ABSTRACT

Deletion of *FMR1* Results in Sex-Specific Changes in Behavior

Suzanne O. Nolan, M.A.

Mentor: Joaquin N. Lugo, Ph.D.

Fragile X Syndrome (FXS) is a neurodevelopmental disorder caused by excessive trinucleotide (CGG) repeats in the *FMR1* gene coding for fragile x mental retardation protein (FMRP). In humans, this disorder is characterized by intellectual disability, as well as other behavioral abnormalities, such as hyperactivity and social behavior abnormalities. Mutations in the *FMR1* gene are found in 2 - 6 % of individuals with Autism Spectrum Disorder (ASD), making it the single largest genetic contributor to ASD. Mouse models of FXS disorder are commonly touted as preferred models for understanding ASD. Furthermore, few studies to date have examined the role of sex in the *FMR1* phenotype. In the present study, we used a systemic *FMR1* knockout in an FVB background strain. We examined the effects of this genetic mutation in males and females homozygous for an *FMR1* mutation on measures sociability, repetitive behaviors, vocalization patterns, anxiety and fear-related learning.

Deletion of *FMR1* Results in Sex-Specific Changes in Behavior

by

Suzanne O. Nolan, B.S.

A Thesis

Approved by the Department of Psychology and Neuroscience

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Charles A. Weaver III, Ph.D., Chairperson

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of

Master of Arts in Psychology

Approved by the Thesis Committee

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Joaquin N. Lugo, Ph.D., Chairperson

_____

Melanie J. Sekeres, Ph.D.

_____

Joseph H. Taube, Ph.D.

Accepted by the Graduate School

December 2016

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J. Larry Lyon, Ph.D., Dean

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ASD	Autism spectrum disorder
CS	Conditioned stimulus
EPM	Elevated plus maze
FAS	Fetal alcohol syndrome
FMRP	Fragile X mental retardation protein
FXS	Fragile X syndrome
GERD	Gastroesophageal reflux disorder
IIPMF	Infrapyramidal mossy fiber terminal fields
KO	Knockout
MANOVA	Multivariate analysis of variance
MWM	Morris water maze
PD	Postnatal Day
PPI	Prepulse inhibition
US	Unconditioned stimulus
UTR	Untranslated region
UVs	Ultrasonic vocalizations
WT	Wild type

ACKNOWLEDGMENTS

I would like to acknowledge and my mentor Dr. Joaquin Lugo for his continued support and guidance during my time at Baylor University and throughout my project. I would also like to thank my colleagues and fellow graduate students that I have worked with including, Gregory Smith, Andy Holley, Samantha Hodges, and Matt Binder. Additionally, I would like to thank all of the undergraduate students that have help me on this project including Conner Reynolds, Ashvini Pandian, Taylor Jefferson, and Brianna Escobar, as well as the many other undergrads that I worked with while at Baylor.I would like to also acknowledge and thank my family, for their never-ending love and support. Additionally, I would like to thank Baylor University and the faculty and staff of Baylor for all of the support I received. Finally, I would like to thank Lee Lowe, and her staff, for their support and dedication to taking care of the animals.

CHAPTER ONE

Introduction

Fragile X Syndrome (FXS) is a neurodevelopmental disorder caused by a trinucleotide (CGG) repeat expansion in the *FMR1* gene coding for FMRP. Mutations in this gene result in subsequent functional silencing of *FMR1* transcription and synthesis of the FMRP protein. FXS is phenotypically characterized by intellectual disability, however symptoms may also include hyperactivity and fidgeting behaviors (R. J. Hagerman, Jackson, Levitas, Rimland, & Braden, 1986). Fragile X syndrome is highly comorbid with other neurodevelopmental disorders, such as attention deficit hyperactivity disorder (Wheeler et al., 2014), epilepsy (Berry-Kravis, 2002) and autism spectrum disorder (ASD) (Clifford et al., 2007). Epidemiological data suggests that *FMR1* mutations constitute the largest genetic contributor to Autism Spectrum Disorder, with a prevalence rate of 2 - 6 % of individuals with Autism Spectrum Disorder (ASD) (Kaufmann et al., 2004). This percentage represents a large portion of monogenic contributors when one considers that other commonly noted single genetic contributors only constitute about one percent each to ASD (i.e. tuberous sclerosis complex (TSC) (P. Baker, Piven, & Sato, 1998), phosphatase and tensin homolog (PTEN) (Buxbaum et al., 2007), and methyl-CpG-binding protein 2 (MECP2) (Moretti & Zoghbi, 2006).

Beyond the epidemiological overlap, FXS and ASD share several behavioral characteristics. ASD is most commonly characterized by three core symptom clusters: abnormal communication skills, social abnormalities, and repetitive behaviors (R.

Hagerman, Hoem, & Hagerman, 2010). Similarly, individuals with FXS show significant communication impairments, such as echolalia and palilalia behaviors (Ferrier, Bashir, Meryash, Johnston, & Wolff, 1991). Concerning sociability, shyness and eye contact avoidance are often noted in these individuals (Kerby & Dawson, 1994), and more extreme, social phobias are a common psychiatric ailment (Cordeiro, Ballinger, Hagerman, & Hessl, 2011). Hand-flapping behavior, a type of repetitive behavior, is commonly noted among individuals with FXS (R. J. Hagerman et al., 1986). However, FXS is most frequently characterized by hyperactivity, mild intellectual disability, as well as delays in motor and language development (R. J. Hagerman, 1997).

Preclinical investigations of FXS and ASD often utilize the *FMR1* genetic knockout mouse model. Previous research has established that deletion of *FMR1* results in downstream changes in a variety of behaviors in the *FMR1* knockout that mirror changes in both FXS and ASD phenotypes. For example, the *FMR1* knockout shows, alterations in social behavior and social communication (Lai et al., 2014; Z.-H. Liu & Smith, 2009) and increased repetitive behaviors (Spencer et al., 2011). Some characteristics of the *FMR1* knockout more accurately represent its relationship with FXS, such as hyperactivity (Ding, Sethna, & Wang, 2014; Pietropaolo, Guilleminot, Martin, D'Amato, & Crusio, 2011; Sorensen et al., 2015), deficits in prepulse inhibition (Frankland et al., 2004), and altered anxiety (Sorensen et al., 2015). While ASD-like characteristics have been shown in the *FMR1* knockout mice, these results have also been shown to be highly variable and strain-dependent (Paradee et al., 1999).

Sex plays a significant role in the overall prevalence and clinical presentation of FXS and ASD. Epidemiological data has estimated the prevalence rate of ASD in males

to be twice that of females (Fombonne, 2003). Similar sex differences in prevalence rates are represented in FXS, with life-time incidence of approximately 1 in 4,000 males as compared to 1 in 8,000 females (Pembrey, Barnicoat, Carmichael, Bobrow, & Turner, 2001). In terms of characteristics of a behavioral phenotype, sex is also a significant contributor to different aspects of both ASD and FXS. In humans with ASD, previous evidence has supported the idea that sex mediates the ASD phenotypic characteristics, with females with ASD showing less stereotypic play and less unusual visual responses than males with ASD (Lord, Schopler, & Revicki, 1982). Both symptomology and symptom severity vary between the sexes in FXS. The symptoms of FXS in males have been shown to be more severe than females with the mutation (Kazdoba, Leach, Silverman, & Crawley, 2014). More broadly, it is generally thought that many X-linked disorders show unequal pattern of phenotype severity, likely owing to the fact that women are more likely only carriers of a single copy of the mutated gene (Germain, 2006). However, there is some evidence that the symptomology of females with mutations is qualitatively different than that of males with mutations. Females with FXS are at a higher risk for schizophrenia and extreme shyness, but the majority of females with mutations are of normal intelligence (Reiss, Hagerman, Vinogradov, Abrams, & King, 1988). Deficits in affective processes are also prevalent among of FXS females (R. J. Hagerman & Sobesky, 1989).

Despite these established differences in both prevalence and phenotypic severity in humans, many studies of the *FMR1* knockout focus exclusively on the effect of males and as such, the impact of deletion of *FMR1* on females is less understood. There are three studies to date that provide evidence that deletion of *FMR1* in females produces

clear and measurable phenotypic alterations, including hyperactivity, learning and memory deficits, and increased sensitivity to seizure stimuli, compared to wildtype *FMR1* phenotype (K. B. Baker et al., 2010; Ding et al., 2014; Qin, Kang, & Smith, 2005). However, there are only three studies that have reported findings with females included, yet the literature base for the *FMR1* knockout in males is vast. Currently, the effect of homozygous deletion of *FMR1* in female mice on repetitive behavior, motor coordination, communication behavior, and social constructs are unknown (Romano, Cosentino, Laviola, & De Filippis, 2016).

The *FMR1* knockout mouse is a well-studied model of the human condition of Fragile X Syndrome and its comorbidity with ASD. However, behavior in the *FMR1* knockout shows high amounts in inherent variability between experiments and background strain. Furthermore, given that sex is shown to significantly impact both severity and profile of symptoms, then it is important to examine the impact of FMR1 deletion in females. However, many dimensions of behavior have yet to be studied in these females. The present study aims to further characterize and reproduce the strain-specific and putative sex-specific effects of deletion of *FMR1*. The focus of the current investigation was on measuring these changes in tests of activity levels, anxiety behaviors, social behaviors, repetitive behaviors, hippocampal and amygdala based memory, as well as sensorimotor gating and motor functioning.

CHAPTER TWO

Review of Literature

The FMR Protein

Fragile X Mental Retardation protein (FMRP) is the gene product of the *FMR1* gene. FMRP is thought to be functionally implicated in synthesis-dependent synaptic plasticity in the nervous system. More specifically, it is an mRNA-binding protein that is involved with the translation of many important synaptic plasticity proteins, including mGLUR5 and GFAP (Fatemi & Folsom, 2011). Therefore, elevations in FMRP translation have been suggested as an indicator of dynamic synaptic plasticity processes, and can be studied through light deprivation paradigms. In this paradigm, rats are raised until P44 in complete darkness. Upon a brief exposure to light, protein levels are assayed. Following this exposure, FMRP levels are elevated transiently in visual cortex dendrites of rats in response to light exposure (Gabel et al., 2004). Mechanistically, it was first thought to bind selectively to mRNA, specifically those involved in synaptic plasticity, and associate with polyribosomes, where it represses translation of certain mRNA transcripts (Penagarikano, Mulle, & Warren, 2007).

While suppression of target mRNA is the most commonly cited role of FMRP, more recent literature suggests that FMRP has other roles in the brain. For instance, FMRP has been reported to function in the rapid activity-regulated transport of mRNA. Transport of mRNA is functionally important in many processes, such as synaptogenesis and plasticity. When FMRP is functionally silenced, dendritic localization of mRNAs in

response to glutamatergic signaling is diminished. Furthermore, suppression of FMRP in wildtype neurons is known to cause altered spine morphology that can be functionally related to dysregulated mRNA transport (Dictenberg, Swanger, Antar, Singer, & Bassell, 2008). Activity-dependent translation of mRNA occurs through activation of mGluR1/5 receptors. Without FMRP, these translation processes become dysregulated, shown in Figure 2.1 below, leading to upregulation of specific dendritic mRNAs, including Arc/Arg3.1, Map1B, and CaMKII (Zalfa et al., 2003). Downstream, these changes can have effects on signaling cascades involved in morphogenesis, such as Rac1 and calbindin (Grossman, Aldridge, Weiler, & Greenough, 2006).

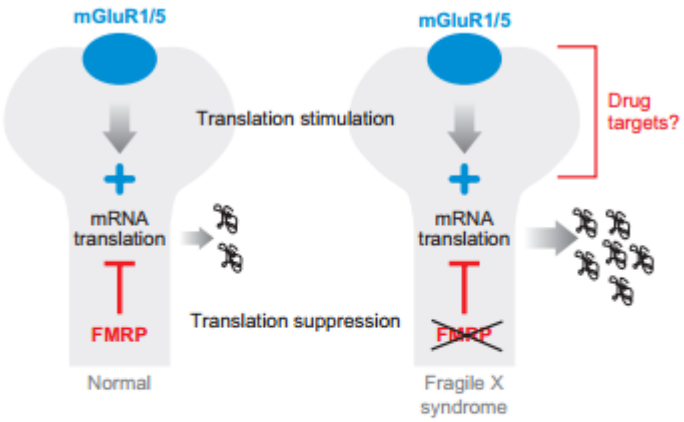

Figure 5

Role of fragile X mental retardation protein (FMRP) in modulating synaptic plasticity. The activation of group I metabotropic receptors (mGluR) stimulates translation of specific mRNAs at synapses. FMRP normally acts as a translational repressor regulating such expression; however, in the absence of FMRP, overexpression of such messages is found.

Figure 2.1. A schematic representation of the role of FMRP in activity-dependent translation. (Penagarikano et al., 2007)

Understanding how FMRP relates to activity-dependent transportation and subsequent translation at the synaptic level can be extrapolated to observable and related

phenomenon, such as adult hippocampal neurogenesis. Neurogenesis is a process carried out by neural stem and progenitor cells throughout life, and is necessary for behaviors such as hippocampal-dependent learning (Gould, Tanapat, Hastings, & Shors, 1999). Areas involved in these processes, i.e. the hippocampus, also show high expression levels of FMRP. Furthermore, it has been shown that loss of FMRP in hippocampal neural stem cells leads to reduced hippocampal neurogenesis, and ultimately learning and memory deficits dependent on these processes, suggesting that FMRP is a necessary component of hippocampal-dependent learning (Guo et al., 2011)

Fragile X Syndrome

History

In 1943, two scientists by the name of Martin and Bell showed that a certain form of intellectual disability (previously classified as mental retardation) was among disorders showing an X-linked inheritance pattern. In this original publication the authors provided a thorough description and pedigree of 11 patients across two generations with marked intellectual disabilities as well as other behavioral abnormalities such as speech difficulties and aggression. The two scientists were so thorough in their description of the neurological disorders that some suggested that this disorder should remain under the name Martin-Bell Syndrome, to credit the scientists who characterized it. In 1969, a scientist named Herbert Lubs developed the first chromosomal test for Fragile X syndrome, formerly known as Martin-Bell Syndrome, though the use of this test was limited in medical settings until the late 1970's. In 1991, with the assistance of a

technique called positional cloning, the *FMR1* gene was identified as the gene disturbed in this syndrome (Yu et al., 1991).

Etiology

Fragile X syndrome (FXS) is a neurodevelopmental disorder caused by a genetic mutation in the *FMR1* gene, which encodes for FMRP. This disorder is caused by an overexpansion in the X-chromosome 5'-untranslated region (UTR) at position Xq27.3 due to a trinucleotide (CGG) repeat (Verkerk et al., 1991). The prevalence of FXS in humans is approximately 1:4000 males and 1:8000 females (Crawford, Acuña, & Sherman, 2001). This disorder does show an X-linked pattern of inheritance, however, it does not show the typical Mendelian pattern of inheritance. Recent evidence suggests that the maternal X chromosome does not always meet criteria for a full mutation. Maternal X chromosomes that carry the *Fmr1* pre-mutation are more likely to expand into the full mutation in meiotic cells, though this instability is still not well understood (Jin & Warren, 2000).

The overexpansion due to trinucleotide repeats in the 5' UTR leads to hypermethylation of the *FMR1* gene and functional silencing of the protein. However, the degree of overexpansion varies by individual, and can be divided into two categories – pre-mutation and full mutation. Individuals with 55 to 200 trinucleotide repeats in this region are said to have the pre-mutation and may display normal to mildly impaired intelligence. Individuals with greater than 200 trinucleotide repeats, considered a full mutation, experience loss of FMRP function, leading to severe intellectual deficiency. This complete loss of this protein is only present in carriers of the full mutation (Devys,

Lutz, Rouyer, Bellocq, & Mandel, 1993). This reasoning suggests that the degree of impairment is directly related to the degree of overexpansion.

Patients diagnosed with FXS show a myriad of impairments beyond intellectual disability, such as hyperactivity, sensorimotor gating abnormalities, and communication problems (R. J. Hagerman & Hagerman, 2002). Before considering how loss of FMRP impacts behavior, it is important to consider the physical and physiological characteristics at play.

Physiological Characteristics

Several common medical conditions among individuals with FXS have been noted, reviewed in (Kidd et al., 2014). The most commonly reported complaint is otitis media, or inflammation of the middle ear, as well as an increased risk of sinus infection commonly seen in children with FXS (R J Hagerman, Altshul-Stark, & McBogg, 1987). Individuals with FXS also frequently report sleep problems and strabismus. Another common concern among children and parents of FXS children is gastrointestinal difficulties. Specifically, gastroesophageal reflux disease (GERD), persistent diarrhea, emesis, and general abdominal discomfort have all been reported at higher than normal rates in FXS (Kidd et al., 2014). Perhaps more serious, it has also been noted that loss of FMRP is related to an increased risk of epilepsy (Qiu, Hao, Li, & Xiong, 2008).

Individuals with FXS have connective tissue dysplasia, a term for the general weakening of the connective tissues, namely bone, ligaments/tendons and skin. This weakness results in higher than normal incidence of flat feet and joint hyperflexibility in individuals with FXS (Hagerman et al, 2002). Secondary to connective tissue dysplasia, craniofacial malformations have also been observed. However, unlike the obvious

craniofacial malformations that have been noted in other developmental disorders, such as fetal alcohol syndrome (FAS) or Down's syndrome, those of individuals with FXS are subtler. Males with FXS have longer and narrower faces, prominent ears, joint hypermobility and feet with flattened arches (R. J. Hagerman, 1997). These facial characteristics are often hard to distinguish at the time of diagnosis, though they may become more apparent with age. All of these physical abnormalities can be related to general connective tissue dysplasia, which is hypothesized to occur secondary to loss of FMRP (Opitz, Westphal, & Daniel, 1984). Craniofacial changes are often present in other such neurodevelopmental disorders and are considered to be "neurological soft signs," serving as signals for underlying structural changes in the central nervous system (CNS).

Changes in Brain Structure

Behavioral impairments often serve as indicators of underlying morphological changes. As mentioned earlier, Fragile X Syndrome is the largest cause of inherited intellectual disability. Other common sources of intellectual disability, such as Down's syndrome, have shown gross morphometric alterations in the brain (Wisniewski, 1990). Thus, it was hypothesized that FXS would show levels of cortical dysgenesis and reduced neuronal growth similar to Down's syndrome. Many early studies did not find alterations in overall gross anatomy (Rudelli et al., 1985). These original studies have been repeated several times with differing results. More recent studies have showed that children with FXS display mostly normal patterns of brain development, but do exhibit increased caudate and thalamic volumes, as well as increased lateral ventricular volumes with age (Eliez, Blasey, Freund, Hastie, & Reiss, 2001).

As will be discussed later, one of the most common phenotypic changes seen in clinical manifestations of FXS is deficits in auditory processing and sensorimotor gating behavior (R. J. Hagerman, 1997). These behavioral changes have been related to alterations in the functional anatomy of a few key brain areas. The medial superior olive is one such structure, known for its role in sound localization (Kulesza Jr, 2007). Work on the cytoarchitecture of the medial superior olive has shown that it is disrupted in FXS, as well as ASD (Kulesza & Mangunay, 2008). Such changes relate functionally to alterations in auditory processing. These deficits in auditory processing have also been related to structural abnormalities of the cerebellum. Early work on the examination of the cerebellum in individuals with FXS reported that the posterior fossa, including the cerebellum and fourth ventricle, showed volumetric decreases that related functionally to changes in auditory processing as well as motor coordination (Mostofsky et al., 1998). These have been characterized on a deeper level as well. Sub-structures alterations in the cerebellum of those with FXS include decreased size of the arbor vita (Ellegood & Crawley, 2015), decreased volume of fastigial nucleus and nucleus interpositus (Ellegood, Pacey, Hampson, Lerch, & Henkelman, 2010), as well as decreased cerebellar vermis size (Reiss, Aylward, Freund, Joshi, & Bryan, 1991).

Structural changes within the temporal lobe have also been related to the observed changes in auditory processing. The superior temporal gyrus, containing the primary auditory cortex, has been implicated in processing the emotionality of facial stimuli as well as auditory processing. Early examination of structural alterations in individuals with FXS also noted decreased volume of the superior temporal gyrus (Reiss, Lee, & Freund, 1994). Changes in the architecture of this gyrus may coincide with changes seen

in the sociability of individuals with FXS. In the same investigation, two other structures of the limbic system, the hippocampus and amygdala, also showed structural alterations. However, whereas all the aforementioned temporal lobe structures have shown volumetric decreases, the hippocampus displays increased size in FXS, while some hippocampal substructures, like intra- and infrapyramidal mossy fiber terminal fields (IIPMF) show volumetric decreases (Mineur, Sluyter, de Wit, Oostra, & Crusio, 2002). Microanatomical changes also reveal hallmark increases in dendritic spine density, namely in the temporal and visual cortices (Irwin et al., 2001). These changes in dendritic field volume may account for the aforementioned increases in volume of the hippocampal field.

Alterations in the frontostriatal circuitry make up the final category of morphological differences. In a seminal study of white matter alterations in FXS females, fractional anisotropy values along this tract were shown to be significantly lower than controls (Barnea-Goraly et al., 2003). Two structures along this pathway have also shown altered volumes. Similar to volumetric increases in the hippocampus, the volume of the basal ganglia is also increased in FXS (Hazlett et al., 2009). These changes can be related to behavioral alterations seen in FXS, such as hyperactivity and increased repetitive behaviors. The origin of this circuit, the prefrontal cortex, also shows functional neuroanatomical changes in this disorder (Kwon et al., 2001), linking these to decreased cognitive flexibility and aggression.

Behavioral Alterations

As was just hinted, changes in these brain structures can be related to a myriad of behavioral indicators related to Fragile X Syndrome. These symptoms include some that

have overlap with ASD, and also some symptoms unique to the FXS diagnosis. The most commonly characterized behavioral alteration in Fragile X is intellectual disability. Other phenotypic changes not related to ASD include anxiety, hyperactivity, impulsivity and even aggression (Tsiouris & Brown, 2004). Moreover, the severity of cognitive and emotional phenotypic ranges from mild to severe, depending of the amount of FMRP produced, which in turn depends on the quantity of repeats and the methylation degree of the gene (Saldarriaga et al., 2014). Autistic-like features include hand flapping, gaze avoidance, tactile defensiveness, and hyperarousal to sensory stimuli (R. J. Hagerman et al., 1986). In addition, individuals with FXS often display impaired social skills that bear resemblance to the ASD phenotype. Other common characteristic of ASD are communication deficits. Language impairments such as echolalia and palilalia are also commonly seen in FXS (Ferrier et al., 1991).

The FXS and ASD Comorbidity

FXS exhibits a high amount of comorbidities, including obsessive-compulsive disorder, anxiety disorders, epilepsy and ADHD (Garber, Visootsak, & Warren, 2008). While FXS is highly comorbid with many neurodevelopmental disorders, the most well-defined comorbidity is the relationship with ASD. Co-examination of these disorders stem from a shared symptomatology. The three major symptom categories of ASD include communication alterations, deficits in sociability, and repetitiveness or resistant to change behavior. Individuals with a FXS diagnosis do share communication deficits, alterations in sociability, and stereotyped behavior as well (R. J. Hagerman et al., 1986). This behavioral overlap leads to a significant portion of diagnostic overlap, with approximately 21 – 50% of individuals with FXS meet criteria for ASD (Moss & Howlin,

2009). The relationship makes sense given that mutations in the *FMR*1 gene are the largest monogenetic contributor to ASD (Reddy, 2005). Notions about the genetics of ASD stemmed from observations of a male-female bias in ASD prevalence, with a diagnosis rate twice as high in males (1 in 68 male children) compared to females (1 in 189) (CDC, 2014). These results are similarly reflected in FXS, with similar disparities between both prevalence and phenotype. FXS occurs in both males and females, with estimates of the prevalence suggesting occurrence at a significantly higher rate in males, approximately 1:5,000 males and 1:10,000 females (Hawkins et al., 2011; Hersh, Saul, & Committee on, 2011).

Phenotypes in both disorders also vary according to sex. In FXS, the symptoms in males are often more severe, thought to be due to compensation in females by the second non-affected X chromosome (Kazdoba et al., 2014). However, some evidence suggested that the female FXS phenotype is also qualitatively different. For example, males show higher prevalence of mental retardation and are more likely to exhibit ASD-like behaviors (Reiss & Freund, 1992). FXS males also more commonly exhibit hyperactivity and inattentiveness (R. J. Hagerman & Sobesky, 1989), as well as social problems, such as extreme eye gaze avoidance (Hall, Lightbody, McCarthy, Parker, & Reiss, 2012). Meanwhile, FXS females are much less likely to be affected by intellectual disability (Ferrando Lucas, Banus Gomez, & Lopez Perez, 2004), yet they show increased rate of schizophrenia (Reiss et al., 1988).

Fragile X Syndrome – The FMR1 Knockout Mouse

History

Owing to the behavioral deficits observed in FXS, an animal model with a rich repertoire of behavior was needed to better understand the biological mechanisms at play. The *FMR1* knockout mouse model of FXS was developed by the Dutch-Belgian Fragile X Consortium (The Dutch-Belgian Fragile et al., 1994). At the time of its inception, little was known about the physiological function of *FMR1*, and the pathology underlying the symptoms seen in FXS. The *FMR1* knockout model has proved invaluable in many aspects of neurodevelopmental research, pharmacotherapeutic research, as well as functional investigations. The *FMR1* gene knocked out in the murine model is 99% homologous in amino acid sequence to that of a human, suggesting it is a valid genetic model (Ashley, Wilkinson, Reines, & Warren, 1993). Interestingly, a transgenic model has also been synthesized, transfected with the human *FMR1* gene leading to overproduction of FMRP (Spencer, Graham, Yuva-Paylor, Nelson, & Paylor, 2008). In these studies, FMRP production led to correction or over-correction of many symptoms typically seen, suggesting this model is truly FMRP-dependent (Peier et al., 2000; Spencer et al., 2008).

FMR1 Knockout as a Model of ASD

Much of the literature concerning the *FMR1* knockout is framed in terms of an ASD-like phenotype, as many studies have sought to prove that deletion in *FMR1* produces an accurate recapitulation of ASD in humans. However, though the gene and its product appear to be relevant in ASD, the behavioral phenotypes show a large degree of

inconsistency across studies. Many have suggested the inherent variability of the phenotype can be traced directly to the background strain. The impact of the mouse strain can greatly influence the behavior of the mouse in different tests. In an exhaustive review of the topic, Crawley et al. (1997) points out that the phenotype of a mutant mice does not necessary reflect only the result of the gene target, but also interactions with background genes and mutations in these genes (Crawley et al., 1997). Thus, when one considers the behavioral phenotype for a mutant line, it is also important to assess these behaviors across strains to form a comprehensive assessment. For example, the review notes that C57BL/6J mice outperform FVB/NJ mice on tests of contextual fear conditioning and Morris water maze. However, these effects are not just limited to complex tasks, but also assessments of basal state, such as anxiety and exploration. Another study showed that subjects of different strains perform markedly different on common behavior paradigms, such as open field and elevated plus maze (Crabbe, 1999). Specifically, they showed that A/J mice show low levels of activity as compared to C57BL/6J mice. Background strain of the mouse can also influence the neurochemistry of the brain. For example, a mutation wherein amyloid plaques are overexpressed in C57B6 mice leads to increased expression of these plaques in the brain of the mice and cognitive deficits (Hsiao et al., 1996). However, when performed on an FVB/N background strain, mutants did not show plaque formation and survivability was low.

Concerning the *FMR1* knockout, the assertion that background strain is at fault for the diversity of findings has been empirically supported by studies utilizing multiple background strains (Pietropaolo et al., 2011). The two main strains of *FMR1* knockout are the C57BL/6 (B6) and FVB background strain. A few studies have yielded a direct

comparison of both strains suggesting that indeed the background strain does influence behaviors, such as hippocampal-dependent memory (Dobkin et al., 2000) and amygdala-based memory (Paradee et al., 1999). However, there does not appear to be a clear separation of behavioral outcomes on these two phenotypes. The influence of strain in the *FMR1* knockout has been a source of trouble for scientists, as well as affected its validity as both a true model of FXS and a model of other related disorders such as ASD. While the relationship has yet to be fully elucidated, the *FMR1* knockout still has provided a wealth of literature that inform preclinical investigations of treatment as well as insight into the biological mechanisms of these disorders.

Behavioral Examinations of the Knockout

Given that the first *FMR1* knockout appeared in 1994, it has been extensively studied over the past two decades, both as a model of FXS and a model of ASD. Owing to its consideration as a model of ASD, many studies of the *FMR1* knockout have been framed by the three common characteristics of ASD, and will be discussed as such below.

Social behavior alterations. The first core characteristic of ASD is deficits in social behavior. Similarly, along with other phenotypic alterations, clinical data suggests that individuals with FXS have a higher than normal incidence of social phobia diagnosis as well as avoidance behaviors (R. J. Hagerman & Hagerman, 2002). Social behavior in animal models of human conditions can be investigated through a variety of methods, such as the three-chambered social task, social partition and tube of social dominance assay. In the three chambered social task, the test mouse is evaluated on how much time is spent investigating both a social stimulus as well an object stimulus. It is expected that

wild-type mice will preferentially explore the novel mouse during the choice phase. In a study examining both phenotypic changes in the *FMR1* knockout, as well as background strain effects, both B6 and FVB strain knockouts showed unexpected normative levels of social interest, when given the chance to interact with a novel conspecific. In a later trial, a new conspecific was introduced and subjects were allowed to choose between the familiar and novel conspecific. It was here that knockouts showed deficits in preference for the novel conspecific (social novelty) (Pietropaolo et al., 2011). This deficit in preference for social novelty is supported in some literature (Mines, Yuskaitis, King, Beurel, & Jope, 2010), while other studies have shown enhanced levels of social interest and interaction (Spencer, Alekseyenko, Serysheva, Yuva-Paylor, & Paylor, 2005). While the results are mixed, it is possible that both enhancement and deficits in social interest levels reflect an autistic-like deficit in social recognition. A more recent study of social behavior in the FXS mouse found that increases in sociability in the three-chambered social task could be corrected for by increased hyperactivity, however deficits in social recognition were also noted in direct observations (Sorensen et al., 2015).

Repetitive and change-resistant behaviors. The second core characteristic of ASD is increased incidence repetitive or perseverative behaviors. As previously mentioned, individuals with FXS often show a distinct behavioral trait called “hand flapping,” which is grouped into a larger phenotypic trait known as repetitive behavior. These behaviors can be studied in murine models through a variety of tasks including the marble burying task (Angoa-Perez, Kane, Briggs, Francescutti, & Kuhn, 2013) and the nose-poke assay (S. S. Moy et al., 2008), as well as the rotorod task (Whishaw, Li, Whishaw, Gorny, & Metz, 2008). While the marble burying and the nose-poke assay are considered standard

tests of repetitive behavior, the rotorod test is more typically regarded as a test of cerebellar coordination and motor ability. Recently, it has been postulated that this motor task is also a display of learned repetitive motor routine. This has been shown through investigations of the striatal circuitry that are related to these movements (Rothwell et al., 2014). However, in order to analyze repetitive behavior in this task, a comprehensive analysis of motor routine (steps) is necessary.

Previous literature in the marble burying test showed an increase in marble burying behavior on a C57BL/6 background strain knockout mouse (Veeraragavan et al., 2011). One study showed that when traditional lines (C57BL/6J, A/J, DBA/2J etc.) are crossed to create six hybrid strains, behavior in the marble burying task is significantly strain-dependent (Spencer et al., 2011). It has been suggested that nose-poke behavior in a hole board task may prove to be sensitive enough to detect phenotypic changes, yet no published work to date has shown this phenotype. It seems that spontaneous displays of repetitive behavior may prove to be difficult to show in this model.

The repetitive behavior phenotype can also be reframed in terms of a "resistant to change" or a cognitive inflexibility phenotype. These perseverative behaviors have been supported by findings of altered morphology in the prefrontal cortex (Bray et al., 2011), as well as deficits in synaptic processes, like long-term potentiation (Martin, Lassalle, Brown, & Manzoni, 2016). This behavior is most often studied using the Morris water maze rehearsal paradigm. In this task, the location of the hidden platform is reversed, and the latency to adjust behavior is examined. The *FMR1* model has shown significant impairments in this aspect of the paradigm in the FVB/N strain as well as a 129/ReJ x C57B/6 (Kooy et al., 1996; Paradee et al., 1999), while another report using an FVB/AntJ

inbred background strain was been unable to recapitulate this phenotype (Leach, Hayes, Pride, Silverman, & Crawley, 2016).

Alterations in communication behavior. The final core characteristic of ASD is deficits in communication behavior. Concerning animal research, one of the most common markers of communication behavior used in rodents is ultrasonic vocalization behavior. Ultrasonic vocalizations (UVs) are a highly conserved phenomenon among several species. In mice, these whistle like noises occur between 30 to 90 kHz frequencies, and follow a clear ontogenetic profile (Branchi, Santucci, & Alleva, 2001). This prelingual communication behavior can be emitted in a variety of situations, including neonatal mice to signal distress and maternal retrieval and in adult mice for mating and aggression. Alterations in ultrasonic vocalizations have been shown in a variety of mouse models, including MeCP2 (Picker, Yang, Ricceri, & Berger-Sweeney, 2006) and Shank mouse models of ASD (Wohr, 2014) suggesting high face validity among neurodevelopmental disorders. As previously mentioned, individuals with FXS do show alterations in communication behavior (Ferrier et al., 1991). Analysis of this behavior in the *FMR1* knockout has revealed several differences between wildtypes and knockouts, specifically on postnatal days 7 and 8 (Lai et al., 2014; Roy, Watkins, & Heck, 2012). However, this behavior has yet to be studied in female knockouts.

Other behavioral abnormalities. Many of the changes noted in the *FMR1* KO mouse are unique to the *FMR1* model, suggesting that this archetype may be more accurately framed as a model of FXS only, rather than a model of ASD. Locomotion behavior is the essential starting place when examining the behavior of any mouse model,

as differences in activity levels can influence testing on multiple subsequent testing paradigms. In previous studies of the *FMR1* knockout on the FVB background strain, activity of *FMR1* knockouts has been significantly higher than their wildtype littermates (Pietropaolo et al., 2011). This effect has been shown to be consistent across both mouse strains (Ding et al., 2014) and between the sexes (K. B. Baker et al., 2010; Ding et al., 2014). Indeed, investigations of this murine behavior accurately mimics the human hyperactivity phenotype (R. J. Hagerman & Hagerman, 2002).

Given the aforementioned clinical data on individuals with FXS, it is also prudent to consider the impact of this genetic mutation on anxiety (Tsiouris & Brown, 2004). Reports of the anxiety phenotype in FXS have shown conflicting results. Early investigations have shown the *FMR1* KO mice to display an increase in anxiety in response to reflected images of mice in the mirrored chamber task (Spencer et al., 2005). This task is based on the assumption that mice will show approach-avoidance behavior when confronted by a mirror. The apparatus consists of an open mirrored cube inside a black Plexiglass box, and another mirror opposite the opening of the cube to create an alleyway. A center mirror ratio is calculated (time spent in the mirrored chamber / time spent in the mirrored chamber + time spent in the mirrored alleyway). This calculation eliminated the time spent in the dark areas, reducing activity level based confounds. The Spencer et al study showed that *FMR1* knockout mice exhibit increased center mirror ratios. However, studies of anxiety behavior in other standard tests of anxiety, such as the elevated plus maze, have shown opposite results (Chen et al., 2013). As a result of several studies on the topic, it is now hypothesized that the *FMR1* mouse shows a dissociated anxiety phenotype in which the KO shows increased social anxiety (apparent

on tests such as three chambered social task) and decreased nonsocial anxiety (apparent on tests such as the elevated plus maze) (Z.-H. Liu & Smith, 2009).

As mutations in the *FMR1* gene constitute the largest form of inherited intellectual disability (Turner, Webb, Wake, & Robinson, 1996), alterations in memory and cognition in the *FMR1* mouse also constitute a large portion of the literature for the *FMR1* knockout. However, investigations of these constructs represent a large spectrum of abilities and should be considered as separate entities. The most commonly tested construct is spatial memory, tested through the Morris water maze paradigm, first described in 1981 (Morris, 1981). This paradigm consists of animals learning to escape a water maze over successive trials through finding a hidden platform. Investigations of this type of hippocampal-dependent memory in the *FMR1* model have shown some degree of impairment. However, much like other examinations of the model, it has proven to be strain dependent. Early studies of behavior on the C57BL/6J strain show no deficits in spatial learning (D'Hooge et al., 1997), while there are spatial memory deficits on an FVB/N background strain (Kooy et al., 1996). A more recent study cohort, bred onto an albino C57BL/6J-*Tyr* $^{c\text{-}Brd}$ background strain, showed robust deficits in place navigation (K. B. Baker et al., 2010). Taken together, the utility of this paradigm seems to come from manipulations of the original protocol to include a reversal task, as previously mentioned, used to assay perseverative or change-resistant behavior.

Due to the probable deficits in hippocampal functioning, the *FMR1* knockout has also been examined in fear conditioning tasks, given that normal functioning hippocampus is also required for adequate performance on contextual fear conditioning tasks. The most commonly examined fear conditioning paradigm is the delay

conditioning task, which is generally thought to be amygdala-dependent. Several examinations of this behavior in this model have shown significant deficits related to the deletion of *FMR1* (D'Hooge et al., 1997; Kooy et al., 1996; Paradee et al., 1999). Contextual fear conditioning is a separate hippocampal-dependent paradigm wherein animals learn an association between the context and a painful foot shock. Examinations of this behavior in the *FMR1* knockout have shown decreased freezing behavior, suggesting a weaker association and a deficit in memory (Ding et al., 2014). Trace fear conditioning has also been examined in the *FMR1* knockout, revealing a subtle deficit in hippocampal memory (Zhao et al., 2005), however this is the only study to examine trace memory.

Perhaps the best characterized clinical observation in FXS individuals has concerned their responsiveness to sensory stimuli, or sensory defensiveness (Miller et al., 1999). It is also interesting to note that recent studies have reported sensitization of the startle and increased prepulse inhibition (PPI) in children that meet DSM criteria for ASD (Madsen, Bilenberg, Cantio, & Oranje, 2014). This behavior is typically categorized as sensorimotor gating in animals, and changes in this behavior are thought to result from aberrant connectivity in sensory circuitry (Ruby, Falvey, & Kulesza, 2015). This behavior is typically tested in animal models through a method known as PPI. Briefly, a weak auditory prepulse stimulus diminishes subsequent responses to a loud startling noise and the degree of attenuation is calculated as the percent inhibition. Investigations of this in both humans with FXS and the *FMR1* knockout model have shown significant enhanced pre-pulse inhibition and reduced startle responding in this behavior (Frankland et al., 2004).

Sex Differences in the KO

Studies of the effects of the deletion of *FMR1* are typically limited to males. However, mounting evidence has suggested that female mutant mice do have some phenotypic characteristics, some of which are different from male *FMR1* knockouts (K. B. Baker et al., 2010; Ding et al., 2014; Qin et al., 2005), reviewed in (Romano et al., 2016). To date, these three studies represent the only investigations on the topic. So far, female *FMR1* knockouts show similar deficits on tests of activity levels, learning and memory (K. B. Baker et al., 2010; Ding et al., 2014), sensorimotor gating (K. B. Baker et al., 2010; Ding et al., 2014) and seizure susceptibility (Qin et al., 2005) . One report noted that while male *FMR1* knockouts exhibit an anxiety phenotype (discussed earlier), females show normal levels of anxiety (Qin et al., 2005) .

Only in recent years has there been a push to include females in empirical research. The National Institutes of Health (NIH) announced an initiative in 2014 to include more females in both clinical and preclinical biomedical investigations (Clayton & Collins, 2014). The bias in the sex of humans and animals alike included in studies is a problem among the greater scientific community. A recent study by Beery and Zucker showed that this bias is especially prevalent in the neuroscience and biomedical science communities (Beery & Zucker, 2011). Studies of FXS and the impact of deletion of *FMR1* are no different. In both molecular and phenotypic investigations of the *FMR1* knockout, females have been considered as an “intermediate phenotype” due to the unaffected second X chromosome and are often not included in investigations. The omission of females broadly across studies is thought to be due to the apparent belief among the scientific community that female mammals have a higher degree of intrinsic

variability perhaps due to estrus cycles. The routine exclusion of females from both human and mammalian basic science studies has significant downstream effects of the quality of medical care for women (Correa-De-Araujo, 2006).

Concerning the *FMR1* knockout literature, several domains of behavior in the female *FMR1* knockout remain unstudied. A recent review pointed out that specifically, female *FMR1* knockout behavior on tests of motor coordination, vocalization behavior, and sociability has yet to be studied (Romano et al., 2016). The same review also pointed out that repetitive behavior has not been investigated in female *FMR1* knockouts. However, the authors of Baker et al., 2010 noted in the discussion that female *FMR1* knockouts showed increased hole poking behavior where male *FMR1* knockouts showed no effect of genotype (K. B. Baker et al., 2010). However, the authors did not report this finding as a main finding of the paper, perhaps due the inconsistency between sexes, and potentially strains. Overall, the effects of deletion of *FMR1* in female animals and the potential sexual dimorphisms remain grossly understudied.

Study Hypotheses

Aim I

An ideal model for ASD should mimic all core features of the phenotype, as well as some secondary features, and this should serve as a framework for evaluating the model. While the relationship of ASD and FXS is established in humans, it has been more difficult to empirically support the classification of the *FMR1* knockout as a model of ASD. As previously mentioned, while strain dependent, examinations of these behaviors in the monogenic FXS model have yielded strong evidence of similar

phenotypes. Beyond the core triad of symptoms, the *FMR1* knockout consistently shows differences in secondary symptomology that mimics that of ASD, such as hyperactivity (Polimeni, Richdale, & Francis, 2005). However, given the considerable effect of strain, the validity of the *FMR1* knockout mouse as a model of ASD is still highly debated among the scientific community. Thus, the first goal of this study is to further evaluate the potential of a null mutation in the *FMR1* gene on an FVB background strain to recapitulate the ASD phenotype.

Aim II

In studies of both ASD and FXS, there is a blatant gap between the depth of characterization of the male and female phenotype. With respect to FXS, the female phenotype is not well understood. Currently there are only three examinations of the female *FMR1* knockout, both of which have yielded evidence that female *FMR1* knockouts show similar phenotypic alterations in startle responding, hyperactivity, and seizure susceptibility (K. B. Baker et al., 2010; Ding et al., 2014; Qin et al., 2005). However, the female *FMR1* knockout has yet to be comprehensively evaluated across all the behavioral paradigms, including social behavior. Thus, the second goal of this study is to better understand the interaction of sex and deletion of *FMR1*.

CHAPTER THREE

Methods and Materials

Animals

Male and female Fmr1^{tm1Cgr} FVB mice originally from Jackson Labs were bred to create wildtype (WT) (+/+) and *FMR1* knockout (KO) (-/-) groups. Subjects were bred and housed at the Baylor University Special Research unit facility. Animals had access to food and water *ad libitum*. A 14-hour light and 10-hour dark (20:00 to 6:00 hr.) diurnal cycle was maintained. All procedures were performed in accordance with Baylor University Institutional Care and Use Committee and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

With the exception of isolation-induced neonatal ultrasonic vocalization testing, all testing was conducted after the mice reached adulthood, approximately 2 months of age. Testing was divided into several cohorts to diversify the sample and to minimize the effects of multiple tests. Each cohort received a battery of behavioral testing that was ordered to minimize test order effects. The first set was tested in open field, elevated plus maze, marble burying, social chamber, and trace fear conditioning. The second cohort was tested on social partition, light dark box, nose poke assay, Morris water maze, passive avoidance and rotorod. A third cohort was tested in the odor habituation/dishabituation test. The details of the cohort schedules are listed in Table 3.1. Detailed information on sample sizes used for the various tasks is in to Table 3.2.

Table 3.1
Cohort Information

Cohort 1	Cohort 2	Cohort 3
Open Field	Light-Dark Task	Odor Discrimination
Elevated Plus Maze	Nose Poke Assay	
Marble Burying	Rotorod	
Social Chamber	Social Partition	
Trace Fear Conditioning	Delayed Fear Conditioning	
	Morris Water Maze	

Table 3.2
Sample Sizes

	Males		Females	
Behavioral Test	$fmr1^{+/+}$	$fmr1^{-/-}$	$fmr1^{+/+}$	$fmr1^{-/-}$
Open Field	17	16	13	16
Elevated Plus Maze	17	16	13	16
Marble Burying	17	16	13	16
Social Chamber	17	15	12	16
Trace Fear Conditioning	17	16	13	16
Light Dark Task	12	16	7	15
Nose Poke Assay	12	14	13	17
Rotorod	12	16	7	14
Social Partition	12	16	13	14
Delayed Fear Conditioning	12	16	7	14
Morris Water Maze	10	16	-	-
Odor Discrimination	12	16	-	-

Behavioral Test	Male $fmr1^{+/+}$	Male $fmr1^{-/-}$	Female $fmr1^{+/+}$	Female $fmr1^{-/-}$
Neonatal UV's – PD9	12	12	12	13
Neonatal UV's – PD10	12	15	12	15
Neonatal UV's – PD11	12	12	11	13
Neonatal UV's – PD12	13	13	13	13
Neonatal UV's – PD13	14	14	11	13
Neonatal UV's – PD14	12	12	12	14

Vocalization Behavior

Isolation Induced Neonatal Ultrasonic Vocalizations

Previous studies have shown that rodent pups emit ultrasonic vocalizations (UVs) in response to maternal separation as a mechanism for signaling retrieval. In the present study we recorded isolation-induced vocalizations to determine if communicative function of UVs were impaired following mutations in the *FMR1* gene. We recorded pup vocalizations on postnatal days (PD) 9-14 in order to characterize the development of pup vocalization behavior. However, each pup was tested only once to reduce the effects of repeated testing. On the testing day mice were first brought into the testing room and allowed to habituate in the home cage for 30 minutes. Following this period pups were removed from their dams and placed in a warmed housing pan with clean bedding maintained at ambient nesting temperature. All pups were then individually placed into a recording chamber where UV detectors were set to 50, 60, 70, and 80 kHz (Mini-3 Detector, Ultra Sound Advice, United Kingdom). The quantity, mean duration, and total

duration of UVs at each frequency were then measured over a 5-minute interval using automatic detection software (Ultravox Software by Noldus, Netherlands). Following testing, pups were returned to the warmed house pan and all mice were returned to their home cage after all mice were tested. No more than 5 mice were tested in any given cohort, making the total time separated from dams less than 30 minutes.

Activity Levels

Open Field

The open field test was performed to evaluate the effect of deletion of *FMR*1 on activity and anxiety levels. The mice were weighed and marked with tail ID quantitys. They were then allowed to habituate to the testing room for at least 30 minutes. The apparatus consisted of a clear plastic arena (40x40x30 cm). The lighting inside the test chamber was approximately 100 lux and the background noise was approximated at 60 dB. Subjects were placed into the testing arena for 30 minutes. Activity levels during the task were analyzed by a computer-operated optical animal activity system (Fusion by AccuScan Instruments, Inc.; USA). This system also measured other exploratory behaviors such as grooming, rearing, clockwise and counterclockwise rotations. To evaluate for anxiety behaviors distance and time spent in the center compared to surround region was compared. The area was cleaned with a 30% isopropyl alcohol solution and dried thoroughly between testing.

Anxiety Behavior

Elevated Plus Maze

The elevated plus maze test was performed to evaluate changes in baseline anxiety levels (Pellow, Chopin, File, & Briley, 1985) The testing room was lit by incandescent lamps (30 lux in the open arm) and the background noise level remained constant at 60 dB. The apparatus was involved four 30 x 5 cm arms positioned 40 cm above the floor and a center platform (5 x 5 cm). Two arms were enclosed with acrylic walls. The apparatus was cleaned thoroughly with a 30% isopropyl alcohol solution between subjects and dried with paper towels. Subjects were recorded for 10 minutes and the Noldus Ethovision video tracking software was used (Ethovision, Netherlands) to score the frequency of entries and the time spent in each of the four arms as well as the center platform. Distance traveled and speed of movement was also assessed. The videos were recorded using Pinnacle video capture software, then scored offline for head-dips in open arms and rearing activity by an experimenter blind to group identity (Corel, Canada).

Light-Dark Task

The light-dark task was conducted as a complement to the elevated plus maze as a measure of anxiety. A clear acrylic test chamber was modified to allow for a black acrylic insert. Time spent in the light portion and the dark portion was then measured using automated software (Fusion by AccuScan Instruments, Inc.; USA). The lighting inside the light test chamber was approximately 100 lux and the background noise was approximated 60 dB. Mice were first allowed to habituate to the testing room for 30

minutes prior to the beginning of testing. Each experimental session was 10 minutes and time spent in each chamber was measured.

Repetitive Behavior

Marble Burying

The marble burying test was performed to evaluate repetitive behavior. The apparatus consisted of a clean Allentown mouse cage (27 x 16.5 x 12.5) filled with sanichip bedding to a height of approximately 3 cm. Twenty black 15 mm glass marbles were placed throughout the cage in an equidistant 4 x 5 pattern (Thomas, Burant et al. 2009). The testing room had a background noise level of 55 dB. Each mouse was individually placed in the testing cage for 30 minutes. The animals were then removed and the quantity of marbles buried at 50%, 75%, 100 % and completely buried was tallied.

Nose Poke Assay

Nose poke behavior was used as a test of repetitive behavior. Subjects were habituated to the testing room for 30 minutes prior to the test. The apparatus consisted of a board inserted into a clear acrylic area (40 x 40 x 30 cm), with 16 equidistant 0.5" holes. A nose poke was counted whenever the nose was extended into the hole as far as the eyes. These were counted by an observer blind to the experimental condition. Each experimental session lasted 10 minutes. The arena was cleaned with 30% isopropyl alcohol between subjects.

Rotorod

Rotorod performance was measured to assess motor learning, as well as repetitive behavior (Rothwell et al., 2014). The apparatus consisted of a rotating rod that accelerated from 5 to 40 rpm over a five-minute trial (Series 8 Rotorod; IITC Inc., Woodland Hills, CA, USA). Subjects were tested for two trials per day for four days of testing with an inter-trial interval of 60 minutes. An experimenter blind to group identity scored the amount of time each animal was able to stay on the rotating rod before falling off. The apparatus was cleaned thoroughly between trials using a 30% isopropyl solution.

Social Behavior

Social Chamber

The social chamber test was performed to evaluate changes in social approach behavior. The mice were placed in a clear acrylic box divided into three chambers, measuring 60 cm x 40.5 cm x 22.5 cm, divided by a 0.25 cm thick acrylic wall. The two outer chambers measured 20.5 cm x 40.5 cm and the middle chamber measured approximately 18.5 cm x 40.5 cm. In the center of each of the dividing walls was a door that was 10 cm x 5 cm. This protocol was previously described in Nadler et al., 2004. Testing was divided into two parts. In part A, the subject was placed in the center chamber and allowed to explore the chamber for 10 minutes. Black wire-mesh cylinders were placed in the posterior corners of the chamber. A tall plastic bottle was placed on top of the cylinder to prevent climbing or overturning of the chamber. The animal was then confined to the middle chamber while the researcher placed the intruder mouse (matched for sex, age and weight) inside one cylinder and a similar sized black block

object in the other. The location of the objects was alternated between subjects to prevent a side bias. The barriers to the side chambers were then removed and they were allowed to explore for 10 minutes. Videos were analyzed offline for time and frequency in each of the three chambers and investigatory behaviors at the cylinders.

Social Partition Task

The social partition task was used to provide a complementary social behavior test to the results of the social chamber task. This task was used to measure the frequency and duration of interacting with a familiar versus an unfamiliar mouse. The following methods have been previously described in Lugo et al., 2014. The animals were housed for 24 hours in a cage divided into two chambers by a clear partition with 0.6 diameter holes. In the other half of the chamber, a sex, age and weight-matched conspecific was placed and animals remained housed together overnight. The following day, the approaches and time spent at the partition by the experimental mice was measured for 5 minutes in three different conditions by computer software to capture duration and frequency of sniffing events (Ethom). The first condition was with the “familiar” mouse it was housed with overnight, the “unfamiliar” condition was with a novel mouse, and then the “familiar 2” condition was the mouse it had been housed with overnight.

Odor Discrimination Test

Methods for this task were previously described in Arbuckle et al., 2015. This test is designed to assay basic olfactory sensory abilities. Two non-social odors were used to prepare this task. The non-social odors were prepared fresh each day. Banana extract and almond extract (100 µl each) were prepared in a 1:100 dilution. A third tube contained

only water. The social odors were prepared on the morning of testing by swabbing the floor of a cage that had not been cleaned for three days. These odors were obtained from members of the same sex as the test subject. Two different cages were used to create the two social odors. They were stored in a covered container. The test mouse was acclimated to a room other than the test room for 45 minutes. Each odor was presented for 1 minute and was repeatedly presented 3 times. Sniffing behaviors were recorded on a stopwatch by a live observer. The inter-trial interval was approximately 1 minute.

Learning and Memory

Trace Fear Conditioning

The conditioned fear task was used to evaluate hippocampal-dependent memory as previously described (Mcllwain, Merriweather et al. 2001). The animal's behavior was recorded using the FreezeFrame 3 software (Coulbourn; Ohio). The chamber consisted of an operant conditioning chamber approximately 26x22x18 cm high with two clear acrylic and two metal sides. The floor consisted of a metal grid enabling it to deliver a mild shock. This chamber was located inside a sound proof chamber. Animals were transported from a separate holding room in cages with sanichip bedding. The chamber was cleaned thoroughly between subjects using 30% isopropyl alcohol. Methods were adapted from Smith et al, 2007 (Smith, Gallagher, & Stanton, 2007). Briefly, on the first day of testing, animals were allowed to freely explore for 12 minutes to obtain baseline information. On the second day of testing, the subject was placed inside the chamber and allowed to explore freely for 4 minutes prior to the CS-US pairings. The conditioned stimulus consisted of a 20 s white noise "tone" (70 dB) followed 18 seconds later (trace

period) by a mild foot shock (2s, .5 mA) as the unconditioned stimulus. Following a 40 second inter-trial interval (ITI) this pairing was repeated. This pairing was repeated a total of 6 times for a total test time of 840 seconds. Behaviors such as freezing, running and jumping were recorded by the observer to ensure the foot shock had been delivered. On test day 3, mice were tested in a new context wherein the floor, chamber shape, sound and smell were altered. During this test day, animals were exposed to four 100 second trials wherein there was a 20 second interval, followed by a 20 second, 70 dB tone presentation, then a 60 second interval before the next trial. Finally, on day 4, animals were placed in the old context and allowed to explore freely for 3 minutes as a test of contextual fear conditioning.

Delayed Fear Conditioning

As a complement to trace fear conditioning, we also evaluated a separate cohort of subjects on the delayed fear conditioning task. This paradigm evaluated amygdala-based fear memories. On the first day of testing, the animals received 2 pairings of a 30 second 80 dB white noise "tone" (the CS) and a 0.7 mA shock stimulus (US) lasting 2 seconds following the CS. Following the second pairing, there was a 20 second interval. This trial lasted approximately 334 seconds. On the second day of the task there were two trials. On the first trial, the animal was placed in the familiar context and allowed to move freely for 300 seconds to evaluate freezing behavior in the original context. After a two-hour period, the animal was presented with a 2nd trial. For the second trial, the context was altered by changing the shape and floor of the chamber as well as a novel odor (vanilla) placed under the floor grid. The animal was placed in a new context for 360 seconds. The first 3 minutes allowed the subject to habituate to a novel context. During

the second 3 minutes of this trial, the animal was presented with the CS tone continuously for 3 minutes and freezing behavior was examined.

Morris Water Maze with Reversal Learning

The Morris water maze was used to examine spatial learning abilities, with the addition of the reversal protocol to examine cognitive flexibility or resistance to change. The methods were adapted from earlier studies of this behavior in the *FMR1* knockout (Paradee et al., 1999). A 1.3 m diameter white pool was filled with water and made opaque through the addition of non-toxic ColorSplash! © liquid tempera white paint (S&S Worldwide, Connecticut). The mice were allowed to acclimate to the room in their holding cages for 30 minutes prior to the onset of testing. Two blocks per day consisting of 4 trials per block were performed for each mouse to test ability to locate a hidden platform. The hidden platform measured 14.5 cm x 14.5 cm and was submerged approximately 2 cm below the water level. After the last trials on the fourth day of testing, the animals were given a probe trial. The probe trial involved removing the platform and allowing the subjects to explore the maze for 60 seconds. The amount of time spent in each quadrant for each trial was recorded using automated tracking software (Ethovision, Netherlands). During the probe trial, the quantity of times the animal crossed the location of the hidden platform and the duration of time in each quadrant was calculated. Testing resumed on day 8 after a 2-day rest period.

On day 8, the platform was placed in the opposite quadrant from the previous location that housed the hidden platform. Testing progressed similarly as before, with two blocks of 4 trials each per day for two days. On the final day of testing, the platform was used to in order to evaluate the mice's visual abilities. The visible platform had a two

tiered platform with one 14.5 cm x 14.5 platform that was submerged 2” below the water level, and a higher tier platform 14.5 cm x 14.5 cm acrylic square that extended 9.5 cm above the lower platform to allow the animal to see the platform. One animal was excluded from analysis due to seizure activity during this task.

Data Analysis

All data was analyzed using GraphPad Software 6.05 (San Diego, CA) or IBM SPSS Statistics 23 (Aramonk, NY). Most results for were evaluated 2 x 2 (Genotype [wildtype, knockout] x Sex [male, female]) analysis of variance (ANOVA) on each variable for the specific test. Any tests that involved multiple measures were analyzed using a two-way repeated measures ANOVA. For all comparisons, the level of significance remained at $p < 0.05$. Animals were monitored throughout the experiment for weight and no significant differences were found.

CHAPTER FOUR

Results

Vocalization Behavior

Neonatal Separation Induced Ultrasonic Vocalizations

MANOVA main effects. We examined the quantity and duration of 50, 60, 70, and 80 kHz calls emitted by the pups across all days with a MANOVA. We used genotype, sex, day, and frequency as independent factors, and the quantity and duration of calls were the dependent measures. Several main effects were noted. Results indicated a main effect of genotype for quantity of calls $F(1,1124) = 3.99$, $p < 0.05$ and for duration of calls $F(1,1124) = 5.22$, $p < 0.05$. The WT mice produced more calls and for a longer duration, as compared to KO counterparts. There was also a main effect of sex for quantity of calls $F(1,1124) = 20.62$, $p < 0.001$ and for duration of calls $F(1,1124) = 22.13$, $p < 0.001$. In general, females emitted more calls and for a longer duration, as compared to male counterparts. Test day also significantly impacted the quantity of calls $F(5,1124) = 17.8$, $p < 0.001$ and duration of calls $F(5,1124) = 12.7$, $p < 0.001$. There were also a main effect of measured call frequency on quantity of calls $F(3,1124) = 48.5$, $p < 0.001$ and duration of calls $F(3,1124) = 149.6$, $p < 0.001$.

MANOVA interactions. There were many interactions found with the MANOVA. The most relevant interaction was between genotype and day for quantity of calls $F(5,1124) = 4.9$, $p < 0.001$ and for duration of calls $F(5,1124) = 3.1$, $p < 0.01$. Sex, test day and genotype also interacted significantly for quantity of calls $F(5,1124) = 2.7$, $p <$

0.05. This interaction was not significant for the duration of calls $F(5,1124) = 1.9$, p = 0.1.

Analysis of sex-specific effects on call quantity. Due to the main effect of sex and the significant interactions observed, subsequent analyses were separated by sex then analyzed the quantity and duration of calls per measured frequency for each individual test day. On PD9, males displayed a genotype effect for the quantity of UVs $F(1,88) = 5.9$, $p < 0.05$, with *FMR*1 WT mice emitting more total UVs than KO mice (Figure 4.1A). Similar to males, females displayed a significant genotype effect $F(1,92) = 24.3$, $p < 0.001$, with *FMR*1 WT mice emitting more total UVs than KO mice (Figure 4.1A).

On PD10, males did not display an effect of genotype $F(1, 100) = 0.291$, $p = 0.60$ (Figure 4.1A). There was also no observed interaction between genotype and measured call frequency $F(3, 100) = 0.52$, $p = 0.7$ in male mice. In contrast, females did display genotype effects $F(1,100) = 5.64$, $p = 0.020$, with *FMR1* WT mice emitting more total UVs than KO mice (Figure 4.1A).

On PD11, neither males $F(1,88) = 1.18$, $p = 0.28$ (Figure 4.1A), nor females $F(1, 88) = 0.02$, $p = 0.88$ displayed an effect of genotype (Figure 4.1A). However, given trending interaction of genotype and measured call frequency $F(3, 88) = 2.63$, $p = 0.054$ in female mice on PD11, we then performed follow-up independent t-tests to determine the specific differences between *FMR*1 WT and KO mice. We found that KO mice emitted fewer 80kHz vocalizations ($M = 94.3 \pm 28.6$) compared to WT mice ($M = 177.6 \pm 22.0$) $t(1,46) = 2.7$, $p < 0.01$. No other statistically significant differences were found for 50, 60, and 70 kHz.

On PD12, males showed a significant genotype effect $F(1,96) = 6.74$, $p < 0.05$, with *FMR1* KO mice emitting more total UVs than WT mice (Figure 4.1A). In contrast, females did not display an effect of genotype $F(1, 96) = 0.232$, $p = 0.63$ (Figure 4.1A).

On PD13, males showed a significant genotype effect $F(1,104) = 5.41$, $p < 0.05$, with *FMR*1 WT mice emitting more total UVs than KO mice (Figure 4.1A). In contrast, females did not display an effect of genotype $F(1, 96) = 0.46$, $p = 0.5$ (Figure 4.1A).

On PD14, males did not display an effect of genotype $F(1,88) = 1.51$, $p = 0.22$ on PD 14 (Figure 4.1A). There was also no observed interaction between genotype and measured call frequency $F(3, 88) = 0.80$, $p = 0.5$ in male mice. Females also did not display an effect of genotype $F(1, 96) = 0.69$, $p = 0.41$ (Figure 4.1A).

Analysis of sex-specific effects on duration of calls. Similar to the above analysis, the data was separated by test day and sex, and the effect of genotype was measured on duration of call behavior. On PD9, males did not display an effect of genotype $F(1, 88) = 1.92$, $p = 0.17$ (Figure 4.1B). However, there was a significant interaction between genotype and measured call duration among males $F(3, 88) = 2.90$, $p < 0.05$. Contrasting from male counterparts, females displayed an effect of genotype $F(1,92) = 24.85$, $p < 0.001$, with female *FMR1* WT mice vocalizing for greater total time than KO mice ($p < 0.001$) (Figure 4.1B).

On PD10, males did not display an effect of genotype $F(1,100) = 0.17$, $p = 0.68$ (Figure 4.1B). However, females displayed a marginally significant effect of genotype $F(1,100) = 3.58$, $p = 0.06$, with female *FMR*1 WT mice vocalizing for greater total time than KO mice (Figure 4.1B).

On PD11, neither males $F(1,88) = 2.2$, $p = 0.14$, nor females $F(1,88) = 0.01$, $p = 0.93$ displayed an effect of genotype (Figure 4.1B). Interestingly, there was a marginal interaction between genotype and measured call duration $F(3,88) = 2.63$, $p = 0.054$ in female mice.

On PD12, males displayed an effect of genotype $F(1,96) = 4.637$, $p < 0.05$, with male *FMR*1 KO mice vocalizing for greater total time than WT mice (Figure 4.1B), results for females did not indicate an effect of genotype $F(1,96) = 0.58$, $p = 0.45$ (Figure 4.1B).

On PD13, males displayed an effect of genotype $F(1,104) = 5.9$, $p < 0.05$, with male *FMR1* WT mice vocalizing for greater total time than KO mice (Figure 4.1B). This genotype effect was not significant in females, $F(1,88) = 0.01$, $p = 0.91$ (Figure 4.1B).

On PD14, neither sex displayed a significant effect of genotype, $F(1,88) = 0.33$, $p = 0.57$, females $F(1,96) = 0.81$, $p = 0.37$ (Figure 4.1B).

Activity Levels

Open Field

To examine differences in activity levels, wildtype and *FMR1* knockout animals, both male and female were tested using the open field task. Total distance moved was examined with a 2 x 2 ANOVA. The ANOVA revealed a main effect of genotype, $F(1, 58) = 7.76$, $p < 0.01$ on distance moved (in cm), with KO's showing increased distance moved (Figure 4.2A). Neither the main effect of sex, $F(1,58) = 0.63$, $p = 0.43$, nor the interaction of sex and genotype, $F(1,58) = 3.49$, $p = 0.07$, were significant. As the interaction was trending, these groups were subdivided into male and female groups

and t-tests with correction for multiple comparisons were run to compare groups (Figure 4.2B). No effect was noted for females $t(1, 28) = 0.52$, $p = 0.61$ with the main differences being noted for males $t(1, 32) = 4.02$, $p < 0.001$.

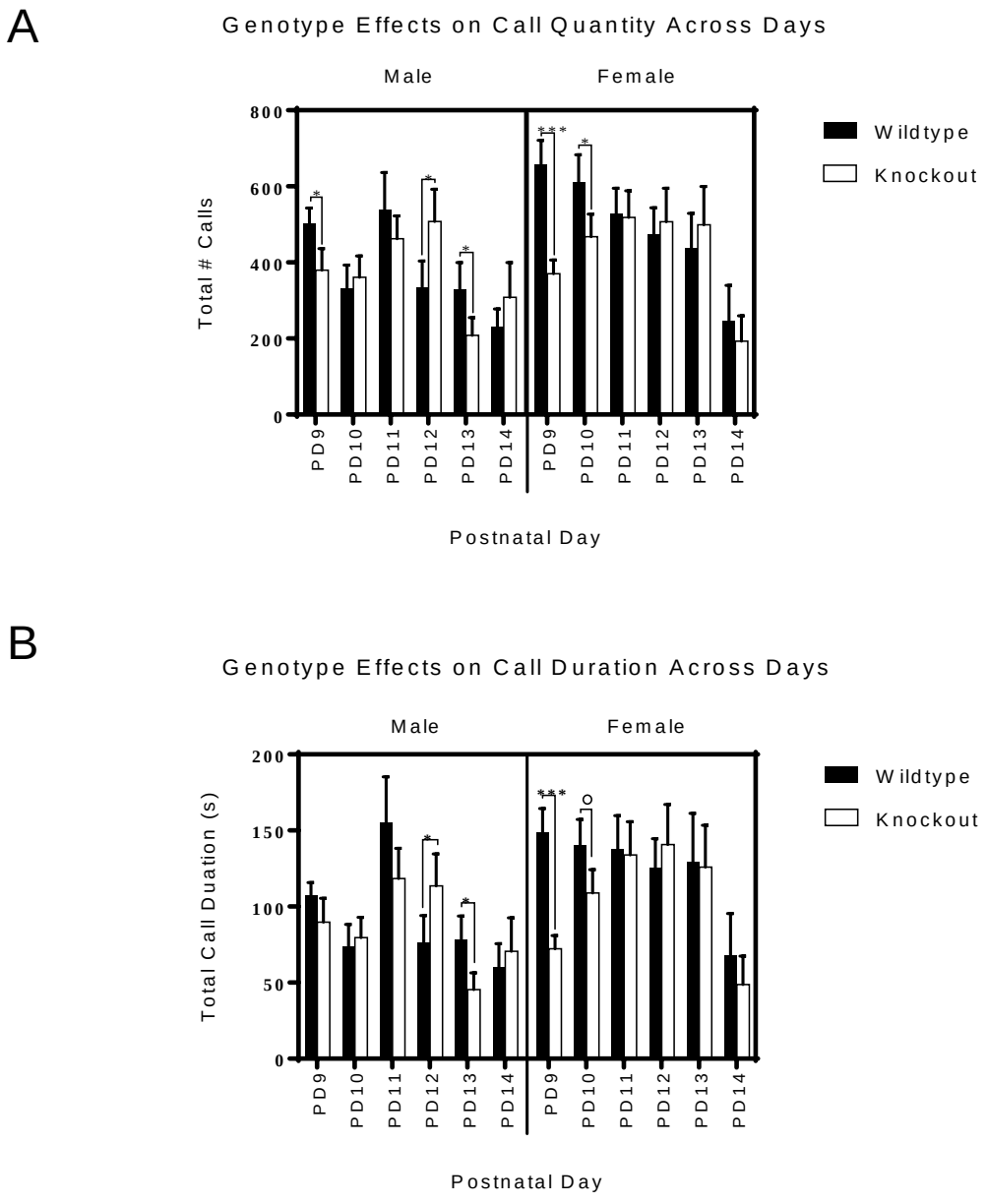

Figure 4.1. Genotype Effects on Call Quantity Across Days. (A) Genotype had a significant effect on call quantity in males on PD9, 12, & 13, and in females on PD9 & 10. (B) Genotype had a significant effect on total call duration in males on PD12 & 13, and in females on PD9. Genotype had a marginally significant effect on total call duration in females on PD10. Data are presented as mean ± standard error of the mean. o = p = 0.05; * = p < 0.05;*** = p < 0.001.

A similar effect was noted for rearing behavior. Two-way ANOVA analysis revealed a main effect of genotype, $F(1, 58) = 4.90$, $p < 0.05$, however no main effect of sex was noted, $F(1,58) = 0.11$, $p = 0.740$. There was a significant interaction of sex and genotype, $F(1,58) = 4.31$, $p < 0.05$. Further investigation of this effect through a Sidak's multiple comparisons test yielded a significant impact of genotype in the males only, $t(1, 32) = 3.14$, $p < 0.05$, Figure 4.2C.

It was also noted that these animals showed differences in time spent performing stereotyped behaviors in the open field, an indicator of repetitive behavior. Two-way ANOVA for sex and group effects testing revealed an overall effect of genotype on stereotyped behavior, $F(1,58) = 8.07$, $p < 0.01$ (Figure 4.2D), with *FMR1* knockouts spending more time engaged in stereotypic behavior. No main effect of sex, $F(1,58) = 0.08$, $p = 0.77$, or interaction of sex and genotype, $F(1,58) = 0.13$, $p = 0.72$, were detected.

Elevated Plus Maze

Differences in overall velocity (cm/s) in the elevated plus maze were also noted. Two-way ANOVA analysis for main effects of genotype and sex revealed a significant main effect of genotype, $F(1, 58) = 14.19$, $p < 0.001$, where *FMR1* knockouts exhibited higher velocity ($M = 5.44 \pm 0.70$) compared to wildtype ($M = 4.80 \pm 0.66$). No significant main effect of sex was noted, $F(1, 58) = 0.18$, $p = 0.67$, nor an interaction, $F(1, 58) = 0.86$, $p = 0.36$.

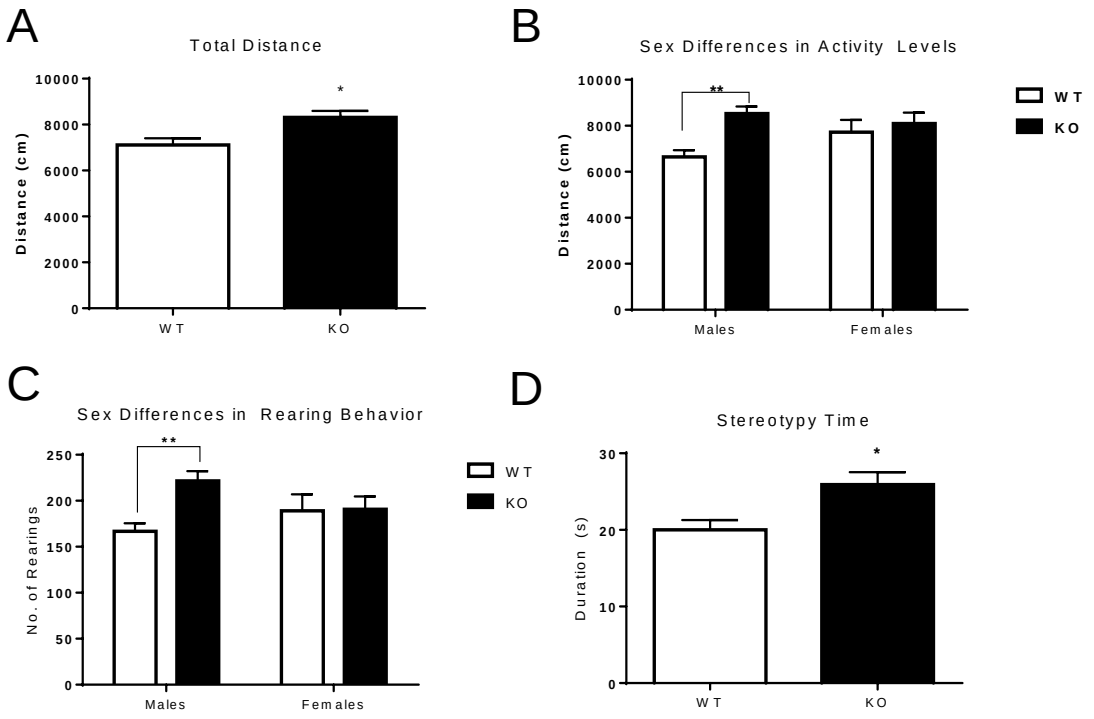

Figure 4.2 A. *FMR1* knockouts showed significant hyperactivity when examining distance moved in the open field task. B. The interaction of sex and genotype was trending at p = 0.07, so groups were subdivided and analyzed. Hyperactivity was only detected in the male knockouts. C. The interaction of genotype and sex was also significant for rearing behavior. Further analyses indicated that males *FMR1* KOs exhibited significantly higher amounts of rearing behavior compared to wildtypes, while this effect was not present in females. D.*FMR1* knockouts also exhibited higher amount of time engaged in stereotyped behaviors. Data are presented as mean ± standard error of the mean. 0 = p = 0.05; * = p < 0.05;*** = p < 0.001

Anxiety Behavior

Elevated Plus Maze

To examine differences in anxiety, as well as activity levels, subjects were also evaluated in the elevated plus maze task. Results were evaluated by a two-way ANOVA on each of the following variables: duration in the open, center, and closed arms. The separate two-way ANOVAs did reveal a main effect of genotype on duration in open arms, $F(1, 58) = 4.14$, $p < 0.05$, with *FMR1* knockouts spending more time than

wildtypes in open arms (Figure 4.3A). There was also a significant effect of sex on this variable, $F(1, 58) = 4.51$, $p < 0.05$, with females spending significantly more time in open arms compared to males (Figure 4.3B). The interaction of these two variables was not significant, $F(1, 58) = 0.77$, $p = 0.38$. No differences were noted for genotype for duration in the closed arm, $F(1,58) = 1.95$, $p = 0.17$, nor a main effect of sex, $F(1,58) = 3.392$, $p = 0.07$, though this effect was trending. The interaction was also not significant for duration spent in closed arms, $F(1,58) = 1.390$, $p = 0.24$. No differences were noted in frequency of visits to the various arms.

Light-Dark Task

Subjects were evaluated in the light-dark chamber task. Results were evaluated by a 2 x 2 ANOVA on duration spent in the light portion and dark portion. The main effect of genotype was not significant for the light portion, $F(1, 49) = 0.91$, $p = 0.76$, nor the dark portion, $F(1, 49) = 0.08$, $p = 0.78$. There was no main effect of sex for the duration in the light, $F(1, 49) = 2.40$, $p = 0.13$, or the dark, $F(1, 49) = 2.61$, $p = 0.11$. The interaction of genotype and sex was not significant, $F(1, 49) = 1.13$, $p = 0.30$, for duration spent in the light portion, or for the duration spent in the dark portion, $F(1, 49) = 0.59$, $p = 0.45$

Repetitive Behavior

Marble Burying

To examine differences in repetitive behaviors, subjects were tested on the marble burying assay. Results were evaluated 2 x 2 ANOVA on total marbles buried. Analysis

revealed no significant main effect of genotype, $F(1,58) = 0.24$, $p = 0.63$, or sex, $F(1,58) = 1.22$, $p = 0.27$, nor an interaction, $F(1,58) = 0.23$, $p = 0.63$ (Figure 4.4A).

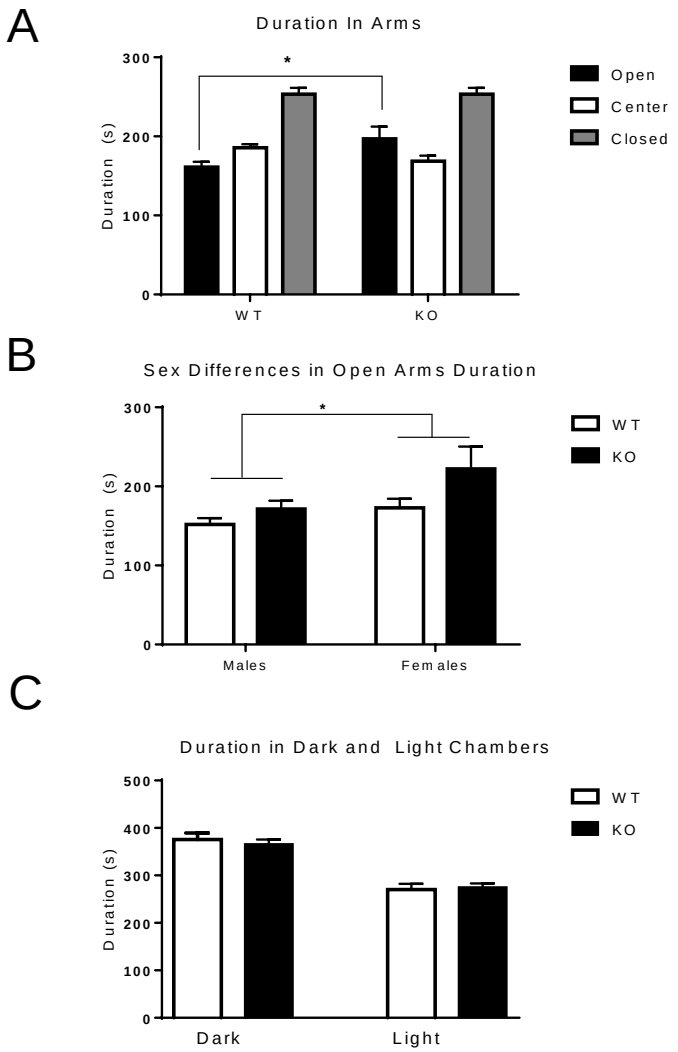

Figure 4.3. A. *FMR1* Knockouts spent significantly more time than wildtypes in the open arm of the elevated plus maze. B. Females across both genotypes spent more time in the open arms of the maze than males. C. No differences were noted in duration spent in the dark and light chambers of the light dark box. Data are presented as mean ± standard error of the mean. 0 = p = 0.05; * = p < 0.05;*** = p < 0.001

Nose-Poke Assay

The nose-poke test was used to determine changes in repetitive behavior. Results were first evaluated with a 2 x 2 ANOVA on the following variables: latency to first hole poke and total holes poked. The results for latency to first nose-poke detected no main effect of genotype, $F(1,55) = 0.91$, $p = 0.34$. There was a significant main effect of sex, $F(1,55) = 6.61$, $p < 0.05$, with males exhibiting a higher latency to the first hole poke than females. There was no interaction of group and sex, $F(1,55) = 1.35$, $p = 0.25$. Results revealed a significant main effect of genotype on total holes poked, $F(1,55) = 5.11$, $p < 0.05$, with *FMR1* knockouts exhibiting more nose-poke behavior (Figure 4.4B). There was no interaction between genotype and sex, $F(1,55) = 2.02$, $p = 0.16$, and sex did not have a significant impact on this variable, $F(1,55) = 0.08$, $p = 0.78$.

When examining hole-type specific results, two-way ANOVAs revealed a few effects on the following independent variables: outer holes, front holes, and corner holes poked. Several main effects of genotype were noted on the following variables: outer holes poked, $F(1,55) = 6.43$, $p < 0.05$, corner holes poked, $F(1,55) = 14.62$, $p < 0.0001$, and quantity of front holes poked, $F(1,55) = 5.34$, $p < 0.05$ (Figure 4.4B). No main effects of sex were detected on any variables. Significant interactions were noted among the following two variables: corner holes poked, $F(1,55) = 7.13$, $p < 0.05$ (Figure 4.4C) and front holes poked, $F(1,55) = 8.22$, $p < 0.01$ (Figure 4.4D). Follow up analyses revealed the following: for corner holes males did not show a genotype difference, $t(1, 25) = 0.79$, $p = 0.68$, while female *FMR1* knockouts showed significantly more corner hole pokes, $t(, 29) = 4.75$, $p < 0.0001$, and for front holes poked a similar effect was noted with males showing no effect, $t(1, 25) = 0.38$, $p = 0.91$, and females exhibiting

significantly more hole pokes, $t(1, 29) = 3.79$, $p < 0.001$. There was a trending interaction of genotype and sex on outer holes poked, $F(1,55) = 0.91$, $p = 0.06$. Follow up analyses revealed the same female-specific genotype effect, with males exhibiting no differences according to genotype, $t(1, 25) = 0.45$, $p = 0.88$, and females showing a genotype-related increase in outer hole poking behavior, $t(1, 29) = 3.23$, $p < 0.01$.

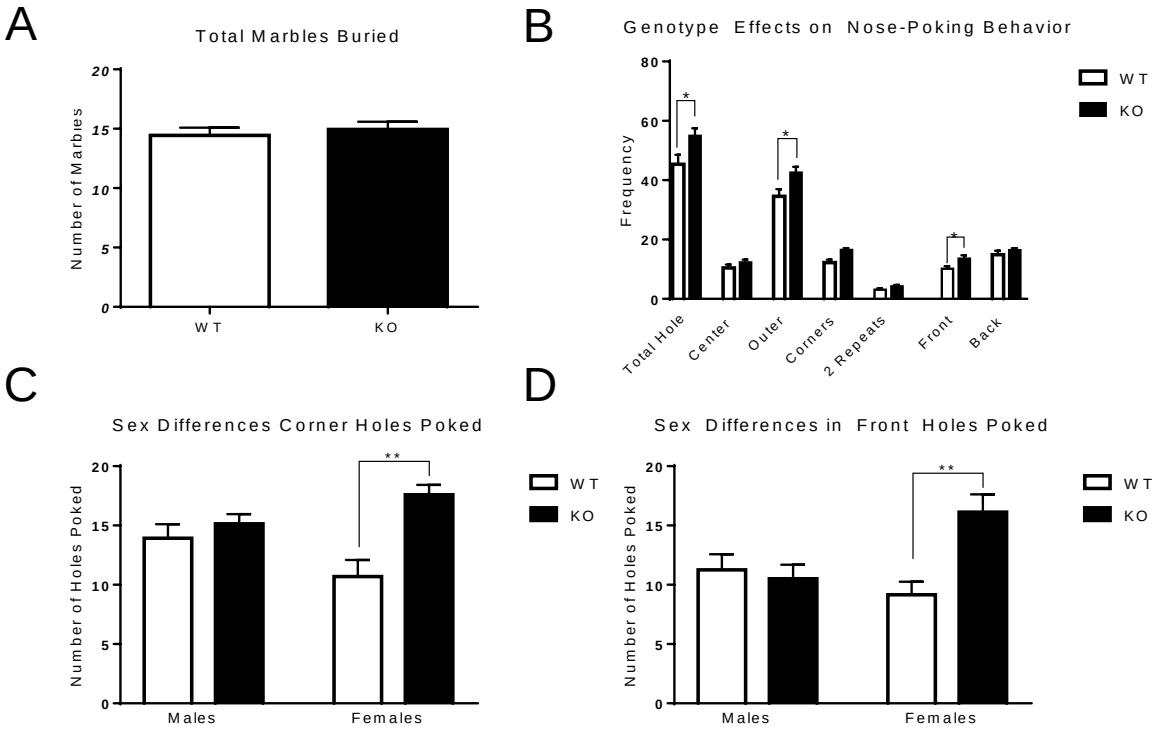

Figure 4.4. A. Knockouts and wildtypes performed similarly on the marble burying task. B. Knockouts exhibited higher amounts on hole poking behavior on total holes poked, outer holes poked and front holes poked. C. A significant interaction of sex and genotype was detected on corner holes poked, and further comparisons revealed that only females knockouts differed significantly from their wildtype counterparts. D. Similar to corner holes, front holes poked showed a female specific effect of genotype. Data are presented as mean ± standard error of the mean. * = $p < 0.05$;** = $p < 0.01$;

Rotorod

To examine changes in motor learning, coordination, and repetitive behavior, subjects were tested in the accelerating rotorod task. A two-way ANOVA with repeated measures revealed some significant effects in

1 knockouts. Tests of within subjects effects indicated a trending interaction of genotype and trial on latency to fall across the 8 trials, $F(1,45) = 1.98$, $p = 0.06$. One way ANOVA's for each trial indicated that on the final trial, KO's exhibited a higher latency to fall, $F(1, 48) = 4.72$, $p < 0.05$ (Figure 4.5A). There was no main effect of genotype, $F(1, 45) = 2.06$, $p = 0.16$. There was a significant main effect of sex, $F(1,45) = 7.41$, $p < 0.01$ (Figure 4.5B), with females exhibiting a higher latency to fall. There was no interaction of sex and genotype, $F(1,45) = 1.7$, $p = 0.20$.

Social Behavior

Social Chamber

Following the marble burying task, a cohort of animals was tested in the three chambered social apparatus. Using offline scoring blind to group, duration in each of the three chambers for phase A and B, as well as time at both cups was recorded. No differences were noted in Phase A. Results for Phase B were evaluated with ANOVA on duration of time in the chamber containing the conspecific (Figure 4.6A) and duration of time at the cup containing the conspecific (Figure 4.6B). Results for the chamber containing the conspecific indicated no overall effect of genotype, $F(1,56) = 1.45$, $p = 0.24$, no main effect of sex, $F(1,56) = 0.02$, $p = 0.88$, nor a significant interaction of sex and genotype, $F(1,56) = 0.29$, $p = 0.60$.

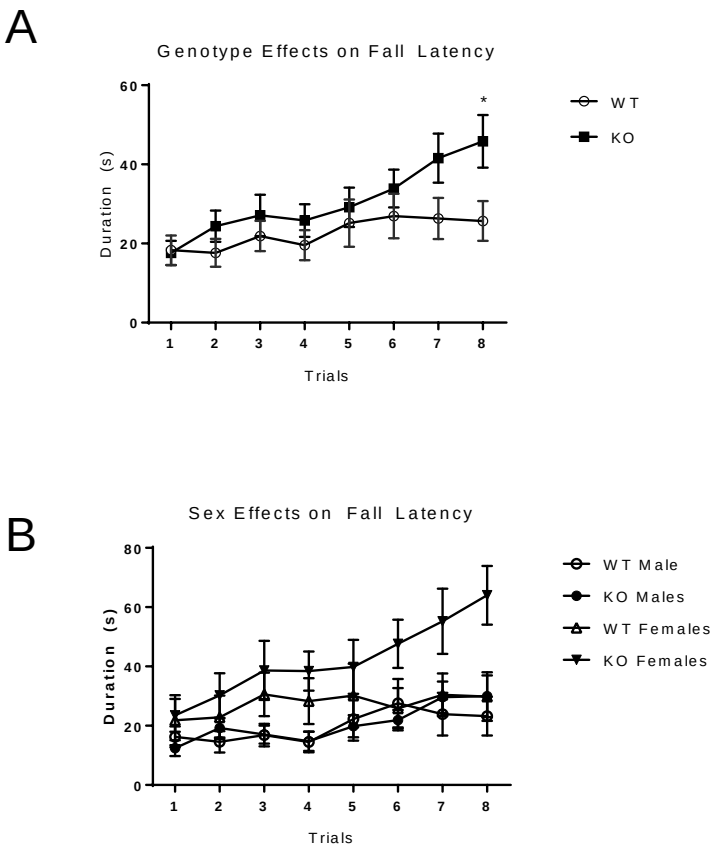

Figure 4.5. A. There was a trending interaction of genotype and trial on latency to fall on the rotorod task, with KO's exhibiting significantly higher fall latency on the final trial. Multiple comparisons indicated the largest effect was on the last trial. B. Females demonstrated a higher latency to fall in the rotorod task than their male counterparts. The effect was especially prevalent in the KO females. Multiple comparisons indicated that female KO's showed enhanced performance on this task. Data are presented as mean ± standard error of the mean. * = $p < 0.05$

Similar results were found for the chamber that housed the novel object. No overall effect of genotype, $F(1,56) = 0.2$, $p = 0.89$, no main effect of sex, $F(1,56) = 0.37$, $p = 0.54$, nor a significant interaction of sex and genotype, $F(1,56) = 0.05$, $p = 0.83$. Results for the impact of these variables on duration of time spent interacting with the cup showed a similar pattern. No main effect of genotype was detected, $F(1,56) = 0.18$, $p = 0.67$, nor a significant impact of sex, $F(1,56) = 0.06$, $p = 0.81$. The interaction of these two variables was also negligible, $F(1,56) = 0.007$, $p = 0.93$. Similar results were found

for the cup that housed the novel object. No overall effect of genotype, $F(1,56) = 1.8$, $p = 0.18$, no main effect of sex, $F(1,56) = 2.2$, $p = 0.14$, nor a significant interaction of sex and genotype, $F(1,56) = 0.44$, $p = 0.51$.

Social Partition Task

As a complement to the three chambered social task, another cohort of animals was tested in the social partition paradigm. Results for the three trials were evaluated 2 x 2 ANOVA with repeated measures across the three trials (Figure 4.6C). Results revealed no significant impact of sex, $F(1, 45) = 0.001$, $p = 0.98$, or genotype, $F(1, 45) = 1.14$, $p = 0.24$, on duration of time spent at the partition. The interaction of sex and genotype was also not significant, $F(1, 45) = 0.29$, $p = 0.59$ across the three trials. The same pattern was noted for frequency of visits across the three trials. The effect of genotype was not significant, $F(1, 45) = 2.09$, $p = 0.16$, nor was the effect of sex, $F(1, 45) = 1.05$, $p = 0.31$. The interaction was also not significant, $F(1, 45) = 0.35$, $p = 0.56$.

Odor Discrimination

To examine potential changes in habituation to social and non-social odors, subjects were tested in the odor discrimination task. Results were analyzed using a repeated measures ANOVA for the impact of genotype on the frequency and duration for each trial (Figure 4.7). Results indicated no impact of genotype on any of the variables, $F(1,26) = 0.46$, $p = 0.51$. There was a main effect of trial presentation $F(14, 364) = 28.7$, $p < 0.001$, but no interaction of genotype and time was found $F(14, 364) = 0.41$, $p = 0.97$.

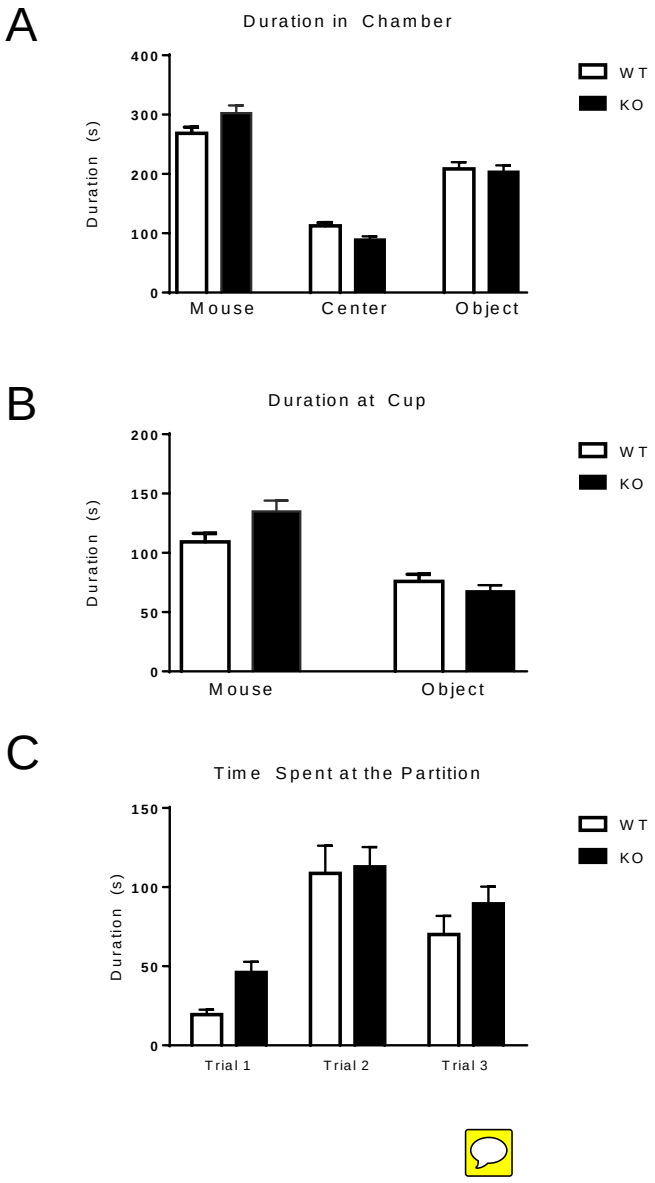

Figure 4.6. A. Social behavior in the three chambered social task is was not different between males and females or by genotype. B. Direct interaction with the cups containing the mouse or the novel object was the same across groups. C. Social behavior across the three trials of the social partition task was not significantly different between groups. Data are presented as mean ± standard error of the mean.

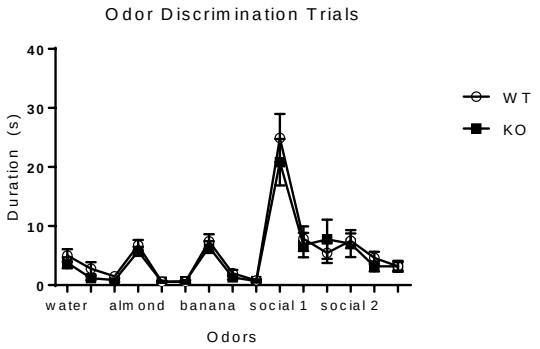

Figure 4.7. To investigate potential changes in habituation to both social and non-social orders, animals were odor discrimination task. Genotype had no effect on habituation and dishabituation to the various odors prese are presented as mean ± standard error of the mean.

Learning and Memory

Trace Fear Conditioning

Following testing for social partition, subjects were evaluated in trace fear conditioning as a test of hippocampal based fear memory. On Day 1, subjects revealed no effect of sex F(1,58) = 0.15, p = 0.9, genotype F(1,58) = 2.0, p = 0.11, or interaction F(1,58) = 0.45, p = 0.83 on any time point (min 1 – 12 or total). On Day 2, results were analyzed using a 2 x 2 ANOVA with repeated measures for the 6 instances of the trace period. A significant main effect of genotype was detected, $F(1, 58) = 6.49$, $p < 0.05$, with *FMR1* knockouts freezing significantly less than wildtype across time (Figure 4.8A). There was a no main effect of sex, $F(1, 58) = 0.000$, $p = 0.99$. No significant interaction of genotype and sex was detected, $F(1, 58) = 0.24$, $p = 0.63$.

On Day 3, cued fear conditioning was tested in a novel environment. During this task, the tone-trace period-ITI bout was repeated four times. Results were first analyzed across the condensed variables for baseline, tone, trace period and ITI. There was no

effect of genotype $F(1,58) = 0.85$, $p = 0.36$, sex $F(1,58) = 1.6$, $p = 0.21$, or genotype x sex interaction $F(1,58) = 0.7$, $p = 0.78$. There was a significant difference in freezing over the 4 instances of the trace period $F(3,174) = 122.8$, $p < 0.001$ and there was a significant interaction between group over the 4 period $F(3,174) = 5.3$, $p < 0.01$. Separate individual t-tests revealed reduced freezing in the KO mice in the trace period $t(1,60) = 2.7$, $p < 0.01$ compared to the WT mice (Figure 4.8B). Results for Day 3 were then analyzed using a 2 x 2 ANOVA with repeated measures across the four trace periods to see if this behavior changed across the four trace periods. A significant main effect of genotype was detected, $F(1,58) = 6.669$, $p < 0.05$ (Figure 4.8C), with *FMR1* knockouts displaying significantly less freezing across time. No main effect of sex was detected, $F(1, 58) = 1.987$, $p = 0.164$. No significant interaction of sex and genotype was detected, $F(1, 58) = 0.686$, $p = 0.411$. Further multiple comparisons revealed significant differences specifically during trace periods 2 and 4.

On Day 4, subjects were returned to the training environment to evaluate hippocampal memory. Results were analyzed using the same repeated measures ANOVA. No significant main effect of genotype, $F(1,58) = 0.3$, $p = 0.64$ (Figure 4.8D), nor a significant main effect of sex, $F(1, 58) = 0.03$, $p = 0.87$. No significant interaction was detected, $F(1, 58) = 0.22$, $p = 0.64$.

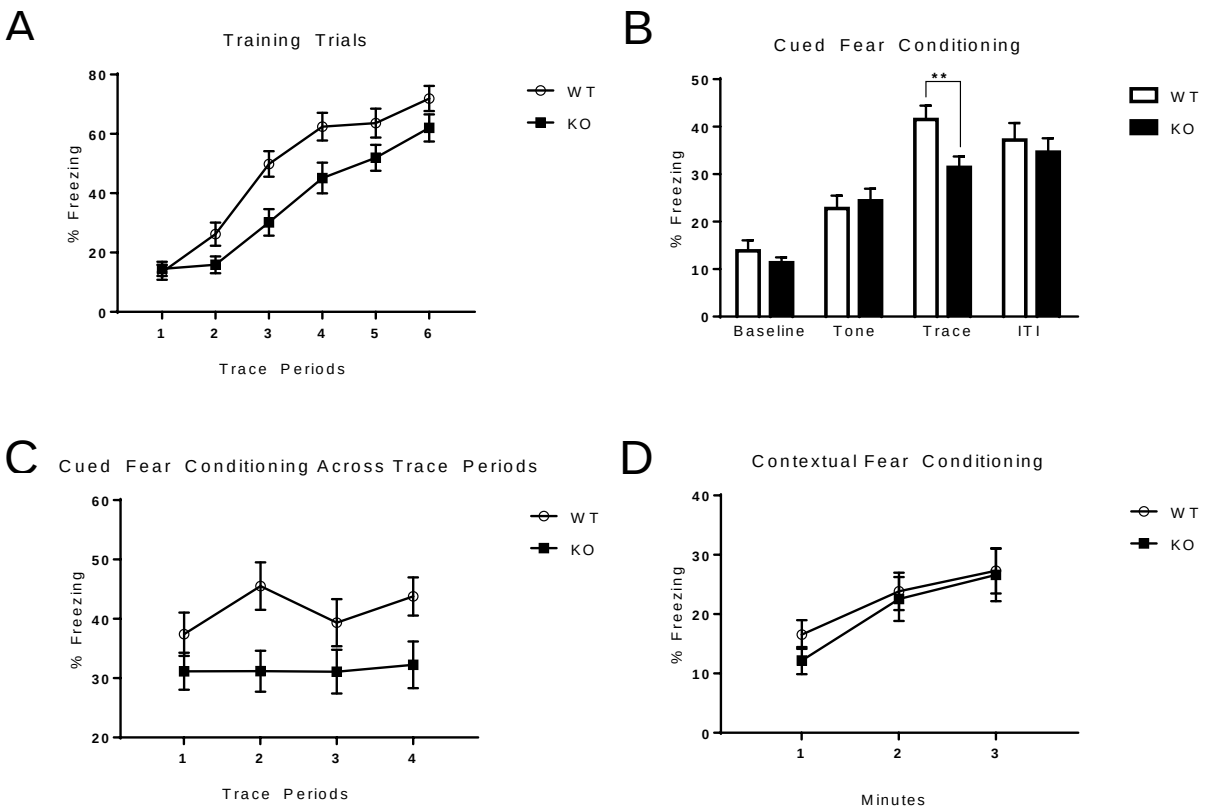

Figure 4.8. A. *FMR1* knockouts exhibited a less freezing behavior during the acquisition of the fear response. B. Knockouts exhibited less freezing during the trace period in a novel testing environment. C. When results for cued fear conditioning were examined across the trace periods, changes in freezing behavior were greatest during the 2nd and 4th trace periods during the same trial. D. Genotype and sex had no impact on behavior on tests of contextual fear conditioning. Data are presented as mean ± standard error of the mean. * = $p < 0.05$; * = $p < 0.01$; *** = $p < 0.001$

Delayed Fear Conditioning

A separate cohort of animals was examined in the delayed fear conditioning task, as a compliment to the trace fear conditioning trials. On Day 1, subjects were presented with repeated pairings of the CS and US stimuli. Results were interpreted using a 2 x 2 ANOVA with repeated measures. The within subjects variable was defined as time with 5 levels: baseline, tone 1, intertrial interval 1, tone 2 and intertrial interval 2. A significant main effect of genotype was detected, $F(1, 45) = 9.73$, $p < 0.01$, with *FMR1* knockouts freezing more over time. There was no main effect of sex, $F(1, 45) = 2.96$, $p = 0.09$.

There was a significant interaction between genotype over the 5 testing periods. Separate independent t-tests found significant difference in freezing at the 1st ITI $t(1,47) = 3.7$, $p < 0.05$, 2nd CS $t(1,47) = 2.6$, $p < 0.05$, and the 2nd ITI $t(1,47) = 2.1$, $p < 0.05$ (Figure 4.9A). There were no differences in freezing at baseline or during the 1st presentation of the CS. Results indicated no major impact of the interaction of sex and genotype, $F(1, 45) = 0.000$, $p = 0.98$.

On Day 2 of testing, animals were placed in a familiar context and freezing behavior was evaluated. Using a repeated-measures ANOVA analysis with repeated measures, a within subjects variable was created for time across the 5 minute bins. Analysis revealed no significant effect of genotype, $F(1, 45) = 0.47$, $p = 0.50$, or sex, $F(1, 45) = 0.48$, $p = 0.49$. There was a trending interaction of sex and genotype, $F(1, 45) = 3.23$, $p = 0.08$ There was a significant interaction between sex x genotype x time $F(4,180) = 2.6$, $p < 0.05$. However, none of the follow up analyses revealed significant differences between the groups (Figure 4.9B).

In the second part of testing for Day 2, animals were placed in an unfamiliar context and freezing behavior to the CS as well as at baseline was evaluated. A repeated measures ANOVA revealed a significant main effect of genotype, $F(1, 45) = 5.05$, $p < 0.05$. The main effect of sex was not significant, $F(1, 45) = 0.20$, $p = 0.66$, nor was the interaction of genotype and sex, $F(1, 45) = 0.92$, $p = 0.34$. There was as significant interaction between genotype over time $F(1,45) = 7.9$, $p < 0.01$, (Figure 4.9C). Further multiple comparisons revealed no significant main effect of genotype at baseline, $F(1, 45) = 0.15$, $p = 0.70$, however during the presentation of the tone, *FMR1* spent significantly less time freezing than wildtypes, $F(1, 45) = 6.83$, $p < 0.05$.

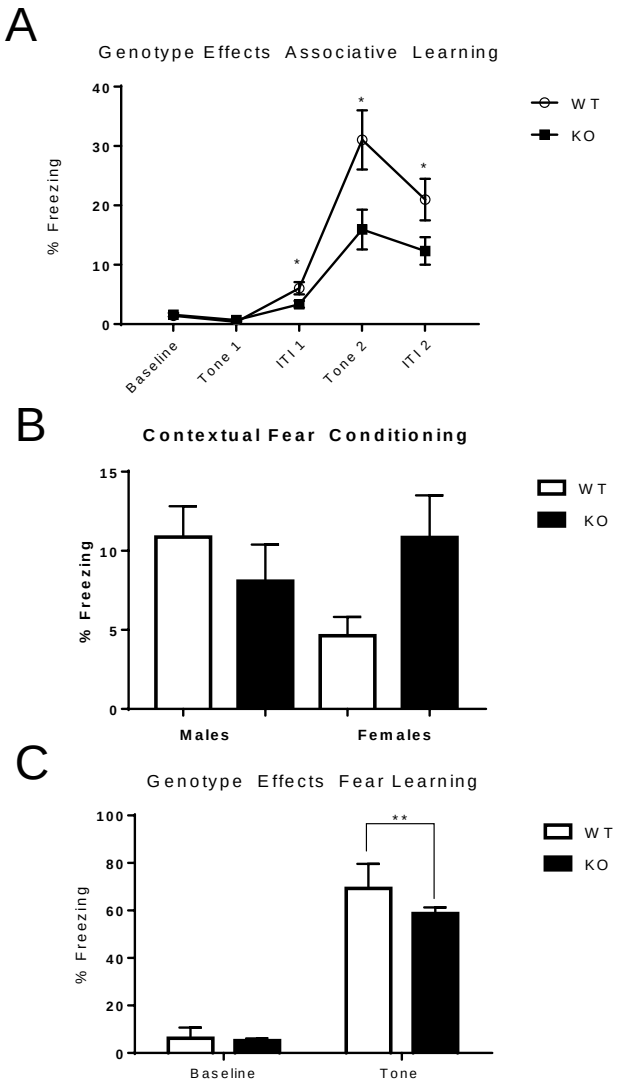

Figure 4.9. A. On Day 1, subjects were presented with 2 pairings of the CS and US. Results indicated main effects of time and genotype, as well as a significant interaction. Multiple t-tests revealed KO presented significantly decreased freezing in response to the first ITI, second CS and second ITI. B. On Day 2, during the first part, there was a significant interaction of sex, genotype and time, however follow up analyses did not reveal any significant differences for contextual conditioning. C. Knockouts exhibited significantly reduced freezing to presentation of the CS in a novel context. Data are presented as mean ± standard error of the mean. $p = 0.05$; * = $p < 0.05$;

Morris Water Maze with Reversal Learning

To investigate the effect of genotype on hippocampal spatial memory, animals were tested in the Morris water maze paradigm. In this task, only males were tested since previous tests in learning and memory did not reveal significant sex differences. A one-way ANOVA was used to analyze swim speed during the 8 learning trials, to ensure no differences in activity levels, and no effect of genotype was reported $F(1, 25) = 0.69$, $p = 0.415$. During the 8 blocks of learning trials (Figure 4.10A), a two-way ANOVA with repeated measures did not reveal any effect of genotype, $F(1, 24) = 0.03$, $p = 0.85$, however the effect of time was significant, $F(7, 168) = 30.15$, $p < 0.0001$. The performance of the mice improved over the 8 blocks. During the probe trial (Figure 4.10B), ANOVA results did not reveal effect of genotype in duration spent in any of the quadrants, $F(1, 92) = 0.00006$, $p = 0.99$. The week following the initial learning trials, animals were tested in a reversal learning paradigm. ANOVA testing revealed a trending main effect of genotype, with KO's showing increased latency to the platform, $F(1, 23) = 3.93$, $p = 0.059$, compared to wildtype mice (Figure 4.10C). There was a main effect across time $F(3,69) = 3.8$, $p < 0.05$; but no group x trial interaction $F(3,69) = 1.2$, $p = 0.29$. Visible platform information was also assessed to ensure differences were not due to deficits in vision. Results were analyzed using repeated measures across the four visible platform trials. No main effect of genotype was noted, $F(1, 24) = 0.28$, $p = 0.60$, nor an interaction of trial x genotype, $F(3, 72) = 0.89$, $p = 0.45$ (Figure 4.10D).

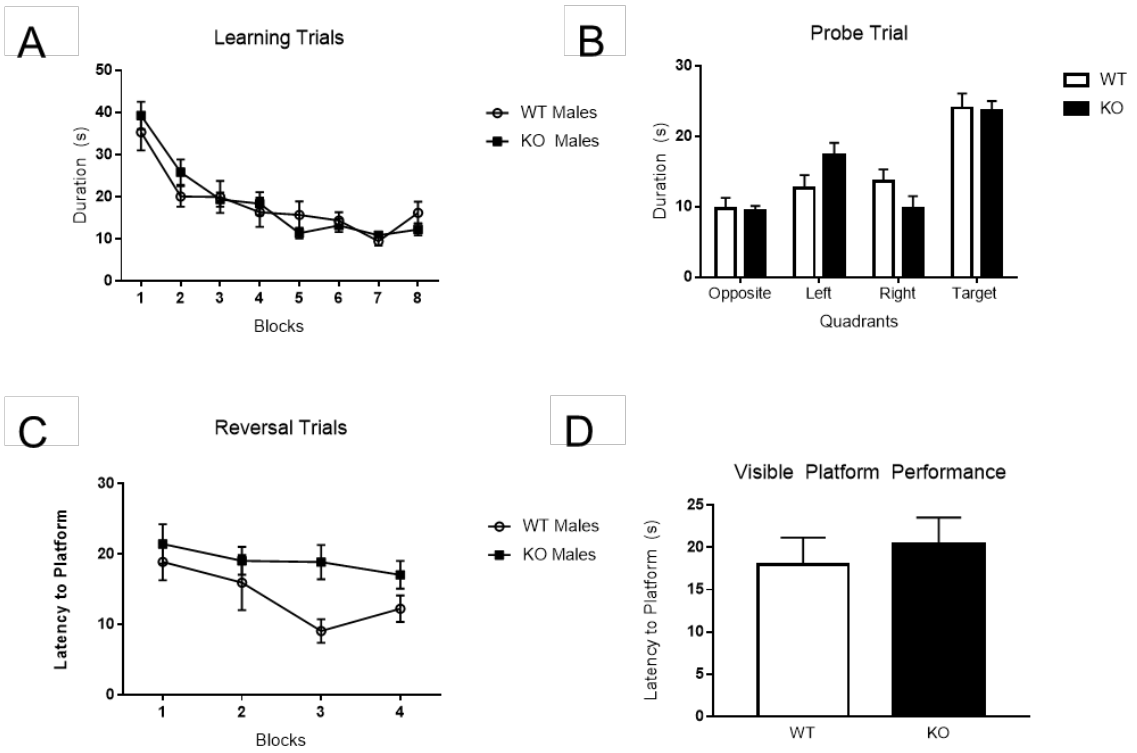

Figure 4.10 A. During the 8 blocks of learning trials, a two-way repeated ANOVA failed to reveal an effect of genotype, while the effect of time was significant. B. During the probe trial, genotype had no effect on duration spent in any quadrant. C. During reversal trials, knockouts performed worse than their wildtype counterparts, though their performance did improve across blocks. D. Performance on the visible platform was not impaired. Data are presented as mean ± standard error of the mean

CHAPTER FIVE

Discussion

Deletion of *FMR1* in a mouse model produces phenotypes that vary between background strains, sexes and methodologies. The present study first aimed to replicate ASD-like behaviors on the FVB background strain. Additionally, clinical data on individuals with FXS suggests that the phenotype varies significantly between the sexes, independent of gene dosage effects. However, to date, few studies have examined the female mouse model of FXS. Our data show that deletion of *FMR*1 produces behavioral changes that are comparable to the clinical population with FXS and ASD individuals. For a summary of findings, see Table A.1 and A.2. Deletion of *FMR1* was associated with sex-specific communication alterations in neonates and alterations in repetitive behavior on the nose poke assay. Specifically, female KO's showed increased repetitive behaviors compared to their female WT counterparts, while males showed no difference. Social behavior in both the three chambered social task and social partition task was unaffected. Deletion of *FMR1* also resulted in deficits in learning and memory in trace fear conditioning, as well as delayed fear conditioning paradigms. As expected, deletion of *FMR*1 did not impact spatial learning in the Morris water maze, however male KO mice showed a trend in an impaired ability to adapt to learning a new location of the hidden platform. Finally, deletion of *FMR*1 was also associated with increased activity levels and decreased nonsocial anxiety in the elevated plus maze. Hyperactivity was only observed in male KO mice.

One core behavioral component of ASD is alterations in communication behavior. The most sophisticated way to study these behaviors in an animal model is through the recording of ultrasonic vocalizations. Mice can emit a variety of vocalizations at a range of 30-120 kHz (Lahvis, Alleva, & Scattoni, 2011). We found that deletion of *FMR1* resulted in shorter and fewer vocalization calls in neonate mice throughout early development. Male KO mice displayed a decrease in vocalizations on specific postnatal days, while female KO mice have a decrease on some days an increase on other days. Previous investigations of vocalization behavior have reported contradicting patterns of UVS in FXS KO male mice. One study recorded vocalizations across PD4, PD7 and PD10 showed that that male PD7 FXS KO pups emit more calls, though no differences were noted on the other days (Lai et al., 2014) Another study using full spectrum analysis methods, which allow investigators deeper analysis into the full range of UVS, revealed genotype specific differences in the types of calls emitted on PD8 (Roy et al., 2012). Neither of the previous studies examined females, and as such this present study represents the first of its kind to examine sexual dimorphisms in the FXS model.

One of the benefits of examining vocalizations across several days during early development is that we have preliminary data highlighting critical days for detecting differences. Full spectrum vocalization analyses could later be used to determine the specific types of calls and physical aspects of these calls altered in male and female KO mice. One limitation of the UV study is portion of the study is that we started to record vocalizations on PD 8. There is concerns the idea that the rate of UV calling follows an ontogenetic profile, reaching a peak around PD6 or PD7, wherein it begins to decline sharply (Elwood & Keeling, 1982). The previous studies of *FRM1* KO mice included

earlier PD time points. Future analyses could expand to these time points in order to attain a fuller picture of spectrographic profiles.

The second core symptom of ASD is increased engagement in repetitive behaviors. In the current study, deletion of *FMR1* resulted in increased nose-poke behavior, but not in the marble burying task. Further analyses indicated that only females showed a genotype-specific difference, while male WT and KOs did not differ significantly on any measure of repetitive behavior. Previous examinations of repetitive behavior phenotypes in have yielded few significant results; moreover, these effects were weak or trending and did not hold up when replicated across several strains. In a seminal article where the investigators created *FMR1* knockout mice in several strains, there was increased marble burying behavior in only one of six tested strains, and the overall effect across strains was not statistically significant (Spencer et al., 2011). In a study published the same year, the deficit in repetitive behavior was only statistically trending (Veeraragavan et al., 2011). Both aforementioned studies were only conducted in male animals. Our study reports the novel finding that female *FMR1* knockouts display increased repetitive behavior in the nose poke task, thus the lack of findings previous may be due to female exclusion. One interesting side note is that in one previous study the authors briefly mention that females engage in increased nose-poke behavior, not present in male *FMR1* knockouts (K. B. Baker et al., 2010). The authors did not show the results, but in the discussion they noted, “Some differences were noted, including effects on hole poking and on circadian activity that were significant only in female KO mice…”.

In the present study, female *FMR1* knockouts also showed increased latency to fall in the accelerating rotorod task. The rotorod task is often regarded as a test of cerebellar coordination and motor ability. Previous research has reported no differences in the latency to fall in the *FMR1* knockout compared to WT (Heulens, D'Hulst, Van Dam, De Deyn, & Kooy, 2012; Peier et al., 2000; Spencer et al., 2011). Phenotypic analyses of another model of ASD, the neuroligin-3 (NLGN-3) knockout mouse, has displayed enhanced spatial learning in the rotorod task, similar to our *FMR1* knockout females (Rothwell et al., 2014). Given that pilot analyses of rotorod performance suggested that several components of the motor routine become less variable with training, the authors suggested that this task may be used to measure repetitive behavior (Rothwell et al., 2014). Using digital recordings of task performance, subject's gait was analyzed by tracking the location of the rear paws, the length of each step and the time between steps from digital recordings of the trials. Variability in these three measures during the first 30 seconds of these trials was measured by standard deviation. The results demonstrated that variability decreased with training in the *FMR1* knockout mouse, and was also negatively correlated with time to fall off the spinning rod. Given this relationship, the authors suggested that latency to fall can be considered an adequate indicator of acquired repetitive behavior.

The authors point out that general hyperactivity could be driving the performance on the rotorod task. The potential confound of hyperactivity is especially salient in the *FMR1* knockout, given the robust findings in the open field task. Yet, if hyperactivity is driving this effect, it should be evident on the first trial, and no differences were shown on trial one. More compelling that hyperactivity is not contributing to the rotorod

behavior is that female *FMR1* knockouts did not show hyperactivity in the open field compared to female WT mice, while they showed enhanced performance in the accelerating rotorod task. In light of the evidence, we hypothesize that the increased repetitive behavior seen in other tasks may be influencing the behavior in the rotorod task, namely the increased latency to fall off the spinning rod. Together with results from the nose poke task, these findings suggest that female *FMR1* knockouts display a clear and distinct behavioral phenotype. Future studies should employ gait tracking analysis to further explore the possibility of rotorod behavior serving as an indicator of acquired repetitive behavior in the *FMR1* female knockout.

The third core symptom of ASD is altered social behaviors, which is the most studied aspect of the *FMR1* knockout. In investigations of the *FMR*1 male knockout, it is often shown that deletion of *FMR1* results in decreased sociability in both social partition and the three chambered social task (Z.-H. Liu & Smith, 2009; S S Moy et al., 2009; Pietropaolo et al., 2011), consistent with ASD symptomology as well as FXS symptomology. However, our model shows no change in investigation time in social tasks, across both sexes. Others have also reported similar social preference in the three chambered social task between WT and KO mice (McNaughton et al., 2008). The discrepancies may be dependent on a variety of environmental and methodological factors. For example, when behavior in the social partition is examined over time bins, *FMR1* knockout mice show initial suppression of social investigation during the first 5 minutes of the task, followed by enhanced investigation in the later part of the task (Spencer et al., 2005). Assays of social behavior in this model appear to be influenced by cage familiarity. On the first day of testing, in an unfamiliar cage, *FMR1* knockouts

exhibited similar time spent at the partition. During the second day *FMR1* knockouts were presented with new unfamiliar partners in the same ("familiar") cage. During the second "familiar cage" trial, knocked reacted differently than wildtypes, and the direction of the effect changed across the 4 time bins. The authors of this study suggested that the social response of the *FMR1* KO mice are dependent on experience. Therefore, it is possible that the lack of alterations seen in the present study may reflect an adaptation to the cage environment. It is also possible that if binned into smaller trials, the effect could be different. Social anxiety is the most common clinical deficit in individuals with FXS, and as such is expected to be detected in characterization of the *FMR1* knockout (Cordeiro et al., 2011). Social anxiety is often assessed through assessment of grooming behavior during social tasks. A significant limitation of this study is a lack of data concerning grooming behavior during these tasks, and future studies should focus on integrating this analysis.

The results in the social tasks were also not associated with deficits in social odor discrimination, as the mice displayed normal habitation and dishabituation to social and non-social odors. We were concerned with the animal's ability to detect non-social odors and social odors because FMRP has been shown to be important for the regulation of neuronal differentiation and is ubiquitously expressed in the olfactory bulb (Scotto-Lomassese et al., 2011). Deletion of *FMR1* results in the elimination of FMRP. Previous work has suggested that FMRP is necessary for olfactory sensitivity, however not necessary for odorant discrimination (Schilit Nitenson et al., 2015). Furthermore, it's possible that FMRP is needed for olfactory discrimination learning, but this does not particularly influence social behavior (Larson, Kim, Patel, & Floreani, 2008). Our study

also supported the notion that WT and KO mice exhibit similar odor detection thresholds (Larson et al., 2008). As such, null results in social paradigms cannot be attributed to deficits in social odor discrimination. However, a limitation of our study is that this behavior was not assessed in female *FMR1* knockouts, though there is no indication of sexual dimorphism on social behaviors.

Hyperactivity remains one of the most commonly reported characteristics of the *FMR1* knockout and is typically assessed through the open field task. Previous research on the FVB background strain have reported fairly consistent levels of hyperactivity as compared to wildtypes, and our data supports this effect in male *FMR1* knockouts (Z. H. Liu, Chuang, & Smith, 2011; Qin et al., 2005). However, in a previous study of the *FMR*1 knockout in the C57BL/6 strain, hyperactivity was not detected (Veeraragavan et al., 2011). The C57BL/6 strain is commonly known to show higher activity levels and this could create a ceiling effect for *FMR1* knockouts on this strain (Crawley et al., 1997). Hyperactivity is a commonly reported clinical characteristic (R. J. Hagerman, 1997), and as such represents an important criterion for a model of FXS. Furthermore, we detected a significant interaction of sex and genotype in the open field task, such that homozygous females did not show hyperactivity like their male counterparts. Previous examinations of this behavior between sexes suggested that female *FMR1* knockouts on the FVB background strain performed similar to males (Qin et al., 2005), C57BL6/J (Ding et al., 2014), and the C57BL/6 albino strain (K. B. Baker et al., 2010). Discrepancies between our findings and the Qin et al paper could be due strictly to methodological differences. For example, their data was analyzed in 6-minute time bins, and they did not report the interaction of sex and genotype in their results, only main effects for each sex. Our data is

congruous with clinical data in humans suggesting that FXS-related hyperactivity in females is less common than in FXS males (Freund, Reiss, & Abrams, 1993).

Anxiety behavior has been a source of discourse in studies of the *FMR1* knockout mouse. Our finding is consistent with other studies of anxiety behaviors in rodent models showing that deletion of *FMR*1 results in reductions of anxiety in the elevated zero maze in the FVB strain (Z.-H. Liu & Smith, 2009) as well as in the open field task on the C57BL/6 strain (Spencer et al., 2005). In the present study, females across both genotypes also displayed lower amounts of anxiety between groups, though sex did not interact significantly with deletion of *FMR1*. Clinical data suggests that women traditionally have higher rates of lifetime diagnosis for anxiety-related disorders, making these results surprising (McLean, Asnaani, Litz, & Hofmann, 2011). These results also represent a reversal of traditional results in animal models, which suggest that females often exhibit higher amounts of anxiety as compared to males (An et al., 2011). One possibility is that the effect is an order effect of testing. All mice had previously been tested in the open field test then tested in elevated plus maze test. Future studies may include a behaviorally naïve group to be tested in the elevated plus maze test.

Decreased non-social anxiety behaviors in mice are surprising considering the human FXS condition. One caveat to the results from the plus-maze data is that the elevated plus maze may not be measuring the type of anxiety that is found in the clinical population. Epidemiological data in humans with FXS show a higher than normal incidence of anxiety related disorders (Gallagher & Hallahan, 2012). One study found that 82.5% of participants with FXS met criteria for at least one anxiety disorder (Cordeiro et al., 2011). However, the three most common anxiety disorders were specific phobia, social phobia,

and selective mutism. Most tests of anxiety for rodents are aimed at measuring behaviors akin to generalized anxiety disorders (GAD) (Z.-H. Liu & Smith, 2009), and therefore could account for differences between clinical data and rodent models. Future experiments could use other types of behavioral tests to examine social anxiety and phobia, such as social fear conditioning (Toth, Neumann, & Slattery, 2001), and perhaps examine ultrasonic vocalizations in adult mice to determine alterations in communication.

As the largest source of inherited intellectual disability, one of the most scrutinized behaviors in the *FMR1* knockout is performance in tasks of learning and memory. On three separate tests of memory, results suggest a significant deficit in the *FMR1* knockout. This effect was present for both males and females for acquisition for learning trials and cued fear conditioning in trace fear conditioning, as well as for delayed fear conditioning. Previous work has shown similar effects in fear conditioning in other strains (Hayashi et al., 2007; Zhao et al., 2005). Similar effects were seen during delayed fear conditioning and have been supported by previous literature (Gu et al., 2002).

One limitation of our study is that females were not tested in the Morris water maze. Previous research has suggested that females do, in fact, show the same deficit in hippocampal spatial memory on the Morris water maze task (K. B. Baker et al., 2010). In the Baker et al., 2010 study males and females showed significant deficits in performance on navigation during the learning trials, however, our results reflect no changes during acquisition. We found moderate effects when the location of the platform was reversed. Methodological differences include that they used a protocol calling for 2 trials per day for 8 days, while our methods included 2 blocks of 4 trials per day for 4 days. A separate

study that used 2 blocks of 4 trials per day for 6 days found no difference during the first portion of the MWM (Paradee et al., 1999).

The first aim of the present study was to replicate the core characteristics of ASD behaviors, given the relationship of the *FMR1* knockout as a model of human Autism Spectrum disorder, in the FVB strain. A common critique of mouse model studies is the reliance on one strain and many of the previous studies have focused on the C57BL/6 strain. We wanted to systemically investigate which ASD-like behaviors were altered in the FVB strain. The results of our study only indicated alterations on two aspects of the most common ASD characteristics, namely communication and repetitive behaviors. Our results also indicated that *FMR1* knockout did mimic many of the behavioral deficits found in human Fragile X syndrome. Indeed, hyperactivity and impaired learning and memory were two robust differences seen in our study. However, as previously discussed these traits are easily influenced by a variety of factors, including genetic background, and even pharmacological agents (Rotschafer, Trujillo, Dansie, Ethell, & Razak, 2012), Our results were consistent with results in the FVB strain as well as some correspondence with data in other strains.

The second aim of the present study was to further characterize the impact of sex on the *FMR1* knockout phenotype. Studies of the impact of deletion of *FMR*1 often fail to include female animals, whether due to avoidance of potentially confounding variables or to the high cost of doubling the cost of animals. Furthermore, if the effect size of the sex differences is small then the experiment may require additional mice to have sufficient statistical power to detect the differences. Previous studies have justified this measure by suggesting that females with deletion of *FMR1* do not show a behavioral phenotype (K.

B. Baker et al., 2010; Ding et al., 2014; Qin et al., 2005). Here we present evidence that females with deletion to *FMR1* display some behavioral alterations similar to male mice and show some behavioral deficits not found in males. Female *FMR1* knockout animals showed increased repetitive behavior on the nose poke assay and rotorod rest, with no genotype differences seen in males. As earlier stated, clinical observations show that FXS females display categorically different phenotypes than males with FXS. Some would ascertain that increased display of repetitive behavior models ASD-like behavior. However, clinical evidence suggests that females with FXS display less incidence of than their male counterparts (Clifford et al., 2007). Recently, evidence has come to light suggesting that FMRP interacts with a TOP3B, a protein thought to be involved in schizophrenia (Stoll et al., 2013), suggesting a link between FXS and schizophrenia. This link has been hypothesized for some time due to clinical data suggesting incidence of schizophrenia-like behaviors and diagnoses, specifically in FXS female carriers (Reiss et al., 1988; Vantalon, Briard-Luginbuhl, & Mouren, 2005). Given this data, we postulate the increased motor behavior on both the rotorod and nose poke assay may model the schizophrenia-FXS comorbidity, with the common symptom of abnormal motor behavior (Ridley, 1994). Enhanced rotorod performance has also been shown in mice lacking heregulin, a specific isoform from the neuregulin family, from the *NRG1* gene (Gerlai, Pisacane, & Erickson, 2000). Mutations in this gene represent the leading schizophrenia susceptibility gene. The two disorders do indeed share common characteristics. Like FXS, schizophrenia is also commonly characterized by deficits in prepulse inhibition and exaggerated startle responding, an aspect of sensorimotor gating behavior (Braff et al., 1978). Previous research has indicated that FXS males and *FMR1* knockout male mice

alike show deficits in sensorimotor gating (Frankland et al., 2004). Further research could examine the comorbidity relationship through an investigation of *FMR1* knockout females in prepulse inhibition. One study on a C57BL/6J background has shown similar enhancements in PPI in males and female *FMR1* knockouts (Ding et al., 2014), however, more studies are needed to replicate this finding across strains.

Failure to investigate and understand the mediational role of sex in the *FMR1* knockout phenotype has broad implications, as it has been shown that wildtype male offspring of heterozygous dams show aspects of the *FMR1* phenotype. Broadly, these findings underline the importance of inclusion of females in preclinical examinations of possible interventions. While some have suggested that the female *FMR1* knockout should be included in studies with males based on similarity, here we wish to highlight the differences. Future research should focus on how these phenotypes could and should be treated separately. For instance, repetitive behavior phenotypes are commonly treated using several different types of drugs, including 5-HT1BR agonists (Ho et al., 2016), 5HT1A partial antagonists (Chugani et al., 2016) and more recently, antioxidants (Hardan et al., 2012). Through the routine exclusion of females from biomedical studies, opportunities are missed to explore potential treatments with a higher possible effectiveness in females.

APPENDIX

APPENDIX A

Table A.1

Genotype Effects

Diagnosis	Behavioral Parameter	Measure	Genotype Effect	Previous Literature on FVB Strain	Previous Literature on Other Strains
ASD Comorbid Symptoms	Communication Behaviors	Neonatal USV's	KO↓, KO↑**	KO ↑[1]	KO ↑[2,3]
	Repetitive Behavior	Marble Burying	-	-	KO ↑[2] KO ↑[4]
		Nose Poke Assay	Female KO ↑	-	KO ↔[5]
		Rotorod	Female KO ↑	-	KO ↔[6,7]
	Social Interaction	Social Chamber	-	KO ↓[8, 9, 10]	KO ↓[9]
		Social Partition	-	-	KO ↓[11] KO ↔[12]
FXS Symptoms	Learning and Memory	Trace Fear Conditioning	KO ↓	KO ↓[13]	KO ↓[14]
		Delayed Fear Conditioning	KO ↓	KO ↔[15]	KO ↓[16]
	Cognitive Inflexibility	MWM Reversal Learning	KO ↓	KO ↔[17] KO ↓ [18]	KO ↓[19]
	Activity Levels	Open Field	KO ↑	KO ↑ [20, 21]	KO ↔[4]
	Anxiety Levels	Elevated Plus Maze	KO ↓	KO ↓[8]	KO ↓[11]

Table A.2

Sex Comparison

Behavior	Males	Females	Previous Literature
Neonatal Communication	KO↓, KO↑**	KO↓	No Data
Repetitive Behavior	-	KO↑	Female KO ↑(K. B. Baker et al., 2010)
Social Interaction	-	-	No Data
Learning and Memory	KO↓	KO↓	No sex difference (K. B. Baker et al., 2010), No sex difference (Ding et al., 2014)
Activity Levels	KO↑	-	No sex difference (K. B. Baker et al., 2010; Qin et al., 2005)
Anxiety Levels	KO↓	KO↓, ♀↓	Male KO ↓ (Qin et al, 2005)

APPENDIX B

Table A.1

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