response assay using an acoustic stimulus was performed, as described previously (Valsamis & Schmid, 2011). Mice were placed in a chamber and exposed to fifty trials of an acoustic stimulus (20 ms, 115 db). *Nex*-cKO^{MALE} mice demonstrated a significant increase in startle response to the acoustic stimuli compared to controls (ANOVA, ****p<0.0001; mc; *p<0.05, **p<0.01; Fig.3-4A).

We also assessed pre-pulse inhibition to the acoustic stimulus by first exposing mice to a pre-pulse that preceded the acoustic stimulus (Valsamis & Schmid, 2011). These trials varied in the intensity of the pre-pulse (75 db or 80db), and the amount of time between the pre-pulse and the acoustic "pulse" stimulus (30ms or 100ms). Additionally, there was one trial that only involved a "pulse" without a pre-pulse. Startle responses to this "pulse-only" trial were used as a baseline, and results from all other trials were normalized to this baseline to determine the remaining startle response of each mouse as a percentage of the baseline. The percentage of pre-pulse inhibition was then calculated by subtracting the remaining startle response from 100% (Valsamis & Schmid, 2011). *Nex*-cKO^{MALE} mice demonstrated impaired pre-pulse inhibition compared to *Nex*-Ctrl^{MALE} mice in three out of the four pre-pulse trials (T-test, *p<0.05, **p<0.01; Fig. 3-4B). Overall, these results demonstrate that in male mice, the early developmental inactivation of *Atrx* in forebrain excitatory neurons results in autistic-like behaviour including over-grooming stereotypies as well as an exaggerated startle response.

3.4 Discussion

With this study, we sought to investigate if the embryonic loss of *Atrx* in forebrain excitatory neurons would results in autistic-like behaviour in male mice. Behavioural tests

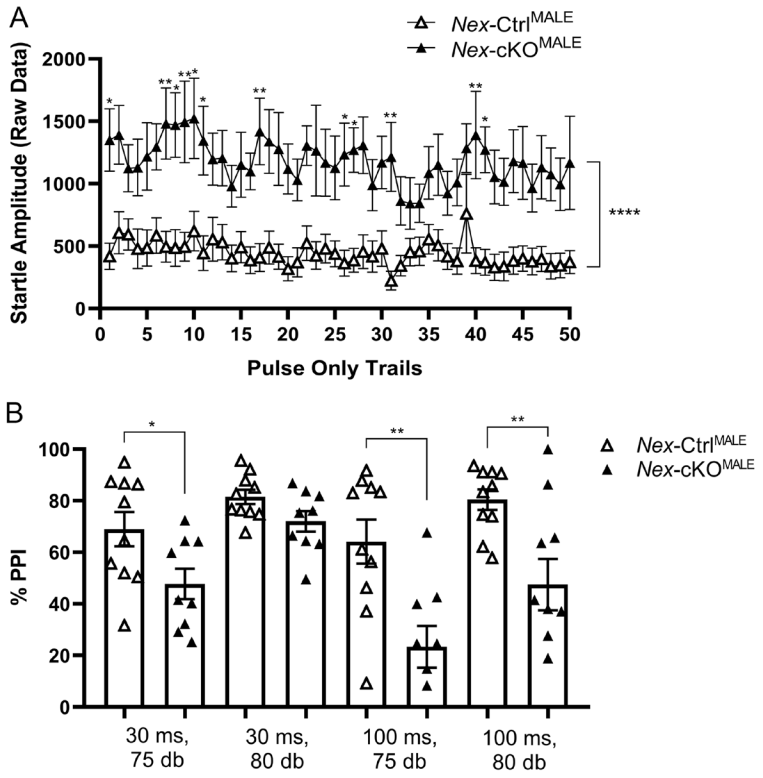

Figure 3-4 *Nex*-cKO^{MALE} mice display an exaggerated startle response to an acoustic stimulus.

A) Quantification of startle responses (recorded in millivolts) to fifty "pulse only" trials of *Nex*-Ctrl^{MALE} (n=10) and *Nex*-cKO^{MALE} (n=9) mice (twANOVA, $F_{(1,850)}$=392.0, ****p<0.0001; mc, *p<0.05, **p<0.01). B) Averaged pre-pulse inhibition for four trial types, varying in interstimulus intervals (30 ms or 100 ms) and pre-pulse intensity (75 db or 80 db). Startle responses for these trials were normalized to results from the "pulse only" trial (baseline), and the percentage of pre-pulse inhibition was calculated by subtracting the remaining startle responses from 100%. (T-test, *Nex*-cKO^{MALE} and *Nex*-Ctrl^{MALE} for each trial type, *p<0.05, **p<0.01). Error bars: ± SEM.

revealed that *Nex*-cKO^{MALE} mice display select behaviour related to autism. When interacting socially with stranger mice, *Nex*-cKO^{MALE} mice display increased tail rattling episodes, a sign of aggression. *Nex*-cKO^{MALE} mice also display an overgrooming stereotype that results in large patches of hairlessness and self-inflicted lesions in some animals. Finally, when exposed to an acoustic stimulus, *Nex*-cKO^{MALE} mice display an exaggerated startle response and impairments in pre-pulse inhibition compared to controls.

Previous behavioural analyses of mice with a postnatal conditional knockout of neuronal *Atrx* using a CamKIICre driver identified changes in olfaction, particularly odor discrimination, upon this loss of *Atrx*. Therefore, we first investigated the ability of *Nex*-cKO^{MALE} mice to habituate to an odor and discriminate between different odors. Through the odor habituation and discrimination assay (Arbuckle et al., 2015), we identified changes in olfaction of *Nex*-cKO^{MALE} compared to controls. *Nex*-cKO^{MALE} spent overall less time smelling multiple odors compared to controls, particularly when first presented with a novel odor following repeated presentation of another, suggesting odor discrimination may be affected. However, although we detected these changes in odor discrimination, *Nex*-cKO^{MALE} did not show an inability to discriminate between a familiar mouse and a novel mouse during the 3-chamber social assay.

Interestingly, when sociability and social novelty were investigated in the 3-chamber social assay, the majority of *Nex*-Ctrl^{MALE} mice did not perform as expected. Several *Nex*-Ctrl^{MALE} mice had a sociability index and memory index score below 0.50, indicating a lack a preference towards the stranger mouse and novel mouse, respectively. We theorize that the sociability of controls may have been altered due to group housing with *Nex*-cKO^{MALE} mice that behave aggressively towards their littermates and often wound the control mice. Mice subjected to consecutive days of defeat by an aggressor mouse during social defeat paradigms show altered motivation for social interactions. Specifically, socially defeated mice often display aversive responses and less time in close proximity to a stranger mouse compared to undefeated controls (Bagot et al., 2015; Berton et al., 2006). *Nex*-Ctrl^{MALE} mice may display poor sociability with stranger mice because they have been socially defeated by *Nex*-cKO^{MALE} littermates. Future studies should be conducted using a cohort of *Nex*-Ctrl^{MALE} mice that are not housed with *Nex*-cKO^{MALE} mice, but rather with

other control mice to allow for the social behaviour of *Nex*-cKO^{MALE} mice to be compared to typically behaving controls.

Although we can only draw limited conclusions regarding the social behaviour of *Nex*-cKO^{MALE} mice due to atypically performing controls, we are able to gain some insight from these behaviour. Results of the 3-chamber social assay demonstrate that *Nex*-cKO^{MALE} mice show typical social behaviour when presented with the two paradigms, demonstrating a preference for a stranger mouse over a novel object, and a novel mouse over a familiar mouse. Based on experiences with handling and husbandry of *Nex*-cKO^{MALE} mice, we anecdotally observed adult *Nex*-cKO^{MALE} mice behave aggressively towards littermates as well as handlers. We indeed noted that *Nex*-cKO^{MALE} mice display more episodes of tail rattling when interacting with a stranger mouse compared to controls. Future studies should further investigate the aggressive behaviour of *Nex*-cKO^{MALE} mice through other assays, such as the resident intruder paradigm (Koolhaas et al., 2013).

The presence of stereotypies as well as alterations in sensory gating and auditory processing are often reported in ASD patients (Ebishima et al., 2019; Takahashi & Kamio, 2018). When assessing *Nex*-cKO^{MALE} mice for the presence of stereotyped or repetitive behaviour, we observed a prominent overgrooming stereotype. Self-grooming behaviour persisted extensively and resulted in patches of hair loss in some adult *Nex*-cKO^{MALE} mice. Other repetitive behaviour was previously examined in the open field assay. During this 120-minute test, *Nex*-cKO^{MALE} mice did not show changes in vertical episode number, however, they travelled a greater distance compared to *Nex*-Ctrl^{MALE} mice, a sign of hyperactivity (Katherine Quesnel, unpublished). Digging and burying behaviour has not yet been assessed due to difficulties in handling *Nex*-cKO^{MALE} mice during the marble burying task. We also investigated if *Nex*-cKO^{MALE} mice display changes in sensory gating to an acoustic stimulus and noted that *Nex*-cKO^{MALE} mice display an exaggerated startle response as well as impaired pre-pulse inhibition compared to controls.

The autistic-like behaviour displayed by *Nex*-cKO^{MALE} mice has been observed in other rodent models with autism-associated genetic alterations (Burrows et al., 2015; Crawley, 2007; Jiang et al., 2018; Jung et al., 2018; Luo et al., 2018; Nuytens et al., 2013; Scott et

al., 2018). Notably, male *Nrxn1α* knockout mice display alterations in pre-pulse inhibition and increased repetitive grooming behaviour (Etherton, Blaiss, Powell, & Sudhof, 2009), as well as increased aggressive behaviour in the presence of other male mice (Grayton, Missler, Collier, & Fernandes, 2013). Additionally, *Shank3*$^{-/-}$ knockout mice have been reported to develop self-inflicted skin lesions due to excessive self-grooming (Peça et al., 2011), similar to was observed with *Nex*-cKO^{MALE} mice.

In conclusion, the conditional loss of *Atrx* in forebrain excitatory neurons during embryogenesis is sufficient to result in autistic-like behaviour, similar to those observed in autism-associated gene knockout mouse models. These results contrast with those obtained with a postnatal loss of *Atrx* in forebrain excitatory neurons described in Chapter 2 (Martin-Kenny & Bérubé, 2020), which do not display autistic-like behaviour. The observed differences between these two models highlights that alterations in autism-associated genes may affect behaviour differently depending on the timing of the dysregulation during pre- and post-natal brain development. Specifically, genes involved in epigenetic regulation, such as *ATRX*, may have a more prominent role in the establishment of neuronal circuitry that occurs early in embryonic brain development, rather than postnatal maintenance (Goodwin & Picketts, 2018; Parikshak et al., 2013). As such, prenatal disruptions to these ASD-associated genes may result in greater alterations to neuronal development, and subsequent behaviour, compared to similar postnatal disruptions. Altogether, these results support *ATRX* as an autism-associated gene and the belief that ASD-risk genes influence key events during early brain development that impact behaviour in adulthood.

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Chapter 4

4 Discussion and Future Directions

4.1 Thesis Summary

This thesis explored whether the timing of ATRX inactivation in neurons could influence the development of autistic-like behaviour in mice. The findings demonstrate that loss of *Atrx* in neurons during embryonic development, but not in juvenile mice, leads to autistic-like behaviour once the mice reach adulthood. Based on these results, we conclude that ATRX has unique roles during embryonic brain development that impacts behaviour in adulthood.

In chapter two I investigated the behaviour of male and female mice with a postnatal deletion of *Atrx* in forebrain excitatory neurons (*Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice). Previous work from the Bérubé lab identified sex-specific learning and memory deficits in *Atrx*-cKO^{MALE} mice that were paralleled by gene expression alterations, synaptic structural changes (Tamming et al., 2020) and reduced hippocampal LTP (Gugustea, Tamming, Martin-Kenny, Bérubé, & Leung, 2019). Based on the growing body of evidence supporting *ATRX* as an autism-associated gene, we further sought to investigate if these mice also presented with autistic-like behaviour. I performed an array of behavioural assays and did not identify changes in social behaviour, the presence of stereotyped or repetitive behaviour, or changes in startle response to an acoustic stimulus in either *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice. Taken together with previous work, these results show that the neuronal loss of *Atrx* several weeks after birth causes hippocampal-dependent learning and memory deficits in male mice but does not result in autistic-like behaviour.

In chapter three I explored if an earlier loss of *Atrx* in telencephalic dorsal neurons during brain development would result in different behavioural changes than the postnatal *Atrx*-cKO^{MALE} model. Given that ASDs are neurodevelopmental conditions, dysregulation of causative genes may have to occur within a specific window of time during brain development to result in behavioural phenotypes related to autism (Abrahams &

Geschwind, 2008; Chen, Peñagarikano, Belgard, Swarup, & Geschwind, 2015; Geschwind, 2011). To investigate this question, we performed an array of behavioural assays on mice with an ablation of *Atrx* in post-mitotic neurons during embryogenesis (*Nex*-cKO^{MALE}). We identified striking differences in several behavioural tasks of adult *Nex*-cKO^{MALE} mice. Notably, these mice displayed aggressive behaviour towards other mice and handlers, presented with an overgrooming stereotype, and exhibited an exaggerated startle response to an acoustic stimulus.

Altogether, this thesis supports evidence that chromatin remodeling proteins, such as ATRX, contribute to the development of autism when disrupted during embryonic brain development. Furthermore, the results suggest different roles for ATRX during pre- and postnatal brain development.

4.2 ATRX loss during development results in distinct behavioural alterations

In humans, hypomorphic mutations in *ATRX* result in ATR-X syndrome, an intellectual disability disorder that presents with cognitive deficits, various developmental delays, and autistic-like behaviour (Gibbons, 2006). Since mutations in *ATRX* have previously been associated with autism and autistic-like behaviour (Aspromonte et al., 2019; Brett et al., 2014; Gibbons, 2006; Gong et al., 2008; Li et al., 2017; Munnich et al., 2019; Sun et al., 2001; Yu et al., 2013), we sought to explore if mice with a conditional loss of *Atrx* would exhibit changes in social behaviour, stereotyped or repetitive behaviour, and alterations in sensory gating.

Several genetically engineered mice with disruptions to the *Atrx* gene in the central nervous system have been previously generated. Generation of a conventional knockout model of *Atrx* is not possible due to the reduced growth capacity of mouse embryonic stem cells upon the loss of *Atrx* (Garrick et al., 2006). Additionally, early inactivation of *Atrx* at the morula stage results in embryonic lethality due to placental defects (Garrick et al., 2006). Similarly, the forebrain specific loss of *Atrx* results in increased DNA replication stress, DNA damage, neuroprogenitor cell death, and early lethality in hemizygous males (Bérubé

et al., 2005). However, there are several approaches that have been used to allow viability and investigate the role of ATRX on behaviour.

When *Atrx* is conditionally deleted in embryonic forebrain neurons, the hemizigous males do not survive, as stated above; however, heterozygous female mice display a mosaic pattern of *Atrx* inactivation due to random X-inactivation. These mice survive to adulthood and exhibit drastic impairments in spatial memory, contextual fear memory, and novel object recognition memory (Tamming et al., 2017). Notably, the presence of autistic-like behaviour was not investigated with these mice. Alternatively, to allow viability and explore behaviour in male mice, the amount of ATRX protein can be reduced rather than generating a knockout. Male mice lacking exon 2 ($Atrx^{\Delta E2}$) have globally reduced ATRX protein levels and are reported to display deficits in novel object recognition, spatial memory, and contextual fear memory (Shioda et al., 2011, 2018). These mice also display withdrawal in social interactions including enhanced passivity, higher escape duration, and decreased social activity such as following and sniffing behaviour (Shioda et al., 2018). To our knowledge, these changes in social behaviour of $Atrx^{\Delta E2}$ mice are the only autistic-like behavioural phenotypes previously explored and reported in an *Atrx* mouse model.

Finally, to bypass early lethality as a result of *Atrx* inactivation in neuroprogenitor cells, cell-type specific ablation can be used to investigate the impact on behaviour. Mice with a postnatal loss of *Atrx* in excitatory neurons of the forebrain are viable into adulthood and males (*Atrx*-cKO^{MALE}) display impairments in contextual memory, spatial learning, as well as spatial long-term memory (Tamming et al., 2020). We investigated adult *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice and failed to identify any behaviour related to autism in our assays (Martin-Kenny & Bérubé, 2020). We questioned if the lack of autistic-like behaviour identified was due to the postnatal timing in which *Atrx* was ablated in this model. To address this question, we used a different Cre driver (NexCre) that directs *Atrx* deletion in postmitotic embryonic neurons. This earlier timing of inactivation led to select autistic-like behaviour in male mice (*Nex*-cKO^{MALE}). By comparing behavioural changes in these various models, it is apparent that ATRX plays a role in learning and memory, and alterations to *Atrx* postnatally can result in learning and memory deficits. However, the role of ATRX in behaviour related to autism appears to result from embryonic abnormalities.

This suggests that ATRX plays distinct roles during pre- and postnatal brain development, and that behavioural changes can vary depending on the timing of *Atrx* ablation in excitatory neurons of the forebrain.

4.3 The role of autism-associated genes during brain development

Studies focused on identifying genes and gene networks associated with ASD susceptibility have pointed to genes known to share commonalities in synaptic, transcriptional, and epigenetic mechanisms (Geschwind, 2011; Loke, Hannan, & Craig, 2015; Voineagu et al., 2011; Yang, Sau, Lai, Cichon, & Li, 2014). To understand how these ASD-associated genes contribute to autism when absent or dysfunctional, several groups have explored their spatial and temporal expression during human brain development. Satterstrom et al. (2020) recently performed large-scale exome sequencing of individuals with ASD to identify genes highly implicated in risk for autism. Notably, X-chromosome ASD genes, such as *ATRX*, were not analyzed in this study. In agreement with previous studies, most ASD-risk genes were found to function in either the regulation of gene expression or in neuronal communication (Satterstrom et al., 2020). The group then investigated the expression profiles of the ASD-risk genes in different brain regions during pre- and postnatal development. These investigations revealed that ASD-risk genes are generally expressed at high levels early in development and are specifically enriched in maturing and mature excitatory cortical neurons (Satterstrom et al., 2020). These results support previously reported analyses showing that ASD genes are preferentially enriched in the mid-fetal prefrontal cortex, and have concentrated expression in layer 5/6 cortical glutamatergic postmitotic neurons (Parikshak et al., 2013; Willsey et al., 2013). Based on these reports, alterations to ASD-risk genes during mid-fetal development in the cortex may heighten the risk of developing autism and autistic-like behaviour. Our results support this concept, since the embryonic loss of a single ASD-risk gene, *Atrx,* in postmitotic telencephalic cortical neurons results in select autistic-like behaviour in mice. The lack of identified autistic-like behaviour when the same gene is deleted in mice that are several

weeks old indicates that cortical and hippocampal excitatory neurons are more susceptible to gene dysregulation during the embryonic stages of brain development.

Cortical and hippocampal development is highly organized, and the sequence and timing of processes such as neurogenesis, neuronal migration, and synaptogenesis are tightly regulated during the pre- and postnatal periods (Budday, Steinmann, & Kuhl, 2015; Stiles & Jernigan, 2010). Studies investigating the expression of ASD-associated genes have identified an enrichment of those involved in epigenetic function during the prenatal period (Goodwin & Picketts, 2018; Parikshak et al., 2013; Satterstrom et al., 2020; Willsey et al., 2013). This includes ASD-associated genes that encode chromatin remodeling proteins such as *CHD8*, *ADNP*, and members of the nBAF complex including *ACTL6B*, *ARID1B*, *SMARCC1*, and *SCARCC2*, among others (Satterstrom et al., 2020; Willsey et al., 2013). The expression of these genes is highest during early to mid-fetal development, during the establishment of neocortical organization and neural circuitry (Goodwin & Picketts, 2018; Parikshak et al., 2013; Satterstrom et al., 2020; Willsey et al., 2013). Processes that are highly active during this time include axon and dendrite growth, as well as the establishment of initial synaptic contacts (Kang et al., 2011; Südhof, 2018). Epigenetic ASD-associated genes have been shown to influence neuronal development and synaptic plasticity through direct and indirect regulation of other ASD-risk genes encoding synaptic molecules (Araujo et al., 2015; Bourgeron, 2015; Iakoucheva, Muotri, & Sebat, 2019; Notwell et al., 2016; Sugathan et al., 2014). Notably, the chromatin remodeler, CHD8, regulates the expression ASD-associated genes encoding synaptic proteins in iPSC neuroprogenitor cells and in *Chd8* mutant mouse models (Cotney et al., 2015; Jung et al., 2018; Platt et al., 2017; Sugathan et al., 2014). Heterozygous mice ($Chd8^{+/-}$) display autistic-like behaviour including alterations in social novelty and anxiety-like behaviour (Platt et al., 2017), and those carrying a heterozygous mutation ($Chd8^{+/N2373K}$) display abnormalities in social communication, anxiety-like behaviour, and elevated self-grooming (Jung et al., 2018). Therefore, alterations to epigenetic ASD-risk genes that encode chromatin remodeling proteins, such as *ATRX*, during early to mid-fetal development can alter synaptic gene expression, leading to abnormal neuronal circuitry formation followed by impaired behaviour (Zoghbi, 2003).

As an epigenetic ASD-associated gene, *ATRX* has been shown to influence gene expression in various ways, including regulating expression of synaptic molecules (Levy, Kernohan, Jiang, & Bérubé, 2014; Tamming et al., 2020). Therefore, the observed autistic-like behavioural changes of *Nex*-cKO^{MALE} mice described in this thesis could result from epigenetic alteration of many synaptic genes, leading to the abnormal establishment of synaptic networks. The lack of autistic-like behaviour observed in *Atrx*-cKO^{MALE} mice suggests that prenatal disruptions to these processes are related to behavioural changes associated with autism, whereas postnatal disruptions do not have the same effect. It is important to note that additional neuronal processes such as cell proliferation, neuroprogenitor cell differentiation, and migration of immature neurons also occur during early to mid-fetal development (Kang et al., 2011). However, AtrxNexCre mice lack *Atrx* in differentiated postmitotic neurons, thus bypassing the reported deleterious effects of ATRX loss-of-function in neural progenitors, including DNA replication stress, DNA damage, and programmed cell death (Bérubé et al., 2005; Seah et al., 2008; Watson et al., 2013).

While initial synaptic contacts are established mostly during embryogenesis, synapse formation continues throughout life in dynamic activity-dependent processes (Südhof, 2018). Specifically, during the early postnatal period in which brain development is still occurring, synapse and dendrite maturation peak in the cortex (Kang et al., 2011). Since these synaptic connections continue to form during postnatal development, ASD-risk genes involved in neuronal communication have high levels of expression between late mid-fetal development and infancy (Satterstrom et al., 2020). Our lab has previously identified numerous ASD-associated genes involved in synapse formation, particularly *Shank2* and *Nlgn4,* that are misregulated upon the loss of *Atrx* (Levy et al., 2014; Tamming et al., 2020). Specifically, in *Atrx*-cKO^{MALE} mice, these changes in gene expression in the adult brain occur in tandem with structural changes in hippocampal excitatory synapses and altered synaptic transmission and plasticity (Gugustea et al., 2019; Tamming et al., 2020). Behaviourally, these mice display impaired learning and memory (Tamming et al., 2020), cognitive processes that involve forming new synaptic connections and strengthening others (Stuchlik, 2014). The contrast in behavioural alterations of *Atrx*-cKO^{MALE} and *Nex*-cKO^{MALE} mice suggests that alterations to synaptic plasticity postnatally can result in

learning and memory changes, however, alterations to initial synaptic connections and neuronal circuitry prenatally must occur early to result in behavioural phenotypes related to autism. Learning and memory has not yet been investigated in *Nex*-cKO^{MALE} mice and should be assessed in the future to gain further insight into the neuron specific role of ATRX in relation to these behavioural changes. Based on the impairments observed in *Atrx*-cKO^{MALE} mice, we predict that the similar ablation of *Atrx* earlier in development will also lead to learning and memory impairments and may even exacerbate these deficits.

4.4 ATRX regulates expression of synaptic genes in the mouse brain

Tamming et al. (2020) have proposed a mechanism as to how the postnatal loss of neuronal *Atrx* in the hippocampus leads to learning and memory impairments in male mice. When present, ATRX binds regulatory sequences at the *miR-137* locus, a microRNA linked to impaired hippocampal-dependent learning and memory and known to regulate expression of genes involved in pre- and postsynaptic function (Olde Loohuis et al., 2015; Siegert et al., 2015). The binding of ATRX promotes the enrichment of the suppressive histone mark H3K27me3 at this site (Tamming et al., 2020). Loss of ATRX causes a reduction of H3K27me3 at the miR-137 locus, resulting in transcriptional upregulation in male mice and dysregulation of a set of synaptic genes, alterations to synaptic structure, leading to reduced LTP and deficits in learning and memory (Gugustea et al., 2019; Tamming et al., 2020). However, less is known regarding the role of ATRX in the cortex, and how the prenatal neuronal loss of *Atrx* results in autistic-like behaviour.

Shioda et al. (2018) recently demonstrated that the altered social behaviour of Atrx$^{\Delta E2}$ mice are related to transcriptional misregulation in the hippocampus resulting in synaptic dysfunction. They showed that in the mouse hippocampus, ATRX normally binds to G-quadruplexes in the CpG islands of the imprinted X-linked lymphocyte-regulated protein 3b (*Xlr3b*) gene, inhibiting its expression by recruiting DNA methyltransferases (Shioda et al., 2018). When expressed, Xlr3b binds to dendritic mRNA and inhibits transport, promoting synaptic dysfunction. Mutant *Atrx* subsequently results in reduced DNA methylation and increased expression of *Xlr3b*, leading to decreased synaptic plasticity and

changes in behaviour (Shioda et al., 2018). However, treatment with 5-aminolevulinic acid (5-ALA), a G-quadruplex binding factor, reduced RNA polymerase II recruitment and repressed *Xlrb3* transcription, improving behavioural deficits (Shioda et al., 2018). Although *Nex*-cKO^{MALE} mice did not display similar withdrawal in social interactions that was reported with Atrx$^{\Delta E2}$ mice, they did show alterations in social behaviour, particularly aggressive social interactions with stranger mice and littermates. In *Nex*-cKO^{MALE} mice, it is likely that the neuronal loss of *Atrx* also results in misregulation of synaptic genes, contributing to the reported autistic-like features. It may be that this mechanism occurs indirectly, through regulation of other RNA-binding molecules such as the models described above (Shioda et al., 2018; Tamming et al., 2020); or rather, occurs by directly regulating expression of genes involved in synapse formation, such as *Nlgn4* (Levy et al., 2014). Further investigation of these questions will elucidate the role of ATRX in neurons during embryonic brain development and moreover, how disruptions to *Atrx* at this time contribute to autistic-like behaviour.

4.5 Autistic-like behaviour of *Nex*-cKO^{MALE} mice

The autistic-like behaviour observed in *Nex*-cKO^{MALE} mice are reflective of behaviour present in individuals with ASD and have been reported in other mouse models with ASD-associated mutations. Firstly, it has been estimated that approximately 68% of ASD patients exhibit aggressive behaviour, often directed towards caregivers (Lai, Lombardo, & Baron-Cohen, 2014; Mazurek, Kanne, & Wodka, 2013). Additionally, patients with ATR-X syndrome have anecdotally been reported to develop aggressive behaviour (Stevenson, 2000). Although aggressive behaviour is commonly reported in ASD patients, many mouse models do not display changes in aggression, or even show increased latency to attack during resident-intruder assays, suggesting decreased aggression (Clipperton-Allen & Page, 2015; El-Kordi et al., 2013). However, increased aggression in mutant male mice has been reported in some instances. Notably, male mice with a knockout of neurexin-1α (*Nrxn1α*$^{-/-}$), a presynaptic cell adhesion molecule, show increased aggressive behaviour towards juvenile and adult conspecific mice (Grayton, Missler, Collier, & Fernandes, 2013). Similarly, male mice harboring a mutation in the postsynaptic cell adhesion molecule neuroligin-3 (*Nlgn3*^{R451C}) display a pronounced increase in aggression (Burrows

et al., 2015). Several brain regions have been implicated in regulating aggressive behaviour, and changes to these regions or the circuitry between them can increase the propensity for aggression. Two of these regions include the amygdala and the prefrontal cortex (Davidson, Putnam, & Larson, 2000; Matthies et al., 2012; Seguin, 2009), both of which are targeted with the NexCre driver (Goebbels et al., 2006).

In addition to aggressive behaviour, ATR-X syndrome patients have also been reported to exhibit repetitive stereotypic movements, such as pill-rolling or hand flapping (Gibbons, 2006). Moreover, repetitive or stereotyped behaviours are one of the core features of ASD (Lai et al., 2014). In rodents, self-grooming is commonly used a model of these repetitive and patterned behaviours (Kalueff et al., 2016). The excessive self-grooming behaviour observed in *Nex*-cKO^{MALE} mice has been reported in other models, including *Nrxn1α*$^{-/-}$ mice (Etherton, Blaiss, Powell, & Südhof, 2009) and several models with alterations to the SHANK family of postsynaptic scaffolding proteins (Guilmatre, Huguet, Delorme, & Bourgeron, 2014; Peça et al., 2011; Sungur, Vörckel, Schwarting, & Wöhr, 2014; Won et al., 2012). Interestingly, *Shank3* knockout mice develop self-inflicted skin lesions due to excessive grooming (Peça et al., 2011), similar to what was observed in several *Nex*-cKO^{MALE} mice. The neocortex has been shown to be involved in the modulation of self-grooming movements, by sending excitatory projections to the striatum and receiving excitatory projections from the thalamus and amygdala (Kalueff et al., 2016). Alterations to the formation of these neuronal circuits in the developing *Nex*-cKO^{MALE} brain due to the loss of *Atrx* may contribute to the observed overgrooming behaviour.

Finally, encompassed within the core features of ASD are alterations in sensory processing, including hypo- or hyper-reactivity to sensory input (Lai et al., 2014). Specifically, reactivity to acoustic stimuli is greater in some individuals with ASD, as demonstrated by exaggerated responses to sounds (Chamberlain et al., 2013; Ebishima et al., 2019; Kohl et al., 2014). Our results show that *Nex*-cKO^{MALE} mice display an exaggerated startle response to acoustic stimuli accompanied by impaired pre-pulse inhibition, a measure of the pre-attentive filtering process known as sensorimotor gating (Luo, Norris, Gordon, & Nithianantharajah, 2018), indicating sensory processing and sensory filtering is affected (Sinclair, Oranje, Razak, Siegel, & Schmid, 2017). Reduced PPI has been reported in some

adults with ASD (Perry, Minassian, Lopez, Maron, & Lincoln, 2007), however, reports have not been consistent, with some studies showing no change in PPI for adults or children with ASD (Kohl et al., 2014; Takahashi, Komatsu, Nakahachi, Ogino, & Kamio, 2016). Similarly, mouse models with ASD related genetic mutations show high variability in acoustic startle responses and PPI based on genetic background and experimental protocol. However, there are other models that demonstrate a similar change in sensory processing and filtering as *Nex*-cKO^{MALE} mice. Specifically, male *Nrxn1α*$^{-/-}$ mice display a small enhancement of startle response and impaired PPI (Etherton et al., 2009). Additionally, mice with a heterozygous deletion of the imprinting center for Prader-Willi syndrome (*PWS-IC*$^{+/-}$) present with increased startle response to acoustic stimulus and impaired PPI (Relkovic et al., 2010). Similar to ATR-X syndrome, Prader-Willi syndrome is a genetic neurodevelopmental disorder characterized by intellectual disability, with many patients present with co-occurring autism or autistic-like behaviour (Dykens et al., 2017). However, the presence of additional autistic-like behavioural changes were not assessed in *PWS-IC*$^{+/-}$ mice (Relkovic et al., 2010). Developmental disruptions of sensory processing can lead to persistent changes in neural circuitries responsible for auditory reactivity (Scott et al., 2018; Sinclair et al., 2017). Ablation of *Atrx* in postmitotic telencephalic cortical neurons during embryonic development may lead to disruptions in connectivity between brain regions involved in auditory processing, including the cortex (Peterson & Hamel, 2019; Sinclair et al., 2017).

4.6 Limitations of the study

While the autistic-like behaviour observed in *Nex*-cKO^{MALE} mice are quite robust, it is necessary to highlight the limitations of this model. Importantly, the AtrxNexCre model results in *Atrx* ablation in excitatory neurons beginning on embryonic day 11.5, as such this model is not a representative model of ATR-X syndrome or autism. Reported *ATRX* mutations associated with ASD, and those that result in ATR-X syndrome, are inherited mutations (Aspromonte et al., 2019; Brett et al., 2014; Gong et al., 2008; Li et al., 2017; Munnich et al., 2019; Yu et al., 2013); therefore, these mutations affect all cell types from the beginning of development. Additionally, these inherited mutations significantly impair ATRX function and protein levels, but do not result in a complete loss of the protein

(Gibbons et al., 2008; Picketts, 1996). While the AtrxNexCre model is not representative of mutations observed in human patients, the use of this model allows us to explore the biological role of ATRX in neurons during prenatal brain development, and the effects on behavioural outcomes. The same limitations exist for the AtrxCaMKIICre model, in which the neuron specific loss of *Atrx* occurs postnatally (Tamming et al., 2020). Particularly, *Atrx*-cKO^{FEMALE} mice used in our study experience a complete loss of ATRX in these forebrain neurons, whereas in humans, females harbouring *ATRX* mutations are typically carriers and are asymptomatic due to skewed X-inactivation (Aspromonte et al., 2019; Brett et al., 2014; Gong et al., 2008; Li et al., 2017; Munnich et al., 2019; Sun et al., 2001; Yu et al., 2013). As such, the clinical relevance of any observed differences in this model are limited. Nevertheless, studying behavioural outcomes of these models allows us to identify functions of ATRX in neurons and compare how the function may differ between pre- and postnatal brain development to affect behaviour.

It is also important to note that our investigation of autistic-like behaviour in this thesis was not comprehensive. There are additional behavioural changes related to autism that we were not able to assess due to various limitations. Due to a lack of available equipment, we were not able to assess behaviour related to communication including deficits in ultrasonic vocalizations emitted during social interactions and by separated pups (Crawley, 2007; Silverman et al., 2010). Additionally, we were not able to perform several behavioural paradigms with *Nex*-cKO^{MALE} mice that were done with *Atrx*-cKO^{MALE} and *Atrx*-cKO^{FEMALE} mice. Specifically, we did not perform the social approach assay, in which test mice freely engage in reciprocal social interactions with stranger mice (Crawley, 2007), due to the aggressive behaviour demonstrated by *Nex*-cKO^{MALE} mice. Similarly, we did not assess digging and burying repetitive behaviour by means of the marble burying assay with *Nex*-cKO^{MALE} mice due to difficulties removing mice from the testing arena without disturbing the results of the test. Therefore, while our analysis of behaviour related to autism was not exhaustive, we identified distinct autistic-like behaviour in *Nex*-cKO^{MALE}.

4.7 Future directions with *Nex*-cKO mice

While it may not be possible to assess all behavioural phenotypes related to autism with *Nex*-cKO^{MALE} mice, there are additional paradigms that can be performed with modifications to account for the complexities of handling *Nex*-cKO^{MALE} mice. Specifically, a modified resident-intruder task to further assess aggressive behaviour with stranger mice should be performed (Crawley, 2007; Silverman et al., 2010). Insistence on sameness is another behaviour related to autism that should be assessed using reversal learning tasks in either the Y-maze or Morris Water Maze, to measure the flexibility of mice to switch from an established habit to a new habit (Silverman et al., 2010). Additionally, we have demonstrated *Nex*-cKO^{MALE} mice show an exaggerated startle response to acoustic stimuli, however, reactivity to other sensory stimuli such as tactile startle should be investigated (Crawley, 2007). Furthermore, due to the relation between *ATRX* mutations and intellectual disability, behaviour related to cognition must be thoroughly investigated and should include assessments of learning and memory and well as anxiety-related traits. Finally, it will be valuable to investigate behaviour of female AtrxNexCre mice, to determine if the identified autistic-like behaviour is sex-specific.

Although the use of a conditional loss of *Atrx* poses limitations, as described above, these models allow for the in-depth analysis of molecular mechanisms underlying alterations to behaviour. The data in this thesis demonstrate that a neuron specific loss of *Atrx* during embryogenesis is sufficient to result in autistic-like behaviour in male mice, thus providing the basis for further exploration of these molecular mechanisms. Specifically, gene expression analysis of the *Nex*-cKO^{MALE} developing and adult cortex will identify synaptic and RNA-binding molecules that are misregulated upon the loss of ATRX. Additionally, electrophysiology studies of the *Nex*-cKO^{MALE} cortex will identify changes in synaptic plasticity that may underly altered neuronal circuity leading to autistic-like behaviour.

Finally, we cannot rule out the impact of mutant *ATRX* in other neuronal cell-types on autism and autistic-like behaviour observed in humans. Both pre- and postnatal cell-type specific knockouts of *Atrx* in astrocytes, oligodendrocytes, microglia, and inhibitory neurons should be investigated to determine the impact on behaviour.

4.8 Thesis Conclusions

Research focused on understanding the mechanisms underlying ASD etiology have identified epigenetic regulators as prominent ASD-associated genes (Geschwind, 2011; Iakoucheva et al., 2019; Lasalle, 2013; Loke et al., 2015; Voineagu et al., 2011; Yang et al., 2014). Although ATRX, a chromatin remodeling protein, has been associated with autism and autistic-like behaviour, little is known regarding how alterations to *ATRX* in the developing brain result in these behavioural changes. In this thesis, I have determined that the embryonic loss of *Atrx* in excitatory neurons of the forebrain results in specific autistic-like behavioural phenotypes in adult male mice. Furthermore, although the postnatal loss of *Atrx* results in deficits in learning and memory, I have shown that it does not lead to behavioural changes related to autism. Altogether, these findings support *ATRX* as an autism-associated gene and suggest that ATRX plays key roles in embryonic excitatory neurons to suppress the development of autistic behaviour. By understanding how disruptions to *Atrx* alter behaviour, we are able to gain insight into how ATRX influences typical neuronal development and function.

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Appendix A: Permission to use published article

The data and text in Chapter 2 has been published in the Journal of Neurodevelopmental Disorders as:

Martin-Kenny, N., & Bérubé, N.G. (2020). Effects of a postnatal *Atrx* conditional knockout in neurons on autism-like behaviours in male and female mice. *J Neurodevelop Disord, 12*(17), 1-11. https://doi.org/10.1186/s11689-020-09319-0

Appendix B: Verification of ATRX loss in *Nex*-cKO^{MALE} mice

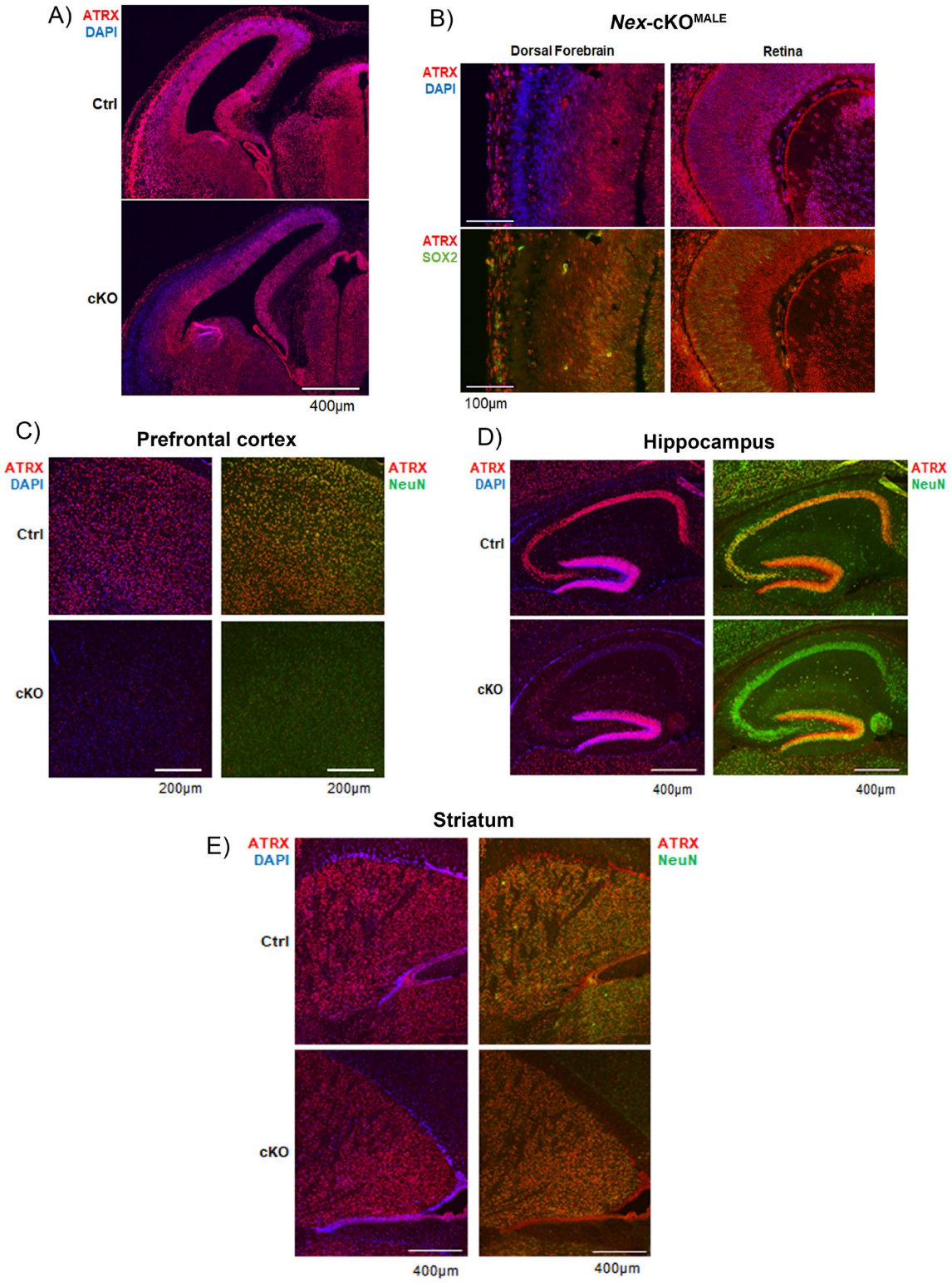

Figure 1 Validation of ATRX loss in postmitotic neurons in *Nex*-cKO^{MALE} mice.

A) Immunofluorescence-based staining of cryosections at embryonic day 13.5 (E13.5) shows a loss of ATRX in differentiating neurons of the dorsal forebrain in *Nex*-cKO^{MALE} mice (5x magnification). B) Co-staining of E13.5 embryonic sections with SOX2, a marker of proliferating cells, shows ATRX loss is specific to SOX2-negative post-mitotic cells in the dorsal forebrain. Images from the retina, a brain region in which the NexCre driver is not expressed, show ATRX is present in both SOX2 positive and negative cells (20x magnification). ATRX: red (1:100; Santa Cruz), SOX2: green (1:100; Sanata Cruz), DAPI: blue. C-D) Immunofluorescence staining of the postnatal day 20 (P20) *Nex*-cKO^{MALE} brain shows a neuron specific loss of ATRX in the prefrontal cortex and hippocampus (10x magnification, 5x magnification). E) Images from the striatum at P20 shows the presence of ATRX in neurons marked with NeuN in both *Nex*-cKO^{MALE} and *Nex*-Ctrl^{MALE} brains (5x magnification). ATRX: red (1:100; Santa Cruz), NeuN: green (1:100; Millipore), DAPI: blue.

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