ATTENTION, AROUSAL REGULATION & INHIBITORY CONTROL IN FMR1 KNOCKOUT MICE: A MOUSE MODEL OF FRAGILE X SYNDROME

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ATTENTION, AROUSAL REGULATION & INHIBITORY CONTROL IN FMR1 KNOCKOUT MICE: A MOUSE MODEL OF FRAGILE X SYNDROME

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Fragile X syndrome (FXS) is the most common inherited form of mental retardation, occurring in roughly 1/4000 males and 1/8000 females. An abnormal expansion of a trinucleotide CGG repeat sequence in the *fmr1* gene results in transcriptional silencing of this gene, which codes for the Fragile X Mental Retardation Protein (FMRP). The loss of FMRP, directly and/or indirectly, gives rise to the FXS phenotype, which includes a characteristic set of anatomic and cognitive/behavioral features. The present studies were designed to test the hallmark areas of dysfunction (i.e. attention, inhibitory control, hyperarousal, and emotional regulation) seen in human FXS and further characterize spared and impaired functions in fmr1 KO mice. The performance of F1 hybrid *fmr1* KO mice (a C57BL/6J x FVB/NJ cross) and wild-type (WT) littermate controls were evaluated on a series of tasks designed to assess inhibitory control and various aspects of attention, Reversal Learning Task, and Associate Learning Task. Regulation of arousal and emotion, two domains affected in FXS, was also evaluated in these tasks by examining the animals' reaction to the unexpected presentation of potent olfactory distractors (in the Distraction task), as well as their reaction to committing an error on the previous trial.

The present studies provided the first evidence that the hallmark deficits in human FXS -- impaired attention, inhibitory control, and arousal regulation – are also impaired in the *fmr1* mouse model of FXS. In addition, these findings demonstrate that attentional dysfunction and impaired inhibitory control are most prominent when task contingencies change and when the animal has just committed an error – situations that arouse or disturb the mice. Analysis of videotapes further demonstrates that arousal regulation is impaired in the *fmr1* KO mice. Additionally, the *fmr1* KO mice were not impaired in associative learning, transfer of learning, or reversal learning. The present results provide strong support for the validity of this animal model for future studies designed to elucidate the pathogenic process in human FXS and to test new therapies.

BIOGRAPHICAL SKETCH

Ji-sook Moon was born in Seoul, Korea, on October 18, 1974. She obtained most of her early formal education from the Seoul Public School system, culminating in her graduation from Yumkyuang Women's High School in February of 1993. In March 1993, she entered Dong-Duk Women's University in Seoul, Korea. She had graduated with a B.S. degree in German Language & Literature in February 1996.

In 1997, Ji-sook began to work for the New Generation Computer & Communication Company located in south Korea. While there, she worked on voice recognition systems, a voice exchange system, and a human vision simulation. She studied the neural mechanisms of several modalities of human perception, including vision, olfaction, and hearing, and worked to develop computer based models of these mechanisms.

Jisook's stidies in New Generation stimulated her desire for a deeper and moe systematic understanding of the neural mechanisms of human brain. She was accepted into the Department of Psychology at Yonsei University, which is considered one of the best programs in Korea. This allowed her the unique combination of being able to study at Yonsei University and to work part time for New Generation C&C. Working and studying at the same time allowed her many opportunities to apply academic knowledge to real-world situations.

To the end, Jisook assisted Dr. Kang in her lab, the Cognitive Neuroscience and Medical Physics Lab at the Seoul National University Hospital. There, she conducted studies on memory and learning in normal human conditions, as well as abnormal states of perception such as epilepsy and dementia. Through cutting-edge imaging methods (i.e. fMRI, PET, and MRI) coupled with some behavioral tests, she furthered her knowledge of functional and structural workings of the human brain

during learning, memorizing, and recognizing. She also ganed insight into how the activity of the brain differs between normal and abnormal individuals. In addition, she also developed and improved her knowledge of visual learning, memory, and brain mechanisms through weekly lab meetings and seminars. This volunteer work in Dr. Kang's lab was a defining moment in Ji-sook's career path; her experiences convinced her that she should pursue a Ph.D. in cognitive neuropsychology.

After her graduation in January 2001, Ji-sook came to Cornell University in Ithaca, New York. There, she began working with Daniel C .Richardson, a graduate student of Dr. Michael J. Spivey. She was primarily responsible for monitoring test subjects during behavioral testing and data entry. The focus of this research involved the encoding of language in the mind as spatial representations grounded in perception and action. This research experience served as a bridge between how research was conducted in Korea and the very different laboratory atmosphere at Cornell.

In April 2001, Ji-sook joined the lab of Dr. Daeyeol Lee, a professor in the Department of Brain & Cognitive Science at University of Rochester. Through seekly reading and discussion meetings, she became acquainted with the quintessential studies and philosophical controversies surrounding the study of brain mechanisms underlying perceptual and motor skill learning and memory. This experience gave Jisook the chance to observe firsthand the inevitable frustration and lots graduate school. Dr. Lee's lab also provided Jisook with herfirst exposure to the statistical analysis of multiple spike trains. This research was also her first exposure to the use of animals in cutting edge research.

In addition to this lab experience, Ji-sook was also admitted to an Undergraduate Workshop on Perception, Action, and Cognition organized by Department of Center for Visual Science at the University of Rochester; she was awarded a research and travel fellowship in June 2001. The workshop provided an

intimate setting in which she was able to integrate her knowledge of human cognition and neural mechanisms as well as deepen her understanding of these issues by interacting with leading researchers in neuroscience, cognitive science and computational brain modeling. She also attended lectures, participated in laboratory demonstrations and interacted with faculties at informal panel discussions and social gatherings. Because the workshop involved faculty from the Departments of Brain and Cognitive Sciences, Neurobiology and Anatomy, Neurology, and Computer Science, it provided Jisook with a broad, inter-disciplinary perspective on cognitive neuroscience.

Recognizing the importance of continued research on cognitive neuroscience, Ji-sook was accepted into the Ph.D program in Division of Nutritional Sciences at Cornell University in September of 2002. Under the guidance of Dr. Barbara J. Strupp Jisook developed a project that would utilize her knowledge of cognition of behavior in normal of abnormal systems. Jisook's research had two primary goals: 1) to determine the nature and underlying neural basis of cognitive dysfunction in mouse models of human developmental cognitive disorders with implications for therapeutic intervention and for elucidating basic brain-cognition relationships 2) to determine the efficacy of nutritional and/or pharmacological interventions in reducing or alleviating the specific cognitive deficits seen in genetic mental retardation syndromes such as Fragile X Syndrome and Down Syndrome. In her work in the Strupp lab, Jisook has been primarily involved in documenting and interpreting behavioral data designed to assess alterations in learning and in selective and sustained attention. She has also collected blood and brain samples from the tested animals, and prepared these samples for atomic absorption spectrophotometry analyses. She has also been responsible for managing undergraduate research assistants and mentoring five honor's undergraduate students.

While pursuing her Ph.D. in behavioral neuroscience, Jisook has also a Master's Degree in Department of Biological Statistics and Computational Biology at Cornell. Under the supervision of Martin Wells, she investigated an improved method for analyzing microarray data. She is submitting a paper "Better Choice of Background Correction and Normalization Methods for cDNA Microarray Data". This work has been a priceless contribution to her training in Biometrics.

To my husband, my daughter Aileen, my parents, my sisters, and brother

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I am not really certain how on Earth I managed to get this education. Many things needed to fall into place. When I look within myself, it is hard to believe that it all happened. However, I have been surrounded by brilliant, supportive, great people at every moment in my life.

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Without question, the most profound contribution to this work is from my advisors. Professor Barbara J. Strupp allowed me to work independently while applying her considerable insight and logical thoroughness to help me clarify my ideas. No words of thanks are enough for her mentorship, guidance, support, and patience. Discussions with her greatly enhanced my education and allowed the completion of this work. Professor Martin T. Wells, as an advisor of my MS degree in Department of Biological Statistics and Computational Biology at Cornell, taught me from his immense experience and was unfailingly generous with his attention to my progress. I also acknowledge Professor Paul Soloway and Danny Manor, a minor member in Genetics and a field representative, respectively, of my special committee, who was very generous and encouraged me. I would like to thank my colleagues in Strupp's lab. Dr. Lori Driscoll, Dr. Mathew Gendle, and Diane Stangle deserve special credit for giving me an idea, mentoring me, training me, and offering valuable discussion.

Special thank Myla Strawderman for expert statistical consultation. She was a practical tutor who taught me how to conduct SAS analyses and how to model complicated data. Caitlyn McNaughton was a great coworker for the mouse project and was particularly helpful and supportive, willing to help with my problems. Kasia was not only my group-mate but also my "writing teacher". She was always quite willing to edit my writings. Special thanks to Tara Lynn Benedetto for working together, helpful discussions, and her support and Mareike Kuypers for her technical support in the lab. I owe them my gratitude for their critical feedback and moral support. I also must thank Dr. David Levitsky for solving technical problems with testing apparatus, maintaining testing apparatus, and writing the programs. I would like to acknowledge the undergraduate honors thesis students, Sara Verosky, Kristie Ota, Marc Weiskopf , May Chen, Shruti Gandhy, Quynh Nguyen, who spent lots of time assisting in data collection, running behavioral tasks, cleaning data, and videocoding animals' behaviors. My experience at Cornell was further enhanced by others at various institutions. I would like to acknowledge my collaboration with Prof. Stephen Ginsberg in NYU. He taught me microdissection and perfusion of mouse brain, and cervical dislocation technique. Special thank Dr. Linda Crnic in Colorado Mental Retardation and Developmental Disabilities Research Center for sending animals to our lab and valuable disscusion.

In the depths of my academic past – prior to coming to Cornell – are countless people that have shaped my persona. Professor Dr. Eunjoo Kang at Seoul National University Hospital opened my eyes to the excitement of research and provided me with the confidence to take these steps. Thanks to all the other colleagues with whom I spent invaluable time at Yonsei University.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE
INTRODUCTION

Fragile X syndrome (FXS) has been known as the most common inherited form of mental retardation, occurring in roughly 1/4000 males and 1/8000 females (Crawford et al., 1999). FXS is caused by an abnormal expansion of a trinucleotide CGG repeat sequence in the *fmr1* gene (Khandjian, 1999; O'Donnell and Warren, 2002), leading to hypermethylation of the promoter region. This genetic alteration results in transcriptional silencing of this gene, which codes for the Fragile X Mental Retardation Protein (FMRP) (Oberle, et al., 1991; Verkerk, et al., 1991; reviewed in O'Donnell and Warren 2002). It is thought that the absence of FMRP is what causes the characteristic symptoms of fragile X. The loss of FMRP, directly and/or indirectly, gives rise to the FXS phenotype, which includes a characteristic set of anatomic and cognitive/behavioral features.

Fragile X syndrome is characterized by a constellation of physical, behavioral and cognitive symptoms. The characteristic physical features of the disease include elongated facial structure, large protruding ears, hyperextensible joints, and in males, macroorchidism (Hagerman, 2002). Behaviorally, fragile X syndrome is associated with a range of impairments, including autistic features, anxiety, hyperarousal, hyperactivity, seizure susceptibility, unstable mood, and social problems that range from social anxiety to autistic behavior (Baumgardner et al., 1995; Cohen et al., 1988; Freund and Reiss, 1993; Hagerman, 2002; Reiss and Freund, 1992). Cognitively, fragile X syndrome is characterized by mental impairment, and deficits in executive function (Mazzocco et al., 1993; Reiss et al., 1995; Sobesky et al., 1994). Females with the disorder typically score in the mildly mentally retarded range while males generally score in the moderate to severe range; only about one-third to half of girls

have significant intellectual impairment, whereas almost all boys suffer from such a deficit. Both males and females with fragile X display relative weaknesses in attention, quantitative skills, and short term memory recall for abstract stimuli (Dykens et al., 1987; Freund and Reiss, 1991; Mazzocco et al., 1993; Miezejeski et al, 1986; Munir et al., 2000).

The molecular biology of the Fragile X Syndrome (FXS)

The length of the repeat region is highly polymorphic in normal individuals, with lengths ranging from approximately 6 to 55 CGGs (Fu et al., 1991). Individuals with between 59 and 200 repeats are classified as having a *premutation*, in which they demonstrate no (or moderate) physical and cognitive/behavioral deficits visually associated with human FXS. Offspring of carrier females have an even further expanded region of CGG repeats to values ranging from 200 to over 2,000 repeats, which are referred to as the *full mutation* (Hagerman, book 2002). This is in contrast to the daughters of the full mutation males all of whom are carriers of the premutation with no more than 200 repeats. This disparity between offspring of carrier females and full mutation males has been elucidated through analysis of sperm samples and such analyses from full mutation males show only premutation-lengthened alleles, indicating that the amplification of the repeats occurs postzygotically (Reyniers et al. 1993). A broad range of sizes of single sperm repeats of premutation males was detected, further indicating that the repeat sequences are unstable after conception (Nolin et al., 1999).

When evaluating the severity of human FXS within families, a phenomenon known as *anticipation* has been observed. That is, the number of affected individuals is greater in later generations than in earlier ones, as the premutation is transmitted

through generations. For example, the brothers of males with full mutations are less likely to be affected than the sons of their daughters. Furthermore, it has been shown that there is a strong familial clustering of repeat size expansion such that the offspring of some female carriers tend to have the full mutation, whereas those of other female carriers tend to be only premutations (Nolin et al., 1994, 1996). Individuals with fewer than 60 repeats have never been found to have had affected male children. Additionally no new mutation, that is an expansion from a normal repeat allele size to a full mutation in one generation, has ever been identified as developing within the first generation (Smits et al., 1993).

The phenotype associated with the expansion of the CGG repeats preceding the *fmr1* gene is considerably variable between sexes**,** as well as among individuals with the mutation. Because the fmr1 mutation that causes fragile X is located on the X-chromosome, females with FXS, who have two X-chromosomes, are typically heterozygous for the condition. In all females, one of the two X-chromosomes in each cell is randomly inactivated early during embryonic development, and remains inactivated in all the cells descending from that cell. Therefore, the severity of FXS in females depends on the proportion of the body's cells in which the wild type (normal) X-chromosome remains active. The production of FMRP is maintained to a certain extent by the remaining unaffected X-chromosome in females. In contrast, males with FXS lack this protein, and therefore are typically more severely affected by the fmr1 mutation.

The inconsistent silencing of the gene in some cells of the body but not others is called "*Mosaicism*", and occurs in both affected males and affected females with FXS. *Mosaicism* is caused by variability in the length of the CGG repeats from cell to cell. A minority of individuals with FXS have a mosaic pattern with some cells showing sizes of less than 200 repeats and partially active gene expression (Rousseau

et al., 1991). *Methylation mosaicism* has also been shown in affected individuals such that a full mutation shows only partial methylation (Nolin et al., 1994). It has been known that *mosaicism* is correlated with a less severe phenotype and a better prognosis of this disorder (McConkie-Rosell et al., 1993; Cohen et al., 1996).

The molecular mechanisms by which the triplet repeat mutational expansions occur are still unclear. However, it has been hypothesized that a form of DNA slippage error or alternative DNA helix formation during replication may be a primary cause (Bowater and Wells 2001; Jin and Warren 2000). Another proposed cause of the CGG repeat expansion is that the absence of the AGG repeats within the 3' end leads to destabilization of the CGG repeat region. Within the normal CGG repeat region are interspersed one (or more than one) AGGs. It has been suggested that the main role of these AGGs is to stabilize the sequence and prevent slippage during replication. Thus, the length of the pure repeat seems to be the major factor determining allele stability (Verkerk et al., 1991). Unstable or premutation alleles have a pure CGG region whereas most control size alleles have multiple AGGs (Eichler et al., 1994; Zhang et al., 1995). This supports the idea that the loss of the AGG within the 3' end results in destabilizing the CGG repeat region and consequently leads to its expansion into the full mutation (Eichler et al., 1994; Zhang et al., 1995).

FMR1 Gene Expression: The FMR1 gene is highly conserved among different species (Verkerk et al. 1991). The murine homolog Fmrp shows about 97% identity in the predicted amino acid sequence with the human FMRP (Ashley et al., 1993). Also, in the mouse and the monkey a heterogeneous subset of proteins with apparent molecular weights between 67 and 80 kDa were observed (Khandjian et al., 1995; Verheij et al., 1995).

In early mouse embryos, expression of the Fmr1 transcripts was observed mostly in the brain and gonads (Hinds et al., 1993). Strong hybridization signals are visible at 19 days gestation throughout all embryonic tissues indicating that Fmr1 expression is turned on during early development of the mouse. Later during development, the levels of Fmr1 mRNA become more specific to the expression, as is found in the adult mouse (Bakker et al., 2000). In human, for example, expression of Fmr1 RNA has been specifically localized in the brain of a nine-week old fetus and expression was mostly found in the nucleus basalis magnocellularis and the pyramidal neurons of the hippocampus in the brain of a twenty five-week-old human fetus (Abitbol et al., 1993). When studied using *in situ* RNA expression studies, high levels of Fmr1 mRNA are found in the brain, testis, ovaries, thymus, esophagus, and spleen whereas moderate or no levels of expression were detected in the kidney, liver, lung, heart, aorta, and muscle tissues of adult mice tissues (Hinds et al., 1993). In the mouse brain, expression was mostly detected in the granular layers of the hippocampus and cerebellum neurons, whereas no expression was observed in the white matter or glial cells (Hinds et al., 1993; Bakker et al., 2000). Additionally, lastly, in the mouse testis, expression was detected in the tubuli (Bachner et al., 1993).

Even though it is not yet known at what point in development the methylation and subsequent loss of fmr1 expression in fragile X patients occurs, it has been suggested that methylation of the FMR1 gene occurs relatively late in the embryo and that the FMR1 gene is expressed from conception onward during early development, even in an affected fetus. There is no crucial role for FMRP in early embryonic development based on the following evidence: 1) The majority of chorion villus samples of a full mutation male fetus have been observed to be undermethylated (Rousseau et al., 1991), 2) in one chorion villus sample at 11 weeks of pregnancy, the FMR1 gene was not methylated and the gene was transcribed, whereas in fetal tissues

at 13 weeks of gestation, nearly complete FMR1 methylation was detected and consequently any mRNA and protein could not be observed during embryonic development (Losekoot et al., 1997; Sutcliffe et al., 1992; Willemsen et al., 1996).

The role of FMRP: Association between FMRP and cognitive function

Because the primary characteristic of FXS is mental retardation, many recent studies have focused on the function of FMRP as it relates to cognitive processes. Information about the causes of cognitive dysfunction in FXS has come from autopsy material. Studies at a neuronal level have found a distinctive dendritic spine phenotype in humans with FXS that suggests that FMRP may play a role in synaptogenesis in early development. Specifically, autopsy studies have found that patients with FXS have significantly long, immature spines and fewer short, mature spines than controls (Rudelli et al., 1985; Hinton et al., 1991; Irwin et al., 2001). In addition, they also have increased spine density as compared to controls of the same age. Although dendritic spine abnormalities are associated with a number of mental retardation syndromes, including mental retardation of unknown etiology (Purpura, 1974), Down's and Rett syndromes (Kaufmann and Moser, 2000), the overabundance of spines seen in human FXS is unusual. This contrast provides support for the hypothesis that FMRP is related to dendritic maturation and pruning. Taken together, FMRP might play an important role in spine morphology resulting in cognitive dysfunction as shown in individuals with FXS.

FMRP is classified under a family of RNA-binding proteins called heterogeneous nuclear ribonucleoproteins (hnRNPs). It has been suggested that hnRNPs are involved in mRNA metabolism since some of them are required for 1) the export of mRNAs from the nucleus. In the light of this role of FMRP, it is

demonstrated that two ribonucleoprotein K homology domains (KH domains) and a cluster of arginine and glycine residues (RGG BOX) within FMRP are target places for a RNA binding 2) their subcellular localization in the cytoplasm (Van de Bor et al., 2004; Gu et al., 2002; Farina et al. 2002) 3) FMRP also forms part of a large messenger ribonucleoprotein (mRNP) complex, which roles are suggested to the transport to the synapse and translation of mRNA in dendrites of neurons. Several lines of evidence have supported this fact such that the abnormal spines in neurons were detected in individuals with FXS (Irwin et al, 2001; Nimchinsky et al, 2001; Greenough et al., 2001) indicating that FMRP is involved in neural plasticity.

To understand its functions at the molecular level, mounting research focuses on finding mRNAs which are targeted by FMRP, and proteins which bind directly or indirectly to the protein as well as the sub-cellular distribution of 'cargo mRNAs' and proteins that bind to FMRP to detect where FMRP arrives to exert its effects. The following sections focus on these roles of FMRP, which are inferred to be related to cognitive phenotypes seen in individuals with human FXS.

FMRP in the nucleus: It is known that FMRP is associated with mRNAs and shuttle them between the nucleus and the cytoplasm by both a functional nuclear localization signal (NLS) and a nuclear export signal (NES), which are two domains in FMRP (Eberhart et al., 1996). Studies with light and electron microscopy (Verheij et al., 1993; Eberhart, et al., 1996; Feng, et al., 1997) support this idea that FMRP interacts with nuclear proteins including the nucleolin, the nuclear FMRP interacting protein (NUFIP), the nuclear/cytoplasmic Y-box-binding protein 1 (YB1/p50) which participates in mRNA transcription, processing and transport from the nucleus, and regulation of mRNA localization, translation and stability in the cytoplasm- the

FXR1P and the FXR2P (Ceman et al., 1999, 2000; Bardoni et al., 2003; Stickeler et al., 2001; Nekrasov et al., 2003).

Another recently suggested role for FMRP in the nucleus is chromatin remodeling (Jin et al., 2004). More specifically it is hypothesized that FMRP could contribute to chromatin remodelling through the RNAi pathway in the nucleus. Several lines of evidence support this hypothesis: 1) *In vitro*, it is suggested that FMRP binds strongly to single-stranded DNA and, to a lesser extent, to double-stranded DNA (Eberhart et al., 1996), 2) it is also suggested that human FMRP is associated with non-coding RNAs (Zalfa et al., 2003) and microRNAs (miRNAs) which regulate mRNA expression and are involved in chromosome methylation (Jenuwein, et al., 2002; Bao, et al., 2004), and 3) FMRP, Argonaute, and miRNAs have also been found in the nucleus, where RNA interference (RNAi)-mediated pathways occur (Jin, et al., 2004; Matzke, et al., 2005).

FMRP in dendritic spines: It has been known that FMRP plays an important role directly/or indirectly in mRNA transport. Several lines of evidence support this fact. FMRP and its mRNA are detected in both dendritic spines and the soma, where neuronal activation is controlled by mGluRs. Furthermore, both protein synthesis and transport in the dendrites control the production of FMRP (Antar, et al., 2004). It is also reported that both *FMR1* and FMRP are transported in the dendritic spine, where translation of both the FMR1 mRNA and other mRNAs are regulated by synaptic activation. mGluR activation depends on the presence of FMRP in dendritic spines. In the absence of FMRP, mGluR activation does not increaseor decrease protein synthesis in synaptoneurosomes (Weiler, et al., 2004). It has been suggested that abnormal mGluR signaling, resulting from FMRP loss, could be responsible for many of the characteristic features of fragile X syndrome, including impaired cognitive

functioning, seizures, anxiety, movement disorders and obesity (e.g., Bear et al., 2004; Bear, 2005). In summary, a loss of FMRP might be related to spine morphology and results in the cognitive dysfunction seen in individuals with human FXS.

Proteins and mRNA that interact with FMRP: In an attempt to understand the mechanism by which the loss of FMRP leads to the phenotype of FXS, there has been an active search for other proteins that might interact with FMRP in the same biochemical pathway as FMRP. Unfortunately, none of the proteins that interact with FMRP has yet been associated with a disease to date. Moreover, none of the genes that encode FMRP-interacting proteins have been linked to hereditary mental retardation that has thus far been linked (Bagni and Greenough, 2005).

FMRP binds to RNA homopolymers and to a subset of transcripts that are found in the brain by domain structures (Ashley, et al., 1993; Zalfa, et al., 2003; Miyashiro, et al., 2003; Adinolfi, et al., 1999; Brown, et al., 2001; Chen et al., 2003), including the two KH domains, an RGG box and an RNA-binding domain in the N-terminal region of the protein (Adinolfi, et al., 2003; Kobayashi et al., 1998). It is proposed that alterations in the metabolism of mRNAs that are important for synaptic structure and function might be associated with the symptoms seen in individuals with human FXS and in the *fmr1* KO mice. Several lines of evidence have shown that there might be an association between phenotypes of FXS and mRNAs that binds to FMRP in neurons. Microarray studies have identified mRNAs including the mRNA for MAP1B that encode proteins which are important for neuronal function, synaptic plasticity and neuronal maturation (Brown, et al., 2001; Zhang et al., 2001). Subsequent studies confirmed these findings; FMRP binds RNAs with G-quartet motifs that FMR1 mRNA contains (Schaeffer, et al., 2001; Darnell, et al., 2001). All mRNAs interacting with FMRP are known to be associated with synaptic function,

supporting the idea that FMRP is strongly related to spine morphology resulting in the cognitive dysfunction seen in individuals with human FXS.

Hypersensitivity to Sensory Stimuli, Hyperarousal, and Hyperactivity

It has been reported that individuals with FXS are hypersensitive to sensory stimuli resulting in hyperarousal and hyperactivity in situations with excess auditory, visual, or tactile stimuli (Hagerman, 2002; Turner et al., 1980; Mattei et al., 1981). It is hypothesized that a hyperarousal behavior seen in individuals with FXS is associated with problems such as tactile defensiveness, hyperactivity, autistic behaviors, aggression, poor eye contact, anxiety, and avoidant behaviors (Cohen et al., 1995). Lines of evidence support this hypothesis. Adult males with FXS given propranol, a beta-blocker that attenuates hyperarousal by reducing heart rate and blood pressure and increasing relaxation, showed less aggression and repetitive behavior (Cohen et al., 1991). Studies with electrodermal reactivity (EDR) which is the sweat response, demonstrated that EDR is enhanced in the males with FXS during eye contact (Belser and Sudhalter, 1995; Miller et al., 1999). Miller et al. demonstrated that individuals with FXS display a generalized sensory defensiveness across sensory modalities in a controlled laboratory paradigm. Specifically, individuals with FXS produced significantly increased electrodermal responses (EDRs) in response to visual, auditory, tactile, olfactory, and vestibular modalities than controls and lower rates of habituation than controls. Furthermore, the pattern of responses to stimulation of individuals with fragile X in one sensory modality predicted responses in the other sensory systems, suggesting a general abnormality in sensory processing. Moreover, the magnitude of EDR responses in individuals with fragile X were highly correlated

with their FMR-protein expression level such that higher levels of FMRP are associated with more normal response patterns (Miller et al., 1999).

A study of heart rate variability in boys with FXS compared to age-matched normal controls further supports hyperarousal in FXS (Boccia and Roberts, 2000). In this study, boys with FXS had a faster heart rate and lower parasympathetic activity compared to controls indicating autonomic dysfunction and another mechanism of hyperarousal in individuals with FXS. Finally, on Conners Rating Score, which is an index of hyperactivity, 73% of prepubertal boys with FXS (Conners 1973; Werry et al. 1975) were scored higher in hyperactivity level, although all showed attention deficits (Hagerman 2002).

Attention

Deficits in attention and impulse inhibition are anecdotally considered to be key symptoms of fragile X, and up to 73% of individuals with fragile X syndrome are diagnosed with attention deficit hyperactivity disorder (Baumgardner et al., 1995). 100% of 14 males with FXS showed attention deficits (Bregman et al., 1998) and Turk (1998) demonstrated that 31 boys with FXS showed significantly higher rates of inattentiveness and fidgetiness compared to controls. More detailed neuropsychological studies showed a unique pattern of attentional deficits such that boys with FXS also have difficulties in divided attention, sustained attention, and inhibitory control or impulsivity compared to controls (Munir et al., 2000; Cornish et al., 2004). The FXS group demonstrated additional problems on tasks associated with executive dysfunction, defined as higher control processes of attention, particularly on measures of planning and organization, attentional shifting, and delayed responding, indicating a frontal lobe dysfunction which is an important brain area for executive

function (Munir et al., 2000). Reiss et al. (1995) found consistent results with Munir et al.'s (2000) finding of possible frontal lobe dysfunction in males with FXS. They reported structural abnormalities in the caudate nucleus in individuals with the disorder. One of the main cortical connections of the caudate is the dorsolateral prefrontal cortex (Selemon and Goldman-Rakic, 1985; Yeterian and Van Hoesen, 1978), which is the cortical area most closely associated with executive functions (Luria, 1966; Shallice, 1982). An increased volume of the caudate nucleus in individuals with an FMR1 mutation was found in magnetic resonance imaging (MRI) and quantitative morphometry studies (Reiss et al., 1995; Eliez et al., 2001). Moreover, analysis of FMR1 protein expression demonstrated that a higher expression of FMR1 was related to a lower (more normal) caudate volume in individuals with the full mutation indicating a dysfunction in the pre-frontal cortex – the basal ganglia circuitry in fragile X syndrome. Another study provided further evidence of alterations in this pathway. Specifically, cerebral blood flow, an index of neural activity, was examined during performance on a task sensitive to prefrontal function, in fragile X females as compared to normal controls (Tamm et al., 2002). During a Stroop cognitive interference task, females with fragile X had a markedly reduced activity level in the prefrontal cortex than controls (Tamm et al., 2002).

Taken together, the results of these studies provide clear evidence that attentional dysfunction, hyperactivity, and inhibitory deficits or impulsivity observed in fragile X are key symptoms of FXS and they are related to abnormal functioning of circuitry involving the prefrontal cortex and basal ganglia (including the caudate).

The Animal Model

Although human studies have proved useful in characterizing the dysfunctions associated with fragile X syndrome, an animal model is needed to understand the mechanisms by which the loss of FMRP leads to cognitive deficits, to elucidate the pathogenic process underlying the disorder, and to develop treatments that might prevent or ameliorate the dysfunctions seen in fragile X syndrome. The animal model will be pivotal for assessing the efficacy of possible treatments prior to testing in humans.

Since no naturally occurring animals for trinucleotide diseases have been described, in 1994, Bakker et al. developed the first fmr1 knockout mouse by homologous recombination in embryonic stem cells. A vector in which exon 5 of the Fmr1 gene was interrupted was used. The mouse is a good animal model in which to study fragile X syndrome because the fmr1 gene is highly conserved across species, with the murine homolog *fmr1* showing a 97% homology in amino acid sequence and 95% sequence identity at the nucleic level (Ashley et al., 1993). The murine Fmr1 gene also has a CGG repeat that is highly polymorphic between different mouse strains, with an average repeat length of 10 CGG repeats. Moreover, the murine expression pattern of the *fmr1* gene at the mRNA and protein level in various tissues is very similar to the human gene (Hinds et al., 1993; Abitbol et al., 1993; Bachner et al., 1993, 1993; Devys et al., 1993;).

Although the mechanism behind the loss of FMRP in the fmr1 knockout mouse differs from that in human fragile X syndrome, the end result is the same: the *fmr1* knockout animals never produce FMRP, and therefore model the absence of FMRP during early development as seen in humans with full mutations, the most

severe cases of fragile X. The knockout mice are viable and healthy and have a normal fertility, as was apparent from normal litter size.

Studies of these mutant mice [often referred to as *fmr1* knockout (KO) mice] have revealed some important areas of correspondence between the phenotypes of humans with FXS and this mouse model: They display macroorchidism (Bakker et al., 1994; Bakker, et al., 2000), abnormal dendritic spines in the hippocampus and cortex (Galvez and Greenough, 2005; Nimchinsky et al., 2001), increased motor activity (Mineur, Sluyter, de Wit, Oostra, and Crusio, 2002), and auditory hypersensitivity (Chen and Toth, 2001, Nielsen, et al., 2002**)** . However, the cognitive/behavioral phenotype of these mice appears to be very subtle (D'Hooge et al., 1997; Dutch–Belgian Fragile-X Consortium 1994; Kooy et al., 1996; Mineur et al., 2000; Yan et al., 2004), at odds with the profound cognitive and behavioral problems that characterize humans with FXS. Commonly-used learning/memory tasks, such as the Morris water maze and the radial arm maze, have either been unable to differentiate the *fmr1* KO mice from controls (Paradee *et al*. 1999; Peier et al., 2000; Dobkin *et al*., 2000) or have revealed very small deficits in the *fmr1* KO mice that are apparent only in some background strains (Hinds et al., 1993; Cianchetti et al., 1991; Bakker et al., 2000; Muneur at al., 2002). Results seemingly contradictory to the phenotype of human FXS have also been reported. For example, in some learning tasks, *fmr1* KO mice performed better than their WT littermates (Frankland et al., 2004; Van Dam et al., 1999). Similarly, in the elevated plus maze, a well-validated test of anxiety for rodents, *fmr1* KO mice either performed similarly to controls (Dobkin et al. 2000), or exhibited a greater preference than controls for the open arms of the maze (Peier *et al*. 2000), a pattern generally interpreted as indicative of reduced anxiety. The available data also indicate discrepancies between *fmr1* KO mice and FXS in prepulse inhibition (PPI), a marker of sensorimotor gating. In a recent study, boys with FXS exhibited reduced

PPI relative to controls, whereas *fmr1* KO mice exhibited greater PPI than their WT littermates (Frankland et al., 2004). These findings, taken together, have raised questions about the validity and utility of this mouse model.

There are several possible explanations for the relative subtlety of the cognitive phenotype and the inconsistencies between studies. One factor, noted above, is that the background strain appears to play an important role in whether or not the mutation produces a phenotype that is different from WT controls (Dobkin et a;., 2000; Moy et al., 2004; Paradee et al., 1999). For example, Paradee et al. (1999) and Dobkin et al. (2000) demonstrated that the inconsistent results from the *fmr1* KO' performance on the Morris water maze were partially due to strain dependence. They conducted studies on a mixed genetic background of primarily C57BL/6 with a 129P2 strain; when Paradee et al. (1999) and Dobkin et al. (2000) examined *fmr1* KO mice on similar C57BL/6-129 hybrid backgrounds, they found mild deficits in their performance on the Morris maze. However, when *fmr1* knockout mice on a pure C57BL/6 background were tested, they found no differences between the *fmr1* KO and the controls on the Morris maze. Further, when *fmr1* KO mice on a FVB/N-129 hybrid background were used, the *fmr1* KO showed slight deficits compared to controls.

It is also possible that the subtlety of the behavioral phenotype reflects species differences in genetic compensation. For example, whereas the two highly conserved paralogs of FMRP, FXR1P and FXR2P, do not seem to compensate for the lack of FMRP in the brain of FXS humans, it is possible that they provide at least partial compensation in the *fmr1* mouse (Hoogenveen et al., 2002; Gu et al., 2003; Bakker et al., 2000; Kooy RF. et al., 2003). Another factor likely contributing to the apparent lack of cognitive dysfunction in the *fmr1* KO mouse is that the hallmark areas of

dysfunction in human FXS have not been studied in the mouse model; these include attention, inhibitory control, and regulation of arousal or emotion.

The present study was designed to test this hypothesis. *fmr1* KO mice and wild-type littermate controls were tested on a series of tasks designed to assess inhibitory control, learning, various aspects of attention (sustained, selective, and divided attention) (see chapter 2 & chapter 4), Reversal Learning Task, and Associate Learning Task (see chapter 3). Regulation of arousal and/or emotion was assessed by examining the animals' reaction to the unexpected presentation of potent olfactory distractors (in the selective attention task), as well as their reaction to committing an error on the previous trial. Reactivity to errors taps both error monitoring (an aspect of executive functioning) (Luu et al., 2000) and emotion regulation (Elliott et al., 1996), two domains affected in human FXS.

The attention tasks and learning tasks described in this thesis are modified versions of the 5-choice serial reaction time task developed by Robbins and colleagues for rats (e.g., Carli et al., 1983; Cole and Robbins, 1987) and recently modified for mice by Humby and colleagues (Humby, et al., 1999). These tasks are similar to ones used to assess various aspects of attention in human subjects, such as Leonard's 5-choice serial reaction time task and the Continuous performance test (reviewed in Robbins, 2002). In the present task series, a brief visual cue was randomly presented after a variable delay from one of the 5 response ports in each trial. The mice were rewarded for making a nosepoke into the illuminated port. Additionally, in the selective attention task, an olfactory distractor was pseudo-randomly presented from one of the ports during the interval prior to onset of the visual cue, on one third of the trials in each testing session. An olfactory learning set task and an olfactory reversal learning task were also tested. The learning set task was further designed to tap

transfer of learning, an area of dysfunction commonly seen in mental retardation syndromes (Campione & Brown, 1984; Campione et al., 1985).

The learning set tasks used in these studies have previously revealed cognitive impairment in animal models of MR syndromes (Strupp et al., 1990; 1994), most notably disease models for which basic learning tasks had not revealed dysfunction (reviewed in Strupp and Levitsky, 1990; Strupp & Diamond, 1996). The Reversal Learning Task was hypothesized to reveal dysfunction in the *fmr1* KO mice for three converging reasons. First, reversal learning taps inhibitory control and adaptability to change, capabilities that are impaired in humans with FXS (Kau et al., 2000; Rogers et al., 2001). Second, reversal learning is dependent on the integrity of the prefrontal cortex (Dias et al., 1996; Remijnse et al., 2005; Smith et al., 2004), a brain region believed to be dysfunctional in human FXS (Tamm et al., 2002; Cornish et al., 2004b; Hagerman, 2002, Menon et al., 2004, Guerreiro et al., 1998). Finally, due to the frustration engendered by the reversal of contingencies and initially high error rate, reversal learning tasks provide an index of emotion regulation, an area of dysfunction in FXS (Hagerman & Sobesky, 1989; Borghgraef et al., 1990; Kerby et al., 1994).

Based on the phenotype of humans with FXS, it was predicted that the *fmr1* KO would exhibit impairments in inhibitory control, sustained attention, and learning problems, as well as heightened reactivity to the olfactory distractors. Furthermore, it was predicted that the disruptive effect of committing an error on the previous trial – seen in WT mice in prior studies in this lab -- would be greater for the *fmr1* KO mice than for controls, due to the impaired arousal regulation and/or affect seen in humans with FXS. Several testing sessions for each animal were videotaped and subsequently coded for various behaviors. The goal of the videotape analyses was to further assess the putative group differences in reactivity to the distractors and/or committing an

error, as well as more generally aid in characterizing the behavioral phenotype of the *fmr1* KO mice.

In the present study, the *fmr1* KO mice were F1 hybrids, produced by crossing female C57Bl/6J mice, heterozygous for the *fmr1* mutation, with normal FVB males for chapter 2 and 3 and by crossing female FVB mice, heterozygous for the *fmr1* mutation, with normal C57Bl/6J males for chapter 4. The strategy of studying the *fmr1* mutation on an F1 hybrid background was followed for several reasons. First, these mice have normal hearing (unlike C57BL/6J mice) and are not blind or susceptible to seizures (unlike FVB/NJ mice) because these deficits are recessive (Johnson et al., 1997; Zheng et al., 1999; Pittler & Baehr, 1991; Goelz et al., 1998). In addition, this procedure produces *fmr1* KO and WT mice from the same litters, thereby equating the intrauterine and postnatal environments of the experimental and control groups. Finally, in light of the pronounced strain differences in startle, anxiety, and performance in various learning tasks, it is risky to draw conclusions about the effects of a given mutation from studies of inbred mice, as background strain effects may greatly accentuate or obscure gene effects (Paradee et al., 1999).

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CHAPTER TWO

ATTENTIONAL DYSFUNCTION, IMPULSIVITY, AND RESISTANCE TO CHANGE IN A MOUSE MODEL OF FRAGILE X SYNDROM

ABSTRACT

On a series of attention tasks, male *fmr1* knockout (KO) mice (F1 hybrids of C57BL/6J X FVB/NJ cross) committed a higher rate of premature responses than WT littermates, with the largest differences seen when task contingencies changed. This finding indicates impaired inhibitory control, particularly during times of stress or arousal. The KO mice also committed a higher rate of inaccurate responses than controls, particularly during the final third of each daily test session, indicating impaired sustained attention. In the selective attention task, the unpredictable presentation of potent olfactory distractors produced a generalized disruption in the performance of the KO mice whereas, for controls, the disruption produced by the distractors was temporally limited. Finally, the attentional disruption seen following an error was more pronounced for the KO mice than for controls, further implicating impaired regulation of arousal and/or negative affect. The present study provides the first evidence that the *fmr1* KO mouse is impaired in inhibitory control, attention, and arousal regulation, hallmark areas of dysfunction in FXS. The resistance to change also seen in these mice provides a behavioral index for studying the autistic features of this disorder.

INTRODUCTION

Fragile X syndrome (FXS), the most common inherited form of mental retardation (Crawford et al., 1999), is caused by expansion of a CGG repeat sequence in the promoter region of the *fmr1* gene (Khandjian, 1999; O'Donnell & Warren, 2002), which leads to transcriptional silencing of this gene (Oberle et al., 1991; Verkerk et al., 1991; reviewed in O'Donnell & Warren 2002). Deficiency of the encoded protein, called the Fragile X Mental Retardation Protein (FMRP), directly and/or indirectly, gives rise to the FXS phenotype. The cognitive dysfunction is not global in nature but rather primarily affects various aspects of executive functioning, such as attention and inhibitory control (Baumgardner et al., 1995; Hagerman, 1996; Lachiewicz et al., 1994; Largo & Schinzel, 1985; Turk, 1998), with up to 73% of affected individuals meeting the diagnostic criteria for Attention Deficit Hyperactivity Disorder (Baumgardner et al., 1995). Other prominent features of FXS include hypersensitivity to sensory stimuli (Baranek & Berkson, 1994; Cohen et al., 1988; Hagerman, 1996; Miller et al., 1999) seizure susceptibility (Musumeci et al., 1999, 2001), emotional difficulties (Borghgraef et al., 1990; Hagerman & Sobesky, 1989; Kerby et al., 1994), and autistic features (e.g., Hagerman, 1996; Lachiewicz et al., 1994). Although there are currently no interventions that can prevent the brain damage in FXS, recent research on FMRP suggests that certain pharmacological interventions, such as mGluR antagonists, could dramatically improve brain development and function in affected individuals (e.g., Bear et al., 2004; Bear, 2005; McBride et al., 2005; Yan et al., 2005). One stumbling block in testing these treatments in the mouse model of FXS [referred to as *fmr1*^{tm1Cgr} or *fmr1* "knockout" (KO) mice] is that the behavioral differences between the KO mice and WT controls

on learning and memory tests have been subtle and strain-specific or nonexistent (Bakker et al., 1994; D'Hooge et al., 1997; Kooy et al. 1996; Mineur et al., 2002; Yan et al., 2004), at odds with the profound cognitive and behavioral problems that characterize humans with FXS. Commonly-used learning/memory tasks, such as the Morris water maze and the radial arm maze, have either been unable to differentiate the *fmr1* KO mice from controls (Dobkin et al., 2000; Paradee et al., 1999; Peier et al., 2000; Yan et al., 2004) or have revealed very small deficits in the KO mice that are apparent only in some background strains (Bakker et al., 2000; Cianchetti et al., 1991; Hinds et al., 1993; Mineur et al., 2002). Results seemingly contradictory with the phenotype of humans with FXS have also been reported. For example, in some learning tasks, *fmr1* KO mice performed better than their WT littermates (Fisch et al., 1999; Frankland et al., 2004; Van Dam et al., 1999). The available data also indicate discrepancies between *fmr1* KO mice and FXS in prepulse inhibition (PPI), a marker of sensorimotor gating: In a recent study, boys with FXS exhibited reduced PPI relative to controls, whereas *fmr1* KO mice exhibited greater PPI than their WT littermates (Frankland et al., 2004). These findings, collectively, have raised questions about the validity and utility of this mouse model (e.g., Yan et al., 2004).

One factor that may contribute to the apparent lack of cognitive dysfunction in the *fmr1* KO mouse is that the most prominent areas of dysfunction in human FXS have not been studied in the mouse model; these include impaired attention, inhibitory control, and regulation of arousal or emotion. The present study was designed to test this hypothesis. The performance of F1 hybrid *fmr1* KO mice (a C57BL/6J x FVB/NJ cross) and wild-type (WT) littermate controls was assessed on a series of tasks designed to assess inhibitory control and various aspects of attention (sustained, selective, and divided attention). These tasks, modified versions of the 5-choice serial reaction time task (Humby et al., 1999), are similar to ones used to assess various

aspects of attention in human subjects, such as Leonard's 5-choice serial reaction time task and the Continuous Performance Test (reviewed in Robbins, 2002). Regulation of arousal and/or emotion was evaluated in these tasks by examining the animals' reaction to the unexpected presentation of potent olfactory distractors (in the Distraction task), as well as their reaction to committing an error on the previous trial. Reactivity to errors taps both error monitoring (an aspect of executive functioning) (Luu et al., 2000) and emotion regulation (Elliott et al., 1996; Luu et al., 2000), two domains affected in FXS.

MATERIALS AND METHODS

Subjects

Breeding of the mice was conducted at the University of Colorado Health Sciences Center, Denver, CO. Breeder pairs of C57BL/6J-*Fmr1*^{tm1Cgr} (B6.129-*Fmr1* ^{tm1Cgr}) (*fmr1* KO) and wild-type (WT) C57BL/6J mice were purchased from Jackson Laboratory, Bar Harbor, ME. In the KO mice, the *fmr1* gene had been disrupted by targeting a transgene to exon 5 with homologous recombination (Bakker et al., 1994). The heterozygous females 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 mice from the same litters. Male offspring (21 WT and 20 *fmr1* KO) from these litters served as subjects in the present experiment. Genotyping was conducted as described in Nielsen et al. (2002).

The strategy of studying the *fmr1* mutation on an F1 hybrid background was followed for several reasons. First, these mice have normal hearing (unlike C57BL/6J

mice) and are not blind or susceptible to seizures (unlike FVB/NJ mice) because these deficits are recessive (Goelz et al., 1998; Johnson et al., 1997; Pittler & Baehr, 1991; Zheng et al., 1999). In addition, this procedure produces *fmr1*- KO and WT mice from the same litters, thereby equating the intrauterine and postnatal environments of the experimental and control groups. Finally, in light of the pronounced strain differences in startle, anxiety, and performance in various learning tasks, it is risky to draw conclusions about the effects of a given mutation from studies of inbred mice, as background strain effects may greatly accentuate or obscure gene effects (Paradee et al., 1999).

At 6-7 months of age, the mice were tested on a passive avoidance task. At 7-8 months of age, the mice were transported to Cornell University for further behavioral testing. At Cornell, the mice were housed singly in polycarbonate cages, with food and water available ad libitum. The mice were housed individually due to previous observations that male mice of this strain, caged in pairs, are prone to fighting when reunited after being removed for testing (Crnic LS, unpublished data). After acclimating to the new environment for several weeks, the mice were placed on a restricted feeding regimen in order to maintain motivation for food reward during the behavioral testing. The daily ration was gradually reduced and then maintained at a level that produced target weights at approximately 80-85% of their pre-restriction weight. A target weight of 80-85% was selected because the animals were somewhat overweight prior to introduction of the food restriction regimen. 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 at UCHSC and Cornell University, both AAALAC accredited institutions.

Apparatus

The mice were tested individually in one of six automated Plexiglas chambers, each controlled by a PC and situated in an insulated, sound-attenuating enclosure. The testing chambers were adapted from the "nine-hole" operant chambers recently developed to assess attention in mice (Humby et al., 1999). The slightly curved rear wall contained five circular response ports, 1 cm in diameter, located 2 cm above the floor and 5mm apart. A nosepoke into any of these ports constituted a response (or choice). Responses to the ports were detected by infrared photodiodes, positioned inside each port, 0.5 cm from the opening. The discriminative visual cues were provided by illumination of green 4 mA light-emitting diodes (LEDs), one embedded on the back surface of each port. Each port also contained a fitting through which scented air could be dispensed. This scented air served as a distractor in the Distraction task. The scented air was produced by passing filtered, compressed air through small bottles of liquid odorant, using solenoid airflow valves and airflow meters. The airflow rate was 1.0 L/min. On the chamber wall opposite the five response ports was an alcove (15 mm wide, 2 cm above the floor) containing the dipper (ENV0302M, MED Associates, Inc., St. Albans, VT) which dispensed the liquid food reward (liquefied AIN-76A, a sweet, nutritionally-complete diet; "Shake and Pour", BioServ, Inc.). Access to the dipper alcove was controlled by a thin metal door, which was activated by a motor located on the outside of the testing chamber. As with the ports, head entries into the alcove were monitored by infrared photodiodes. A nosepoke into this alcove port was required to initiate each trial. Each chamber was fitted with an exhaust system, which transported the air from the chamber directly to the room exhaust ventilator system at a rate of four complete air changes per minute. All automated events (door opening, dipper movement, responses, etc.) within each

chamber were timed, controlled, and recorded by custom programs written in QBASIC.

For videotaping, each chamber was equipped with a wide-angle infrared video camera and infrared LED light source attached to the ceiling directly over the center of each testing chamber. The camera allowed full view of the mouse at all times. Each camera was connected to a separate VHS VCR. An array of infrared LEDs, positioned outside the Plexiglas chamber but within viewing range of the camera, provided information about the various events during each trial (e.g., location of the visual cue, demarcation of the intertrial interval, presentation of the distractor, whether a response was correct or incorrect, access to liquid reward).

Behavioral testing

The training tasks began when the mice were 8 months of age. Testing on the four attention tasks was conducted when the mice were 10 months of age. Behavioral testing and coding of the videotapes were conducted by individuals blind to the genotype of the animals.

Training tasks

The mice first completed a four-stage training procedure designed to shape the general response sequence required for completion of each trial in the subsequent tasks. These training stages are described in a prior report (Driscoll et al., 2004). Briefly, the mice learned that the door to the dipper alcove would be raised at the start of each trial and that a nosepoke into the dipper port, followed by a nosepoke into one of the five response ports, would produce the delivery of 0.04 ml of the liquid diet in

the dipper alcove. These four training phases were mastered in approximately 8-10 sessions.

Each mouse was then trained on a five-choice visual discrimination task. In this task, one of the five port LEDs was illuminated on each trial; the mouse was rewarded for making a nose-poke into the illuminated port. After reaching the learning criterion (mean: 11 sessions), each mouse progressed through 4 subsequent visual discrimination tasks, all of which were identical in concept but with progressively shorter cue durations. The cue durations were 5.0, 2.0, 1.4, and 1.0s; the mice received these durations for 3, 10, 10, and 5 sessions, respectively. These tasks were designed to establish stable performance and prepare the mice for the subsequent attention tasks. For additional details on this task series, see Driscoll et al., 2004.

All testing equipment was thoroughly cleaned and dried following the testing of each mouse, using Odormute (R.C. Steele Co, Brockport, NY), a detergent containing an enzyme that removes olfactory cues (including pheromones).

Attention task 1: learning to wait for the cue

The mice were then tested on four visual attention tasks that were all identical in terms of the basic rules and procedures but which entailed different cue durations, delays prior to cue onset, and/or presentation of olfactory distractors (see Table 1).

Several types of errors were possible. A premature response was recorded if the mouse responded to any of the response ports before onset of the visual cue. A response to an incorrect port following cue presentation was tallied as an inaccurate response. An omission error was scored if the mouse initiated the trial but did not respond to any of the five response ports within 5 s of cue onset, indicative of missing the visual cue. Following any of these types of errors, a 5-s time-out period was imposed. These time-out periods were signaled by the illumination of a 2-W

houselight on the ceiling of the chamber. A time-out was also imposed following a "nontrial", the term given to trials in which the alcove door was raised at trial onset but the mouse did not enter the alcove in the following 60 s; nontrials were very rare, however. A 5-s intertrial interval separated adjacent trials. All trials on which the mouse made an initiation poke into the dipper alcove (regardless of the outcome of the trial) were defined as response trials. Each session was terminated after 30 min or 70 response trials, whichever came first. The mice were tested on this task for 8 sessions.

Table 2-1. Cue parameters for the four visual attention tasks, in the order in which they were administered.

Task	Cue Duration	Pre-cue delay	Distractor (yes or no)
Attention Task 1	1.0 s (constant)	0, 2, 4 s (variable)	no
Sustained Attention Task	0.8, 1.0, 1.6 s (variable)	0, 2, 4 s (variable)	no
Baseline Task	1.0 s (constant)	2, 3 s (variable)	no
Distraction Task	1.0 s (constant)	2, 3 s (variable)	Yes (⅓ of trials per session)

Sustained Attention Task

The Sustained Attention Task was a variation of the preceding task in which both the pre-cue delay and the cue duration varied randomly across trials (see Table 1). Each combination of correct response port (1-5), pre-cue delay (0 s, 2 s, and 4 s), and cue duration (0.8, 1.0, and 1.6 s) was presented an approximately equal number of times in each session. The mice were tested for 20 sessions on this task. The design of this task was based on a similar task that we developed for assessing sustained attention in rats (e.g., Gendle et al., 2003; Morgan et al., 2001; 2002; Stangle et al., submitted).

Distraction task

The mice were then tested on a variation of the preceding visual attention tasks that included the unpredictable presentation of potent olfactory distractors. This task was designed to tap selective attention and reactivity to salient stimuli. The design of this task was based on a selective attention task for rats (e.g., Gendle et al., 2004; Stangle et al., submitted). Immediately prior to the Distraction Task, the mice were tested on a Baseline Task that was identical to the Distraction Task in terms of the visual cue parameters (pre-cue delays and stimulus duration) but did not include olfactory distractors (see Table 1).

The disruption produced by the unpredictable presentation of the olfactory distractors was assessed in two ways. First, within the Distraction Task, performance on the trials with distractors (Distraction trials) was compared to performance on the trials without distractors (Nondistraction trials). In addition, performance on the Nondistraction trials of the Distraction Task was compared to performance on the Baseline Task. This comparison provided an index of the extent to which the

unpredictable presentation of the olfactory distractors produced a generalized disruption of performance that extended to the Nondistraction trials.

For both the Baseline and Distraction Tasks, cue duration was constant across trials (1 s) but the pre-cue delay varied randomly between 2 or 3 s (in addition to the 1 s "turnaround" time). For the Distraction Task, one of 9 different olfactory stimuli was pseudo-randomly presented on one third of the trials, from one of the five response ports either 1 or 2 s before the visual cue. The nine scents used for these distractors were lemon, hazelnut, apricot, butter, anise, raspberry, maple, coconut, and almond. The liquid odorants were made by diluting artificial flavorings (McCormick, International Flavors and Fragrances, Inc.) with propylene glycol. Scented air was produced by passing filtered, compressed air through small bottles of these scented liquids, using solenoid airflow valves, as described above. All parameters of visual cue and distractor presentation were balanced for each testing session; these included the location of the visual cue, duration of the pre-cue delay (2 or 3 s), the timing of the distractor relative to the visual cue (1 or 2 s prior to cue onset), and the response port from which the distractor was emitted.

Diet and Control of Motivation

As noted above, the animals were maintained at 80-85% of their ad libitum weights throughout the study to maintain motivation for the food rewards during testing, and ensure an approximately equal number of trials for all mice. On each testing day (6 days per week), the number of calories obtained during testing was subtracted from the total caloric allotment, and the remainder was fed as chow (ProLab 1000; Purina, Inc.) in the home cage directly after testing. On non-testing days, each mouse was given 0.4 ml of the liquid diet plus the remainder of the ration in chow in its home case. The goal was to provide the maximal daily caloric intake

that would still maintain adequate motivation for 60-70 trials during each daily test session.

Videotape Coding

All sessions of the Baseline and Distraction Tasks were recorded. A coder, blind to the genotype of the animals, scored four test sessions for each mouse: the last two sessions of the baseline task and the first two sessions of the Distraction task. The frequency and duration of four behaviors were quantified: wall climbing, grooming, jumping, and exploring (defined below). Also coded was the location of the behavior (the side of the chamber containing the response ports vs. the side containing the dipper).

Reliability of the behavioral ratings was determined prior to proceeding with the coding. For these reliability analyses, eight sessions of session 2 of the Distraction Task were pseudo-randomly selected (the eight sessions were balanced by box and treatment). To determine the intra-rater reliability, the coder scored each of the eight sessions twice (with time elapsed between recoding of the same session), and the results of the first round of coding were correlated with those of the second. To assess inter-rater reliability, the same eight sessions were coded by another coder, and the results from both coders were correlated. Coding of the remaining 156 sessions commenced only after high levels of inter- and intra-rater reliability were achieved ($r > 0.9$ for all behavioral measures).

Statistical Analyses

The data were analyzed using a generalized linear mixed model (GLIMMIX) which correctly handles non-normal data and the repeated measures for each animal

(Wolfinger & O'Connell, 1993). All statistical analyses were conducted using SAS 8.2 (SAS Institute, Cary, NC) for Windows 2000.

The following performance measures were analyzed: percent correct, percent inaccurate responses, percent premature responses, percent omission errors, and percent non-trials. For each of these dependent measures, means were calculated for each animal for each testing condition, defined by the following variables (as appropriate for the task characteristics): pre-cue delay, stimulus duration, distraction condition (Distraction vs. Nondistraction trials), Session-block (blocks of testing sessions, defined below), Trial-block (blocks of trials within each test session; defined below) and outcome of the previous trial (correct or error). The analyses were conducted on these means. The models used for these analyses included the aforementioned variables plus Genotype (*fmr1*-KO and WT) and all relevant higher-order interactions. However, simpler models were used in cases where the outcome was rare for that task, to obtain more observations for each mean.

Nonparametric techniques were used to analyze the dependent measures for the video data due to non-normality of the distributions. Specifically, Wilcoxon rank-sum tests were used to analyze between-condition differences and Genotype-by-condition differences for the dependent measures. Dependent measures were analyzed as a percentage of time spent for a given behavior, or time spent on a given behavior divided by time spent on all other behaviors multiplied by 100. Some analyses were conducted on difference scores, which were created by subtracting each animal's mean percentage of time spent for a given dependent measure during one condition from the mean percentage time spent during another condition. The dependent measures included percent time grooming, percent time wall-climbing and mean number of jumps.

For measurements of body weight and daily food intake, a t-test was performed.

RESULTS

Body weight: The body weights of the groups did not differ ($t_{(39)=}$ -1.28, n.s.).

Daily food intake: There was no effect of Genotype on mean daily food intake ($t_{(39)}$= -1.58, n.s.).

Non-trials and Dipper Latency: The KO and WT mice did not differ in the rate of nontrials for any of the tasks (all $p > 0.3$), nor for the latency to retrieve the liquid reinforcer following a correct response (all $p > 0.9$). These findings indicate that motivation to solve the tasks was comparable for the two groups.

Performance on the Attention tasks: In all of the tasks, performance was significantly affected by the pre-cue delay (better performance at shorter delays) and the outcome of the prior trial (impaired performance on trials following an error relative to trials following a correct response). Finally, in those tasks where cue duration and distraction condition varied across trials, these factors also produced consistent and significant effects for all outcome variables (i.e., better performance for trials with longer cue durations and without distractors). However, to streamline the presentation of results, these effects are only discussed within the context of describing the genotypic differences.

1. Attention Task 1 (first task with pre-cue delays):

Premature responses (responses made prior to cue onset): The analysis of percent premature responses revealed a main effect of Genotype ($F_{(1,52.5)} = 5.02$, $p = 0.03$). As depicted in Figure 1, the KO mice committed 30% more premature responses than controls, indicative of impaired inhibitory control.

Percent Correct, Omission Errors, Inaccurate Responses: For Attention Task 1, there were no significant differences between the *fmr1* KO mice and the WT controls for percent correct, percent omission errors, or percent inaccurate responses.

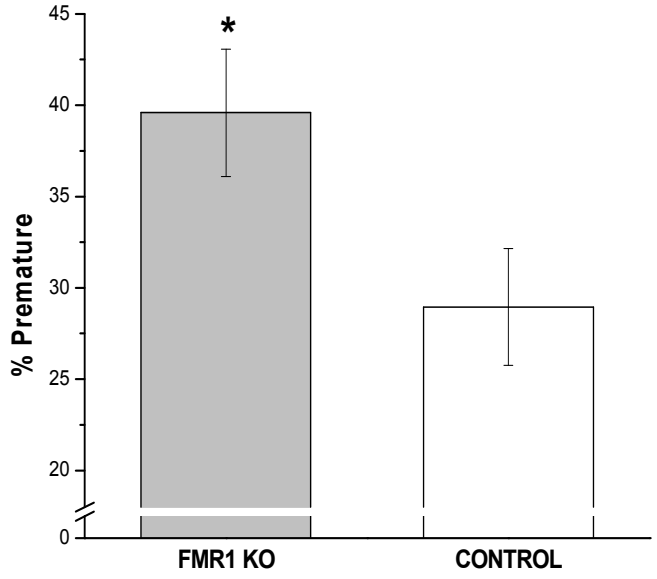

Figure 2-1: During Attention Task 1, the first task in which a delay was imposed prior to cue onset, the KO mice committed a higher rate of premature responses than controls, indicative of impaired inhibitory control. Bars represent the mean (+/- SEM).

2. Sustained Attention Task:

Percent Correct: The analysis of the 20 sessions on the Sustained Attention Task revealed a significant interaction between Genotype and Delay ($F_{(2,2693)} = 5.26$, $p = 0.005$). The two genotypes did not differ at the 0 second delay, whereas the *fmr1* KO mice performed more poorly than the controls on trials with a 2 or 4 s pre-cue delay. This decline in performance across delays was driven primarily by the increase in premature response rate, described below and depicted in Figure 2.

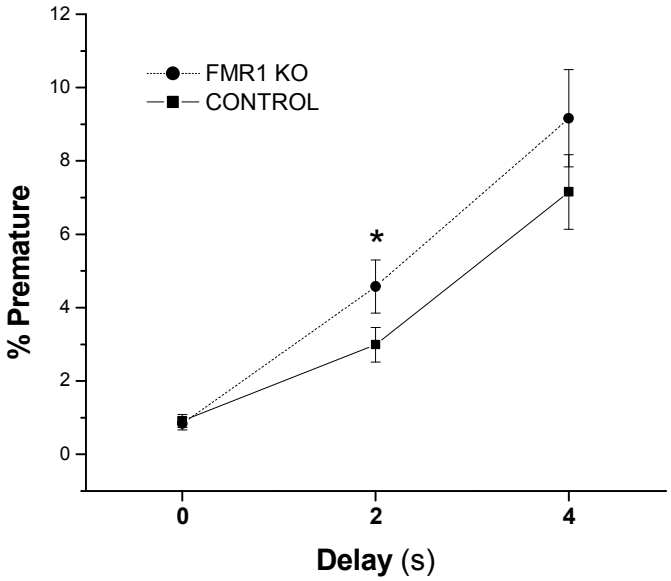

Figure 2-2: Premature responses in the Sustained Attention task: As the pre-cue delay increased from 0 to 2 s, the rate of premature responses increased to a greater extent for the KO mice than for controls, indicating impaired inhibitory control. Bars represent the mean (+/- SEM).

Inspection of average performance across the 20 test sessions revealed that the group difference for overall percent correct was most pronounced on the first session of the task. The KO group rapidly improved, with only subtle differences being

apparent later in testing. One possible explanation for this pattern is that the KO mice, like humans with FXS (Kau et al., 2000) and autism (Rogers et al., 2001), have difficulty dealing with change. Note, however, that the only difference between the Sustained Attention task and the prior attention task (Attention Task 1) was that cue duration now varied randomly from trial to trial, rather than being constant; all other characteristics were identical. To directly test the effect of changing this one aspect of the task, an additional analysis was conducted which included the final session of Attention Task 1 and the first session on the Sustained Attention Test, so that these two consecutive test sessions could be statistically compared.

This analysis revealed a significant interaction of Genotype and Task ($F_{(1,118)}$ = 5.01, p = .027; Fig. 3, top panel): The performance of the *fmr1* KO mice and controls did not differ on the final session of Attention task 1 (p = 0.7), whereas that of the KO mice was significantly lower than WT controls on the first session of the Sustained Attention Task (p = 0.01). Comparisons of within group performance across these two test sessions revealed a significant drop for the KO group ($p < 0.0001$) but not for the controls (p = 0.12).

Premature Responses: The analysis of percent premature responses for the 20 sessions of the Sustained Attention Task revealed a significant interaction of Genotype and Delay ($F_{(2, 76.1)}$ = 3.26, p = 0.04; Figure 2); the increase in premature response rate from trials with a 0 s pre-cue delay to those with a 2 s delay was marginally significantly greater for the KO mice than for controls (p = 0.06), indicative of impaired inhibitory control. The increase from 2 to 4 s was comparable for the two groups. Group differences were significant only at the 2 s delay, due to the greater variance seen at the 4 s delay.

An additional analysis of premature responses was conducted to compare the final session of Attention Task 1 and the first session of the Sustained Attention Task. This analysis revealed a significant interaction of Genotype and Task ($F_{(1,36.7)} = 5.82$, $p = 0.02$; see Figure 3, lower panel). The rate of premature responses increased significantly across these two sessions for the KO mice ($p = 0.0001$), but not for the controls ($p = 0.52$).

Inaccurate Responses: The analysis of percent inaccurate responses revealed a main effect of Genotype [$F(_{1,38}) = 4.16$, $p = .048$], and a marginally significant two-way interaction between Genotype and Trial-block ($F(_{2,1012}) = 2.58$, $p = 0.06$]. Overall, the *fmr1* KO mice committed a higher percentage of inaccurate responses than the controls ($p = 0.048$). The borderline interaction between Genotype and Trial-block ($p = .06$) reflected that the impairment of the KO mice, relative to controls, was most pronounced in the final block of trials in each testing session (trials 51-70). In this final block of trials, the KO mice, on average, committed a higher rate of inaccurate responses than controls ($p = 0.008$). Although the average difference in inaccurate response rate was relatively small (3.4%), a comparison of the distributions of the two groups suggests that this difference would translate into a functionally important deficit for affected children. As seen in Fig. 4, only 23.8% of the control mice had scores above the overall median, whereas 63.1% of the KO mice had scores above this value.

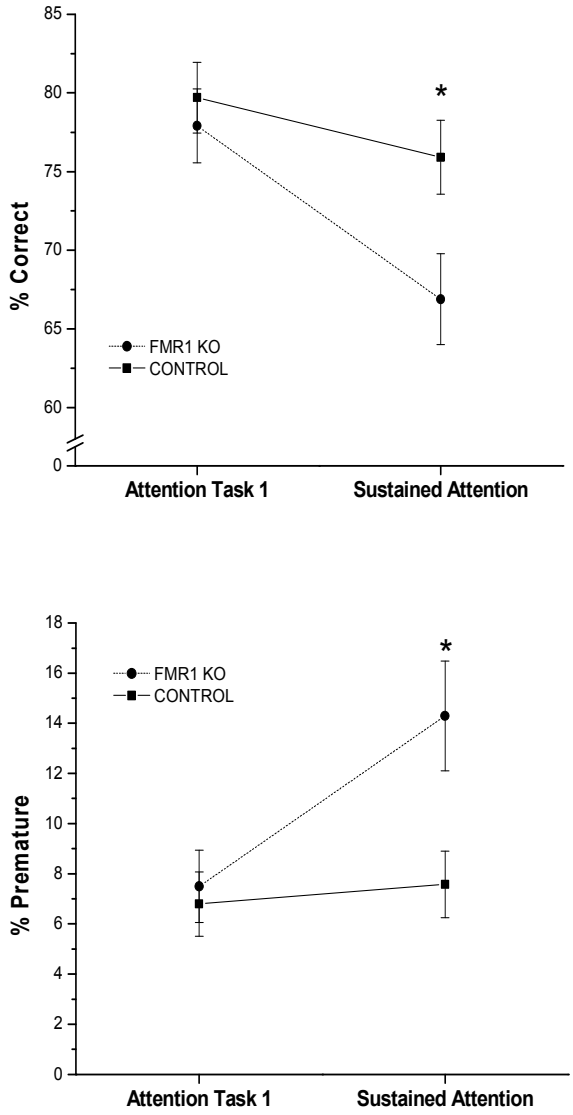

Figure 2-3: Top panel: The drop in performance from the final session of Attention Task 1 to the first session of the Sustained attention task (consecutive sessions) was significantly greater for the KO mice than for controls (p=0.027). Lower panel: This more pronounced drop in performance for the KO mice was largely due to an increase in premature responses. Bars represent the mean (+/- SEM).

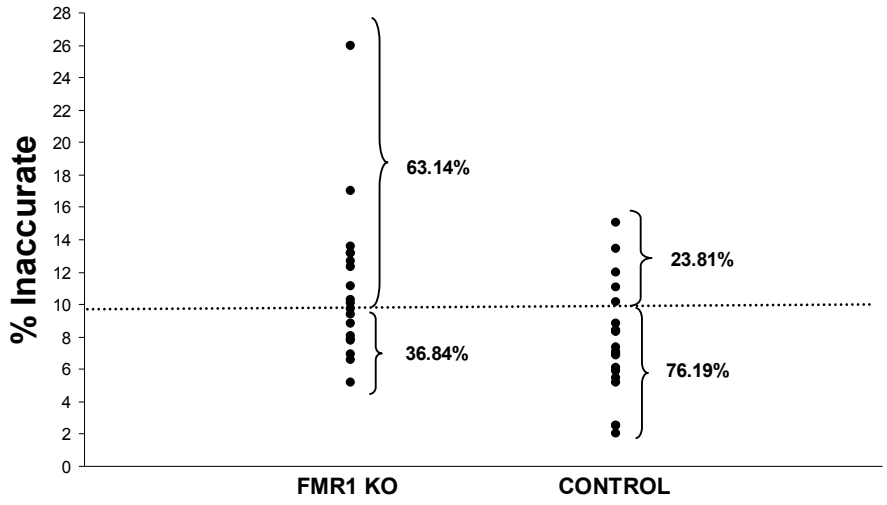

Figure 2-4: Average percent inaccurate responses during the third block of trials in each session (trials 51-70) of the Sustained Attention task. Each dot represents one mouse. The dotted line denotes the overall median. Only 23.8% of the control mice had scores above the overall median, in comparison to 63.1% of the mutant mice.

3. Baseline Task (prior to the Distraction Task): The WT and KO mice did not differ for percent correct, percent premature responses or percent inaccurate responses. The only error type that revealed a genotype-related effect was percent omission errors; the analysis of this measure revealed a significant interaction of Genotype and Previous Trial Outcome ($F_{(1, 430)}$= 7.49, p=0.006). Although both groups made more omission errors on trials following an error than on trials following a correct response, this increase in omission errors for trials following an error was greater for the KO animals than for the controls (see Fig. 5). This finding indicates that the disruptive effect of committing an error was more pronounced for the KO mice than for the controls.

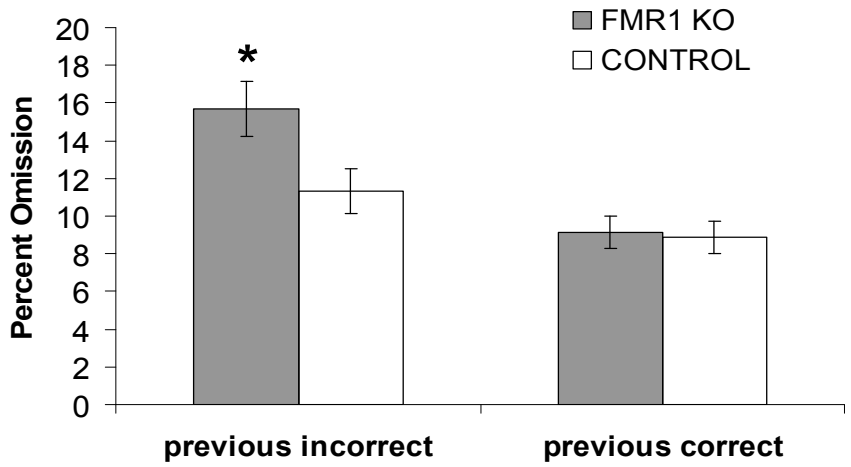

Figure 2-5: Percentage of omission errors for trials following an error vs. trials following a correct response, during the Baseline task. Committing an error on the previous trial increased the rate of omission errors to a greater extent for the KO mice than for controls (Genotype X Previous Trial Outcome, $p = 0.003$). *, $p = 0.006$, KO vs. control for trials following an error. Bars represent the mean (+/- SEM).

4. Distraction Task: A preliminary analysis used a 7-level "session-block" variable to assess the change in performance across the 20 sessions of testing on this task (3 sessions in each of the first 6 blocks, and 2 sessions in the final block). This analysis revealed a significant interaction between Distraction condition and session-block ($F_{(6,1928)} = 3.09$; $p = 0.005$), reflecting the fact that the difference in performance between the Distraction and Non-distraction trials was most pronounced during the first Session-Block and was constant, at a slightly lower value, for the remaining 6 sesssion blocks. Therefore, subsequent models used a two-level session-block variable in which the first three sessions were designated "Session-Block 1" and the final 17 sessions were designated "Session-Block 2".

Percent Correct: For percent correct, neither the main effect of Genotype ($F < 1.0$) nor the interaction between Genotype and Distraction condition ($F_{(1, 53)} = 3.00$, $p = 0.09$) was significant. However, significant interactions were found between Genotype and Session-block ($F_{(1, 37.4)} = 5.92$, $p<0.02$), and Genotype, Session-block and Distraction condition ($F_{(1, 458)} = 11.97$, $p = 0.0006$; see Fig. 6). This three-way interaction reflected the fact that the two genotypes differed significantly only on the Non-distraction trials during session block 1 ($p= 0.01$). The two groups did not differ in performance on the Non-distraction trials in session block 2, nor on the Distraction trials in either block of sessions, despite the profound disruption produced by the distractors (relative to Nondistraction trials) in both blocks of sessions ($p < 0.0001$).

Additionally, the interaction of Genotype and Previous Trial Outcome was marginally significant ($F_{(1, 36.7)} = 3.4$, $p = 0.07$). Although both groups performed more poorly on trials following an error than on trials following a correct response (p's = 0.0001, 0.003, for the KO and WT mice, respectively), this error-induced drop was more pronounced for the KO mice than for controls, as described above for the Baseline task.

The fact that the KO mice were impaired relative to controls during the Non-distraction trials of session-block 1, whereas their overall performance did not differ from controls during the Baseline task (identical trial characteristics) suggests that, for the KO mice, the unpredictable presentation of the distractors produced a generalized disruption in performance that extended beyond the Distraction trials into the Non-distraction trials. An additional analysis was conducted in which the Non-distraction trials of session-block 1 in the Distraction task were directly compared to the final session of the Baseline Task. This analysis revealed a significant interaction between Genotype and Trial type ($F_{(2,91.5)} = 4.02$, $p = .02$; Figure 6), the latter term being a 3-level variable designating the three trial types: (1) Baseline Task, (2) Non-distraction

trials in the Distraction Task, and (3) Distraction trials in the Distraction Task. This interaction reflected the fact that the two groups differed only for the Non-distraction trials of the Distraction Task. They did not differ during the Baseline Task, which involved identical stimulus parameters to the Non-distraction trials of the Distraction Task. In addition, the KO mice performed significantly less well during the Non-distraction trials of the Distraction Task than during the last session of the Baseline task (p= 0.01; see Fig 6), which was not the case for the controls.

Inaccurate Responses: The analysis of percent inaccurate responses did not reveal a main effect of Genotype ($F_{(1, 38)} < 1.0$; ns), but the 2-way interaction of Genotype and Session-block ($F_{(1, 38.9)} = 4.25$, $p = 0.04$) and the three-way interaction of Genotype, Session-block and Distraction condition ($F_{(1, 491)} = 16.61$, $p = 0.01$) were significant. The 3-way interaction reflected the same pattern of findings seen for percent correct; namely that the two genotypes differed in the rate of inaccurate responses only for the Non-distraction trials in session-block 1 ($p < 0.0001$). They did not differ on Distraction trials in either Session-block, nor on Non-distraction trials for session-block 2. Because the analysis of percent inaccurate responses also implicated a generalized disruption in performance due to the unpredictable presentation of the distractors, the same type of analysis was conducted as described above for percent correct, which included the final session of the Baseline task and the first session block of the Distraction Task. This analysis revealed a significant interaction between Genotype and Trial type ($F_{(2,94.5)} = 3.99$, $p = .02$; Fig. 6), reflecting the identical pattern seen for percent correct.

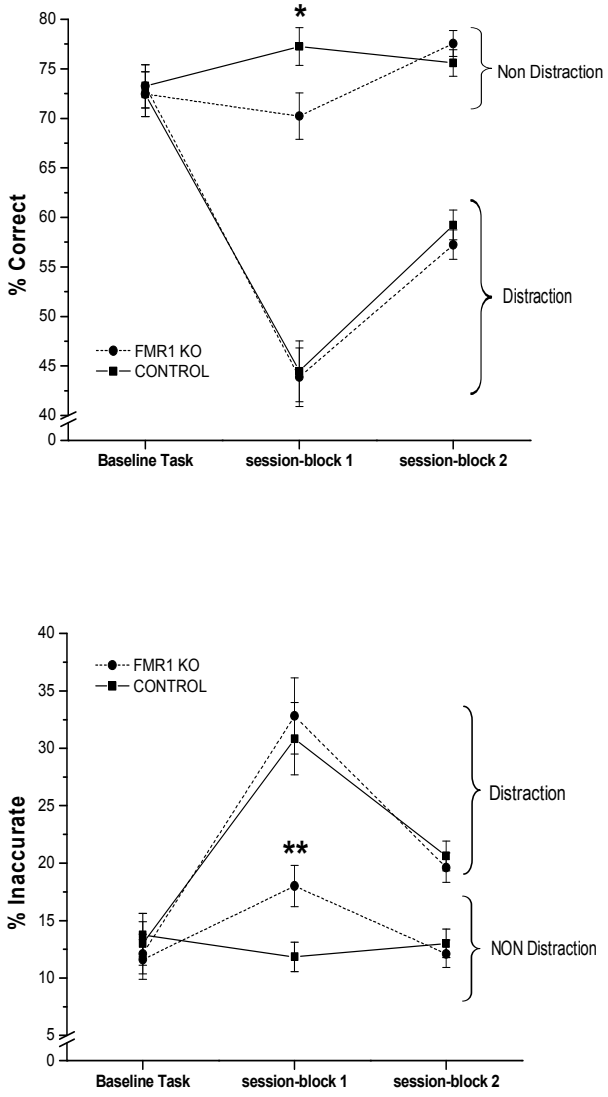

Figure 2-6: Performance on the final session of the Baseline task compared to the two blocks of sessions in the Distraction task. Top panel: The KO mice performed significantly less well ($p<0.01$) than controls during the first three sessions on the Distraction task (session block 1), specifically on the trials without olfactory distractors (the Non-Distraction trials). Lower panel: This pattern was driven largely by an increased rate of inaccurate responses (responses made to an incorrect port after cue onset). Note that omission errors and premature responses are counted as errors but are not considered "inaccurate responses". Bars represent the mean (+/- SEM).

The two groups differed only for the Non-distraction trials of the Distraction Task, not for the final session of the Baseline Task nor the Distraction trials. For the KO mice, the rate of inaccurate responses increased significantly from the final session of the Baseline task to the Non-Distraction trials during session-block 1 of the Distraction Task ($p = 0.006$) but not for the controls ($p = 0.32$).

Premature Responses: A borderline effect of Genotype was detected ($F_{(1, 41.1)} = 3.71$, $p = 0.06$), reflecting the fact that the *fmr1* KO animals tended to commit more premature responses than controls in this task. Because the two groups of mice did not differ for this measure in the Baseline Task, this finding indicates a generalized disruption in response to the unpredictable presentation of the olfactory distractors, which then manifested as deficient inhibitory control. The fact that the interaction of Genotype and Distraction condition was not significant ($F_{(1, 115)} = 1.25$; $p = 0.26$) indicates that the increase in premature responses was not limited to the distraction trials, supporting this interpretation.

5. Analysis of the videotapes

Due to apparatus malfunction, videotapes were not available for all animals. For these analyses, the sample size was 12 WT and 13 KO mice.

Wall-climbing increased in both groups in response to the presentation of the distractor ($p < .05$) and on trials following an error ($p < .01$), indicating that this behavior was reflective of disruption experienced by the animal. The increase in wall-climbing on distraction trials was comparable for the two groups, but the increase observed on Nondistraction trials (relative to the Baseline Task) was significantly greater for the KO mice than for the WT controls ($p = 0.05$). As seen in Figure 7, 62% of the KO mice had difference scores greater than zero, whereas only 25% of the

controls had a difference score greater than this value. This finding provides further evidence that the unpredictable presentation of the distractors produced a more generalized disruption in performance for the KO mice than for the controls.

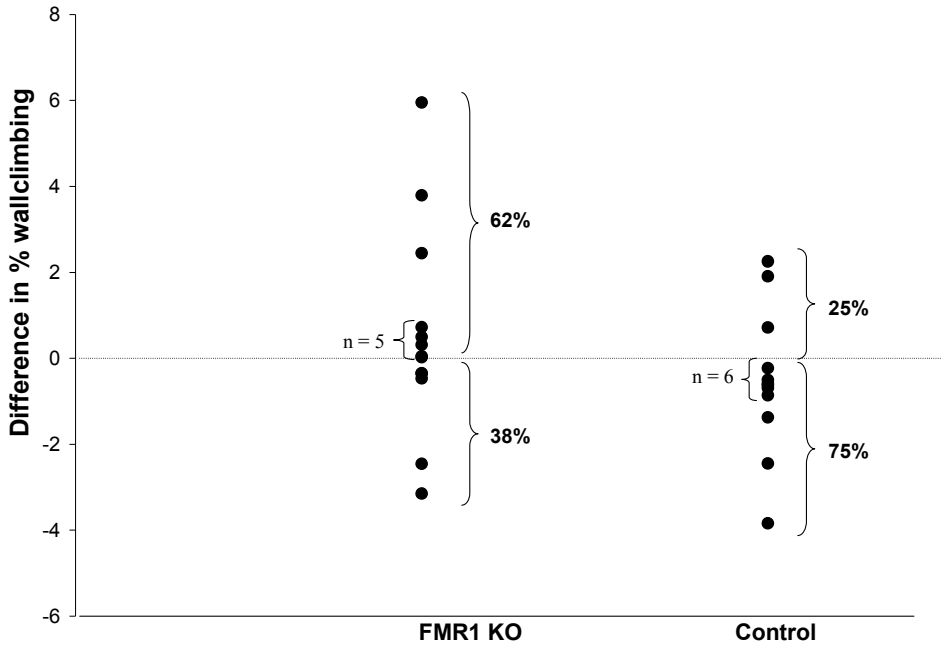

Figure 2-7: Difference in wall-climbing between the Baseline task and the Nondistraction trials of the Distraction task. The increased wall-climbing observed on Nondistraction trials (relative to the Baseline task) was significantly greater for the *fmr1* KO mice than controls (p= 0.05). 62% of the KO mice had difference scores greater than zero, in contrast to 25% of the controls. This finding provides further evidence that the unpredictable presentation of the distractors produced a more generalized disruption in performance for the *fmr1* KO mice than for the WT controls. Note that the overall rate of wall-climbing was relatively low (median = 4% per trial, during the Baseline Task); thus, a difference score of 2% represents a 50% increase in this behavior.

DISCUSSION

The present findings implicate impairments in inhibitory control and attention in the *fmr1* mutant mice, which were most pronounced during the first few sessions of a new task, immediately following a change in task characteristics. This pattern – whereby inhibition deficits and attentional dysfunction become manifest under times of arousal and when confronted with changing task demands – recapitulates findings from humans with FXS whereby overstimulation and difficulty with change leads to loss of behavioral control and attentional dysfunction (Cornish et al., 2001; Hagerman, 1996; Mazzocco et al., 1993; Merenstein et al., 1994; Munir et al., 2000; Schapiro et al., 1995). The evidence for dysfunction in each of these domains is delineated below.

Impaired inhibitory control

A prominent aspect of the *fmr1* mutant phenotype revealed in this task series is impaired inhibitory control or impulsivity. In Attention Task 1, the first task in which a delay was imposed between trial initiation and cue presentation, the KO mice committed 30% more premature responses than the WT controls, indicative of an impaired ability to withhold responding. This deficit was transient, however; the two groups did not differ in premature response rate by the final testing session on this task. In light of this pattern, impaired learning of the task contingencies could be responsible for the higher rate of premature responses; i.e., learning that now, on some trials, the cue would be presented after a delay. However, the normal learning rate of these same mice in a series of olfactory discrimination and reversal learning tasks, as well as an olfactory learning set task (Moon et al., in prep), suggests that basic associative ability is not impaired in these mice, consistent with prior studies of F1

hybrid *fmr1* mutant mice (e.g., Yan et al., 2004). Based on the pattern of results in the present series of tasks (discussed in more detail below), a more likely explanation is that the neural systems underlying inhibition are abnormal in the KO mice, but that deficient inhibitory control is evident only under conditions that arouse and/or disturb the animals, such as when task characteristics change. In the present case, Attention Task 1 was the first task in which the mice were required to wait on some trials for the cue to be presented, a profound change in task characteristics that was accompanied by a drop in reinforcement from 80% correct (on the prior training tasks) to 10% correct in the early sessions on Attention Task 1. The videotape data from the Baseline and Distraction Tasks, described below, support the inference that changing task contingencies produced arousal in all mice, but that the WT mice were better able to regulate this increased arousal than the KO mice.

This interpretation is supported by the fact that two other instances of impaired inhibitory control re-emerged when task characteristics changed. The analysis directly comparing performance on the final session of Attention Task 1 to the first session of the Sustained Attention Task (consecutive sessions) revealed a pronounced increase in premature response rate for the KO mice, whereas no change was seen for the controls across these two sessions. Because the two groups had not differed in premature response rate for the final session of Attention Task 1, the increased premature response rate of the mutant mice early in the Sustained Attention Task appears to result from the slight change in task characteristics. The subtlety of the change in task parameters is notable: The only characteristic that differentiated the two tasks was that cue duration varied randomly across trials in the Sustained Attention Task, whereas it was constant in Attention Task 1; the 4 pre-cue delays were identical in the two tasks, as were the basic contingencies. Note too that the poorer performance of the KO mice on this first session was not specific to any cue duration; premature responses are, by

definition, independent of the duration of the visual cue on a given trial. Thus, the subtle change in task characteristics appears to have generally disrupted the KO mice, which manifested as impaired inhibitory control. A slight increase in premature response rate of the KO mice, relative to controls, was also evident throughout the Distraction Task. Because this was not seen in the prior Baseline Task, this re-emergence of an increased premature response rate appears to reflect the arousal caused by the unpredictable presentation of the olfactory distractors.

Impaired attention

The Sustained Attention Task placed the greatest demand on sustained attention or vigilance, as the pre-cue delays were often long, and the cue duration was variable and sometimes very brief. As noted above, the most robust differences between the KO and WT mice in this task were seen on the first session, indicative of difficulty in dealing with the change in task characteristics. This performance drop on session 1 was primarily driven by the increase in premature response rate and is therefore indicative of impaired impulse control, rather than impaired attention per se. However, analysis of performance across the 20 sessions on this task revealed evidence for attentional dysfunction in the KO mice: They committed a higher rate of inaccurate responses than controls overall, with group differences being most pronounced during the final third of each testing session, a pattern that specifically indicates impaired sustained attention.

Two other instances of impaired attention in the KO mice appear to be the indirect result of impaired arousal regulation and inhibitory control. First, during the first three sessions of the Distraction Task, the KO mice committed a significantly higher rate of inaccurate responses than the WT mice, specifically on the trials without distractors. Impaired accuracy was not seen later in the task, following some degree

of habituation to the olfactory stimuli, nor during the Baseline Task, a task that was identical to the Non-distraction trials of the Distraction Task. Thus, whereas the distractors disrupted performance of the WT mice only on the Distraction trials, they disrupted performance of the KO mice on both the Distraction and Non-distraction trials. This generalized disruption seen in the KO mice seems to reflect two factors: (1) the arousal caused by the change in task characteristics (as seen in the transition from Attention task 1 to the Sustained Attention task), mirroring the impaired ability to deal with change seen in humans with FXS and autism (Kau et al., 2000; Rogers et al., 2001); and (2) hypersensitivity to potent sensory stimuli, a prominent feature of humans with FXS (Baranek & Berkson, 1994; Cohen et al., 1988; Hagerman, 1996; Miller et al., 1999). Studies of humans with FXS suggest that sensory processing alterations not only tax cognitive function but also lead to emotional arousal which is incompatible with focused attention (Cornish et al., 2004; Hagerman, 1996; Merenstein et al., 1994).

The videotape data provide converging evidence that the KO mice were more aroused by the olfactory distractors and/or change in task characteristics than the controls. Wall-climbing increased early in the Distraction Task, relative to the Baseline Task for both groups of mice, indicating that the rate of this behavior provides an index of the arousal produced by the unpredictable presentation of the olfactory stimuli. For this measure, the increase (relative to the Baseline Task) was similar for the two groups on the distraction trials, but was significantly greater for the KO mice than for Controls on the Non-distraction trials, indicative of generalized arousal, as seen for accuracy of responding.

Analysis of performance as a function of the outcome of the prior trial (correct or incorrect) revealed another instance of attentional dysfunction that appears to be secondary to impaired regulation of arousal or negative affect. Performance of both

groups was significantly disrupted by committing an error; i.e., all types of errors increased on trials following an error, relative to trials that followed a correct response. Whereas this basic pattern was seen for both groups, the increase in error rate on post-error trials was more pronounced for the KO mice than for controls in both the Baseline and Distraction tasks. Although some studies with human subjects have reported an exceptionally low error rate on trials following an error (e.g., Laming, 1979; Robertson et al., 1997), indicating the operation of an executive, error-correction system, localized to the anterior cingulate cortex (see Bush et al., 2000; Fernandez-Duque et al., 2000), the finding uniformly seen in our rodent studies -- increased error rate on post-error trials (Gendle et al., 2003, 2004; Morgan et al., 2001, 2002)-- has also been reported in some human studies (e.g., Elliott et al., 1996; Rabbitt & Rogers, 1977). This pattern likely reflects a dominant influence of the emotional reaction engendered by committing an error. Consistent with this view, an electrophysiological measure of error detection, termed the error-related negativity (ERN), varies as a function of individual differences in negative affect and emotionality (Luu et al., 2000). Similarly, depressed subjects exhibit a more pronounced increase in error rate on post-errors trials than non-depressed subjects (Elliott et al., 1996). Thus, the current finding that the attentional disruption seen on post-error trials was more pronounced for the KO mice than WT controls provides converging evidence for dysregulation of affect in the KO mice (for additional discussion, see Strupp & Beaudin, 2006).

Although these latter two instances of impaired performance in the KO mice seem most appropriately viewed as the indirect consequence of impaired arousal regulation and/or inhibitory control, it is notable that the resulting impairment was attentional in nature. This inference, based in part on the characteristics of these tasks, gains support from other findings from this same cohort of mice (Moon et al., in prep).

In an olfactory reversal learning task, the KO mice exhibited more pronounced behavioral disruption than controls when the contingencies were reversed, and the reinforcement rate consequently dropped from 80% to 0% correct. Coded videotapes of the mice performing this task revealed that all mice exhibited higher rates of wall-climbing early in the task (relative to later in the task, after the contingencies had been mastered), and that this early behavior change was more pronounced for the KO mice. However, neither response accuracy nor learning rate differentiated the groups in this task. Thus, in a task without attentional demands, the impaired arousal regulation of the KO mice did not impair performance, in contrast to the attention tasks described here, where the animals were required to wait for, and then detect, brief visual cues, unpredictable in onset time and location.

CONCLUSIONS AND IMPLICATIONS

This study provides the first evidence that the hallmark deficits in human FXS – impaired attention, inhibitory control, arousal regulation, and adaptability to change– are also seen in the *fmr1* KO mouse model of FXS. The present findings also demonstrate that impaired regulation of arousal or affect appears to be a critical factor underlying the appearance of impaired attention and inhibitory control in the KO mice.

Although the nature of the dysfunction observed here provides strong encouragement for the validity of this mouse model, large deficits were seen only for a session or two, immediately following a change in task characteristics; the lasting deficits (i.e., seen across days or weeks of testing) were relatively small in magnitude. This aspect of the findings may indicate that a neural system playing a key role in FXS symptomatology is not used in a comparable way in the mouse. For example, as noted by Yan et al. (2004), if dysfunction of the neocortex is central to the deficits in human

FXS, it is possible that the functional effects of this type of damage might be much less significant for the mouse, which has a much smaller and less complex neocortex. It is also possible that certain characteristics of the present tasks may have led to an underestimate of the degree of impairment of the mutant mice in terms of inhibitory control and attention. Each of these tasks assessed the ability to attend to a single stimulus in relatively calm, non-complex, testing conditions. Moreover, each task was administered for many sessions, allowing the characteristics of the task to become rote and predictable. These characteristics contrast with everyday life in which the cues to which we must attend occur amidst a complex background, with new stimuli constantly entering our perceptual world. Based on the present findings that the attentional and inhibitory control deficits of the mutant mice were most pronounced at the beginning of each new task, particularly when confronted with novel distractors, it is likely that these mice would be more impaired, relative to controls, if their attentional abilities were tested in a more complex environment and the contingencies of the tasks were frequently changed; i.e., conditions that more closely approximate the complexity of the real world. Nonetheless, the fact that significant deficits in these key domains were detected in the present study, despite these task design limitations, provides strong support for the validity of this animal model.

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CHAPTER THREE

IMPAIRED AROUSAL REGULATION IN A MOUSE MODEL OF FRAGILE X SYNDROME

ABSTRACT

This study was designed to further assess cognitive and affective functioning in a mouse model of Fragile X syndrome (FXS), the *fmr1* ^{tm1Cgr} or *fmr1* "knockout" (KO) mouse. KO mice and wild-type littermate controls were tested on Learning Set and Reversal Learning tasks. The KO mice were not impaired in associative learning, transfer of learning, or reversal learning, based on measures of learning rate. However, analysis of videotapes of the Reversal Learning Task revealed that (1) for both genotypes, wall climbing and activity level were higher during the first two sessions of the task than during the final two sessions (after the new contingencies had been mastered), indicating that the initial higher rate of these behaviors reflected heightened arousal produced by reversal of the contingencies and low rate of reinforcement; and (2) the increase in both behaviors early in the task was more pronounced for the KO mice than for controls; group differences in these behaviors were not seen in the final sessions of the task. This pattern of findings indicates that arousal regulation is impaired in the KO mice. Although this dysfunction did not slow learning in the current tasks, other findings from this same cohort of mice (Moon *et al.*, submitted) demonstrated that impaired arousal regulation significantly disrupted performance in attention tasks where the target cue was brief and presented unpredictably. As dysregulation of arousal and affect is a prominent feature of FXS, the present findings provide additional support for the validity of this mouse model.

INTRODUCTION

Fragile X syndrome (FXS) is the most common inherited form of mental retardation (Crawford *et al.*, 1999) and the most common known cause of autism (Hagerman, 2002; Mazzocco *et al.*, 1997). Expansion of a CGG repeat sequence in the promoter region of the *fmr1* gene (Khandjian, 1999; O'Donnell & Warren, 2002) leads to transcriptional silencing of this gene (Verkerk *et al.*, 1991; reviewed in O'Donnell & Warren, 2002). The resulting deficiency of the encoded protein, called the Fragile X Mental Retardation Protein (FMRP), directly and/or indirectly gives rise to the FXS phenotype. The cognitive dysfunction is not global in nature but rather primarily affects various aspects of executive functioning, such as attention and inhibitory control (Baumgardner *et al*., 1995; Hagerman, 1996; Lachiewicz *et al*., 1994; Largo & Schinzel, 1985; Turk, 1998), with up to 73% of affected individuals meeting the diagnostic criteria for Attention Deficit Hyperactivity Disorder (Baumgardner *et al.*, 1995). Other prominent features of FXS include hypersensitivity to sensory stimuli (Baranek & Berkson, 1994; Cohen *et al.*, 1988; Hagerman, 1996; Miller *et al*., 1999), autistic features (Lachiewicz *et al*., 1994), impaired regulation of arousal and/or emotion (Borghgraef *et al.*, 1990; Hagerman & Sobesky, 1989; Kerby & Dawson, 1994), social anxiety (Cornish *et al*., 2004a; Hagerman, 2002; Reiss & Freund, 1992), and seizure susceptibility (Musumeci *et al*., 1999, 2001).

The current treatments for FXS focus on providing symptomatic relief, such as methylphenidate for the ADHD symptoms and SSRI's for anxiety; no treatments are clinically available that target the cascade of events leading from loss of FMRP to aberrant brain development. However, recent findings concerning the role of FMRP suggest that it may be possible to develop treatments to intervene in this process and thereby normalize brain development in individuals with FXS. For example, one

conceptualization implicates excessive activity at group 1 metabotropic glutamate receptors (mGluRs) as the cause of many, if not all, of the FXS symptoms, including cognitive dysfunction, anxiety, and increased seizure susceptibility (Bear, 2005; Bear *et al*., 2004). It follows therefore that treatment with mGluR antagonists, such as MPEP, may dramatically improve outcome in this syndrome. Support for this hypothesis has been provided by two studies, one concerning the *fmr1* knockout (KO) mouse model of FXS (Yan *et al*., 2005), and one using a Drosophila model of the syndrome (McBride *et al*., 2005). One stumbling block to further testing this theory and the potential clinical efficacy of such drugs is that cognitive dysfunction – which is central to the phenotype of FXS – has been very difficult to demonstrate in animal models. Commonly-used learning and memory tasks, such as the Morris water maze and the radial arm maze, have either been unable to differentiate the *fmr1* KO mice from controls (Dobkin *et al*., 2000; Paradee *et al.*, 1999; Peier *et al*., 2000; Yan *et al*., 2004) or have revealed very small deficits in the KO mice that are apparent only in some background strains (Bakker *et al*., 1994; Cianchetti *et al*., 1991; Hinds *et al*., 1993; Mineur *et al*., 2002). Results seemingly contradictory with the phenotype of humans with FXS have also been reported. For example, in some learning tasks, *fmr1* KO mice performed better than their WT littermates (Fisch *et al*., 1999; Frankland *et al*., 2004; Van Dam *et al*., 2000).

One factor that may contribute to the apparent lack of cognitive dysfunction in the *fmr1* KO mouse is that the most prominent areas of dysfunction in human FXS have not been assessed, notably including attention, inhibitory control, regulation of arousal or emotion, and resistance to change. The present study was designed to test this hypothesis. The performance of F1 hybrid *fmr1* KO mice (a C57BL/6J x FVB/NJ cross) and wild-type (WT) littermate controls was compared on a series of visual attention tasks (described in Moon et al., submitted), an olfactory learning set task, and

an olfactory reversal learning task; the latter two are described in the present report. The learning set task was included to tap transfer of learning, an area of dysfunction commonly seen in mental retardation syndromes (Campione & Brown, 1984; Campione *et al*., 1985). Learning set tasks have previously revealed cognitive impairment in animal models of MR syndromes (Strupp *et al*., 1990; 1994), notably including disease models for which basic learning tasks had not revealed dysfunction (reviewed in Strupp & Levitsky, 1990; Strupp & Diamond, 1996). The reversal learning task was hypothesized to reveal dysfunction in the *fmr1* KO mice for three converging reasons: First, reversal learning taps inhibitory control and adaptability to change, capabilities that are impaired in humans with FXS (Kau *et al*., 2000; Rogers *et al*., 2001). Second, reversal learning is dependent on the integrity of the prefrontal cortex (Dias *et al*., 1996; Remijnse *et al.*, 2005; Smith *et al*., 2004), a brain region believed to be dysfunctional in FXS (Cornish *et al*., 2004b; Guierreiro *et al*., 1998; Hagerman, 2002; Menon *et al*., 2004; Tamm *et al*., 2002). Finally, due to the frustration engendered by the reversal of contingencies and initially high error rate, reversal learning tasks provide an index of emotion regulation, an area of dysfunction in FXS (Borghgraef *et al.*, 1990; Hagerman & Sobesky, 1989; Kerby & Dawson, 1994). The reversal learning task was videotaped to provide richer information on putative genotypic differences in the regulation of arousal than provided by the automated performance measures alone. In a prior study of a mouse model of Down syndrome and Alzheimer's Disease (the Ts65Dn mouse), videotape coding revealed that committing an error frequently led to stereotypic jumping in these trisomic mice (Driscoll *et al.*, 2004), one of the few demonstrations of naturally-occurring stereotypic behavior in a mouse model of a mental retardation (MR) syndrome, despite the fact that such behaviors are a hallmark of many human MR syndromes (Branford

et al., 1998; Miller & Jones, 1997). This finding demonstrates the importance of videotape coding as an adjunct to the collection of automated performance measures.

MATERIALS AND METHODS

Subjects

Breeding of the mice was conducted at the University of Colorado Health Sciences Center, Denver, CO. Breeder pairs of C57BL/6J-*Fmr1*^{tm1Cgr} (B6.129-*Fmr1* ^{tm1Cgr}) (*fmr1* KO) and wild-type (WT) C57BL/6J mice were purchased from Jackson Laboratory, Bar Harbor, ME. In the KO mice, the *fmr1* gene had been disrupted by targeting a transgene to exon 5 with homologous recombination (Bakker *et al.*, 1994). The heterozygous females 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 mice from the same litters. Male offspring (15 WT and 13 *fmr1* KO) from these litters served as subjects in the present experiment. Genotyping was conducted as described in Nielson *et al.* (2002).

The strategy of studying the *fmr1* mutation on an F1 hybrid background was followed for several reasons. First, these mice have normal hearing (unlike C57BL/6J mice) and are not blind or susceptible to seizures (unlike FVB/NJ mice) because these deficits are recessive (Goelz *et al.*, 1998; Johnson *et al.*, 1997; Pittler & Baehr, 1991; Zheng *et al.*, 1999). In addition, this procedure produces *fmr1*- KO and WT mice from the same litters, thereby equating the intrauterine and postnatal environments of the experimental and control groups. Finally, in light of the pronounced strain differences in startle, anxiety, and performance in various learning tasks, it is risky to

draw conclusions about the effects of a given mutation from studies of inbred mice, as background strain effects may greatly accentuate or obscure gene effects (Paradee *et al*., 1999).

At 6-7 months of age, the mice were tested on a passive avoidance task (data not shown). At 7-8 months of age, the mice were transported to Cornell University for further behavioral testing. At Cornell, the mice were housed singly in polycarbonate cages, with food and water available ad libitum. The mice were housed individually due to previous observations that male mice of this strain, caged in pairs, are prone to fighting when they are reunited after being removed for testing (Crnic, L.S., unpublished data). After acclimating to the new environment for several weeks, the mice were placed on a restricted feeding regimen in order to maintain motivation for food reward during the behavioral testing. The daily ration was gradually reduced and then maintained at a level that produced target weights at approximately 80-85% of their pre-restriction weight. A target weight of 80-85% was selected because the mice had been on *ad libitum* feeding throughout their lives, resulting in somewhat elevated levels of adiposity.

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 at UCHSC and Cornell University, both AAALAC accredited institutions.

Apparatus

The mice were tested individually in one of six automated Plexiglas chambers, each controlled by a PC and situated in an insulated, sound-attenuating exterior chamber. The testing chambers were adapted from the "nine-hole" operant chambers recently developed to assess attention in mice (Humby *et al.*, 1999). The slightly

curved rear wall contained five circular response ports, 1 cm in diameter, located 2 cm above the floor and 5mm apart. Only three of the five ports were operative for the olfactory learning tasks described in the present report: the far right, far left, and center ports. For these tasks, scented air served as the discriminative cues and was projected from these three ports on each trial. The scented air was produced by passing filtered, compressed air through small bottles of liquid odorant, using solenoid airflow valves and airflow meters. The airflow rate was 1.0 L/min.

On the chamber wall opposite the five response ports was an alcove (15 mm wide, 2 cm above the floor) containing the dipper (ENV0302M, MED Associates, Inc., St. Albans, VT) which dispensed the liquid food reward (liquefied AIN-76A, a sweet, nutritionally-complete diet; "Shake and Pour", BioServ, Inc.). Access to the dipper alcove was controlled by a thin metal door, which was activated by a motor located on the outside of the testing chamber.

The mice initiated each trial by making a nosepoke into the dipper alcove port. Then, following a 1 s "turn-around" time, the olfactory cues were simultaneously presented from the three response ports. A nosepoke into any of these three ports constituted a response (or choice). Nosepokes into the response ports and the dipper alcove were detected by infrared photodiodes, positioned inside each port, 0.5 cm from the opening.

Each chamber was fitted with an exhaust system, which transported the air from the chamber directly to the room exhaust ventilator system at a rate of four complete air changes per minute. All automated events (door opening, dipper movement, responses, etc.) within each chamber were timed, controlled, and recorded by custom programs written in QBASIC.

For videotaping, each chamber was equipped with a wide-angle infrared video camera and infrared LED light source attached to the ceiling directly over the center of

each testing chamber. The camera allowed full view of the mouse at all times. Each camera was connected to a separate VHS VCR. An array of infrared LEDs, positioned outside the Plexiglas chamber but within viewing range of the camera, provided information about the various events during each trial (e.g., demarcation of the intertrial interval, presentation of the olfactory cue, whether a response was correct or incorrect, access to liquid reward).

Behavioral testing

At 8 months of age, the mice were administered a four-stage training procedure designed to shape the general response sequence required for completion of each trial in the subsequent tasks. These training stages are described in a prior report (Driscoll *et al*., 2004). Briefly, the mice learned that the door to the dipper alcove would be raised at the start of each trial and that a nosepoke into the alcove port, followed by a nosepoke into one of the five response ports, would produce the delivery of 0.04 ml of the liquid diet in the dipper alcove. These four training phases were mastered in approximately 8-10 sessions. The mice were then tested on a series of visual discrimination and attention tasks, lasting approximately 6 months (described in Moon *et al*., submitted).

The tasks described in the present report were initiated when the mice were 20-22 months of age. For each of these three olfactory discrimination tasks, three different odors were presented on each trial, one from each of the three operative ports (middle, far left and far right). The odor emitted from each port was randomly determined, but balanced for the test session.

The first task, a Learning Set Task, comprised two 3-choice simultaneous olfactory discrimination tasks, administered sequentially, to assess basic associative ability and transfer of learning between versions of the same task (different exemplars).

Two different sets of odors were used: (1) rum-pineapple-peach (set A); and (2) lime-vanilla-pear (set B). Half of the mice of each genotype were tested on set A first, half on set B first; then each mouse was tested on the alternate set. In addition, within each of the two sets, the correct odor was pseudo-randomly assigned but counterbalanced across genotypes and order of odor sets. Within set A, the possible correct odors were rum or pineapple; within set B, the possible correct odors were lime or vanilla. For each of the two olfactory discrimination tasks within the Learning Set Task the learning criterion was 80% correct for two of three consecutive test sessions.

Testing on the Reversal Learning Task was initiated for each mouse in the test session immediately following mastery of the Learning Set Task. For this task, the odor that had been correct in the second olfactory discrimination of the Learning Set Task was now designated as incorrect, and one of the previously incorrect odors was now the correct odor (i.e., associated with reward). The learning criterion was the same as for the Learning Set Task.

For all of these tasks, the mouse initiated each trial by making a nosepoke into the dipper alcove, after which the door closed. These nosepokes were required to initiate each trial but did not produce a reinforcement. After a 1-s "turn around time" (allowing the mouse to face the response ports), the three olfactory cues were emitted simultaneously from the response ports. The scented air was projected continuously for 5 s or until a nosepoke response was made. A nosepoke into the port emitting the correct odor was rewarded by 5 s access to the liquid reinforcer in the dipper alcove. An incorrect response was followed by a 5-s time-out period, signaled by the illumination of a 2-W houselight on the ceiling of the chamber. A time-out was also imposed following a "nontrial", the term given to trials in which the alcove door was raised at trial onset but the mouse did not enter the alcove in the following 60 s; nontrials were very rare. A 5-s intertrial interval separated adjacent trials. All trials

on which the mouse made an initiation poke into the dipper alcove (regardless of the outcome of the trial) were defined as response trials. Each session was terminated after 30 min or 70 response trials, whichever came first.

All testing equipment was thoroughly cleaned and dried following the testing of each mouse, using Odormute (R.C. Steele Co, Brockport, NY), a detergent containing an enzyme that removes olfactory cues (including pheromones).

Videotape Coding

All sessions of the olfactory reversal task were videotaped, and the first two sessions and the last two sessions on this task were coded for various behaviors, described below. The first two sessions, immediately after the change in contingencies, were considered most likely to reveal genotypic differences in regulation of arousal or affect. The final two sessions on the task, after task contingencies had been mastered and reinforcement rate was high, were also scored to ascertain whether group differences in the early sessions, if observed, were specific to arousing conditions, or were uniform throughout the task.

A coder scored each of these sessions for four behaviors: jumping, grooming, exploring, and wall-climbing. Frequency, duration, and location (right vs. left side of the chamber) of each behavior was recorded, using a computer program developed for these tasks. An index of activity level (hyperactivity) was also created, which denoted the number of times the mouse traversed from one side of the chamber to the other during each trial.

Reliability of the behavioral ratings was determined prior to proceeding with the coding of the Reversal Learning Task. These reliability analyses were based on the coding of session 4 of the Learning Set Task for eight pseudo-randomly selected mice; the eight sessions were balanced by testing chamber and genotype. To

determine intra-rater reliability, the coder scored each of the eight sessions twice (with time elapsed between recoding of the same session), and the results of the first round of coding were correlated with those of the second. To assess inter-rater reliability, the same eight sessions were coded by another trained coder, and the results from both individuals were compared. Coding of the reversal learning sessions commenced only after high levels of intra and inter-rater reliability were achieved ($r > 0.9$) for all behavioral measures.

Behavioral testing and coding of the videotapes were conducted by individuals blind to the genotypes of the animals.

Statistical Analyses

Statistical analyses were conducted on a Cornell University mainframe computer using the Statistical Analysis System (SAS; SAS Institute, Inc., Carey, NC), version 9.1.

Learning rate measures for the Learning Set Task and the Reversal Learning Task were 1) sum of errors to criterion and 2) sum of trials to criterion. These measures were analyzed using a mixed models analysis of variance procedure (SAS, Proc mixed) to account for the repeated observations on each animal.

An additional analysis was conducted for the Reversal Learning performance data, to permit direct comparison with the videocoding data, which were available for only the first two sessions and the final two sessions. For each of these four sessions, mean percent correct responses was calculated for each animal for each testing condition, defined by the following variables: Genotype, Previous Trial Outcome [correct or incorrect], and Session number. The analysis was conducted on these

means using a generalized linear mixed models procedure for conducting repeated measures analyses of variance with non-normal data (PROC GLIMMIX in SAS).

PROC GLIMMIX was also used to analyze video coding data, using the same variables as listed above with the exception that for some analyses (described in the results), the four sessions were grouped into two or three blocks. These analyses, too, were conducted on means calculated for each animal for each session or session-block.

T-tests were used to compare body weight and daily food intake of the two genotypes. For each of these analyses, a mean was calculated for each animal for the first and last testing sessions, and than the group means were calculated and compared.

RESULTS

Body Weight: The body weights of the groups did not differ ($t_{(27)}$= 0.70, n.s).

***Daily Food Intake*:** There was no effect of Genotype on mean daily food intake ($t_{(27)}$ = 1.17, n.s.).

Learning Set Task:

Analysis of errors to criterion revealed a significant effect of task (task 1 vs. task 2; $F_{(3, 38)}$ = 4.80, p = 0.006). The mice learned the second task faster than they learned the first task, reflecting successful transfer of learning. There was no effect of the particular sets of olfactory cues or the order in which the two sets were administered. The analysis did not reveal a main effect of Genotype ($F_{(1, 38)}$ = 0.42, p = 0.52; see Figure 1), or an interaction of Genotype and Task ($F_{(1, 38)}$ = 0.00, p = 0.96), indicating that the two groups did not differ in transfer of learning.

Analysis of trials to criterion revealed the same results.

Reversal Learning Task (Automated Performance Data):

An analysis was conducted to compare learning rate on the Reversal Learning Task with that of the second task of the Learning Set Task (i.e., original learning of the odor triplet used in the Reversal Learning Task). The analysis of errors to criterion revealed that the Reversal Learning Task took significantly longer to learn ($F_{(1, 38)}$ = 17.48, p = 0.0002), indicating that the mice had difficulty changing their behavior after a reversal of the contingencies. This analysis did not reveal either a main effect of Genotype ($F_{(1, 47)}$ = 0.04, p = 0.84; see Figure 1], or an interaction between Genotype and Task ($F_{(1, 38)}$ = 0.57, p = 0.45). The analysis of trials to criterion revealed the same results.

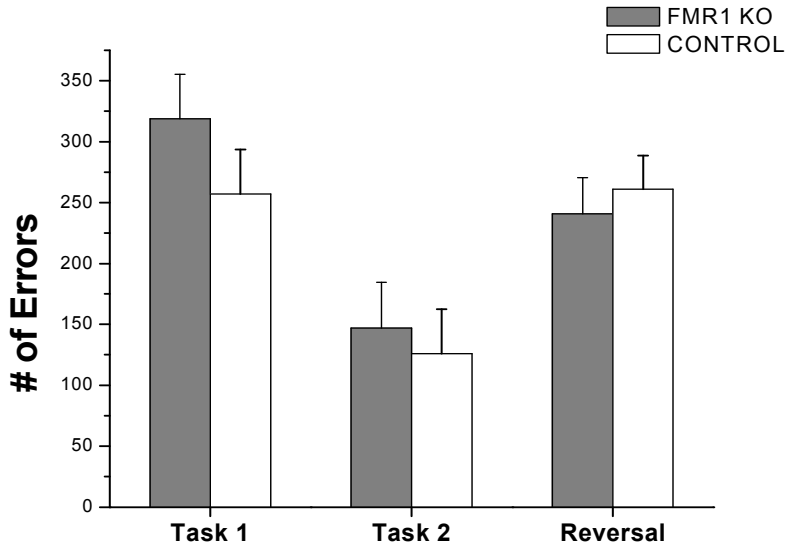

Figure 3-1. Mean (+/- SE) errors to criterion for the Learning Set Task (Tasks 1 and 2) and the Reversal Learning Task. Learning rate did not differ by genotype for any of these tasks.

For comparison with the videotape data, an additional analysis was conducted on performance (percent correct responses) during the first two sessions and last two sessions of the Reversal Learning Task. This analysis revealed a significant effect of Session ($F_{(3, 78.2)}$ = 189.30, $p<0.0001$; see Figure 2), reflecting the improvement in performance across these four sessions. Again, there was no effect of Genotype ($F_{(1,28.3)}$ = 0.1, p = 0.74), or an interaction between Genotype and Session ($F_{(3, 78.2)}$ = 0.06, p = 0.98).

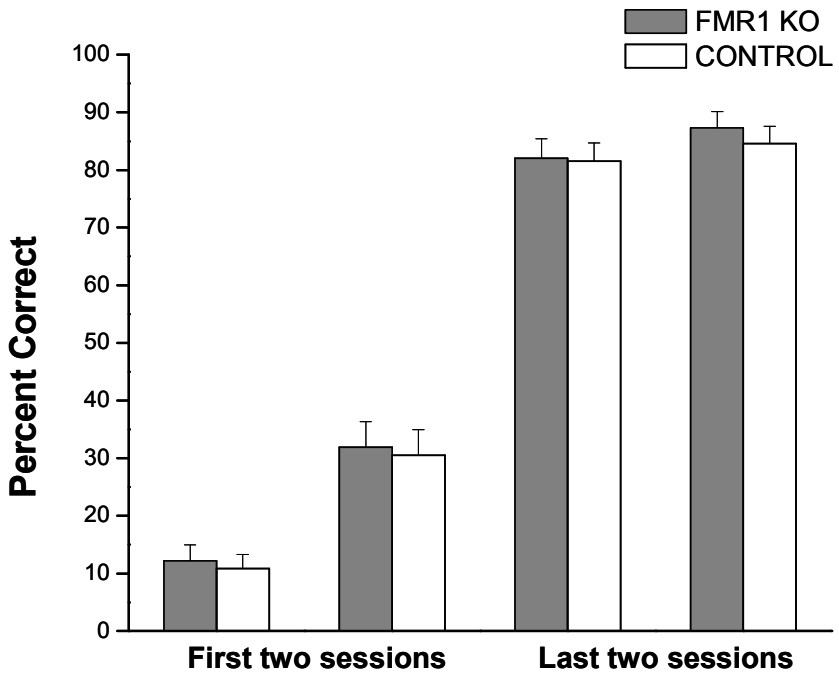

Figure 3-2. Mean (+/- SE) percent correct for the first two and last two sessions of the Reversal Learning Task. No genotypic differences were seen for any session.

Reversal Learning Task (Coded Videotapes)

Due to technical difficulties, videotape data were available for only 19 animals (10 WT and 9 KO mice).

A square root transformation was applied to the data for wall climbing, jumping, exploring, and grooming to normalize the distributions. For the analyses of these four behaviors, sessions 1 and 2 were collapsed into 'Session-block 1", and the final two sessions were collapsed into "Session-block 2" because for these dependent measures, sessions 1 and 2 did not differ from each other and sessions 3 and 4 did not differ from each other. For the analysis of activity level, only the final two sessions were collapsed, because sessions 1 and 2 differed significantly from each other.

***Hyperactivity*:** The dependent measure used to analyze activity level was [(total number of transitions) / (total number of trials)] as the dependent measure. A significant main effect of Previous Trial Outcome was found ($F_{(1,\ 104)} = 34.01$, $p<0.0001$), indicating that the mice were significantly more active on trials immediately following an error than on trials following a correct response. There was also an interaction between Session-block and Previous Trial Outcome ($F_{(2,104)} = 8.34$, $p = 0.0004$) indicating that the increase in activity level on trials following an error was more pronounced during the first two sessions than during the final session-block. Finally, a marginal interaction of Session-block and Genotype ($F_{(2,29.1)} = 2.97$, $p = 0.06$) revealed a different pattern of activity level across the three session-blocks for the two genotypes. As seen in Figure 3, the initial elevation in activity level was more prolonged for the KO mice than for the WT controls, with group differences being significant during the second session ($p = 0.05$). This pattern suggests impaired regulation of arousal in the KO mice.

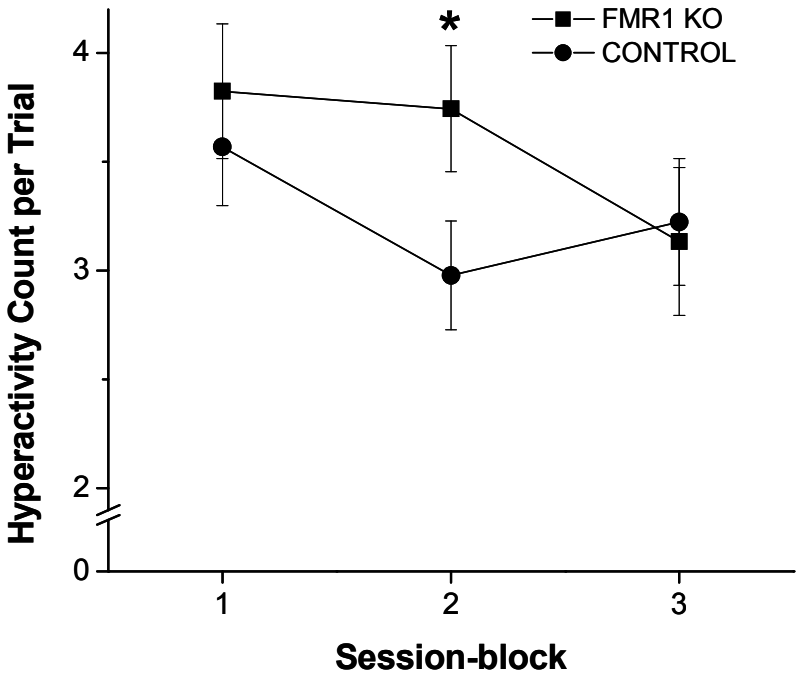

Figure 3-3. Mean (+/- SE) hyperactivity count per trial for the KO mice and littermate controls for the Reversal Learning Task. The initial elevation in activity level was more prolonged for the KO mice than for the WT controls, with group differences being significant during the second session. *, $p < 0.05$.

Wall Climbing: The dependent measure used to analyze wall climbing was [(total duration of wall climbing) / (total number of trials)]. The analysis revealed significant main effects of Session-block ($F_{(1,17.9)} = 34.19$, $p < 0.0001$) and Previous Trial Outcome ($F_{(1,17.9)} = 24.98$; $p < 0.0001$), indicating that the mice wall-climbed significantly more during the first Session-block than during the final Session-block, and on trials following an error relative to trials following a correct response. In addition, a significant interaction of Genotype and Session-block was found ($F_{(1, 17.9)} = 4.69$; $p = 0.04$; see Figure 4), demonstrating that the increase in wall-climbing during

Session-block 1 was significantly more pronounced for the KO mice than for controls. During Session-block 1, the KO mice wall-climbed significantly more than controls ($p = 0.007$), whereas genotypic differences were not seen during Session-block 2 ($p = 0.97$).

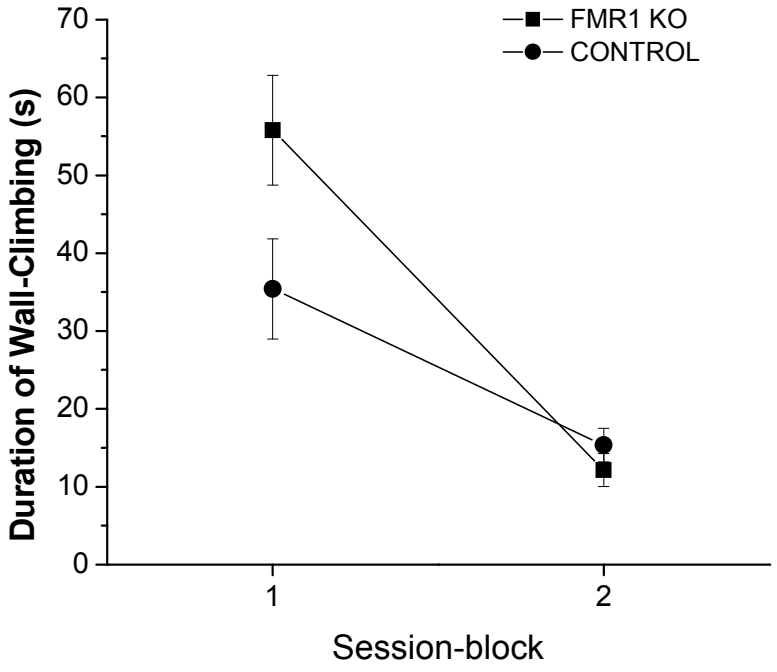

Figure 3-4. Mean (+/- SE) duration of wall-climbing across the two session-blocks of the Reversal Learning Task. The heightened wall-climbing during session-block 1 was significantly more pronounced for the KO mice than for controls. **, $p = 0.007$.

Exploring: The dependent measure used to analyze exploratory behavior was [(total duration of exploring)/(number of trials)]. The analysis revealed a significant effect of Session-block ($F_{(1, 54)} = 7.28$, $p = 0.009$) and Previous Trial Outcome ($F_{(1,54)} = 8.89$, $p = 0.004$). Exploratory behavior was greatest during the first Session-block and on

trials that followed an error. There was no main effect of Genotype ($F_{(1, 17.4)} = 2.59$, $p = 0.13$) nor interactions involving Genotype.

Grooming: The dependent measure used to analyze grooming was [(total duration of grooming)/(number of trials)]. There was a significant main effect of Previous Trial Outcome ($F_{(1,60.6)} = 9.85$, $p=0.003$), indicating that the mice groomed more on trials that followed an error than on those that followed a correct response. Grooming was not affected by Genotype ($F_{(1,26.5)} = 1.41$, $p=0.25$) or Session-block ($F_{(1, 60.6)} = 0.18$, $p = 0.67$), nor were there any interactions involving Genotype.

Jumping: The dependent measure used to analyze jumping behavior was [(sum of instances of jumping)/ (number of trials)]. The analysis revealed an effect of Session-block ($F_{(1, 13.7)} = 6.48$, $p=0.02$), but did not reveal effects of Previous Trial Outcome ($F_{(1, 13.7)} = 0.73$, $p = 0.4$), Genotype ($F_{(1, 2.33)} = 0.00$, $p = 0.99$), nor any interactions involving Genotype.

DISCUSSIONS

The present study assessed associative learning, transfer of learning, reversal learning and regulation of arousal in *fmr1* KO mice and WT littermate controls. Measures of learning rate did not reveal genotypic differences for any of these aspects of learning. However, analysis of videotapes of the mice performing the Reversal Learning Task provided evidence of impaired regulation of arousal in the KO mice. Each of these findings is discussed below.

The *fmr1* KO mice did not differ from WT littermate controls in the rate at which they mastered the initial olfactory discrimination task, indicating that basic associative learning is intact in these mutant mice. This finding is consistent with the normal learning rate seen in many different types of tasks in previous studies with the *fmr1* KO mouse (Bakker *et al*., 1994; Mineur *et al*., 2002; Yan *et al*., 2004).

Both groups learned the second olfactory discrimination task more quickly than the first, demonstrating significant transfer of learning across different exemplars of the same basic task. Moreover, the magnitude of this learning transfer was comparable for the two genotypes. Yan and colleagues (2004) reached a similar conclusion, based on a different type of task and a different method for assessing learning set formation. To our knowledge, these are the only two studies that have assessed learning set formation in either the *fmr1* KO mouse or humans with FXS. This finding further defines the cognitive phenotype of the *fmr1* KO mouse and clarifies similarities and distinctions between this animal model and models of other MR syndromes. For example, rats exposed to hyperphenylalaninemia early in life (models of maternal or classic PKU) do not exhibit impaired associative learning but are impaired, relative to controls, in the ability to transfer learning across similar tasks (Strupp *et al*., 1990; 1994), as commonly seen in mentally retarded humans (e.g., Campione *et al*., 1985; reviewed in Strupp *et al.*, 1994). There is growing evidence that different MR syndromes, while all characterized by low IQ, exhibit very different cognitive profiles in terms of spared and impaired functions (e.g., Bellugi *et al.*, 2000; Cornish *et al*., 2004a; Greydanus & Pratt, 2005; Wang & Bellugi, 1994). One cannot rule out the possibility that the *fmr1* KO mouse might exhibit impaired transfer of learning in some other types of tasks, but the available data indicate that this aspect of cognition is intact in this mouse model and, by extension, in humans with FXS. If so, this finding would add to the growing evidence that the dysfunction in FXS is quite

specific, limited primarily to aspects of executive functioning (including inhibitory control and attention), arousal regulation, and social behavior (see Cornish *et al*., 2004c; Hagerman *et al*., 2002).

In the present study, the *fmr1* KO mice also performed comparably to WT controls on the reversal learning task. This finding was unexpected because several lines of evidence, reviewed in the introduction section of this paper, led to the expectation that this type of learning would be impaired in this mouse model. In particular, the absence of genotypic differences in the duration of perseverative responding to the previously correct cue – an inference based on analyses of overall learning rate (trials to criterion; Figure 1) and percent correct responses during sessions 1 and 2 (see Figure 2) – appears inconsistent with some prior findings concerning FXS. Specifically, males with FXS committed a higher number of perseverative errors in the Wisconsin Card Sorting Task (WCST) than controls (Cornish *et al.*, 2001; Mazzocco *et al.*, 1993), and repeatedly responded to target stimuli in a visual search task, under conditions in which only the first response was "correct" (Wilding *et al*., 2002). There are several possible reasons for these apparently disparate findings. First, the tasks are quite different, despite the fact that all can be impaired by perseverative responding. For example, the WCST task assesses shifting between sets of predictive cues (i.e., extra-dimensional shifting), whereas reversal learning tasks tap the ability to reverse a previously learned contingency within a single dimension. Increased perseverative errors in the WCST can reflect impaired selective attention, rather than inflexibility or deficient inhibitory control; as such, the findings are not necessarily contradictory. Another factor that may have been instrumental in these different outcomes is the nature of the reinforcement used coupled with the motivational state of the subjects. In the human studies, the only consequence of committing an error is the feedback that the response

is incorrect. In contrast, in the present Reversal Learning Task, perseverative responding to the previously correct due leads to strings of nonrewarded trials, which may severely curtail this type of responding, since the animals are food-restricted and are rewarded for correct responses with food. These task characteristics may preclude the detection of deficient inhibitory control in the *fmr1* KO mice, notwithstanding the existence of dysfunction in this area in these mice, as demonstrated in a series of attention tasks (described below).

Although no prior studies of *fmr1* KO mice have assessed reversal learning within the context of a discrimination task (as used in the present study), several previous studies have compared *fmr1* KO mice and WT controls in maze tasks where the location of the escape location was moved following initial learning, termed a "reversal". Some of these studies reported that the KO mice were impaired in reversal learning (Bakker *et al.*, 1994; D'Hooge *et al*., 1997; Kooy *et al*., 1996; Paradee *et al*., 1999; Van Dam *et al.*, 2000), whereas others found no differences between the *fmr1* KO mice and controls (Paradee *et al.*, 1999; Yan *et al*., 2004). However, these apparently discrepant results can be reconciled by a consideration of the background strains. Those instances in which reversal learning deficits were reported for the *fmr1* mutant mice seem to be due to alleles of the 129 strain segregating with the *fmr1-tm1Cgr* mutation and/or the presence of modifying genes of the 129 strain influencing the *fmr1* KO phenotype (for additional discussion, see Paradee *et al*., 1999; Yan *et al*., 2004). Reversal learning was uniformly unimpaired in *fmr1* KO mice when the mutation was studied on highly backcrossed C57BL/6J or F1 hybrid backgrounds that excluded most 129/ReJ alleles, consistent with the present findings.

Despite the absence of genotypic differences in learning rate in the Reversal Task in this study, the videotape data provided evidence that the *fmr1* KO mice were, in fact, less proficient than controls in regulating the arousal produced by the reversal

of contingencies. The mice in both groups exhibited higher levels of activity and wall-climbing during the initial sessions of the task than during the final sessions, indicating that the initial high rate of these behaviors reflects the heightened arousal and/or frustration created by the reversal of contingencies and low rate of reinforcement. Therefore, the finding that this initial increase in both behaviors was more pronounced for the KO mice indicates that they were impaired in regulating the arousal produced by the reversal of contingencies and low reinforcement. A similar pattern was seen for this same cohort of mice when they transitioned between two visual attention tasks, the latter of which included, for the first time, unpredictable olfactory distractors (Moon *et al.*, submitted). During the first few sessions on this new task, wall-climbing increased in both groups on the trials with distractors, but only the KO mice exhibited this increase in wall-climbing on the trials without distractors, indicating impaired regulation of the arousal produced by the potent olfactory distractors. These findings correspond well with the profile described by Cornish and colleages (2004a, p.14) for FXS: "…males with FXS react more strongly than those without FXS to many forms of environmental and social stimuli and the hyperarousal that results can take an unusually long time to abate. As a result, individuals with FXS are prone to long periods of sustained hyperarousal…".

The observed pattern of results for activity level in the Reversal Learning Task provides new insight into the nature of the hyperactivity reported previously for these mice (in some studies) and, by extension, perhaps in humans with FXS. In the present study, an effect of genotype on activity level was seen only during times of heightened arousal, and thus should be viewed as an indirect effect of impaired regulation of arousal or emotion. This pattern of findings may help explain why some prior studies of *fmr1* KO mice have found effects of the mutation on activity level (Bakker et al., 1994; Peier et al., 2000) whereas others have not (Nielson *et al*., 2002).

Whether or not impaired arousal regulation of the *fmr1* KO mice affects the accuracy of responding in cognitive tasks appears to depend on task characteristics. It did not impair accuracy in the olfactory Reversal Learning Task described in the present report whereas it appears to be at least partially responsible for the impaired performance of these same mice in a series of visual attention tasks (Moon *et al*., submitted). In these attention tasks, the *fmr1* KO mice performed less well than their WT littermate controls under very specific testing conditions, including: (1) trials immediately following an error; and (2) the first few sessions of a new task when the characteristics of the visual cue had changed slightly (e.g., cue duration became variable) or when potent olfactory distractors were now presented unpredictably. The increased arousal created by each of these conditions disrupted attention and inhibitory control in both groups, but to a greater extent for the *fmr1* KO mice. The finding that conditions which increase arousal uncovered genotypic differences in performance in these attention tasks whereas it did not do so in the present Reversal Learning Task likely reflects differences in task demands. In these attention tasks, the visual cue was brief and presented after a delay on some trials, thus requiring both inhibitory control and sustained attention. In contrast, in the Reversal Learning Task, the discriminative olfactory cues were presented immediately after trial initiation and were continuously available until a response was made. The pattern of findings in this series of attention tasks indicates that impaired regulation of arousal in the KO mice disrupts attention and inhibitory control, which then lowers performance in tasks which tap these functions. An interesting parallel was noted in a recent study involving humans with FXS: Deficits in inhibitory control were more pronounced for tasks that required higher attentional capacity (Cornish et al., 2004b).

Impaired regulation of arousal and/or emotion in humans with FXS and in the *fmr1* KO mouse may relate to dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis. In FXS, cortisol levels exhibit a prolonged recovery from psychosocial stress, and bedtime levels of corticosterone are elevated (Hessl *et al*., 2004; Wisbeck *et al*., 2000). These data are consistent with the finding of decreased hippocampal glucocorticoid (GC) receptors in the *fmr1* mouse model (Miyashiro *et al*., 2003), as a decrease in GR would be expected to delay return of corticosterone to basal levels.

CONCLUSION

In sum, although the KO mice did not differ from WT littermate controls in the rate of learning any of the tasks in the present study, their behavioral response to the stress of the reversal was more pronounced than that of controls. This evidence for dysregulation of arousal and/or emotion in the *fmr1* KO mouse is consistent with the results of a series of attention tasks previously administered to these mice, although in the latter case, this dysregulation led to impaired task performance (Moon *et al.*, submitted). These findings support the validity of this mouse model of FXS, as problems in the regulation of arousal and emotion are believed to underlie many of the behavioral symptoms of FXS (e.g., Cornish *et al*., 2004b). This study lays the ground work for future studies designed to examine possible pathways leading to impaired brain development in FXS and test potential therapies.

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CHAPTER FOUR

IMPAIRED ATTENTION, IMPULSIVITY, AND RESISTANCE TO CHANGE IN A MOUSE MODEL OF FRAGILE X SYNDROM

ABSTRACT

A study recently completed in this laboratory demonstrated that the most prominent areas of dysfunction in Fragile X syndrome (FXS) -- impaired attention, inhibitory control, and arousal regulation – are also seen in the *fmr1* "knockout" mouse model of this disease. Although the pattern of spared and impaired functions observed in the *fmr1* knockout (KO) mice in this prior study provides strong encouragement for the validity of this mouse model, the differences between the *fmr1* KO mice and littermate controls were most pronounced during the first few sessions on each new task; differences significantly diminished or disappeared with continued testing on each task. The present study was designed to build on these findings, with the goal of producing larger and more lasting group differences, a pre-requisite for tests of putative therapeutic agents. After a period of training on three attention tasks, F1 hybrid *fmr1* KO mice (FVB/NJ- *Fmr1*^{tm1Cgr}× C57BL/6J cross) and wild-type (WT) littermate controls were tested on a series of attention tasks in which the task characteristics changed daily. In all of these tasks, one of the five response ports was briefly illuminated on each trial, and the mouse was rewarded for making a nosepoke into the illuminated port. The task parameters which changed daily included the duration(s) of light cue presentation, the duration(s) of the delay prior to light cue presentation, as well as the presence or absence of olfactory distractors. The results

revealed attentional dysfunction in the KO mice throughout the entire 15 weeks of testing, a much longer period of time than in the prior study. The magnitude of the impairment was largest in two conditions: (1) during the initial three attention tasks when the mice were learning to wait for and attend to brief cues, and (2) throughout the entire testing period on trials specifically on trials for which the target visual cue was very brief, placing the greatest demands on sustained and focused attention. This study lays the groundwork for future studies designed to assess the efficacy of putative therapeutic agents.

INTRODUCTION

Fragile X syndrome (FXS) is considered to be the common inherited form of mental retardation, and the most common known cause of autism (Hagerman, 2002; Mazzocco *et al.*, 1997). FXS is caused by an abnormal expansion of a trinucleotide CGG repeat sequence in the *fmr1* gene (Khandjian, et al., 1999; O'Donnell and Warren, 2002), leading to hypermethylation of the promoter region and consequently transcriptional silencing of this gene, which codes for the Fragile X Mental Retardation Protein (FMRP) (Oberle, et al., 1991; Verkerk, et al., 1991; reviewed in O'Donnell and Warren 2002). The absence of FMRP, directly and/or indirectly, gives rise to the FXS phenotype including a characteristic set of anatomic and cognitive/behavioral features. The characteristic physical features of the disease include elongated facial structure, large protruding ears, hyperextensible joints, and in males, macroorchidism. Individuals with FXS also display mild to severe mental retardation, with notable deficits in language and executive function, autistic features, anxiety, hyperarousal, social withdrawal, hyperactivity, and seizure susceptibility

(Baumgardner et al., 1995; Cohen et al., 1988; Freund and Reiss, 1993; Hagerman, 1996; Reiss and Freund, 1992).

Mounting research has revealed that the behavioral/cognitive dysfunction in FXS is not global in nature but rather primarily affects various aspects of executive functioning, such as attention and inhibitory control, as well as emotion regulation and reactivity to sensory stimuli. Difficulties with attention and impulse control are considered to be core symptoms of FXS (Largo and Schinzel, 1985; Baumgardner et al., 1995; Hagerman RA, et al., 1987; Lachiewicz et al., 1994; Turk et al., 1998), with up to 73% of FXS individuals meeting the diagnosis for Attention Deficit Hyperactivity Disorder (Baumgardner et al., 1995). Hypersensitivity to sensory stimulation is also characteristic of individuals with FXS, commonly giving rise to autistic-like behaviors, tactile defensiveness, and gaze avoidance (Hagerman, 1996; Lachiewicz et al., 1994). Consistent with these clinical reports, individuals with FXS have been found to display aberrant electrodermal responses (EDRs) in response to sensory stimulation (Baranek & Berkson, 1994; Cohen et al., 1998; Miler et al., 1999). It has been suggested that altered sensory processing in FXS not only taxes cognitive functioning, but also leads to emotional arousal, which is incompatible with focused attention (Merenstein et al., 1996; Hagerman, 1996).

Problems in emotion or affect are also viewed as central to the profile of FXS. Individuals with FXS are generally described as shy, socially withdrawn, anxious and emotional (Kerby et al., 1994, Borghgraef M et al., 1990). One possible mechanism for dysregulation of affect or arousal in FXS is dysfunction within the hypothalamic-pituitary-adrenal (HPA) axis. Reported HPA abnormalities in FXS include higher bedtime levels of corticosterone (Wisbeck et al., 2000), slow recovery of cortisol levels following psychosocial stress (Hessl et al., 2002b), and decreased glucocorticoid receptor density (Miyashiro et al., 2003). It has been proposed that

disruptions in the HPA axis may be responsible for the social anxiety and hypersensitivity to stimuli seen in individuals with Fragile X (Hessl et al., 2004).

Growing knowledge about the roles of FMRP in brain function (Hessl et al., 2004; O'Donnell & Warren, 2002; Willemsen, 2004) provides several plausible mechanisms by which a deficiency of the protein might lead to aberrant brain development and impaired cognitive and affective functioning. For example, one prominent theory, often referred to as the "The mGluR theory of Fragile X syndrome", posits that abnormal mGluR signaling, resulting from FMRP loss, could be responsible for many of the characteristic features of fragile X syndrome, including impaired cognitive functioning, seizures, anxiety, movement disorders and obesity (e.g., Bear et al., 2004; Bear, 2005). However, tests of these putative links depend on studies with an animal model, as do subsequent efforts to translate this knowledge into possible therapies. A murine model of FXS has been developed in which the *fmr1* gene has been inactivated by homologous recombination (Bakker, et al., 1994). The mouse is well-suited as a model for FXS because the *fmr1* gene is highly conserved across species, with the murine homolog of the *fmr1* gene showing a 97% homology in amino acid sequence with the human gene (Ashley, et al., 1993). Moreover, the murine expression pattern of *fmr1* gene at the mRNA and protein level in different tissues is very similar to the human gene (Hinds, et al., 1993).

Studies of these mutant mice [often referred to as *fmr1* knockout (KO) mice] have revealed some important areas of correspondence between the phenotypes of humans with FXS and this mouse model: They display macroorchidism (Bakker et al., 1994; Bakker, et al., 2000), abnormal dendritic spines in the hippocampus and cortex (Galvez and Greenough 2005; Nimchinsky et al., 2001), increased motor activity (Mineur, et al., 2002), and auditory hypersensitivity (Chen and Toth, 2001, Nielsen, et al., 2002) . However, it has been much more difficult to demonstrate correspondence

in terms of the cognitive and emotional dysfunction. Commonly-used learning/memory tasks, such as the Morris water maze and the radial arm maze, have either been unable to differentiate the *fmr1* KO mice from controls (Paradee *et al*. 1999; Peier et al., 2000; Dobkin *et al*., 2000) or have revealed very small deficits in the *fmr1* KO mice that are apparent only in some background strains (Hinds et al., 1993; Cianchetti et al., 1991; Bakker et al., 2000; Muneur eat al., 2002). Results seemingly contradictory with the phenotype of human FXS have also been reported. For example, in some learning tasks, *fmr1* KO mice performed better than their WT littermates (Frankland et al., 2004; Van Dam et al., 1999). Similarly, in the elevated plus maze, a well-validated test of anxiety for rodents, *fmr1* KO mice either performed similarly to controls (Dobkin et al. 2000), or exhibited a greater preference than controls for the open arms of the maze (Peier *et al*. 2000), a pattern generally interpreted as indicative of reduced anxiety. The available data also indicate discrepancies between *fmr1* KO mice and FXS in prepulse inhibition (PPI), a marker of sensorimotor gating: In a recent study, boys with FXS exhibited reduced PPI relative to controls, whereas *fmr1* KO mice exhibited greater PPI than their WT littermates (Frankland et al., 2004). These findings, taken together, have raised questions about the validity and utility of this mouse model.

One factor most likely contributing to the apparent lack of cognitive dysfunction in the *fmr1* KO mouse is that the hallmark areas of dysfunction in human FXS have not been studied in the mouse model; these include attention, inhibitory control, and regulation of arousal or emotion. A prior study recently completed in this lab was designed to test this hypothesis. *fmr1* KO mice and wild-type littermate controls were tested on a series of tasks designed to assess inhibitory control, various aspects of attention (sustained, selective, and divided attention), associative learning, transfer of learning, and reversal learning. Regulation of arousal and/or emotion was assessed by

examining the animals' reaction to the unexpected presentation of potent olfactory distractors (Moon et al., 2006a, 2006b), and the frustration produced by the reversal of contingencies in the Reversal Learning Task, as well as their reaction to committing an error on the previous trial. Reactivity to errors taps both error monitoring (an aspect of executive functioning) (Luu et al., 2000) and emotion regulation (Elliott et al., 1996), two domains affected in FXS (Borghgraef et al., 1990; Hagerman & Sobesky, 1989; Kerby & Dawson, 1994). Briefly, this study demonstrated that the most prominent areas of dysfunction in human FXS -- impaired attention, inhibitory control, and arousal regulation – are also seen in the *fmr1* mouse model of FXS. Notably, attentional dysfunction and impaired inhibitory control were most pronounced immediately following a change in task characteristics or on trials immediately following an error– situations that arouse or disturb the mice. This pattern of results corresponds well with the results of studies of humans with FXS, in which impairments in attention and inhibitory control have been postulated to be secondary to alterations in the regulation of affect or emotion (Cornish et al., 200?). Notably, too, the difficulty in dealing with change seen in the KO mice recapitulates one of the autistic features commonly seen in humans with FXS.

Although the pattern of spared and impaired functions observed in the *fmr1* KO mice in the prior study provides strong encouragement for the validity of this mouse model, the differences between the *fmr1* KO mice and littermate controls were most pronounced during the first few sessions on each new task; differences significantly diminished or disappeared with continued testing on each task. Prior to undertaking tests of putative pharmacological interventions, it would be optimal to have larger, more lasting, group differences. Only with large group differences can one assess whether the pharmacological intervention produces complete or partial amelioration of a specific function. For this reason, the present study was designed to build on these

findings, with the goal of producing larger and more lasting group differences. Specifically, after the mice had mastered the basic rules of a 5-choice serial reaction time task, they were tested for 12 weeks with the specific characteristics of the task changing daily. The specific characteristics that varied daily included the duration(s) of the delay prior to cue presentation, the duration(s) of the duration of the visual cue, and the presence of unpredictably presented olfactory distractors. This task series is referred to here as "The Loop" because a fixed sequence of six tasks was repeated several times.

In addition to the automated performance measures that were collected, the performance of the mice was also videotaped and then coded by individuals blind to the genotypes of the mice. It was hypothesized that if the KO mice were impaired in effectively regulating the arousal produced by the daily change in task characteristics, this would be apparent in their behavior (e.g., increased wall-climbing, grooming, activity level), as seen following reversal of task contingencies in the study noted above (Moon et al., submitted).

GENERAL 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 FVB/NJ-^{tm1Cgr} mutant female mice that had been 12+ generations backcrossed to inbred FVB/NJ normal males purchased from The Jackson Laboratories (JAX). These heterozygous females were then bred with normal C57BL/6J males (Jackson Laboratory, Bar Harbor, ME) to produce male KO and WT hybrid (C57Bl x FVB) mice. Male offspring (21 WT and 20 *fmr1* KO) from these litters served as subjects in the present experiment. The mice were shipped to Cornell

at about 3 of months of age. Reciprocal cross (C57BL male x FVB female) was used in the present study since FVB strain mice are better breeders.

The strategy of studying the *fmr1* mutation on an F1 hybrid background was followed for several reasons. First, these mice have normal hearing (unlike C57BL/6J mice) and are not blind or susceptible to seizures (unlike FVB/NJ mice) because these deficits are recessive (Johnson et al., 1997; Zheng et al., 1999; Pittler & Baehr, 1991; Goelz et al., 1998). In addition, this procedure produces *fmr1*- KO and WT mice from the same litters, thereby equating the intrauterine and postnatal environments of the experimental and control groups. Finally, in light of the pronounced strain differences in startle, anxiety, and performance in various learning tasks, it is risky to draw conclusions about the effects of a given mutation from studies of inbred mice, as background strain effects may greatly accentuate or obscure gene effects (Paradee et al. 1999).

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 at UCHSC and Cornell University, both AAALAC accredited institutions.

Genotyping*:* The mice were genotyped at the University of Colorado Health Sciences Center for the presence of the *fmr1* ^{tm1Cgr} mutation as described in Nielsen and colleagues (2002).

Apparatus: The mice were tested individually in one of six automated Plexiglas chambers, each controlled by a PC and situated in an insulated, sound-attenuating enclosure. The testing chambers were adapted from the operant chambers recently developed to assess attention in mice (Humby, Laird, Davies, & Wilkinson, 1999).

The slightly curved rear wall contains five circular response ports, 1 cm in diameter, located 2 cm above the floor and 5mm apart. A nosepoke into any of these ports constitutes a response (or choice). Responses to the ports are detected by infrared photodiodes, positioned inside each port, 0.5 cm from the opening. The discriminative visual cues are provided by illumination of green 4mA light-emitting diodes (LEDs), one embedded on the back surface of each port. Each port also contains a fitting through which scented air can be dispensed. This scented air serves as a distractor in the Distraction task. The scented air was produced by passing filtered, compressed air through small bottles of liquid odorant, using solenoid airflow valves and airflow meters. The airflow rate is 1.0 L/min. On the chamber wall opposite the five response ports is an alcove (15 mm wide, 2 cm above the floor) containing the dipper (ENV0302M, MED Associates, Inc., St. Albans, VT) which dispenses the liquid food reward (liquefied AIN-76A, a sweet, nutritionally-complete diet; BioServ, Inc.). Access to the dipper alcove is controlled by a thin metal door, which was activated by a motor located on the outside of the testing chamber. As with the ports, head entries into the alcove were monitored by infrared photodiodes. A nosepoke into this alcove port is required to initiate each trial. Each chamber is fitted with an exhaust system, which transports the air from each chamber directly to the room exhaust ventilator system at a rate of four complete air changes per minute. All automated events (door opening, dipper movement, responses, etc.) within each chamber are timed, controlled, and recorded by custom programs written in QBASIC.

For videotaping (described below), each chamber was equipped with a wide-angle infrared video camera and infrared LED light source attached to the ceiling directly over the center of each testing chamber. The camera allowed full view of the mouse at all times. An array of infrared LEDs, positioned outside the Plexiglas chamber but within viewing range of the camera, provided information about the

various events during each trial (e.g., location of the visual cue, demarcation of the intertrial interval, presentation of the distractor, whether a response was correct or incorrect).

Behavioral testing

Training tasks: Behavioral testing began when the mice were 5 months of age. The mice first completed a four-stage training procedure designed to shape the general response sequence required for completion of each trial in the subsequent tasks. These training stages are described in a prior report (Driscoll et al., 2004). Briefly, the mice learned that the door to the dipper alcove would be raised at the start of each trial and that a nosepoke into the dipper port, followed by a nosepoke into one of the five response ports, would produce the delivery of 0.04 ml of the liquid diet in the dipper alcove. These four training phases were mastered in approximately 8-10 sessions.

Five-Choice Visual Discrimination Task: Each mouse was then trained on a five-choice visual discrimination task. The metal door at the dipper alcove was raised at the onset of each trial. A nosepoke into the alcove initiated each trial. Following a "turn-around time" of 1 s, an interval provided on all trials to allow the mouse time to turn around and face the response ports, 1 of the 5 LEDs in the response ports was illuminated for 32 s or until the mouse made a response. A nosepoke into the illuminated port was the correct response, and was rewarded by 5 s access to the dipper alcove. Each of the five ports was illuminated for an equal number of times within each testing session. Following a error (response to a non-illuminated port), a 5-s time-out period was initiated, signaled by the illumination of a 2-W houselight on the ceiling of the chamber. A time-out was also imposed following a "nontrial", the

term given to trials in which the alcove door was raised at trial onset but the mouse did not enter the alcove in the following 60 s; nontrials were very rare, however. A 5-s intertrial interval separated adjacent trials. All trials on which the mouse made an initiation poke into the dipper alcove (regardless of the outcome of the trial) were defined as response trials. Each session was terminated after 30 min or 70 response trials, whichever came first. This task was administered until the mouse reached a criterion of 80% correct for two out of three consecutive sessions, each containing at least 50 response trials.

Visual Discrimination Tasks with progressively shorter cue durations

Four subsequent visual discrimination tasks were administered, all of which were identical to the previous task but with progressively shorter cue durations. These tasks were designed to establish stable performance and to prepare the mice for subsequent attention tasks. The cue durations for each task were 5.0 s, 2.0 s, 1.4 s, and 1.0 s, and the mice received these durations for 3, 10, 10, and 10, sessions, respectively. The goal of these tasks was establish stable performance and prepare the mice for the subsequent attention tasks.

Several types of errors were possible. If the mouse responded to any of the ports prior to cue presentation (termed a premature response), the trial was terminated. A response to an incorrect port following cue presentation was also considered to be incorrect, as was a trial on which the mouse did not respond to any of the five response ports within 5 s of cue onset, indicative of missing the cue. Following any of these types of errors, a 5-s time-out period was imposed.

Pre-LOOP Attention tasks with variable pre-cue delays: Before moving on to "The Loop" Task, the mice were tested on 2 additional attention tasks that were identical to the prior tasks but with variable pre-cue delays. These tasks were designed to allow the mice to learn that the light cue might, on some trials, be presented after a delay and that a nosepoke into a response port prior to cue illumination would be incorrect and terminate the trial.

Pre-Loop task with variable pre-cue delays #1 (delays of 2 and 3 s): In this task, a delay of varying duration (2, & 3s) was randomly imposed between trial initiation (nosepoke into dipper alcove) and onset of the visual cue (in addition to a "turnaround" time of 1 s, which was imposed on all trials). The number of presentations of each combination of pre-cue delay and response port (1-5) was balanced across the session. The duration of visual cue illumination was constant for all trials (1 s). Because this was the first task in which a delay was imposed before cue onset, performance on this task largely reflected the ability of the mice to learn to wait (i.e., inhibitory control). This task was designed to assess inhibitory control, and prepare the mice for the subsequent attention tasks. This task was administered for 5 sessions.

Pre-Loop task with variable pre-cue delays #2 (delays of 0, 2, and 4 s): This task was identical to the prior task but with a different set of pre-cue delays (0, 2, and 4 s). This task was presented consecutively for 10 sessions.

"The LOOP" Attention series

The mice then began testing on the "Loop task", in which one of six different attention tasks (defined in Table 4-1) was presented each day, with the stipulation that no task was presented for two consecutive sessions (see Table 4-2). The average age at the onset of this task was 10 months. A fixed 18 –session sequence of tasks (listed in Table 2) was repeated four times, for a total of 72 sessions. The basic rules and procedures were the same for all tasks (described above), but the cue parameters (pre-cue delay, duration of cue illumination, presence or absence of distractors) varied between tasks (see Table 4-1). For all tasks, the various levels of each parameter (pre-cue delay, cue duration, distraction condition) and their combinations were presented an equal number of times per session, and balanced for each of the five response ports.

Distraction Task in the LOOP: The mice were tested on a variation of the preceding visual attention tasks that included the unpredictable presentation of scented air from one of the five response ports during the interval prior to visual cue onset, on one third of the trials within each session. This task was designed to tap selective attention and reactivity to salient stimuli. Assessment of the disruption produced by the unpredictable presentation of the olfactory distractors was assessed. Performance on the trials with distractors (Distraction trials) was compared to performance on the trials without distractors (Nondistraction trials).

Table 4-1. Cue parameters for the five visual attention tasks.

Task	Total # of Sessions	Cue Duration	Pre-cue delay	Distractor
Attention Task 1	12 sessions	1.0 s (constant)	0, 2, 4 s (variable)	No Distractor
Sustained Attention Task 1	8 sessions	0.8, 1.0, 1.6 s (variable)	0, 2, 4 s (variable)	No Distractor
Sustained Attention Task 2	8 sessions	0.8, 1.0, 1.6 s (variable)	0, 4, 6 s (variable)	No Distractor
Sustained Attention Task 3	8 sessions	0.6, 0.8, 1.0 s (variable)	0, 4, 6 s (variable)	No Distractor
Baseline Task	12 sessions	1.0 s (constant)	2, 3 s (variable)	No Distractor
Distraction Task	24 sessions	1.0 s (constant)	2, 3 s (variable)	Yes (⅓ of trials per session)

Table 4-2. Task series for each "LOOP". This Loop was repeated 4 times, for a total of 72 sessions.

Session	Task
Session 1	Attention Task 1
Session 2	Sustained Attention Task 1
Session 3	Distraction Task
Session 4	Baseline Task
Session 5	Sustained Attention Task 2
Session 6	Distraction Task
Session 7	Attention Task 1
Session 8	Sustained Attention Task 3
Session 9	Distraction Task
Session 10	Baseline Task
Session 11	Sustained Attention Task 1
Session 12	Distraction Task
Session 13	Attention Task 1
Session 14	Sustained Attention Task 2
Session 15	Distraction Task
Session 16	Baseline Task
Session 17	Sustained Attention Task 3
Session 18	Distraction Task

Cue duration was constant across trial (1 s) but pre-cue delay varied randomly between 2 or 3 s (in addition to the 1 s "turnaround" time) which are identical with parameters of Baseline Task. Presentation of scented air was pseudo-randomly presented on one third of the trials, from one of the five reposne ports either 1 or 2 s before the visual cue.

The nine scents used for the distractors were lemon, hazelnut, apricot, butter, anise, raspberry, maple, coconut, and almond. The liquid odorants, artificial flavorings (McCormick, International Flavors and Fragrances, Inc.), were diluted with propylene glycol. Scented air was produced by passing filtered, compressed air though small bottles of these scented liquids, using solenoid airflow valves.

This task was administered for a total of 24 sessions; 6 sessions within each Loop (moon please verify).

Sustained Attention Task 1 in the LOOP: This task had pre-cue delays of 2 and 3 s, and a constant cue duration of 1 s; it was identical to the Pre-Baseline Task (see Table 1). This task had the same cue parameters as the Distraction task, and therefore provided a reference condition to assess any generalized disruption produced by the olfactory distractors in the Distraction Task (i.e., performance disruption that extended beyond the trials with the distractors to those that did not include distractors).This task was administered for a total of 12 sessions; 3 sessions within each Loop.

Sustained Attention Tasks 2, 3, 4, and 5 were all variants of the Sustained Attention Task 1, but with different combinations of pre-cue delays and cue durations (see Table 1).

All testing equipment was thoroughly cleaned and dried following the testing of each mouse, using Odormute (R.C. Steele Co, Brockport, NY), a detergent containing an enzyme that removes olfactory cues (including pheromones). All

behavioral testing and coding of the videotapes was conducted by individuals blind to the genotype of the animals.

Videotape Coding: All sessions of the "Loop task" were recorded.

Diet and Control of Motivation: As noted above, the animals were maintained at 80-85% of their *ad libitum* weights throughout the study to maintain motivation for the food rewards during testing, and ensure an approximately equal number of trials for all mice. On each testing day (6 days per week), the number of calories obtained during testing was subtracted from the total caloric allotment, and the remainder was fed as chow (ProLab 1000; Purina, Inc.) in the home cage directly after testing. On non-testing days, each mouse was given 0.04ml of the liquid diet plus the remainder of the ration in chow in its home case. The goal was to provide the maximal daily caloric intake that maintained adequate motivation for 60-70 trials during each daily test session.

Statistical Analyses

The automated performance data from the attention tasks were analyzed using a generalized linear mixed model (GLIMMIX) which correctly handles the repeated measures analyses of variance with non-normal data on each animal (Wolfinger & O'Connell, 1993). All statistical analyses were conducted using SAS 9.1 (SAS Institute, Cary, NC) for Windows 2001.

The overall percent correct, percent inaccurate responses, percent premature responses, percent omission errors, and percent non-trials were each analyzed but only the percent correct measurement is presented to simplify the presentation of the results.

For each of these measures, a mean was calculated for each animal for each testing condition, defined by the following variables (as appropriate for the task characteristics): pre-cue Delay, stimulus Duration, Distraction condition (Distraction vs. Nondistraction trials), Session-block (blocks of testing sessions), Trial-block (blocks of trials within each test session) and Outcome of the Previous Trial (correct or incorrect). The models used for these analyses included the aforementioned variables plus Genotype (*fmr1*-KO and WT) and all relevant higher-order interactions.

A nonparametric technique, the Wilcoxon rank-sum test, was used to analyze the dependent measures for the video data due to non-normality of the distributions. The dependent measures, including percent time grooming, percent time wall-climbing and mean number of jumping, were analyzed as a percentage of time spent for given behavior, or time spent on a given behavior divided by time spent on all other behaviors multiplied by 100 (For more detail, see Moon et al., 2006a). T-tests were used to compare body weight and daily food intake of the two genotypes. For each of these analyses, a mean was calculated for each animal for the first and last testing sessions, and than the group means were calculated and compared.

RESULTS

Body weight*:* The body weights of the groups did not differ ($t_{(40)}$= 0.48, n.s.).

Daily food intake*:* There was no effect of genotype on mean daily food intake ($t_{(40)}$= -0.51, n.s.).

Automated Performance Data for the Attention Tasks: Percent correct was calculated for each testing condition by dividing the number of correct trials in that

condition (e.g., block of trials across sessions, previous trial outcome, block of testing sessions, etc) by the total number of response trials in that condition, and then multiplying by 100.

Several experimental variables produced significant and consistent effects for all outcome measures for a given task. In all of the tasks, performance decreased with decreasing cue duration, increasing pre-cue delay, on trials with distractors, and on trials following an error (relative to trials following a correct response (all $p < 0.0001$). Performance also improved across the 10-11 weeks of testing ($p < 0.0001$). Performance also varied across each daily test session (i.e., main effect of "Trial-Block", a 3-level variable corresponding to trials 1-20, 21-50, and 51-70, with performance generally being best in the middle of each daily test session ($p < 0.0001$). However, to streamline the presentation of results, these effects will not be presented below in the results for each task, only the genotype-related effects will be presented.

Pre-Loop Visual Discrimination Task with 1 s Cue Duration (problem 59 – this is just for me, Moon – not to include later): Overall, the *fmr1* KO mice committed a lower percentage of correct responses than the controls (main effect of Genotype, $F_{(1,35.4)} = 12.41$, $p=0.001$, see Figure 1). A three way interaction between Genotype, Trial-block, and Session-block was also detected ($F_{(4,646)} = 2.43$, $p < .05$), reflecting the fact that although the KO mice performed more poorly than the WT controls throughout the three session-blocks (three blocks of ? sessions each), the portion of each daily testing session for which they were most impaired (relative to controls) varied somewhat across the three sessionblocks.

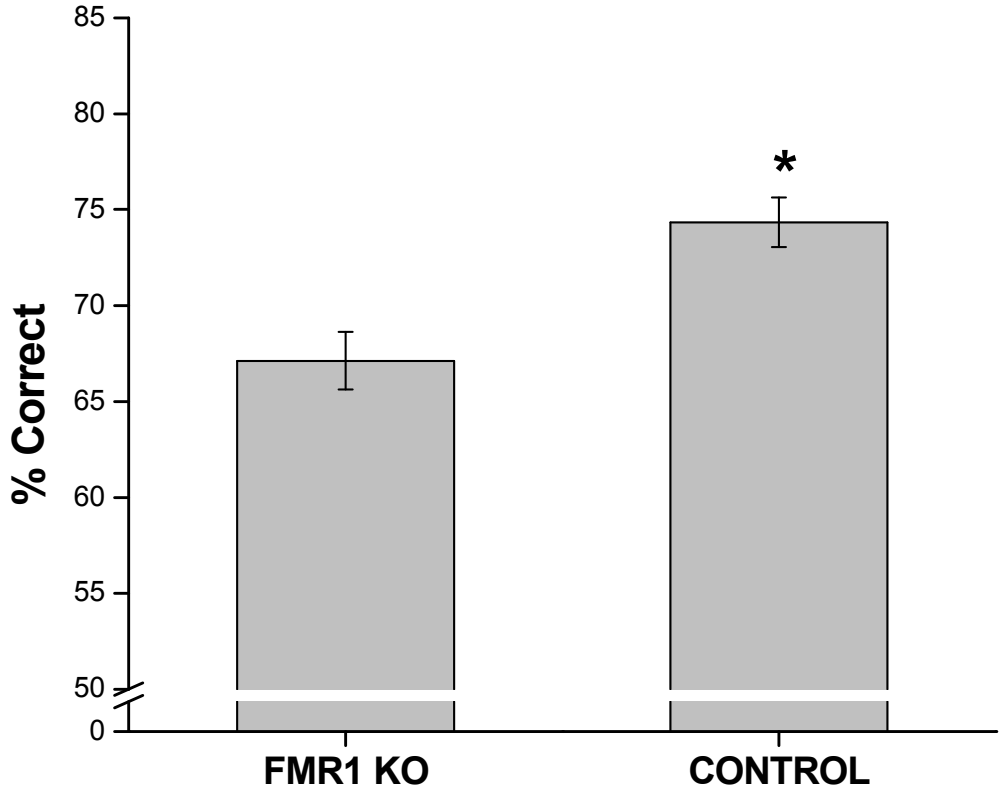

Figure 4-1: During Pre-Loop Visual Discrimination Task, the *fmr1* KO mice committed a lower percentage of correct responses than the controls. Bars represent the mean (+/- SEM). * represents p=0.001.

Pre-LOOP Attention Tasks

Pre-Loop task with variable pre-cue delays #1 (delays of 2 and 3 s): (pre-loop 50): The main effect of Genotype ($F_{(1,40.5)} = 4.88$, $p = 0.03$) and the interaction between Genotype and Session-block ($F_{(1, 263)} = 7.81$, $p = 0.0056$) were significant. As seen in Figure 2, the KO mice performed significantly less well than controls during the first three sessions on this task (Session-block 1; $p = 0.0036$), whereas performance of the two groups had converged by the final sessionblock (final two sessions) ($p = 0.25$). The impairment of the KO mice early in this task appeared to be due to a difficulty in

learning to wait for the cue. The two groups had not differed in performance during the final session of the preceding Visual Discrimination Task with 1 s cue duration.

The analysis of the 5 sessions on this task also revealed a borderline interaction between Genotype and Delay ($F_{(1,262)} = 3.66$, $p = 0.057$, see Figure 3). The KO mice performed less well than controls on trials with the 2 s pre-cue delay ($p=0.009$) but not on trials with the 3 s delay ($p=0.15$). This pattern, although perhaps counterintuitive, suggests that both groups had difficulty learning to wait for 3 s, but only the KO mice had difficulty with the 2 s delay

Pre-Loop task with variable pre-cue delays #2 (delays of 0, 2 and 4 s): The analysis of the 10 sessions on this task revealed a significant interaction between Genotype and Session-block ($F_{(2,305)} = 4.07$, $p = 0.018$). Both groups performed very poorly during the early sessions, due to the inclusion of a relatively long pre-cue delay (4 s) on some trials. However, the WT mice were significantly more proficient in mastering this new task demand than the KO mice, with a significant difference being detectable by the final block of sessions ($p=0.04$; see Figure 4).

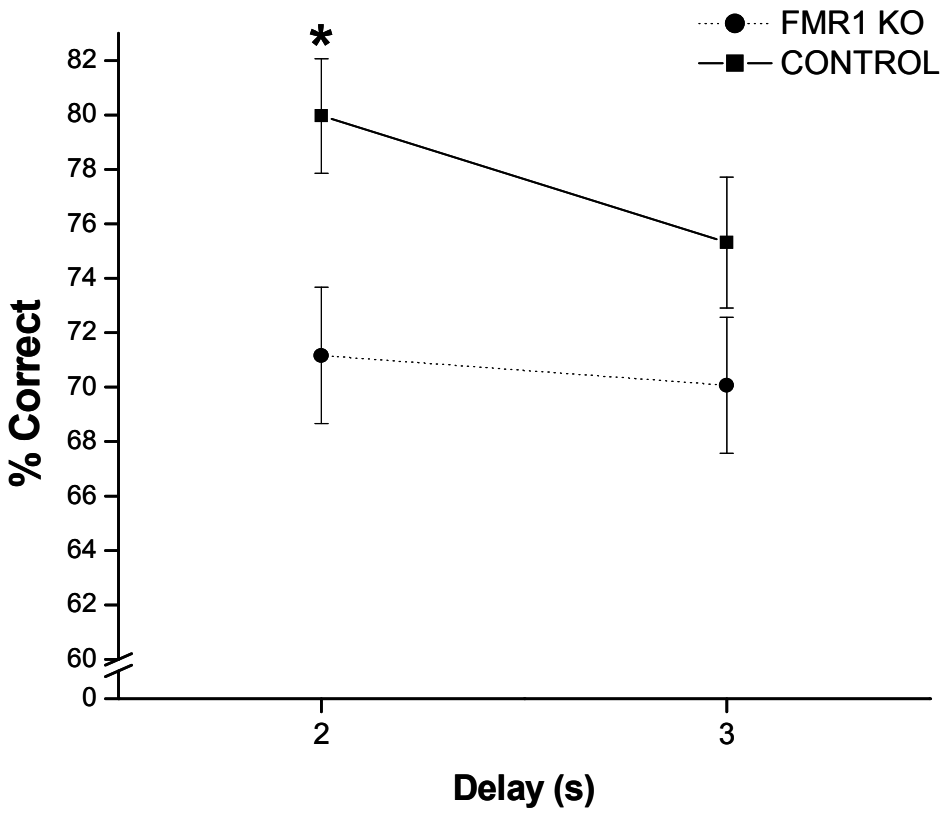

Figure 4-2: During Pre-Loop task with variable pre-cue delays # **1,** the *fmr1* KO mice committed a lower percentage of correct responses than the controls in the Session-block 1 whereas two groups are not significantly different in the Session-block 2. Bars represent the mean (+/- SEM). * represents p=0.0036.

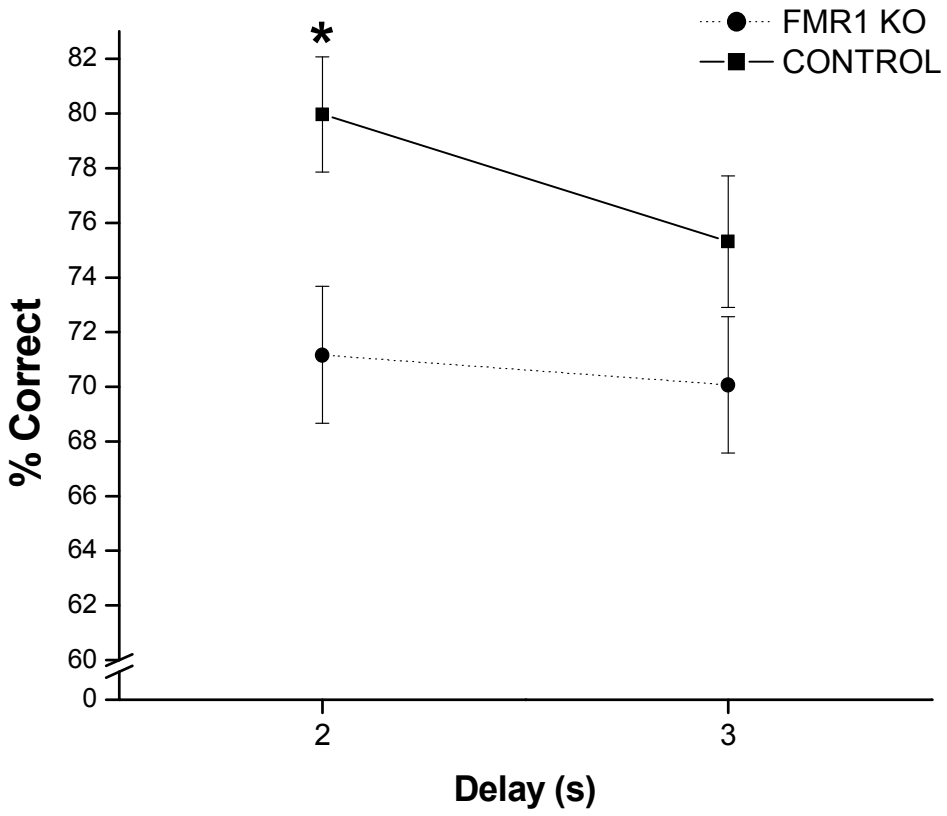

Figure 4-3: During Pre-Loop task with variable pre-cue delays # **1,** the *fmr1* KO mice performed less well than controls on trials with the 2 s pre-cue delay but not on trials with the 3 s delay. Bars represent the mean (+/- SEM). * represents p=0.009.

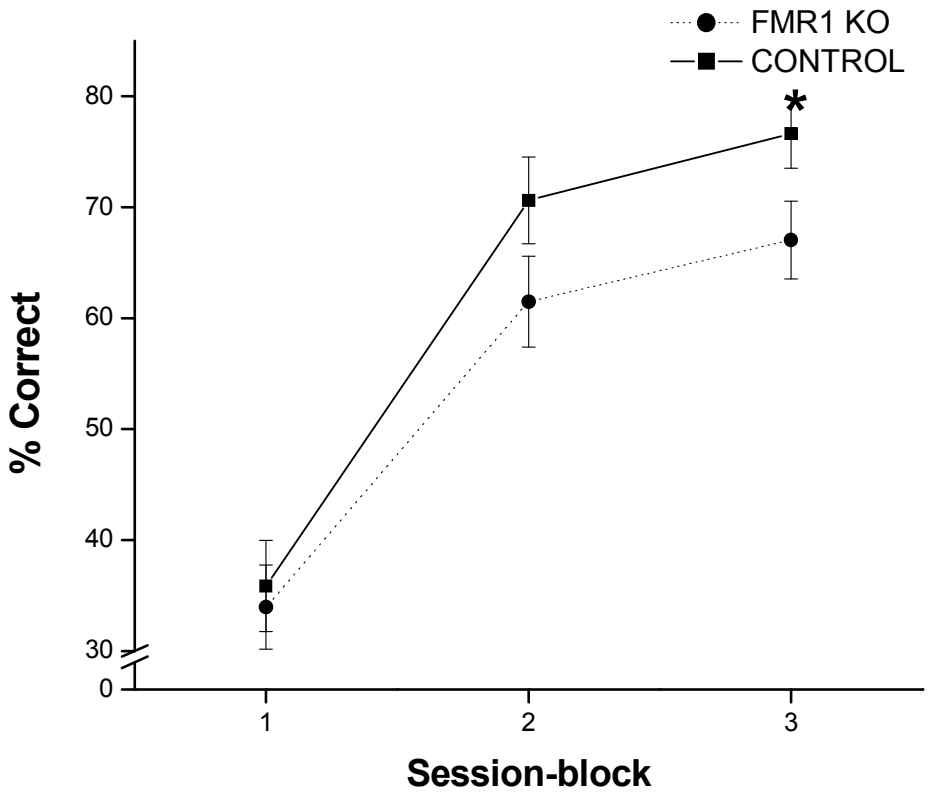

Figure 4-4: During Pre-Loop task with variable pre-cue delays # 2**,** the WT mice were significantly more proficient in mastering this new task demand than the KO mice, with a significant difference being detectable by the final block of sessions whereas both groups performed very poorly during the early sessions, due to the inclusion of a relatively long pre-cue delay (4 s) on some trials. Bars represent the mean (+/- SEM). * represents p=0.04.

Attention Tasks within "The LOOP"

Each task presented in the Loop was analyzed separately because of the different cue parameters of the various tasks. It should be emphasized that none of the sessions for each task were sequential; the variable "session-block" included in these

analyses captures changes in the level of performance for a given task across the 11 weeks that the Loop task was administered.

The results for (need new names I think) Attention task1 and the Baseline task are not presented here since there were few genotype-related findings and most of them were redundant, indicating that both groups had mastered these tasks findings by the time when they start to run LOOP tasks.

1. Sustained Attention Task 1 (149) (pre-cue delays: 0, 2, 4 s; cue durations: 0.8, 1.0, 1.4 s): The analysis of the 8 sessions on Sustained Attention Task 1 revealed a significant interaction between Genotype and Delay ($F_{(2,663)} = 7.44$, $p = 0.0006$, see Figure 5). Whereas the two genotypes did not differ at the 0 second delay (p=0.68), the *fmr1* KO mice performed more poorly than the controls on trials with 2 s (p=0.09) and 4 s (p=0.02) delays, indicative of impaired impulse control and sustained attention. The absence of group differences at the 0 s delay allows one to exclude motivation, visual acuity, and motoric factors as causes of the poorer performance of the KO mice.

A significant interaction between Genotype and Session-block ($F_{(1,665)} = 5.89$, $p = 0.01$, see Figure 6) was also found, reflecting the fact that the KO mice performed less well than controls during the first 4 sessions on the task (session-block 1; p=0.05) but not during the final four sessions (p=0.62).

2. Sustained Attention Task 2 (150) (pre-cue delays: 0, 4, 6 s; cue durations: 0.8, 1.0, 1.4 s): A significant interaction was found between Genotype and Delay ($F_{(2,1042)} = 3.73$, $p = 0.02$), reflecting the fact that the drop in performance from trials with no delay prior to cue presentation to those with a 2 s delay was significantly steeper for the KO mice than for controls ($p = 0.01$), whereas the drop from the 2 s delay to the 4 s delay was comparable for the two groups.

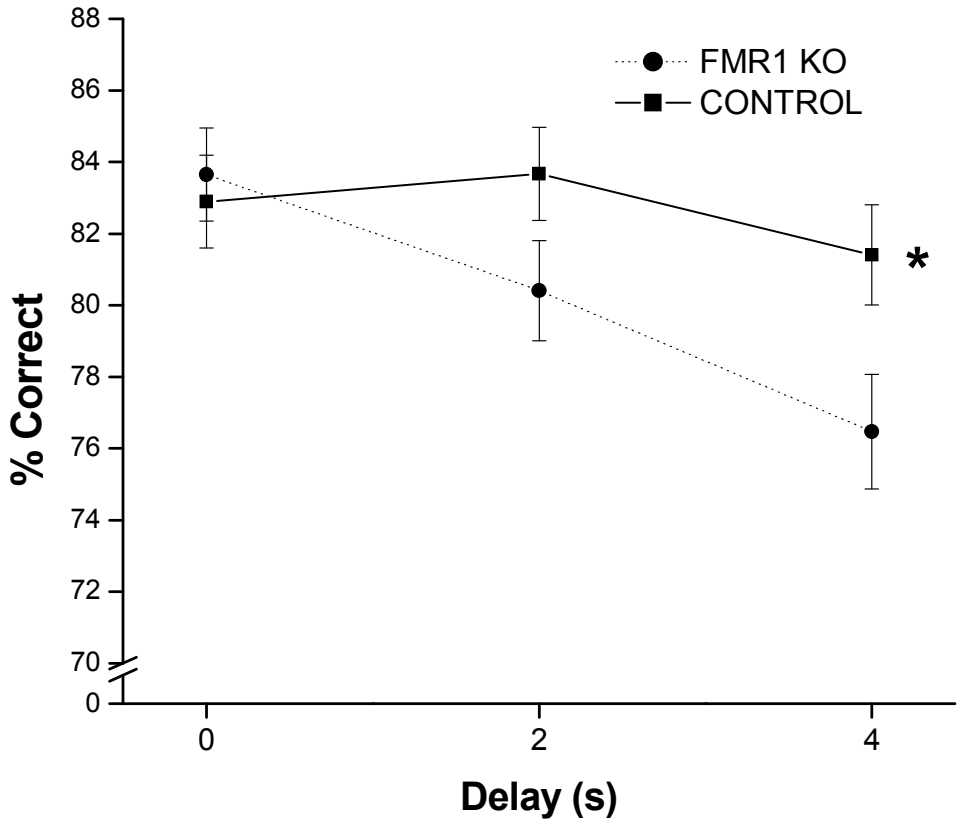

Figure 4-5: During Sustained Attention Task 1, as the delay increased from 0 to 2s, the *fmr1* KO mice showed decrease in performance ($p<0.0001$) whereas the WT mice did not show decrease in performance ($p =0.08$), leading to a marginally significant group difference in 2s Delay ($p=0.09$). In the longest delay condition, 4 s Delay, the *fmr1* KO mice performed more poorly than the controls. Bars represent the mean (+/- SEM). * represents $p=0.02$.

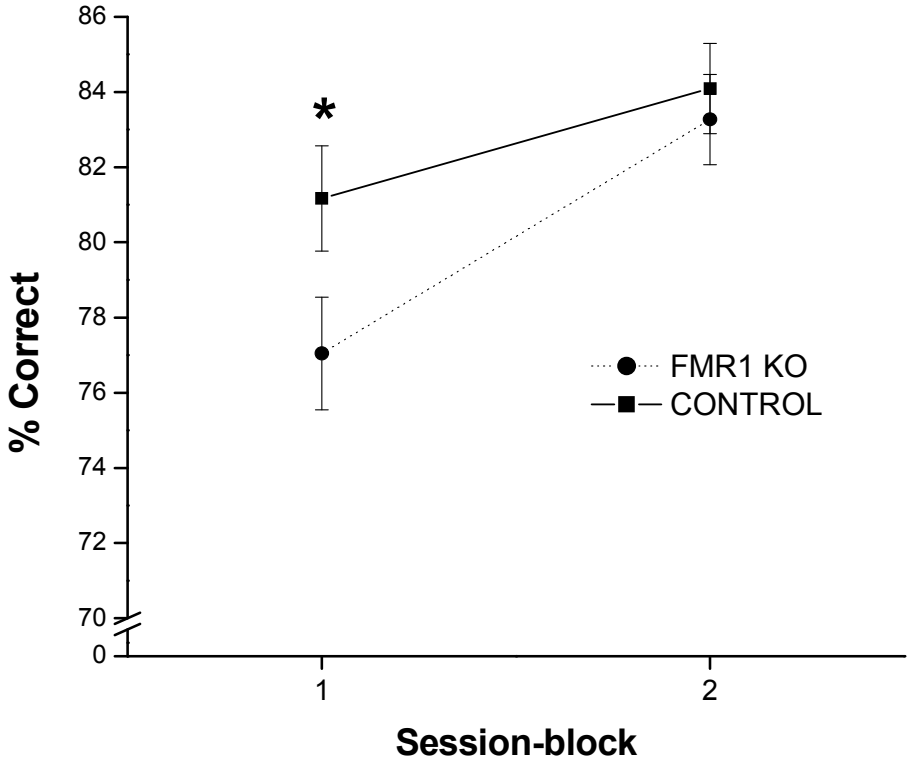

Figure 4-6: Performance on the first session of the Sustained Attention Task 1: the *fmr1* KO mice performed less well than controls during the first 4 sessions on the task (session-block 1) but not during the final four sessions (sessuib-block 2). Bars represent the mean (+/- SEM). * represents p=0.05.

3. Sustained Attention Task 3 (151) (pre-cue delays: 0, 4, 6 s; cue durations: 0.6, 0.8, 1.0 s): The analysis of the 8 sessions on Sustained Attention Task 3 revealed a significant interaction between Genotype and Duration ($F_{(2,988)}$ = 3.01, p = 0.04, see Figure 7). Whereas the two genotypes did not differ on trials for which the cue was presented for 1 s (p=0.68), the *fmr1* knockout mice performed more poorly than the controls for trials with the 0.6 and 0.8 s cues (p=0.01, p=0.08, respectively). This pattern, whereby impaired performance of the KO mice was seen only on trials with

briefest cues, provides strong support for attentional dysfunction. The intact performance of the KO mice for trials with the 1 s cue again rules out motivational or other performance factors as causes of their inferior performance, as well as group differences in understanding of task rules.

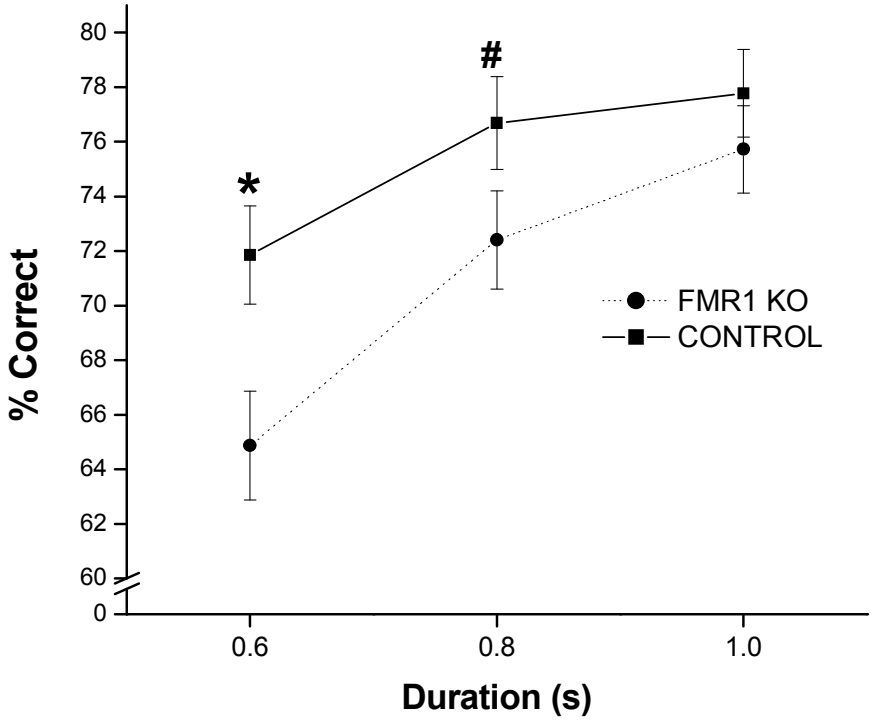

Figure 4-7: During Sustained Attention Task 3, whereas the two genotypes did not differ on trials for which the cue was presented for 1.0 s, the *fmr1* KO performed more poorly than the controls for trials with the 0.6 and 0.8 s cues. Bars represent the mean (+/- SEM). * represents p=0.01 and # represent p = 0.08.

4. Distraction task: Neither the main effect of Genotype ($F_{(1, 41.6)}$= 2.00, p = 0.16) nor the interaction between Genotype and Distraction condition ($F_{(1, 897)}$= 2.38,, p = 0.12) was significant. However, the interaction of Genotype, Distraction, and Previous Trial

Outcome was significant ($F_{(1, 897)} = 4.76$, p=0.03, Figure 8). Both groups performed more poorly on trials following an error than on trials following a correct response, but the magnitude of this error-induced performance disruption was greater for the KO mice than for controls specifically on trials with a distractor presented. This pattern suggests that committing an error on the prior trial, coupled with presentation of the potent olfactory cue, produced greater disruption for the KO mice than for controls.

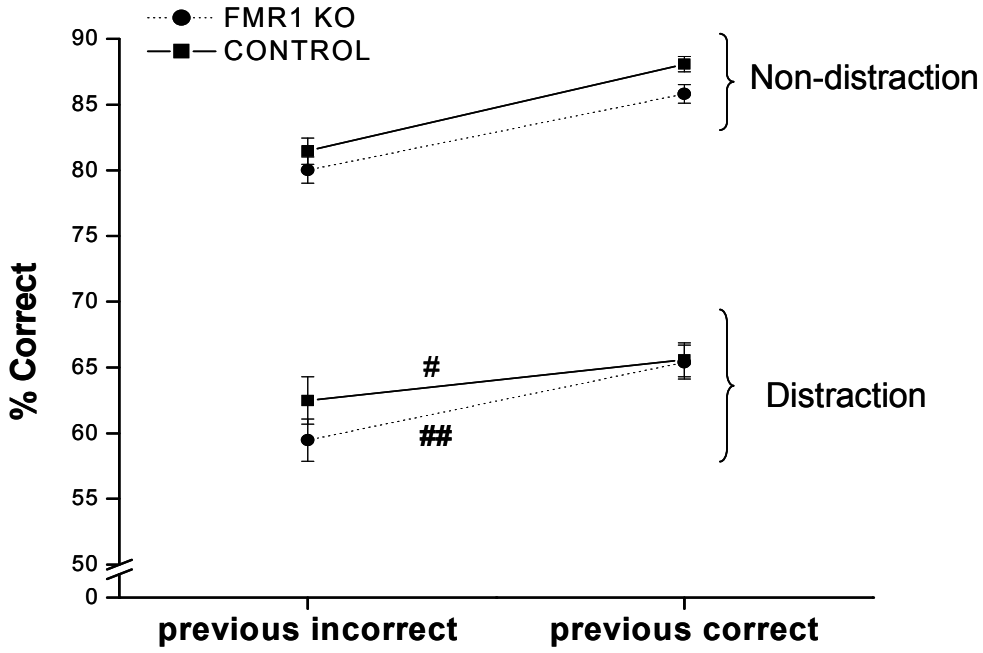

Figure 4-8: Percentage of correct responses for trials following an error vs. trials following a correct response, during the Distraction task in the LOOP. Committing an error on the previous trial decreased the rate of correct responses to a greater extent for the fmr1 KO mice than for controls (Genotype x Distraction x Previous Trial Outcome) specifically on trials with a distractor presented. However, for both groups, committing an error on the previous trial decreased the rate of correct responses. Bars represent the mean (+/- SEM). # represents p=0.01 and ## represents p=0.004.

On the trials without distractors, both groups performed more poorly when the prior trial was an error than when the prior trial was a correct response ($p<0.0001$ for each case), but the magnitude of this effect was comparable for the two groups.

Additionally, a significant interaction was detected between Genotype, Distraction, and Previous Task (the task administered on the previous session) ($F_{(2, 397)} = 4.29$, $p = 0.01$). For trials without distractors (Nondistraction trials), the performance of the *fmr1* KO mice was impaired relative to that of controls during when the prior task was Sustained Attention Task 3 ($p=0.03$), which was the most demanding attention task (shortest cue durations). In contrast, during Distraction trials, the KO group was impaired relative to controls when tested immediately following the Sustained Attention Task 1 ($p=0.01$), whereas no impairment was shown when the prior task was Sustained Attention 1 or 2 ($p > 0.9$).

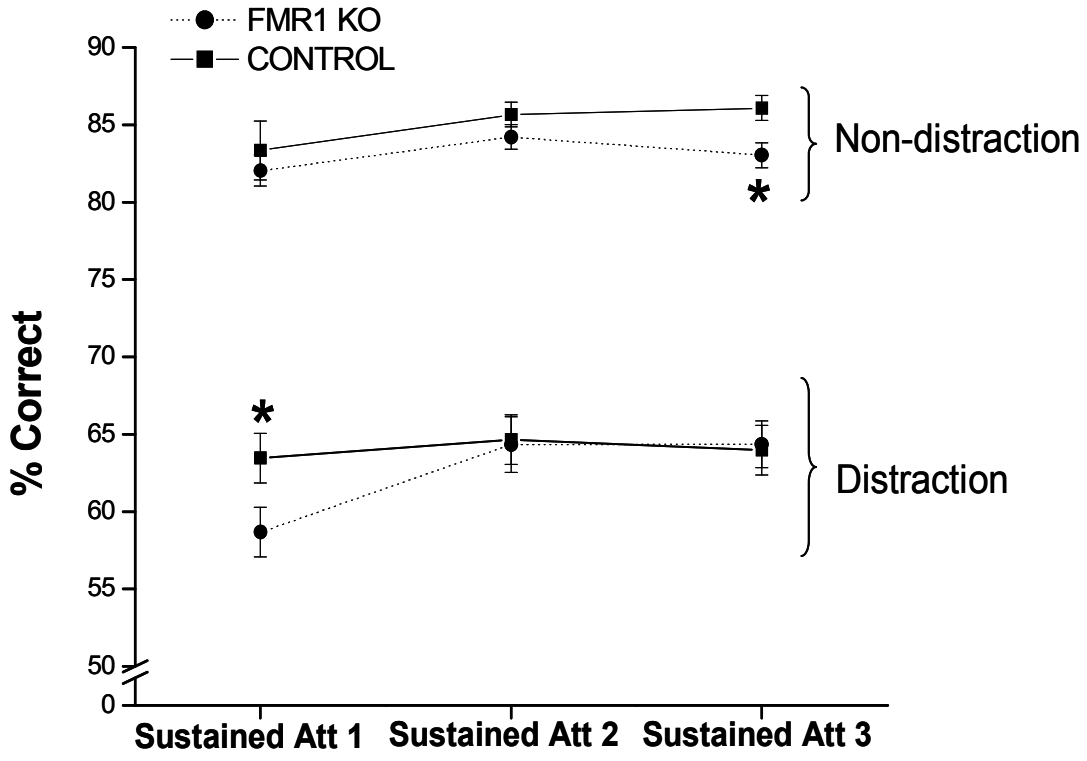

Figure 4-9: Percentage of correct responses following the task administered on the previous session during the Distraction task in the LOOP. For trials without distractors (Nondistraction trials), the performance of the *fmr1* KO mice was impaired relative to that of controls during when the prior task was Sustained Attention Task 3. In contrast, during Distraction trials, the KO group was impaired relative to controls when tested immediately following the Sustained Attention Task 1, whereas no impairment was shown when the prior task was Sustained Attention 1 or 2 ($p > 0.9$). Bars represent the mean (+/- SEM). * represents $p < 0.05$.

DISCUSSION

This study replicates and extends our prior findings that *fmr1* KO mice exhibit impaired attention and inhibitory control, prominent features of FXS. Notably, this study demonstrated dysfunction in these two domains in the KO mice across the entire 15 weeks of testing, a much longer period of time than in the prior study.

The KO mice were significantly impaired in the final three training tasks administered prior to the Loop task; i.e., the visual discrimination task with 1-sec cue duration and the two tasks with variable pre-cue delays). This was the period of training when the mice were learning to wait for and detect brief visual cues. The impairment of the KO mice during this phase suggests deficits in both attention and inhibitory control.

Converging evidence for dysfunction in these two domains was provided by the pattern of results seen for the three sustained attention tasks administered within the Loop task. These tasks placed the greatest demand on focused attention and inhibitory control, as the pre-cue delays were often long and cue duration was variable and sometimes very brief. In Sustained Attention Tasks 1 and 2, the performance of the KO mice fell more on trials with the longer-pre-cue delays (relative to trials with no delay) than did that of the WT littermate controls, indicating deficient inhibitory control and attentional dysfunction. In addition, a novel variant of this attention task which included briefer cues and longer pre-cue delays (Sustained attention Task 3) revealed a robust deficiency in focused attention. The *fmr1* KO mice were impaired relative to controls on trials with the two briefest cues (0.6 and 0.8 s) but not on trials with a slightly longer cue (1.0 s). This pattern rules out various alternative explanations for the impaired performance in this task, including alterations in motivation, motoric ability, and understanding of task contingencies, thereby strongly

implicating attentional dysfunction. This finding is an important contribution of this study, as the most prominent impairment seen in the KO mice in our prior study was deficient inhibitory control, although subtle attentional dysfunction had also been demonstrated.

The Distraction task also revealed subtle attentional dysfunction of the KO mice on trials following an error. For both groups of mice, performance was significantly lower on trials following an error than on trials following a correct response, suggesting that the affective response to committing an error and/or not receiving reward disrupted attention. For trials that both followed an error and included potent olfactory distractors, performance was impaired to a greater extent for the KO mice than for controls, although group differences were relatively small in magnitude. This finding replicates our prior finding that the *fmr1* KO mice were significantly more likely than controls to miss the cue on trials following an error (Moon et al., under review). This pattern of findings is consistent with the proposal that impaired attention and inhibitory control in FXS are often secondary to dysregulation of arousal (e.g. Cornish et al., 2004, Kerby et al., 1994, Borghgraef M et al., 1990). Dysregulation of the hypothalamic-pituitary-adrenal axis has been reported for humans with FXS (Wisbeck et al., 2000) and the *fmr1* KO mouse model (Markham et al., 2006).

Humans with FXS also tend to be resistant to change, one of the autistic features commonly seen in this syndrome. *Fmr1* KO mice also exhibit this characteristic, based on the performance disruption seen in the prior study each time task characteristics changed even slightly. The results of the present study provide indirect support for this area of dysfunction, as deficits in attention and inhibitory control were seen throughout the 15 weeks of testing in contrast to the very transient nature of these deficits in our prior study. For example, when Sustained Attention task

1 was administered in this prior study, the KO mice exhibited a robust impairment relative to controls only during the first session on the task, immediately after task characterstics had changed. For the remaining 19 sessions on the task, only very subtle impairment was evident, primarily at the end of each daily testing session. In contrast, when this task was administered in the present study, the KO mice exhibited a robust impairment relative to controls throughout the first 4 sessions on the task (spanning 6 weeks of testing), and continued to show impairment throughout the entire 12 weeks of testing on this task for trials with the longest pre-cue delays. This difference in results for this task in the two studies implies that the KO mice had greater difficulty than controls in dealing with the daily change in task characteristics. This inference is supported by the fact that the KO mice also exhibited deficits in attention and inhibitory control in Sustained Attention tasks 2 and 3 and the Distraction task throughout the entire Loop series (12 weeks of testing). One potential caveat to this interpretation, however, should be noted; i.e., that that the mice used in the present study were the products of a reciprocal cross of the parental strains (C57/BlJ and FVB/??), relative to the subjects in the prior study. It remains possible that the more lasting dysfunction in the present study could reflect phenotypic characteristics inherent to this reciprocal cross instead of, or in addition to, the design differences between the two studies.

SUMMARY AND IMPLICATIONS

In sum, the present study revealed deficits in attention and inhibitory control in the *fmr1* KO mice throughout the entire 15 weeks of testing, a much longer period of time than in the prior study. The magnitude of the impairment was largest in two conditions: (1) during the initial three attention tasks when the mice were learning to wait for and attend to brief cues, and (2) throughout the entire testing period on trials specifically on trials for which the target visual cue was very brief, placing the greatest demands on sustained and focused attention. This pattern of findings recapitulates the evidence from humans with FXS, wherein overstimulation and difficulty with change leads to loss of behavioral control and attentional dysfunction (Cornish et al., 2001; Hagerman, 1996; Mazzocco et al., 1993; Merenstein et al., 1994; Munir et al., 2000; Schapiro et al., 1995). This study lays the groundwork for future studies designed to assess the efficacy of putative therapeutic agents.

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CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

Even though a number of researchs on the mouse model of FXS have been conducted, surprisingly the hallmark symptoms of dysfunction in human FXS have not been studied in a mouse model; these include attention, inhibitory control, and regulation of arousal or emotion. The present studies were designed to test this hypothesis and further characterize spared and impaired functions in *fmr1* KO mice. The performance of F1 hybrid *fmr1* KO mice (a C57BL/6J x FVB/NJ cross or reciprocal cross) and wild-type (WT) littermate controls were assessed on a series of tasks designed to assess inhibitory control and various aspects of attention (sustained, selective, and divided attention), Reversal Learning Task, and Associate Learning Task. Regulation of arousal and emotion, two domains affected in FXS, was also evaluated in these tasks by examining the animals' reaction to the unexpected presentation of potent olfactory distractors (in the Distraction task), as well as their reaction to committing an error on a previous trial (in all tasks).

The present studies provided the first evidence that the hallmark deficits in human FXS -- impaired attention, inhibitory control, and arousal regulation – are also impaired in the *fmr1* mouse model of FXS. In addition, these findings demonstrated that attentional dysfunction and impaired inhibitory control were most prominent when task contingencies changed and when the animal had just committed an error – situations that arouse or disturb the mice. Reactivity to errors taps both error monitoring (an aspect of executive functioning) (Luu et al., 2000) and emotion regulation (Elliott et al., 1996; Luu et al., 2000), two domains affected in FXS (see chapter 2, 4). Additionally, the *fmr1* KO mice were not impaired in associative

learning, transfer of learning, or reversal learning, based on measures of learning rate whereas the analysis of videotapes of the Reversal Learning Task indicated that arousal regulation was impaired in the *fmr1* KO mice (see chapter 3). The present results provide strong support for the validity of this animal model for future studies designed to elucidate the pathogenic process in FXS and test new therapies.

Difficulties with change: Deficit in Regulation of Arousal and Emotion

Studies of humans with FXS have postulated that alterations in cognitive functioning may be secondary to alterations in the regulation of affect or emotion, but empirical support has been lacking (Mazzocco et al., 1993; Merenstein et al., 1994; Schapiro et al., 1995; Hagerman, 1996; Munir et al., 2000; Cornish et al., 2001). The results of the present studies support this inference such that impaired regulation of arousal or affect appears to be a critical factor underlying the appearance of impaired attention and inhibitory control in the KO mice (see chapter 2). In addition, these findings demonstrated that attentional dysfunction and impaired inhibitory control were prominent when task contingencies changed and when the animal had just committed an error – situations that aroused or disturbed the mice (see Chapter 2). This interpretation is further supported by the fact that, in a series of attention tasks (chapter 2) series, two other instances of impaired inhibitory control re-emerged when the performances in two adjacent tasks were directly compared. For example, the analysis directly comparing premature response rate on the final session of Attention Task 1 to the first session of the Sustained Attention Task (consecutive sessions) revealed a significant interaction of genotype and task. The rate of premature responses increased significantly across these two sessions for the *fmr1* KO, but not for the controls. Because the two groups had not differed in premature response rate for the final session of attention task 1, the increased premature response rate of the

mutant mice early in the Sustained Attention Task appeared to result from the slight change in task characteristics. The subtlety of the change in task parameters is notable: the only characteristics that differentiated the two tasks were that cue duration varied randomly across trials in the Sustained Attention Task, whereas those were constant in Attention Task 1; the 4 pre-cue delays were identical in the two tasks, as were the basic contingencies. It should be also noted that the poorer performance of the *fmr1* KO mice on this first session was not specific to any cue duration; premature responses were, by definition, independent of the duration of the visual cue on a given trial. Thus, the subtle change in task characteristics appeared to have generally disrupted the *fmr1* KO mice, which was manifested as an impaired inhibitory control. A slight increase in premature response rate of the *fmr1* KO mice, relative to controls, was also evident throughout the Distraction Task. Because this was not seen in the prior Baseline task (identical but without distractors), this re-emergence of an increased premature response rate appeared to be due to the arousal or stress of the unpredictable presentation of olfactory distractions (see chapter 2).

Finally, these findings suggested that one factor likely contributing to the difficulty encountered in previous studies in detecting cognitive deficits in *fmr1* KO mice might have been that the selected tasks primarily tapped learning and memory, rather than attention, inhibitory control, or the interface between affect and cognition.

Support for the suggestion that this disruption in performance early in the distraction task reflected impaired regulation of arousal in the *fmr1* KO mice was provided by the analysis of videotapes of the mice performing these tasks. Wall-climbing increased for all mice from the baseline task to the first block of sessions in the Distraction task (supporting the inference that wall-climbing is one index of arousal). The increase in wall-climbing on Distraction trials was comparable (for the two groups) in both groups but the increase observed on Nondistraction trials (relative

to the Baseline task) was significantly greater for the *fmr1* KO mice than the controls. The 77% of the *fmr1* KO mice had the difference score greater than the overall median score, whereas only the 33% of the controls had the difference score greater than this value. This finding (provides) provided further evidence that the unpredictable presentation of the distractors produced (a) more generalized disruption in performance for the *fmr1* KO mice than that for the controls.

Furthermore, the analysis of videotapes of the Reversal Learning Task (see chapter 3) gave an additional evidence that arousal regulation was impaired in the *fmr1* KO mice when the task contingences were reversed. Two lines of findings supported this idea such that (1) for both genotypes, wall climbing and activity level were higher during the first two sessions of the task than during the final two sessions (after the new contingencies had been mastered), indicating that the initial higher rate of these behaviors reflected heightened arousal produced by reversal of the contingencies and low rate of reinforcement; and (2) the increase in both behaviors early in the task was more pronounced for the KO mice than for the controls; group differences in these behaviors were not seen in the final sessions of the task.

An additional analysis of performance as a function of the outcome of the prior trial (correct or incorrect) revealed another instance of impaired regulation of arousal or affect. Performance of the mice in both groups was significantly disrupted after committing an error; (i.e.,) all types of errors increased on trials following an error, relative to trials that followed a correct response. Whereas this basic pattern was seen in both groups, the increase in omission errors on trials following an error was more pronounced for the *fmr1*KO mice than for the controls in both Baseline and Distraction tasks (for Baseline task: genotype and previous trial outcome). Although some studies with human subjects (have) reported an exceptionally low error rate on trials following an error (e.g., Laming, 1979; Robertson et al., 1997), indicating the

operation of an executive, error-correction system, localized to the anterior cingulate cortex (Bush et al., 2000), other studies (Rabbitt & Rodgers, 1977; Elliott et al., 1996) reported the same findings as those seen in our rodent studies, i.e. increased error rate on post-error trials (e.g., Gendle et al., 2004; Morgan et al., 2001, 2002). This pattern likely reflects the dominant influence of the emotional reaction engendered by committing an error. Interestingly, depressed subjects exhibit a more pronounced increase in the error rate on post-errors trials than non-depressed subjects (Elliott et al., 1996). More generally, an electrophysiological measure of error detection, termed the error-related negativity (ERN), varies as a function of individual differences in negative affect and emotionality (Luu et al., 2000). Thus, the current finding that the attentional disruption seen on post-error trials was more pronounced in the fmr1-KO mice than the WT controls provides a converging evidence for dysregulation of affect in these mutant mice. In light of these findings, the third study (chapter 4) demonstrated that the group difference could be lasting by changing task contingencies daily called LOOP task. This task makes the *fmr1* KO mice keep in a high arousal level, consequently the group difference gets bigger for a long period of time.

Taken together, the present studies demonstrated that impaired attention in the *fmr1* KO mice was the indirect consequence of impaired arousal regulation and/or inhibitory control which was shown in studies of humans with FXS have been postulated that alterations in cognitive functioning may be secondary to alterations in the regulation of affect or emotion (Mazzocco et al., 1993; Merenstein et al., 1994; Schapiro et al., 1995; Hagerman, 1996; Munir et al., 2000; Cornish et al., 2001).

Impaired Attention

Although, as noted above, impaired attention in the *fmr1* KO mice seem most appropriately viewed as the indirect consequence of impaired arousal regulation and/or inhibitory control, it should be emphasized that the resulting impairment was attentional in nature. Several findings from this mouse model of FXS supported this idea.

Firstly, the Sustained Attention task placed the greatest demand on sustained attention or vigilance, as the pre-cue delays were variable and relatively long, and the cue duration was variable and often very brief. As noted above, the most robust differences between the *fmr1* KO and WT mice in this task were seen on the first session, indicative of difficulty in dealing with a change in task characteristics. This performance drop on session 1 was primarily driven by the increase in premature response rate and is therefore indicative of impaired impulse control, rather than attention per se. However, throughout the 20 sessions of testing on this task, the *fmr1* KO mice exhibited a more subtle, but significant, reduction in accuracy relative to controls, an effect that was most pronounced towards the end of each test session, indicative of impaired sustained attention.

Specifically, in an olfactory reversal learning task, the *fmr1* KO mice exhibited more pronounced behavioral disruption than controls when the contingencies were reversed, and the reinforcement rate consequently dropped from 80% to 0 % correct. Specifically, coding of videotapes of the mice performing this task revealed that all mice exhibited higher rates of activity and wall-climbing early in the task (relative to later in the task, after the contingencies had been mastered), and that this early behavior change was more intense for the KO mice. However, neither accuracy of responding nor learning rate differentiated the groups in this task. Thus, in a task without attentional demands, the impaired arousal regulation of the KO mice did not

impair performance, in contrast to the attention tasks described here, where the animals were required to wait for, and then detect, brief visual cues, unpredictable in onset time and location.

FUTURE DIRECTION

The present studies revealed significant impairment in attention, inhibitory control, and arousal regulation in the *fmr1* KO mice. They would provide the needed model system for testing pharmacological interventions. We plan to test the ability of various pharmacological interventions to normalize brain development and ultimate cognitive and affective functioning. We plan to start with the mGluR5 antagonist, MPEP, based on the evidence that the consequences of group I metabotropic glutamate receptor activation are exaggerated in the absence of FMRP, likely reflecting altered dendritic protein synthesis. It has been hypothesized that abnormal mGluR signaling could be responsible for diverse psychiatric and neurological symptoms in FXS, including delayed cognitive development, seizures, anxiety, movement disorders, and obesity (reviewed in Bear et al., 2004; Bear, 2005; Yan et al., 2005; McBride et al., 2005). If this theory is correct, early developmental treatment with mGluR5 antagonists, such as MPEP, should normalize brain development; continued treatment with such drugs may allow normal brain function in adulthood. Such studies in the mouse model hold great promise for identifying a treatment that can ameliorate, or perhaps prevent, the profound dysfunction now associated with this single gene defect. In addition, such studies will provide information about the role of FMRP, a protein of great interest to basic scientists interested in neuronal plasticity and brain function.

Although several other labs are currently testing MPEP in the *fmr1* KO mouse, the proposed research will offer critical information not provided by these ongoing studies. It is possible that the efficacy of a given treatment, such as MPEP, may vary across the different functional areas of impairment (e.g., learning, seizure susceptibility, anxiety, hyperactivity, attention, inhibitory control, arousal regulation), which in turn relate to different underlying neural systems. For this reason, it is important to assess whether each putative therapeutic agent ameliorates dysfunction in each of these areas; conclusions drawn about efficacy based on only one task or area of dysfunction may not generalize to other areas of impairment. This view is based on the success of a recent study in which we assessed the ability of the chelating agent, succimer, to lessen the cognitive and affective dysfunction produced by a short period of early developmental lead exposure (Stangle et al., 2003; submitted; Beaudin et al., 2005; submitted). The lead-induced dysfunction in this study was comparable in magnitude to that seen in our prior study with *fmr1* KO mice, and the study was successful in showing a lasting benefit of the early succimer treatment, and in revealing that the magnitude of the benefit varied as a function of the different areas of dysfunction produced by the early lead exposure.

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