SOCIAL BEHAVIOR IN FMR1 KO MICE: A MODEL OF FRAGILE X SYNDROME

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ABSTRACT

The *fmr1* "knockout" (KO) mouse is a model for fragile X syndrome, the most common form of heritable mental retardation (Hagerman, 2002). The present study was designed to further assess the validity of this mouse model. Specifically, in light of the prominence of social anxiety in the human FXS phenotype, the present study assessed various aspects of social behavior in fmr1 KO mice and wild-type (WT) littermates. A three-chambered apparatus was used to assess: (1) the preference for being near a novel conspecific vs being alone; and (2) the preference for a novel conspecific vs. a familiar one. In the first phase, experimental subjects were exposed to a restrained unfamiliar male mouse in one of the side compartments. Subsequently, in the second phase, a second unfamiliar male mouse was restrained in the opposite compartment. In both phases, square crossings, the time spent in each area, sniffing each restraining cage, rearing, grooming and wall climbing were measured. The results revealed that both the KO and WT mice preferred to be near a novel conspecific rather than to be alone; the magnitude of this effect was comparable in the two genotypes. Preference for a novel conspecific over a familiar conspecific was also seen in both groups, but only when the stimulus mouse was a preferred animal. When a non-preferred stimulus mouse was the novel animal, both groups showed a diminished novelty preference, but the magnitude of this effect varied by genotype. Under this condition, the WT mice showed a more pronounced negative reaction to the non-preferred mouse than did the KO mice. In addition, two other genotypic differences indicated that the KO mice may have been more anxious than controls in these social encounters: (1) a greater proportion of the KO mice had high total grooming times; and (2) the average duration of nose contact with the stimulus mouse was significantly shorter for the KO mice than for controls. These results provide

further support for the validity of this mouse model, although future studies are needed to more fully characterize the social behavior alterations in the *fmr1* KO mouse.

BIOGRAPHICAL SKETCH

Caitlyn Harris McNaughton was born in Lancaster, Pennsylvania, daughter of D. Alfred and Deborah Owens. She received her early education at Donegal High School, in Mount Joy, Pennsylvania. At Donegal, she was a member of the National Honor Society, the Math Club, and the Lancaster Youth Symphony. She participated in several theater productions and led the cello section in the school's orchestra. Caitlyn graduated from Donegal High School in 2000 ranked at fourth in a class of 150 by grade point average. After high school, Caitlyn attended Franklin and Marshall College, Lancaster, Pennsylvania, as a Biological Foundations of Animal Behavior major and Environmental Studies minor. While at F&M, Caitlyn assisted in the lab of Dr. Charles Heyser, conducting research on the effects of drugs on rodent behavior. For this research, Caitlyn was awarded the Hackman Research Scholar's grant and the Howard Hughes Medical Institute Scholarship. Under Dr. Heyer's supervision, Caitlyn designed and completed an Honor's thesis entitled "The Effects of Scopolamine on Overshadowing and Maze Learning in Rats," funded by the Undergraduate Psi Chi Research Grant. Caitlyn was a member of the Psi Chi Psychology Honors Society and the Phi Beta Kappa honors society. Outside of her academic pursuits, Caitlyn was a resident assistant and a member of the F&M orchestra, the F&M Philharmonia and the F&M dance company. Caitlyn graduated *summa cum laude* from F&M in 2004, ranked tenth in a class of 480 for grade point average. At graduation, Caitlyn was awarded the Rawnsley Science Prize, for top science student, and the Roger and Elizabeth Thompson Award for Animal Behavior. The following fall, Caitlyn was accepted into Cornell University's Psychology Department with a Teaching Assistantship. In her two years at Cornell, Caitlyn assisted Dr. Barbara Strupp in behavioral neuroscience research on mice genetically

modified to have Down's Syndrome or Fragile X Syndrome. Caitlyn designed and implemented a project quantifying social behavior of mice with Fragile X Syndrome, the results of which are described in this thesis. Outside of academia, Caitlyn enjoyed hiking in the areas surrounding Ithaca and indulging in her many hobbies: quilting, knitting, spinning wool, and playing cello.

After graduation, Caitlyn plans to teach high school biology in Lancaster county, Pennsylvania. It is her hope to spread the joy and wonder of science to the next generation, and encourage young adults to pursue research in biology.

To my husband, Elijah, for his love, patience

and never-ending support.

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LIST OF ABBREVIATIONS

AAALAC – Association for Assessment and Accreditation of Laboratory Animal Care

DSM – IV – Diagnostic Statistical Manual, fourth edition

FMRP – Fragile X mental retardation protein

FX – Fragile X

FXS – Fragile X syndrome

FS – Fragile X flies

IQ – Intelligence quotient

KO – Knockout

LTD – Long term depression

LTP – Long term potentiation

mGluR – Metabotropic glutamate receptor

MPEP – 2-methyl-6-phenylethynyl-pyridine

MRI – Magnetic resonance imaging

NIH – National Institute of Health

SRO – Surprising reward omission

WT – Wildtype

CHAPTER ONE

INTRODUCTION

Fragile X syndrome (FXS) is the most common form of heritable mental retardation. It affects both males and females, but because it results from a mutation of a gene on the X chromosome, a greater number of males are affected (1/4000 males vs. 1/8000 females; Turner et al, 1996). The disorder is caused by an abnormal expansion of a CGG trinucleotide repeat in the fragile region of the X chromosome (Xq27.3). The expansion causes hypermethylation of a CpG island, effectively silencing transcription of the *fmr1* gene (fragile x mental retardation gene). The resulting lack of the encoded protein, FMRP (fragile X mental retardation protein) leads to abnormal brain development and cognitive dysfunction.

On a gross anatomical level, the brains of humans with FXS are subtly different from normal brains. MRI (magnetic resonance imaging) studies have found that humans with FXS tend to have an increased brain weight, a smaller cerebellar vermis , larger lateral ventricles, a larger caudate nucleus and a larger hippocampus (Hagerman, 2002, Reiss et al, 1991, Reiss et al, 1995). These slight differences have not been reliably linked to the phenotype of the disorder. Humans with FXS also have abnormal dendritic connections. As a normal nervous system develops, an excess of synapses are formed. As development continues, the synapses that are unneeded are pruned away leaving dendrites that have shorter spines with large synaptic areas. Humans with FXS have an increased proportion of dendritic spines in the cortex that are long and thin, characteristic of immaturity. In addition, these dendrites exhibited reduced synaptic contact area and the spines were denser than normal in the distal regions. These abnormalities are indicative of improper synaptic pruning and maturation and suggest a role of FMRP at the synaptic level during development(Abrams & Reiss, 1995, Churchill et al, 2002, Irwin et al, 2001).

The cognitive and behavioral phenotype is more severe in males than in females. Males have one X chromosome that is activated in nearly every cell of the body. If that X chromosome has the *fmr1* mutation, then FMRP will be lacking throughout the body. This results in the severe phenotype presented in males. Females, on the other hand, inherit two X chromosomes, only one of which can be activated in each cell. In most cases, the *fmr1* mutation is present on only one of these chromosomes, leading to a mosaic pattern of expression. The proportion of mutated X chromosomes that is expressed relative to the normal X chromosomes directly determines the severity of the phenotype in females, resulting in great variability (Mazzocco, 2000).

Physically, FXS manifests itself most prominently in a long, narrow face, wide, everted ears and macroorchidism (enlarged testes). Other physical features that may be present include a prominent forehead, hyperextensible joints, velvety skin (due to lack of ridges, particularly on the hands), a high, arched palate, flat feet and muscular hypotonia (Hagerman, 2002, Terracciano et al, 2005).

In males with FXS, mental retardation is present in all cases. IQ can range from 20 – 70, though this number declines with age during early development. This decline is not due to degeneration, but to the widening gap in functioning levels between FX boys and their peers. Global adaptive skills, as measured by the Vineland Adaptive Behavior Scale, decline in parallel with IQ (Fisch et al, 1996, Maes et al, 1994). These measures remain stable in adulthood (Einfeld et al, 1999).

In addition to mental retardation (e.g. low IQ score), FXS is characterized by specific cognitive dysfunctions. FX males show deficits in visual - motor coordination, manual dexterity and non-verbal pattern imitation as measured by the McCarthy Scales of Children's Abilities (Maes et al, 1994). In addition, deficits in sequential memory and processing, particularly in relation to abstract or meaningless

information, have been demonstrated (Cornish et al, 2004, Maes et al, 1994, Zigler & Hodapp, 1991). Problems with language and communication such as perseverative speech, abnormalities in form and content of speech, limited imaginative speech and deficits in non-verbal communication skills as measured by the Neuropsychiatric Developmental Interview are also common (Reiss & Freund, 1992).

Attention deficits and hyperactivity are extremely prevalent in males with FXS; 80% meet the DSM-IV criteria for Attention Deficit Hyperactivity Disorder. More specifically, FX males have deficits in selective and sustained attention as measured by visual search and visual vigilance tasks, respectively (Munir et al, 2000). In the same tasks, FX males also showed an inability to inhibit responses, leading to perseverative responses, and problems shifting visual attention (Wilding et al, 2002).

FXS is considered to be the most common known cause of autism; however, only 16-25% of males with FXS meet the full DSM-IV criteria for autism (Kau et al, 2002). Autistic features are present in all males with FXS, but the severity of these symptoms ranges from mild to severe (full autism). Common motoric autistic features include hand flapping when excited or anxious, hand biting and rocking (Cornish et al, 2004, Hagerman, 2002, Reiss & Freund, 1992). FX males also exhibit hypersensitivity to stimuli in all domains, taking a long period of time to recover after being overstimulated (Cornish et al, 2004, Hagerman, 2002, Reiss & Freund, 1992). Changes in routine or situation, new experiences and social interactions also tend to upset males with FXS (Hatton et al, 2002, Reiss & Freund, 1992). The key features that differentiate FX males that meet the full criteria for autism from FX males that do not are attachment behavior to caregivers and social avoidance. FX males without full autism show normal attachment to caregivers, do not avoid social interactions, can perceive facial signals and have no difficultly in taking the perspective of others

(Hagerman, 2002, Reiss & Freund, 1992, Zigler & Hodapp, 1991). FX males with autism are impaired in these domains.

Social anxiety in FX males is most often manifested in gaze aversion. Cohen et al (1991) showed that FX males selectively avoid eye contact with another person; gaze aversion is not due to communication inability or inattention. The characteristics of social behavior in FX males change as they mature. Young boys with FXS (under 6 years old) show the greatest dysfunction in play and leisure activities compared to age matched controls, and relatively better interpersonal relations and coping skills. Boys with FXS greater than 9 years old, however, show the opposite pattern; they show the greatest dysfunction in interpersonal relations and relatively better play/leisure skills. It should be noted, however, that all of these skills were below normal for all age groups (Fisch et al, 1999). Cohen (1995) hypothesized that hyperarousal and hypersensivity to sensory stimuli are the underlying cause of social anxiety in FXS, although no studies have been conducted to investigate this hypothesis further.

Currently, no treatments or interventions are available to treat FXS as an entire disorder. Medications are prescribed to treat the various symptoms; no treatments are available that attempt to normalize brain development. Common prescriptions include stimulants for hyperactivity, impulsivity and attention problems, and anti-depressants (such as selective serotonin reuptake inhibitors) for perseverative behaviors and anxiety. More severely affected boys may be prescribed anti-psychotics to control aggression and extreme problem behaviors (Berry-Kravis & Potanos, 2004). An ideal treatment is one that would correct brain development rather than treat the resulting symptoms. However, this type of treatment depends on knowledge of the mechanism by which the lack of FMRP causes abnormal neural development.

The role of FMRP in the development of the nervous system is unclear. FMRP is expressed in brain tissues throughout development, most notably in the granular

layers of the hippocampus and the cerebellum. It is able to bind mRNA in the synapses and is expressed after activation of metabotropic glutamate (mGlu) receptors. The abnormalities in dendritic spines of humans with FXS suggest a role of FMRP in dendritic pruning and plasticity (Abrams & Reiss, 1995, Churchill et al, 2002). Recent work by Bear et al (2004) provides evidence for a link between FMRP, long term depression (LTD) and the mGlu receptors. LTD and its counterpart LTP (long term potentiation) are thought to be involved in memory storage and learning. Perhaps more importantly, LTD may play a role in activity-mediated synaptic pruning during development. In many areas of the brain, LTD requires activation of mGlu receptors; this activation leads to post-synaptic activation of protein-synthesizing mRNA. FMRP is a protein that is produced rapidly in synapses following activation of the mGlu receptors; without FMRP, LTD was highly exaggerated following mGluR activation. This data indicates a role of FMRP in the inhibition of LTD at the synaptic level. The hypothesis is that FMRP inhibits mRNA translation following mGlu activation, effectively decreasing LTD and perhaps other neural functions. In humans without FMRP, this model suggests that mGluR – mediated protein synthesis is over active, resulting in functional impairments. For example, memory formation in the amygdala mediates anxiety responses; a dysfunction in LTP or LTD in this area could lead to the anxiety and autistic behaviors seen in FX males (Bear et al, 2004). If this model of FXS is correct, then potential treatments could be developed. If FMRP serves an inhibitory function of mGlu activation, an mGlu receptor antagonist such as MPEP (2-methyl-6-phenylethynyl-pyridine) may normalize brain development in people who lack FMRP.

This hypothesis for neural dysfunction in FXS needs to be tested in animals before it can be applied to humans. Experimental drugs cannot be given to humans without first testing their potential efficacy and safety. While *in vitro* experiments are

invaluable, animal testing is the best predictor for safety and efficacy in humans. However, to test drugs in animals for efficacy, the animals must first exhibit symptoms that mimic the human condition. The *fmr1* knockout (KO) mouse was developed for this purpose (Bakker et al, 1994). The human *fmr1* gene has a 97% identical amio acid sequence to the murine analog (Ashley, 1993). Using mouse embryonic stem cells, exon 5 of the *fmr1* gene was interrupted, inactivating the gene. These stem cells were subsequently transplanted into blastocysts and implanted in female mice. The female offspring of these females were crossed with normal C57Bl/6J males to ensure the transmission of the mutated X chromosome (Bakker et al, 1994). Since then, research with *fmr1* KO mice has been conducted to determine correspondence with FX humans.

Fmr1 KO mice show physical abnormalities that correspond well to the human condition, such as macroorchidism and abnormal dendritic spines in the cortex and hippocampus (Bakker et al, 1994, Comery et al, 1997, Mineur et al, 2002). Behavioral and cognitive correspondence has been more difficult to demonstrate, however. Hyperactivity was found in most (Bakker, et al, 1994; Mineur, et al, 2002, Peier, et al, 2000), but not all studies (Nielsen, 2002, Yan et al, 2004). Typical learning and memory paradigms have lead to conflicting results; differences that were found between genotypes tended to be subtle. In the Morris and cross-shaped water mazes, no differences between genotypes were found in initial acquisition or performance over time. A few studies found small differences between genotypes during the reversal phase of this task (Bakker et al, 1994, D'Hooge, 1996, Kooy et al, 1996, Van Dam et al, 2000), but these differences were not replicated by other labs (Dobkin et al, 2000, Paradee et al, 1999, Peier et al, 2000, Yan et al, 2004). For other memory tasks, such as the 8-arm radial maze and the olfactory sequence working memory task, no differences were found between the genotypes (Yan et al, 2004). Surprisingly, in a

number of learning tasks *fmr1* KO mice have been found to perform better than WT (wildtype) mice (Fisch et al, 1999).

A possible explanation for these discrepancies is differences in the background strain of the mice used. Dobkin et al (2000) found that *fmr1* KO mice with an inbred C57Bl/6J background strain performed better in a Morris water maze than did *fmr1* KO mice with an inbred FVB background. Both of these background strains exhibited macroorchidism and had similar responses to a fear conditioning paradigm. The authors suggested that the C57 strain may have a greater propensity for learning and memory than the FVB strain. Likewise, Paradee et al (1999) found differences between *fmr1* KO mice from the inbred C57Bl/6J strain and the inbred 129/ReJ strain on the Morris water maze task, but no differences in conditioned fear response. Inbred mouse strains are used to limit genetic variability; however, inbred strains of mice suffer from recessive defects. The FVB strain, for instance, has recessive alleles for retinal degeneration and albinism. The current study uses an F1 hybrid cross between C57Bl and FVB strains. The hybrid limits the transmission of recessive defects and limits the amount of influence exerted by any one particular background strain.

One possible explanation for the inability to find a robust behavioral phenotype is that the studies described above were not designed to assess the core features of FXS, such as attention impairments, impulsivity and social anxiety. It is these areas that differentiate FXS from other forms of mental retardation. In addition, these symptoms are evident even when mental impairment is mild. Currently, our lab has been assessing selective attention, sustained attention, inhibitory control and emotional reactivity in *fmr1* KO mice and preliminary results have been encouraging.

Social anxiety has been studied very little in *fmr1*KO mice, with only two studies published to date. Spencer et al (2005) administered a battery of tests designed to assess social anxiety and social interaction characteristics using the C57Bl/6J strain.

In a dominance test, two male mice were placed in opposite ends of an opaque tube and allowed to approach each other. Whichever mouse backed out of the tube was considered the 'loser'. Spencer et al found that male *fmr1* KO mice were more likely to lose the dominance test if the partner mouse was unfamiliar to them. However, if matched against a cagemate, KO mice won as many matches as WT mice. To assess social interaction, mice were exposed to each other in a small cage separated by a wire mesh screen. The frequency of approaching the screen and the time spent at the screen were measures of social interest. WT and KO mice showed a similar degree of interest in familiar mice when they were in either a familiar or novel environment. KO and WT mice also did not differ in social interest when exposed to unfamiliar mice in an novel environment, but KO mice exhibited longer latencies and shorter initial time at the screen than WT mice when presented with unfamiliar mice in a familiar environment. This suggests that the relative novelty of both the partner mouse and the environment has an effect on the social behavior of the KO mice. Finally, in a direct social interaction test (two mice allowed to directly interact with one another), both KO and WT showed similar social behaviors toward other mice.

Mineur et al (2006) used a different approach to study social behavior in *fmr1* KO mice. Male *fmr1* KO were allowed to interact with an overiectomized female for a total of 8 minutes, after which they were exposed to a novel female. The frequency of social behaviors was measured as an indicator of social interest. *Fmr1* KO mice were found to have a lower frequency of social behaviors over all trials with the 'familiar' female. Upon introduction of the novel female, KO mice failed to show an increase in social interest as was exhibited in WT mice.

The current study was designed to further characterize the social behavior of male *fmr1* KO mice. The paradigm was based on a design developed by Nadler et al (2004) and was different than the tests used by Spencer et al (2005) and Mineur et al

(2006) in several ways. The paradigm was comprised of two distinct phases, one designed to assess the preference of the experimental mouse for the presence of an unfamiliar male mouse (vs. no animal), and the second designed to assess the preference of the experimental mouse for the presence of a novel male mouse over a familiar male mouse. The stimulus mice were confined using round, wire cages to prevent aggressive acts from occurring between the experimental and stimulus mice. The apparatus was three-chambered and allowed the experimental mice the choice to be alone or in the presence of another animal. Social investigation and preference were measured with the time spent in the area directly adjacent to the stimulus mouse and the time spent in nose contact with the stimulus cages. All sessions were videotaped to allow detailed behavioral coding at a later time. Behavioral coding allowed us to characterize the social interactions as well as measure other behaviors that occurred during the session. We hypothesized that *fmr1* KO mice would exhibit abnormal social interactions when compared their WT littermates.

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CHAPTER TWO

SOCIAL BEHAVIOR IN *FMR1* KO MICE: A MODEL OF FRAGILE X SYNDROME

Fragile X syndrome (FXS) affects 1/4000 males and 1/8000 females, making it the most common form of heritable mental retardation and the most common known cause of autism (Hagerman, 2002; Turner et al, 1996). It is caused by the expansion of a CGG repeat in the *fmr1* gene of the X chromosome (Pieretti et al, 1991). Individuals with the full mutation have greater than 200 repeats; normal individuals have no more than 50 (Fu et al, 1991). The expansion results in hypermethylation of a CpG island which functionally silences transcription of the gene, leaving individuals with FXS without the encoded protein: FMRP (fragile x mental retardation protein) (Feng et al, 1995; Oberle et al, 1991;).

FMRP is expressed in brain tissue, with the highest concentrations in the granular layers of the hippocampus and the cerebellum (Abrams & Reiss, 1995). Although its precise function is unknown, the presence of RNA binding sites and its pattern of expression have led researchers to hypothesize that it may have a role in protein production at the synaptic level (Churchill et al, 2002; Comery et al, 1997;). In addition, persons with FXS have long, thin dendrites, suggestive of immaturity or a lack of proper pruning. It has been hypothesized, therefore, that FMRP also plays a crucial role in the stabilization and elimination of synapses during development (Comery et al, 1997; Irwin et al, 2001).

FXS affects the two genders differently. Females tend to be less severely affected than males, and the phenotype in females is highly variable. These gender differences are explained by the fact that females have two X chromosomes, only one of which is activated in each cell. Generally, females only have the full mutation on

one of the X chromosomes, and the ratio of the activation of the two X chromosomes determines the severity of the phenotype. Because males inherit a single X chromosome, the variability in phenotype is much less (Mazzocco, 2000).

Males with the full mutation are invariably mentally retarded (i.e., IQ between 20-70), with a very specific constellation of cognitive and affective problems. Males with FXS show impairments in communication skills, visuo-motor skills, and sequential working memory (Cornish et al, 2004; Hatton et al, 2002; Maes et al, 1994; Terracciano et al, 2005; Zigler & Hodapp, 1991). Deficits in selective attention, sustained attention and inhibitory control are also prominent; 80% of boys with FXS meet the DSM-IV criteria for attention deficit hyperactivity disorder (ADHD) (Cornish et al, 2004; Hagerman, 2002; Hatton et al, 2002). Autistic behaviors in FX boys are present in a continuum from very mild to severe; all meet some aspects of the DSM-IV criteria for autism, though only approximately 25% of FX boys meet the full criteria for autism (Bailey, 1998). The most common autistic features in FX boys that do not meet the full criteria include repetitive motoric behaviors (hand flapping, hand biting, etc), sensitivity to sensory stimuli of all modalities, and social anxiety (Cornish et al, 2004; Hagerman, 2002; Reiss & Freund, 1992).

Social anxiety in FX males is most often characterized by gaze aversion (Hagerman, 2002). Cohen, et al (1991) showed that the gaze aversion exhibited by FX males was not due to inattention or communication inability, but rather was a selective aversion to eye contact with another person. Despite their aversion to eye contact, the majority of boys with FXS (those that do not meet the full criteria for autism) do not avoid social interactions and are able to perceive facial cues and emotions of others (Hagerman, 2002). Fisch, et al (1999) showed that as FX boys matured, they became more proficient at play and leisure activities, but less proficient at interpersonal relationships. It should be noted, however, that scores in both categories were below

normal. In addition, social interaction appears to cause an increase in anxiety levels in individuals with FXS as evidenced by behaviors such as hand flapping, hand biting, perseverative speech and running (Cohen, 1995).

Currently, no treatments exist that target the abnormal neural development that is the basis for FXS; rather, current treatments attempt to provide symptomatic relief, such as the use of methylphenidate for the ADHD symptoms and selective serotonin uptake inhibitors (SSRIs) for relief of anxiety and perseverative behaviors (Berry-Kravis & Potanos, 2004). Such treatments, while effective in relieving these specific symptoms in approximately 70% of patients, only address a subset of the areas that are impaired. In order to develop and test more effective treatments – ideally ones that might normalize brain development -- an animal model of FXS must be used. In 1994, a mouse model of FXS was developed in which the *fmr1* gene was inactivated by homologous recombination (Bakker et al, 1994). Since that time, numerous studies have been conducted to determine the degree of correspondence between these mice (termed *fmr1* "knockout" (KO) mice) and humans with FXS. These studies have yielded mixed results: Correspondence has been found for various anatomical features such as abnormal dendrites in the visual cortex and hippocampus (Comery et al, 1997; Mineur et al, 2002) and macroorchidism (Bakker et al, 1994). Some instances of hyperactivity have also been reported (Bakker et al, 1994; Mineur et al, 2002; Peier et al, 2000). In contrast, it has been more difficult to demonstrate correspondence between humans with FXS and *fmr1* KO mice in the cognitive and affective domains; differences between the two genotypes have been subtle at best. Research using the Morris water maze and the plus maze, common learning and memory paradigms, have either failed to find differences between the two genotypes (Dobkin et al, 2000; Paradee et al, 1999; Peier et al, 2000; Yan et al, 2004) or have found very slight deficits in the *fmr1* KO mice (Bakker et al, 1994; D'Hooge et al, 1996; Kooy et al,

1996; Van Dam et al, 2000). In addition, research with some learning tasks has reported that *fmr1* KO mice perform better than their WT controls, contradictory to the human phenotype (e.g. Van Dam et al, 2000). Background strain of the subject mice may be at least partly responsible for these contradictory results. Recent work has shown that background strain of the *fmr1* KO mice has a profound effect on whether or not genotypic differences are seen for behavioral endpoints (Dobkin et al, 2000; Paradee et al, 1999). However, a more likely reason for the difficulties in demonstrating behavioral and cognitive deficits in *fmr1* KO mice is that the majority of studies that have been conducted with *fmr1* KO mice were designed to assess learning and memory, areas that are not prominently affected in FXS. In contrast, very few studies have assessed the hallmark areas of dysfunction in FXS such as attention, inhibitory control and social behavior.

Perhaps surprisingly, in light of the profound social problems in humans with FXS, very little work has been done studying the social behavior of the *fmr1* KO mouse; only 2 papers have been published to date. Spencer, et al (2005) administered a battery of tests designed to assess whether *fmr1* KO and WT mice differ in social anxiety. They found that male KO mice were more likely to back down in a dominance tube task than male WT mice, specifically when the partner was an unfamiliar mouse. Genotypic differences were not found, however, if the partner was a cagemate. Spencer, et al also found that WT and KO male mice were equally interested in social interaction, but that if the KO mice were presented with a novel male mouse in a familiar environment, they had longer latencies to approach the novel mouse than did WT mice, perhaps indicative of social anxiety. More recently, Mineur, et al (2006) found that male KO mice had a lower frequency of social interactions with ovariectomized females overall than did WT mice. In addition, male KO mice, unlike

WT controls, failed to show an increase in frequency of social behaviors when presented with a novel female.

The current study was designed to further characterize social behavior in *fmr1* KO mice using a paradigm developed by Nadler, et al (2004). Specifically, a three-chambered apparatus was used to expose experimental mice to unfamiliar male conspecifics, enclosed in a small wire cage. The paradigm is comprised of two phases: the first designed to measure the preference for being near an unfamiliar mouse vs. no animal, and the second designed to measure the preference for a novel mouse vs. a familiar mouse. Detailed videocoding allowed us to characterize the quality as well as the quantity of social interactions. We hypothesized that *fmr1* KO mice would exhibit altered social interactions when compared to WT mice.

The present study studied male *fmr1* KO and WT littermates that were F1 hybrids of a C57Bl x FVB cross. This procedure eliminated recessive genetic defects that are common in inbred background strains, such as blindness.

MATERIALS AND METHODS

Subjects:

Breeding of the mice was conducted at the University of Colorado Health Sciences Center, Denver, CO. Females heterozygous for the *fmr1* mutation were obtained by breeding C57BL/6J-^{tm1Cgr} mutant female mice that had been 12+ generations backcrossed to C57BL/6J with inbred C57BL/6J normal males purchased from The Jackson Laboratories (JAX). These females were then bred with normal FVB/NJ males (Jackson Laboratory, Bar Harbor, ME) to produce male KO and WT hybrid (C57Bl x FVB) mice. Male offspring (20 WT and 20 *fmr1* KO) from these litters served as subjects in the present experiment. Mice were between the ages of 10 and 12 months at the time of testing and ranged in weight from 40 grams to 65 grams.

All mice were housed with one or two littermates in clear Plexiglas containers (30cm L x 20cm W x 13cm H) with corncob bedding, cardboard shelters and cotton nesting material. Free access to food (Purina Rat Chow) and water was provided throughout the study. Lights were turned on daily at 0100 and off at 1300 with all testing taking place between 1500 and 1700. Temperature and humidity were kept at a constant level throughout the study (21˚C ± 1˚; 40% ± 10%). All procedures used in these experiments adhere to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees Cornell University, an AAALAC accredited institution.

Stimulus mice:

Two wildtype mice were used as the stimulus mice. One of these mice (referred to as M1) was 11 months at the time of testing. The other mouse (M2) was 12 months at the time of testing. These mice were housed singly in the same room as the experimental subjects

Apparatus:

The apparatus used in this study was similar to the one used by Nadler et al (2004). In brief, it was a rectangular chamber made of transparent Plexiglas that was divided into three equal sized compartments (Figures 1 & 2). Each chamber was 20cm L x 40.5 cm W x 22 cm H. Circular openings (3.5 cm in diameter) in the dividing walls provided access to the two side compartments. The floor of the apparatus was covered with corncob chips that were changed between animals. The apparatus was illuminated with a 25 watt red light bulb. Sessions were videotaped using a VC- 22P black and white pinhole board camera suspended overhead (Spy Camera Specialists, Inc, Congers, NY).

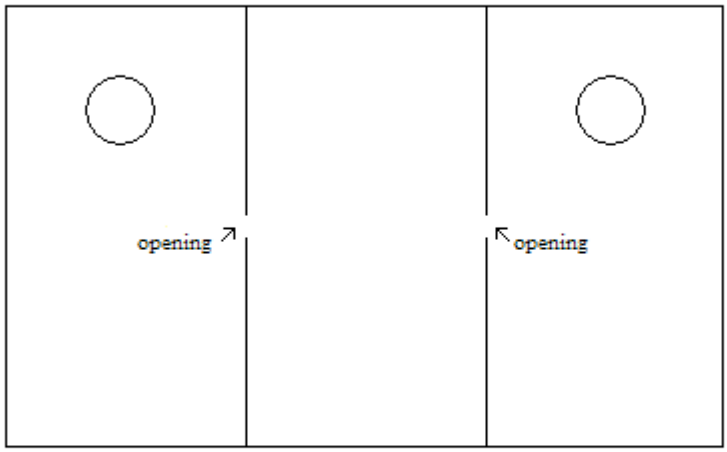

Figure 1. Diagram of the apparatus used. The circles represent the cages used to restrain the stimulus mice. The line breaks represent the openings between the chambers.

Figure 2. Photograph of the apparatus.

Each stimulus mouse was enclosed in a small, overturned, circular cage with bars spaced 1 cm apart (Galaxy Cup, Spectrum Diversified Designs, Inc, Streetsboro,

OH). The cage was 11 cm in height and 10 cm in diameter at the bottom. On top of this small restraining cage was a clear, glass beaker, which weighed the cage down and prevented the experimental subjects from climbing on top. The cages allowed nose and paw contact, but prevented fighting.

OdorMute (Hueter Toledo, Inc, Bellevue, OH), an enzymatic cleaner designed to eliminate organic odors, was used to clean the cages between trials to prevent odor cues from previous subjects influencing subsequent subject behavior.

Procedure:

Habituation. Stimulus mice were confined to the small wire cages for 30 minutes each day 7 days prior to the start of the study to habituate them to being restrained.

The 40 experimental subjects (20 WT, 20 KO) mice were allowed to freely explore the entire apparatus for 25 minutes on the day before testing commenced. The small cages were present during habituation, but did not enclose any stimulus mice. Following habituation, the experimental mice were placed in a clean home cage without their cagemates and were single housed for 24 hours before the day of testing. This avoided territory conflicts between cagemates that might arise if one mouse was removed for testing and then reintroduced to the home cage.

Testing. Testing was comprised of three phases that were conducted consecutively on the same day for a total of 25 minutes of testing.

Phase 1 – Re-habituation (5 minute duration): The experimental subject was placed in the center chamber and allowed free access to the entire apparatus for 5 minutes. The wire cages were present, but empty.

Phase 2 – Sociability (10 minute duration): An unfamiliar male mouse was introduced into one of the two small wire cages; location (left or right compartment) was counterbalanced across treatments. Half of the mice of each genotype received M1 as the stimulus mouse, and half received M2 as the stimulus mouse. The cage in the opposite chamber was present but empty. While the stimulus mouse was being placed under the wire cage, the experimental subject was confined to the center chamber. The experimental mouse was subsequently allowed to explore the entire apparatus for 10 minutes. The goal of this phase was to assess whether the experimental mouse preferred to be with an unfamiliar conspecific or an empty cage, as well as to observe the nature of the social interactions that took place.

Phase 3 – Preference for social novelty (10 minute duration): A second mouse was introduced into the cage on the opposite side of the apparatus. The stimulus mouse that had been present during phase 2 is referred to as the "familiar" mouse, whereas the mouse introduced in phase 3 is termed the "novel" mouse. For the experimental mice exposed to M1 in phase 2, M2 was the novel mouse and vice versa. While the second stimulus mouse was being placed under the restraining cage, the experimental mouse was confined to the center chamber. The experimental mouse was subsequently allowed to freely explore for 10 minutes. The goal of this phase was to assess whether the experimental mouse preferred to be with a novel conspecific, or a familiar conspecific.

Behavior coding:

All trials were videotaped for later coding. For coding, the apparatus was split into 18 identical squares. The 4 squares surrounding the cage on each side were termed "Social" areas and all other squares were termed "Non-social" areas. The coded behaviors included nose contact with cage, rearing on cage, grooming and

climbing (defined in Table 1). These behaviors were coded for both duration and location. In addition, square crossings were recorded to quantify activity level. All videocoding was done via keyboard and mouse using coding software on a PC. Two people trained to videocode were rated for intra-rater reliability (how similar the same person coded one session twice) and inter-rater reliability (how similar the two people coded the same session). Reliability was measured using Spearman correlation analysis; data were not considered accurate until both inter-rater and intra-rater reliabilities were measured as equal to or greater than 90% for each behavior in each phase.

Table 1. The behaviors that were coded from the videotaped sessions.

Behavior	Operational Definition
Square Crossing	All four of the subject's paws cross into a new square
Nose contact with cage	The subject's nose in contact with the wire cage
Rearing on cage	The subject rising onto hind legs with front paws in contact with the glass beaker atop the wire cage
Grooming	The subject's paws or mouth in contact with any part of his own body
Climbing	The subject rising onto hind legs in any context other than in contact with the wire cage

Statistics:

All statistical analyses were conducted using SAS v9.1 (SAS Institute, Cary, NC) for Windows 2000 Professional. Group means for nose contact with cage, square crossings, rearing on cage, grooming and climbing were compared using a two-tailed Student's t-test. When more than two independent group means were compared, a

general linear model was used with pairwise comparisons of the least square means to determine the statistical significance of group differences. For example, this type of analysis was conducted for the various measures of social interest in phase 2, where there were two subgroups for each of the two genotypes, corresponding to the two different stimulus mice. When assessing paired (within-subject) data, such as the difference in time spent with the novel and familiar stimulus mice in phase 3, a paired t-test was performed on the differences of interest. In cases where the assumption of normality was violated, a Wilcoxon Rank Sum test was used in place of the two-sample t-test and the Wilcoxon Signed Rank test was used in place of the paired t-test.

In phase 3, a relative novelty preference score for each of the dependent measures was calculated as $\frac{novel - familiar}{novel + familiar}$, which took into account the individual behavior of each experimental mouse. This measure also had the benefit of normalizing the data, which allowed parametric analyses to be conducted.

RESULTS

One WT animal, clearly an outlier, was excluded from all analyses because he remained in one corner throughout phase 2 of the test.

Phase 1- Habituation:

During the habituation phase, there were no differences between genotypes for any of the measures.

Phase 2- Social interaction test:

Nose Contact with Cage. All animals had more nose contact with the cage restraining the stimulus mouse (termed "social nose contact") than nose contact with the empty cage (termed "non-social nose contact"), regardless of side (left or right) or

stimulus mouse (M1 or M2, see Figure 3). The difference between the average social nose contact and average non-social nose contact was significantly greater than zero (t_{38} = 15.08, p<0.0001) reflecting a preference for the stimulus mouse (vs. the empty cage). The two genotypes did not differ for this measure (Wilcoxon Rank Sum Test, p = 0.9). The specific stimulus mouse affected the amount of social nose contact: Total social nose contact time was significantly greater for those mice having M1 as the stimulus mouse than for those mice having M2 as the stimulus mouse ($F_{1,35}$ = 6.02, p = 0.019, see Figure 4). There was no significant difference between genotypes ($F_{1,35}$= 0.42, ns), nor was there an interaction between genotype and stimulus mouse ($F_{1,35}$ = 0.00, ns).

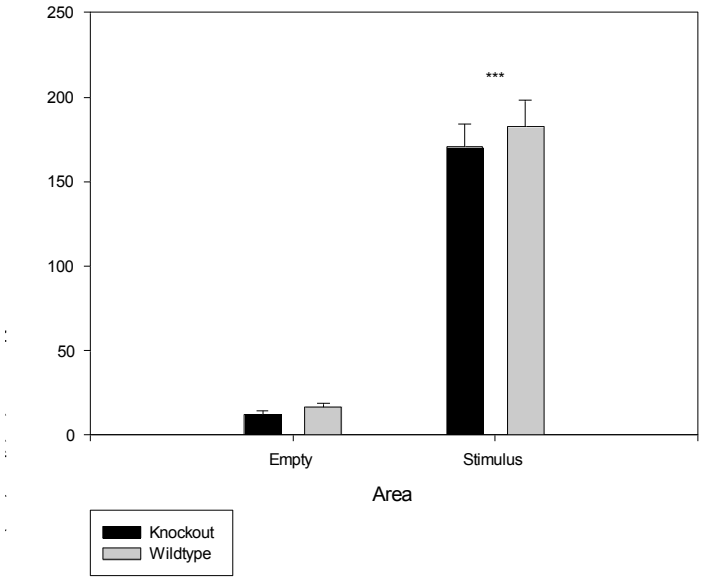

Figure 3. The effect of the presence of a stimulus mouse on nose contact time with the restraining cages. *** denotes a significance level of p< 0.0001 between areas.

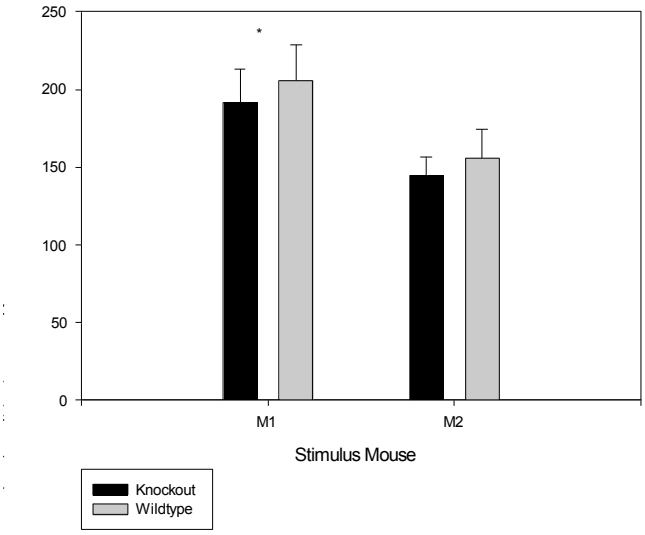

Figure 4. The effect of stimulus mouse on nose contact time. * denotes a significance level of $p < 0.05$ between groups.

Time in each area. For this analysis, the apparatus was split into 3 discrete areas: 1) the 4 squares immediately adjacent to the cage enclosing the stimulus mouse, 2) the corresponding 4 squares around the empty cage, and 3) the 10 squares that comprised the rest of the apparatus (2 squares in each of the sides and the 6 squares in the center section of the apparatus), termed the "non-cage" area. All animals spent the greatest amount of time in the area around the cage enclosing the stimulus animal, and the least amount of time in the area around the empty cage (Figure 5). The time spent in the non-cage area was intermediate. A paired t-test found that more time was spent with the stimulus cage than with the empty cage ($t_{38} = 11.80$, $p<0.0001$) or the non-cage area ($t_{38} = 7.04$, $p<0.0001$) reflecting a preference for being with the stimulus animal. Furthermore, more time was spent in the non-cage area than with the empty cage ($t_{38} = -4.36$, $p<0.0001$). There was no difference between genotypes in any of the three comparisons (Wilcoxon Rank Sum Test, $p = 0.38$, $p=0.92$, and $p = 0.25$, respectively). Contrary to time spent in nose contact with the stimulus cage, the time

spent in the area of the stimulus cage did not vary as a function of stimulus mouse (M1 vs M2) ($F_{1,35} = 1.57$, p=0.22, see Figure 6), genotype ($F_{1,35} = 0.09$, p = 0.76), nor the interaction of these two factors ($F_{1,35} = 0.51$, p = 0.48).

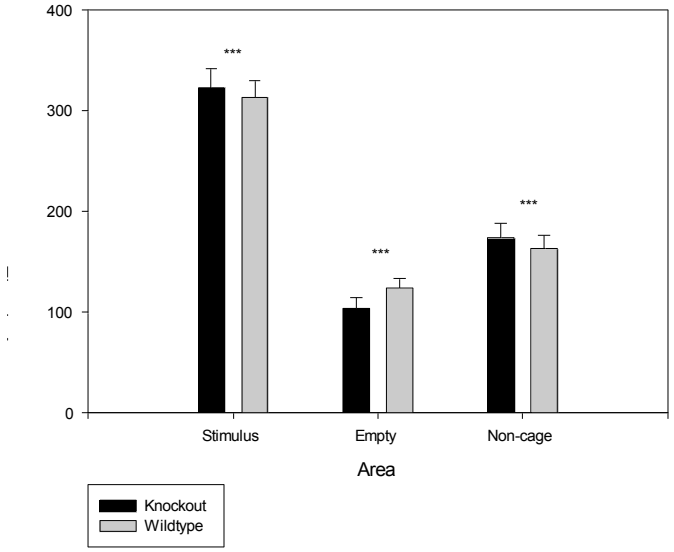

Figure 5. The mean time subjects spent in each area. *** denotes a significance of p<0.0001 between areas.

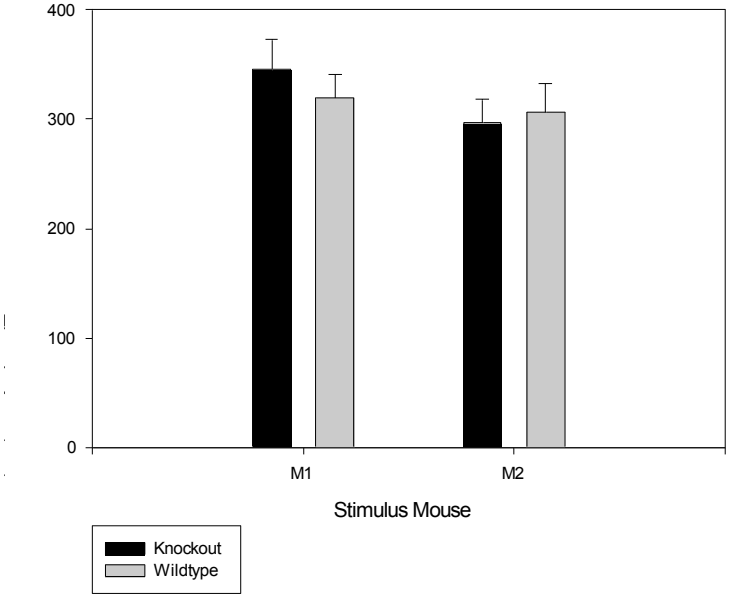

Figure 6. The effect of stimulus mouse on the time spent in the area of the model mouse as a function of genotype.

Grooming. There was a difference in the distribution of the grooming scores for the two genotypes. Seventy-five percent of the KO animals had grooming times that were greater than the population median groom time (12.5 seconds), whereas only 30% of WT animals had a total groom time that was greater than this value (Fisher's Exact Test, p=0.025, Figure 7).

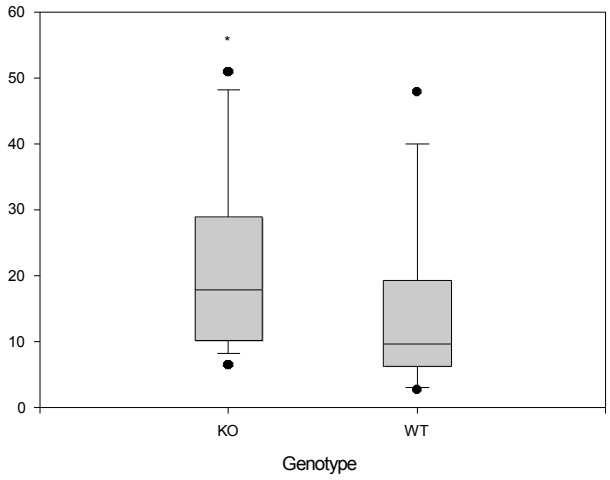

Figure 7. The distribution of mean groom times as a function of genotype. * denotes a significance level of $p<0.05$.

Average Duration of Nose Contact. The average duration of each bout of social nose contact was calculated as $\frac{Total\ duration\ of\ nose\ contact}{Total\ number\ of\ bouts\ of\ nose\ contact}$. A general linear model found a significant effect of genotype ($F_{1, 36} = 4.14$, $p = 0.04$, $d = 0.53$) and a significant effect of stimulus mouse ($F_{1,36} = 13.5$, $p = 0.0008$), but no interaction between these factors ($F_{1,35} = 2.09$, $p=0.16$) . The contrasts revealed that KO animals had significantly shorter average duration of nose contact bouts than did WT animals. In addition, animals that were exposed to M1 had significantly longer average bouts of nose contact than animals exposed to M2 (Figure 8).

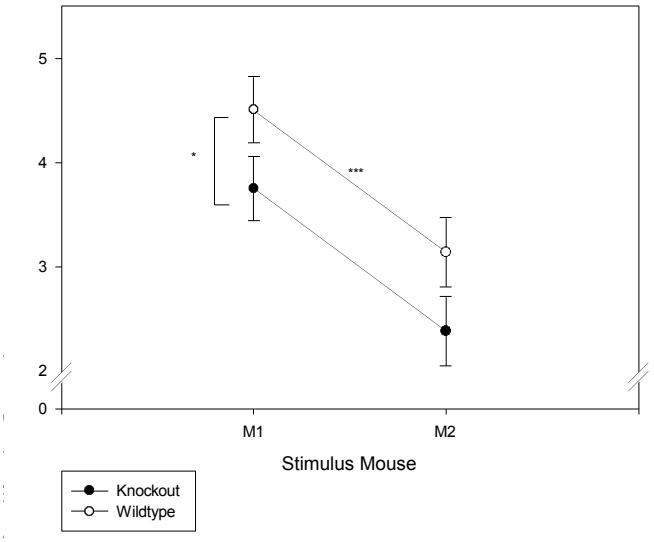

Figure 8. The effect of stimulus mouse on average duration of nose contact. * denotes a significance level of $p<0.05$ and *** denotes a significance level of $p<0.0001$.

Phase 3 - Preference for Social Novelty:

Nose Contact with Cage. Subjects that had the preferred stimulus mouse (M1) as the novel animal in phase 3 had a greater amount of novel nose contact than familiar nose contact (Figure 9); the difference between the two means was significantly different from zero with a paired t-test ($t_{17} = 3.96$, $p = 0.001$). For this subgroup of subjects, there was no significant difference between genotypes (Wilcoxon Rank Sum test, $p = 0.73$). In contrast, this novelty preference was not seen for animals that had the not-preferred mouse (M2) as the novel animal: the novel nose contact time minus the familiar nose contact time was not significantly different from zero ($t_{20} = 1.18$, $p = 0.25$). Again, there was no significant difference between genotypes (Wilcoxon Rank Sum test, $p = 0.35$).

An index of social novelty preference that took into account individual differences in total nose contact (difference between familiar and novel nose contact,

divided by total nose contact), called a relative novelty preference score, revealed the same pattern of results (data not shown).

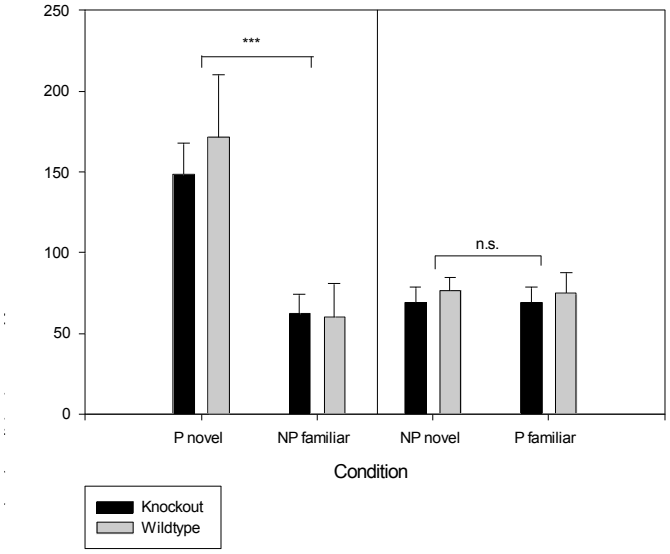

Figure 9. The effect of stimulus mouse on nose contact time as a function of genotype. P refers to the preferred stimulus mouse (M1) and NP refers to the non-preferred stimulus mouse (M2). *** denotes a significance level of $p<0.001$.

Time in Area. A similar pattern was seen for time in area, in terms of novelty preference. Those subjects that had the preferred stimulus mouse (M1) as the novel animal spent a significantly greater amount of time with the novel animal than with the familiar animal (Figure 10). A paired t-test found the difference between the means to be significantly different than zero ($t_{17} = 4.21$, $p = 0.0006$) but no differences were found between genotypes (Wilcoxon Rank Sum test, $p = 0.79$) The subjects that had the non-preferred stimulus mouse (M2) as the novel animal, however, did not spend a greater amount of time with the novel than with the familiar animal ($t_{20}= -0.758$, $p = 0.46$). There was no significant difference between genotypes (Wilcoxon Rank Sum test, $p = 0.70$).

As seen for nose contact time, analysis of the relative novelty preference score for time in area (i.e., a difference score that took into account individual differences in total time spent with both stimulus mice) showed the same pattern of results (data not shown).

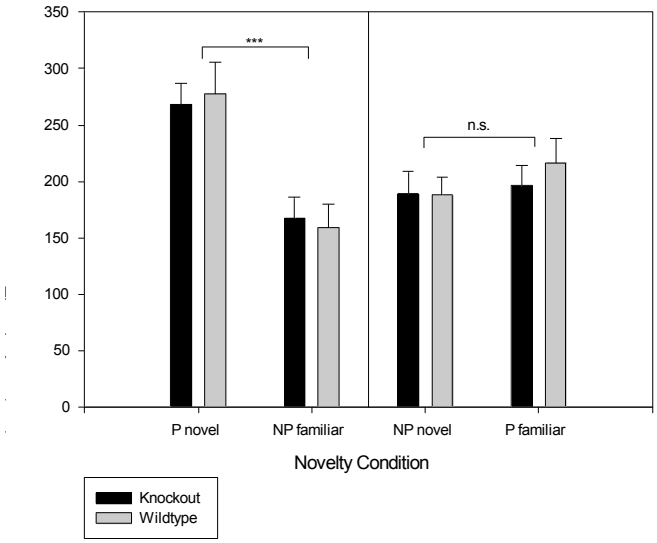

Figure 10. The effect of stimulus mouse on the amount of time spent in the area of the cages. P refers to the preferred stimulus mouse (M1) and NP refers to the non-preferred stimulus mouse (M2). *** indicates a significance level of $p<0.001$.

Average Duration of Nose Contact. Whether or not a novelty preference was seen for this measure varied as a function of the specific stimulus mouse that was novel. Subjects that had the preferred stimulus mouse (M1) as the novel stimulus had significantly longer average duration of novel nose contact times than familiar nose contact times ($t_{17} = 3.62$, $p = 0.0021$). In contrast, subjects that were exposed to the non-preferred stimulus mouse (M2) as the novel stimulus had similar average duration of nose contact times for both the novel and familiar mice ($t_{21}= -0.139$, $p=0.89$, see

Figure 11). A borderline effect of genotype was seen for this latter subgroup of mice (Wilcoxon Rank Sum test, $p = 0.07$); the pattern is described below.

The relative novelty preference score for average duration of nose contact revealed a similar pattern of results, but because this measure normalized the data, a parametric analysis could be conducted. A general linear model found a main effect of specific stimulus mouse ($F_{1,35} = 35.99$, $p<0.0001$) as well as a borderline interaction between genotype and stimulus mouse ($F_{1,35} = 2.92$, $p = 0.09$). There was no main effect of genotype ($F_{1,35} = 0.83$, $p=0.37$). Contrasts revealed that whereas the two genotypes did not differ when the preferred mouse was novel, both exhibiting a strong novelty preference, a borderline genotypic effect was seen for those subgroups for which the not-preferred stimulus mouse (M2) was novel ($p=0.06$). As can be seen in Figure 12, when the non-preferred stimulus mouse was novel, the WT animals actually showed a slight preference for the familiar mouse, whereas a slight novelty preference was still seen for the KO mice. This pattern suggests that the negative reaction to the non-preferred stimulus mouse, seen in both genotypes, was more pronounced for the WT mice than for the KO mice.

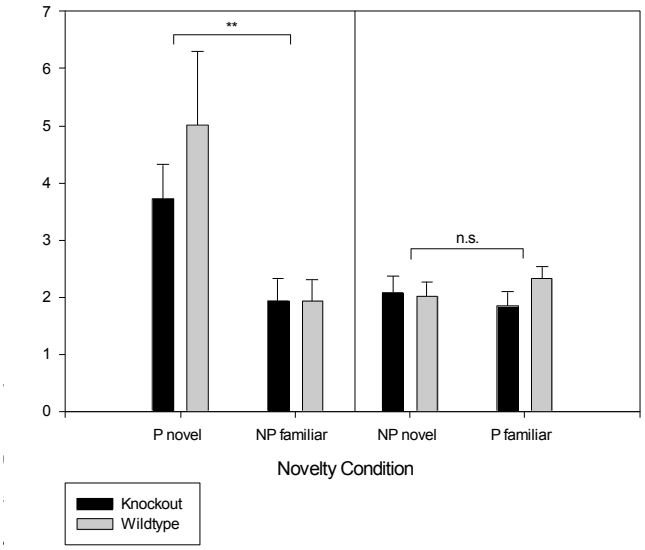

Figure 11. The effect of stimulus mouse on average duration of nose contact. P refers to the preferred stimulus mouse (M1) and NP refers to the non-preferred stimulus mouse (M2). ** indicates a significance level of $p<0.005$.

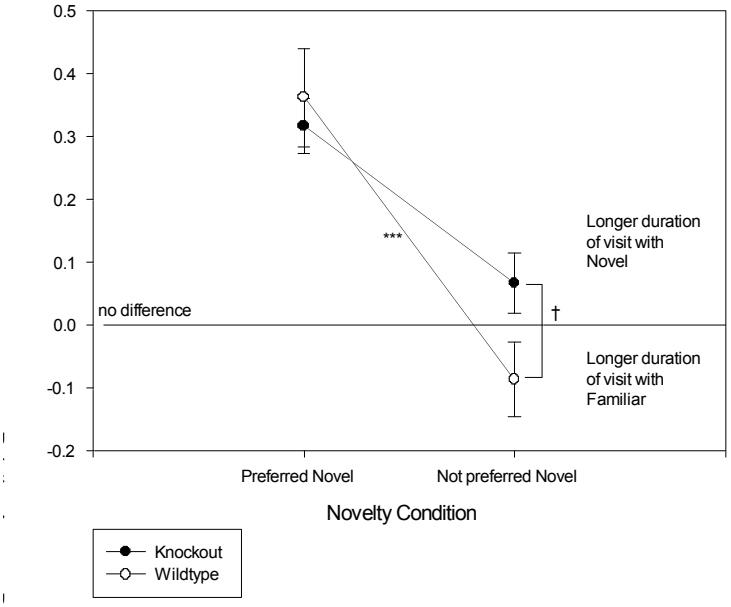

Figure 12. The effect of stimulus mouse on the novelty effect as measured by the relative average duration of nose contact as a function of genotype. *** denotes a significance level of $p<0.0001$. † denotes a significance level of $p=0.06$.

DISCUSSION

Social anxiety and abnormal social interactions are hallmark deficits of Fragile X Syndrome; however, few studies have been performed on the social behavior of *fmr1* KO mice. The current study was designed to further characterize the social interactions of *fmr1* KO mice in comparison to their WT littermates.

Some characteristics of the social interactions of the mice varied by genotype. During phase 2 (preference for conspecific vs. empty cage), the average duration of nose contact with the stimulus mouse was shorter for the *fmr1* KO mice than for the WT mice. One explanation could be hyperactivity; i.e. that the *fmr1* KO mice could not stay in nose contact with the stimulus mouse for long periods of time because of an impulse to keep moving. However, because the present study did not find a difference in square crossings in any phase, this explanation is unlikely. Another possible explanation is that *fmr1* KO mice were aroused to a greater extent by the interaction than were the WT subjects and could not stay in constant contact with the stimulus mouse. This explanation is suggested by behavior of humans with FXS. Individuals with FXS are hypersensitive to sensory stimuli in all modalities. Cohen (1995) hypothesized that hyperarousal and hypersensitivity to sensory stimuli may be the basis of abnormal social behavior in humans with FXS. If this deficit was reproduced in the *fmr1* KO mouse, it is reasonable to believe that while they may not avoid social interaction, they may be more aroused by it. In essence, the shorter average duration of nose contact time could reflect this hypersensitivity to sensory stimuli.

The possibility of hyperarousal when exposed to the stimulus mouse is further supported by the finding that a greater proportion of the *fmr1* KO mice exhibited high levels of grooming than the WT mice. Prior findings from this lab have revealed that (1) grooming levels increased in both *fmr1* KO and WT mice immediately following

an error in discrimination tasks or when contingencies changed and error rates were correspondingly increased, demonstrating that grooming rate can serve as an index of increased arousal or negative affect; and (2) that under these conditions, the increase in grooming rate was more pronounced for *fmr1* KO mice than for controls, indicating impaired regulation of arousal or negative affect in these mice (Moon et al, 2004; Moon et al, 2005). In light of these prior findings, the present finding that a greater proportion of *fmr1* KO mice exhibited high levels of grooming when first presented with a strange mouse but not during phase 1 (re-habituation) indicates that this behavioral difference may be due to the social interaction specifically. The increased arousal seen in the *fmr1* KO mice following the introduction of an unfamiliar mouse may correspond to social anxiety seen in FXS humans when meeting a stranger. This finding did not extend to phase 3 (preference for a novel stimulus vs. a familiar stimulus); one possible explanation for this is that the introduction of a second unfamiliar mouse may be less arousing than the introduction of the first unfamiliar stimulus mouse.

During phase 3, genotypic differences emerged in relation to the specific characteristics of the two stimulus mice. Whereas both genotypes exhibited a similar preference for a novel conspecific over a familiar one when the novel animal was the preferred mouse, genotypic differences emerged when the novel mouse was the non-preferred stimulus mouse. In this case, the novelty preference was decreased in both groups but to a significantly greater extent in the WT mice. This suggests that WT mice had a greater negative reaction than KO mice to the non-preferred stimulus mouse. A possible interpretation is that KO mice were less able than the controls to distinguish between positive and negative social interactions. Fisch, et al (1999) found that adult humans with FXS are impaired in interpersonal relationships skills.

Notwithstanding these differences in social behavior, *fmr1* KO mice exhibited normal behavior in many respects when compared to WT littermates. In phase 2, KO mice, similar to WT mice, spent the greatest amount of time in the social area and had a greater amount of social nose contact than empty nose contact. Both of these measures indicate that the subjects preferred to be near a novel conspecific rather than alone. In phase 3, the *fmr1* KO and WT mice exhibited a similar preference for a novel conspecific relative to a familiar conspecific when the novel animal was the preferred stimulus mouse. Hagerman (2002) reports that males with FXS do not avoid social interactions, but that the quality of the interactions are abnormal. This seems to be paralleled in the *fmr1* KO mice; the data in this study support that *fmr1* KO mice seek out social interactions and show a normal novelty preference, but that the characteristics of the social interactions tend to be abnormal when compared to WT littermates.

The current study did not find a significant difference in activity level between the *fmr1* KO mice and the WT mice in any of the three phases. The majority of the studies on this topic have reported *fmr1* KO mice to be hyperactive in an open field (Bakker et al, 1994; Mineur et al, 2002; Peier et al, 2000). However, Yan et al (2004) and Nielsen et al (2002) reported no difference between the two genotypes in activity levels in either a cross shaped maze or an open field. One possible explanation for these discrepancies is that both Yan et al and Nielsen et al used F1 hybrids of a C57Bl x FVB cross, as did the present study, whereas the other three groups used the C57Bl/6J background strain. However, prior findings from this lab have detected hyperactivity in the same F1 hybrid KO mice following a change in task contingencies, suggesting that the level of arousal or negative affect created by a situation may affect the degree to which these mice exhibit hyperactivity.

Similarities and differences were found when comparing the current study to the two previous studies on social behavior in *fmr1* KO mice. Like Spencer et al (2005), KO mice in the present study had an interest in unfamiliar male mice. Spencer and colleagues found that *fmr1* KO mice exhibited abnormal social behavior only in one specific situation: when the subjects were exposed to a novel male mouse in a familiar environment. In this situation, these investigators found that the fmr1 KO mice had longer latency to approach the stimulus mouse and social investigation increased over time. In the present study, the experimental subjects were also exposed to novel male conspecifics in a familiar environment (assuming that the habituation regimens used here created "familiarity" of the environment), but the pattern of results reported by Spencer et al were not seen. There were no differences in latency time to approach the stimulus mouse, nor was there a change in investigation over time. These differences in results could be because the apparatus that we used was much larger than the cages that Spencer et al used and experimental mice needed to travel a greater distance to approach the stimulus mouse. Also, experimental mice in this study needed to go through a small hole in order to be in the same chamber as the stimulus mouse; the animals in Spencer et al's study were already in the same area as the stimulus mouse. A further difference between the current methodology and Spencer et al's that may explain the discrepant results is the strain of mouse used. Spencer et al used backcrossed C57Bl/6J mice as the background of their *fmr1* KO mice while the current study studied an F1 hybrid mouse of a C57Bl x FVB cross. As noted in the Introduction, background strain has been found to be an important factor in prior studies of the fmr1 KO mouse.

Mineur et al (2006) found that *fmr1* KO mice exhibited less interest in social interactions than did their WT counterparts, and did not exhibit the usual preference for a novel conspecific over a familiar one. Not only did the current study find that

fmr1 KO mice showed normal interest in an unfamiliar male mice, as did Spencer et al (2005), but it also found that *fmr1* KO mice had greater social exploration (nose contact) of a novel mouse over a familiar mouse. These discrepancies may be explained by differences in methodology. Firstly, the stimulus mice used by Mineur and colleagues were ovariectomized females from a different background strain than the experimental mice, whereas the present study and that conducted by Spencer et al (2005) used WT males of the same background strain as the experimental mice. It is possible that *fmr1* KO mice react differently to ovariectomized females than to males. Furthermore, Mineur et al allowed the stimulus and experimental mice to freely interact during the test session, whereas stimulus mice in the present study were restrained by wire cages and only the experimental mice could initiate social contact. It may be that having the stimulus mouse restrained allowed the experimental mice more opportunity to initiate social contact without the interference of the stimulus mouse's reaction. Mineur et al also coded for behaviors that require two animals interacting (e.g. mutual grooming, climbing over/under each other), behaviors that were not possible in the current paradigm given the restraining cage. It is possible that *fmr1* KO mice are abnormal in these specific behaviors while not showing a deficit in social interest of a restrained mouse. Finally, Mineur et al, like Spencer et al (2005), used a pure C57Bl/6J background strain for the experimental subjects, which differed from the F1 hybrids used in this study. The difference in strain could account for or contribute to the discrepant results.

Coded behaviors in the present study provided more detailed information about the social interactions between the stimulus and experimental mice than an automated version of this apparatus would have provided. The paradigm used in the present study was developed by Nadler et al (2004) and used by Moy et al (2004). Nadler et al measured both the time spent sniffing the restraining cages and the time spent in each

of the three compartments and found that the two measures were significantly correlated. The authors concluded that the time spent in each of the three compartments was an accurate indicator of social preference; for that reason, Moy et al measured only time in the three compartments using an automated version of the apparatus. The present study, however, found social nose contact to be a more sensitive measure of social interaction; in phase 3, nose contact time revealed genotypic differences in reaction to the two stimulus mice that were not reflected in the time spent in the three compartments.

Prior studies that have used this paradigm have either not evaluated (Moy et al, 2004) or have not found (Nadler et al, 2004) an effect of the specific mice used as stimulus animals. However, despite the restraining cages, the stimulus mice in the current study had a clearly quantifiable effect on the behavior of the experimental mice. Present findings demonstrate that the specific stimulus mice are an important variable and may be useful in characterizing the phenotypes, as seen here.

Summary and Conclusions

The current study was designed to characterize social behavior in *fmr1* KO mice in order to more completely assess the validity of this mouse model of fragile X syndrome. These mice showed a normal preference for the presence of an unfamiliar conspecific over being alone and showed a preference for a novel mouse over a familiar one. These preferences are paralleled in human males with FXS in that they do not avoid social interaction. However, FX humans show abnormal social interactions. This study found subtle differences between the two genotypes in social interactions. Most notably, the average duration of social nose contact was shorter for the KO animals than for the WT animals, suggesting shorter, more frequent visits to the stimulus mouse. Humans with FXS exhibit hypersensitivity and hyperarousal to

sensory stimuli of all modalities; social interactions have been shown to trigger stereotypic behaviors indicative of anxiety and arousal (Cohen, 1995). The interaction with another male mouse may have caused an increase in arousal in the *fmr1* KO mice that resulted in shorter visits with the stimulus mouse. A greater proportion of *fmr1* KO mice were above the median groom time in phase 2, further supporting the idea that the KO mice were more aroused by the introduction of an unfamiliar male mouse than were the WT mice. Finally, the KO mice reacted to a lesser extent than the WT mice to the non-preferred stimulus mouse in phase 3; this may reflect an impairment in appropriately characterizing the quality of a social interaction. In sum, these findings suggest that fmr1 KO mice may be more aroused and/or anxious in social situations than their WT littermates, and may be impaired in characterizing the quality of a social interaction. These findings exhibit correspondence with the behavior of FXS humans and thereby support the validity of this mouse model of fragile X syndrome.

APPENDIX

A list of the statistical analyses that were conducted in both phase 2 and phase 3 and were not discussed in Chapter 2 because of a lack of significant results.

- Latency to leave center chamber
- Latency to approach stimulus animal(s)
- Initial side choice
- Initial side choice as a function of phase 2 stimulus mouse
- Latency to approach stimulus animal(s) as a function of initial side choice
- Latency to approach stimulus animals across phases
- Time spent in the "small areas" of the compartments containing the stimulus animal(s)
- The proportion of time spent next to the stimulus animal(s) and time spent everywhere else in the apparatus as a function of experimental animal
- The distribution of time spent in various behaviors by genotype
- Total time wall climbing and rearing combined
- The correlative relationship between time in nose contact and frequency of nose contact
- Nose contact over time as a function of genotype and stimulus mouse
- Activity over time as a function of genotype and stimulus mouse
- Grooming over time as function of genotype and stimulus mouse

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CHAPTER THREE

CONCLUSIONS

The present study found subtle differences in social behavior between *fmr1* KO and WT mice. The pattern of effects suggests that KO mice may be aroused to a greater extent by the introduction of an unfamiliar male mouse, and that KO mice have a more difficult time distinguishing between a positive and a negative social interaction.

Paradigmatic strengths

This study was the first to measure social behavior of male *fmr1* KO mice in a paradigm in which the initiation of social contact was completely reliant upon the experimental mouse. A social interaction necessarily involves at least two individuals, but the initiation of a social interaction is reliant upon only one individual. The wire cages restraining the stimulus mice ensured that the initiator was the experimental animal rather than the stimulus animal. This limited, but did not eliminate, the influence the stimulus animal had on the experimental animal.

Furthermore, the wire cages limited the types of social behaviors in which the two animals could engage. Direct social interactions that require the participation of two animals, such as crawling over each other (as used in Mineur et al, 2006), were not possible. These behaviors, however, depend as much on the behavior of the stimulus animal as on the behavior of the experimental animal, leading to results that may or may not be attributable to the experimental animals The behaviors that were coded in the present study relied solely on the experimental animal, not on the stimulus animals. For example, nose contact with the cage could occur whether or not the stimulus animal was facing the experimental animal. Coding behaviors that

depended only on the experimental animal also limited, but did not eliminate, the influence the stimulus animal had on the experimental animal.

The wire cages were used to prevent aggressive contact, but to allow nose and paw contact between the animals. These cages were designed to provide opportunities for social interaction without endangering either mouse. The cages differed from a screened partition (as used in Spencer et al, 2005) in that the experimental mice in this paradigm could investigate the stimulus mouse from all directions and social interaction did not depend upon the presence of the stimulus mouse at the partition. The wire cage was large enough so that the stimulus mouse could turn around, but too small for the stimulus mouse to retreat from the experimental mouse. Therefore, all approaches and nose contact directed toward the cages could reliably be considered 'social interaction' with another mouse.

Another strength of this paradigm was the design of the apparatus. Because the apparatus was three-chambered, the center chamber was always free of stimulus mice. This allowed the experimental mice to avoid the stimulus mice if they preferred. Furthermore, since access to the two side chambers was restricted to two small holes, the experimental mice could not wander into the side chambers randomly. This further strengthens the assumption that when an experimental mouse spent the majority of his time in the area directly around the stimulus cage, he has exhibited a preference for the presence of the other mouse.

Paradigmatic weaknesses

The sensitivity of behavioral coding was not optimal in this paradigm. In order to view the entire apparatus at once, a camera was suspended overhead. This inevitably reduced the size and resolution of the experimental mice and the stimulus cages. Whereas gross behaviors such as nose contact and rearing were possible to

code, more detailed behaviors were not. In particular, the coder noticed a frequently occurring behavior that appeared to be paw contact between the two mice in a "grappling" fashion. However, because of poor resolution and the overhead angle, this behavior could not reliably be coded. Behaviors like nose to nose contact could not be coded for the same reasons. Cameras at floor level that are directed at the stimulus cages would make it possible to code precise behaviors involved in the social interactions. This would provide a wealth of information on the characteristics and the quality of the social interactions. In addition, given the difference in the behavior of the experimental mice toward the two stimulus mice, coding the behavior of the stimulus mice underneath the wire cage would give more information about the types of interactions that are negative for the experimental mice.

Despite this weakness, the advantages of this paradigm make it well suited for examining social behaviors in *fmr1* KO mice. If the social anxiety and abnormal social interactions that are evident in FX humans are also characteristic of *fmr1* KO mice, then an ideal paradigm would focus on social behaviors that are under the control of the experimental mice. Measuring behaviors in a direct social interaction that are necessarily a response to the stimulus mouse may mask or distort the nature of the social dysfunction. Furthermore, a partition separating the stimulus and the experimental mice creates a situation in which social interactions are dependent upon the presence of both animals. It would be difficult to interpret results if the stimulus mouse does not approach the partition, though this is rarely coded. The wire restraining cages permit the experimental animal to control all social interactions, allowing a detailed analysis of the social behavior of the experimental animal as independent from the stimulus animal as possible.

Methodological contributions

This study contributed in an unexpected way to methodological research. Despite the restraining cages, the specific stimulus mice had a profound impact on the behavior of the experimental mouse. Previous studies using stimulus mice either did not address this issue (e.g. Spencer et al, 2005, Mineur et al, 2006) or did not find an effect of the specific stimulus mice that were used (e.g. Nadler et al, 2004). The stimulus mice in the present study were randomly chosen, but were the same genotype, gender and age as the experimental mice. Within the first week, however, the experimenter noticed a difference in temperament in the two mice. M2 acted in an aggressive manner toward the experimenter, ran away from her when she tried to pick him up, and scuffled in a frantic manner when she restrained him with the wire cage. M1, on the other hand, did not run from the experimenter when she picked him up and was calm while being restrained. In addition, the behavior of the experimental mice was quantifiably different in the presence of the two different stimulus mice, as outlined in chapter 2. The difference between the specific stimulus mice, while unexpected, was a benefit. Differences emerged between the two genotypes in phase 3 in response to the two stimulus mice; WT mice had a greater negative reaction to the non-preferred stimulus mice than did KO mice. The differences found between genotypes in relation to the specific stimulus mice may be more generalizable to the real world than differences found in the two other studies on social behavior in *fmr1* KO mice (Mineur et al, 2006; Spencer et al, 2005). No two people are identical; FX humans are bombarded with different people every day. The reactions to different types of people may give more information about the nature of their social anxiety than responses to people that are very much the same. Varying the stimulus mice used in social behavior paradigms may add a level of generalizability to the research. It is

important, however, to counterbalance between stimulus mice to avoid confounding factors.

Further phenotyping of the fmr1 KO mice

In the effort to find behavioral and cognitive correspondence between the *fmr1* KO mouse and FX humans, creative paradigms need to be used to assess the hallmark features of FXS. While mental retardation is the presenting feature of FXS, IQ scores can be affected by a number of cognitive deficits. In FXS, the most prominent features are attention deficits, impulsivity, hyperactivity, and social anxiety. The difficulty to date in demonstrating cognitive and behavioral correspondence between *fmr1* KO mice and FX humans is not surprising given that the majority of studies conducted with the *fmr1* KO mice have used classic learning and memory paradigms, such as the Morris water maze and the radial arm maze. Instead, more research should be focused on characterizing attention deficits, impulsivity and social anxiety. The current study has addressed the need for research on social behavior. Several paradigms are in existence currently that could be used to assess the other features discussed. The 5-choice serial reaction time task was developed in 1977 to assess sustained attention in rats (Robbins, 2002). The task has subsequently been adapted for mice and is extremely versatile (Humby et al, 1999). It has been modified to assess not only sustained attention, but also selective attention, inhibitory control, emotional reactivity and cognitive flexibility. In the basic task, a mouse needs to monitor 5 response ports for a light cue. If the mouse nosepokes into the port in which a light was presented, it is rewarded with food. The accuracy of the responses measures sustained attention (e.g. how well the mouse paid attention to the five ports while waiting for the light cue). To measure inhibitory control, the delay before the light cue is presented is varied; if the mouse nosepokes in any port before the light appears, it does not receive

a food reward. Selective attention can be measured by adding distractors, such as white noise or odors, to the chamber while the mouse is waiting for the light cue. Emotional reactivity can be measured in a number of ways. The first is to examine performance on a trial following a trial in which the mouse made an error. If the animal is more likely to make another error after an error on the previous trial, it is an indication of negative affect or arousal. Another method to assess emotional reactivity is to use surprising reward omission (SRO). On infrequent, random trials throughout the session, the food reward is omitted, regardless of the accuracy of the response. Omitting the food reward when the mouse expects to receive one could cause a decrease in performance if the animal is affected negatively. How well the mice recover after a reward omission is a measure of affective control. Finally, cognitive flexibility is measured using extra dimensional shifts. In this paradigm, the visual cue is not always predictive of a food reward; the spatial location of the port or a particular scent could be used to indicate the correct port. The predictive cue is shifted in a random order across days, forcing the mouse to adapt to which cue is predictive for a given day. Animals that have poor cognitive flexibility will perseverate with a cue that had been predictive the day before even if it is making errors.

The 5-choice serial reaction time task is an attractive paradigm for use with *fmr1* KO mice. It is extremely versatile and can be adapted to suit particular research hypotheses. In particular, the paradigms that I have briefly described tap the hallmark deficits seen in FX humans. Recent work in our lab using this paradigm (Moon et al, 2004, 2005) has been extremely encouraging. Specifically, we have found that *fmr1* KO mice have a greater number of premature responses in the sustained attention task, indicative of a lack of inhibitory control. In addition, *fmr1* KO mice have a greater drop in performance than WT mice when task contingencies are changed, suggestive of cognitive inflexibility, or disruption as a result of change. Finally, as described in

chapter 2, we have found that KO mice exhibit a greater increase in grooming and activity level following a change in contingencies in an olfactory reversal learning task, indicative of impaired regulation of arousal or affect. These findings, while subtle in magnitude, provide further evidence of correspondence between *fmr1* KO mice and FX humans.

Pharmacological interventions and future directions

Strong correspondence needs to be demonstrated between *fmr1* KO mice and FX humans before pharmacological interventions or treatments designed to normalize brain function can be tested. Current treatments, described in chapter 1, target the symptoms of FXS rather than the underlying neural deficit. Without a robust deficit as a baseline, treatment with drugs would be meaningless. This underlies the importance of pursuing the appropriate paradigm to demonstrate the correspondence that is hypothesized to exist.

The most attractive candidate for treatment is MPEP (2-methyl-6-phenylethynyl-pyridine). One current hypothesis is that in FXS, the metabotropic glutamate (mGlu) receptors are overactive due to the lack of FMRP (Bear et al, 2004), thereby causing insufficient inhibition of mRNA and an overproduction of proteins involved in long term depression (LTD). MPEP acts as an mGlu receptor antagonist; the hypothesis is that by antagonizing the mGlu receptors, brain function will normalize in brains that lack FMRP. Further, it is hoped that if MPEP is administered prenatally, it will prevent abnormal development that is a result of the lack of FMRP. This hypothesis has been tested in two recent studies, discussed below.

The first study used a *Drosophila* model of fragile X syndrome (McBride et al, 2005). Flies have an *fmr1* homologue (called *dfmr1*) which can be deactivated to create a KO fly (termed FS flies). FS flies do not court virgin females to the extent that

normal flies do; McBride et al (2005) found that treatment with MPEP, either as a larvae or as an adult, restored normal courting behavior in FS flies. FS flies also have an immediate recall and a short term memory deficit as compared to WT flies; treatment with MPEP ameliorated both of these deficits. While these results may not be directly applicable to the human condition, they are encouraging in the fact that MPEP reversed all effects of the *dfmr1* KO phenotype, suggesting the MPEP is a good candidate for FX treatment.

The second study used *fmr1* KO mice of different background strains, including an F1 hybrid of a C57xFVB cross (Yan et al, 2005). Yan et al (2005) found that MPEP was effective in reducing the frequency and severity of audiogenic seizures. Perhaps more importantly, they also found that MPEP reversed abnormal open field behavior. Prior to application of MPEP, Yan et al (2005) found that *fmr1* KO mice spent a greater amount of time in the center area of an open field than did WT mice. After an injection of MPEP, *fmr1* KO mice spent an equivalent amount of time in the center area of the open field. This indicates that MPEP has the ability to reverse at least some of the abnormal phenotypes present in the *fmr1* KO mice. These two studies provide results that support the hypothesis that MPEP may be a likely candidate for treatment of FXS in humans. What is needed now is the demonstration of robust deficits in the cognitive and affective realms in the *fmr1* KO mouse, so that potential treatments, like MPEP, can be tested. The next few years of research in fragile X syndrome should be prove to be extremely exciting.

Conclusions

The present study assessed social behavior in *fmr1* mice. Subtle differences were found between the genotypes, particularly relating to the specific characteristics of the social interactions and the degree of anxiety created by social interactions. In

addition, *fmr1* KO mice showed a less pronounced negative reaction to a stimulus mouse that was non-preferred by all animals. With more sensitive measurements, it is likely that a reliable difference in social behavior can be demonstrated between the two genotypes in this paradigm. Following the demonstration of a behavioral deficit in *fmr1* KO mice that corresponds to human deficits, pharmacological treatment can be tested, most likely using MPEP. In conclusion, the results from this study are encouraging, and more work needs to be done in order to find a treatment for fragile X syndrome.

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