Rescue of Fragile X Syndrome Phenotypes in *Fmr1* KO Mice by the Small Molecule PAK Inhibitor FRAX486.

by

Bridget M. Dolan

Submitted to the Department of Biology
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN BIOLOGY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

November 2011

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Abstract

Autism is a diverse and complex family of disorders, and its prevalence is on the rise: 1 in 110 children have autism. There is no effective treatment for the symptoms which often include language and communication deficits, repetitive behavior, intellectual disability, epilepsy, attention deficits, and hyperactivity. The quest for a cure is challenging due to the heterogeneity of the disorder, but also because more than 90% of cases of autism are idiopathic, meaning the cause is unknown.

Fortunately, one cause of autism has been discovered: silencing of a single gene causes an autism-like disorder called Fragile X Syndrome (FXS). The knowledge of the genetic basis of FXS allowed for the development of a mouse model of autism. The *fmr1* knockout (KO) mouse displays phenotypes similar to symptoms in the human condition – including hyperactivity, repetitive behaviors, and seizures. Humans and mice share not only behavioral expression of the disease, but also analogous abnormalities in the density and morphology of dendritic spines – the sites of connections between neurons and critical substrates for learning. Abnormal dendritic spines is a common feature in FXS, idiopathic autism, and intellectual disability. Thus, this neuroanatomical abnormality may contribute to disease symptoms and severity.

Here we take a hypothesis-driven, mechanism-based approach to the search for an effective therapy for FXS. We hypothesize that a treatment that rescues the dendritic spine defect may also ameliorate behavioral symptoms. Thus, we targeted a protein that regulates spines through modulation of actin cytoskeleton dynamics: p21-activated kinase (PAK). In a healthy brain, PAK and FMRP – the protein product of *fmr1* – antagonize one another to regulate spine number and shape. Inhibition of PAK with a strategy utilizing mouse genetics reverses spine abnormalities as well as cognitive and behavioral symptoms in *fmr1* KO mice, as we demonstrated in our previous publication. This discovery highlights PAK as a potential target for drug discovery research.

In this thesis work, we build on this finding to test whether the small molecule FRAX486 – selected for its ability to inhibit PAK – can rescue behavioral, morphological, and physiological phenotypes in *fmr1* KO mice. Our results demonstrate that

seizures and behavioral abnormalities such as hyperactivity, repetitive movements, and habituation to a novel environment can all be rescued by FRAX486. Moreover, FRAX486 reverses spine phenotypes in adult mice, thereby supporting the hypothesis that a drug treatment which reverses the spine abnormalities can also treat neurological and behavioral symptoms.

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Acknowledgments

Many people contributed, both scientifically and personally, to this thesis work. I am grateful to all of them. Here, I would like to thank a few in particular.

The Tonegawa lab is a special place where big dreams and epic curiosity live. I thank my thesis supervisor, Susumu Tonegawa, for fostering this environment and bringing together an exceptional group of bright and dedicated minds. Thank you, Susumu, for encouraging me to ask the important questions and giving me the freedom to follow my dreams, even when they led me out of academia and into the world of science policy.

The spirit of science promoted by Susumu is best exemplified by two exceptional people: Arvind Govindarajan and Alex Rivest.

It was after sitting in on Arv's thesis defense in the fall of my first year of graduate school, that I wanted to join the Tonegawa lab. The breadth and depth of Arv's knowledge and passion for discovery amazed me then and continues to now. Arv is not only the smartest person I know, but also one of my best friends. He is generous, trustworthy, energetic, and the life of any party. While he moved on from MIT to embark on a new career a year ago, he has continued to serve as my primary mentor.

Scientific curiosity lives in Alex. You can hear it in his lab meetings and see it in his photographs of the stars. He has inspired and supported me, giving me the courage to express myself in science and in life. He is patient but timely, and provided feedback on every thesis chapter within 24 hours. His intellectual and emotional support helped me through the most challenging times in graduate school and was critical in the writing of this thesis.

Jennie Young has acted as a mentor and teacher to me and many of the other blossoming, female scientists in the lab since we both joined in the summer of 2005. I have tremendous respect for Jennie, who provided critical scientific expertise and constructive criticism, in particular on the analysis and interpretation of physiology experiments and the oral thesis presentation.

Junghyup Suh is the quiet leader of the group. He is an impeccable writer, pre-

senter, and scientist.

Greg Lin is an exceptional undergraduate student at Carnegie Mellon University who dedicated summer and winter breaks to working in the lab. He is an independent worker and a great problem solver, who made significant contributions to my research projects.

I would like to give a special thanks to additional wonderful colleagues at MIT, especially:

- Mansuo Hayashi for initiating the PAK project and taking me under her wing to get me started in the lab.
- Shu Ying Huang for her friendly and gentle ways of building community (and for great teamwork in tissue culture).
- Arek Hamalian for accelerating the rate of academic science.
- Emily Hueske for standing as a pillar of GraSA, the T-lab graduate student group that graduated 4 Ph.D.s in one year.
- Amy LeMessurier for expertise in physiology analysis.
- Dina Rooney for diligent work in the mouse colony week after week.
- Candy Carr for managing the transgenic facility with humor and an iron fist.
- Sean Perry for being our very own computer whiz.
- Derek Buhl and Dan Bendor for sound production.
- Betsey Walsh, Janice Chang, and Nick Polizzi from the Biology Education Office for assisting the department's graduate students with excellence, sponsoring inspiring events during MIT's IAP, and making great thesis defense posters.

In addition, I am grateful for the opportunity to work with excellent collaborators:

- Sergio Duron, David Campbell, Ben Vollrath, and Chris Rex, Afraxis.
- B.S. Shankaranarayana Rao, Dept of Neurophysiology, National Institute of Mental Health and Neuro Sciences, India.
- Se-Young Choi and Hui-Yeon Ko, Dept of Physiology, Seoul National University School of Dentistry.

I want to express my sincere appreciation to my Thesis Committee for all the time they dedicated to my graduate education: David Housman (chair), Troy Littleton, Li-Huei Tsai, and Damon Page (Scripps Florida), as well as former members Morgan Sheng and Mark Bear.

And finally, I am deeply thankful for my family. My parents have always encouraged me to pursue excellence and given me the tools required to reach the top. They have selflessly provided me with a loving home and served as my role models in more ways than they know. It was wonderful to share my Ph.D. experience with them, Ellen, and my fan club from around the country who flew in to make my defense a special day: Julie, Larry, Kristen, Travis, Matt, Grace, and Gerry. Thanks for the laughs, love, and patience. I am thrilled to start the next chapter of my life with all of you by my side.

This thesis is dedicated to my grandmother, Margaret Dolan.

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List of Abbreviations (A - E)	
ACSF	artificial cerebral spinal fluid
AD	Alzheimer's Disease
ADF	actin-depolymerization factor
ADHD	attention deficit and hyperactivity disorder
ADME	absorption, distribution, metabolism, and excretion
AGS	audiogenic seizure
AID	auto-inhibitory domain
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
ASD	autism spectrum disorder
B6	C57BL6
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
CCG	cytosine, cytosine, guanine
CNS	central nervous system
CYFIP	cytoplasmic FXR interacting protein
dFmr1	*Drosophila* homolog of the gene *fmr1*
DHPG	dihydroxyphenylglycine
DMSO	dimethyl sulfoxide
dMT	double mutant (*fmr1* KO; *dnPAK* TG)
dn	dominant negative
dFXR	*Drosophila* homolog of the protein FMRP
EDR	electrodermal response
EGFP	enhanced green fluorescent protein
ERK	extracellular-signal-regulated kinase

List of Abbreviations (F - M)	
fEPSP	field excitatory postsynaptic potential
fmr1	fragile X mental retardation 1 gene
***fmr1* KO**	FVB.129P2-Fmr1^{tm1Cgr}/J
FMRP	fragile X mental retardation protein
FRET	fluorescence resonance energy transfer
FVB	FVB.129P2-*Pde6b*$^{+}$ *Tyr*$^{c-ch}$/AntJ; aka FVBS/Ant (Errijgers et al. 2007)
FXPOI	Fragile X associated primary ovarian insufficiency
FXR1P, FXR2P	mammalian autosomal homologs of the protein FMRP
FXS	Fragile X Syndrome
FXTAS	Fragile X tremor/ataxia syndrome
HRP	horse radish peroxidase
IC$_{50}$	concentration at which the activity of a specific enzyme is inhibited by 50%
ICc	inferior colliculus
IEG	immediate-early gene
KH	K homology
KO	knockout
LC/MS/MS	liquid chromatography with tandem mass spectrometry
LIMK	LIM kinase
L-LTP	late phase of long-term potentiation
LTD	long-term depression
LTP	long-term potentiation
MAP1B	microtubule-associated protein 1B
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase

List of Abbreviations (M - Z)	
mGluR	metabotropic glutamate receptor
miR	microRNA
mRNP	messenger ribonucleoprotein complex
mTOR	mammalian target of rapamycin
NCE	new chemical entity
NMDA	*N*-methyl-d-aspartate
p	postnatal day
PAK	p21-activated kinase
PBS	phosphate buffered saline
PDD-NOS	pervasive developmental disorder not otherwise specified
PDGF	platelet-derived growth factor
PI3K	phosphoinositide 3-kinase
PK	pharmacokinetics
PKC	protein kinase C
PP-LFS	paired-pulse low-frequency stimulation
PSD	postsynaptic density
SEM	standard error of the mean
SSRI	selective serotonin reuptake inhibitor
STDP	spike timing-dependent plasticity
TBS	theta-burst stimulation
TG	transgenic
VEGF	vascular endothelial growth factor
veh	vehicle alone control
WT	wildtype

Chapter 1

Introduction

1.1 Overview.

Neurodevelopmental disorders like autism and Fragile X Syndrome have profound effects on individuals, families, and society. Unfortunately, the prevalence of autism spectrum disorder (ASD) – an umbrella diagnosis which includes classic autism, Rett Syndrome, Asperger's Syndrome, and often Fragile X Syndrome – is on the rise. In the 12 years from 1997 to 2008, the prevalence of autism increased by 289.5% such that 1 in 110 children age 3 to 10 in the U.S. have an ASD (Boyle et al. 2011). There is no cure for these diseases, but clinicians and scientists are working together to develop drugs to treat not only the symptoms but also the cell signaling and neuroanatomical defects thought to underlie the disease. This thesis describes a small molecule – selected for its ability to inhibit the actin cytoskeleton remodeling protein p21-activated kinase (PAK) – and the discovery of its therapeutic potential. The novel PAK inhibitor FRAX486 rescues dendritic spine defects, seizures, hyperactivity, excessive repetitive behaviors, and loss of habituation in an animal model of FXS.

ASD is a diverse and complex family of disorders with multiple causes, at least 90% of which are unknown (Abrahams and Geschwind 2008). The heterogeneity of ASD, both in terms of its causes and symptoms, makes drug discovery challenging. It is clear that genetic vulnerabilities play a significant role in its etiology. Fortunately, one of the genes responsible for some cases of ASD is known: *fragile X mental retardation*

1 (fmr1). FXS, caused by the silencing of a single gene, is the most common inherited form of both autism and intellectual disability, formerly known as mental retardation. With a simple, known cause, FXS is a tractable disease and the subject of much research ranging from basic neurobiology to drug development that may eventually benefit families and individuals with FXS, idiopathic ASD, and intellectual disability.

In this introductory chapter, I introduce FXS and the animal model with an analogous mutation and similar phenotypes. This mouse model of FXS has been a valuable tool for understanding the disease on multiple levels – molecular, biochemical, cellular, anatomical, and behavioral – all of which are discussed in this chapter. Humans and mice share not only behavioral expression of the disease, but also analogous abnormalities in the density and structure of dendritic spines, the sites of connections between neurons and critical substrates for behavior and learning. Interestingly, abnormal dendritic spine morphology has also been reported in cases of idiopathic ASD and intellectual disability. Thus, it is hypothesized that atypical spine density and shape may contribute to the cognitive and behavioral disease symptoms.

From this hypothesis comes the prediction that therapies that rescue the spine defect may also rescue some or all of the behavioral phenotypes. Dendritic spine morphology is controlled by the actin cytoskeleton. In this way, important regulators of actin dynamics may be good drug targets for therapies for neurodevelopmental disorders. This chapter provides background information on critical components of the signal transduction pathways required for spinogenesis and morphological plasticity. Here I highlight research supporting the hypothesis that inhibition of a specific kinase in the actin remodeling pathway – PAK – may be sufficient to reverse symptoms in the mouse model of FXS.

Finally, drug discovery is a challenging but vitally important endeavour. The empirical approach to drug discovery – focused on safety and efficacy in humans, but blind to the underlying biology – has been sidelined for a mechanism-based, targeted approach. The last section of this introductory chapter discusses the principles and challenges of modern, hypothesis-driven therapeutic strategies, in particular as they relate to FXS and the kinase PAK.

1.2 Fragile X Syndrome.

Fragile X Syndrome (FXS) is the leading inherited cause of both intellectual disability – a term synonymous with and now preferred to mental retardation – and autism known today (Turner et al. 1996)(Reddy 2005). Decades of research, including the visualization of an unusual constriction, or fragile site, on the X-chromosome, culminated in the discovery of the causative mutation: the expansion of a trinucleotide repeat upstream of a single gene, *fmr1* (Lubs 1969)(Harrison et al. 1983)(Verkerk et al. 1991). Its prevalence, known genetic etiology, and relationship with common but less well understood neurodevelopmental disorders – including autism spectrum disorder (ASD), attention deficit and hyperactivity disorder (ADHD), and learning disabilities – make FXS a valuable model for understanding the neurobiology of disease and developing targeted treatments (Berry-Kravis et al. 2011).

1.2.1 Genetics of Fragile X Syndrome.

Men are more likely to have intellectual disabilities than women, a fact that was recognized by doctors and scientists in the beginning of the last century. In 1943, a seminal study on a family with 11 intellectually disabled males described the first evidence of an X-linked form of inherited mental retardation and named it Martin-Bell Syndrome (Martin and Bell 1943). The pedigree analysis was consistent with X-linked recessive inheritance, as approximately 50% of the unaffected females transmitted intellectual disabilities to 50% of their sons. However, two facts conflicted with recessive inheritance: First, the grandfathers of the affected males did not show signs of cognitive impairment. Second, two females (the mothers of afflicted sons) exhibited mild intellectual disabilities. This phenomenon – the increase in severity of disease with successive generations – became known as the Sherman paradox (Sherman et al. 1985). The paradox was resolved a few years later following the discovery of the gene responsible for the disease later named FXS (Fu et al. 1991)(Verkerk et al. 1991).

The next breakthrough came in 1969, with the karyotyping of a family spanning four generations. The long arm of the X chromosome in all four affected males and

two unaffected females contained a constriction that resembled a break or fragile site (Lubs 1969). The fragile site was only seen when cells were grown under conditions of folate or thymidine depletion. It is a region of chromatin that fails to compact normally during mitosis – presumably because it is the last to be transcribed or is not transcribed – and therefore is prone to breakage. This initial observation of a "fragile X" was corroborated by a cytogenetic evaluation of the intellectually disabled males from the original family described by Martin and Bell (Richards et al. 1981). The precise location of the fragile site was identified with scanning electron microscopy and mapped to position q27.3 on the X chromosome (Harrison et al. 1983). Nearly a decade later, with the advent of new tools in molecular biology, a CpG island and a trinucleotide repeat were identified in this region on the X chromosome that were methylated and expanded in affected males, respectively (Heitz et al. 1991)(Yu et al. 1991).

Finally, in 1991, the gene was isolated, characterized, and named *fragile X mental retardation 1* (*fmr1*)(Verkerk et al. 1991). There are 17 exons spanning 38 kb of the X chromosome in the *fmr1* gene, and *fmr1* is expressed in the brain and throughout the body of unaffected individuals (Eichler et al. 1993)(Verkerk et al. 1991)(Hinds et al. 1993). The Sherman paradox was resolved with the discovery that the CCG trinucleotide repeat was expanded to variable lengths, the expansion increased with successive generations, and this increase correlated with an escalation in the severity of symptoms (Fu et al. 1991). A healthy individual has 6 - 54 CCG trinucleotide repeats. The *fmr1* gene becomes mutated through a two-step process. The first mutation results in the expansion of the CCG sequence to 55 - 200 repeats (called a premutation), and the person is considered a carrier and has no clinical symptoms or mild symptoms. The second generation of the mutation, results in an affected individual with more than 200 repeats (called a full mutation). The premutation only expands into a full mutation when transmitted by a female to her offspring, as the expansion process involves a recombination event with the other X chromosome. The full mutation results in hypermethylation of the CpG island and transcriptional silencing of the *fmr1* promoter with the consequent loss of gene expression (Devys

et al. 1993).

While originally thought to be a recessive disorder, geneticists noticed that ~1/3 of carrier females showed mild symptoms including learning difficulties, emotional and social problems, depression, and occasionally mild cognitive impairment (Tassone et al. 2000)(Cornish et al. 2005). This led to the realization that FXS is not recessive, but rather an X-linked dominant disorder with reduced penetrance in females as a result of X-inactivation (Sherman et al. 1985). Female carriers of the premutation – even those considered cognitively and emotionally healthy – may experience problems later in life. Twenty-eight percent of female carriers suffer from premature ovarian failure and early menopause, a condition called Fragile X associated primary ovarian insufficiency (FXPOI) (Cronister et al. 1991)(Sullivan et al. 2005)(Welt et al. 2004). Male premutation carriers are at risk for developing a neurological condition called Fragile X tremor/ataxia syndrome (FXTAS) – characterized by ataxia, tremor, dementia, autonomic dysfunction, and Parkinsons-like symptoms – usually by age 50 (Hagerman et al. 2004)(Tassone et al. 2004). Thus, even premutation carriers may be symptomatic.

1.2.2 Symptoms of Fragile X Syndrome.

Cognitive Symptoms

Intellectual disability, formerly called mental retardation, is the hallmark feature of FXS. While preschool age children with FXS show an IQ in the borderline to average range, intellectual growth slows in childhood and adolescence relative to their peers. In this way, FXS boys progressively fall behind in school. By adulthood, most FXS men have an IQ in the 40s – compared to the mean score of 100 – with specific deficits in visual-spatial skills, attention, and executive function (Hagerman 1997). Females with the full mutation tend to have learning disabilities, with 25% of females with cognitive problems serious enough to be characterized as intellectual disability (de Vries et al. 1996). Men with the full mutation and complete methylation have a mean IQ of 41, while men with the full mutation but less than 50% methylation

have a mean IQ of 88 (Merenstein et al. 1996). In this way, the IQ of an individual depends on the methylation pattern of his DNA, which impacts expression of *fmr1* and production of Fragile X Mental Retardation Protein (FMRP)(Warren and Ashley 1995).

Physical Symptoms

In addition to the cognitive features of FXS, a number of physical symptoms are common. The typical triad of features in FXS adult males includes a long face, prominent ears, and enlarged testes – a condition known as macroorchidism (Hagerman 1997). Also common are flat feet, high arched palate, and connective tissue abnormalities like hyperextensible finger joints. Of all the physical features, it has been suggested that large ears is the feature specifically associated with FXS but not other causes of intellectual disability (Guruju et al. 2009).

Behavioral Symptoms

Males with FXS have characteristic behavioral features. Hyperactivity and attentional problems are present in 89% of FXS boys, while perseverative language and restrictive, repetitive behaviors such as hand-flapping occur in 95% of boys (Merenstein et al. 1996)(Hagerman 1997). FXS males display poor eye contact, excessive shyness, and anxiety. Anxiety disorders are often seen in both males and females with this syndrome and include selective mutism, social phobia, specific phobias, as well as generalized anxiety (de Vries et al. 1996)(Sullivan et al. 2007)(Hagerman et al. 2009). Aggression occurs in at least ~30 - 50% of males and can be accompanied by impulsivity, hand biting, and tactile defensiveness (Hagerman et al. 2009). Females with FXS tend to have milder and more variable symptoms. They often exhibit executive function and attention deficits, even when their IQ is within the normal range (Hagerman et al. 2009)(de Vries et al. 1996). Visual-perceptual deficits are also notably affected in these females. Finally, social deficits and social anxiety are problematic in FXS females and can lead to shyness and selective mutism (Berry-Kravis et al. 2011).

Neurological Symptoms

FXS males experience the world differently. Hyperarousal and strong reactions to tactile, visual, olfactory, or auditory stimuli in the environment can lead to tactile defensiveness – defined as avoidant, aversive, or negative responses to typically neutral stimuli of any sensory modality (Hagerman and Hagerman 2002)(Kinnealey et al. 1995). Sensitivity to visual stimuli or visual avoidance presents itself in over 90% of FXS males, including high functioning males of normal intelligence (Merenstein et al. 1996). Auditory hypersensitivity is also a common sensory-perceptual abnormality in individuals with FXS as well as other types of autism. Its prevalence ranges from 15 - 100% in patients on the autism spectrum (in clinical research tests 15 - 40%, in parent questionnaires 16 - 100%, in teacher questionnaires $\sim$ 30%)(Gomes et al. 2008).

To characterize the sensory responses of FXS patients in a controlled and quantitative way, Miller et al. established a laboratory paradign to record patterns of skin responses to various environmental stimuli (Miller et al. 1999). The magnitude of the skin (aka dermal) response is used as a metric of physiological or psychological arousal. The electrodermal responses (EDRs) of FXS patients were significantly different than those of neurotypical humans. FXS EDRs were of greater size and more frequent, with lower rates of habituation (Miller et al. 1999). These finding gave rise to the theory that there is a physiologically based enhancement of the experiences and reaction to sensations in FXS patients compare to neurotypicals. Corroborating evidence of a biological basis comes from EEG recordings in the somatosensory and auditory corticies of FXS patients, demonstrating increases in cortical excitability (Ferri et al. 1994)(Musumeci et al. 1999).

While epilepsy is not common with other types of intellectual disability, seizure susceptibility affects many males with FXS and other types of autism with a prevalence of 13 - 18% and 25%, respectively (Berry-Kravis 2002)(Hara 2007). Many seizures are grand mal or generalized events in which the whole brain is involved from the onset, while other episodes may be more subtle, partial complex or partial

motor seizures which start at a specific focal area (Musumeci et al. 1999). Seizures are characterized by muscle tension followed by convulsions, a types of seizure called tonic-clonic. If uncontrolled, status epilepticus – a state of persistent seizure – is rare but has a likely fatal outcome. While the pathogenesis of seizures in FXS is not fully understood, they can be precipitated by environmental stimuli (Hagerman and Hagerman 2002). Abrupt violent attacks not induced by such stimuli may be temporal lobe or partial complex seizures (Hagerman and Hagerman 2002).

1.2.3 FXS Causes Autism with Incomplete Penetrance.

Biological complexity and heterogeneity contribute to the etiology of autism. While the cause of most cases of autism remains unknown, multiple lines of evidence demonstrate that autism has a strong genetic basis. In families with a child on the autism spectrum, the risk that the next sibling will have the disorder is 25 - 50 times greater than for the general population (Abrahams and Geschwind 2008)(DiCicco-Bloom et al. 2006). Twin studies present even more compelling evidence that autism is a heritable disorder: concordance rates are 70 - 90% for monozygotic twins and 0 - 10% for dizygotic twins. This evidence has led many researchers to search for the autism susceptibility loci.

To date, FXS is the most common known single gene cause of autism, responsible for 2 - 6% of all cases (Hagerman et al. 2010). However, it is also important to note that FXS causes autism with incomplete penetrance: 30% of males with FXS have classic autism (sometimes called autistic disorder) and an additional 30% have pervasive developmental disorder not otherwise specified (PDD-NOS) (Hagerman et al. 2010). PDD-NOS includes cases where the criteria for classic autism have not been met due to late age of onset, atypical symptomotology, or sub-threshold symptomotology (American Psychiatric Association 1994). Of the remaining 40% of FXS patients, the majority have one or more autistic feature. There is evidence that patients with additional neurological medical problems such as seizures, have an increased risk of having classic autism compared to patients with FXS alone (Garcia-Nonell et al. 2008). In addition, males and females with FXS and low IQs have

a higher chance of qualifying for an autism diagnosis than their more intellectual counterparts (Hagerman et al. 2010).

ASD and FXS are heterogeneous disorders in terms of which symptoms an individual presents as well as the severity of these symptoms. This variability is likely the result of differences in genetic background effects, prenatal and postnatal environment, and the interaction of the two. In other words, the interrelationships among behavioral, cognitive, and attentional deficits in FXS are modulated by added environmental influences and additional genetic irregularities (Hagerman et al. 2010)(Spencer et al. 2011). There are a few examples of patients with FXS plus additional pathological mutations – such as Down syndrome, Tourette syndrome, other sex chromosome disorders, allelic variants of the serotonin transporter, and gene expression changes related to Prader-Willi phenotype (Garcia-Nonell et al. 2008)(Stevens et al. 2010)(Hessl et al. 2008)(Nowicki et al. 2007). Their symptoms tend to be more severe or more diverse than patients with FXS alone. Males with the later condition – Prader-Willi phenotype and FXS – have severe obesity, excessive hunger, hypogenitalia, and a higher prevalence of autism than males with FXS alone (Nowicki et al. 2007). The prenatal and postnatal environment is also likely to impact disease expression. Environmental influences on the symptoms of FXS and their severity are just beginning to be explored and likely include exposure to toxins, perinatal asphyxia, and abuse or neglect (Hagerman et al. 2010).

1.2.4 Neuroanatomical Basis for Cognitive and Behavioral Disorders.

Dendritic Spine Abnormalities in Intellectual Disability

A breakthrough came in the quest to understand the neurobiological basis of intellectual disabilities, FXS, and autism with autopsy studies dating back to the 1970s. While gross anatomy was intact, abnormalities were present on the level of dendritic spines, the main sites of excitatory synaptic input for neurons. Using the Golgi staining technique developed by Camillo Golgi in the 19th century and used by Ramoñ

y Cajal to make seminal observations in neurobiology, detailed neuronal anatomy can be visualized in brain slices from postmortem tissue or biopsies (Ramoñ y Cajal 1909). The first few studies analyzed cortical neurons in infants and children with moderate to profound intellectual disability due to chromosomal trisomy (as in Down Syndrome) or of unknown etiology (Marin-Padilla 1972)(Purpura 1974). In most cases, abnormally long, thin, and tortuous spines were observed on apical dendritic segments in cortical tissue, and some of these spines appeared entangled with neighboring spines. A second type of spine abnormality was observed in a profoundly cognitively challenged 12 year old with normal karyotypes: some apical dendritic segments had an almost complete absences of spines (Purpura 1974).

Spine Phenotypes in FXS

The long and thin spine morphology has also been observed in patients with FXS. The initial autopsy study in FXS analyzed the cortex of a 62 year old man with moderate intellectual disability and found abnormally long, thin, and tortuous spines described as "synaptic immaturity," and reminiscent of the brains of children with trisomy (Rudelli et al. 1985). The same phenotype was reported in two new FXS cases in 1991. This study also counted spines, but found no neuronal density differences in neocortical layers II - VI of cingulate and temporal association areas (Brodmann's areas 23 and 38) (Hinton et al. 1991). Increased spine density was reported for the first time in a study of neurons in the temporal and visual cortex of three FXS patients. In addition to displaying a density phenotype, these FXS neurons also exhibited more long spines and fewer shorter spines. The authors postulated that a spine maturation and elimination defect was responsible (Irwin et al. 2001).

Dendritic Spine Abnormalities in ASD

Just last year, spine analysis was conducted in a large-scale study of postmortem brains of ASD males (Hutsler and Zhang 2010). Pyramidal neurons of the temporal, frontal, and parietal lobes had increased spine density in layer II/III neurons. In layer V neurons, the density phenotype was evident in only one region, the temporal

cortex. As the scientists had detailed medical histories of all 10 ASD males, they were able to look for correlations between spine density and multiple medical or behavioral symptoms. Level of cognition was a predictor of density abnormalities, as the more severely intellectually disabled patients had the highest spine densities, providing support for the hypothesis that abnormal spine density may be the cause of intellectual disability (Hutsler and Zhang 2010).

Additional, Preliminary Observations in Brain Anatomy

While previous studies did not observe abnormalities in brain weight or volume in FXS, recent cross-sectional, *in vivo* neuroimaging studies have reported differences in the volumes of specific brain regions in young FXS boys compared to both ASD patients without FXS and healthy subjects (Wilson et al. 2009)(Hazlett et al. 2009)(Hoeft et al. 2010)(Hoeft et al. 2011). However as the findings of these studies – three of which were conducted by the same laboratory – are conflicting, more research and perhaps better techniques are required before conclusions can be made with confidence.

1.3 Animal Models of FXS.

1.3.1 Engineering of a Mouse Model of FXS.

The knowledge that such remarkable cognitive, behavioral, and neuroanatomical defects are caused by the silencing of a single gene, has led to the emergence of a research field to study the neurobiology and synaptic mechanisms of FXS and neurodevelopmental diseases more broadly. *Fmr1* is highly conserved between humans and mice, with nucleotide and amino acid homology of 95% and 97% respectively, including the upstream trinucleotide repeat (Ashley et al. 1993). Furthermore, expression patterns of *fmr1* mRNA are similar in tissue distribution and developmental time course in humans and mice (Hinds et al. 1993)(Hergersberg et al. 1995).

These facts, and the knowledge that FXS is caused by what is functionally a

null mutation in the causative gene, suggested that a knockout mouse would produce a valid and applicable animal model for the human condition. With this in mind, the Dutch-Belgian Fragile X Consortium engineered a mouse model of FXS through the inactivation of murine *fmr1* by a homologous recombination event that interrupted exon 5 with a neomycin cassette, resulting in a functional knockout of *fmr1* in 129/OlaHsd embryonic stem cells introduced into the C57BL6 (B6) background strain (and subsequently crossed with the FVB/N strain by Dr. Oostra)(Dutch-Belgian Fragile X Consortium 1994). Since then, this mouse and numerous *in vitro* studies of the function of the product of the gene *fmr1*, fragile X mental retardation protein (FMRP), have provided important insights into the regulation and function of cellular and synaptic mechanisms in health and in disease.

1.3.2 Phenotypes in *Fmr1* Knockout Mice.

Background Genetics Influence Presentation of FXS

As was mentioned in the previous section on humans with FXS and autism, there is heterogeneity in the presentation of these complex disorders, likely due to genetic background effects, prenatal and postnatal environment, and the interaction of nature and nurture (Hagerman et al. 2010). Thus, not all patients express the same combination of symptoms, nor do symptoms present themselves to the same extent. This is also true in the mouse model of the disease. Historically speaking, most behavioral studies on the mouse model of FXS have been conducted on a pure B6 or FVB strain (reviewed in (Bernardet and Crusio 2006)), though recent studies have explored how behavioral phenotypes are modified by genetic strain (Spencer et al. 2011). Some of the strain differences are highlighted below.

Cognitive Symptoms

While intellectual disability is a prominent feature of FXS in humans, it is inconsistently reported in the mouse model of the disease (reviewed in (Bernardet and Crusio 2006)). Genetic background has been shown to alter the cognitive phenotype in *fmr1*

KO mice (Spencer et al. 2011). For example, Dobkin et al. reported a learning deficit in a visual-spatial task – the hidden platform version of the Morris Water Maze – in *fmr1* KO mice on the FVB/N-129 hybrid background but not on the B6 background (Dobkin et al. 2000). If a learning or memory deficit is present in the mouse model of FXS, it is highly sensitive to both genetic background and environmental factors which may differ between animal facilities. In accordance with this, our laboratory observed a trace fear conditioning deficit in B6 *fmr1* KO mice (Hayashi et al. 2007), which could not be replicated when the laboratory switched buildings and obtained a modern, ventilated rack system for housing laboratory mice. Similarly, other labs have reported phenotypes in learning and memory tasks that could not be repeated (Kooy et al. 1996)(Yan et al. 2004)(Zhao et al. 2005)(Dolen et al. 2007). In summary, *fmr1* KO mice have a subtle or no cognitive deficit, and genetic background and environmental factors significantly alter behavioral responses in different strains of mice (Spencer et al. 2011).

Physical Symptoms

Like males with FXS, *fmr1* KO mice display enlarged testes, a condition known as macroorchidism (Dutch-Belgian Fragile X Consortium 1994). There is no obvious effect on facial features or connective tissue as is seen in humans with FXS, at least none that are detectable to human observers (Hagerman 1997).

Behavioral Symptoms

The mouse model of FXS displays a number of behavioral symptoms, some of which are consistent with predictions based on humans with FXS and autism. Increased locomotion is a reliable and reproducible phenotype in *fmr1* KO mice on multiple genetic backgrounds (Dutch-Belgian Fragile X Consortium 1994)(Mineur et al. 2002)(Spencer et al. 2011) (reviewed in(Bernardet and Crusio 2006). This increased activity, usually assayed in novel environments, can also be interpreted as increased exploratory behavior or decreased anxiety. In particular, *fmr1* KO mice often spend more time in the center of a brightly illuminated arena than their wildtype (WT)

counterparts, though background strain impacts the expression of this phenotype (Yan et al. 2004)(Hayashi et al. 2007)(Spencer et al. 2011). Finally, restrictive and repetitive behaviors – one of the core criteria for a diagnosis of autism in humans – are seen more often *fmr1* KO mice than WT controls. These stereotopies can manifest themselves as episodes of excessive grooming, including repetitive forearm gestures in *fmr1* KO mice, movements that resemble the distinctive hand flapping observed in FXS humans (Hayashi et al. 2007)(Lewis et al. 2007). Cognitive perseveration has also been investigated, and was assayed as the rate of extinction following training to swim to a platform in a few versions of the Morris Water Maze. Three studies found that *fmr1* KO mice had significantly longer latencies to find the platform when it had been moved from the original position (Dutch-Belgian Fragile X Consortium 1994)(Kooy et al. 1996)(D'Hooge et al. 1997), while two studies found no differences between WT and *fmr1* KO (Paradee et al. 1999)(Van Dam et al. 2000). Finally, abnormal social interactions have been observed in *fmr1* KO mice in a few tests – including a mirrored chamber test, tube test of social dominance, social interest, and social recognition tests – and phenotypes are influenced by genetic background (Spencer et al. 2005)(Mineur et al. 2006)(Spencer et al. 2011).

Neurological Symptoms

Fmr1 KO mice display idiosyncratic responses to sensory stimuli. Phenotypes have been reported in assays of auditory startle response, prepulse inhibition, and classical delay eye-blink conditioning (Chen and Toth 2001)(Nielsen et al. 2002)(Qin et al. 2005)(Koekkoek et al. 2005). Recently, when tested for analgesia-related responses, *fmr1* KO on multiple genetic backgrounds exhibited an increased latency to show a hindlimb response in the hotplate assay, suggesting they may be less sensitive to pain (Spencer et al. 2011), though this may be strain dependent as *fmr1* KO mice on the FVB background were found to perform normally (Zhao et al. 2005). Finally, increase seizure susceptibility is a robust and reliable phenotype in *fmr1* KO mice (see Chapter 3 for a thorough literature review) (Chen and Toth 2001)(Yan et al. 2004)(Yan et al. 2005)(Musumeci et al. 2007)(Dolen et al. 2007). Seizures are

induced by extremely loud sounds and are sensitive to genetic modifiers. Therefore, they are called audiogenic seizures (AGS). Genetic modifiers allow for the induction of seizures in *fmr1* KO mice of any age on the FVB background (Chen and Toth 2001)(Yan et al. 2004)(Yan et al. 2005). In contrast, there is a three day window in which AGS can be reliably induced in B6 *fmr1* KO mice (Chen and Toth 2001)(Dolen et al. 2007). WT mice seldom, or in some studies never, have seizures of this type.

Dendritic Spine Phenotype

In postmortem cortical tissue of young and old males with FXS, dendritic spines are abnormally long, thin, and abundant (Rudelli et al. 1985)(Hinton et al. 1991)(Irwin et al. 2001). This increase in immature dendritic spines and decrease in mature, stubby shaped spines has also been observed in Golgi staining of pyramidal neurons in the cortex of *fmr1* KO mice (Comery et al. 1997)(Irwin et al. 2002)(Hayashi et al. 2007). This important discovery demonstrates that the mouse model resembles the human condition on a cellular and neuroanatomical level. When considered in combination with the behavioral and neurological phenotypes which are analogous in the *fmr1* KO mouse and FXS humans, this finding highlights the high degree of similarity of the disease symptoms between *fmr1* KO mice and FXS humans, an important concept in therapeutic research described as the face validity of an animal model (Crawley 2007). This provides hope that the mouse model may have predictive validity, that is, therapies that are effective in the animal model may also prove effective in humans with FXS.

The details of the presentation of the spine phenotype in *fmr1* KO mice have been extensively studied. Dr. William Greenough's laboratory conducted a number of Golgi staining studies on neurons in various cortical regions in *fmr1* KO mice on both the B6 and FVB backgrounds. The initial studies were conducted in adult mice (8 - 16 weeks old), and all showed increased density and/or proportion of long spines in the mouse model of FXS, a phenotype analogous to that in the human condition (Comery et al. 1997)(Irwin et al. 2002). Then, Dr. Karl Svoboda's group challenged the universality of this finding throughout development by investigating spines on 7, 14, and 28 day old

mice using viral expression of EGFP and a 2-photon microscope. In 7 day old mice, Nimchinsky et al. observed a 33% spine density increase in primary somatosensory cortex of *fmr1* KO mice compared to WT mice, however this phenotype was not observable in 14 or 28 day old mice (Nimchinsky et al. 2001). This inspired Greenough et al. to assess spine phenotypes in younger mice. Using the traditional Golgi method, they replicated Nimchinsky et al.'s finding in young *fmr1* KO mice (25 days old) while also confirming their previous work, demonstrating that 73 day old *fmr1* KO mice have phenotypes in apical spine density and shape as well as basal spine shape (Galvez and Greenough 2005). In summary, *fmr1* KO mice have a higher density of spines, as well as a shift from mature to immature morphology, in the first week after birth and throughout adulthood, however there is a period in between when the phenotype is not evident. These studies support the idea that FMRP promotes maturation of the developing cortex, and therefore processes such as spine maturation and pruning are disrupted or delayed in *fmr1* KO mice.

More recently, subfields of the hippocampus have also be analyzed for spine phenotypes. More immature spines were observed in *fmr1* KO mice in hippocampal CA1 neurons. Golgi staining by Greenough's group revealed longer spines in *fmr1* KO CA1 neurons than in controls (Grossman et al. 2006). DiOlistic labeling and confocal microscopy by a laboratory in The Netherlands reported an increase in filopodia in CA1 (15.6% more filopodia in *fmr1* KO than WT), but only a 0.4% increase in overall protrusion density (Levenga et al. 2011). No differences were observed in CA3 pyramidal neurons (Levenga et al. 2011). Spine density on dentate gyrus granule cell dendrites was higher in *fmr1* KO mice than in WT mice, and this phenotype was evident in young, adolescent, and adult mice (Grossman et al. 2010). Thus the spine phenotypes observed in the hippocampus were different than and often smaller in magnitude than those present in the cortex of *fmr1* KO mice. These findings suggest that FMRP is required for the processes of spine maturation and pruning in multiple brain regions and that the specific pathology and its developmental expression is brain sub-region specific.

1.3.3 Synaptic Plasticity in *Fmr1* KO Mice.

Hippocampal Long-term Depression

In the hippocampus of the mouse model of FXS, exaggerated metabotropic glutamate receptor (mGluR)-dependent long-term depression (LTD) has been consistently reported (Huber et al. 2002). This forms the basis for "the mGluR theory of fragile X" – discussed in detail in later sections of this chapter – in which there is runaway or poorly regulated protein synthesis in response to mGluR signaling in the *fmr1* KO mouse and presumably in humans with FXS (Bear et al. 2004). This type of persistent plasticity can be induced by either electrical stimulation (paired-pulse low-frequency stimulation, PP-LFS) or chemical stimulation (brief application of mGluR agonist DHPG). It activates protein synthesis, internalization of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, and spine morphology changes. On the other hand, hippocampal NMDA (N-methyl-d-aspartate) receptor-dependent LTD is normal in *fmr1* KO mice.

Hippocampal Long-term Potentiation

Until recently, it was thought that mGluR-dependent LTD was the only synaptic plasticity deficit in the hippocampus in these mice. Initial studies demonstrated that classically defined hippocampal CA1 long-term potentiation (LTP) – induced by intense, high frequency stimulation, triggered by calcium influx through NMDA receptors, and recorded extrcellularly – and short-term potentiation (STP) were normal in 5 - 26 week old *fmr1* KO mice (Godfraind et al. 1996)(Paradee et al. 1999)(Li et al. 2002)(Larson et al. 2005). Recently three studies have utilized modified protocols and uncovered LTP-related defects in the hippocampus of *fmr1* KO mice. First, conventional stimulation of Schaffer Collaterals with 10 burst theta trains elicited normal LTP, while threshold levels of stimulation – thought to be more physiologically plausible – unmasked a plasticity deficit in the hippocampus of *fmr1* KO mice (Lauterborn et al. 2007). This phenotype was rescued by brain-derived neurotrophic factor (BDNF), a neurotrophin that activates cytoskeleton reorganization pathways

in dendritic spines (Lauterborn et al. 2007). Similarly, a pairing or spike-timing protocol induced LTP in the hippocampus of 2 week old WT mice, however LTP was reduced by ~50% in *fmr1* KO mice (Hu et al. 2008). This second type of LTP impairment involved a selective loss of synaptic delivery of AMPA receptor subunit GluR1, a subunit usually restricted from synapses but driven into the postsynaptic density (PSD) by signaling of the small GTPase Ras. In this way, enhanced Ras signaling restored LTP in the hippocampus of *fmr1* KO mice (Hu et al. 2008). Finally, while mGluR-dependent priming of LTP was comparable in WT and *fmr1* KO CA1 neurons, it no longer required protein synthesis in *fmr1* KO mice (Auerbach and Bear 2010). This is consistent with the observation that basal protein synthesis was elevated in *fmr1* KO (Qin et al. 2005).

Cortical Long-term Potentiation

Numerous studies have investigated LTP in cortical slices from *fmr1* KO mice. Reduced or abnormal LTP was recorded in the *fmr1* KO in the somatosensory cortex, olfactory cortex, visual cortex, prefrontal cortex, and temporal cortex (Li et al. 2002)(Zhao et al. 2005)(Larson et al. 2005)(Desai et al. 2006)(Meredith et al. 2007)(Wilson and Cox 2007)(Hayashi et al. 2007). These studies used a variety of stimulation protocols, brain regions, mouse strains, and mouse ages (Figure 6-1). In the neocortex of 10 - 18 day old pups, mGluR5-dependent LTP was normal but NMDA receptor-dependent, spike timing-dependent plasticity (STDP) was significantly impaired (Desai et al. 2006). In the prefrontal cortex of slightly older mice (3 - 4 weeks old), STDP was not so much absent, but rather, the threshold for LTP induction was increased (Meredith et al. 2007). This plasticity abnormality was accompanied by compromised calcium signaling through L-type calcium channels. As local concentrations of calcium vary depending on spine volume and neck width, the authors linked the altered calcium signaling to the long, thin dendritic spine morphology characteristic of pyramidal neurons in the cortex of *fmr1* KO mice and FXS humans (Meredith et al. 2007).

Protocol	Region	Age	Genetic background	Results	References
Tetanic stimulation	Anterior somatosensory (frontal neocortex)	8-10 weeks	C57BL6	Reduced LTP	Li et al. 2002
TBS (10 burst)	Anterior Piriform Cortex (primary olfactory)	phenotype only in mice >6 months	C57BL6	Reduced LTP	Larson et al. 2005
Pairing protocol (80 pulses, 2 Hz, +30 mV)	Anterior cingulate cortex and lateral amygdala	6-8 weeks	FVB.129P	Loss of LTP	Zhao et al. 2005
Tetanic stimulation	Visual cortex (L5)	p13-25	FVB.129P	Reduced LTP	Wilson and Cox 2007
Spike-timing	Prefrontal cortical (L2/3)	p14-23, p53-100	C57BL6	LTP is not absent, but the threshold for induction is increased	Meredith et al. 2007
Spike-timing Pairing protocol	Somatosensory cortex (L5)	p10-18	C57BL6	Loss of STDP	Desai et al. 2006
TBS (100 Hz)	Temporal cortex (L2/3)	2-3 months	C57BL6	Reduced LTP	Hayashi et al. 2007

Figure 1-1: **Cortical LTP Literature.** A number of published studies report decreased LTP in the cortex of *fmr1* KO mice. The experiments vary in terms of stimulation protocols, cortical regions, mouse age, and genetic background.

TBS, theta-burst stimulation; STDP, spike timing-dependent plasticity; LTP, long-term potentiation; p, postnatal day.

Amygdalar LTP

LTP in the lateral amygdala (LA) shares an important property with LTD in the hippocampus: it is mGluR5-dependent (Lee et al. 2002). Thus, the mGluR theory predicts that LTP should be enhanced in LA in *fmr1* KO mice. Interestingly, the opposite result was observed: mGluR-dependent LTP was impaired at thalamic inputs to LA (Suvrathan et al. 2010). Consistent with this observation, surface expression of AMPA receptor subunit GluR1 was decreased in LA in *fmr1* KO mice. Surprisingly, these deficits were not corrected by mGluR antagonist MPEP. However, MPEP did correct presynaptic phenotypes, in particular it restored presynaptic release probability. Additional studies are required to examine the divergent prediction and observations.

These electrophysiology findings suggest that FMRP, the protein product of *fmr1*, plays a role in synaptic plasticity in multiple brain regions, and that the specific deficit depends on the cellular context.

1.3.4 *Drosophila dFmr1* Mutants.

Drosophila melanogaster is also a valuable genetic model system for the study of intellectual disability, and in particular FXS. The fly genome harbors a single homolog of *fmr1* called *dFmr1* with an amino acid sequence identity of 35% and overall similarity of 56% (Wan et al. 2000)(Zhang et al. 2001)(Gao 2002). *Drosophila* Fragile X Related Protein (dFXR) is comprised of two K homology (KH) domains and exhibits similar RNA binding properties to mammalian FMRP (Wan et al. 2000). The introduction of a point mutations analogous to I304N – selected because severe FXS symptoms have been reported in a human with this mutation – in each of these KH domains acted as a loss-of-function mutation and produced the first fly model of FXS (Wan et al. 2000). Several additional loss-of-function mutants have been generated using fly genetic techniques (Zhang et al. 2001)(Dockendorff et al. 2002)(Lee et al. 2003).

Behavioral Symptoms

Consistent with the knowledge that one *Drosophila* protein, dFXR, is thought to play the role of mammalian FMRP and its two autosomal homologs, FXR1P and FXR2P, phenotypes observed in the *Drosophila* model of FXS are typically more severe or even different than seen in the mouse model and FXS humans (Zarnescu et al. 2005). Circadian clock defects in *dFmr1* nulls lead to erratic activity patterns stunted with brief hyperactivity, consistent with sleep disturbances and hyperactivity in FXS humans (Dockendorff et al. 2002). Phenotypes are seen in young and adult *Drosophila*: Mutant larvae display abnormal crawling behavior (Xu et al. 2004), and courtship behavior is reduced in male *dFmr1* mutants (Dockendorff et al. 2002).

Axon and Dendrite Phenotype

Studies on the cellular level highlight the role of *dFmr1* as a regulator of synaptic morphology and function (reviewed in (Zarnescu et al. 2005)). Most neurons in the peripheral and central nervous system of *dFmr1* nulls display elaborate and overgrown axons and dendrites (Zhang et al. 2001)(Gao 2002)(Lee et al. 2003). In particular, at the *Drosophila* neuromuscular junction – a metabotropic type synapse – *dFmr1* null mutants exhibit an increase in the number of synaptic boutons and an elaboration of synaptic terminals (Zhang et al. 2001).

This has been compared to the dendritic spine phenotype in the cortex of FXS humans and *fmr1* KO mice and corroborates the theory that dFXR and FMRP may play critical roles in neurite pruning (reviewed in (Zarnescu et al. 2005)). In the mushroom body learning and memory center – the fly equivalent of the hippocampus – axon pruning depends on dFXR expression and sensory experience (Tessier and Broadie 2008). This is complimentary to a discovery in the mouse cortex, where visual experience drives expression of *Fmr1*, and *fmr1* KO mice deprived of sensory input display deficits in cortical plasticity (Gabel et al. 2004)(Dolen et al. 2007).

Abnormal Signaling: Protein Synthesis and Actin Polymerization

The phenotypes observed in the *Drosophila dFmr1* null are mimiced in mutants of the *Drosophila* homolog of MAP1B, a microtubule-associated protein called Futsch (Zhang et al. 2001). dFXR binds Futsch mRNA, as well as the mRNA of other cytoskeleton regulators including Rac1 and Profilin. dFXR, which is part of a large messenger ribonucleoprotein (mRNP) complex, is believed to negatively regulate the translation of these mRNAs (Lee et al. 2003)(Reeve et al. 2005). Another component of this mRNP complex is the closely related protein cytoplasmic FXR interacting protein (CYFIP). *CYFIP* interacts both biochemically and genetically with *fmr1*, and there is evidence that CYFIP antagonizes *fmr1* function by acting as an effector for *Rac1* (Schenck et al. 2003)(Castets et al. 2005). In this way, CYFIP may act as a bridge between protein synthesis and cytoskeleton remodeling. To regulate the actin cytoskeleton, CYFIP modulates the activity of WAVE1 which stimulates the Arp2/3 complex. This results in actin nucleation – the rate-limiting step in actin polymerization – thus creating filaments that grow at their barbed ends (Mullins et al. 1998). Arp2/3-mediated actin polymerization is required for the dendritic spine morphogenesis or enlargement involved in LTP, while Arp2/3 inhibition may lead to actin depolymerization and LTD (Nakamura et al. 2011). Thus, there is some evidence that FMRP interacts with the actin polymerization pathway in *Drosophila.*

In summary, phenotypes observed in the *Drosophila* model of FXS are consistent, albeit sometimes more severe, with expectations given knowledge of the mouse and human FXS phenotypes. In this way, *Drosophila dFmr1* nulls are useful models for understanding the biological basis of this disease and testing therapeutics.

1.4 Morphological Plasticity Is Required for Normal Brain Function.

Fragile X Syndrome, autism, and intellectual disability are complex disorders with multiple causes and heterogeneous presentation of symptoms. Amazingly, however, on

the neuroanatomical level, they all have something in common: immature dendritic spines. Cortical neurons from FXS patients and *fmr1* KO mice have long, thin, and tortuous dendritic spines and increased spine density. These modifications in spines may be responsible, or partially responsible, for the complex symptoms of FXS and other disorders, but the precise mechanisms are still a mystery. A deeper understanding of spine morphology in health and in disease provides insights into the molecular mechanisms that are disrupted in neurodevelopmental disorders and leads to the identification of novel therapeutic targets.

In this section, I will provide background information on dendritic spines and explain research conducted in our laboratory that identified a novel target, based on the correlation between abnormal spines and neurodevelopmental disorders, and validated its therapeutic potential with a genetic rescue strategy. Then I will present detailed information on this protein target – a kinase called PAK – and what is known about it in mice, including upstream and downstream components of the signal transduction pathways.

1.4.1 Memory Storage Depends on Structural Plasticity.

The immense capacity and specificity our of memory system depends on structural changes that occur in the brain in response to experience. Dendritic spines are the morphological specializations that receive neuronal communications, and changes in these spines are thought to be the cellular correlate of learning and memory (Yuste and Bonhoeffer 2001). Reciprocal changes in the structure and function of dendritic spines impact the integration of activity-dependent input at the level of individual synapses and dendritic branches (Govindarajan et al. 2011)(reviewed in (Bourne and Harris 2008)). In this way, enduring plasticity – measured in the form of LTP or LTD – typically requires the modulation of actin dynamics in dendritic spines (Fukazawa et al. 2003)(Matsuzaki et al. 2004)(reviewed in (Bramham 2008)). While the induction of LTP initially promotes a brief period of actin depolymerization (Ouyang et al. 2005), the maintenance phase of LTP involves enduring spine enlargement (Kim and Lisman 1999)(Fukazawa et al. 2003). Accumulating evidence suggests that

actin polymerization is required for the expansion of the PSD and enlargement of dendritic spines observed in LTP, while the shrinkage of spines is associated with LTD (Okamoto et al. 2004) (reviewed in (Bourne and Harris 2008)). The shrinkage of spine heads or elongation of spines associated with LTD requires depolymerization of actin (Chen et al. 2004).

The actin dynamics required for bidirectional structural and function plasticity are mediated by actin-binding proteins and the signaling pathways upstream of them (Tada and Sheng 2006). Profilin promotes activity-dependent actin polymerization and therefore facilitates LTP-induced actin assembly and the stabilization of actin that results in sustained spine enlargement (Ackermann and Matus 2003). Cofilin depolymerizes actin filaments. LTP induces inhibition of cofilin via phosphorylation by the p21-activated kinase (PAK) / LIM kinase (LIMK) signaling pathway, thus promoting polymerization and spine enlargement (Fedulov et al. 2007)(Chen et al. 2007).

1.4.2 p21-Activated Kinase.

This thesis work, and the genetic rescue strategy that preceded it, is based on the hypothesis that a therapeutic strategy that rescues the dendritic spine phenotype will also reverse behavioral and neurological symptoms. Our approach targets one kinase that plays an important role in the actin dynamics the underlie spine morphology: PAK.

Introduction to p21-Activated Kinase

p21-activated kinase (PAK) is a family of serine-threonine kinases that regulate neuronal morphology and cognitive function. Initially identified in a screen for Rho GTPase binding partners in the rat brain, PAK is an effector of Rac and Cdc42 that has been implicated in mental retardation and Alzheimers disease (Manser et al. 1994)(Allen et al. 1998)(Zhao et al. 2006).

This family, composed of two groups of PAKs defined by homology, is enriched

in the nervous system. Group I – composed of PAK 1, 2, and 3 – contains an auto-inhibitory domain (AID) in its N-terminal and has been better studied than group II, which includes PAK 4, 5, and 6. AID acts in trans to suppress the catalytic activity of group I kinases. Overexpression of AID alone is sufficient to inhibit activity of endogenous PAK1, 2, and 3, and therefore can serve as a dominant negative (dn) when exogenously expressed /shortciteFrost98. Experiments utilizing this *dnPAK*, as well as knockout experiments, have been useful in characterizing the roles of PAK1, 2, and 3 in the mammalian brain.

Lessons from Knockout Mice

Familial X-linked mental retardation has been linked to mutations in the *PAK3* gene (Allen et al. 1998). Based on the results of hippocampal culture experiment in which *PAK3* has been knocked down, it has been hypothesized that the *PAK3* mutation leads to intellectual disabilities through misregulation of actin cytoskeleton dynamics. This misregulation results in the formation of abnormally elongated dendritic spines and filopodia-like protrusions which are accompanied by a reduction in the number of mature synapses and a deficit in LTP (Boda et al. 2004). Therefore it was surprising to researchers to discover that despite a hippocampal late-phase LTP deficit, hippocampal dendritic spines and performance on most learning and memory tasks are normal in the *PAK3* KO mouse (Meng et al. 2005). While levels of other PAK isoforms were not abnormally high in this mouse, one cannot rule out the possibility of compensation and functional redundancy.

In contrast, the *PAK4* KO is embryonic lethal, most likely due to a defect in the fetal heart, suggesting that PAK4 is critical to life (Qu et al. 2003). In addition, the *PAK4* KO embryos suffered from defects in neural tube development. Similarly, the KO of *PAK2* results in embryonic lethality at an early stage (Marlin et al. 2011).

The KO of *PAK1*, the best studied isoform in the brain and one that is expressed throughout development, produced normal gross brain anatomy and normal hippocampal spines and synapse size (Asrar et al. 2009). Interestingly, a deficit was observed in hippocampal LTP in these mice. To understand the mechanism of this

reduction in synaptic plasticity, authors analyzed basal filamentous-actin (F-actin) density and activity-dependent activation of cofilin in cultured hippocampal neurons. Both were abnormally low in the *PAK1* KO, suggesting that PAK1 regulates hippocampal synaptic plasticity through mechanisms involving the activation of cofilin, presumably through LIMK, and regulation of F-actin density (Asrar et al. 2009).

Genetic deletions of *PAK1* and *PAK3* individually did not impact brain development, neuronal morphology, nor performance on most memory and learning tasks. To investigate whether this was because of functional redundancy between these proteins, Huang and colleagues engineered a double KO mouse. While these mice were born healthy, with normal brain size and structure, postnatal brain growth was severely stunted (Huang et al. 2011). By adulthood, these mice exhibited a significant reduction in brain volume, simplified hippocampal and cortical neurites, and smaller, thinner hippocampal spines, but no change in spine density compared to WT mice. Consistent with the structural abnormalities, functional deficits were observed on the physiological and behavioral level. Basal synaptic transmission was enhanced, a confusing finding given the dramatic reduction in dendritic arbors and axons, and bidirectional synaptic plasticity was reduced in the hippocampus. A battery of behavioral assays were run, demonstrating hyperactivity, increased anxiety, and learning acquisition deficits. These findings support the hypothesis that group I PAKs regulate postnatal brain growth including dendritic arbor, dendritic spine, and axon formation, and are necessary for proper cognitive function.

Spine Morphology Is Regulated by Actin Remodeling Proteins Like PAK

Before many of these knockout mice were engineered, a former post-doctoral fellow in our laboratory, Dr. Mansuo Hayashi, manipulated PAK activity in a region- and age-specific manner (Hayashi et al. 2004). Dr. Hayashi decreased PAK activity, and therefore manipulated the actin cytoskeleton signaling pathway, in the forebrain of adult mice, as she was interested in whether morphological changes in spines and synapses underlie cognition. In this mouse line with a dominant-negative version of PAK *dnPAK*, PAK kinase activity is decreased to ~59% of wildtype (WT) level

in the cortex and ~62% of WT level in the hippocampus. The residual level of active, phosphorylated PAK (P-PAK) in the hippocampus of the *dnPAK* TG mice was similar to the endogenous level of P-PAK in the cortex of WT mice, because normally hippocampal levels are two-fold higher than cortical levels. Expression of the *dnPAK* transgene did not occur until adulthood, as it was under the control of the α-CaMKII promoter.

Transgenic mice with reduced PAK activity (*dnPAK* TG) have fewer dendritic spines in the cortex than WT mice, which is consistent with PAKs function as a promoter of spine formation and/or growth. *dnPAK* TG mice have a greater percentage of larger, perforated synapses, perhaps to compensate for the decreased spine density. These larger synapses are accompanied by enhanced cortical LTP and reduced cortical LTD. In this way, *dnPAK* TG mice have opposite morphology and electrophysiology phenotypes to those in the *fmr1* KO mouse.

Disrupting PAK Signaling Impairs Memory Consolidation

Next a battery of behavioral tests was conducted. Motor activity, pain perception, motor coordination, and anxiety were normal in the mutant mice. To determine whether the structural and functional deficits in *dnPAK* TG mice lead to impaired performance on cognitive tasks, Hayashi et al. conducted learning and memory assays. The *dnPAK* TG mice learned as quickly as WT mice in training sessions for two spatial memory tasks: the hidden platform version of the Morris water maze and contextual fear conditioning. When tested soon after the training session, the *dnPAK* TG mice performed as well as WT littermates, suggesting that short-term memory was intact. Deficits became obvious when mice were tested again later. *dnPAK* TG mice were significantly impaired in the Morris water maze at the probe test 21 days after training, suggesting that the consolidation phase of memory was disrupted by inhibition of PAK activity. Similarly, *dnPAK* TG mice did not remember the noxious context 24 hours after training in the fear conditioning assay. In this way, forebrain specific inhibition of PAK activity impaired the consolidation phase of memory formation in adult mice. These data support the hypothesis that cortical

dendritic spine plasticity and bidirectional synaptic modifiability are required for the retention of spatial memories in mice.

Partial Inhibition verses Knockout of PAK Activity in the Adult Mouse Brain.

In summary, in a transgenic mouse expressing a dominant negative form of PAK (*dnPAK* TG), PAK activity it decreased by ~60% in the cortex and hippocampus of adult mice (Hayashi et al. 2004). These mice have fewer dendritic spines in the cortex, and these spines are frequently short with large PSDs. In accordance with this, cortical LTP is enhanced while cortical LTD is deficient. As long-term synaptic plasticity is thought to be the cellular correlate of learning and memory, it is not surprising that these mice have intact short-term memory but impaired long-term memory.

Interestingly the dendritic spine phenotype does not mirror that reported in the double knockout for *PAK1* and *PAK3* (Huang et al. 2011). There are two important differences in the genetic strategies: First, PAK activity is only reduced in the adult in our *dnPAK* TG mouse, and therefore it is present to allow for healthy brain development. Second, PAK activity is decreased but not abolished in our *dnPAK* TG mouse. In the hippocampus, residual PAK activity is sufficient to maintain normal spine morphology. In the double knockout for *PAK1* and *PAK3*, there is more likely to be aberrant signaling due to compensatory mechanisms as neither PAK1 nor PAK3 are ever expressed. In summary, PAK is a critical regulator of dendritic spine morphology, and inhibition of PAK can disrupts spine shape and density in the cortex and hippocampus.

1.4.3 Opposite Phenotypes Beget a Therapeutic Strategy.

The discovery that structural and functional plasticity phenotypes are opposite in *dnPAK* TG mice and *fmr1* KO mice led to the hypothesis that *PAK* and *fmr1* may antagonize each other in neurons to regulate dendritic spines. From this hypothesis

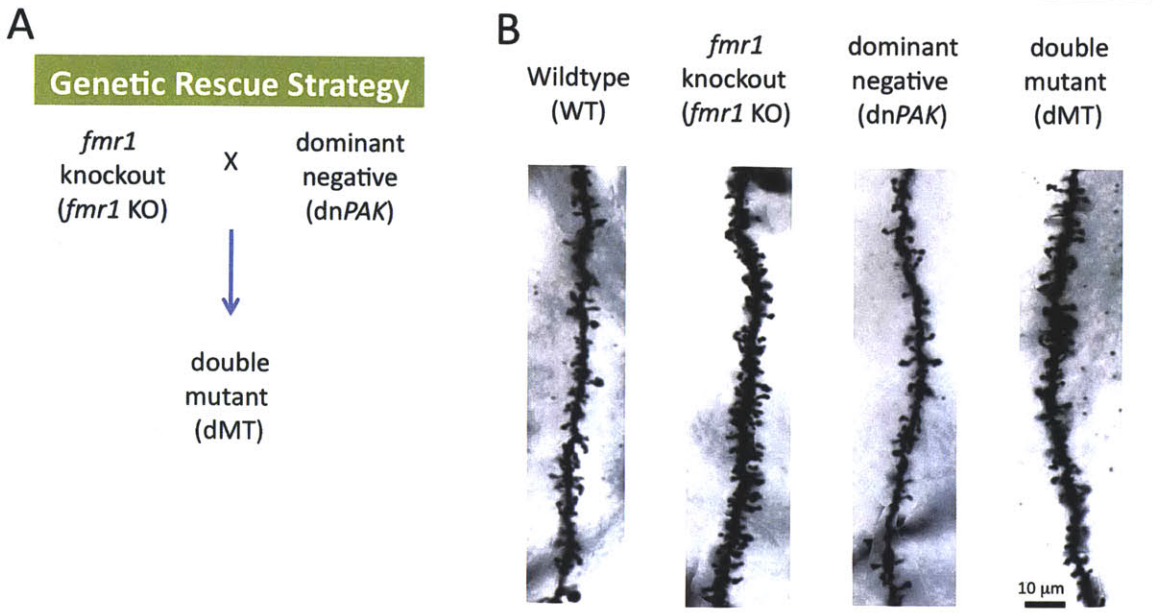

Figure 1-2: **Experimental Strategy and Dendritic Spine Results.**
A. Genetic rescue strategy. Our therapeutic strategy involved a genetically engineered mouse line: *dnPAK*. A cross of this *dnPAK* TG with the *fmr1* KO mouse produces four offspring, one of which is the double mutant *fmr1* KO; *dnPAK* TG (dMT). **B. Inhibition of PAK reverses the spine abnormalities.** Golgi staining of cortical dendrites reveals a spine density and morphology phenotype in *fmr1* KO mice that is opposite to that observed in *dnPAK* TG mice. Expression of the *dnPAK* transgene on the *fmr1* KO background produced spines that resembled those in WT mice in both density and morphology.
Data was published in PNAS ((Hayashi et al. 2007)).

came the prediction that dampening PAK activity in the *fmr1* KO mouse would reverse the dendritic spine and synaptic plasticity phenotype, and that the restoration of normal spine structure and synaptic plasticity would be sufficient to ameliorate behavioral phenotypes.

To test this hypothesis, our laboratory used the *dnPAK* construct to inhibit PAK activity in the forebrain of adult *fmr1* KO mice (Hayashi et al. 2007). To this end, we crossed a male *dnPAK* TG mouse with a female heterozygous for *fmr1* KO, both on the C57BL6 (B6) background (Figure 1-2). This breeding scheme produced offspring of four genotypes: WT, *dnPAK* TG, *fmr1* KO, and *dnPAK* TG; *fmr1* KO double mutants (dMT).

Rescue of Spine Phenotype

To assess the effect of PAK inhibition on the spine morphology phenotype in *fmr1* KO mice, we quantified the number of spines per 10 μm segment of dendrite for a total of 100 μm, where segment 1 was closest and segment 10 was farthest from the soma. *dnPAK* TG mice displayed a decrease in spine density compared to WT mice in segments 1 - 4. *fmr1* KO mice displayed an increase in spine density in segments 1 - 6. In the dMT mice, spine density was comparable to WT controls in all segments except 7 and 8. As the spine density was highest in *fmr1* KO mice in segments 3 - 5, rescue was most impressive in these three segments. In summary, inhibition of PAK activity rescued increased spine density in the temporal cortex of *fmr1* KO mice (Figure 1-2).

Rescue of Reduced Cortical LTP

To determine whether partial inhibition of PAK is sufficient to restore synaptic plasticity in *fmr1* KO mice, we induced LTP in cortical slices and measured postsynaptic responses. PAK inhibition rescued reduced cortical LTP in *fmr1* KO mice. As LTP is thought to be the cellular correlate of learning and memory, this result supported the prediction that PAK inhibition would also rescue cognitive and perhaps behavioral deficits in the mouse model of FXS.

Rescue of Cognitive and Behavioral Phenotypes

To determine whether PAK inhibition increased cognitive function in *fmr1* KO mice, dMT mice had to be challenged in a task in which *fmr1*KO mice did not perform as well as WT mice. Since humans with FXS have attentional deficits in addition to intellectual disability, a version of fear conditioning that requires attention - trace fear conditioning - was conducted. This was the first demonstration that *fmr1* KO mice and *dnPAK* mice respond similarly to WT mice on the training day, but cannot recall the memory one day later. dMT mice also showed a learning deficit on the testing day, however it was less pronounced than the single mutants.

In an exploration assay in a novel, brightly illuminated arena, *fmr1* KO mice displayed phenotypes in three metrics: distance traveled, number of stereotyped behaviors, and time spent in the center of the arena. In all three of these metrics, dMT mice performed similar to WT mice. This finding indicates that a decrease in PAK activity in the forebrain of adult *fmr1* KO mice is sufficient to reverse the phenotypes of hyperactivity, perseveration, and hypoanxiety.

Summary

Through this series of experiments in transgenic mice, we demonstrated that abnormalities in *fmr1* KO mice are ameliorated at both the cellular and behavioral levels by inhibition of the catalytic activity of the actin cytoskeleton remodeling kinase PAK. This discovery suggests that the PAK signaling pathway is a novel intervention site for the development of therapeutics for humans with FXS, autism, and intellectual disability. However, this demonstration utilized a genetic strategy, and drug therapy is favored in humans. Therefore, this thesis builds on this work to report a novel small molecule inhibitor of PAK and test its efficacy in the mouse model of FXS.

1.5 Therapeutics for FXS.

In the two decades since the causative gene was cloned, numerous discoveries have been made regarding the molecular mechanisms and neuroanatomical abnormalities that characterize humans and mice lacking expression of *fmr1*. Now the challenge is to apply this knowledge of the basic neurobiology of this specific disease to the development of targeted treatments to ameliorate the behavioral symptoms of FXS, and perhaps intellectual disability and autism by other causes.

1.5.1 Empirical Approach to Drug Discovery.

Drug discovery is not a new concept. For thousands of years, humans have known the therapeutic potential of natural products, like poppy seeds or willow tree bark. This premodern approach was simple: It focused exclusively on efficacy and safety

without concern for mechanism of action (reviewed in (Enna and Williams 2009)). The Greek physician Hippocrates described headache relief and fever reduction from the bitter powder of the willow tree in writings from the 5th century BC. Nearly two and a half millennia later, Bayer's chemists isolated the active ingredient – salicylic acid – modified it to reduce gastric side effects, and sold it as aspirin in 1899. Similarly, morphine, a powerful analgesic, was isolated from opium in 1804, industrially produced by Merck in Germany in the 1820s, and modified to be commercially sold as a cough suppressant by the Bayer Company of Germany in 1898, under the trade name Heroin (Meldrum 2003). All of this was done without knowledge of the target in the human body. Finally, the first barbituates – central nervous system depressants with sedative and hypnotic properties – were synthesized by German chemist Adolf von Baeyer at the beginning of the 20th century, (Lopez-Munoz et al. 2005). The mechanism of action, and in the earliest examples even the chemical compound itself, was unknown when these three drugs were introduced for human use. In this way, an empirical approach led to the discovery of the original CNS drugs.

1.5.2 All Eyes on the Therapeutic Target.

In the high-throughput era following the genomic revolution, the approach to the discovery of novel therapeutics is different. Now, scientists focus on a hypothesis-driven, mechanism-based approach. Today's drug discovery emphasizes target identification and the discovery of a new chemical entity (NCE) that acts on this preselected target site. It requires good knowledge of the physiology of the target and a hierarchical integration from *in vitro* cellular and tissue studies, all the way to animal models that reasonably predict human responses. Already, some advances have been achieved in the treatment of various forms of cancer because of the human genome project and our improved understanding of the biology of disease (reviewed in (Preskorn 2011)).

This "molecular medicine" approach begins with the identification of patients with similar or identical presentation of the disorder (reviewed in (Krueger et al. 2011)). This can be challenging with psychiatric and neurological disorders like ASD which are highly heterogeneous in behavioral symptoms, but it is most important with these

diseases, as complex genetic (and environmental) interactions are likely to be responsible. The "splintering" of what originally appeared to be one illness into multiple, more specific syndromes will increase the chance of discovery of the underlying mechanism causing the disease. It is important to note that while overlapping but distinct disorders are best considered independently at this first stage of the drug discovery process, a therapeutic intervention may successfully ameliorate symptoms in some or all related disorders. In this way, we hope that the identification of the causative gene in FXS will leads to the development of a drug to treat not only FXS but also autism and intellectual disabilities more generally.

A similar approach has been proposed in the development of therapeutics for Alzheimer's Disease (AD). There are autosomal dominant forms of AD, and while these may only account for ~1% of all cases of AD, understanding the genetic basis of cases has shed light on the biology of the sporadic forms of AD (Preskorn 2011). A number of genes associated with autosomal dominant AD have been identified, thus inspiring theories about the biogenesis of AD and new directions in AD therapeutics research.

A deep understanding of the genetic basis of the disease – be it FXS or AD – then allows for the creation of animal models. Basic neurobiology research in these animal aims to uncover the molecular, cellular, and tissue level neuropathology that is associated with behavioral symptoms. Then molecular targets, such as receptors or proteins in a critical signal transduction cascade, may be identified for therapeutic intervention. Genetic or small molecule approaches are validated first in *in vitro* assays and later in the animal model.

In Vivo Pharmacology

Pharmacology studies *in vivo* serve to evaluate compounds on a broad range of pharmaceutical topics, notably toxicology, pharmacokinetics (PK), and efficacy (Li and Zhao 2007). A general toxicology study evaluates adverse drug effects *in vivo* to determine whether the compound is safe and at what clinical dose range. If toxicity or unwanted side-effects are observed, further studies are necessary to determine

whether the negative effects are mechanism-based, and therefore an issue with the target itself, or off-target based, and thus could be addressed by a more specific small molecule.

Simply stated, pharmacokinetics (PK) is the study of what the body does to a drug. More specifically, it is an assessment of the absorption, distribution, metabolism, and excretion (ADME) of drugs in animals and humans. The fraction of drug that is absorbed determines a drug's bioavailability. Absorption properties – such as the mechanism and rate of entrance into blood circulation – are affected by the administration route and the dose. While oral administration is strongly preferred for human consumption, there are many options for dosing routes in rodents including intra-peritoneal (IP), subcutaneous (SC), intravenous (IV), etc. (Li and Zhao 2007). Pharmaceutical formulation development is an integral part in the drug discovery and development process and can be optimized depending on the route of administration as well as the optimum *in vivo* exposure at the target site and the molecular properties of the drug that influence solubility. The chemical and physical stability of the formulation also impacts the distribution of the drug throughout the fluids and tissues of the body.

In order to target a compound to the CNS, formulation must be selected to enable the drug to be transported across the blood-brain barrier (BBB), most likely through the endogenous transport systems within the capillary endothelium protecting the brain (Pardridge 2007). This is a huge challenge for drug discovery for CNS disorders, as only $\sim$2% of small molecule therapeutics can cross the BBB. While drugs could be administered via intraventricular cannulae in the rodent brain for proof of concept, a compound must penetrate the BBB in order to be effective in humans. Two additional properties play important roles in pharmacokinetics. Metabolism is the irreversible breakdown or transformation of the original compound into metabolites, a process the usually takes place in the liver. Metabolites may be pharmacologically active or inert. Finally, excretion is the elimination of the drug from the body via excretion, usually through the kidneys.

Once new drugs are evaluated for safety, side-effects, and pharmacokinetics, it

is time to evaluate therapeutic efficacy. While previous steps in the pharmacology study could be conducted in control or wildtype animals, efficacy is best evaluated in assays directly related to the disease phenotype. It is here that a valid animal model becomes critical.

Validity in Animal Models.

Effective animal models are critical for drug discovery research. Models are evaluated based on three criteria: whether the cause of the disease is the same in the animal as in humans (construct validity), whether the presentation of the disease symptoms is the same in the animal as in humans (face validity), and whether a treatment that works in animals will also be effective in humans (predictive validity) (Silverman et al. 2010). The *fmr1* KO mouse has high construct validity as it is caused by a functionally identical genetic mutation that results in a lack of expression of FMRP. It has high to moderate face validity, since the all of the phenotypes in the mouse model have strong analogies to the endophenotypes of the human syndrome, even if all of the features of FXS in humans – in particular intellectual disability – are not recapitulated in *fmr1* KO mice. In this way, the mouse model of FXS shares the pathogenesis and pathophysiology underlying the human illness. (Interestingly, this was not the case in the development of the some of the most successful CNS drugs of the 1980s and 1990s, including selective serotonin reuptake inhibitors (SSRIs), identified through the Porsolt learned helplessness model of depression (Porsolt et al. 1977)(reviewed in (Borsini and Meli 1988).) Finally, the predictive validity of the mouse model of FXS has yet to be determined.

Evaluating Drug Efficacy in Mice and Humans

The development of appropriate assays to test the efficacy of drug treatments is a critical part of the drug discovery process. Initial *in vitro* screens identify compounds that bind to and inhibit the immediate function of target proteins. Cell culture or slice physiology assays can evaluate secondary or longer term effects, like impacts on spine morphology or synaptic plasticity. Finally, *in vivo* studies can determine

whether a single compound or combination of drugs is sufficient to correct or at least ameliorate phenotypes in an animal model. As was mentioned previously, these assays can test for phenotypes in mice that are also relevant for the human condition, and in this way have face validity. In a properly designed experiment, there is a vehicle alone control, the experimenter is blind to treatment condition as well as genotype, and both the disease model and wildtype control littermates are assayed.

These principles carryover into human studies. Well designed clinical trials will be double-blind and placebo-controlled, though this has not been the case in previous studies in FXS humans (reviewed in (Krueger et al. 2011)). In theory, studies should include patients with the disease, as well as age-matched neurotypicals, however the necessity of this is controversial. Treatments which have profound impacts on disease states are also likely to have significant and perhaps adverse effects on healthy subjects as a result of the same mechanism that produced benefit (Preskorn 2011). However, the impact that a small molecule has on healthy patients need not be relevant to its potential to improve the lives of patients with moderate to severe disease symptoms.

Critical Periods for Altering the Course of Illness

FXS and developmental disorders like autism may alter the trajectory of brain development starting early on in life. Activity-dependent circuit or synapse formation is thought to have a critical period. In this way, an important question in the drug discovery field relates to whether at some point it is too late to correct the defects. Is spine morphology or spine density plastic throughout life? Are abnormalities reversible even after brain connectivity is established? In an optimistic scenario, therapeutic intervention will be sufficient to reverse all or nearly all of the deficits, even in adults (Krueger et al. 2011). However, if a critical time window exists, pharmacological approaches introduced after this critical period may be ineffective. In a third and hopeful scenario, interventions introduced later in life will slow or prevent increases in the severity or progression of symptoms, though they will be unable to reverse the damage that has already been done. Thus, it is important to identify if a time window exists, and if so, when therapeutic intervention will be most successful.

Furthermore, it is wise to consider the type of therapeutic strategy best suited for a particular stage of disease progression in FXS, autism, or intellectual disability, as some therapeutic interventions alter the course of the illness, while other treatments aim to mask symptoms.

Our genetic rescue strategy involved expression of *dnPAK* in the *fmr1* KO mouse by the age of 8 weeks, but not as early as 3 weeks (Hayashi et al. 2007). In this case, therapeutic intervention in the adult mouse was sufficient to rescue or partially rescue behavioral, morphological, and physiological symptoms, suggesting that there is not a critical window in development for effective treatment of FXS in mice. This makes our PAK strategy promising for both children and adults with FXS and related neurodevelopmental disorders.

1.5.3 Hypothesis-Based Therapeutic Strategies for the Treatment of FXS.

In this thesis work, we target the actin cytoskeleton signaling pathway to reverse the dendritic spine deficit in *fmr1* KO mice. While we focus on inhibition of the kinase activity of PAK, other proteins in this pathway may be equally good targets. Here is a summary of the principles and challenges in the development of kinases inhibitors, followed by the basic biology of additional actors which could be targeted to rescue the dendritic spine deficit.

Targeting Kinases in Human Diseases

Protein kinases have been one of the most studied classes of drug targets in recent years. Dysregulation of kinase activity has been implicated in a number of human diseases, ranging from cancer to bipolar disorder (Zhang et al. 2009)(Catapano and Manji 2008). As of January 2009, approximately 30 distinct kinase targets were under development to the level of Phase I clinical trial and 80 inhibitors had been advanced to some stage of clinical evaluation, almost exclusively for the treatment of cancer (Zhang et al. 2009). Small molecule kinase inhibitors, which tend to be fewer than

1,000 Daltons, are natural or synthetic compounds that inhibit particular protein kinases. The vast majority of these small molecules act through ATP-competitive interactions with the catalytic pocket, though a few non-ATP competitive kinase inhibitors have been discovered which target unique allosteric sites (Janne et al. 2009). While ATP-binding sites are well conserved across the 518 mammalian kinases – a subgroup of the proteins encoded by the human genome referred to as the human "kinome" – inhibitors of a specific kinase (or at least a small number of kinases) can be developed and refined using modern approaches in medicinal chemistry, including high-throughput screening using biochemical or cellular assays and analog synthesis (Zhang et al. 2009). Higher specificity decreases off-target effects which otherwise can produce unwanted side-effects due to the inhibition of unintended cellular targets.

However, several of the most successful clinically approved small molecules are potent inhibitors of multiple kinases at clinically relevant doses (Janne et al. 2009). For example, dasatinib is a synthetic small molecule used for the treatment of chronic myeloid leukemia which inhibits all nine members of the Src family of kinases as well as the oncogenic Abl-Bcr tyrosine kinase and Bruton's tyrosine kinase, yet shows fewer side effects in patients than conventional chemotherapy (Sprycel; Bristol-Myers Squibb)(Zhang et al. 2009). Similarly, sunitinib – a drug marketed as Sutent by Pfizer for the treatment of cancers of the stomach or esophagus – acts on cellular signaling through inhibition of multiple receptor tyrosine kinases including the receptors for fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and KIT. It is the simultaneous inhibition of PDGF receptor and VEGF receptor that is thought to lead to tumor shrinkage in renal cell carcinoma (Janne et al. 2009). Finally, imatinib – sold by Novartis since 2001 under the name Gleevec – the second kinase inhibitor approved for use in humans, works through broad specificity for a cluster of kinase targets – including PDGF receptors, Bcr, Abl, KIT, colony stimulating factor 1 receptor, and breast cancer-resistant protein – rather than through high affinity for a single target (Chico et al. 2009).

As kinases are usually components of complex, interconnected signal transduction cascades, an approach that targets the cascade at multiple points can be more effec-

tive. In the case of PAK, an inhibitor that also inhibits downstream effectors – such as LIMK or MEK – could provide the desired pharmacological effect *in vivo*. In the next section, I describe the cellular signaling pathways upstream and downstream of PAK to highlight a number of potential targets in the PAK cascade.

Upstream Activators of PAK Signal Transduction Pathways

A number of studies in cell lines and cultured neurons have investigated the signal transduction pathways upstream of PAK activation. First there are the canonical PAK activators, Rac and Cdc42, which promote spine formation, enlargement, and maintenance (Manser et al. 1994)(Penzes and Jones 2008). These small GTPases are molecular switches turned on by growth factors, including BDNF and PDGF, or NMDA receptors, and they act through a variety of downstream effector proteins. Rac and Cdc42 bind PAK, resulting in a conformational change that allows the kinase to transition from an inactive dimer to a catalytically competent state (reviewed in (Bokoch 2003)). Next, phosphorylation of the critical Thr423 residue in the activation loop of PAK occurs, either via autophosphorylation or by an exogenous kinase such as phosphoinositide-dependent protein kinase 1 (PDK1), which is downstream of phosphatidylinositol 3-kinase (PI3K) (King et al. 2000). Another protein regulated by PI3K, Akt/PKB, phosphorylates PAK1 on a different residue, Ser21, which in turn decreases its binding affinity for adapter proteins and stimulates its kinase activity through a GTPase-independent mechanism (Tang et al. 2000).

As the site of active PAK in the cell is critical to its impact on actin dynamics, a number of proteins bind PAK directly or act indirectly through Rac to localize this signaling pathway. Nck and Grb2 are small adapter proteins which respond to growth factor signaling and physically associate with the active receptor tyrosine kinases. These adapters can recruit PAK to the membrane. Similarly, G-protein-coupled receptor kinase-interacting protein 1 (GIT1) functions to localize PAK interacting exchange factor (PIX), a guanine nucleotide exchange factor (GEF), to dendritic spines, thereby targeting Rac activity (Zhang et al. 2003). Local activity-dependent regulation of dendritic spine plasticity through PAK signaling is also induced by the

intercellular adhesion-like molecules ephrinB and their EphB receptors. EphB receptor activation induces translocation of the Rho-GEF kalirin to synapses where it activates Rac1 and its effector PAK (Penzes et al. 2003). Using this mechanism, the ephrin/EphB receptor signals through PAK to control multiple aspects of neuronal development (reviewed in (Penzes and Jones 2008)). In this way, there are many proteins in the PAK pathway, some of which may be appropriate targets to small molecule inhibitors.

Not only is the location of PAK activity important, but the magnitude and duration of PAK activity is critical for proper cellular signaling. Endogenous negative regulators serve to limit PAK activity. These include cyclin-dependent kinase 5 (Cdk5) – though it is controversial whether Cdk5 inhibits PAK1 through phosphorylation of Thr212 or an unknown mechanism – protein kinase A (PKA), and protein phosphatases (Sananbenesi et al. 2007)(Howe and Juliano 2000)(reviewed in (Arias-Romero and Chernoff 2008)).

Downstream Targets and Actin Dynamics

There is increasing evidence that PAK is an important signaling protein that contributes to a variety of cellular signaling pathways critical for prevention of cancer as well as normal brain development and function. PAK function achieves these goals through its regulatory roles in cell motility, growth, and transformation in cancer, as well as the formation and stabilization of dendritic arbors and axons in the brain. A key mechanism for regulation of these cellular process is regulation of cytoskeleton dynamics.

PAK orchestrates the cytoskeleton remodeling that accounts for the establishment and plasticity of synaptic connections, and thus PAK impacts cognitive function (Nikolic 2008). But before the details of PAK's impact in the brain were understood, numerous studies were conducted in mammalian cell lines. The first evidence that PAK might be involved in cytoskeleton dynamics came in several papers from Dr. Gary Bokoch's laboratory in 1997: Immunofluorescence analysis of fibroblast cell lines stimulated by PDGF, insulin, mechanical insult, or transformation by v-Src

demonstrated a redistribution of PAK1 from the cytosol to actin structures at the leading edge of lamellae and membrane ruffles (Dharmawardhane et al. 1997). In the second study, micro-injection of activated PAK1 in quiescent fibroblasts resulted in the formation of membrane ruffles, lamellipodia, and filopodia (Sells et al. 1997). Similar effects of PAK1 were demonstrated in neuronal cell lines a year later in which membrane-targeted PAK1 induced growth cone-tipped neurite formation (Daniels et al. 1998).

PAK has been proposed to act through up to 12 substrates to regulate the actin cytoskeleton, a few of which have been extensively studied (reviewed in (Arias-Romero and Chernoff 2008)). PAK1 phosphorylates LIM kinase (LIMK) on Thr509 of the kinase activation loop and increases LIMK mediated phosphorylation of the actin-regulatory protein cofilin (Edwards et al. 1999). Small actin-binding proteins of the cofilin/actin depolymerizing factor (ADF) family are important regulators of the cycle of actin polymerization and depolymerization required for cell motility. LIMK catalyzes the phosphorylation of cofilin, thereby inactivating its F-actin depolymerizing activity and allowing for the accumulation of stable actin filaments. In WT cells, cofilin cycles between a phosphorylated (inactive) and nonphosphorylated (active) state (Delorme et al. 2007). The consequences of this cycling on F-actin dynamics and downstream effects on neuronal morphology can be complex. Phosphorylation of cofilin inhibits it depolymerization and severing activity, thereby promoting F-actin polymerization. On the other hand, active cofilin directly depolymerizes F-actin, but indirectly promotes polymerization by modifying F-actin kinetics, increasing the free pool of G-actin, and increasing the polymerization-competent free barbed ends (Delorme et al. 2007). Thus, precise regulation of cofilin activity through the PAK/LIMK signaling pathways is required for proper activity-dependent actin dynamics and the resulting changes in dendritic spines.

There is mounting evidence that LIMK and cofilin play significant roles in the developing nervous system through regulation of processes such as neuronal migration, axonal motility, and the establishment of functional synaptic connections (reviewed in (Nikolic 2008)). For example, in LIMK-1 KO mice, where increased cofilin-dependent

actin depolymerization is hypothesized to result in increased turnover of the actin architecture, there was an abnormal phenotype in the morphology of both spines and growth cones. LIMK-1 KO mice have abnormalities in spine shape, though normal spine density and length, in pyramidal neurons in layer V of the visual cortex and CA1 layer of the hippocampus (Meng et al. 2002). The smaller spine heads and smaller PSDs observed in these mice allow for enhanced hippocampal LTP, presumably because there is more room for growth in small spine heads than more mature PSDs, but also enhanced LTD, which the authors interpreted as suggesting that LIMK-1 is also involved on the presynaptic side, presumably in sustained neurotransmitter release. When examined behaviorally, LIMK-1 KO mice displayed hyperactivity, enhanced freezing in hippocampal-dependent delay fear conditioning, and impairments in reversal learning in the Morris Water Maze. In summary, genetic and physiological evidence supports the hypothesis that LIMK-1 is critically involved in spine morphogenesis and synaptic function via regulation of the actin cytoskeleton, and that these processes are essential for normal cognitive function. Therefore, LIMK is a potential therapeutic target for disorders characterized by abnormal dendritic spines.

Activated PAK also regulates cytoskeleton activity through signaling to promote cellular relaxation. It does so through two substrates: myosin II regulatory light chain (MLC) and MLC kinase (MLCK) (reviewed in (Bokoch 2003)). Myosins are actin-activated motor proteins that convert the energy of ATP hydrolysis into force between actin and myosin filaments. They are responsible for actin-based motility and may act to stabilize the actin network at the leading edge of lamellipodia in fibroblasts and by extension at spines in neurons (Zhang et al. 2005). In response to upstream Rac signaling in cultured hippocampal neurons, PAK directly phosphorylates MLC on Ser19 (Chew et al. 1998). This phosphorylated form of MLC leads to increased actomycin contractility and corresponding increased spine and synapse formation (Zhang et al. 2005). A less well studied PAK1 substrate, filamin A, is an actin-binding protein that functions as a structural component of cell adhesion sites where it serves as a scaffold for signaling molecules in the Rho/Rac/Cdc42 pathway (Vadlamudi et al. 2002). In this way, PAK regulates the actin cytoskeleton through at least four

substrates.

Several lines of evidence have implicated PAKs in the regulation of another critical component of the cellular architecture: microtubules. Studies of microtubule dynamics have been conducted primarily in non-neuronal tissue and involved the coordination of cytoskeletal events required for cell division (reviewed in (Bokoch 2003)). However, stathmin, also known as oncoprotein 18 (OP18), is a microtubule destabilizing protein which is known to be inactivated by PAK1 phosphorylation in neurons (Wittmann et al. 2004). Expression of two OP18 family members in cultured hippocampal neurons enhanced axonal branching and increased growth cone spreading, respectively (Nikolic 2008). Through these multiple kinase substrates and interacting partners, PAK functions as a signaling node, coordinating upstream signaling with multiple effector proteins to control cytoskeleton dynamics in a localized fashion in neurons.

Downstream Targets and MAPK Signaling

In addition to its established role in cytoskeleton regulation, PAK also contributes significantly to regulation of the mitogen-activated protein kinase (MAPK) cascade. In neurons, the MAPK signaling pathway is activated by stimuli associated with synaptic activity and plasticity including neutrotrophins – nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3), or neurotrophin-4/neurotrophin-5 (NT-4/5) – calcium influx through channels such as the NMDA receptor, and calcium-independent signaling through mGluRs (McAllister et al. 1999) (West et al. 2001)(Wang et al. 2007). Abnormal signaling through mGluRs has been implicated in FXS and is discussed further in the next section of this chapter.

The intracellular MAPK cascade involves the sequential activation of four levels of signaling proteins: small GTPases (Ras and Rac), MAPK kinase kinases (Raf or MEKKs), MAPK kinases (MEKs), and MAPKs (Wang et al. 2007). The connection of PAK to the MAPK pathway is particularly interesting, because PAK phosphorylates Raf1 and also MEK1, thereby activating the pathway at two sites (Frost et al. 1997). Phosphorylation of MEK1 on Ser298 by PAK is necessary for effi-

cient activation of MEK1 and is central to the organization and localization of active Raf-MEK1-MAPK signaling complexes (Slack-Davis et al. 2003).

The role of MAPK signaling in the regulation of transcription and translation in neurons is well established (Treisman 1996)(Kelleher et al. 2004). Active MAPKs phosphorylate specific transcription factors like CREB and Elk in response to particular extracellular signals (Wang et al. 2007). Similarly, MAPK activity is required for inducible phosphorylation of multiple factors that play central roles in the initiation of protein synthesis, including ribosomal protein S6, eukaryotic initiation factor 4E (eIF4E), and its bind protein (4E-BP1) (Kelleher et al. 2004). Experimental evidence suggests that these functions of the MAPK signaling pathway are essential for hippocampal synaptic plasticity and long-term memory formation. For example, the late phase of hippocampal LTP (L-LTP) and hippocampal memory are disrupted in a mouse engineered to express a dominant-negative form of MEK1 in the postnatal forebrain (Kelleher et al. 2004).

1.5.4 Abnormal Signaling in *Fmr1* KO Mice.

Interestingly, the MAPK pathway has been implicated in the pathogenesis of FXS in mice. To fully comprehend the disease state and design targeted therapeutics, it is important to understand to the function of *fmr1* and its protein product FMRP in healthy neurons. While the general role of FMRP in normal synaptic functioning has been illuminated by studies of humans and mice without this protein, studies of normal cells and their lysates have been more effective at establishing the precise molecular mechanisms by which FMRP acts. Following this background information, we return to abnormal PAK and MAPK signaling in the *fmr1* KO mouse and how this information has led to the identification of drug targets.

Fragile X Mental Retardation Protein.

FMRP is widely distributed in various tissues types with notable expression in the brain – particularly in neurons of the cortex, hippocampus, and cerebellum – and

testes (Devys et al. 1993). It is an RNA binding protein and part of a family of heterogeneous nuclear ribonucleoproteins (hnRNPs) that regulate multiple steps of RNA biogenesis and metabolism. Through two KH domains, one arginine-glycine-glycine (RGG) box, and an N terminus with high affinity for RNA, FMRP binds preferentially to specific mRNAs – ~4% of those expressed in the human fetal brain – and transports them to dendrites (Siomi et al. 1993)(Ashley et al. 1993)(Brown et al. 1998).

The importance of the RNA binding activity is underscored by the severe, detrimental effects of a point mutation at an isoleucine residue in the second KH domain that abolishes RNA binding (Siomi et al. 1994)(Zang et al. 2009). A patient with this mutation has severe intellectual disability (IQ below 20), focial seizures, facial dysmorphisms, flat feet, and impressive macroorchidism (De Boulle et al. 1993). The N terminal domain is important for an additional reason: it has been implicated in protein-protein interactions, including homodimerization and heterodimerization with its autosomal homologs FXR1P and FXR2P (Zhang et al. 1995).

While FMRP associates with polysomes and mRNPs, the precise details and functions of FMRP have yet to be elucidated (Bassell and Warren 2008). Sucrose gradient fractionation studies have shown that FMRP is in the same fraction as translation machinery, however these findings have been controversial and contradictory, as some studies show FMRP with inactive ribosomes while others localize FMRP with actively translating polyribsomes (Khandjian et al. 1995)(Eberhart et al. 1996)(Zalfa et al. 2003)(Stefani et al. 2004)(Aschrafi et al. 2005). FMRP is thought to act as a translational repressor of specific, bound mRNAs including those encoding MAP1b, a microtubule-associated protein critical for microtubule and axon stabilization; SAPAP3/4, postsynaptic scaffolding proteins that bind PSD-95; NAP-22, a 22 kD neuronal tissue-enriched acidic protein that associates with calmodulin; Munc13, a SNARE-associated protein involved in neurotransmitter release; Rab6-interacting protein; and Sema3F, an axon guidance factor (reviewed in (Bassell and Warren 2008))(Brown et al. 2001)(Darnell et al. 2001).

In response to activity – mimicked *in vitro* by depolarization of cultured neurons

by KCl – FMRP rapidly localizes to dendrites (Antar et al. 2004). This response is dependent upon signaling through mGluRs, but does not require protein synthesis. The activity-dependent trafficking of FMRP and the RNA and proteins with which it associates – together known as an FMRP granule – results in colocalization of this granule with F-actin rich structures containing ribosomes, ribosomal RNA, and *MAP1B* mRNA (Antar et al. 2005). As *MAP1B* encodes a protein important for microtubule and actin stabilization, FMRP may play a role in the regulation of cytoskeleton dynamics important for activity-dependent structural and functional plasticity. Interestingly, dendritic spines are elongated in culture through a protein synthesis-dependend mechanism after stimulation of mGluRs, and authors described the resulting spines as resembling those seen the FXS brain (Vanderklish and Edelman 2002) (Vanderklish and Edelman 2005).

FMRP Regulation of Local Translation

Not only does FMRP colocalize with and bind to mRNA, but it also represses mRNA translation *in vitro*. The first demonstrations of FMRP as a translational repressor – conducted in microinjected Xenopus oocytes and rabbit reticulocyte lysate – were received with skepticism, as the "mRNA" reporters were not endogenous (Laggerbauer et al. 2001)(Li et al. 2001)(reviewed in (Darnell 2011)). Additional studies provided support for the involvement of FMRP in regulation of protein synthesis, though they arrived at different conclusions with respect to the mechanism of translational repression (Lu et al. 2004)(Muddashetty et al. 2007)(Zalfa et al. 2003). Recent work by the Darnell laboratory, designed to preserve endogenous interactions between FMRP and its neuronal mRNA targets, started with *in vivo* crosslinking and immunoprecipitation before moving to an *in vitro* translation assay (Darnell 2011). This study concluded that FMRP reversibly stalled ribosomes on its target mRNA during the elongation phase, therefore acting as a translation brake after the initiation of protein synthesis for a subset of synaptic proteins.

Other studies – initially conducted in *Drosophila* and human cell lines – suggest that FMRP is involved in the microRNA and RNA interference (RNAi) pathways

(Caudy et al. 2002)(Ishizuka et al. 2002). The idea that FMRP may regulate the translation of specific mRNAs through the microRNA pathway is an intriguing hypothesis for rapid and reversible regulation of protein synthesis. Edbauer et al. showed that miR-125b and miR-132 associate with FMRP in the mouse brain and have opposite effects on dendritic spine morphology and synaptic physiology in hippocampal cultures (Edbauer et al. 2010). They utilized hippocampal neurons again to demonstrate that mRNA for NMDA receptor subunit NR2A is a target of miR-125b, and then returned *in vivo* to demonstrate that NR2A mRNA associates with FMRP. Overall, while progress has been made in the field, the precise mechanism(s) by which FMRP modulates mRNA translation are still under investigation.

Absence of FMRP Leads to Excess Protein Synthesis

Excess synthesis of proteins is generally agreed to be a consequence of FMRP deficiency, however there is no clear consensus whether this is truly limited to a few specific proteins or a more general phenomenon (compare (Lu et al. 2004)(Muddashetty et al. 2007) to (Dolen et al. 2007)). Evidence supporting high basal levels of synthesis of unidentified proteins in *fmr1* KO mice came in a seminal *in vivo* study that utilized a sensitive technique for quantifying translation in living mice. *Fmr1* KO mice were found to have significantly higher rates of protein synthesis in 26 of the 75 brain regions examined, mostly in the thalamus, hypothalamus, and hippocampus (Qin et al. 2005). Of the cortical regions analyzed only the frontal association and posterior parietal areas were significantly affected by genotype. This *in vivo* phenotype was reproduced *in vitro* in hippocampal slices (Dolen et al. 2007). This study went on to demonstrate that increased basal protein synthesis was not limited to a few proteins, but rather it includes unidentified proteins of a variety of molecular weights. In summary, basal rates of protein synthesis are higher in *fmr1* KO mice compared to WT littermates.

Aberrant Signaling Impacts Translation in *Fmr1* KO Mice

Although it is clear that basal translation is increased in *fmr1* KO mice, the mechanism through which the absence of FMRP leads to elevated production of proteins is controversial. In WT neurons, Group 1 mGluRs signal through a few pathways to stimulate protein synthesis (reviewed in (Proud 2007)). Phosphoinositide 3-kinase (PI3K) signals through Akt to mammalian target of rapamycin (mTOR) which positively and negatively regulates distinct components of the translation machinery. Extracellular-signal-regulated kinase (ERK) and p38 MAPK both signal through MAPK signal-integrating kinase (Mnk) to activate eukaryotic initiation factors 4 E and F (eIF4E/F). Through these pathways, stimulation of mGluR1/5 promotes protein synthesis in WT cells.

Two early reports suggested that Akt and mTOR signaling in the hippocampus were activated in *fmr1* KO mice following DHPG (dihydroxyphenylglycine) stimulation of mGluRs (Hou and Klann 2004)(Sharma et al. 2010), however a recent, seminal study found otherwise. When a different acute slice preparation was utilized – one in which the *in vivo* phenotype of increased basal rates of translation in *fmr1* KO mice was replicated – the subtleties of the phenotype were uncovered (Osterweil et al. 2010). Basal and even DHPG-evoked ERK and PI3K signaling were normal in hippocampal slices from *fmr1* KO mice. Activity did not induce excess protein synthesis, presumably because elevated basal translation occluded further increases (Osterweil et al. 2010). Instead, the translation machinery was more sensitive to mGluR1/5 signaling in *fmr1* KO mice.

ERK1/2 as a Therapeutic Target

Hypersensitivity to mGluRs and downstream ERK1/2 may be responsible for some phenotypes in the mouse model of FXS. In this way, it is not surprising that acute injection of an inhibitor of ERK1/2 – a member of the MAPK pathway previously mentioned as a potential target for drug development – prevents AGS in *fmr1* KO mice (Osterweil et al. 2010). Unfortunately, due to the poor solubility of this drug

(SL 327), it had to be delivered in a vehicle composed of 50% DMSO (dimethyl sulfoxide) – a concentration too toxic for repeated treatments – and therefore additional behavioral assays were not conducted. Fortunately, this study highlights the therapeutic potential of targets like ERK1/2 in the MAPK signaling pathway for the treatment of seizures and potentially other phenotypes in *fmr1* KO mice.

Aberrant Activity-Dependent Regulation of Protein Synthesis.

Potentially more problematic than the elevated basal protein synthesis is the aberrant activity-dependent protein synthesis in the *fmr1* KO mouse. To understand the impact that it might have on mice and men with FXS, let us first review the significance and mechanisms of activity-dependent regulation of translation in neurons.

A prevailing view in neuroscience is that the cellular and molecular mechanisms that underlie synaptic plasticity also form the basis of learning and cognition. Synaptic plasticity – the ability of the brain to structurally and functionally modify its synaptic connections in response to activity patterns – can be measured by the ability of neurons to strengthen (long-term potentiation, LTP) or selectively weaken (long-term depression, LTD) responses to presynaptic activity. Neuronal responses to a repeated, strong stimulus strengthen over time, the phenomenon of LTP, and this process is dependent on NMDA receptor activation and AMPA receptor trafficking (Frenkel et al. 2006). Some types of LTD also require NMDA receptor activation, as well as postsynaptic calcium influx, activation of a phosphatase cascade, and AMPA receptor endocytosis (reviewed in (Massey and Bashir 2007)). In addition, a second type of LTD is induced through activation of G-coupled glutamate receptors, the most well characterized of which are the group 1 mGluRs – mGluR1 and mGluR5 (reviewed in (Dolen and Bear 2008)). Both gene expression and new protein synthesis play important roles in LTP and LTD (Bear 1996).

Interestingly, as stimulus selectivity and synaptic specificity are thought to be critical components of encoding memories with accuracy, it is not surprising that translation is thought to happen locally (Bear 1996)(Klann and Dever 2004)(Steward and Schuman 2001). Polyribosomes, mRNA, and other critical components of the

machinery for protein synthesis are positioned at the base of dendritic spines, endowing individual synapses with the capacity to control synaptic strength through the local synthesis of proteins (Steward and Schuman 2001). This translation machinery can be activated through mGluR signaling at synapses in the CA1 region of the hippocampus and modify the strength of synaptic connections within minutes (Huber et al. 2000). Interestingly, FMRP is one of the proteins synthesized in response to the stimulation of mGluRs with the receptor agonist DHPG in synaptoneurosomes (Weiler et al. 1997). This led to the hypothesis that FMRP is required for mGluR-dependent LTD, a theory which was tested using the *fmr1* KO mouse (Huber et al. 2002).

The mGluR Theory of Fragile X Syndrome

Instead of impaired mGluR-dependent LTD, however, LTD was discovered to be selectively enhanced in the hippocampus and cerebellum of *fmr1* KO mice (Huber et al. 2002)(Koekkoek et al. 2005). In this way, FMRP may normally serve as a brake on mGluR-dependent protein synthesis, thereby limiting the expression of LTD. This led Dr. Huber, Dr. Bear, and Dr. Warren to propose "the mGluR theory of Fragile X" in which there is runaway or poorly regulated protein synthesis in response to mGluR signaling in the *fmr1* KO mouse and presumably in humans with FXS (Bear et al. 2004). The mGluR theory predicts that dampening mGluR activity in the *fmr1* KO mouse will restore activity-dependent protein synthesis and therefore reverse phenotypes in this mouse model (reviewed in (Krueger et al. 2011)). Numerous studies *in vitro* and *in vivo* tested this prediction and demonstrated that a reduction in signaling through the mGluR pathway – via small molecule antagonists or genetic manipulations that decrease expression of mGluR1 and/or mGluR5 – can reverse mutant phenotypes in the *fmr1* KO mouse (Yan et al. 2005)(Dolen et al. 2007)(de Vrij et al. 2008)(Levenga et al. 2011)(Thomas et al. 2011).

The mGluR theory is the predominant theory in FXS research, though it does not explain the dendritic spine phenotype observed in mice and humans lacking expression of *fmr1*. Thus, the most effective therapeutic strategy may require dual targeting of

the PAK / spine morphology and mGluR / protein synthesis pathways.

Clinical Trials Test the Efficacy of mGluRs Inhibitors in Humans.

Clinical trials are already underway to test therapeutic interventions in adults and adolescents with FXS (reviewed in (Krueger et al. 2011)). The mGluR theory of fragile X was originally tested with the compound fenobam, a selective, noncompetitive mGluR5 antagonist which acts at an allosteric modulatory site which it shares with 2-methyl-6-phenylethynyl-pyridine (MPEP), the protypical selective mGlu5 receptor antagonist (Porter et al. 2005). Fenobam was discovered for its anxiolytic activity before a molecular target was identified. Six male and six female FXS patients, all adults, received a single dose of fenobam in this open-label phase II clinical trial (Berry-Kravis et al. 2009). Serious adverse effects in relation to altered CNS function were not reported – in contrast to a previous report of 4 weeks of treatment 4 times a day which cited hallucinations, vertigo, paraethesias ("pins and needles" sensation of the skin), and insomnia as side effects – though sedation was a side effect in one male and two female patients. Pharmacokinetic studies revealed that plasma levels peaked 2 - 3 hours following oral administration. Calm behavior and improvement in prepulse inhibition (PPI) was evident in a subset of drug treated patients within an hour of fenobam dosing. While these findings were promising, the study was not conducted blind.

Last year, a different mGluR antagonists called acamprosate – approved for the treatment of alcoholism – was tested in three patients with FXS and autism (Erickson et al. 2010). During a 21 week treatment course, patients showed improved linguistic communication. Recently, a 6 week open-label study was conducted with riluzole, a compound hypothesized to inhibit glutamate release, block excitotoxic effects of glutamate, and increase postsynaptic $GABA_A$ receptor function. This study assayed repetitive and compulsive behaviors as well as ERK activation in the periphery as a biomarker. While abnormal ERK activation was corrected by riluzole, only one of six patients showed signs of clinical improvement. While accumulating evidence suggests that mGluR5 antagonists are not a panacea for FXS, three additional compounds are

in phase I or II clinical trials in the U.S. or Europe (Krueger et al. 2011).

1.5.5 Local Protein Synthesis and Actin Dynamics Work Together to Promote LTP and LTD.

As inhibition of mGluR signaling through the use of mGluR antagonists may not be sufficient to treat symptoms in FXS patients, it is worth returning to the basic neurobiology of healthy neurons as well as neurons lacking expression of *fmr1*. In this way, the molecular medicine approach to drug discovery allows for, and perhaps requires, feedback between clinical trials in humans and laboratory research on molecular mechanisms.

Actin Plays a Role in Dendritic Translation

Persistent forms of synaptic change, such as LTP and LTD, require both dendritic mRNA translation and cytoskeletal dynamics. Research suggests that these two requirements are not distinct, but rather interrelated and moreover, interdependent (Vanderklish and Edelman 2005). The design of effective therapeutics for complex disorders like FXS, autism, and intellectual disability may require an understanding of the diverse molecular mechanisms and their relationship in neurons.

Let us consider mRNA translation in neurons. Large assemblies of mRNA, translation machinery, and related proteins including FMRP are transported down dendrites via microtubules in response to synaptic signaling (Antic et al. 1999)(Bassell and Singer 1997)(Antar et al. 2005). These mRNA granules are heterogeneous and not translationally competent, as they only contain trace amounts of leucine-tRNA and the translation factors eIF4E and eIF4GI (Krichevsky and Kosik 2001). It is by virtue of interactions with actin at individual spines that they associate with specific synapses and the missing pieces of the protein synthesis machinery (Antic and Keene 1998). In support of this, mRNA, tRNA, ribosomal subunits, and translation factors are known to bind to F-actin, and an ordered distribution of these components on the cytoskeleton promotes protein synthesis (Bassell 1993)(Gavrilova et al. 1987)(Lenk

et al. 1977)(Takizawa et al. 1997).

Further support for the role of F-actin in local protein synthesis comes from LTP studies in hippocampal slices. For example, the synthesis of the atypical protein kinase C (PKC) isoform PKMγ, which normally follows LTP-inducing stimulation, is blocked by inhibition of actin polymerization via latrunculin B treatment (Kelly et al. 2007). Furthermore, local F-actin dynamics are critical for the localization of Arc mRNA at active synapses (Huang et al. 2007). In this way, the actin-based cytoskeleton organizes and contributes to the differential regulation of mRNAs in response to signaling (Vanderklish and Edelman 2005).

Dendritic Translation Impacts Structural Plasticity

Similarly, local protein synthesis may act on actin dynamics. Accumulating evidence supports the theory that translation within dendrites and spines controls long-term functional and structural plasticity through modulation of actin polymerization (reviewed in (Bramham 2008)). Advances in imaging technology have allowed for ultrastructural studies with resolution high enough to see enlargement of spines in organotypic hippocampal slice cultures in real-time (Tanaka et al. 2008). LTP and spine enlargement occurred in response to glutamate uncaging at individual synapses paired with postsynaptic stimulation. Both functional and structural plasticity were affected by incubation with protein synthesis inhibitors. More specifically, long-lasting changes but not the immediate increase in spine size, were prevented by inhibition of translation. Thus, sustained spine enlargement and the stabilization of the actin cytoskeleton that underlies it require protein synthesis.

Arc Regulates Actin Polymerization Through Cofilin

Evidence suggests that the group of proteins upregulated by stimulations that induce long-term plasticity includes modulators of the cytoskeleton. The immediate-early gene Arc (aka Arg3.1), recently shown to be required for mGluR-dependent LTD in the hippocampus, has been well studied for its role in NMDA receptor-dependent LTP (Guzowski et al. 2000)(Plath et al. 2006)(Park et al. 2008)(Bramham 2008). Arc is

a spectrin-like molecule that binds actin filaments, and interacts with the endocytic machinery to regulate trafficking of AMPA receptors (Chowdhury et al. 2006)(Shepherd et al. 2006). In addition, there is evidence that Arc regulates the dynamics of actin through cofilin. Inhibition of Arc in hippocampal slices, a perturbation that reverses LTP even 2 hours after its induction, induced the rapid dephosphorylation and causative activation of cofilin (Messaoudi et al. 2007). As a result, nascent F-actin was lost at synaptic sites. The opposite was also shown to be true in this study: the F-actin stabilizing drug, jasplakinolide, prevented Arc inhibition from disrupting LTP. Taken together, these findings couple activity-dependent expression of Arc to spine enlargement via actin cytoskeleton dynamics as mechanisms underlying enduring LTP (Bramham 2008).

Summary

Dendritic translation and actin dynamics are required for sustained forms of synaptic plasticity and presumably the consolidation of memory. These processes are not independent but rather closely coupled. While they may converge at more than one point – including the MAPK pathway highlighted previously – accumulating evidence suggests that the PAK / cofilin signaling pathway is likely an important convergence point.

1.5.6 PAK Signaling in FXS.

This thesis work is built on the observation that abnormal dendritic spine density and shape are associated with cognitive and behavioral symptoms in humans with FXS, autism, and intellectual disability. Furthermore, a therapeutic strategy that corrects the spine deficit may also ameliorate the behavioral and neurological phenotypes. We have identified PAK as a novel therapeutic target for the treatment of neurodevelopmental disorders, and our demonstration of rescue with a dominant negative version of PAK validates this target (Hayashi et al. 2007). While a precise explanation of the molecular mechanism of this rescue is beyond the scope of this thesis work, in

this final section of the introductory chapter, I explore potential interactions between *PAK* and *fmr1*.

FMRP downregulates the PAK signaling pathway. There are a few possible explanations for the mechanism of genetic interaction between *fmr1* and *PAK*. First, FMRP may normally function to downregulate PAK or another member of the PAK signaling pathway. In this way, FMRP may either repress the translation of PAK mRNA (or a related mRNA) or inhibit the kinase activity of PAK. Thus, the absence of FMRP may result in an increase in PAK and its kinase activity, therefore leading to an increase in actin dynamics, actin filament stabilization, and/or spine number.

To test whether levels of PAK protein were unusually high in the brains of *fmr1* KO mice, Dr. Christopher Rex and Dr. Lulu Chen Western blotted for total PAK1 and PAK3 in whole-cell hippocampal homogenates from *fmr1* KO mice and WT mice (Chen et al. 2010). They did not observe a difference in basal protein levels in the hippocampus. Interestingly, they did find a significant increase when they compared synaptically localized PAK3 in stratum radiatum of CA1 of *fmr1* KO mice to WT mice. These data suggest that the absence of FMRP may lead to an accumulation of PAK at the PSD, poised to respond to incoming signaling. This localization of PAK would likely increase the fraction of PAK that is activated following synaptic activity. In this way, excessive PAK signaling may occur in response to synaptic activity in *fmr1* KO mice, and therefore partial inhibition of PAK would rescue this aberrant signaling.

In support of the view that FMRP antagonizes the PAK signaling pathway, experiments in *Drosophila* provide evidence for a genetic interaction between *dFmr1* and PAK activator *dRac1*. In this case, the fly ortholog of CYFIP1/2 provides a bridge between actin remodeling and protein synthesis by acting as a dRac1 effector to antagonize *dFmr1* (Schenck et al. 2003). Furthermore, experiments in murine fibroblasts demonstrate that FMRP binds Rac1 mRNA and interferes with Rac1-induced actin remodeling (Castets et al. 2005). Thus accumulating evidence suggests that FMRP downregulates the PAK signaling pathway.

PAK negatively regulates FMRP. Alternatively, PAK may normally physi-

cally interact with FMRP to negatively regulate FMRP activity. PAK may achieve this either through its kinase activity (i.e. it may phosphorylate FMRP on a serine or threonine residue) or by helping to localize FMRP to specific synapses in an activity-dependent fashion. PAK acts as a scaffold to recruit other proteins – in particular Akt – to the membrane, so in theory it could localize FMRP also (Higuchi et al. 2008). In support of this, we have shown that PAK immunoprecipitates FMRP, and that this binding is direct and dependent on the KH domains of FMRP (Hayashi et al. 2007). While this potential mechanism may explain how PAK and FMRP interact in WT neurons, it does not explain how inhibition of PAK can compensate for lack of FMRP.

FMRP and PAK act in parallel pathways. By crossing a *dnPAK* TG male with a *fmr1* KO female, Dr. Hayashi was able to demonstrate rescue of FXS-like phenotypes, suggesting a genetic interaction between *fmr1* and *PAK*. The result of this cross was a dMT that resembled neither parent, but rather appeared more like WT mice. In this way neither gene is obviously epistatic, though the results are complicated by the fact that PAK is only partially inhibited by the dominant negative. This suggests that rather than be in the same cell signaling pathway, FMRP and PAK act in separate but parallel pathways, converging on the same downstream effector or process. This downstream process may related to actin dynamics or dendritic translation.

FMRP has been shown to regulate the cytoskeleton in mice and flies. FMRP associates with mRNA encoding MAP1B, a protein important for microtubule and actin stabilization (Menon et al. 2008). In the absence of FMRP, MAP1B translation is misregulated and the developmentally programmed MAP1B decline in expression in the hippocampus is delayed (Lu et al. 2004). The result is excess MAP1B and rigid stability of microtubules in *fmr1* KO neurons. The *Drosophila* homolog *dFmr1* regulates the microtubule-associated protein Futsch to control synapse development at the neuromuscular junction, suggesting that *fmr1* regulation of cytoskeletal components is evolutionarily conserved (Zhang et al. 2001). While PAK primarily regulates the actin cytoskeleton, three of its effectors are microtubule associated proteins:

microtubule destabilizing protein stathmin/Op18 (Wittmann et al. 2004); tubulin cofactor B (TCoB), a protein that promotes formation of microtubule organizing centers (Vadlamudi et al. 2005); and dynein light chain (DLC), a cargo adapter with the actin-dependent motor myosin V (Espindola et al. 2000). In this way, FMRP and PAK signaling pathways may converge to regulate the cytoskeleton.

Both PAK and FMRP regulate the mitogen-activated protein kinase (MAPK) pathway, albeit in opposite directions. FMRP normally serves as a break on MAPK-dependent protein synthesis. In accordance with this, ERK1/2 signaling is overactive in *fmr1* KO mice, and new findings indicate that this is because the translation machinery is hypersensitive to ERK1/2 activation (Osterweil et al. 2010). PAK also activates the MAPK cascade by phosphorylating two proteins upstream of ERK1/2: Raf1 and MEK1 (Frost et al. 1997). In this way, inhibition of PAK kinase activity could counteract the hypersensitive ERK1/2 signaling cascade to rescue the protein synthesis phenotype in *fmr1* KO mice (Hayashi et al. 2007).

1.6 Conclusion.

While FXS and autism may be complex and heterogenous disorders, they share a common cellular phenotype. Abnormal dendritic spine morphology has been consistently reported in humans with FXS, autism, and intellectual disability. Moreover, the severity of the spine phenotype is correlated with the severity of cognitive defects in patients with ASDs. These observations support the hypothesis that correction of the spine deficit may ameliorate cognitive and/or behavioral symptoms in patients with neurodevelopmental disorders. A valid animal model is required to test novel therapeutics that target the spine phenotype. Since the gene responsible for FXS is known, scientists in the Dutch-Belgium Consortium were able to mimic the genetic cause of FXS in mice. The resulting mutant mouse – the *fmr1* KO mouse – shares the neuroanatomical phenotype and some of the behavioral phenotypes with humans with this condition.

Our laboratory used the *fmr1* KO mouse to test the hypothesis that specific

inhibition of PAK kinase activity in the adult brain is sufficient to reverse FXS-like phenotypes. Our results validated this theory and propelled us to search for a small molecule inhibitor of the kinase PAK. After two years of testing small molecule kinase inhibitors, we discovered a potent inhibitor of PAK. Chapter 2 of this thesis identifies this novel PAK inhibitor – an ATP-competitor known as FRAX486 – characterizes its targets, and demonstrates that it is an excellent drug for *in vivo* studies in mice. Chapter 3 presents data from Golgi-stained cortical neurons of WT and *fmr1* KO mice treated with FRAX486, demonstrating that this small molecule PAK inhibitor rescues the spine density phenotype. In chapter 4, I will describe the audiogenic seizure phenotype, and show that treatment with FRX486 decreases seizures in *fmr1* KO mice. Chapter 5 describes results from the open field assay, highlighting the impact that FRAX486 has on FXS-like phenotypes of hyperactivity and perseverative behaviors like excessive, repetitive grooming and circling. The final data section – chapter 6 – describes electrophysiology experiments conducted in the cortex of *fmr1* KO mice. Finally, I will discuss the implications of these findings and address outstanding issues in the field.

Chapter 2

Characterization of a Novel p-21 Activated Kinase (PAK) Inhibitor.

Contributions

The compound FRAX486 was identified by Dr. Sergio Duron, Dr. Ben Vollrath, and David Campbell, founders and employees of Afraxis, Inc., a company that was started to carry out the work described in "PAK Modulators," a patent held by Bridget M. Dolan, Susumu Tonegawa, and Mansuo Hayashi.

Kinases were selected for the *in vitro* kinase assays by Dr. Sergio Duron and Bridget M. Dolan. The kinase assays were outsourced to Invitrogen^{TM}.

For the pharmacokinetic experiments, drug delivery, tissue collection, and brain homogenizations were conducted by Bridget M. Dolan, and LC/MSMS was outsourced to Dr. Jon Gilbert at Apredica, a Cyprotex company.

Drug treatments and measurements of body and testicle weight were performed by Bridget M. Dolan.

2.1 Summary

Genetic rescue experiments demonstrated that p21-activated kinase (PAK) inhibition in the adult is sufficient to rescue symptoms of Fragile X Syndrome (FXS) in the mouse model of the disease. This discovery, made in our laboratory (Hayashi et al. 2007), is striking as it is an illustration of a therapeutic intervention that can mask, or even reverse, disease phenotypes that began in development and persisted into adulthood. It provides hope for adults who have suffered from FXS and related neurodevelopmental disorders their whole lives.

However, in order to translate this basic science discovery in mice into a potential therapeutic for humans, the gene therapy approach must be transformed into a pharmacological solution. Protein kinases are druggable targets, and since small molecule inhibitors have been identified for other kinases, pharmacological inhibition of PAK is an *a priori* feasible cure for FXS. In this chapter we report the identification and characterization of a small molecule PAK inhibitor: FRAX486. Here we show the potency and specificity of FRAX486 *in vitro*, as well as the pharmacokinetic properties of FRAX486 *in vivo*.

2.2 Introduction

The reversible phosphorylation of proteins is a fundamental regulatory mechanism for cellular processes controlling everything from embryonic development to cell death. Protein kinases, the enzymes that catalyze the transfer of phosphate groups from adenosine triphosphate (ATP) to protein substrates, are therefore essential components of life for all organisms. Furthermore, an increasing body of evidence implicates the faulty regulation of kinases in various diseases that lack effective therapies, including cancer and neurological disorders. As a result, kinase-targeted drugs offer the promise of future therapies, and there is intense interest in the pursuit of small molecule kinase inhibitors.

Inhibition of PAK with a Dominant Negative Transgene

PAKs are effectors of the small GTPases Rac and Cdc42, involved in a range of biological processes in cancer cells and neurons (Bokoch 2008). This family of serine-threonine kinases contains two groups: group I includes PAK 1, 2, and 3 and group II includes PAK 4, 5, and 6 (reviewed in (Bokoch 2003). Group I PAKs are effectively inhibited by expression of a peptide that includes the auto-inhibitory domain (AID) found in the N-terminal (amino acids 83 to 149 of PAK1 or 78 to 146 of PAK3) (Zhao et al. 1998). Our laboratory expressed this autoinhibitory domain *in trans* in a mouse, producing a dominant negative *dnPAK* transgenic (TG) line (Hayashi et al. 2004). Expression of *dnPAK* was sufficient to rescue symptoms in a mouse model of autism, the *fmr1* KO mouse (Hayashi et al. 2007).

Inhibition of PAK with a Small Molecule

While the dominant negative strategy is extremely useful and highly specific, it has limitations. In particular, in its current form, it is difficult to translate our discovery in mice into a useful therapeutic in humans, as it would be necessary to introduce the peptide or DNA construct into every neuron we wished to affect. A small molecule would be more useful for clinical studies in humans. Luckily, PAK is a kinase, and like other kinases, it is a druggable target because the conserved ATP-binding pocket can accommodate small molecules (Deacon et al. 2008). Of course, a strategy that targets this conserved site must also be evaluated for kinase selectivity, as a broad spectrum kinase inhibitor may have unwanted side-effects.

To our knowledge there is no potent and selective ATP-competitive inhibitor for PAK. A selective, cell-permeable, small molecule inhibitor that targets the PAK1 activation mechanism has been identified (Deacon et al. 2008). While it is particularly impressive due to its high degree of specificity – inhibiting only 9 of 214 kinases tested (4% of total) in a large-scale screen – it has not been shown to work *in vivo*. Most small molecules which show efficacy *in vitro* are not effective *in vivo*, especially when the target tissue is the brain.

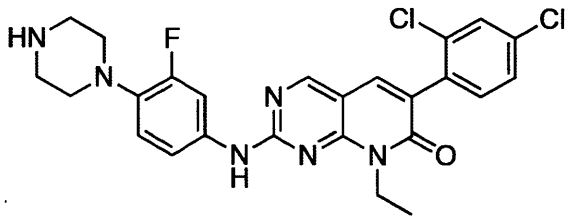

Figure 2-1: **Structure of FRAX486.** The chemical structure of the small molecule 6-(2,4-dichlorophenyl)-8-ethyl-2-(3-fluoro-4-(piperazin-1-yl)phenylamino)pyrido[2,3-*d*]pyrimidin-7(8*H*)-one, a compound provided by Afraxis.

A large obstacle to central nervous system (CNS) drug discovery efforts is blood-brain barrier (BBB) penetration. It is estimated that nearly 100% of large molecule neurotherapeutics and 98% of small molecule drugs are excluded from the CNS, as they cannot cross the BBB (Pardridge 2005). Thus, it is important to evaluate BBB penetration capabilities early on in the drug discovery process for disorders like FXS and idiopathic autism. In this chapter we investigate the pharmacological properties of FRAX486 in mice.

2.3 Results

2.3.1 FRAX486 Is a Novel Inhibitor of Group I PAKs.

Afraxis, a startup company inspired by our genetic rescue study, licensed our patent on the use of PAK modulators in the treatment of FXS and other neurodevelopmental disorders (For a summary of the patent, see Appendix 8-1). In the search for an inhibitor of the kinase PAK, Dr. Sergio Duron and colleagues at Afraxis screened a library of compounds for small molecules with high affinity for PAK's ATP binding site. This site, also called the ATP fold, is highly conserved and is the motif in other kinases that has historically been successfully targeted to generate drugs and candidate compounds (Chico et al. 2009). The structure of the lead compound for *in vivo* experiments as of 2010, an ATP-analog named FRAX486, can be found in Figure 2-1.

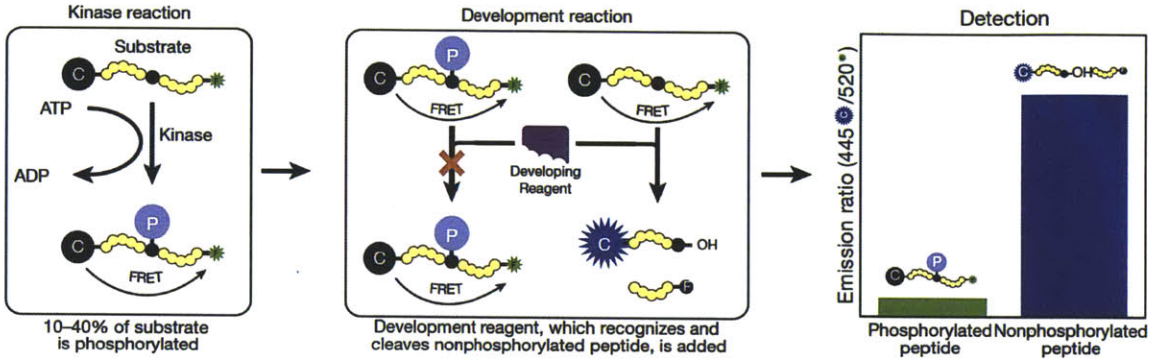

Figure 2-2: **Schematic of the *in vitro* Kinase Assay Used to Characterize FRAX486.** Invitrogen's Z′-LYTE™assay is a non-radioactive, functional assay that utilizes an optimized peptide substrate that is terminally labeled with a FRET fluorophore pair that is more sensitive to cleavage in a non-phosphorylated state than in a phosphorylated state. In the kinase reaction (left panel) the optimized substrate is incubated with the full-length kinase and FRAX486 (not shown). Kinase that is not inhibited by FRAX486 transfers the γ-phosphate of ATP to the target serine, threonine, or tyrosine on the synthetic peptide. The development reaction (middle panel), in which a site-specific protease is added, relies on the differential sensitivity of phosphorylated verses non-phosphorylated peptides to proteolytic cleavage. A phosphorylated peptide is protected from cleavage and therefore the coumarin (donor fluorophore which emits at 445 - 460 nm) and fluorescein (acceptor fluorophore which emits at 520 - 535 nm) are positioned for fluorescence resonance energy transfer (FRET), and a low coumarin to fluorescein emissions ratio is observed (445 nm / 520 nm; green bar in right panel). In contrast, a non-phosphorylated substrate is cleaved and therefore primarily gives off a coumarin emission signal, resulting in a high emissions ratio (blue bar in right panel). The percent inhibition is calculated from the emission ratio and takes into account experimentally determined minimum and maximum ratios.

To characterize the inhibitory capacity of the novel small molecule *in vitro*, Afraxis employed Invitrogen to conduct a Z′-Lyte^{TM} biochemical assay. Z′-Lyte^{TM} is a non-radioactive, fluorescence resonance energy transfer (FRET) based assay that relies on differential sensitivity of phosphorylated and non-phosphorylated peptide substrates to proteolytic cleavage. It utilizes synthetic peptides optimized for each kinase that are terminally tagged with the fluorophores coumarin (donor) and fluorescein (acceptor). For a more detailed explanation of the theory and methods of the Z′-Lyte^{TM} kinase assay, refer to Figure 2-2.

To determine whether FRAX486 inhibits group I and/or group II PAKs *in vitro*, the Z-Lyte^{TM} functional assay was conducted using 10 titrations of FRAX486 for 4 PAK isoforms. The group I PAKs – PAK1, PAK2, and PAK3 – were inhibited by FRAX486 with low nanomolar potency (Figure 2-3A-C), whereas PAK4 – a group II PAK – had a half maximal inhibitory concentration (IC_{50}) of nearly 800 nM (Figure 2-3D and Figure 2-4). This represents the discovery of a novel, potent inhibitor of group I PAKs.

2.3.2 FRAX486 Has Additional Targets.

Since the ATP-binding site of protein kinases is highly conserved, drugs which target this motif may inhibit more than one kinase. By determining the inhibitory profile of FRAX486 against a series of kinases, we can identify additional targets which may be beneficial for therapeutic intervention or deleterious due to opposing or toxic off-target effects. To screen for additional kinases which are inhibited by 100 nM FRAX486, Afraxis ran the Invitrogen Z′-LYTE^{TM}kinase assay across a panel of 106 kinases, that is 20% of the subset of genes in the human genome that encode kinases (aka human kinome). Twenty-one of the 106 kinases were inhibited by 80% or more, including PAK1. An additional nine enzymes were inhibited by 50% or more, including PAK2 and PAK3.

A more in depth assay was performed for the subset of inhibited kinases that are expressed in high levels in the brain and/or play important roles in neurons (Figure 2-2 and Figure 2-5). This assay was used to calculate the IC_{50} values for multiple

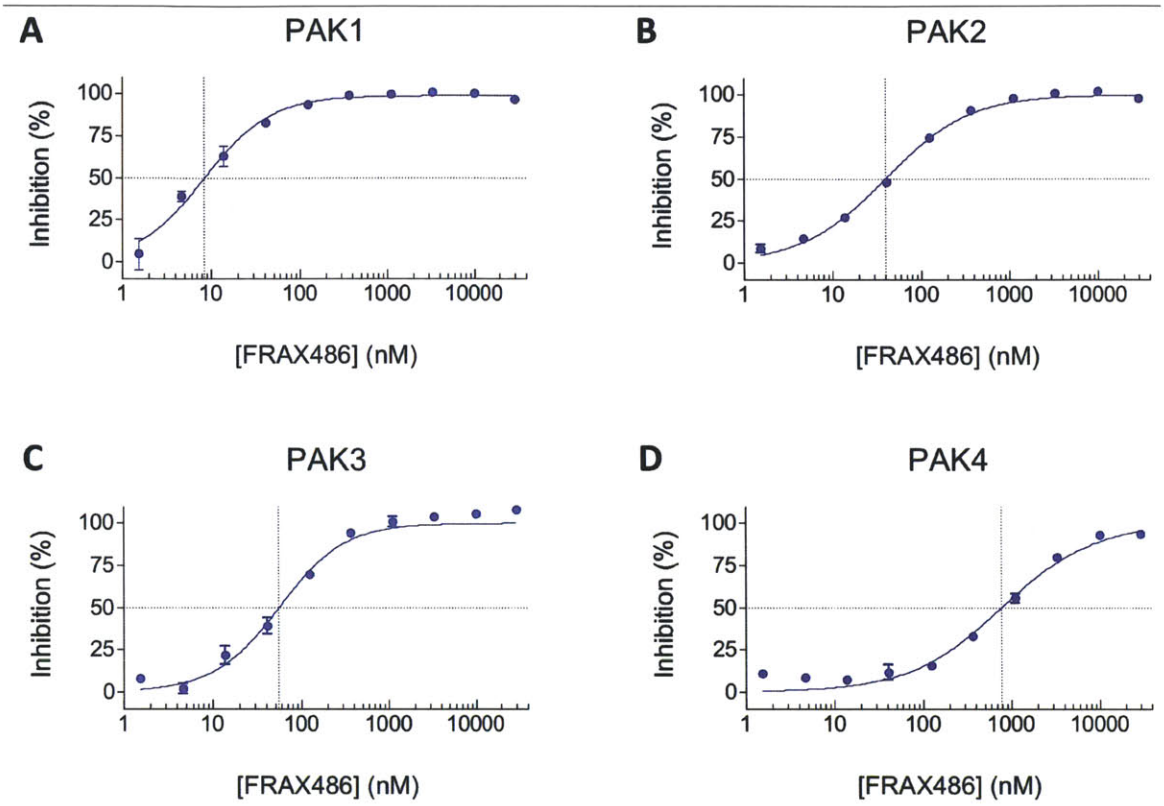

Figure 2-3: **FRAX486 Is an Inhibitor of Group I PAKs *in Vitro*.**
In vitro efficacy of the putative PAK inhibitor, FRAX486. *In vitro* kinase assay tested the ability of full-length PAK isoforms to phosphorylate an optimized peptide substrate. **(A-C)** FRAX486 inhibits group I PAKs with nanomolar potency. **(D)** FRAX486 is a weak inhibitor of PAK4, a group II PAK.

Invitrogen's Z′-LYTE^{TM}biochemical assay was performed for 10 point titrations of FRAX486. Dose-response data is presented as percent inhibition of kinase activity on semi-log plots. Values are mean +/- SEM (n = 2). Sigmoidal dose-response curves with variable slope were fit to the data with the constraints that the bottom of the curve must equal zero and the top of the curve cannot surpass 100% (GraphPad Prism).

Kinase	IC_{50} (nM)
PAK1	8.253 +/- 0.04480
PAK2	39.45 +/- 0.02929
PAK3	55.31 +/- 0.05157
PAK4	779.3 +/- 0.08978

Figure 2-4: **FRAX486 Inhibits Group I PAKs with Nanomolar Potency.** IC_{50} values are concentrations at which each PAK isoform is inhibited by 50%. Values are mean +/- SEM (n = 2) derived from the data in Figure 2-3.

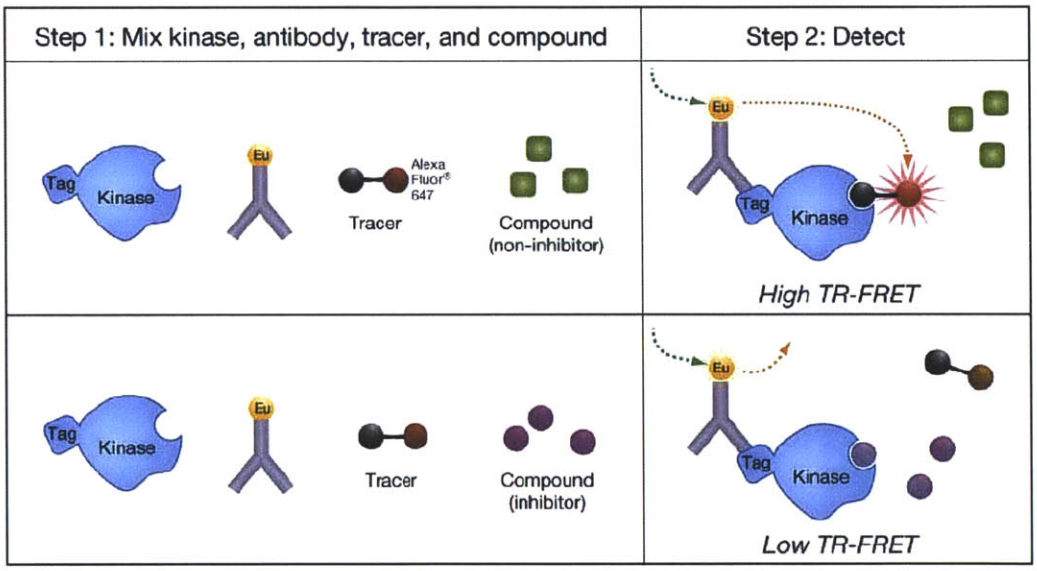

Figure 2-5: **Schematic of the *in Vitro* Binding Assay Used to Characterize FRAX486.** Invitrogen's LanthaScreen™Eu kinase binding assay is a competition binding assay that measures displacement of the compound by a kinase tracer. In step 1, the tagged kinase is mixed with an europium (Eu) labeled, anti-tag antibody, the tracer, and the compound. If the compound does not bind the kinase, then the tracer will bind the kinase. In the detection step, the tracer, fluorescently labeled (Alexa Fluor®647) ATP-competitor, will act as an acceptor fluorophore, resulting in high FRET and a corresponding high tracer to antibody emission ratio. However, if the compound is an effective competitive inhibitor, it will out compete the tracer for binding to the kinase. In this case, there will be low FRET and a low emission ratio.

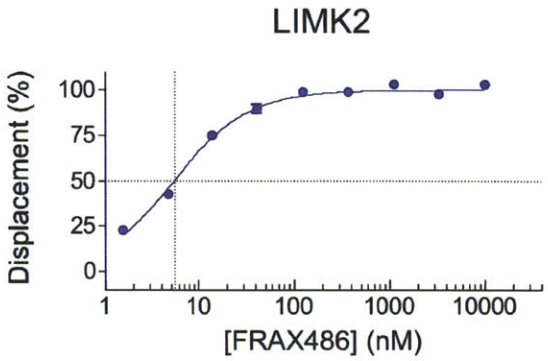

Figure 2-6: **FRAX486 Inhibits PAK Substrate LIMK2.**

kinase targets. This includes LIMK2, the PAK substrate which phosphorylates cofilin to prevent actin depolymerization (Figure 2-6). Thus, FRAX486 targets the PAK signaling pathway at more than one point. In addition, the kinases B-Raf, ErbB4, MEK1, MEK2, RET, and Src bound to and/or were inhibited by FRAX486 in the Invitrogen^{TM}assays. The concentration of FRAX486 required to inhibit half of the activity of these kinases was in the low nanomolar range (Figure 2-7 and Figure 2-8). In this way, FRAX486 is a potent inhibitor of PAK1, PAK2, PAK3, LIMK2, B-Raf, ErbB4, MEK1, MEK2, RET, and Src, some of which may be valuable targets for therapeutic intervention.

2.3.3 FRAX486 Has Excellent Pharmacokinetic Properties for *in Vivo* Treatment.

A great obstacle to drug discovery research for central nervous system (CNS) diseases is effective blood-brain barrier (BBB) penetration. The BBB, a neuroprotective shield which consists of tight junctions between neuronal endothelial cells around capillaries, restricts the passage of drugs between the bloodstream and the brain. Since only ~2% of small molecule drugs can cross the BBB (Pardridge 2005), it was important to determine whether FRAX486 achieves optimum *in vivo* exposure at the target tissue – the brain – prior to conducting a more in depth characterization of this

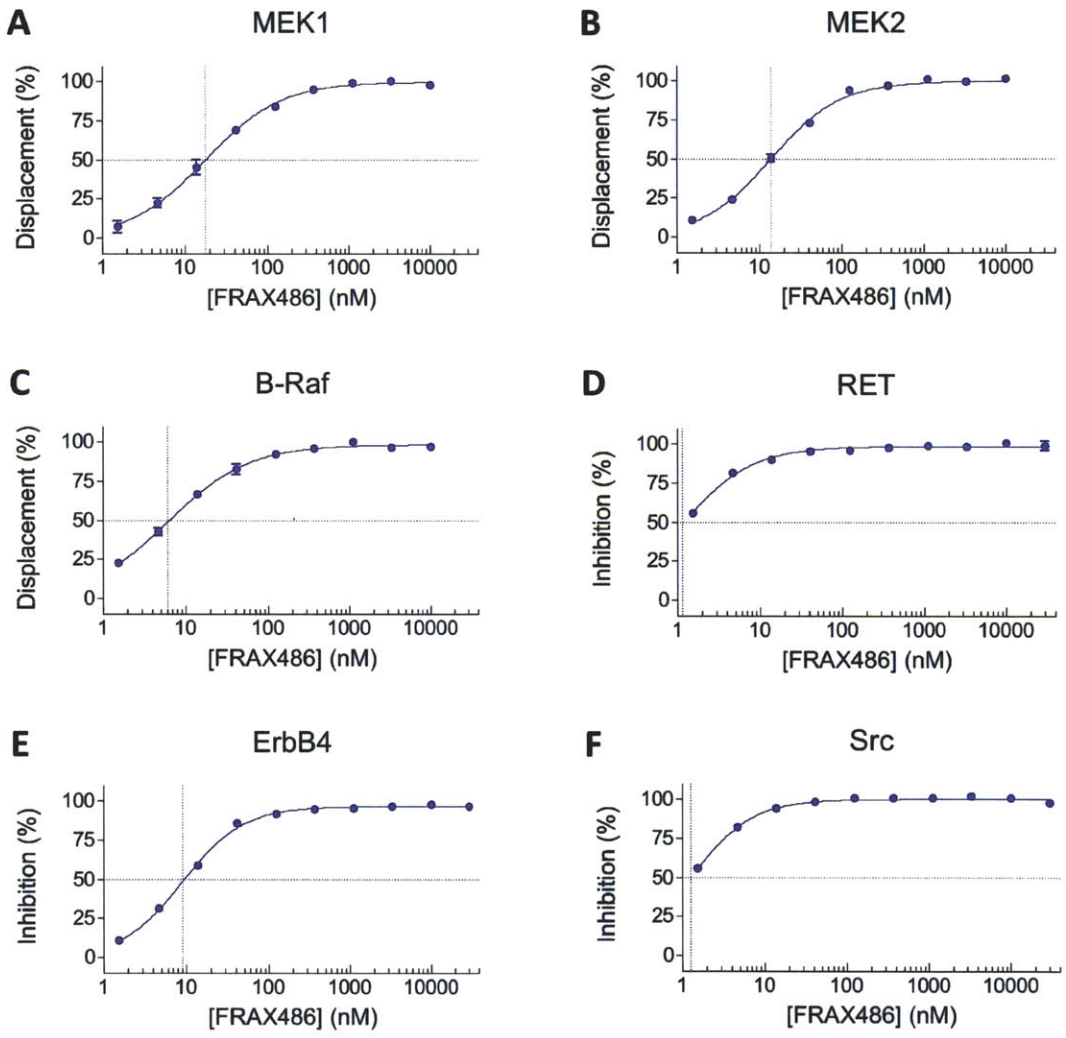

Figure 2-7: **FRAX486 Inhibits MEK1, MEK2, B-Raf, RET, ErbB4, and Src *in Vitro*.**

Kinase	IC_{50} (nM)	Format
LIMK2	5.405	Binding
MEK1	17.61	Binding
MEK2	13.72	Binding
B-Raf	6.116	Binding
RET	1.138	Kinase
ErbB4	8.903	Kinase
Src	1.267	Kinase

Figure 2-8: **Kinases Inhibited by FRAX486 *in Vitro*.**
IC_{50} values are concentrations at which each kinase is inhibited by 50%. Values are mean +/- SEM (n = 2) derived from the data in Figure 2-7.

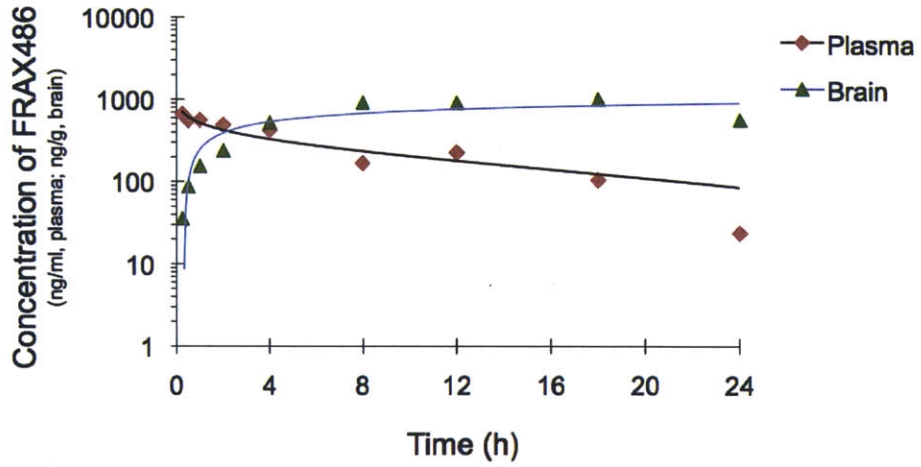

Figure 2-9: **Brain Levels of FRAX486 Are High by 4 h and Remain High for at least 24 h Following Drug Administration.** Pharmacokinetic (PK) profiles of plasma (ng/ml) and brain concentrations (ng/g) of FRAX486 at different time points following subcutaneous injection of 20 mg/kg FRAX486.

Values are plotted on a logarithmic scale. Values are mean +/- SEM (n = 2 - 3).

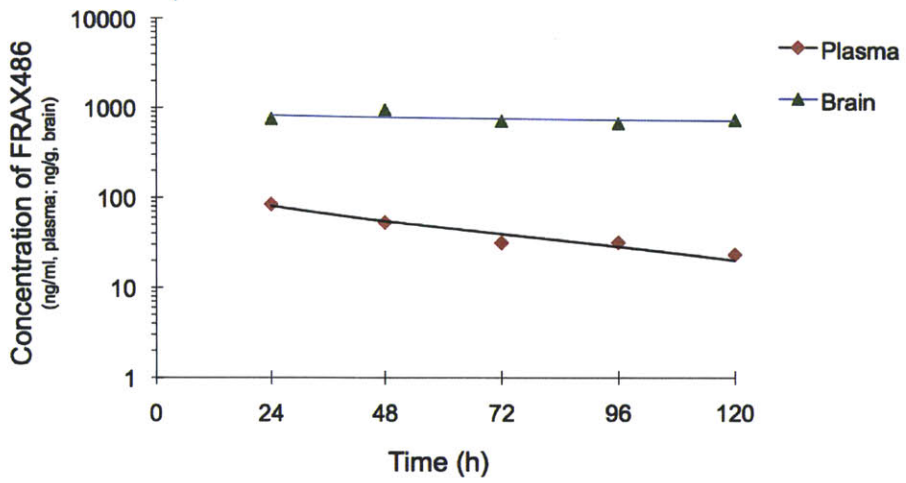

Figure 2-10: **Brain Levels of FRAX486 Are Steady and Do Not Accumulate with Daily Administration.** Pharmacokinetic (PK) profiles of plasma (ng/ml) and brain concentrations of FRAX486 (ng/g) 24 h following 1, 2, 3, 4, or 5 daily subcutaneous injections of 20 mg/kg FRAX486.

Values are plotted on a logarithmic scale. Values are mean +/- SEM (n = 3 - 4).

novel kinase inhibitor. In addition, a drug candidates potential for long-term success depends upon the effect the body has on the drug, the so called pharmacokinetic properties, which are affected by *in vivo* absorption, tissue distribution, metabolism and excretion.

To determine whether FRAX486 is suitable for *in vivo* experiments, we conducted pharmacokinetics studies. In the initial experiment, wildtype mice of the FVB.129P2 background strain (referred to as WT throughout this thesis) received a single subcutaneous injection of 20 mg/kg FRAX486. Brain and blood samples were collected at various time-points following drug administration. One hour after drug administration, brain levels of FRAX486 reached 155 +/- 25.5 ng/g, equivalent to 301 +/- 50.0 nM and therefore approximately 36 times the IC_{50} for PAK1, 7.6 times the IC_{50} for PAK2, and 5.4 times the IC_{50} for PAK3. They remained at least this high for the duration of the experiment (Figure 2-9). These results demonstrate that FRAX486 is a compound that crosses the BBB at levels that will inhibit PAK. Based on our knowledge from *in vitro* studies, these should be therapeutically useful concentrations.

Therefore this compound is suitable for experiments that target the CNS.

Additional insights can be learned from this data set. To determine the optimal time-course for mouse behavior assays, we compared levels of FRAX486 across time-points. Plasma levels of FRAX486 were highest at the initial time-point (15 min) and remained over 100 ng/ml (~200 nM) for up to 18 h (Figure 2-9). As expected, the time-course for brain levels involved a slower ascent. FRAX486 levels rose in the brain until reaching a ceiling at 8 h, maintained near 951 +/- 27.0 ng/g (1.84 +/- 63.5 μM) from 8 h to 18 h, and began to decrease at 24 h. These data suggest that the maximal beneficial effects of FRAX486 may begin 8 h after administration.

To determine whether daily dosing results in steady state levels of drug or whether drug accumulates in the body, samples were taken from mice that received daily injections of 20 mg/kg FRAX486 for up to 5 days. Each blood and brain sample was taken 24 h following the ultimate injection (Figure 2-10). Plasma levels of FRAX486 remained consistent from day-to-day (one-way ANOVA: plasma, $p = 0.1285$; brain, $p = 0.7928$). In this way, once a day dosing of 20 mg/kg FRAX486 via subcutaneous injection results in steady state levels of FRAX486 in the brain.

2.3.4 FRAX486 Does Not Impede Normal Growth.

A successful drug candidate will have minimal side effects on the physiology and behavior of the human or mouse receiving the treatment. To determine whether inhibition of PAK with FRAX486 has negative impacts on overall health and normal growth, we tracked weight changes in mice that received 20 mg/kg FRAX486 injections daily for 21 days. FRAX486 did not impede normal weight gain in mice (Figure 2-11).

2.3.5 FRAX486 Does Not Impact Phenotypes Outside of the Brain.

Humans and mice with FXS suffer from enlarge testes, a condition called macroorchidism. To determine whether FRAX486 acted outside the brain to correct the macroorchidism

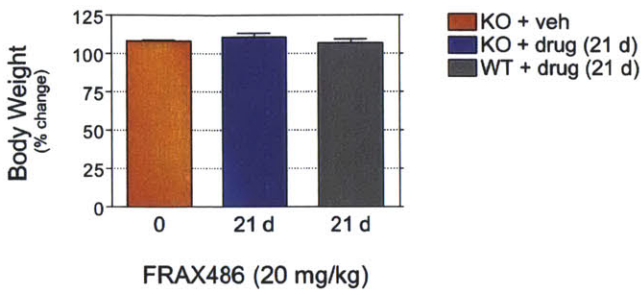

Figure 2-11: **Treatment with FRAX486 Does Not Affect Body Weight.** Daily administration of 20 mg/kg FRAX486 for 21 days does not impact normal increases in body weight, one indication of good health. Mice were 8 weeks old at the start of drug treatment. *fmr1* KO + veh, n = 5; *fmr1* KO + drug, n = 6; WT + drug, n = 5.

phenotype, we compared the weight of testes of drug or vehicle treated *fmr1* KO mice and WT littermates. Testes were larger in *fmr1* KO mice when compared to WT mice. This phenotype was not rescued by 21 days of treatment with FRAX486 (20 mg/kg)(Figure 2-12). Therefore we conclude that FRAX486 does not alter the macroorchidism phenotype in the mouse model of FXS.

2.4 Discussion

2.4.1 Hypothesis-driven, Targeted Approach Led to the Discovery of a Novel Kinase Inhibitor.

Advances in our understanding of the molecular basis of disease and the neuronal phenotypes in humans and mice lacking FMRP allowed us to design a targeted drug discovery process. Our target – PAK – is a critical regulator of the actin cytoskeleton and dendritic spines. The work presented here describes the identification of a novel inhibitor of group I but not group II PAKs. It is an extremely potent PAK inhibitor, with an IC_{50} value in the low nanomolar range. This potency is particularly impressive when compared to other kinase inhibitors. For example, Deacon et al. identified the first specific chemical inhibitor of group I PAKs – IPA-3 – in a screen of 33,000 small molecular weight compounds (Deacon et al. 2008). IPA-3 has a PAK1 IC_{50} of 2.5

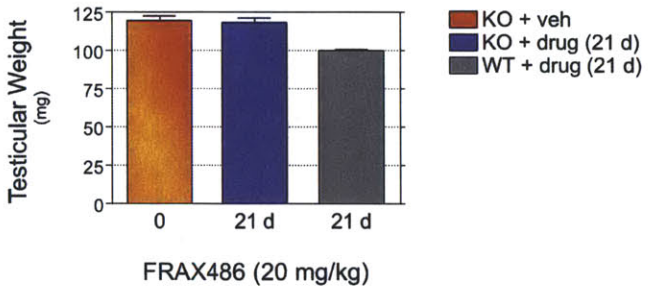

Figure 2-12: **Macroorchidism in *Fmr1* KO Mice Is Not Rescued by FRAX486.** Mice were administered daily subcutaneous injections of 20 mg/kg FRAX486 or vehicle alone control for 21 days. 1 (in the case of WT) or both (in the case of KO) testes were collected and weighed. Consistent with the literature of human and murine males without *fmr1* KO, *fmr1* KO + veh had enlarged testes when compared to WT + drug. Treatment of *fmr1* KO with FRAX486 did not rescue this abnormality.

fmr1 KO + veh, 119.7 +/- 2.6, n = 12 testes from 6 mice; *fmr1* KO + drug, 118.3 +/- 3.3, n = 11 testes from 6 mice; WT + drug, 100.2 +/- 0.88, n = 6 testes from 6 mice. Mean values and SEM are listed (two-way ANOVA: Interaction, $p = 0.8197$; Genotype, $p < 0.0001$; Treatment, $p = 0.8197$; Bonferroni posttests show significant effect of genotype but not drug treatment.)

μm, which means that our PAK1 inhibitor is 300 times more potent.

In addition to potency, another important characteristic of kinase inhibitors is selectivity. The previously mentioned PAK inhibitor – IPA-3 – was lauded for its high degree of specificity, as it only inhibits 4% of kinases by more than 50% at a drug concentration of 10 μm (Bokoch 2008). This is especially impressive when compared to clinically successful kinase inhibitors like Gleevec by Novartis (imatinib), which is described as "selective" for Bcr-Abl though it inhibited 12% of kinases in the same assay (Deacon et al. 2008). In this way, while FRAX486 is not a specific inhibitor of 1 kinase at the exclusion of all other kinases, neither are most kinase inhibitors used *in vivo* and perhaps all kinase inhibitors currently approved for use in humans.

Afraxis has an active drug discovery program and is developing additional small molecules from the FRAX486 scaffold, as well as other base scaffolds, in search of an equally potent but more selective group I PAK inhibitor. It will be useful to test additional PAK inhibitors, even those which are not highly selective but have kinome selectivity profiles different than FRAX486, in the *in vivo* assays described in

subsequent chapters. If phenotypes are inhibited by a variety of small molecule PAK inhibitors, this will lend credence to our hypothesis that PAK is an important target for the treatment of FXS.

2.4.2 There May Be Therapeutic Value in Inhibiting Additional Targets.

Interesting, some of the unexpected targets of FRAX486 may also have therapeutic potential. First, three of the targets – LIMK, MEK, and B-Raf – are important substrates of PAK. Thus, inhibiting these kinases may be redundant to inhibiting PAK. This is especially true with LIMK, the protein that links PAK to cofilin and actin cytoskeleton remodeling. Next, with the exception of LIMK, all other targets converge on the MEK/ERK pathway. As this signaling cascade is hypersensitive or even overactive in *fmr1* KO mice, this may be of added benefit to a strategy for the treatment of FXS. In order to reverse the maximum number of symptoms, it may be most effective to target the excess protein synthesis as well as the dendritic spine phenotype. Finally, all of these kinases have been linked to human diseases (Figure 2-14). In particular, LIMK kinase has been associated with Williams syndrome and Alzheimers disease (AD), while MEK/ERK has been linked to autism. Therefore a kinase inhibitor like FRAX486 may have broad application to a number of human disorders.

Let us consider the likely effect of inhibition of each of these targets one-by-one (see Figure 2-13 and Figure 2-14 for details and references).

LIMK Is a Critical Effector of PAK

LIMK, a serine kinase with two isoforms, is expressed in the brain and testes (Bernard 2007). It is an important effector of PAK, and therefore inhibition of LIMK will be redundant to inhibition of PAK (Edwards et al. 1999). LIMK has been linked to a number of diseases including Williams syndrome, AD, and cancer (Frangiskakis et al. 1996)(Heredia et al. 2006). In Williams syndrome, a heterozygeous deletion of LIMK1

Kinase	Downstream of PAK?	Linked to protein synthesis?	Potentially responsible for rescue?	Has it been linked to human disease?	Inhibitors in clinical trials or approved for humans?
LIMK	Yes Phosphorylation by PAK activates LIMK	No	Yes	Williams syndrome, Alzheimer's disease, cancer	No
MEK	Yes Phosphorylation by PAK activates MEK1	Yes Upstream of ERK	Yes	Autism, cancer, diabetes, inflammatory disease	No
Ret	No	Yes Upstream of Ras/ERK, PI3K/Akt, and p38/MAPK	Unlikely. Ret mutations predominantly affect PNS ganglia	Cancer (GOF; colorectal, thyroid), Hirschsprung disease (LOF)	Imatinib (Gleevec; Novartis), Sorafenib (Nexavar; Bayer), Selumetinib (AstraZeneca),
B-Raf (aka Raf-1)	Yes Phosphorylation by PAK activates Raf	Yes Upstream of MEK/ERK	Yes	Cancer (melanoma and others), neuro-cardio-facial-cutaneous syndromes	Sorafenib (Nexavar; Bayer), PLX4032/RG7204 (Plexxikon/Roche), GSK 2118436; GlaxoSmithKilne)
ErbB4	No	Yes Upstream of MAPK and PI3K	Unlikely.	Cancer (breast, prostate), schizophrenia	No
Src	No	Yes Upstream of Ras/Raf/MEK	Unlikely.	Cancer (breast and others)	Bosutinib (Pfizer), AZD-0530 (AstraZeneca), Dasatinib (Bristol-Myers Squibb), JNJ-26483327 (Johnson & Johnson), XL-228 (Exelixis), KXO-1 (Kinex)

Figure 2-13: **Therapeutic Potential of Additional FRAX486 Targets.**

CA = catecholaminergic, dn = dominant negative, FC = fear conditioning, GOF = gain-of-function, HP = hippocampal, KO = knockout, LOF = loss-of-function, MWM = Morris water maze, PSD = postsynaptic density

and neighboring genes leads to cognitive deficits including visuospatial impairment as well as vascular disease. In AD, LIMK1 is overactive in areas of the brain with AD pathology. Interestingly, inhibition of cofilin phosphorylation can prevent neuronal degeneration in an AD model. In this way, a small molecule like FRAX486 that inhibits LIMK may have broad therapeutic potential.

Inhibition of MEK1/2 May Rescue Excess Protein Synthesis in *fmr1* KO Mice

Activation of the MAPK pathway triggers a rich network of signaling pathways including gene regulation, translation, cell proliferation, cell motility, and cell survival (Gerits et al. 2007). PAK1 activates two proteins in this pathway: Raf1 and MEK1 (Frost et al. 1997). Thus, FRAX486 inhibits MEK1 directly and indirectly through its actions on PAK and Raf1. Abnormal signaling of the MAPK pathways has been linked to a number of conditions including autism, cancer, inflammatory disease, and diabetes (Yang et al. 2011). Therefore, while an inhibitor of MEK1/2 may be useful for treating various conditions, it may also have various side effects.

The mGluR theory of FXS is based on the observation that basal levels of protein synthesis are elevated in *fmr1* KO mice (Qin et al. 2005). Recent work has demonstrated that the translation machinery in *fmr1* KO mice is hypersensitive to ERK1/2 signaling (Osterweil et al. 2010). Therefore a treatment that decreases ERK1/2 signaling will decrease the abnormally high levels of protein synthesis in *fmr1* KO, and this may be sufficient to reverse some or all of the FXS-like phenotypes. Consistent with this prediction, treatment of hippocampal slices with ERK1/2 inhibitors *in vitro* decreases protein synthesis in *fmr1* KO and WT slices (Osterweil et al. 2010). *In vivo* administration of this inhibitor is sufficient to prevent audiogenic seizures in juvenile *fmr1* KO mice.

It would be interested to investigate whether FRAX486 acts in part by rescuing the excess protein synthesis in *fmr1* KO mice. Such an experiment could be conducted first using the *in vitro* method mentioned above, then using the *in vivo* quantitative autoradiographic L-[1-14C]leucine method of Dr. Carolyn Beebe Smith (Qin et al. 2005).

B-Raf Is a Common Target for Cancer Therapeutics

B-Raf (aka Raf-1) plays important roles throughout the body and specifically in nervous system development, where it regulates neuron differentiation and axon growth

Kinase	Behavior phenotype in KO or dn mouse?	Spine phenotype in KO mouse?	LTP phenotype in KO slices?	Reference
LIMK	Hyperactivity, increased freezing in delay FC, deficit in reversal learning in MWM	Smaller spine heads and smaller PSDs	Enhanced HP LTP and LTD	(Bernard, 2007), (Frangiskakis, 1996)
MEK	Deficit in recall phase of MWM, impaired contextual FC	NA Inhibitors block spine dynamics in vitro	Impaired L-LTP	(Kelleher, 2004), (Yang, 2011), (Gerits, 2007)
Ret	NA	NA	NA	(Li, 2006)
B-Raf (aka Raf-1)	Deficit in training phase of MWM, impaired contextual discrimination		Impaired HP LTP	(Chen, 2006), (Tartaglia, 2010)
ErbB4	Hypoactivity, altered cue use in MWM	NA shRNA decreases size of presynap-tic inputs	Inhibitor depotentiates LTP in HP	(Buonanno, 2010) (Yarden, 2001) (Krivosheya, 2009) (Golub, 2004)
Src	NA	NA	NA	(Kalia, 2004)

Figure 2-14: **KO Phenotype of Additional FRAX486 Targets.** CA = catecholaminergic, dn = dominant negative, FC = fear conditioning, GOF = gain-of-function, HP = hippocampal, KO = knockout, LOF = loss-of-function, MWM = Morris water maze, PSD = postsynaptic density

(Zhong et al. 2007). Oncogenic B-Raf has been linked to cancer, and B-Raf inhibitors are therapeutically successful at treating cancer, in particular melanomas.

B-Raf is downstream of PAK and upstream of MEK/ERK signaling. Thus, FRAX486 inhibition of B-Raf is redundant to its inhibition of PAK and MEK. In this way, FRAX486 inhibits the MAPK signaling pathway through multiple distinct targets.

ErbB4 Signals Through MAPK in GABAergic Interneurons

ErbB4 is a receptor tyrosine kinase that has been implicated in a variety of cellular processes and a few clinical conditions including schizophrenia and cancer (reviewed in (Buonanno 2010)). ErbB4 plays a critical role in the CNS in development and adulthood as the major receptor for neuregulin 1 (NRG1). In development, NRG1-ErbB signaling mediates migration of neurons and pathfinding of axons, as well as differentiation, migration, and development of neurons (Krivosheya et al. 2008). It triggers an intracellular cascade of signaling events that act through MAPK and PI3K. In this way, ErbB4 signaling converges on the same MAPK pathway as the previously mentioned targets of FRAX486. However, there is a significant difference in ErbB4 mediated signaling, as ErbB4 expression is restricted to GABAergic interneurons (Krivosheya et al. 2008). Several lines of evidence support the hypothesis that an impairment of GABAergic transmission may contribute to the development of autism (Pizzarelli and Cherubini 2011). A dysfunction of GABAergic signaling in early development may lead to neuronal circuits with an imbalance of excitation and inhibition. If severe, this could be responsible for some of the cognitive and behavioral abnormalities characteristic of autism (Pizzarelli and Cherubini 2011). A treatment which increases inhibitory drive in *fmr1* KO mice may compensate for the imbalance, however this strategy may require activation rather than inhibition of ErbB4.

Src Upregulates NMDA Receptor Activity

First identified as an oncogene, Src is a nonreceptor tyrosine kinase expressed in abundance in neurons (Kalia et al. 2004). Proliferation and differentiation of neurons is under the control of Src during development. In addition, Src has been implicated in the adult brain where it phosphorylates and therefore modulates NMDA receptor activity. Src acts to upregulate the activity of NMDA receptors and thereby influence NMDA receptor-mediated LTP in the hippocampus (Xu et al. 2008). As *fmr1* KO mice express an LTP deficit in the cortex, a small molecule that further dampens LTP may have detrimental effects on neuronal activity. Therefore it is important to

investigate the impact that FRAX486 has on LTP, and that experiment was conducted as part of this thesis work.

2.4.3 Conclusion

Knowledge of the molecular mechanism of FXS has allowed for the emergence of a field studying the neurobiology and synaptic mechanisms of disease and designing targeted treatments. Taking a hypothesis-based, targeted approach we search for inhibitors of the actin cytoskeleton remodeling protein PAK. Here, for the first time, we present the discovery of a small molecule inhibitor of group I PAKs. With this potent PAK inhibitor we return to the mouse model of FXS and test the hypothesis that inhibition of PAK is an effective strategy for the treatment of morphological, neurological, and behavioral phenotypes in *fmr1* KO mice.

2.5 Materials and Methods

2.5.1 Animals.

Adult male *Fmr1* KO mice (FVB.129P2-*Fmr1*^{tm1Cgr}/J) and age-matched WT mice (FVB.129P2-*Pde6b*$^{+}$ *Tyr*$^{c-ch}$/AntJ, sometimes abbreviated FVBS/Ant where "S" is for sighted (Errijgers et al. 2007)), of the genetic background which includes the wild-type *Pde6b* allele so the mice do not suffer from blindness due to retinal degeneration, 10 to 12 weeks old were used for pharmacokinetic assays and 10 to 17 weeks were used for the Rotarod performance test(Jackson Labs). Animals were housed in groups of two to five per cage with food and water freely available. Mice were kept on a 12-h light/dark cycle and cared for in accordance with the standards of the Massachusetts Institute of Technology Committee on Animal Care and in compliance with National Institutes of Health guidelines.

2.5.2 FRAX486 and Pharmacokinetics.

FRAX486 is a yellow powder of molecular weight 513.4. FRAX486 was dissolved in 20% hydroxypropyl-β-cyclodextrin vehicle (Sigma-Aldrich, St. Louis, MO). A 2 mg/ml solution of drug or vehicle alone control was administered via subcutaneous injection at 20 mg/kg in a volume proportional to the animals weight (typically 300 - 400 μl). Single injections were given 4 - 5.5 h prior to the Rotarod and at various intervals prior to tissue collection for pharmacokinetic experiments: Blood and brain samples were collected at 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 18 h, and 24 h following drug administration. Forebrain tissue was weighed, fast frozen and homogenized in 2x volumes of cold PBS. Levels of FRAX486 were determined via LC/MS/MS (Apredica, Watertown, MA). We thank Dr. Sergio Duron from Afraxis, Inc. for providing us with this kinase inhibitor.

2.5.3 *In vitro* Kinase Assays.

Invitrogen's SelectScreen Biochemical Kinase Profiling Service utilized a Z'-LYTE^{TM}functional biochemical assay.

As is depicted in Figure 2-2, Z'-LYTE^{TM}employs a fluorescence-based screening technique to detect kinase activity in the presence of vehicle alone (1% DMSO) and FRAX486 at a single concentration or at 10 titrations. Synthetic Ser/Thr peptide substrates optimized for each kinase were used in a 1 h kinase reaction. Full-length kinase (e.g. 2.71 - 30.8 ng PAK1, 0.29 - 6 ng PAK2, 1.75 - 20 ng PAK3, or 0.1 - 1 ng PAK4) was incubated with 2 μM peptide substrate in 50 mM HEPES pH 7.5, 0.01% BRIJ-35, 10 mM MgCl2, 1 mM EGTA in the standard assay (aka direct format). Then protease was added, which preferentially cleaves non-phosphorylated peptide. Since the peptides were terminally tagged with a donor fluorophore (coumarin, emits at 445 - 460 nm) and an acceptor fluorophore (fluorescein, emits at 520 - 535 nm), quantitation of cleavage, which is inversely proportional to kinase activity, relied on detection of a FRET signal and calculation of an emission ratio.

The assay conditions were modified for 8 kinases for which there are no syn-

thetic direct substrates: B-Raf, B-Raf V599E, MAK2K1 (MEK1), MAP2K2 (MEK2), MAPK10 (JNK3), MAPK8 (JNK1), MAPK9 (JNK2), and Raf1 (cRaf) Y340D Y341D. In this version of the screen, called the cascade assay, the kinase of interest is used to activate an inactive downstream kinase which in turn phosphorylates its optimized synthetic peptide or activates a third kinase in the signaling pathways which in turn phosphorylates its peptide substrate. For example in the Z′-LYTE^{TM}cascade assay for B-Raf, B-Raf activates MAPK2K1 (MEK1) which in turn activates MAPK1 (ERK2), resulting in the phosphorylation of Invitrogen peptide Ser/Thr 03.

Utilizing the FRET-based technology, FRAX486 was screened at 100 nM with 106 kinases to generate an inhibitory profile. For the 10 titration versions, FRAX486 was serially diluted and screened a 1.53 nM, 4.60 nM, 13.7 nM, 41.0 nM, 123 nM, 370 nM, 1.11 μM, 3.33 μM, 10 μM, and 30 μM. Dose-response data was plotted on semi-log charts as percent inhibition of kinase activity. Values are mean +/- SEM (n = 2). Sigmoidal dose-response curves with variable slope were fit to the data with the constraints that the bottom of the curve must equal zero and the top of the curve cannot surpass 100% (GraphPad Prism). For each kinase, the half maximal inhibitory concentration (IC_{50}) was calculated.

Invitrogen's SelectScreen Kinase Profiling Service utilized a LanthaScreen ^{TM}Eu Binding Assay.

As described in Figure 2-5B, LanthaScreen^{TM}Eu Binding Assays rely on the binding and displacement of a fluorescent tracer to the kinase of interest in the presence of FRAX486 at 10 titrations or vehicle alone (1% DMSO). This assay was run for four kinases: LIMK2, MEK1, MEK2, and B-Raf. The tracer / antibody emission ratio for 10 FRAX486 concentrations was used to calculate percent displacement. The values (Mean +/- SEM, n = 2) were graphed as semi-log plots, and sigmoidal dose-response curves with variable slope were fit to the data and used to calculate IC_{50} values.

Chapter 3

Rescue of Dendritic Spine Excess in *Fmr1* KO Mice by FRAX486.

Contributions

The work described in this chapter was a collaboration between Bridget M. Dolan and Dr. B. S. Shankaranarayana Rao, Additional Professor, Department of Neurophysiology, National Institute of Mental Health and Neuro Sciences, India. Experimental design, drug delivery, tissue collection and data analysis was conducted by Bridget M. Dolan. Golgi staining, camara lucida drawings, and spine counts were conducted by the laboratory of Dr. Rao.

3.1 Summary

The neuroanatomical hallmark of mental retardation, and in particular Fragile X Syndrome (FXS), is abnormal dendritic spines. Long, thin, and tortuous spines are extraordinarily abundant on dendrites of cortical neurons in FXS humans and *fragile X mental retardation 1* knockout (*fmr1* KO) mice compared to neurotypical humans and wildtype (WT) mice. We hypothesized that treatment of the dendritic spine phenotype might address the cellular cause of cognitive and behavioral symptoms associated with FXS.

To rescue the dendritic spine phenotype, we inhibit the critical cytoskeleton remodeling protein PAK. Inhibition of PAK via a dominant negative transgene was sufficient to rescue the dendritic spine phenotype in our previously published genetic rescue experiment. In this chapter we build on that discovery to investigate whether treatment with a small molecule PAK inhibitor – FRAX486 – also rescues the dendritic spine phenotype *in vivo*. Here we demonstrate that FRAX486 reverses the abnormal spine phenotype in cortical neurons in *fmr1* KO mice, and that rescue can occur within 8 h of the initial treatment.

3.2 Introduction

3.2.1 An Abundance of Immature Dendritic Spines in FXS.

A breakthrough in our understanding of the underlying pathology of FXS came with the postmortem analysis of brain tissue of FXS patients. While gross anatomy was intact, abnormalities were present on the level of dendritic spines, the main sites of excitatory synaptic input for neurons. Dendritic spines on pyramidal neurons in the neocortex of FXS patients were abnormal when compared to age-matched control subjects (Irwin et al. 2001)(Hinton et al. 1991)(Rudelli et al. 1985). That is, the spines were long, thin, and tortuous in morphology on apical dendrites of pyramidal cells in layers III and V of parieto-occipital neocortex and in the pyramidal layer of allocortex (Rudelli et al. 1985). In addition, the density of spines was higher in FXS

patients (Irwin et al. 2001). The density phenotype was first observed in the visual and temporal cortices on distal segments of the dendrites: 50 μm or more from the soma on the apical dendritic shaft and 37.6 μm or more from the soma on the basal dendrites (Irwin et al. 2001). The consistency of the effects of FXS across cortical regions and layers is striking and suggests a very specific morphological deficit in this syndrome. Increased spine density and increased numbers of long and immature-like spines are the most salient neuropathologies described in association with FXS so far and seem likely to underlie the cognitive, behavioral, or neurological deficits associated with it (Irwin et al. 2001). For example, these data suggest that excitatory synapse number is elevated in FXS, providing a potential mechanism for the increased incidence of seizures in FXS males.

In addition to the increase in excitatory synapse density, aberrant spine morphology also distinguished the FXS brain tissue from normal human brain tissue (Irwin et al. 2001). Abnormally long dendritic spines with immature architecture – characteristic of early development or lack of sensory experience – were observed in the adult FXS brain tissue (Hinton et al. 1991)(Rudelli et al. 1985). This is interesting because while there is an iconic dendritic spine architecture – a spine head with a postsynaptic density (PSD) connected by a narrow neck to dendritic shaft – the morphology of any given spine can change within days or even minutes in healthy brains (reviewed in (Cingolani and Goda 2008))(Govindarajan et al. 2011). In this way, the diversity of dendritic spines observed at any one time is thought to be a temporal snapshot of a dynamic phenomenon (Parnass et al. 2000). This dynamic phenomenon, governed by rearrangements of the actin cytoskeleton, may be dysfunctional in neurons of patients with FXS and other types of mental retardation.

3.2.2 Abnormal Dendritic Spines in *Fmr1* KO Mice.

The spine phenotype observed in the postmortem cortical tissue has largely been corroborated by studies in the mouse model of the disease. However, the existence and magnitude of the spine phenotype varies based on developmental period, mouse strain, and brain region, highlighting the complex and multifactorial regulation of

spines. Several studies of Golgi-stained adult neocortical neurons agree that there is an increase in the number of immature spines and a decrease in the number of mature spines, however, only a subset of these see an increase in overall spine density (Comery et al. 1997)(Irwin et al. 2002)(Hayashi et al. 2007).

One hypothesis based on the increase in spine density is that neurons without FMRP lack the ability to prune unnecessary synaptic connections. Pruning is a process that eliminates excess spines during development thereby contributing to the refinement of brain circuitry. In support of this hypothesis, Dr. William Greenough's laboratory conducted numerous studies which demonstrated that a reduction in spine density during development does not occur in the somatosensory whisker barrel cortex of *fmr1* KO mice in comparison to normal mice, where improperly located dendritic spines are withdrawn during development (Galvez and Greenough 2005).

Work from other laboratories has further defined the dendritic spine phenotype in *fmr1* KO mice. The stabilization of dendritic spines was investigated with *in vivo* time-lapse imaging with 2-photon microscopy of layer II/III neurons in the barrel field of the somatosensory cortex of neonatal *fmr1* KO mice. This work demonstrated a developmental delay in the downregulation of spine density that normally accompanies a rapid phase of fine-tuning of intracortical circuitry (Cruz-Martin et al. 2010). Interestingly, this study also reported an increase in the number of dendritic protrusions gained or lost in *fmr1* KO mice, suggesting increased dynamics and/or decreased stabilization of dendritic spines in this mutant. These findings support a second hypothesis, originally proposed based on the increase in the proportion of long, thin spines in human and mouse postmortem tissue: dendritic spines do not mature in FXS. There may be a failure in the transition of filopodia, the earliest dendritic protrusions, to mature spines in the absence of FMRP (Comery et al. 1997).

3.2.3 Defective Signaling in the PAK Pathway in *Fmr1* KO Neurons.

Another signaling pathway, a critical regulator of spine size and turnover, is impaired in *fmr1* KO mice. At the University of California, Irvine, scientists investigated the stimulation-induced activation of synaptic Rac1 and its effector PAK. They demonstrated that theta-burst stimulation increased the activation of Rac1 and the number of synapses associated with phosphorylated PAK in WT but not *fmr1* KO hippocampal slices (Chen et al. 2010). Interestingly, while the initial polymerization of actin in spines occurred in *fmr1* KO neurons, these activity-induced actin filaments did not stabilize. The hypothesis that Rac/PAK signaling is required for the stabilization of activity-dependent rearrangements in the actin cytoskeleton is corroborated by work in WT rat hippocampal slices. In this preparation, inhibitors of Rac or PAK – in this case the PAK inhibitor IPA-3 that was described in the previous chapter – had no obvious effect on basal synaptic transmission or the initial expression of LTP. However, both inhibitors blocked the stabilization of the new actin architecture which normal occurs within 30 - 120 s of theta-burst stimulation (Rex et al. 2009)(Chen et al. 2010). These results provide direct evidence that physiological activation of the PAK signaling pathway, a primary actin regulator in adult spines, is impaired in the absence of FMRP. Furthermore, the number of synapses associated with high levels of PAK3 is increased by ~50% in hippocampal neurons in *fmr1* KO mice. This accumulation of synaptic PAK could result in overactive PAK signaling or could provide negative feedback to the Rac/PAK signaling pathway.

3.2.4 Genetic Rescue of Spine Phenotype.

Correction of the defects in physiologically driven actin dynamics and stabilization may be sufficient to reverse the phenotypes in *fmr1* KO mice. To test this hypothesis, we crossed the *fmr1* KO mouse to our *dnPAK* mouse so that PAK activity was decreased by ~40% in the forebrain of adult mice. This decrease in PAK signaling was sufficient to reverse the abnormalities in dendritic spine density and morphology

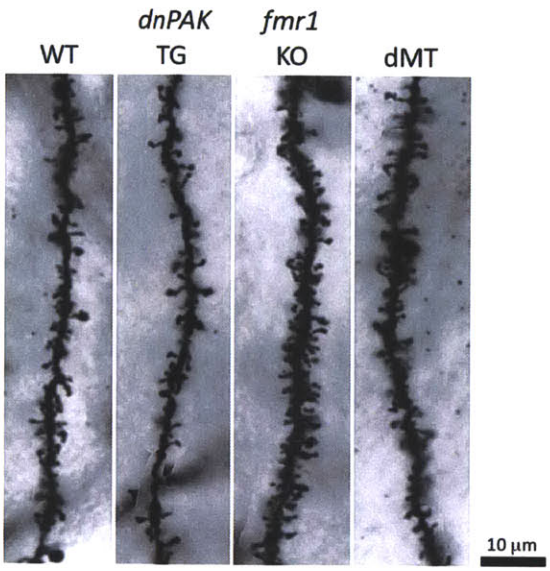

Figure 3-1: **Expression of *dnPAK* in *Fmr1* KO Rescues Spine Phenotype.** Golgi staining of representative segments of apical dendrites on layer II/III pyramidal neurons in the temporal cortex of 11 week old B6 mice. Spine density was lower in *dnPAK* TG mice compared with WT mice in segments 1 - 4, whereas it was higher in *fmr1* KO mice compared with WT mice in segments 1 - 6. Crossing the *dnPAK* TG and *fmr1* KO mouse lines produced a double mutant (dMT) in which the spine phenotype was rescued in all 6 of these segments.

Wildtype C57BL6, WT; dominant negative *PAK* transgenic, *dnPAK* TG; *fmr1* x *dnPAK* double mutant, dMT. On each primary apical dendritic branch, 10 consecutive 10 μm long dendritic segments were analyzed to quantify spine density. Segment 1 is most proximal to the soma, and segment 10 is farthest from the soma. Data was published in PNAS (Hayashi et al. 2007).

in the cortex (Figure 3-1). In this thesis, we take the next step to determine whether *in vivo* administration of PAK inhibitors is sufficient to reverse the spine deficit in adult *fmr1* KO mice and how quickly this reversal can occur.

3.3 Results

Abnormalities in dendritic spines, the anatomical substrates for excitatory synapses in the brain, have been observed in humans with various forms of mental retardation including FXS. This is not surprising as spine morphology and spine dynamics are

thought to underlie memory and learning. To test whether a small molecule PAK inhibitor is sufficient to rescue the spine phenotype in the mouse model of FXS, we treated *fmr1* KO mice with FRAX486 and examined its impact on cortical spine density. The results of this experiment are presented in this chapter.

3.3.1 Visualization of Dendritic Spines with Golgi Staining.

For over one hundred years, neuroscientists have used the Golgi-Cox staining method to visualize neurons and the details of their dendrites (Ramoñ y Cajal 1909)(Ramon-Moliner 1970). We used this reliable, classic technique to quantify the spine density in pyramidal neurons in the mouse cortex. For the spine analysis we focused on neurons in cortical layers II/III of the mouse temporal lobe, so that results here could be compared to our previous spine studies (Hayashi et al. 2004)(Hayashi et al. 2007) as well as the synaptic plasticity experiments described in a coming chapter. Please refer to Figure 3-2 to see the location in the mouse cortex and a sample camera lucida tracing of a layer II/III pyramidal neuron.

Spines were counted in a total of 10 segments of 10 μm each, and the segments were numbered 1 to 10, proximal to distal with respect to the cell body. Apical and basal dendrites were analyzed separately, as they occupy distinct cortical layers and may be targeted by axons originating in different regions of the cortex (Spratling 2002). This segregation of inputs may have functional significance and may impact the information-processing for these neurons. In addition, the primary apical dendrites observed in this study originate 50 - 100 μm from the cell body towards the pial surface, in contrast to the primary basal dendrites which originate near to the cell body. Our previous study observed a phenotype in apical but not basal dendrites. This analysis also showed that the absolute apical spine density in *fmr1* KO was highest in segments 3 through 5. In contrast the *dnPAK* transgenic mouse had the opposite phenotype – a decrease in spine density compared to WT mice – and it was most pronounced in apical segments 3 and 4. Crossing these mutant mouse lines resulted in a rescue of the dendritic spine density phenotype at all of these segments. For these reasons, we were particularly interested in analyzing the spine results from

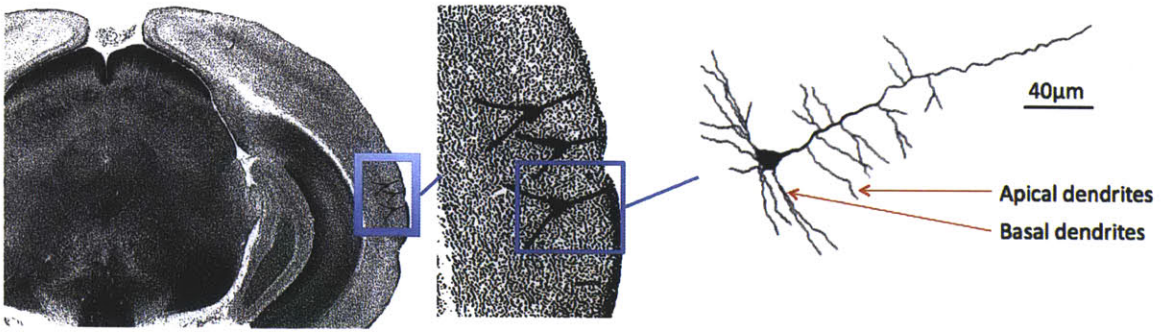

Figure 3-2: **Golgi Staining of Cortical Neurons.** Pyramidal neurons of the temporal cortex were identified in coronal slices of the brains of 11 week old adult WT and *fmr1* mice through comparison to temporal cortex association area A in Figures 62 - 67 of the mouse brain atlas (Franklin and Paxinos 1997). Golgi-stained layer II/III pyramidal neurons were traced with camera lucida. Apical and basal dendrites on layer II/III pyramidal neurons were divided into 10 μm segments, with segment 1 closest to the soma and segment 10 farthest from the soma, and the number of dendritic spines in each segment was counted.

segments 3, 4, and 5 on the apical dendrites.

3.3.2 Rescue of Dendritic Spine Phenotype in *Fmr1* KO by FRAX486.

To determine whether a single treatment or prolonged administration of FRAX486 could rescue the dendritic spine phenotype in *fmr1* KO, we delivered subcutaneous injections of 20 mg/kg FRAX486 to mice once or daily for 21 days and collected brain tissue 8 hours after the ultimate drug treatment. Golgi staining showed that in cortical neurons of *fmr1* KO mice, the mean density of apical dendritic spines was greater than in WT neurons in segment 3 (WT + veh vs. KO + veh: *, $p < 0.05$)(Figure 3-3). This spine phenotype was rescued by treatment with FRAX486 as early as 8 hours after a single treatment, as spine density was decreased to levels similar to WT mice but significantly lower than untreated *fmr1* KO mice (WT + veh vs. KO + drug: ns; KO + veh vs. KO + drug: ***, $p < 0.001$). Spine density was also decreased in segment 4 of neurons in *fmr1 KO* mice by FRAX486 treatment for 8 hours (KO + veh vs. KO + drug: *, $p < 0.05$). Rescue was also observed following

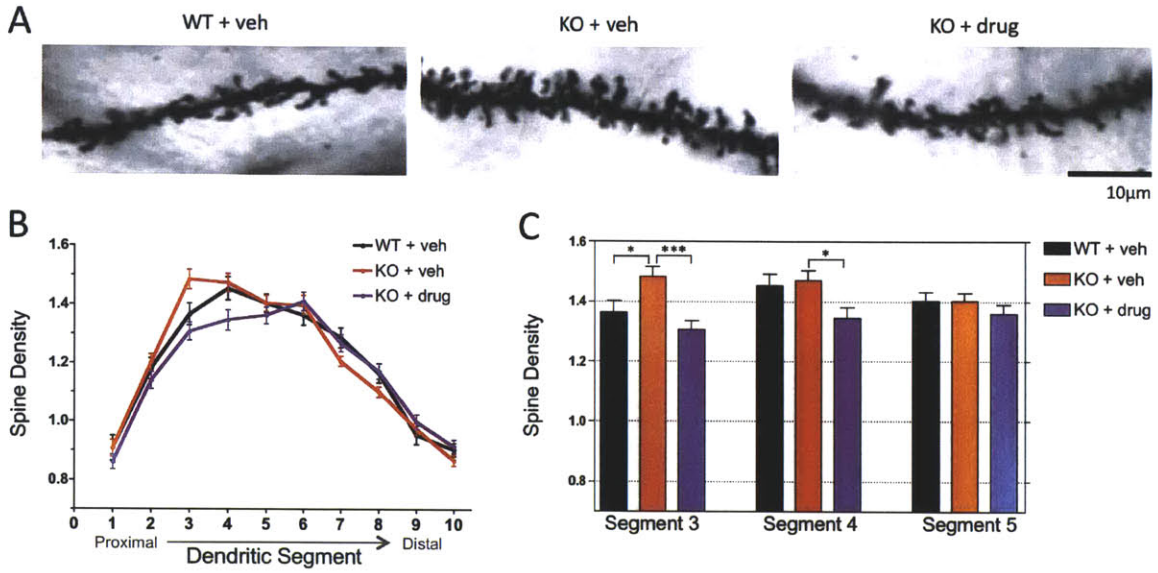

Figure 3-3: **FRAX486 Rescues Increased Density of Apical Dendritic Spines in *Fmr1* KO Mouse. (A) Representative apical dendritic segments** of layer II/III pyramidal neurons from WT + veh, *fmr1* KO + veh, and *fmr1* KO + drug, where FRAX486 was administered via a single subcutaneous injection 8 h prior to tissue collection. **(B) Dendrites were divided into 10 segments** of 10 μm each based on distance from the soma (proximal to distal, left to right). A phenotype was observed in *fmr1* KO + veh compared to WT + veh control. This phenotype was rescued by treatment with FRAX486. **(C) Statistical analysis of data from segments 3, 4, and 5.** Spine density was significantly higher in *fmr1* KO + veh compared to WT + veh control in segment 3. Drug treatment decreased spine density in *fmr1* KO such that the density in *fmr1* KO + drug was significantly less than that of *fmr1* KO + veh in segments 3 and 4 but similar to WT + veh in all segments. No differences were observed in segment 5.

WT + veh (8 h)(n = 40 neurons; 4 mice), *fmr1* KO + veh (8 h)(n = 60 neurons; 6 mice), *fmr1* KO + drug (8 h)(n = 60 neurons; 6 mice). One-way ANOVA comparing all groups for each segment: segment 3, $p = 0.0004$; segment 4, $p = 0.0193$; segment 5, $p = 0.4980$. Results of Tukey multiple comparison posttest comparing 2 genotype/treatment groups at each segment are represented as * on graph (C): *, $p < 0.05$; ***, $p < 0.001$.

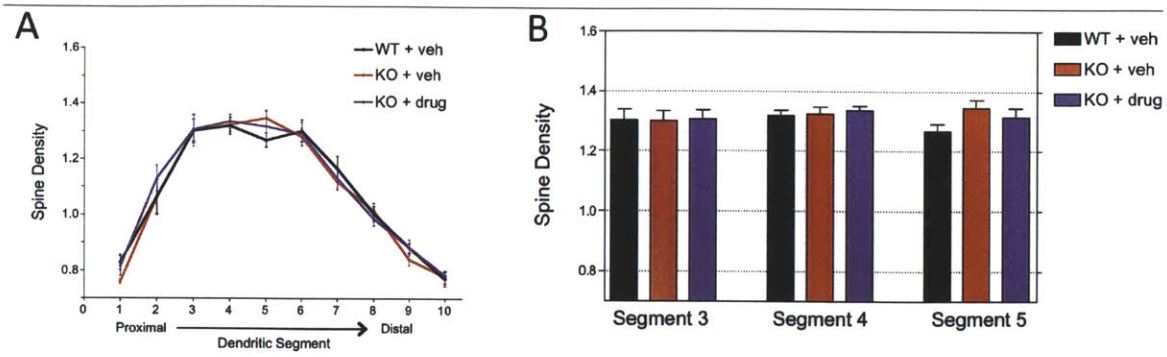

Figure 3-4: **Basal Spine Density Is Not Abnormal in *Fmr1* KO Mouse.**
(A) Basal dendritic segments of layer II/III pyramidal neurons from WT + veh, *fmr1* KO + veh, and *fmr1* KO + drug, where FRAX486 was administered via a single subcutaneous injection 8 h prior to tissue collection. Dendrites were divided into 10 segments of 10 μm each based on distance from the soma (proximal to distal, left to right).
(B) Statistical analysis of data from segments 3, 4, and 5 shows no difference between genotype and treatment groups.

WT + veh (8 h)(n = 40 neurons; 4 mice), *fmr1* KO + veh (8 h)(n = 60 neurons; 6 mice), *fmr1* KO + drug (8 h)(n = 60 neurons; 6 mice). One-way ANOVA comparing all groups for each segment: segment 3, p = 0.9929; segment 4, p = 0.8300; segment 5, p = 0.1211.

prolonged administration of FRAX486. Following 21 days of treatment with PAK inhibitors, the spine density phenotype was rescued in segment 3 (WT + veh vs. KO + drug (21 d): ns, $p > 0.05$; KO + veh vs. KO + drug (21 d): **, $p < 0.01$) and segment 4 (WT + veh vs. KO + drug (21 d): ns, $p > 0.05$; KO + veh vs. KO + drug (21 d): *, $p < 0.05$)(data not shown). To our surprise, there was no statistical difference between 8 hours or 21 days of treatment with FRAX486, suggesting that maximum rescue can occur rapidly (KO + drug (8 h) vs. KO + drug (21 d): ns, $p > 0.05$). In summary, the small molecule PAK inhibitor FRAX486 ameliorated spine abnormalities in the mouse model of FXS.

Consistent with our previous publication, a spine density phenotype was not observed in the first 100 μm of primary basal dendrites (Figure 3-4). In addition, FRAX486 did not impact spine density in these basal dendrites in *fmr1* KO or WT cortical neurons (Figure 3-4 and Figure 3-5).

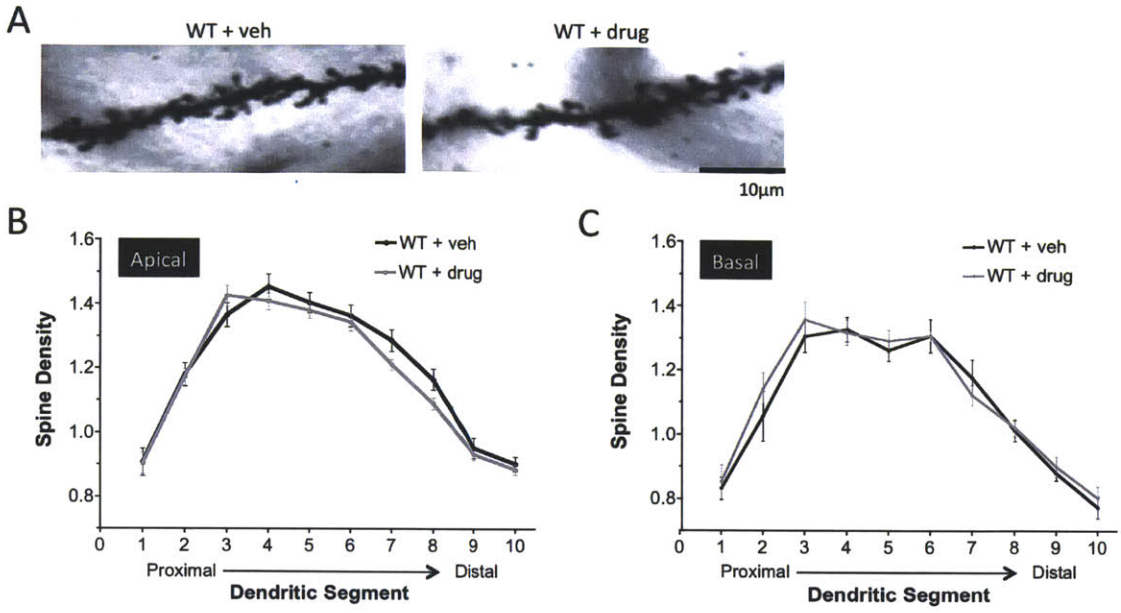

Figure 3-5: **FRAX486 Has No Effect on Spine Density in Control Mice.** **(A) Representative apical dendritic segments** of layer II/III pyramidal neurons from WT mice treated with vehicle control or FRAX486, where drug was administered daily for 21 days. **(B) Apical dendrites.** No difference in spine density was observed in any of the 10 dendritic segments. **(C) Basal dendrites.** No difference in spine density was observed in any of the 10 dendritic segments.

WT + veh (1 d)(n = 40 neurons; 4 mice), WT + drug (21 d) (n = 50 neurons; 5 mice).

3.3.3 Spine Density Does Not Change in WT Mice Treated with FRAX486.

To determine whether the impact of a small molecule PAK inhibitor was specific to the abnormally excessive spine density in *fmr1* KO cortex or could decrease even healthy levels of dendritic spines in normal mice, we tested FRAX486 on WT mice. WT littermates received subcutaneous injections of 20 mg/kg FRAX486 for 21 days. The kinase inhibitor FRAX486 did not alter dendritic spine density in pyramidal neurons in layer II/III of the temporal cortex of WT mice (WT + veh vs. WT + drug: ns, $p > 0.05$)(Figure 3-5). These finding support the notion that FRAX486 is specifically rescuing the *fmr1* KO deficit and not simply decreasing spine density irrespective of genotype.

3.4 Discussion

There is increasing evidence that cortical dendritic spine deficits are the common, underlying cause of abnormal cognitive or behavioral symptoms in multiple diseases. Based on this, we believe that rescuing the dendritic spine deficit is key to treating the cognitive and behavioral symptoms in FXS, and may be broadly applicable to mental retardation, autism spectrum disorders, as well as seizures of unknown etiology. Toward this end, in this thesis work we demonstrate that *in vivo* a single injection or prolonged treatment of adult *fmr1* KO mice with the PAK inhibitor FRAX486 is sufficient to reverse the spine density phenotype. We propose that small molecule PAK inhibitors like FRAX486 may reverse the spine abnormality in humans with FXS too.

3.4.1 Dendritic Spines in Temporal Cortex.

Our studies focused on the spine density in the mouse temporal cortex. This cortical area was selected for a number of reasons. First, our previous studies in B6 mice demonstrated that *fmr1* KO mice have a spine phenotype in this cortical region and that it could be reversed (Hayashi et al. 2007). Second, an LTP phenotype which is consistent with the spine abnormalities has been observed in *fmr1* KO mice on the B6 background strain (Hayashi et al. 2007). Third, humans with autism show spine density increases in the most cortical layers in this region of the cortex (Hutsler and Zhang 2010). Fourth, the temporal cortex is an area of the brain involved in the sensation of sound, processing speech and language, object recognition, face recognition, and emotional recognition as well as the associated social-emotional functioning (Baron-Cohen et al. 1999)(Critchley et al. 2000). As autism is a disorder characterized by language delays and social deficits, it is not surprising that the temporal cortex may have anatomical abnormalities. Three studies in children with autism found that cerebral blood flow in the temporal lobe is inversely proportional to the severity of autism symptoms (Gendry Meresse et al. 2005)(Ohnishi et al. 2000)(Zilbovicius et al. 2000). Furthermore, seizures in *fmr1* KO mice – the subject of the

next chapter – are induced by intense sounds. Finally, historically, reports have used similar Golgi staining techniques to observe changes in rats in dendritic branching in the same cortical region analyzed in this thesis – the temporal cortex – as a result of rearing in a complex environment (Greenough et al. 1973).

However, *in vivo* morphological plasticity has also been demonstrated in other brain regions. For example, rearing in enriched environments is correlated with plasticity in the primary somatosensory cortex (Coq and Xerri 1998), hippocampus (Fiala et al. 1978)(Moser et al. 1994)(Rampon et al. 2000), amygdala (Nikolaev et al. 2002), striatum (Comery et al. 1995), and the cerebellum (Greenough et al. 1986). Stressful environments – like chronic restraint for 21 days – can also induce changes, increasing dendritic complexity in the basolateral amygdala (Johnson et al. 2009). Thus, if we analyzed spine morphology in other cortical regions of *fmr1* KO mice treated with vehicle or FRAX486 we would expect to get equally interesting results. In addition, while we did not see a change in spine density in the WT mice treated with drug, we cannot rule out the possibility that PAK inhibitors like FRAX486 may decrease spine density in other brain regions in WT mice.

3.4.2 Counting Spines and Measuring Spine Length.

While spine density is a common metric for spine analysis following Golgi staining of cortical neurons, so is spine morphology. While the mouse model of FXS displays spines of increased length, expression of *dnPAK* in otherwise WT mice leads to decreases in spine length (Hayashi et al. 2007). By crossing these mice in our genetic rescue study, we demonstrated that the spine length abnormalities canceled one another out. That is, inhibition of PAK in the forebrain of *fmr1* KO mice was sufficient to rescue the spine length phenotype to levels indistinguishable from WT. In this way, it would be very interesting to continue the analysis of spines in our dataset to include measurements of spine length in drug-treated *fmr1* KO mice. This metric was part of the original experimental design but was not completed by our collaborator, presumably because it is a labor intensity process. Additionally, we do not know whether an effect of FRAX486 in WT mice would be evident if spine length

was compared.

Our observation of increases in spine density in *fmr1* KO mice and a rescue by PAK inhibition is consistent with Greenough's hypothesis that a spine pruning deficit is responsible for the FXS-like phenotypes in *fmr1* KO mice. However, as we did not analyze spine shape, we cannot comment on the second hypothesis proposed in the introductory section of this chapter: filopodia fail to mature into spines in the absence of FMRP.

3.4.3 Is There a Developmental Window for Spine Rescue?

Our analysis was conducted on the brains of 11 week old mice. Some of these mice received daily injections starting at 8 weeks old, while others received a single injection 8 hours before tissue colection. While many of the previously reported studies were conducted in neonatal or juvenile mice or rats (Greenough et al. 1973)(Fiala et al. 1978), a few report structural plasticity in adult rodents. In particular, there is precedent for spine and dendrite changes in adult rats in noncortical brain regions, such as the hippocampus and amygdala. The stress study mentioned above – demonstrating chronic stress increases dendritic complexity in pyramidal neurons of the basolateral amygdala – was conducted in adult rats (Johnson et al. 2009). Interesting, this stress-induced change in dendrite architecture could be averted by treatment with lithium, a mood stabilizing drug for bipolar disorder thought to have neuroprotective powers. In this way, there is precedent for a pharmacological treatment preventing dendritic abnormalities associated with a disease state.

Next, in the paper describing the CA1 region of the hippocampus, spine density was significantly increased on pyramidal neurons after 14 - 30 days of spatial training of adult rats in a hippocampal-dependent task (Moser et al. 1994). In this way there is precedent for spine changes in adult rodent pyramidal neurons *in vivo*, as well as pharmacological prevention of stress-induced dendritic remodeling. Our finding – that FRAX486 reverses the spine density phenotype in adult *fmr1* KO mice (8 - 11 weeks old) – is also promising, as it suggests that treatment of FXS patients may be possible even into adulthood. That is, there is not a developmental window in which

treatment must occur in order to ameliorate FXS symptoms and therefore improve quality of life by rescuing dendritic spine abnormalities.

3.4.4 Rapid Reversal of Spine Abnormalities.

Our data suggest that when spine density is pathologically high, spine elimination can happen rapidly. This is a novel finding *in vivo*, as the Golgi studies mentioned previously examined the dendrites of mice after 14, 21, or 30 days of exposure to novel environments or chronic stress but not look at earlier time points. This is mainly because of practical limitations due to the tedious nature of Golgi staining – a procedure which in which brains are treated in successive staining solutions for 2 - 4 months – and spine counting – hand counting of tens of thousands of dendritic protrusions – which has limited the number of time points and/or conditions that could be compared in a single experiment. Novel techniques for staining and counting are under development at Afraxis which would allow us to conduct a thorough study of the time course of spine density and morphology changes following *in vivo* administration of FRAX486 in addition to novel PAK inhibitors currently under development. It would be fascinating to use these new techniques to track the rate of reversal of the spine phenotype and compare it to the rate of rescue of the behavioral symptoms. This would provide further evidence about whether structural plasticity causes, is caused by, or is simply correlated to changes in behavior.

While these limitations exist in *in vivo* studies, *in vitro* preparations have been used to demonstrate that rapid activity-dependent changes can occur in dendritic structure. Organotypic cultured hippocampal slices from rat brain and 2-photon imaging of CA1 neurons expressing enhanced green fluorescent protein have been used to demonstrate changes in the density of protrusions on apical dendrites (Maletic-Savatic et al. 1999). Increases in protrusion density were significant as early as 20 minutes after tetanic stimulation. By 40 minutes, the total density of protrusions had increased by 19 +/- 4%. Authors also characterized the mechanism of this spine density increase, demonstrating that it is NMDA receptor-dependent and input specific, occurring only close to activated parts of the dendrite. While many of these

new filopodia may be transient, 27% of the new protrusions developed a bulbous head within 1 hour, suggesting they may be precursors of mature spines (Maletic-Savatic et al. 1999). In this way, rapid changes in spine density on pyramidal neurons have been reported in cultured neurons (Ziv and Smith 1996)(Maletic-Savatic et al. 1999).

3.4.5 Spine Deficit in Genetic verses Pharmacological Rescue Strategies.

While the goal of our study was to rescue the excessive spine density phenotype in the mouse model of FXS, it is also interesting to learn how inhibition of PAK impacts dendritic spines in WT mice. Through the generation of a targeted genetic tool – the forebrain-specific *dnPAK* TG mouse that targets all three group I PAKs – previous scientists in our laboratory were able to knockdown PAK1 activity by ~62% and ~59% of WT levels in the cortex and hippocampus, respectively, of B6 mice and observe the impact on dendritic spines (Hayashi et al. 2004). This study focused on pyramidal neurons in two regions of the brain – the CA1 area of the hippocampus and layer II/III of the cortex – as their synaptic plasticity and dendritic spine morphology has been extensively studied (Diamond et al. 1994)(Tsien et al. 1996)(Trachtenberg et al. 2000). In the hippocampus, there was no significant difference in spine density, dendrite length, or the number of dendritic branch points in the *dnPAK* TG mouse compared to control mice. However, in the temporal cortex, mean spine density was decreased by ~22% compared to WT mice. The lack of a phenotype in the hippocampus and the significant phenotype in the cortex are interesting, given that PAK activity was decreased by approximately the same amount in these two brain regions but consistent with the plasticity phenotypes observed in slice electrophysiology experiments. Hayashi et al. reasoned that the necessary threshold for PAK activity may be higher in the cortex than in the hippocampus for normal neuronal function. Interestingly, this mouse exhibited behavioral deficits consistent with predictions given the regional specificity of the spine and plasticity phenotypes. Behavioral deficits did not occur in the early stages of hippocampal-dependent learning tasks, but rather

in the consolidation/retention phases when the memory is presumably transferred to the cortex. For future studies of morphology and synaptic plasticity involving the inhibition of PAK, we decided to focus on the region already known to be affected: the temporal cortex.

Given the finding that a genetic inhibition strategy that knocks down the activity of PAK decreases spine density in otherwise WT mice, we were initially surprised to discover that our small molecule PAK inhibitor did not affect spine density in the temporal cortex of WT mice, even when mice were treated for 21 days. To understand why this might be the case, consider three ways our pharmacological strategy differs from the genetic strategy.

First, the length of PAK inhibition *in vivo* was not carefully controlled in the *dnPAK* mouse and therefore may have been different than the subcutaneous injections in the pharmacological case. By 8 weeks of age, PAK is inhibited by ~59% in the *dnPAK* cortex, but expression of the transgene may occur up to 4 weeks prior to that. To test if additional time is required to impact spine density in WT mice, future studies should treat with FRAX486 for more than 3 weeks.

Second, the experiments were conducted on different mouse strains. The background strain of mice has significant effects on behavior, morphology, and physiology (Spencer et al. 2011). Future studies with small molecule PAK inhibitors could address this concern by treating WT and *fmr1* KO mice on both B6 and FVB backgrounds.

Finally, while the *dnPAK* transgene is highly specific for group I PAKs, binding to the catalytic domain and blocking autophosphorylation and the subsequent activation of kinase activity, pharmacological approaches are inherently more complicated, as almost all kinase inhibitors target the ATP binding pocket which is highly conserved among kinases. In this way, we cannot rule out the possibility that the inhibition of a kinase other than PAK is responsible for protecting spine density in WT mice. Replicating these experiments with additional drugs that inhibit PAK, but do not inhibit other targets of FRAX486, would provide stronger support of the hypothesis that the inhibition of PAK kinase activity is sufficient to rescue deficits in the *fmr1*

KO mouse.

On this note, whether it is through PAK or another kinase that FRAX486 acts in the mouse brain, the data in this chapter demonstrate that FRAX486 reverses the spine density phenotype in the *fmr1* KO mouse. Now we can go forward and test whether a drug treatment that rescues the spine phenotype is sufficient to ameliorate seizures, hyperactivity, and other abnormal behaviors in the mouse model of FXS.

3.5 Materials and Methods

3.5.1 Drug Delivery.

The powder FRAX486 (Afraxis) was dissolved in 20% hydroxypropyl-β-cyclodextrin vehicle (Sigma-Aldrich). A 2 mg/ml solution of drug or vehicle alone control was administered via subcutaneous injection at 20 mg/kg in a volume proportional to the animals weight (typically 300 - 400 μl). Daily injections were given every 24 h for 21 days with the ultimate injection administered 8 h prior to tissue collection.

3.5.2 Golgi Analysis.

The brains of drug treated 11 week old littermates were prepared and analyzed via the Golgi-Cox technique. Dendrites of layer II/III pyramidal neurons of the temporal cortex were analyzed in 10 consecutive 10 μm segments to quantify spine density. WT + veh (1 d)(n = 40 neurons; 4 mice), WT + drug (21 d)(n = 50 neurons; 5 mice); KO + veh (1 d)(n = 60 neurons; 6 mice), KO + drug (1 d)(n = 60 neurons; 6 mice); KO + drug (21 d)(n = 60 neurons; 6 mice).

Chapter 4

Rescue of Audiogenic Seizures in *Fmr1* KO Mice by FRAX486.

Contributions

The work described in this chapter was performed by Bridget M. Dolan.

4.1 Summary

Seizures occur in conjunction with Fragile X Syndrome (FXS) and autism in up to a quarter of children with these disorders. Increased susceptibility to audiogenic seizures (AGS) is a robust and reliable phenotype in the *fragile X mental retardation 1* knockout (*fmr1* KO) mouse. This mouse provides an opportunity to explore mechanisms of seizures and neuronal hyperactivity in a disorder for which the molecular pathophysiology is unique and specific, and therefore is considered a valuable animal model of epilepsy and seizures. While the pathogenesis of seizures in FXS is unknown, we hypothesize that it is related to the excess of dendritic spines observed in both humans and mice with FXS. To test whether rescue of the spine density phenotype is sufficient to prevent seizures, we treated *fmr1* KO mice with a kinase inhibitor that reverses the morphological phenotype. Here we tested whether brief or prolonged treatment with FRAX486 is sufficient to rescue the AGS susceptibility in *fmr1* KO mice. Our results demonstrate that both single and daily injections of FRAX486 reduce AGS susceptibility, and that rescue is dose-dependent.

4.2 Introduction

4.2.1 Seizures in FXS Humans.

Epilepsy, a chronic disorder characterized by seizures, is the most common neurological abnormality in FXS (Hagerman and Hagerman 2002). While epilepsy is not widespread in other kinds of intellectual disability, it affects many males with FXS and other types of autism. Studies report 13 - 18% of FXS individuals and 25% of autistic individuals have seizures (Berry-Kravis 2002)(Hara 2007). Manifestation of seizures is age-dependent and usually dissipates after puberty (Hagerman and Stafstrom 2009). However, in a study of 168 FXS patients, 17% had seizures and 25% of this fraction continued to have seizures in adulthood (Musumeci et al. 1999). In this way, seizures are often but not always age-dependent in FXS humans.

Of those FXS boys with seizures, nearly half experience generalized tonic-clonic

seizures, formerly known as grand mal seizures (Musumeci et al. 1999). Tonic-clonic seizures are serious and involve loss of consciousness, tensing of skeletal muscles, and the rapid muscle contraction and relaxation characteristic of convulsions. More commonly, epileptic episodes involve partial seizures which originate in one region of the brain. In simple partial seizures – seen in 25% of FXS seizure patients – the seizures remain localized, and the individual may experience changes in sensations, motor control, or ability to speak without loss of consciousness. In complex partial seizures – seen in 89% of FXS seizure patients – consciousness is also affected (Musumeci et al. 1999). Such seizures have the potential to be devastating.

The pathogenesis of this symptom of FXS is unknown, however it is interesting to note that sub-threshold abnormal brain activity has also been observed in individuals with FXS. Electroencephalographic (EEG) recordings have found spike wave discharges in the centrotemporal regions of the brain in three quarters of FXS individuals with seizures and one quarter of FXS individuals who have not displayed obvious seizures (Berry-Kravis 2002)(Musumeci et al. 1999). The spike pattern on EEG can be useful in identifying children at high risk for seizures and in counseling families regarding possible seizure likelihood and anticonvulsant medication management.

Interestingly, increases in cortical excitability have been observed by EEG in the somatosensory and auditory cortices of FXS patients (Ferri et al. 1994)(Hagerman et al. 1999). This is consistent with the hypersensitivity to auditory, visual, or tactile input which sometimes leads to defensive actions designed to avoid what some FXS and autism patients experience as painful stimuli. This sensory defensiveness is hypothesized to be the underlying cause of anxiety, hyperactivity, and impulsiveness – behavioral states which drive patients to social isolation and exacerbate social interaction and communication problems (Hagerman and Hagerman 2002)(Miller et al. 1999). In this way, a therapy that ameliorates abnormal brain activity could treat multiple autism-related symptoms.

4.2.2 Seizures in the Mouse Model of FXS.

Seizures Are Induced by Loud Auditory Stimuli in *Fmr1* KO Mice

The quest for a treatment for seizures and sensory integration abnormalities would benefit from a relevant animal model. Consistent with the enhanced sensitivity to auditory, visual, or tactile stimulation in FXS patients, *fmr1* KO mice display increased responsiveness to auditory stimuli, observed as increases in the amplitude of sound-induced responses in the brain (Rojas et al. 2001)(reviewed in (Chen and Toth 2001)). Similarily, a robust and reliable phenotype in the *fmr1* KO mouse is increased susceptibility to AGS (Musumeci et al. 2007). These seizures are specifically induced by intense auditory stimuli, as *fmr1* KO mice are not more sensitive to chemical convulsants (bicuculline, PTZ or kainic acid) than WT mice, and handling alone does not induce seizures (Chen and Toth 2001). In the mouse model, seizures are generalized, convulsive (tonic-clonic), complex seizures evoked by a loud auditory stimulus (siren, bell, or white noise of 115-125 dB).

Multiple Brain Regions Are Activated by AGS

Examination of the rodent brain following seizures has shed some light onto areas of the brain involved in seizure activity. In AGS-susceptible Wistar rats, a well studied animal model for the genetically-based seizure susceptibility, considerable evidence indicates that activation of the inferior colliculus (ICc) is required for the initiation of AGS (Raisinghani and Faingold 2003). This is not surprising given that the ICc is the principal midbrain nucleus of the auditory pathway and a relay station for almost all ascending and descending auditory information (Shore 2009). To compare the brain regions activated by an auditory stimulus in mice with and without a seizure response, Dr. Chen and Dr. Toth examined expression of the immediate early gene (IEG) c-Fos, as the regional distribution of IEGs can indicate the specific structures involved in seizure propagation (Chen and Toth 2001). While ICc was indeed activated in mice that experienced seizures, it also showed c-Fos expression in WT mice that did not have a seizure response. In contrast, the dorsal nucleus of lateral lemniscus

(DNLL) of the brainstem and in the posterior intralaminar nucleus (PIL) of the thalamus showed c-Fos increases in *fmr1* KO mice but not WT mice. In summary, the brainstem, midbrain, and thalamus are all activated by AGS, but only the brainstem nucleus DNLL and the thalamic nucleus PIL were specifically activated in mice that experience seizures.

Complex Factors – Some Genetic – Impact the Age of AGS Susceptibility

A robust AGS phenotype has been reported in *fmr1* KO mice compared to WT litermates on multiple background strains (Chen and Toth 2001)(Yan et al. 2004)(Musumeci et al. 2007)(Dolen et al. 2007). The degree of seizure susceptibility, however, varies by background strain and also by age. On the C57BL6 (B6) background, seizure susceptibility is limited to a short time window in development: 19 - 21 days after birth (Chen and Toth 2001)(and data not shown). During this three day period, 72.2% of B6 *fmr1* KO mice have tonic-clonic seizures compared to 0% of their WT litermates (Dolen et al. 2007). The age dependence of seizures is considerably broader in the FVB.129P2 and FVB/NJ background strains and in the C57BL/6J x FVB/NJ F1 hybrid (Yan et al. 2005) (Chen and Toth 2001). The Bauchwitz laboratory found that seizures were induced in FVB/NJ *fmr1* KO mice as young as 14 days postnatal and as old as 13 weeks, however by 22 weeks of age, seizures were no longer induced in their AGS assay (125 dB; 1.8 - 6.3 kHz)(Yan et al. 2005). In contrast, Dr. Chen and Dr. Toth did not observe convulsions before 10 weeks of age in *fmr1* KO mice on the FVB/NJ or FVB.129P strains (115 dB, 2 - 20 kHz)(Chen and Toth 2001). At 10 - 12 weeks, 57.7% of *fmr1* KO mice displayed seizures, and at 20 - 34 weeks, 70% of *fmr1* KO mice were affected.

In addition, in these background strains the precise time window of susceptibility varies depending on the type of noise (siren, bell or white noise), the intensity of the stimulus (115, 120 or 125 dB), and the laboratory in which the experiments were conducted. Chen and Toth used a white noise of 115 dB on the FVB.129P2 and FVB/NJ backgrounds and reported that seizures did not develop before 10 weeks of age, peaked from 20 - 34 weeks of age, and never occurred in WT control mice (Chen

and Toth 2001). The Bauchwitz laboratory used a siren of 125 dB on the FVB/NJ background and reported that seizures occurred as early as 14 days postnatal, peaked at 21 days postnatal, stopped by 21 weeks of age, and occurred in 18% of WT mice (Yan et al. 2004). Musucemi et al. used a bell of 120 dB on an unspecified FVB background strain and reported that seizures occurred at all of the ages tested (17, 22, 35, 45, and 60 days), seizure incidence varied by age (15.4%, 55.0%, 50.0%, 63.6%, and 58.3%, respectively), and WT litermates occasionally suffered from seizures (0%, 36.8%, 4.2%, 0%, and 7.3%, respectively)(Musumeci et al. 2007). In this way, it is important to determine the age-dependent seizure incidence for each protocol and mouse strain.

4.2.3 Investigations into the Underlying Mechanisms of AGS.

The mechanisms by which the absence of expression of the protein product of the *fmr1* gene, the fragile X mental retardation protein (FMRP), leads to increased susceptibility to sound-induced seizures are only beginning to be understood. Interestingly, the AGS phenotype seems to be extremely and specifically sensitive to the re-introduction of FMRP via the human *fmr1* transgene. In *fmr1* KO mice with additional copies of the human *fmr1* gene or *fmr1* cDNA, the epileptic phenotype is partially or completely rescued, respectively (Musumeci et al. 2007). This is interesting given the time course of FMRP expression in the neonatal hippocampus where protein levels are high at postnatal days 4, 7, and 14 but decrease by postnatal day 28. FMRP expression may be required for normal brain function – including prevention of AGS – in the first 3 weeks of life, when seizure susceptibility is highest in B6 *fmr1* KO mice.

Seizures and Signaling Through mGluRs and $GABA_A$Rs

Investigations of related signaling pathways have sought to increase our understanding of the underlying mechanism. First, in support of the metabotropic glutamate receptor (mGluR) theory of FXS, reduction in mGluR5 expression is sufficient to rescue

the seizure phenotype (Dolen et al. 2007). Similarly, antagonists of mGluR5 reduce seizure incidence in a dose-dependent fashion (Yan et al. 2005). Second, alterations in the excitatory/inhibitory balance have been reported in *fmr1* KO mice. In layer IV neurons of the neocortex of *fmr1* KO mice, a decrease in excitatory drive onto inhibitory neurons and increased intrinsic excitability of excitatory neurons lead to hyperexcitable circuits (Gibson et al. 2008). Additionally, downregulation of GABA receptor subunits has been reported at both the mRNA and protein level in *fmr1* KO neurons, resulting in increased excitability in the mouse hippocampal and neocortical circuitry (reviewed in (Hagerman et al. 2009)). In support of this, an allosteric modulator of the $GABA_A$ receptor – ganaxolone – has delivered promising results in pediatric and adolescent patients with refractory epilepsy, though to our knowledge it has not been tested in FXS humans or *fmr1* KO mice (Pieribone et al. 2007).

Spine Density and Seizure Susceptibility

Finally, the dendritic spine abnormalities characteristic of humans and mice with FXS may contribute to seizure susceptibility. Excessive numbers of dendritic spines may lead to excessive neuronal excitation and spiking (Vieregge and Froster-Iskenius 1989). In support of this, the developmental time course of the seizure phenotype in *fmr1* KO mice on the B6 background mirrors the spine density phenotype in the cortex. That is, spine length and density increases are most pronounced in neonatal *fmr1* KO neurons, but disappear by 4 weeks of age (Nimchinsky et al. 2001). This spine phenotype time course is controversial, however, and density and morphology phenotypes have also been reported in adult *fmr1* KO mice (Comery et al. 1997)(Irwin et al. 2002)(Hayashi et al. 2007).

In this thesis we set out to test the hypothesis that amelioration of the dendritic spine phenotype through inhibition of the PAK signaling pathway may prevent the AGS phenotype in *fmr1* KO mice. This is the first time we were able to test this hypothesis, as our genetic strategy was not applicable since the *dnPAK* transgene was on the B6 background strain and was not expressed until the window of seizure susceptibility in this strain had passed.

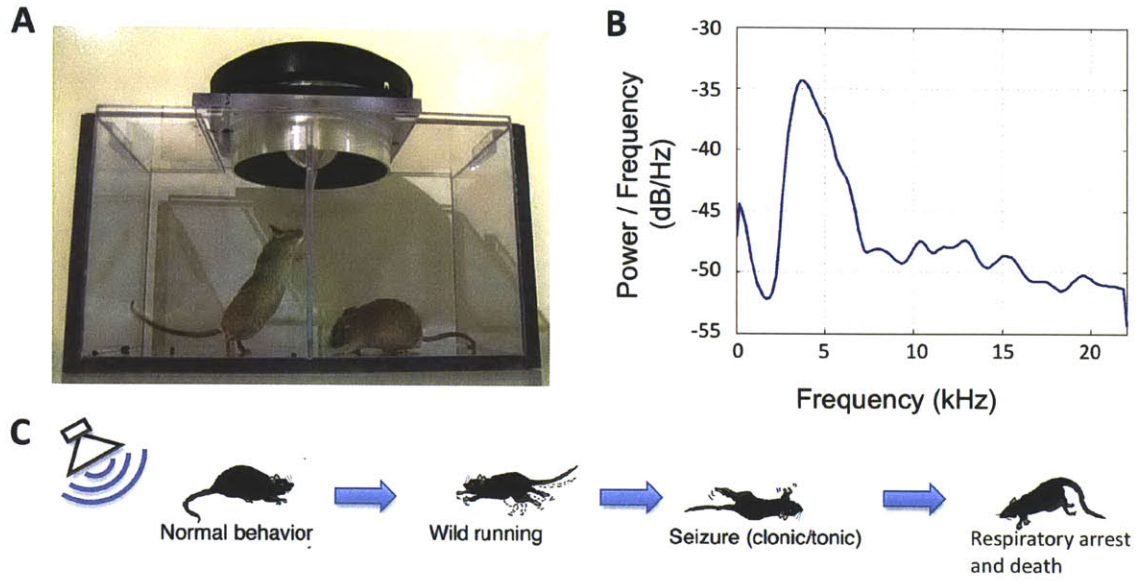

Figure 4-1: **Audiogenic Seizure Assay.** (A) A clear acrylic box with two identical square chambers (13 cm x 13 cm x 16 cm) divided by a clear partition was designed for the AGS assay. The auditory stimulus (124 dB siren, modeled after the RadioShack Personal Alarm used in (Dolen et al. 2007) was played by a speaker in the top of the box. A sound meter was used each day to confirm the intensity of the stimulus. (B) Peak frequency spectrum for auditory stimulus. (C) *Fmr1* KO mice have a sequential seizure response to a loud auditory stimulus. Later phases never occur without preceding phases. First, there is a phase of wild running and jumping which generally occurs 30 - 40 s from the onset of the siren and lasts about 10 - 15 s. Wild running is followed by clonic convulsions, in which the mouse falls on its side and its extremities twitch, and tonic hindlimb tensing and extension (the clonic and tonic phases together are know as tonic-clonic seizures). Finally, while some *fmr1* KO mice recover, for many, respiratory arrest leads to death.
Cartoon modified from (Raisinghani and Faingold 2005).

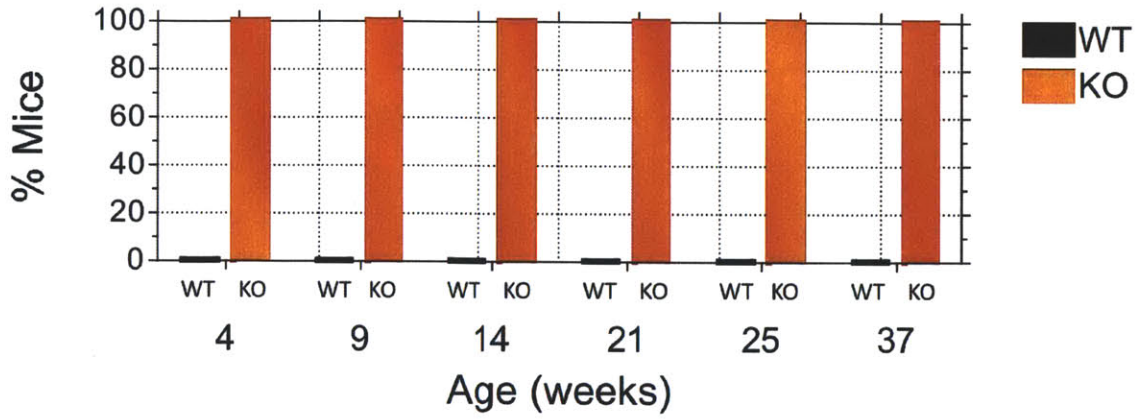

Figure 4-2: **Audiogenic Seizures Are Not Age-Dependent in *Fmr1* KO Mice of the FVB.129P2 Background Strain.** Regardless of age, *fmr1* KO mice had AGS in response to a 124 dB siren. In contrast, WT mice never displayed a seizure phenotype.

4.3 Results

We took advantage of the robust sensitivity to AGS in the *fmr1* KO mouse to test the hypothesis that pharmacological inhibition of PAK can rescue an autism-related phenotype in an animal model of the disease. To do so, we constructed a clear, two-chambered apparatus and generated a loud siren-like noise following protocols from the laboratories of Dr. Robert Bauchwitz and Dr. Mark Bear and described in detail in Figure 4-1 (Yan et al. 2005)(Dolen et al. 2007). Briefly, two male mice were put in separate chambers of a clear acrylic box. After a 2 min acclimation period, a 124 dB siren was played from a speaker in the ceiling of the box. The siren's frequency profile is plotted in Figure 4-1B. *Fmr1* KO mice, but not WT mice, exhibited wild running, tonic-clonic seizures, and often respiratory arrest leading to death. To determine whether acute or prolonged treatment with FRAX486 could rescue AGS phenotypes, we treated mice with vehicle, a single injection of FRAX486, or 5 daily injections of FRAX486, and assayed seizure susceptibility 2 or 8 h following the ultimate injection. The results are summarized in Figure 4-3 and described in the subsequent sections.

Genotype	Days (d)	Time (h)	FRAX486 (mg/kg)	n	Wild Running			Seizure			Death		
					%	p	χ^2	%	p	χ^2	%	p	χ^2
KO	1		0	15	100			100			53		
		2	10	13	92	0.137	1.20	92	0.137	1.20	15	0.0183*	4.37
			20	10	100	1	NA	90	0.106	1.56	0	0.0026**	7.84
			30	12	17	<0.0001***	19.85	17	<0.0001***	23.20	0	0.0013**	9.10
		8	10	15	80	0.0339*	3.33	80	0.0339*	3.33	13	0.0101*	5.40
			20	12	25	<0.0001***	16.88	25	<0.0001***	16.88	0	0.0013**	9.10
			30	5	0	<0.0001***	20.00	0	<0.0001***	20.00	0	0.0175*	4.44
	5		0	27	100			96			44		
		2	10	13	100	1	NA	100	0.2411	0.49	54	0.2885	0.31
			20	32	81	0.0088**	5.64	81	0.0088**	5.64	34	0.2147	0.62
		8	10	4	75	0.0041**	6.88	50	0.002**	8.54	25	0.231	0.54
			20	26	54	<0.0001***	16.11	42	<0.0001***	18.32	19	.0247*	3.87
WT			0	37	0	<0.0001***	79.00	0	<0.0001***	75.09	0	<0.0001***	23.59

Figure 4-3: **Incidence of Audiogenic Seizures in *Fmr1* KO Mice Treated with FRAX486 or Vehicle Control and WT Mice.** *Fmr1* KO mice have a sequential seizure response to a loud auditory stimulus. There is an early phase of wild running and jumping, followed by clonic seizures, tonic hindlimb extension, and finally respiratory arrest and death. WT mice do not have audiogenic seizures (AGS). This table is a summary of the data presented in all subsequent figures.

Days = number of daily injections; Time = delay between ultimate injection and assay; n = number of mice; % = percentage of animals; p value = *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; χ^2 test was used to compare incidence in KO + veh (i.e. 0 mg/kg) and KO + FRAX486 treated for 1 or 5 d, and KO + veh and WT (includes n = 11 veh injected and n = 26 not injected); NA (not applicable), as χ^2 test is impossible when both values are 100%.

4.3.1 The Seizure Phenotype Is Not Age-Dependent in *Fmr1* KO Mice.

Since the AGS phenotype is most reliable on the FVB strains, we used FVB.129P2-*fmr1*tm1Cgr (*fmr1*KO) and the WT controls derived from this mutant from Jackson Labs for our experiments. First we conducted experiments with mice of different ages to determine the developmental profile of AGS susceptibility with our experimental conditions. Our results demonstrate that all *fmr1* KO mice displayed wild running and tonic-clonic seizures at all ages tested (4, 9, 14, 21, 25 and 37 weeks of age) (Figure 4-2). In contrast, WT mice were not susceptible to audiogenic seizures. Although WT mice, like *fmr1* KO mice, exhibited a startle response to the onset of the noise, these control mice never exhibited wild running, seizure, or respiratory arrest. In this way, our AGS set-up provided robust and reliable data that clearly distinguished between mutant and control mice between 4 and 37 weeks of age.

4.3.2 AGS Severity in *Fmr1* KO Mice Is Reduced by Brief Treatment with FRAX486.

To test whether a brief exposure to the PAK inhibitor FRAX486 is sufficient to rescue some or all of the phases of AGS, we injected *fmr1* KO mice with 10, 20, or 30 mg/kg FRAX486 or vehicle alone as a control. As FRAX486 crossed the blood-brain barrier at low levels within an hour of subcutaneous injection but may require additional time to enter neurons and inhibit PAK, we conducted our first assay 2 h after subcutaneous administration (Figure 4-9). The percentage of *fmr1* KO mice that exhibited wild running and jumping was not affected by 10 or 20 mg/kg FRAX486 ($p = 0.137$; $p = 1$, respectively), but was significantly reduced by the highest dose in this experiment, 30 mg/kg ($p < 0.0001$) (Figure 4-4A and 4-3). A similar trend was observed for the seizure phase, which consists of clonic convulsions and tonic hindlimb extension. *Fmr1* KO mice treated with 10 or 20 mg/kg were indistinguishable from vehicle controls ($p = 0.137$; $p = 0.106$, respectively), but 30 mg/kg was sufficient to prevent seizures in all but 17% of mice ($p < 0.0001$) (Figure 4-4B). In this way, prevention of

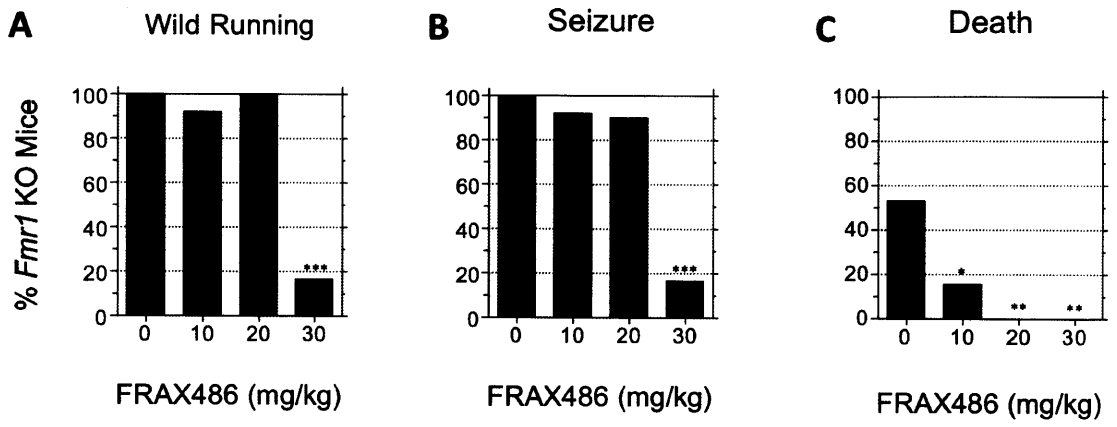

Figure 4-4: **Brief Exposure to FRAX486 Reduces Audiogenic Seizure Severity in *Fmr1* KO Mice.** *Fmr1* KO mice have a sequential seizure response to a loud auditory stimulus: wild running and jumping (A), clonic seizures (B), respiratory arrest and death (C). Single injections of 0, 10, 20, or 30 mg/kg FRAX486 were administered 2 h prior to the assay. (A) Wild running was induced in *fmr1* KO administered 0, 10, or 20 mg/kg, but prevented in all but 17% administered 30 mg/kg. (B) Seizure was induced in *fmr1* KO administered 0, 10, or 20 mg/kg, but prevented in a statistically significant percentage administered 30 mg/kg (17%). (C) Respiratory arrest and death was the end result for 53% of untreated *fmr1* KO, but survival was significantly increased by 10, 20, and 30 mg/kg FRAX486 administration.

Percentage of *fmr1* KO animals exhibiting each phase of the seizure response is plotted. χ^2 test was used to compare incidence in drug treated mice to that of vehicle control: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

wild running and seizure is dose-dependent.

Seizure severity is measured by frequency of mortality from respiratory arrest (Yan et al.. 2005). All three doses of FRAX486 completely or partially reduced the percentage of mice that died in response to the auditory stimulus (10 mg/kg, $p = 0.0183$; 20 mg/kg, $p = 0.0026$; 30 mg/kg, $p = 0.0013$)(Figure 4-4C). Therefore, we conclude that brief exposure to FRAX486 was sufficient to reduce AGS severity in *fmr1* KO mice.

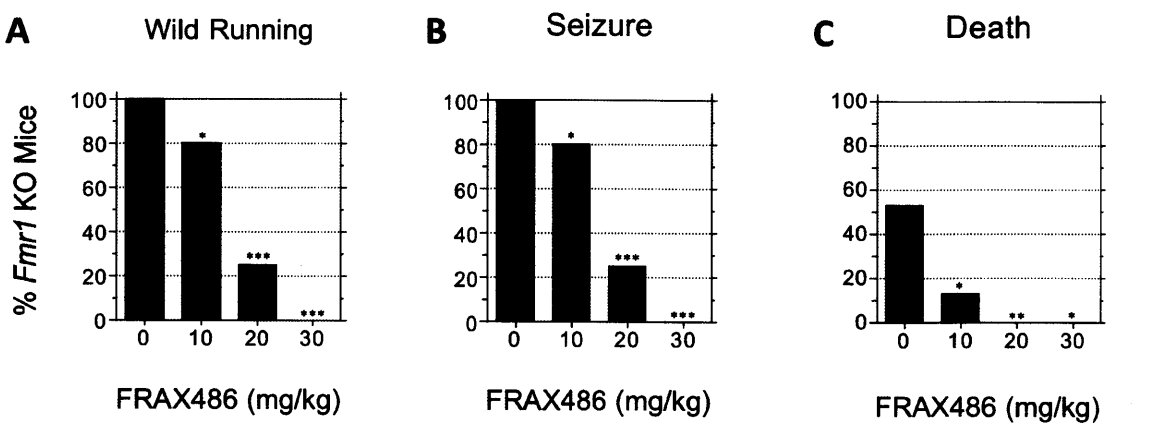

Figure 4-5: **A Single Dose of FRAX486 Reduces Incidence of Sound-Induced Wild Running, Seizure, and Death in *Fmr1* KO Mice.** Single injections of 0, 10, 20, or 30 mg/kg FRAX486 were administered to *fmr1* KO mice 8 h prior to the assay. There was a dose-dependent reduction in the incidence of wild running (A), seizure (B), and death (C).

χ^2 test was used to compare incidence in drug treated mice to that of vehicle control: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

4.3.3 A Single Administration of FRAX486 Is Sufficient to Rescue the Audiogenic Seizure Phenotype.

While a 2 h exposure to 10 or 20 mg/kg FRAX486 was sufficient to prevent death in some or all *fmr1* KO mice, respectively, it did not rescue the earlier phases of AGS. To determine whether a longer exposure to a single dose of FRAX486 is sufficient to prevent wild running and seizure, we repeated the experiment with a 8 h time point. In addition to an increase in the length of brain exposure to FRAX486, this time point also resulted in approximately four-fold higher brain levels of drug than present during the 2 h assay (Figure 4-9). The incidences of wild running and seizure were reduced from 100% to 80% by 10 mg/kg, to 25% by 20 mg/kg, and completely abolished by 30 mg/kg ($p = 0.0339$; $p < 0.0001$; $p < 0.0001$, respectively)(Figure 4-5A, B). A dose-dependent decrease in the percentage of *fmr1* KO mice that died as a result of respiratory arrest was also observed (10 mg/kg, $p = 0.0101$; 20 mg/kg, $p = 0.0013$; 30 mg/kg, $p = 0.0175$)(Figure 4-5C). In summary, a single dose of FRAX486

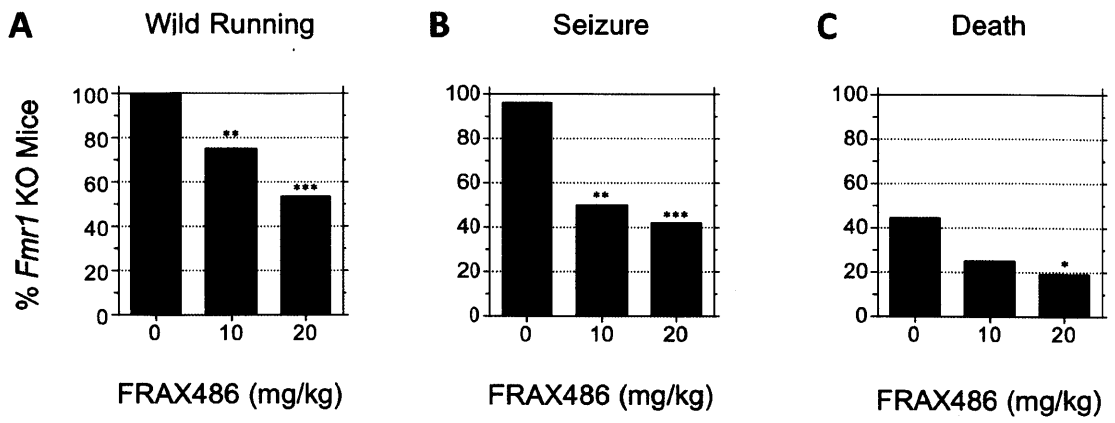

Figure 4-6: **Daily Administration of FRAX486 Reduces AGS Susceptibility in 8 h Assay.** Five daily injections of 0, 10, or 20 mg/kg FRAX486 were administered, the last of which was given to *fmr1* KO mice 8 h prior to assay. Incidence of wild running (A) and seizure (B) were reduced by both doses. Incidence of respiratory arrest and death (C) was reduced by 20 mg/kg but not 10 mg/kg.

χ^2 test was used to compare incidence in drug treated mice to that of vehicle control: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

is sufficient to rescue the AGS phenotype in *fmr1* KO mice.

Eight hours after drug administration, the portion of the mice that received the highest dose (30 mg/kg) appeared sluggish. Specifically, these mice were sedentary, remaining in one place in the home cage more than littermates, and did not huddle with siblings while sleeping. In addition, one mouse died and a second was moribund by 24 hours after an injection of 30 mg/kg FRAX486. These signs of toxicity, which were not observed at the low or intermediate doses, led us to discontinue the administration of this dose and carefully monitor mice for signs of sedation in future experiments.

4.3.4 Prolonged Administration of FRAX486 Reduces Seizure Incidence in *Fmr1* KO Mice.

The most effective treatment for epilepsy in humans would prevent seizures throughout a patient's life. Therefore we determined whether the observed effect was specific

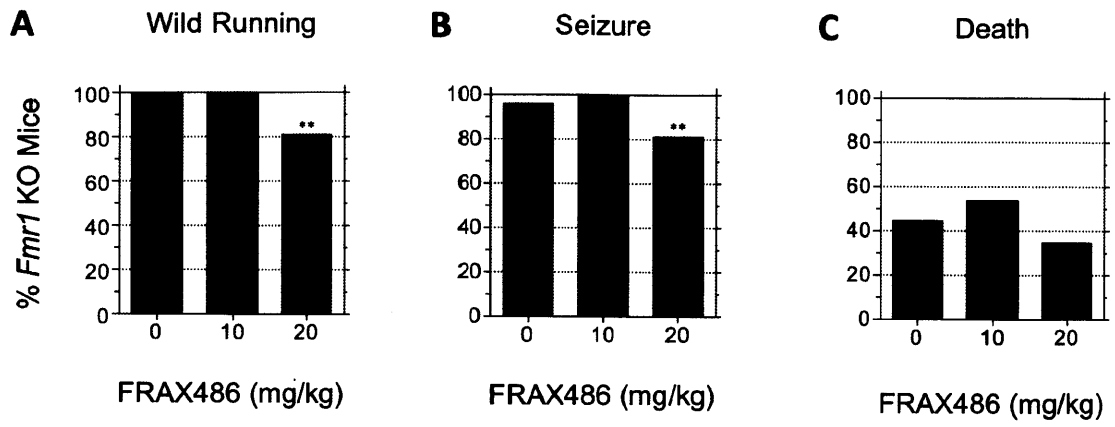

Figure 4-7: **Wild Running and Seizure Incidence Is Partially Rescued 2 h Following the Last of 5 Daily Injections of 20 mg/kg FRAX486.** Five daily injections of 0, 10, or 20 mg/kg FRAX486 were administered, the last of which was given 2 h prior to assay. Incidence of wild running and seizure was reduced to 81% by 20 mg/kg, but not impacted by 10 mg/kg. Neither dose was sufficient to reduce frequency of mortality.

χ^2 test was used to compare incidence in drug treated mice to that of vehicle control: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

to acute administration or whether prolonged drug administration also reduced AGS incidence in the mouse model of FXS. To do so, we treated mice daily for 5 days and conducted the assay 2 and 8 h after the ultimate injection. At the 8 h time point, 10 and 20 mg/kg reduced the incidence of wild running ($p = 0.0041$; $p < 0.0001$, respectively) and seizure ($p = 0.002$; $p < 0.0001$, respectively)(Figure 4-6). Death was prevented in all but 19% of *fmr1* KO mice that received 20 mg/kg, a statistically significant rescue when compared to the 44% of vehicle treated mice that proceeded from seizure to respiratory arrest and death ($p = 0.0247$). The effect of the lower dose on survival did not reach statistical significance, in part because it was only administered to 4 mice ($p = 0.231$). 2 h following the ultimate injection, 5 days of 20 mg/kg was sufficient to reduce the incidence of wild running and seizure ($p = 0.0088$; $p = 0.0088$, respectively), though the lower dose (10 mg/kg) did not have an effect on the AGS phenotype in *fmr1* KO mice ($p = 1.0$; $p = 0.2411$, respectively)(Figure 4-7).

In this way, acute and prolonged administration of FRAX486 reduced seizure

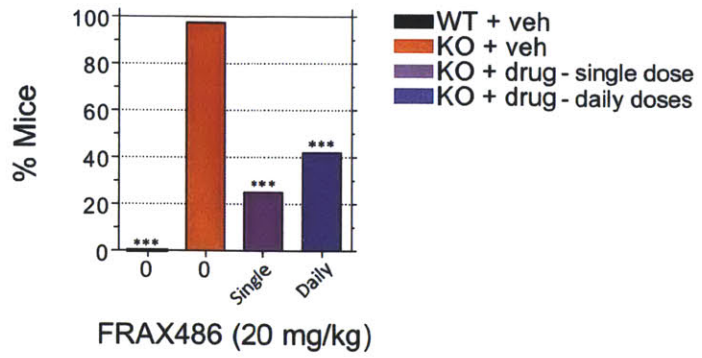

Figure 4-8: **Audiogenic Seizure Susceptibility Is Reduced by Acute or Daily Administration of 20 mg/kg FRAX486.** While WT mice do not have audiogenic seizures, nearly all *fmr1* KO mice suffer from seizures. Administration of 20 mg/kg FRAX486 as a single dose or daily doses for 5 d significantly reduces seizure incidence in the 8 h assay. Color coding for genotype, dose, and number of days of injections matches that used in figures in subsequent thesis chapters.

χ^2 test was used to compare incidence in drug treated KO mice or WT mice to that of vehicle treated KO mice: ***, $p < 0.001$.

incidence. A summary of the data for 8 h at 20 mg/kg is provided in Figure 4-8, and demonstrates that a small molecule PAK inhibitor reduces the incidence of AGS in the mouse model of FXS. As this dose was the most effective at rescuing the AGS phenotype, it was used for all subsequent behavioral experiments.

4.4 Discussion

Seizures are the most common neurological abnormality in humans with FXS (Hagerman and Hagerman 2002). In addition, seizure susceptibility is present in the mouse model of this disease, where it is a robust phenotype that can be reliably reproduced in the laboratory. To determine whether inhibition of PAK may be an effective strategy for the treatment of FXS, we tested whether FRAX486 could protect *fmr1* KO mice from sound-induced seizures. Our results demonstrate rescue of audiogenic seizures in *fmr1* KO mice, and support the hypothesis that inhibition of PAK may be sufficient to rescue neuological abnormalities *in vivo*.

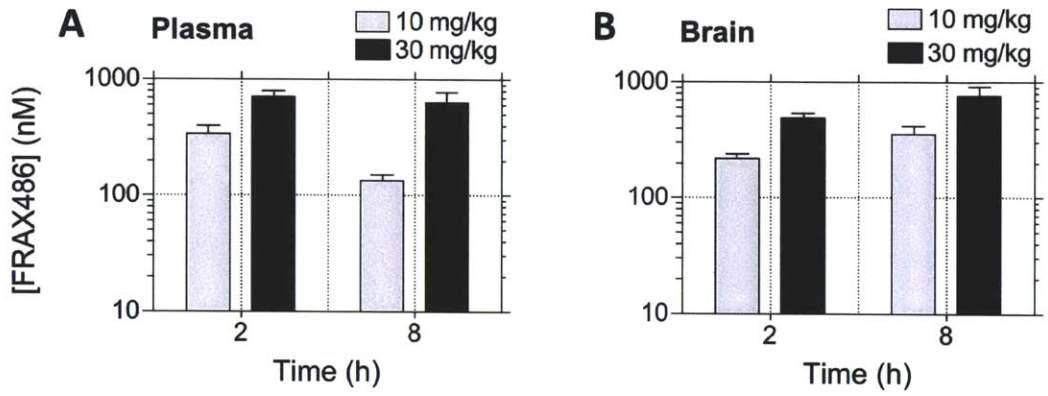

Figure 4-9: **Concentration of FRAX486 in the Plasma and Brain at the Time of the AGS Assay.** Blood and brain samples were collected from *fmr1* KO mice administered a single dose of 10 or 30 mg/kg FRAX486. These samples were collected immediately after the AGS assay, and therefore approximate FRAX486 levels in the body during the test.

For the 2 h assay, plasma levels were 338.8 +/- 61.30 nM (n = 5) and 704.4 +/- 95.56 nM (n = 10) and brain levels were 219.3 +/- 20.28 nM (n = 6) and 494.4 +/- 45.15 nM (n = 10) for 10 mg/kg and 30 mg/kg, respectively. For the 8 h assay, plasma levels were 135.7 +/- 17.91 nM (n = 6) and 630.0 +/- 149.2 nM (n = 5) and brain levels were 354.3 +/- 63.69 nM (n = 6) and 768.0 +/- 141.3 nM (n = 6) for 10 mg/kg and 30 mg/kg, respectively.

4.4.1 Seizure Susceptibility Was Not Age-Dependent in Our Assay.

There is a wealth of literature on the significance of age in seizure susceptibility in FXS humans and *fmr1* KO mice. In males with FXS, the manifestation of seizures is most pronounced in childhood and often dissipates after puberty, that is, in at least 3/4 of seizure sufferers (Hagerman and Stafstrom 2009)(Musumeci et al. 1999). In mice, the significance of age in seizure susceptibility is most pronounced on the B6 background strain where the window of susceptibility last only three days (Chen and Toth 2001)(Dolen et al. 2007). While we initially set-up our seizure assay with the intention of using the B6 background strain, we found the phenotype too unreliable (data not shown). We discovered a more robust AGS phenotype in *fmr1* KO mice on the FVB background strain. This is not surprising given that a number of laboratories have demonstrated a seizure phenotype in adult *fmr1* KO mice of multiple ages on this genetic background (Yan et al. 2005)(Yan et al. 2004)(Chen and Toth 2001)(Musumeci et al. 2007). In our assay – which utilized a siren-like noise of 124 dB – seizure susceptibility was not age-dependent.

4.4.2 AGS Susceptibility Varies Slightly between Single Doses and Prolonged Administration.

Subtle differences were observed in the results from single injections and 5 days worth of daily treatments of FRAX486. While the dose of 20 mg/kg does not impact wild running or seizure incidence 2 hours following a single injection, it does decrease the incidence of these AGS stages following 5 days of treatment. This could be related to observations in our pharmacokinetic study that brain levels of FRAX486 are higher 24 hours after injection than 2 hours after injection (Figure 2-9). Thus, 2 hours following the 5th treatment, residual FRAX486 from the 4th treatment will increase the available amount of this PAK inhibitor. Alternatively, structural changes – including decreases in spine density – may require more than 2 hours of FRAX486 treatment, and this may account for the decrease in wild running and seizure incidence

observed following multiple treatments.

A couple observations can be made from the data collected 8 hours following a single or multiple treatments. First, 5 treatments has a more profound effect on wild running and seizure incidence than 1 treatment for the lowest dose: 10 mg/kg. A higher dose – 20 mg/kg – has a statistically equivalent impact on wild running and seizure compared to vehicle after a single or multiple doses, however the percentage of animals rescued is fewer following multiple rounds of FRAX486. In this way, prolonged treatment may be required before a low dose is maximally effective, while the effectiveness of a higher dose may wane over time.

4.4.3 High Doses of FRAX486 Can Be Detrimental to Mice.

As was mentioned in the results section, the 30 mg/kg dose had negative effects on mice. In most cases this was limited to sluggishness, but in a couple of cases it was fatal or near fatal. It is unclear whether this is related to the inhibition of PAK or an off-target effect of the drug. Either way, all subsequent experiments were conducted with a lower dose of FRAX486.

4.4.4 Spine Density and AGS.

Increased spine density and increased numbers of long and immature-like spines are the most salient neuropathologies described in association with FXS so far and seem likely to underlie neurological deficits associated with the disease (Irwin et al. 2001). Increased spine density suggests that excitatory synapse number is elevated in FXS. Increased excitatory synaptic transmission may be the mechanism for the increased incidence of seizures in FXS males. In this way, a therapeutic strategy that targets the spine phenotype is likely to rescue the seizure phenotype.

However, as our study did not analyze spine density in the brain regions shown to be specifically activated by AGS, we cannot definitively link increased spine density to increased seizure susceptibility. A study of c-Fos expression in the brain of *fmr1* KO mice verses WT mice indicates that the brainstem nucleus DNLL and the thalamic

nucleus PIL are specifically activated, however spine density has not been measured in these subregions of the brain (Chen and Toth 2001).

4.5 Materials and Methods

4.5.1 Animals.

Adult male *fmr1* KO mice (FVB.129P2-Fmr1^{tm1Cgr}/J) and age-matched WT controls (FVB.129P2-*Pde6b*$^{+}$Tyr $^{c-ch}$/AntJ) were used of the genetic background which includes the wildtype *Pde6b* allele so the mice do not suffer from blindness due to retinal degeneration (Jackson Labs). After the initial experiment which utilized mice 1 - 9 months old to test age dependence, all subsequent audiogenic seizure experiments used mice 2 - 5 months old. Animals were housed in groups of two to five per cage with food and water freely available. Mice were kept on a 12-h light/dark cycle, and experiments were conducted during the second half of the light cycle. Experiments were performed according to protocols approved by the Massachusetts Institute of Technology Committee on Animal Care and in compliance with National Institutes of Health guidelines.

4.5.2 Drug Delivery.

The yellow powder FRAX486 (aFRAXis) was dissolved in 20% hydroxypropyl-β-cyclodextrin vehicle (Sigma-Aldrich). A 1, 2, or 3 mg/ml solution of drug or vehicle alone control was administered via subcutaneous injection at 10, 20, or 30 mg/kg, respectively, in a volume proportional to the animals weight (typically 300-400 μl). Single injections were given at 2 h or 8 h prior to experimentation. Daily injections were given every 24 h for 5 days with the ultimate injection administered 2 h or 8 h prior to the assay.

4.5.3 Audiogenic Seizure Assay.

As described in Figure 4-1, a clear acrylic box with two identical square chambers (13 cm x 13 cm x 16 cm) divided by a clear partition was designed and built for the assay. A mouse was placed on either side of the partition and the two mice were acclimated to the apparatus for 2 min. Then a speaker in the top of the box presented an auditory stimulus (124 dB siren) until seizures occurred but not longer than 5 min. Mouse behavior was observed for wild running and jumping, seizure, and cardiac arrest leading to death.

4.5.4 Pharmacokinetics.

Immediately after the assay, the animal was anesthetized with isoflurane. Retrorbital blood samples were collected into heparinized tubes and spun down to isolate the plasma. Mice were decapitated, and brains were dissected out and fast frozen in liquid nitrogen. Later brain samples were homogenized in 2x PBS, and levels of FRAX486 were determined via LC/MS/MS by Apredica, a Cyprotex company.

Chapter 5

Rescue of Hyperactivity and Repetitive Behaviors in *Fmr1* KO Mice by FRAX486.

Contributions

The work described in this chapter was performed by Bridget M. Dolan.

5.1 Summary

The mouse model of Fragile X Syndrome (FXS) displays the autism-like phenotypes of hyperactivity and restrictive or repetitive behaviors. Results from the genetic rescue strategy we described in our previous work demonstrated that partial inhibition of PAK kinase activity in the forebrain of adult *fragile X mental retardation 1* knockout (*fmr1* KO) mice was sufficient to ameliorate these phenotypes (Hayashi et al. 2007). To determine whether a pharmacological version of this strategy – the small molecule PAK inhibitor FRAX486 – is effective at reversing hyperactivity and repetitive movements in *fmr1* KO mice, we treated mice with FRAX486 and conducted the open field assay. As FRAX486 rescued the seizure phenotype in these mice after a single treatment or 5 daily injections – as demonstrated in the previous chapter of this thesis – we administered FRAX486 for 1 and 5 days in this assay. *Fmr1* KO mice that did not receive drug displayed hyperactivity – measured by distance traveled, time moving, and number of movements – higher levels of repetitive movements – including stereotypical motor behaviors and circling – and a lack of habituation to a novel environment. Treatment with the kinase inhibitor FRAX486 rescued these abnormalities, providing evidence that inhibition of PAK is a potential therapeutic strategy for the treatment of neurodevelopmental disorders.

5.2 Introduction

5.2.1 Behavioral Symptoms in Autism and FXS.

Autism is known to have genetic etiology, however as no reliable biomarkers have been identified, it is a behaviorally defined disorder. To be diagnosed with autism, a patient must display impairments in social interaction, delays or impediments in language, and restrictive or repetitive behaviors. These are the three core criteria as defined by the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association 1994). An ideal animal model of autism and related disorders would display strong correlates to the human endophenotypes (Silverman et al. 2010).

In addition to the core criteria, hyperactivity and attentional deficits are often significant problems for boys with autism spectrum disorders (ASDs) and in particular FXS. In fact, attention deficit hyperactivity disorder (ADHD) is the most common comorbid DSM-IV diagnosis among FXS boys (Backes et al. 2000). In this way, it is not surprising that increased locomotion is the most consistently assayed and reported phenotype in the mouse model of FXS (Dutch-Belgian Fragile X Consortium 1994)(Peier et al. 2000)(Mineur et al. 2002)(Spencer et al. 2005).

5.2.2 Hyperactivity in *Fmr1* KO Mice.

Abnormally high levels of motor activity were first observed in an exploratory behavior test conducted by the Dutch-Belgian Fragile X Consortium. In the original paper describing the mouse model of FXS, the authors reported that *fmr1* KO mice explored more than their WT littermates in a 10 min light-dark transition test (Dutch-Belgian Fragile X Consortium 1994). They inferred heightened exploratory behavior from an increase in movements in and between two chambers, measured by interference with infrared beams. Consistent with this finding, increased locomotion is a reliable phenotype in the *fmr1* KO mouse as assayed in the open field test (Peier et al. 2000)(Mineur et al. 2002)(Qin et al. 2002)(Hayashi et al. 2007). In this assay a single mouse is introduced to a novel, spacious environment, and his movement is tracked in distance and time spent moving.

5.2.3 Investigation of Anxiety in *Fmr1* KO Mice.

Increased exploratory behavior is likely due to hyperactivity, however abnormal levels of anxiety may also be a contributing factor. In the open field assay, mice naturally tend to remain close to the walls and avoid the brightly illuminated open space in the center of the arena. This behavior, called thigmotaxis, is increased in some mice, and this increase is interpreted as an indication of heightened anxiety. Some studies report that *fmr1* KO mice spend more time in the center and conclude that this means they are less anxious (Spencer et al. 2005)(Peier et al. 2000). However, the opposite

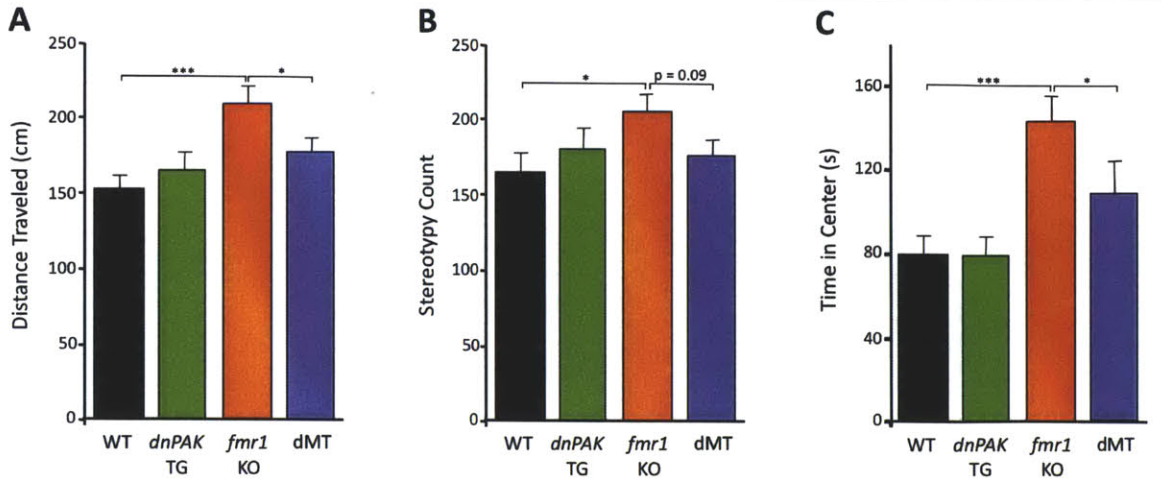

Figure 5-1: **Inhibition of PAK with a Dominant Negative Transgene Rescues Behavioral Abnormalities.** Several behavioral abnormalities were associated with *fmr1* KO mice. In the open field assay, *fmr1* KO mice exhibited increased locomotion, increased stereotopies, and decreased center time when compared to WT littermates. The behavior of *dnPAK* TG mice and dMT mice was similar to WT in all three parameters in the 10 min assay. **(A) Distance traveled.** *Fmr1* KO traveled significantly farther than WT. This locomotion phenotype was rescued in dMT, as these values are not significantly different than WT. **(B) Stereotopy count.** *Fmr1* KO exhibited a higher number of repetitive behaviors than WT. dMT exhibited comparable performance to WT in stereotopy counts. **(C) Time in center of arena.** *Fmr1* KO spent more time in the center of the open field than WT, and this abnormality was rescued by inhibition of PAK.

Wildtype C57BL6, WT, n = 10; dominant negative *PAK* transgenic, *dnPAK* TG, n = 10; *fmr1* KO, n = 11; double mutant *dnPAK* TG; *fmr1* KO, dMT, n = 11. Mean values from Fisher's least significant difference post hoc analysis are presented with error bars representing SEM for comparisons of each group to WT and *fmr1* KO to dMT: $*$, $p < 0.05$; $**$, $p < 0.01$; $***$, $p < 0.001$. Data was published in PNAS (Hayashi et al. 2007).

phenotype has also be reported in *fmr1* KO mice (Restivo et al. 2005). In summary, anxiety to a novel environment is a subtle phenotype that is not consistently observed in the mouse model of FXS. As increased locomotion has been observed in almost all reports of the open field assay, hyperactivity may be a more robust and therefore better correlate of the human phenotype to test the effects of small molecule inhibitors (Peier et al. 2000) (Mineur et al. 2002).

5.2.4 Restrictive and Repetitive Behaviors in FXS and *Fmr1* KO Mice.

Restricted or repetitive behavior is one of three behavioral domains required for the diagnosis of autism in humans (American Psychiatric Association 1994). These behaviors include repetitive motor actions – like hand flapping or the opening and closing of doors – as well as insistence on routines or rituals – a more complex category that has a distinct cognitive component. Like autistic humans, some mice display spontaneous stereotyped behaviors. In our animal models, it is easier to test for the lower order motor actions (Lewis et al. 2007). In mice, these stereotopies can manifest themselves as repetitive motor behaviors such as circling, backflips, and jumping, and these behaviors may continue for hours. In addition, mice can exhibit unusually long episodes of self grooming and repetitive forelimb movements resembling the distinctive hand stereotypies (e.g., hand-wringing, waving, and clapping) (Lewis et al. 2007). These actions may be observed in an animal's homecage or in the previously described open field test.

5.2.5 Rescue of Behavioral Phenotypes with *dnPAK*.

As we demonstrated in our previous work, *fmr1* KO mice displayed a higher count of stereotopies, such as repetitive motions during grooming, than WT controls (Hayashi et al. 2007). Interestingly, inhibition of PAK activity in the forebrain of adult *fmr1* KO mice, via introduction of a dominant negative *PAK* transgene, returned stereotopy counts to levels comparable to WT mice (reproduced here in Figure 5-1B). In our open field assay stereotypy count is the number times a mouse interferes with the same infrared beam (or a set of beams), as this is thought to occur many times in a few seconds during a bout of intense grooming, repetitive forelimb motions, and head bobbing. Genetic rescue was also observed in the two previously discussed open field phenotypes: hyperactivity and anxiety, measured as total distance traveled and time in the arena's center (Figure 5-1A, C).

These novel findings suggest that inhibition of PAK kinase activity is sufficient to

treat autism-like behavioral symptoms in the mouse model of FXS. The aim of the research presented in this chapter was to assay whether pharmacological inhibition of PAK also reverses autism-like behaviors in *fmr1* KO mice.

5.3 Results

To understand the therapeutic potential of small molecule PAK inhibitors, we conducted the open field assay on *fmr1* KO mice that had been administered FRAX486 or vehicle control and compared the results to those of WT littermates. Mice were administered a single dose or daily doses for 5 d of 20 mg/kg FRAX486. While mice were group housed with two to four littermates until this point, when given the ultimate dose, they were separated into individual cages so that recent social interactions would not affect behavior. The assay was conducted 4.5 - 5.5 h later, at which point brain concentration of FRAX486 exceeded 1 μM and was therefore more than 120 times the IC_{50} for PAK1, 25 times the IC_{50} for PAK2, and 18 times the IC_{50} for PAK3 (Figure 2-3 and 2-4).

In this behavioral test, a single mouse is placed in the center of a large, open arena and allowed to explore for 30 min. A series of sensors track the animal's horizontal and vertical movements, and the data is organized into 1 min bins. The data can then be analyzed to assess hyperactivity, restrictive or repetitive behaviors, and anxiety, three areas in which phenotypes have been reported in the literature for both mice and humans with FXS (Peier et al. 2000)(Mineur et al. 2002)(Qin et al. 2002)(Hayashi et al. 2007)(Hagerman and Hagerman 2002)(Berry-Kravis 2002).

5.3.1 FRAX486 Reverses Hyperactivity in *Fmr1* KO Mice.

To demonstrate that *fmr1* KO mice display increased locomotor activity and to determine whether this hyperactivity phenotype can be rescued by FRAX486, we compared results for total distance traveled, time spent moving, and number of horizontal movements. Reports in the literature, highlight the importance of the later half of the 30 min exploration period, as this is the time when WT mice tend to habituate

to the novel environment, but *fmr1* KO mice continue to be active (Qin et al. 2005). For this reason, data is presented first for each 1 min bin and then as the mean plus standard error of the mean (SEM) of minutes 18 to 30.

As shown in Figure 5-2A, the patterns of distance traveled differ for *fmr1* KO mice and their WT littermate controls. First, *fmr1* KO + veh traveled a greater distance in minutes 18 to 30 compared with WT + veh ($p < 0.01$) (Figure 5-2B). This phenotype was rescued by treatment with FRAX486 for 5 d ($p < 0.01$; Figure 5-2B) or 1 d ($p < 0.001$; data not shown), as drug treated *fmr1* KO mice traveled a distance indistinguishable from WT controls ($p > 0.05$). Similarly, *fmr1* KO + veh spent more time moving than WT + veh ($p < 0.05$), and this abnormality was successfully reversed by treatment with FRAX486 for 5 d ($p < 0.05$; Figure 5-2C, D) or 1 d ($p < 0.01$; data not shown).

Finally, the number of distinct horizontal movements made by *fmr1* KO mice was significantly greater than that for WT + veh ($p < 0.01$), and treatment with FRAX486 was effective at remedying this display of hyperactivity (Figure 5-2E, F). In summary, the mouse model of FXS displayed a hyperactivity phenotype that was rescued by the small molecule PAK inhibitor FRAX486.

5.3.2 FRAX486 Reduces Activity in WT Mice.

To determine whether the effects of FRAX486 on activity and repetitive behaviors is specific to *fmr1* KO mice, we also treated WT mice with FRAX486 and performed the open field. Our results demonstrate that WT mice were also impacted by FRAX486 treatment. In particular, they traveled less distance, spent less time moving, and made fewer horizontal movements (Figure 5-2).

5.3.3 FRAX486 Does Not Impair Motor Performance or Balance.

To determine whether the general decrease in activity could be a result of sedation, we assayed motor coordination and balance in drug treated mice. *Fmr1* KO mice

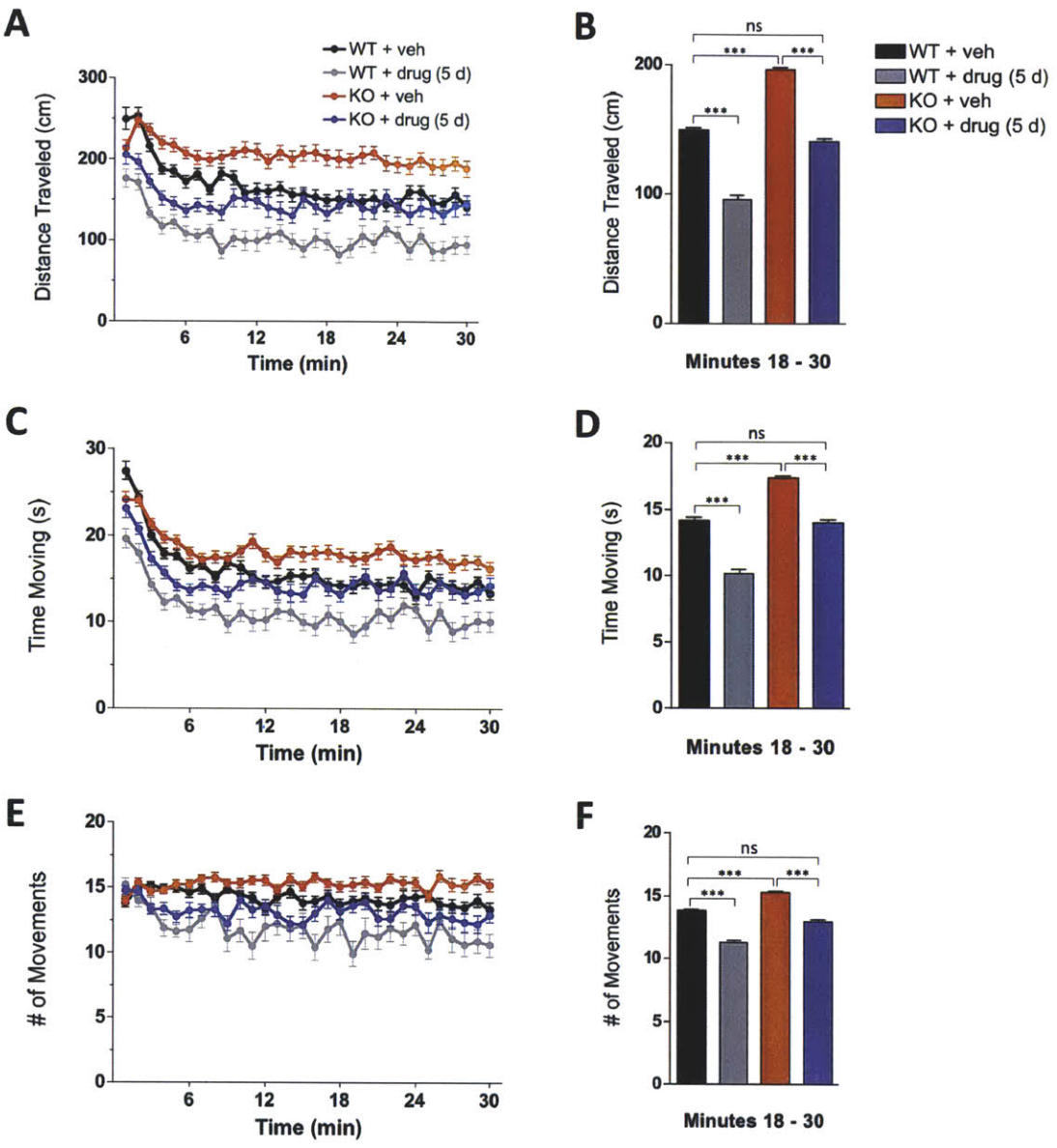

Figure 5-2: **FRAX486 Reverses Hyperactivity in *Fmr1* KO Mice.**
Fmr1 KO mice were more active in the open field test than WT littermates. This hyperactivity was rescued by administration of FRAX486 for 5 d. In all three measures, the mutant phenotype manifested itself towards the latter half of the 30 min exploration session.
(A-B) Distance traveled. In min 18 to 30, *fmr1* KO + veh traveled a greater distance than WT + veh. However, this increase in motor activity was reversed by treatment with FRAX486, as these values are not significantly different than WT + veh, though they are significantly less than values for *fmr1* KO + veh. **(C-D) Time moving.** In min 18 to 30, *fmr1* KO + veh spent significantly more time moving than WT + veh and drug treated *fmr1* KO. *Fmr1* KO + drug did not differ from WT + veh. **(E-F) Number of movements.** In min 18 to 30, *fmr1* KO + veh made significantly more horizontal movements than WT + veh and *fmr1* KO treated with drug. Move numbers from *fmr1* KO + drug were similar to WT + veh.

WT + veh, n = 61; WT + drug (5 d), n = 29; *fmr1* KO + veh, n = 62; *fmr1* KO + drug (5 d), n = 50. (A, C, E) Mean values and SEM analyzed in 1 min bins are shown. (B, D, F) One-way ANOVA with post hoc Tukey-Kramer multiple comparison test for min 18 to 30: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ns = not significant.

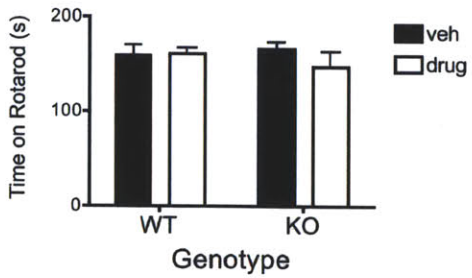

Figure 5-3: **Treatment with FRAX486 Does Not Impair Motor Coordination.** The rotarod performance test was utilized to assay balance and motor coordination in mice treated with 20 mg/kg FRAX486. WT and *fmr1* KO mice were administered a single dose of FRAX486 or vehicle, and 4 h, 4.5 h, and 5.25 h later the mice participated in three trials on the accelerating rotarod. Rodents naturally try to stay on the rotating cylinder. Latency to fall off is measured in each of three trials. Each trial lasted a maximum of 180 s, and the average for each animal was used for the statistics.

WT + veh, 155 +/- 3.0 s, n = 7; *fmr1* KO + veh, 161 +/- 6.6 s, n = 12; WT + drug, 164 +/- 6.7 s, n = 11; *fmr1* KO + drug, 147 +/- 6.0 s, n = 9. Mean values and SEM are listed (two-way ANOVA: Interaction, p = 0.3298; Genotype, p = 0.7437; Treatment, p = 0.4290; Bonferroni posttests show no effect of genotype or drug treatment.)

and WT littermates were administered 20 mg/kg FRAX486, and 4 h later the first of three rotarod tests commenced. Mice were allowed to recover for 30 - 40 min between tests, therefore the final rotarod test was given ~5.25 h after injection of FRAX486. All four groups of mice (WT + veh, WT + drug, KO + veh, KO + drug) performed well on the rotarod test. In light of these findings, we concluded that FRAX486 does not impair motor performance and balance, and therefore it is unlikely that the rescue effect observed in the audiogenic seizure or open field assay is simply an artifact of sedation.

5.3.4 FRAX486 Restores Habituation to a Novel Open Environment in *Fmr1* KO Mice.

WT animals adjust their behavior in response to a novel environment over time, a process called habituation. Locomotor activity is the most important parameter of habituation in the open field experiment (Brenes et al. 2009). WT + veh traveled a significantly greater distance in the first 5 min of open field exploration than they did

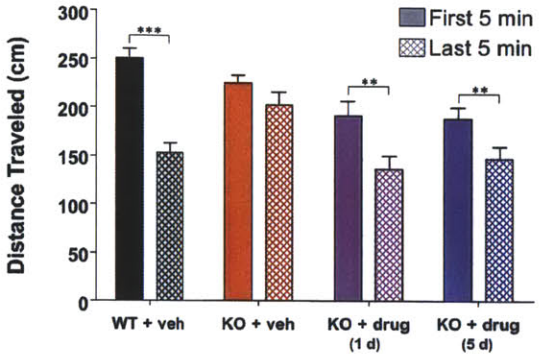

Figure 5-4: **FRAX486 Restores Habituation to a Novel Open Environment in *Fmr1* KO Mice.** WT + veh traveled a significantly greater distance in the first 5 min than in the last 5 min (***, $p < 0.0001$). *Fmr1* KO + veh traveled similar distances in these 5 min time periods (ns, $p = 0.0856$). FRAX486 administration for 1 or 5 d restored habituation to *fmr1* KO (**, $p < 0.0052$; **, $p < 0.0091$, respectively).

Mean values and SEM are shown. One-tailed, unpaired t test; ***, $p < 0.0001$.

in the last 5 min of a 30 min assay ($p < 0.0001$) (Figure 5-4). However, this was not the case for *fmr1* KO + veh as the distance traveled did not change over time (ns, $p = 0.0856$). The lack of habituation observed in these mice was rescued by FRAX486 for 1 or 5 d ($p < 0.0052$; $p < 0.0091$, respectively).

5.3.5 FRAX486 Reverses Repetitive Behaviors in *Fmr1* KO Mice.

In humans, repetitive behaviors – which include motor stereotopies, repetitive use of objects, and excessive adherence to ritualized patterns of behavior – are one of three criteria for a diagnosis of autism (American Psychiatric Association 1994). Like humans, some mice have a tendency towards restrictive or repetitive movements. To determine whether *fmr1* KO mice exhibit these abnormal behaviors and to assess the ability of the novel PAK inhibitor FRAX486 to treat these autism-like symptoms, we counted stereotopies and revolutions in small circles in the open field. Stereotypy count is quantified as the number times a mouse interferes with the same infrared

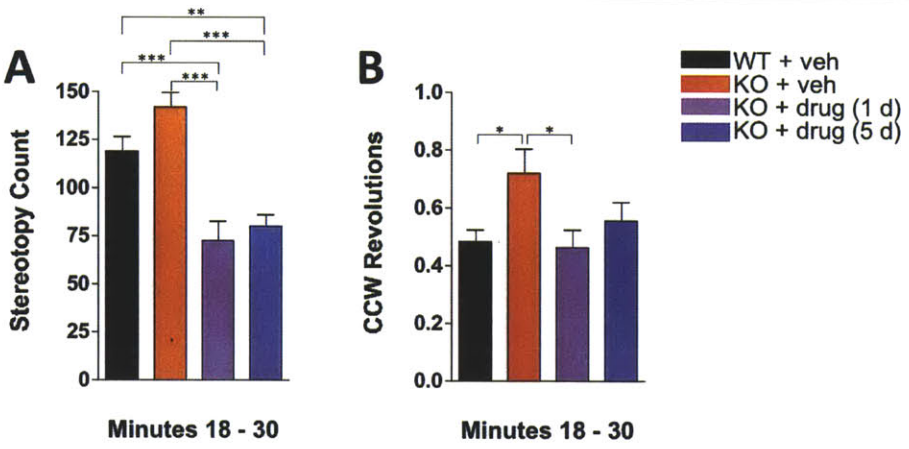

Figure 5-5: **FRAX486 Reverses Repetitive Behaviors in *Fmr1* KO Mice.** *Fmr1* KO mice exhibited an increase in the number of restrictive and repetitive movements over WT controls, however these behaviors were reversed by FRAX486 treatment for 1 or 5 d (WT + veh, n = 26; *fmr1* KO + veh, n = 24; *fmr1* KO + drug (1 d), n = 18; *fmr1* KO + drug (5 d), n = 22). **(A) Stereotopy count.** *Fmr1* KO + veh tended to display more stereotypical movements than WT controls. Levels did not reach statistical significance in the Tukey-Kramer multiple comparison test, however the unpaired t test, suggested the trend was significant (one-tailed, p = 0.0162; asterisks not shown). Administration of FRAX486 reversed this trend, resulting in counts of stereotopies that were significantly lower than those for both WT + veh and *fmr1* KO + veh. **(B) Counter clockwise revolutions (CCW).** *Fmr1* KO + veh circled CCW significantly more than control littermates. Drug treatment reversed this phenotype, returning counts to values similar to WT + veh.

Mean values and SEM are shown (one-way ANOVA with post hoc Tukey-Kramer multiple comparison test for 1 min bins of min 18 to 30: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

beam (or a set of beams) in a bout of stereotypic activity that typically involves behaviors like grooming and head bobbing.

As with the locomotion measurements, differences between *fmr1* KO and controls were most pronounced in minutes 18 to 30. *Fmr1* KO + veh exhibited a trend towards higher levels of stereotopy counts when compared to WT + veh (Figure 5-5). The difference between these two genotype + veh conditions was not statistically significant when compared using a one-way ANOVA, but was statistically significant when compared using an unpaired t test (p = 0.0162). This upward trend in repetitive behaviors was reversed by drug treatment with FRAX486, and in fact, the resulting stereotopy counts were significantly lower than observed in both *fmr1* KO + veh (p <

0.001; $p < 0.001$, respectively) and WT + veh ($p < 0.001$; $p < 0.01$, respectively). This was also the case for WT mice treated with FRAX486, which displayed stereotopy counts similar to those of *fmr1* KO mice (data not shown).

Similarly, *fmr1* KO + veh revolved in the counter clockwise (CCW) direction significantly more than WT + veh ($p < 0.05$)(Figure 5-5). This phenotype was rescued by a single injection of FRAX486, as CCW revolutions were significantly lower than in *fmr1* KO + veh ($p < 0.05$) but similar to CCW revolutions in WT + veh. While the 5 d treatment showed a trend towards rescue, it was not significantly different than either *fmr1* KO + veh or WT + veh. Drug treatment also decreased CCW circling in WT mice (data not shown).

In summary, the mouse model of FXS exhibited restrictive and repetitive behaviors, and FRAX486 reversed this phenotype.

5.3.6 *Fmr1* KO Mice Do Not Display an Anxiety Phenotype.

Shyness and social anxiety are observed in even the mildest cases of FXS (Merenstein et al. 1996), however, the phenotype is different and less consistent in the mouse model of the disease. While some groups report that *fmr1* KO mice do not have a phenotype in the open field or elevated plus assay (Nielsen et al. 2002), others report that they are less anxious (Mineur et al. 2002). While this observation may seem opposite to what has been reported in FXS humans, it is important to consider that humans experience social anxiety while these murine behavioral tasks test anxiety to a physical environment.

Time in the Center of an Open Field. To measure generalized anxiety, we analyzed the amount of time mice spent in the center of the arena in the open field assay. Since WT mice habituate to a novel environment, and thereby become less anxious over time, it is especially informative to compare time in the center during the first 10 min of the test. No difference was observed between *fmr1* KO + veh and WT + veh in the first 10 min or the entire 30 min period (Figure 5-6A). While a single administration of FRAX486 did not affect behavior, 5 d of drug treatment resulted in less time in the center when compared to *fmr1* KO + veh ($p < 0.001$) or WT +

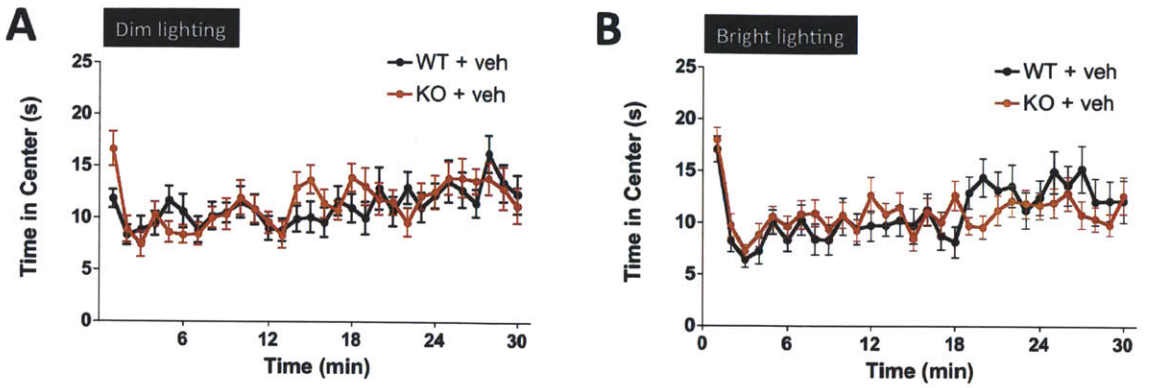

Figure 5-6: ***Fmr1* KO Mice Do Not Display an Anxiety Phenotype.** While some studies report that *fmr1* KO mice spent more time than WT littermates in the center of an open field, this open field experiment did not reveal a hypoanxiety phenotype when performed in either a dimly lit arena (A) or a brightly lit arena (B). *Fmr1* KO + veh spent a similar amount of time in the center of the arena as WT + veh.

Dim lighting: WT + veh, n = 26; *fmr1* KO + veh, n = 24. Bright lighting: WT + veh, n = 20; *fmr1* KO + veh, n = 30. Mean values and SEM are shown.

veh ($p < 0.001$), suggesting increased anxiety (data not shown). However, since the *fmr1* KO + veh did not display the sometimes reported hypoanxiety phenotype, it is difficult to make conclusions about the drug's effect.

Time in the Center of a Brightly Illuminated Open Field. Since lighting conditions may affect the expression of anxiety in a novel open environment, we conducted a second set of experiments with *fmr1* KO + veh and WT + veh under brighter illumination. Still, no differences were observed in center time between these genotypes, suggesting that under multiple conditions, *fmr1* KO mice are not more anxious in open spaces than WT controls (Figure 5-6B).

Additional Indicator Suggests No Anxiety Phenotype in *Fmr1* KO Mice. In this round of experiments, we also counted the number of fecal boli released by the mice during the 30 min exploration period, as more fecal boli may suggest higher anxiety. There was no difference between *fmr1* KO + veh and WT + veh in this measurement (Figure 5-7). In summary, even in brighter lighting conditions, no difference was observed between *fmr1* KO and WT controls in anxiety

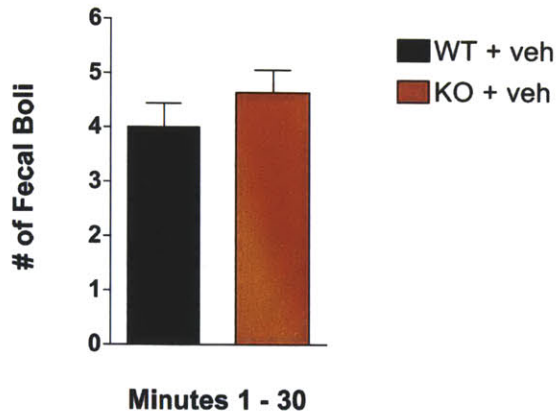

Figure 5-7: **Additional Indicator Suggests No Hypoanxiety in *Fmr1* KO Mice.** Number of fecal boli released by mice in brightly illuminated arena is an additional metric of anxiety. *Fmr1* KO + veh produce a similar number of fecal boli as WT + veh, providing another indication of normal levels of anxiety.

WT + veh, n = 24; *fmr1* KO + veh, n=22. Mean values and SEM are shown (two-tailed unpaired t test for all 30 min: p = 0.3139).

measures in the open field.

5.4 Discussion

In this chapter we demonstrate that administration of FRAX486 in adult *fmr1* KO mice is sufficient to rescue phenotypes analogous to behaviors observed in patients with FXS and idiopathic autism (Figure 5-8). Our data are consistent with the behavioral rescue observed with the *dnPAK* transgene and validate the hypothesis that inhibition of PAK is a valid therapeutic strategy for countering various symptoms of FXS and autism.

5.4.1 FRAX486 Decreases Locomotion in All FVB Mice.

FRAX486 ameliorates hyperactivity in *fmr1* KO mice, but interestingly, this effect is not specific for this mutant. In some ways this is surprising, given that this PAK inhibitor decreased spine density in the temporal cortex of *fmr1* KO mice but not WT littermates. This could be interpreted to mean that spine rescue is not the

mechanism, or at least not the only mechanism, through which FRAX486 decreases locomotion. However, the spine analysis was not comprehensive. While we counted spines, we did not analyze spine morphology. In the *dnPAK* mouse, spine morphology is significantly altered. There is a higher proportion of short, large spines and a corresponding decrease in the numbers of long, filopodia-like spines (Hayashi et al. 2004). In this way, FRAX486 may influence spine morphology in both WT and *fmr1* KO mice, and future studies should consider this. Additionally, spine density or shape changes may occur in other regions of the brain following FRAX486 treatment, including brain areas more closely coupled to locomotion and exploration of a novel environment than the temporal cortex. Future studies, for example, could analyze spine density and shape in motor cortex as well as the hippocampus.

There is precedent in the FXS field for therapeutic treatments that impact both *fmr1* KO mice and WT mice. A recent study by Dr. Richard Paylor's group tested the mGluR theory by treating *fmr1* KO mice and B6 WT littermates with 2 inhibitors of group I mGluRs: mGluR1 antagonist JNJ and mGluR5 antagonist MPEP (Thomas et al. 2011). These scientists compared total distance traveled during a 20 min period of exploration of the open field in the VersaMax Animal Activity Monitoring System, the same behavioral testing set-up used in our experiments. While JNJ did not affect locomotion in *fmr1* KO mice, it did significantly decrease distance traveled in WT mice. MPEP had the opposite effect, increasing activity levels in *fmr1* KO mice at doses of 10 mg/kg and higher and in WT mice at doses of 40 mg/kg and higher. In this way, while neither of the mGluR antagonists decreased activity in *fmr1* KO mice, they did impact locomotion in both WT and *fmr1* KO mice.

There may be an additional explanation for the impact of FRAX486 on WT mice: these mice may also be hyperactive. A research study published by Paylor's group earlier this year investigated the impact that genetic background differences have on performance in behavioral tasks in both WT and *fmr1* KO mice (Spencer et al. 2011). Of the 6 genetic backgrounds tested, mice with genes from the FVB strain (F1 cross of B6 and FVB/NJ) were by far the most active in the open field. This is consistent with other published data on the influence of this specific genetic strain

on mouse behavior (Errijgers et al. 2007). When compared to a pure B6 strain, the B6 x FVB/NJ mice traveled ~40% more and made vertical movements ~50% more (numbers estimated based on data in bar graphs). The effect is even greater when B6 x FVB/NJ was compared to three of the other genetic backgrounds. Hyperactivity was observed for both WT and *fmr1* KO mutants on this background strain. Thus, as WT and *fmr1* KO mice on the FVB strain are hyperactive, FRAX486 may be correcting abnormally high levels of activity in all FVB mice. Future experiments could test whether FRAX486 also impacts activity levels in WT mice of a less active strain, such as the B6 genetic background.

Finally, we sought to test whether FRAX486 may be decreasing activity levels due to sedation. As was mentioned in the previous chapter, sluggishness was sometimes observed in drug treated mice, particularly at the 30 mg/kg dose. This was much less common at 20 mg/kg and was never observed at 10 mg/kg. The data presented in this chapter was collected from mice administered FRAX486 at a dose of 20 mg/kg. In order to address the possibility that the decrease in activity was due to sedation from the drug, we assessed the impact of FRAX486 on motor coordination and balance. Using an accelerating Rotarod treadmill, we demonstrated that 20 mg/kg FRAX486 had no significant effect on performance. This suggests that the decrease in locomotion observed in WT and *fmr1* KO mice treated with FRAX486 is not likely due to sedation.

5.4.2 Investigation of Anxiety in *Fmr1* KO Mice.

Phenotypes in the open field assay – in particular center stay time – are sometimes interpreted as related to anxiety. This is an attractive interpretation in a field aimed at treating autism and FXS patients, many of whom are anxious, particularly in social settings. In the open field test, more anxious mice are thought to avoid open spaces and remain close to the walls, a behavior called thigmotaxis. This behavior is especially evident during the first few minutes of exploration. As WT mice generally prefer to stay in the perimeter of a novel environment, any increase in the amount of time spent in the center of the arena is considered an indication of low anxiety. Some

HUMANS	MICE		
FXS Symptom	**Behavioral Assay**	**Phenotype in *fmr1* KO?**	**Rescued by FRAX486?**
Hyperactivity	**Increased locomotion**	**Yes**	**Yes**
Attention deficit	**Habituation to open field**	**Yes**	**Yes**
Restrictive and repetitive behaviors	**Bouts of stereotyped grooming or circling**	**Yes**	**Yes**
	Repetitive digging in marble burying task	**No**	NA
	Perseveration and cognitive inflexibility in 8-arm radial maze	**No**	NA
Anxiety	**(Hypo)anxiety: Increased time in center of an open field**	**No**	NA

Figure 5-8: **Summary of Behavioral Phenotypes Assayed in *Fmr1* KO Mice.** In this chapter, the open field apparatus was used to assay a variety of behaviors in mice (shown in bold). Additional mouse behavior tasks – including marble burying and the radial maze – were conducted, however since a phenotype was not observed in *fmr1* KO mice in these assays, FRAX486 was not administered. Importantly, FRAX486 rescued all behavioral phenotypes in the mouse model of FXS.

reports in the literature suggest that *fmr1* KO mice are hypoanxious, as they spent significantly more time in the center than WT controls and traveled a significantly greater portion of their distance in the center versus the perimeter (Spencer et al. 2005). This is opposite to what one would expect based on symptoms in humans. In our assay, we did not observe a change in the amount of time spent in the center of the open field in *fmr1* KO mice compared to WT mice. This is consistent with findings from a two-chamber light-dark assay. A collaboration among the laboratories of Dr. David Nelson, Dr. Richard Paylor and Dr. Stephen Warren demonstrated that *fmr1* KO mice and WT mice spent a similar amount of time in the light verses the dark chamber (Peier et al. 2000).

To assess anxiety-related responses and therefore tease out the contributions of hyperactivity and anxiety to the previously described phenotype, the laboratory of Dr. Wim Crusio used an alternative mouse behavior assay, the elevated plus maze (Mineur et al. 2002). The elevated plus maze consists of a plus-shaped apparatus

with two enclosed and two open long, narrow arms connected by a square center area. More anxious mice avoid the open arms, confining their movements to the enclosed arms. No significant difference in arm entries or percent of time spent in the open arms was observed between WT and *fmr1* KO mice (Mineur et al. 2002) (Nielsen et al. 2002). This suggests that hypoanxiety is not the cause of the increased exploratory behavior previously described in the light-dark transition test (Dutch-Belgian Fragile X Consortium 1994).

5.4.3 Perseverative Behaviors in FXS.

In the open field assay, *fmr1* KO mice exhibit repetitive motor actions, including stereotyped grooming and circling. In humans, the phenotype extends beyond the motor realm into the cognitive sphere where it manifests itself as insistence on sameness and cognitive inflexibility. An ideal therapeutic for FXS and idiopathic autism would ameliorate repetitive behaviors, thoughts, and language. Therefore we tested *fmr1* KO mice in additional assays, including the marble burying test of repetitive digging behavior (Thomas et al. 2009) and the eight-arm radial maze (Devenport et al. 1983). We did not observe a phenotype in these assays, and therefore we did not pursue them further to determine whether FRAX486 changes behavior (Figure 5-8).

5.4.4 PAK Signaling during Exploratory Behavior.

Let us turn to another phenotype observed in our experiments: *Fmr1* KO mice do not habituate to a new environment. This phenotype is not regularly reported in the literature, though a closer look at published studies suggests it is a trend (Qin et al. 2002)(Zhao et al. 2005). In particular, expression of this phenotype appears to depend on background strain, as Spencer et al. observed a lack of habituation in *fmr1* KO mice on the B6 x FVB/NJ background but not on other background strains (Spencer et al. 2011). Loss of habituation could be due to a number of factors, the simplest of which is hyperactivity. Furthermore, decreased habituation could be due

to decreased attention to visuospatial cues. This explanation is consistent with two features of the human condition. First, ADHD is a common diagnosis among FXS patients, affecting ~74% of FXS boys (Backes et al. 2000). Therefore, attention deficits may exist in the mice as well. Consistent with a deficit in attention, our previous study reported a phenotype in *fmr1* KO mice on the B6 background strain in the trace fear conditioning task – a version of tone conditioning that is sensitive to attention-distracting stimuli and depends on the integrity of the anterior cingulate cortex (Hayashi et al. 2007)(McEchron et al. 1998)(Han et al. 2003). Second, humans with FXS as well as Williams Syndrome – a disorder linked to the PAK substrate LIMK – have specific deficits in visuospatial tasks. Thus, lack of attention, specifically to visuospatial information, may account for the decrease in habituation. Interestingly, *fmr1* KO mice did not display a phenotype in the eight-arm radial maze, a task that requires working memory and therefore attention (Figure 5-8).

Beyond Williams Syndrome and LIMK, there is additional evidence that the PAK signaling pathway is involved in exploratory behavior, and in particular, the formation of a memory of the explored area. Phosphorylated cofilin (P-cofilin) levels are known to increase in dendritic spines in the hippocampus of rats during exploration of a novel environment (Fedulov et al. 2007). The spines with dense P-cofilin immunoreactivity displayed significantly larger synapses than P-cofilin negative neighboring spines. Additionally, drug treatments that prevent the increase in P-cofilin – such as NMDA receptor antagonist CPP – also block memory for the explored environment. Taken together, these results suggest that phosphorylation of cofilin – an event downstream of PAK activity – and actin-dependent synapse enlargement are induced by exploration of a novel environment and required for the formation of a memory of this space. It would be interesting to see whether these cellular processes are disrupted in the hippocampus of *fmr1* KO mice during exploration of the open field.

5.5 Materials and Methods

5.5.1 Animals and Drug Delivery.

Animals.

Adult male *fmr1* KO mice (FVB.129P2-Fmr1^{tm1Cgr}/J) and age-matched WT controls (FVB.129P2-*Pde6b*$^{+}$Tyr $^{c-ch}$/AntJ, sometimes called FVBS/Ant (Errijgers et al. 2007)) were used of the genetic background which includes the wildtype *Pde6b* allele so the mice do not suffer from blindness due to retinal degeneration (Jackson Labs). Animals 3 - 5 months of age were housed in groups of two to five per cage with food and water freely available. Mice were kept on a 12-h light/dark cycle, and experiments were conducted during the second half of the light cycle. Experiments were performed blind to genotype and drug treatment. All experiments were in accordance with protocols approved by the Massachusetts Institute of Technology Committee on Animal Care and in compliance with National Institutes of Health guidelines.

Handling.

Animals were handled for 2 min per day for 3 days prior to open field testing. On the morning of testing, they were transferred to new cages and housed individually so that social interactions would not interfere with activity or anxiety levels in the open field.

Drug Delivery.

The powder FRAX486 (aFRAXis) was dissolved in 20% hydroxypropyl-β-cyclodextrin vehicle (Sigma-Aldrich). A 2 mg/ml solution of drug or vehicle alone control was administered via subcutaneous injection at 20 mg/kg in a volume proportional to the animals weight (typically 300 - 400 μl). Single injections were given 4.5 - 5.5 h prior to experimentation. Daily injections were given every 24 h for 5 days with the ultimate injection administered 4.5 - 5.5 h prior to the assay.

5.5.2 Behavioral Assay.

The open field assay was performed in a VersaMax activity monitor chamber with the associated VersaDat software (Accuscan Instruments, Columbus,OH). A series of sensors track the animal's horizontal and vertical movements, and the data is organized into 1 min bins. The data are then be analyzed to assess hyperactivity, restrictive or repetitive behaviors, and anxiety. Criteria was established to remove outlier mice based on extreme hyperactivity or adverse effects of FRAX486: Mice that scored more than two standard deviations from the mean in total distance traveled over the 30 min period were removed (3 WT + veh, 2 KO + veh, 1 KO + drug (5 d)). Mice that did not travel horizontally for 5 consecutive minutes were considered sluggish and perhaps sick (3 WT + drug (5 d), 1 KO + drug (5 d), 6 KO + drug (1 d)). These mice were not counted in the statistics.

5.5.3 Rotarod Performance Test.

The Rotarod treadmill consists of a motor-driven, computer-controlled, rotating cylinder divided up into five test zones. One mouse is placed in each test zone on the cylinder while it is stationary such that they are facing away from the eventual direction of the rotation. When all mice are in position, the clock starts and the cylinder begins to rotate. Initial rotation is slow (1 revolution / 12 s) and accelerates in intervals every 40 s. The latency to fall or jump off the rotating cylinder is measured for each mouse (maximum of 3 min). Each mouse is given three trials which are separated by 30 - 40 min, and the average latency for each mouse is used for comparison.

Chapter 6

Cortical Synaptic Transmission and Long-term Potentiation in the *Fmr1* KO Mouse

Contributions

The work described in this chapter was a collaboration between Bridget M. Dolan and two scientists in the Department of Physiology, Seoul National University School of Dentistry, Korea: Dr. Se-Young Choi, Associate Professor, and Hui-Yeon Ko, Ph.D. Candidate. The experimental design and data analysis was a collaboration among all three scientists. The cortical LTP experiments were conducted by Hui-Yeon Ko.

6.1 Summary

The small molecule PAK inhibitor FRAX486 prevents seizures, hyperactivity, and repetitive behaviors and restores habituation to a novel environment in the mouse model of Fragile X Syndrome (FXS). In addition, FRAX486 rescues the dendritic spine phenotype on pyramidal neurons in the cortex of *fragile X mental retardation 1* knockout (*fmr1* KO) mice. We hypothesized that the structural deficit in dendritic spines may be accompanied by a functional deficit in basal synaptic transmission and/or synaptic plasticity. In this chapter we investigate whether *fmr1* KO mice have an impairment in long-term potentiation (LTP), and if so, whether this defect could be rescued by the novel PAK inhibitor FRAX486. Here we show that the mouse line used for all previous experiments – the *fmr1* KO on the FVB mouse strain – does not have a basal transmission or LTP phenotype in layer II/III neurons of the temporal cortex, and that normal synaptic function is not affected by FRAX486. We cannot rule out the possibility that an LTP phenotype exists in other regions of the brain, and that FRAX486 might rescue impairments in those regions.

6.2 Introduction

6.2.1 Intellectual Impairments in Humans But Not Mice with Null Mutations in *Fmr1*.

FXS is sometimes called Fragile X Mental Retardation, as it is the most common known genetic cause of intellectual disability, affecting 1 in 4000 males and 1 in 8000 females. Adult males with FXS usually have moderate to severe intellectual disability with specific deficits in cognitive functions requiring visual-spatial skills, attention, or executive function (Hagerman 1997).

Surprisingly, while the mouse model of the disease has been extensively studied in a large variety of learning and memory assays, a reproducible cognitive phenotype has not been uncovered (reviewed in (Bernardet and Crusio 2006). Despite the lack of a cognitive deficit, many laboratories have used the *fmr1* KO mouse to investigate

what is thought to be the cellular correlate of learning and memory – the phenomena of long-term potentiation (LTP) and long-term depression (LTD).

6.2.2 LTP in the *Fmr1* KO Mouse.

Using a variety of protocols, abnormal phenotypes have been observed by some laboratories in cortical electrophysiology in the *fmr1* KO mouse. Reduced or abnormal LTP has been reported in the somatosensory cortex, olfactory cortex, visual cortex, prefrontal cortex, and temporal cortex (Li et al. 2002)(Zhao et al. 2005)(Larson et al. 2005)(Desai et al. 2006)(Meredith et al. 2007)(Wilson and Cox 2007)(Hayashi et al. 2007). Interestingly, there is no standard protocol for LTP studies in the *fmr1* KO mouse (Figure 6-1).

The original report of cortical plasticity in the *fmr1* KO mouse was conducted on the B6 background where white matter or layer VI at the base of the anterior somatosensory cortex was stimulated and responses from layer IV/V were recorded. One hundred minutes after the third tetanic stimulation, field excitatory postsynaptic potential (fEPSP) amplitudes were ~172% of baseline in WT slices but only ~110% in *fmr1* KO slices (Li et al. 2002). In this experiment, mice were 8 - 10 weeks old. In a subsequent study of cortical LTP in these mice, a different groups of scientists demonstrated that LTP is only abnormal in mice greater than 6 months old and is most pronounced in mice greater than 12 months old. These experiments were conducted in a different cortical region – the primary olfactory (anterior piriform) cortex – an area that has LTP more similar to that of the hippocampus than other cortical regions because of its three layer structure and recurrent network (Larson et al. 2005). In a third study, a couple of cortical plasticity protocols were tested in the somatosensory cortex, the same region investigated in Li et al., however mice were only 10 - 18 days old. In the mGluR5-dependent LTP protocol, intrinsic plasticity was normal in neonatal *fmr1* KO mice, but in the NMDA receptor-dependent LTP protocol, LTP was entirely absent (Desai et al. 2006).

Finally, cortical LTP experiments have been conducted in *fmr1* KO mice on a different background strain: FVB.129P (Zhao et al. 2005)(Wilson and Cox 2007)(Fig-

Protocol	Region	Age	Genetic background	Results	References
Tetanic stimulation	Anterior somatosensory (frontal neocortex)	8-10 weeks	C57BL6	Reduced LTP	Li et al. 2002
TBS (10 burst)	Anterior Piriform Cortex (primary olfactory)	phenotype only in mice >6 months	C57BL6	Reduced LTP	Larson et al. 2005
Pairing protocol (80 pulses, 2 Hz, +30 mV)	Anterior cingulate cortex and lateral amygdala	6-8 weeks	FVB.129P	Loss of LTP	Zhao et al. 2005
Tetanic stimulation	Visual cortex (L5)	p13-25	FVB.129P	Reduced LTP	Wilson and Cox 2007
Spike-timing	Prefrontal cortical (L2/3)	p14-23, p53-100	C57BL6	LTP is not absent, but the threshold for induction is increased	Meredith et al. 2007
Spike-timing Pairing protocol	Somatosensory cortex (L5)	p10-18	C57BL6	Loss of STDP	Desai et al. 2006
TBS (100 Hz)	Temporal cortex (L2/3)	2-3 months	C57BL6	Reduced LTP	Hayashi et al. 2007

Figure 6-1: **Previous Studies on Cortical LTP in *Fmr1* KO Mice.**
A number of studies have been published which demonstrate a reduction in LTP in cortical regions in *fmr1* KO mice. The experiments vary in terms of stimulation protocols, cortical regions, mouse age, and genetic background. To our knowledge, none of these protocols has been reproduced by another laboratory.

TBS, theta-burst stimulation; STDP, spike timing-dependent plasticity; LTP, long-term potentiation; p, postnatal day.

ure 6-1). These studies also changed the age, brain region, and stimulation protocol, so it is difficult to make a proper comparison of effects of background strain. In summary, an LTP deficit has been observed in some studies of the *fmr1* KO mouse and the particulars of the LTP protocol affect the phenotype.

6.2.3 Investigate the Effect of FRAX486 on Cortical Physiology.

In this chapter we investigate whether *fmr1* KO mice have an impairment in basal synaptic transmission or LTP, and whether FRAX486 affects synaptic transmission

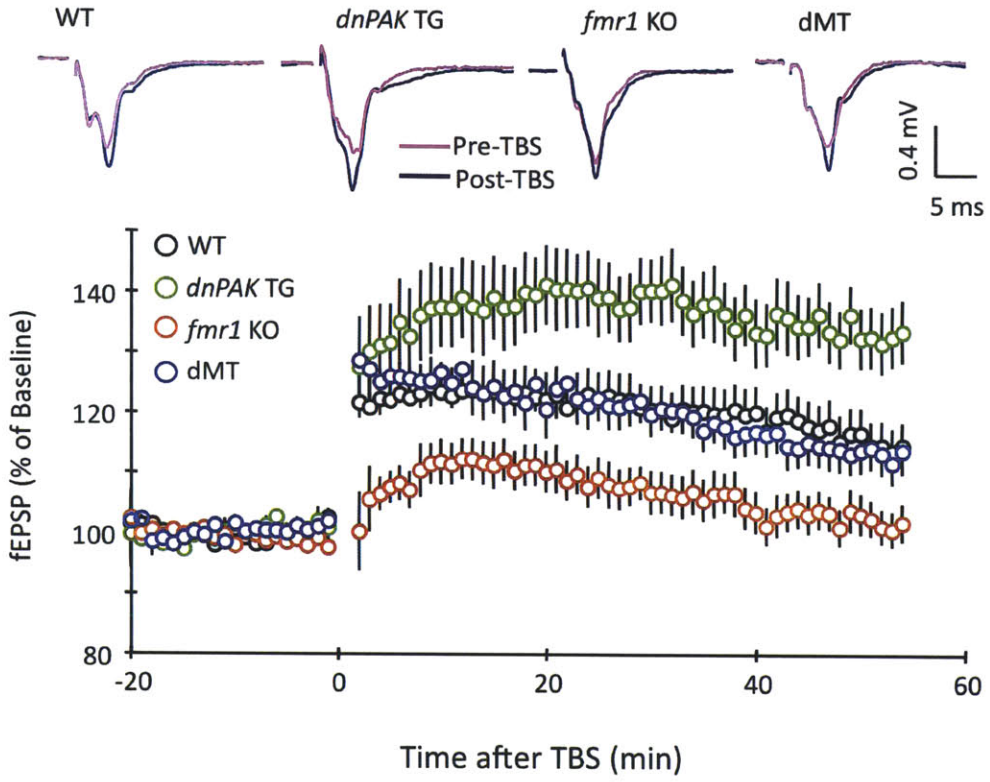

Figure 6-2: **Expression of *dnPAK* in *Fmr1* KO Rescues Cortical LTP Phenotype.** Cortical LTP was induced by TBS at 100 Hz in layer IV and recorded in layer II/III of the B6 mouse temporal cortex. LTP was enhanced in *dnPAK* TG mice compared with WT mice ($p < 0.05$), whereas LTP was reduced in *fmr1* KO mice compared with WT mice ($p < 0.05$). Crossing the *dnPAK* TG and *fmr1* KO mice produced dMT in which the LTP phenotype was rescued ($p > 0.05$). An overlay of representative traces taken during baseline recording and at the final minute poststimulation is shown for each genotype.

Wildtype C57BL6, WT, n = 17 slices, 11 mice; dominant negative *PAK* transgenic, *dnPAK* TG, n = 13 slices, 11 mice; B6 *fmr1* knockout, *fmr1* KO, n = 17 slices, 11 mice; *fmr1* x *dnPAK* double mutant, dMT, n = 13 slices, 9 mice. Data was published in PNAS (Hayashi et al. 2007).

or plasticity in *fmr1* KO mice or WT littermates. Based on our genetic rescue experiments conducted with the *dnPAK* TG mouse (Figure 6-2, we hypothesize that inhibition of PAK with a small molecule inhibitor will be sufficient to rescue an LTP deficit in *fmr1* KO cortex.

6.3 Results

6.3.1 Synaptic Transmission and Plasticity in the FVB *Fmr1* KO Mouse.

Basal Synaptic Transmission Is Normal in Cortical Slices from FVB WT and *Fmr1* KO Mice Treated with FRAX486

Before investigating differences in LTP, we assessed whether the kinase inhibitor FRAX486 interferes with or enhances normal synaptic transmission. Coronal brain slices containing temporal cortex were prepared and incubated in 2 μM FRAX486 dissolved in artificial cerebrospinal fluid (ACSF) or 0.02% DMSO in ACSF vehicle alone control starting 2 h prior to electrophysiology experiments. Extracellular recordings in the temporal cortex layer II/III were taken following stimulation of layer IV. The amplitude of the fEPSP was determined in response to a range of presynaptic stimulus intensities (Figure 6-3).

Basal synaptic transmission did not differ between WT and *fmr1* KO cortex. Here we demonstrate that a 2 h incubation in FRAX486 does not perturb normal synaptic transmission for either genotype ($p = 0.8871$)(Figure 6-3). This suggests that FRAX486 is not toxic to neurons.

Normal Cortical LTP Is Not Disrupted by FRAX486 in FVB WT or *Fmr1* KO Mice

Next, to assess whether synaptic plasticity was altered by the novel kinase inhibitor FRAX486, we induced LTP in cortical slices incubated for 2 h prior to the start of the experiment with 2 μM FRAX486 or vehicle control. Following the protocol utilized

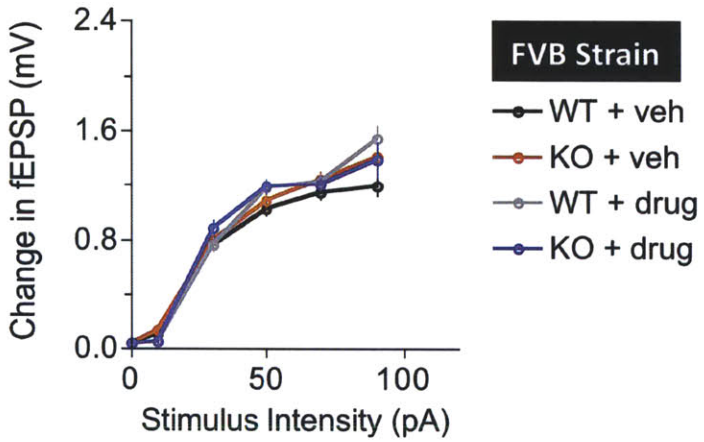

Figure 6-3: **FRAX486 Does Not Interfere with Basal Synaptic Transmission.** Input-output curves plot the changes in field excitatory postsynaptic potential (fEPSP) amplitude and their corresponding presynaptic stimulus intensity for slices of temporal cortex from FVB WT and *fmr1* KO mice treated with DMSO vehicle alone control (WT + veh and KO + veh) or 2 μM FRAX486 (WT + drug and KO + drug). Neither genotype nor drug treatment affects basal synaptic transmission.

FVB WT + veh, n = 41; *fmr1* KO + veh, n = 47; WT + drug, n = 33; *fmr1* KO + drug, n = 34. Mean values and SEM are shown (two-way repeated measures ANOVA to test effect of genotype and drug treatment: FVB, p = 0.8871).

in our previous study in B6 mice, we administered theta-burst stimulation (TBS) at 100 Hz and quantified the evoked population response as the amplitude of the fEPSP, since in the cortex, the initial slope is contaminated by the presynaptic fiber volley. To our knowledge, this is the first investigation of LTP in the temporal cortex of *fmr1* KO mice on the FVB background.

Since we have previously demonstrated that LTP is impaired in this region of the brain in *fmr1* KO mice on the B6 background (Hayashi et al. 2007), and since others have found that FVB.129P mice are deficient in LTP in other regions of the cortex (Zhao et al. 2005)(Wilson and Cox 2007), we hypothesized that LTP would be reduced in *fmr1* KO mice on the FVB.129P2 (FVB) background. (FVB.129P2 differ from the FVB.129P strain only in that they contain the gene that prevents retinal degeneration.) The results of our experiment, however, do not support this hypothesis. Instead, both WT and *fmr1* KO mice on the FVB background exhibited normal levels of LTP in slices from mice age 5 - 6 weeks (Figure 6-4) and 15 - 16 weeks (data not shown). These findings suggest that the LTP phenotype in *fmr1* KO mice is sensitive to the genetic background of the mouse strain and perhaps also age and/or stimulation protocol. Nonetheless, we continued with the experiment and tested the effect of the compound FRAX486 on synaptic plasticity.

To determine whether a 2 h incubation in the novel kinase inhibitor FRAX486 is sufficient to enhance or depress plasticity, we treated both FVB WT and *fmr1* KO slices with the drug and induced LTP by TBS. Here we show that normal LTP was not disrupted by the drug treatment in either genotype on the FVB background (Figure 6-4). This suggests that FRAX486 does not have detrimental effects on normal synaptic plasticity. This finding allows us to go forth with future studies without fear of toxicity. Now we describe experiments that extend our investigation into another mouse strain with the null mutation for *fmr1*.

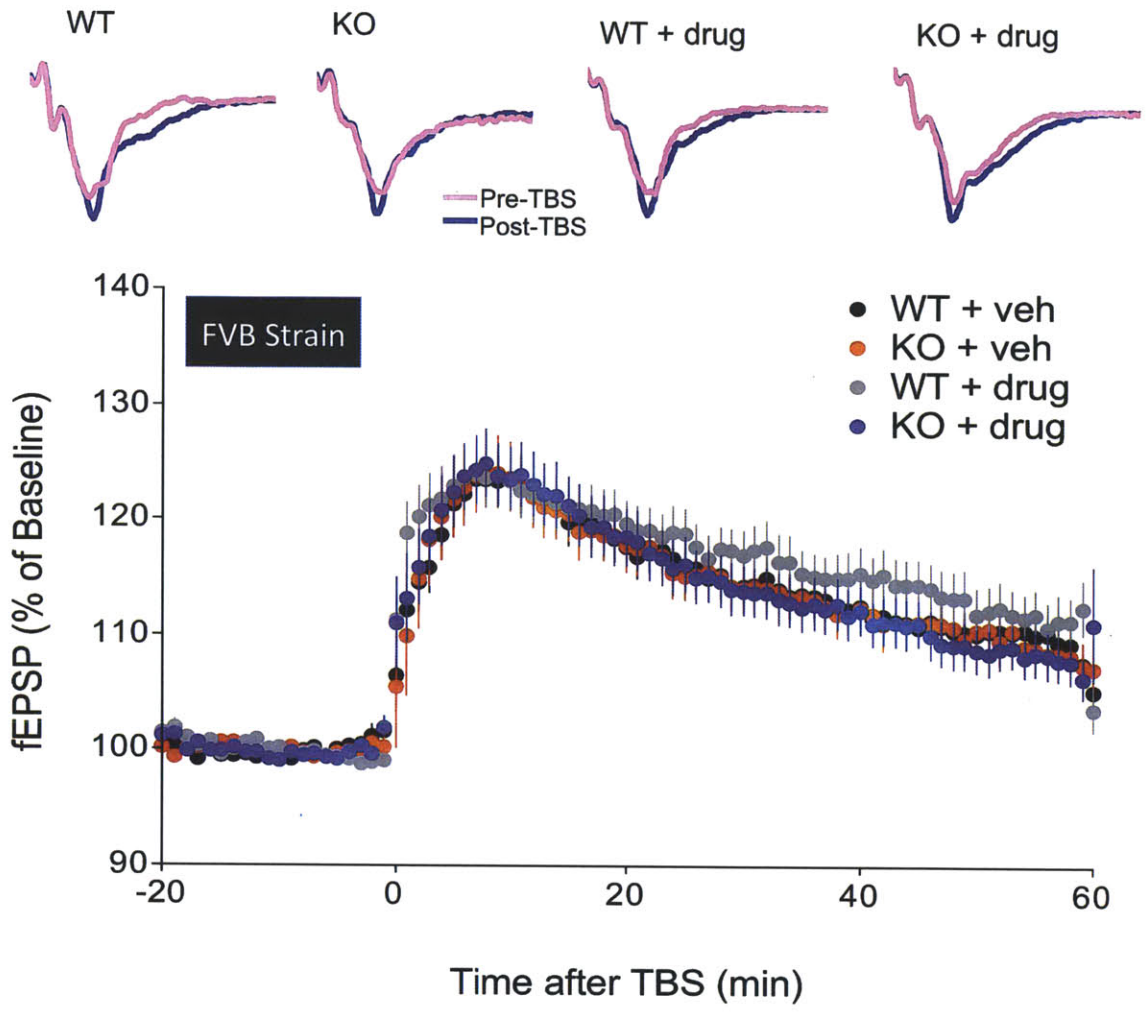

Figure 6-4: **Cortical LTP Is Not Disrupted by FRAX486 in FVB WT or *Fmr1* KO Mice.** LTP was induced by TBS in slices from the temporal cortex of 5 - 6 week old WT and *fmr1* KO mice. Responses, plotted as the amplitude of the fEPSP, were similar for WT and *fmr1* KO slices incubated in the control solution (veh). Brief incubation (2 h) in the novel PAK inhibitor FRAX486 did not alter normal LTP in either genotype.

FVB WT + veh, n = 49 slices, 25 mice; *fmr1* KO + veh, n = 31 slices, 17 mice; WT + drug, n = 48 slices, 23 mice; *fmr1* KO + drug, n = 39 slices, 15 mice. Mean values and SEM are shown (two-way ANOVA for responses at last minute of recording: Interaction, p = 0.1435; Genotype, p = 0.3933; Drug treatment, p = 0.1302; Bonferroni posttests show no significant effect of genotype or FRAX486 treatment).

6.3.2 Synaptic Transmission and Plasticity in the B6 *Fmr1* KO Mouse.

Genotype, But Not Acute FRAX486 Incubation, Impacts LTP

The absence of a synaptic plasticity phenotype in the cortex of *fmr1* KO mice on the FVB background, while consistent with the lack of a cognitive deficit, makes it impossible to test whether FRAX486 is beneficial to mice with deficits in cortical LTP. If we conducted additional LTP experiments with different aged mice, brain regions, and protocols, we might uncover a phenotype in FVB *fmr1* KO mice. Given that a central goal in this thesis was to examine FRAX486's effect on a number of phenotypes in the mouse model of FXS, we repeated the physiology experiments in slices from *fmr1* KO mice on the B6 background, where we have previously demonstrated an LTP phenotype.

First we determined whether genotype or FRAX486 treatment impacted normal synaptic transmission. Consistent with our findings on the FVB background strain, basal synaptic transmission in B6 *fmr1* KO mice was similar to that of WT mice, and FRAX486 does not affect synaptic output ($p = 0.8939$). The latter finding gives us additional confidence that FRAX486 is not detrimental to synaptic signaling and therefore does not appear toxic to neurons.

Then we compared responses to TBS in B6 WT and *fmr1* KO mice. Here we discovered that genotype impacts LTP. That is, LTP was impaired in the cortex of B6 *fmr1* KO mice when compared to WT littermates (Figure 6-6). Statistical comparisons were conducted for the final minute of recording (two-way ANOVA with variable genotype and treatment for responses 60 minutes after TBS: Interaction, $p = 0.0972$; Genotype, $p = 0.0436$; Drug treatment, $p = 0.1418$), as was done in our 2007 *PNAS* paper, and also as repeated measures of the last 10 minutes of recording, which is also common in the LTP literature (two-way ANOVA with repeated measures for minutes 51 - 60 after TBS: WT + veh vs. KO + veh, $p = 0.0344$). The results of both statistical comparisons confirmed that on the B6 background there was a synaptic plasticity phenotype in the *fmr1* KO mouse when compared to WT litermates.

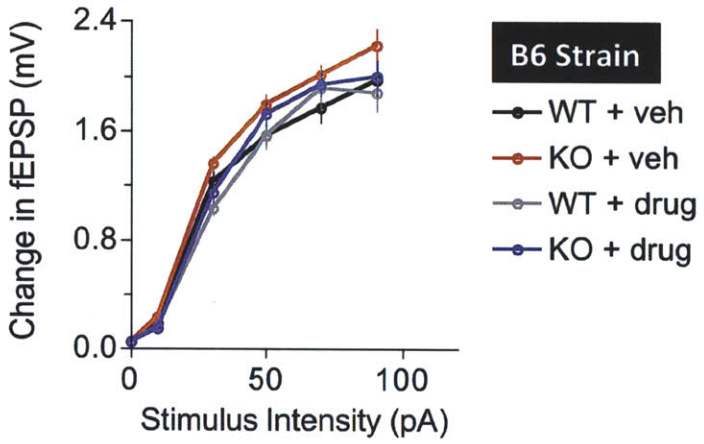

Figure 6-5: **FRAX486 Does Not Interfere with Basal Synaptic Transmission.** Input-output curves plot the changes in field excitatory postsynaptic potential (fEPSP) amplitude and their corresponding presynaptic stimulus intensity for slices of temporal cortex from B6 WT and *fmr1* KO mice treated with DMSO vehicle alone control (WT + veh and KO + veh) or 2 μM FRAX486 (WT + drug and KO + drug). Neither genotype nor drug treatment affects basal synaptic transmission.

B6 WT + veh, n = 19; *fmr1* KO + veh, n = 28; WT + drug, n = 16; *fmr1* KO + drug, n = 27. Mean values and SEM are shown (two-way repeated measures ANOVA to test effect of genotype and drug treatment: B6, p = 0.8939).

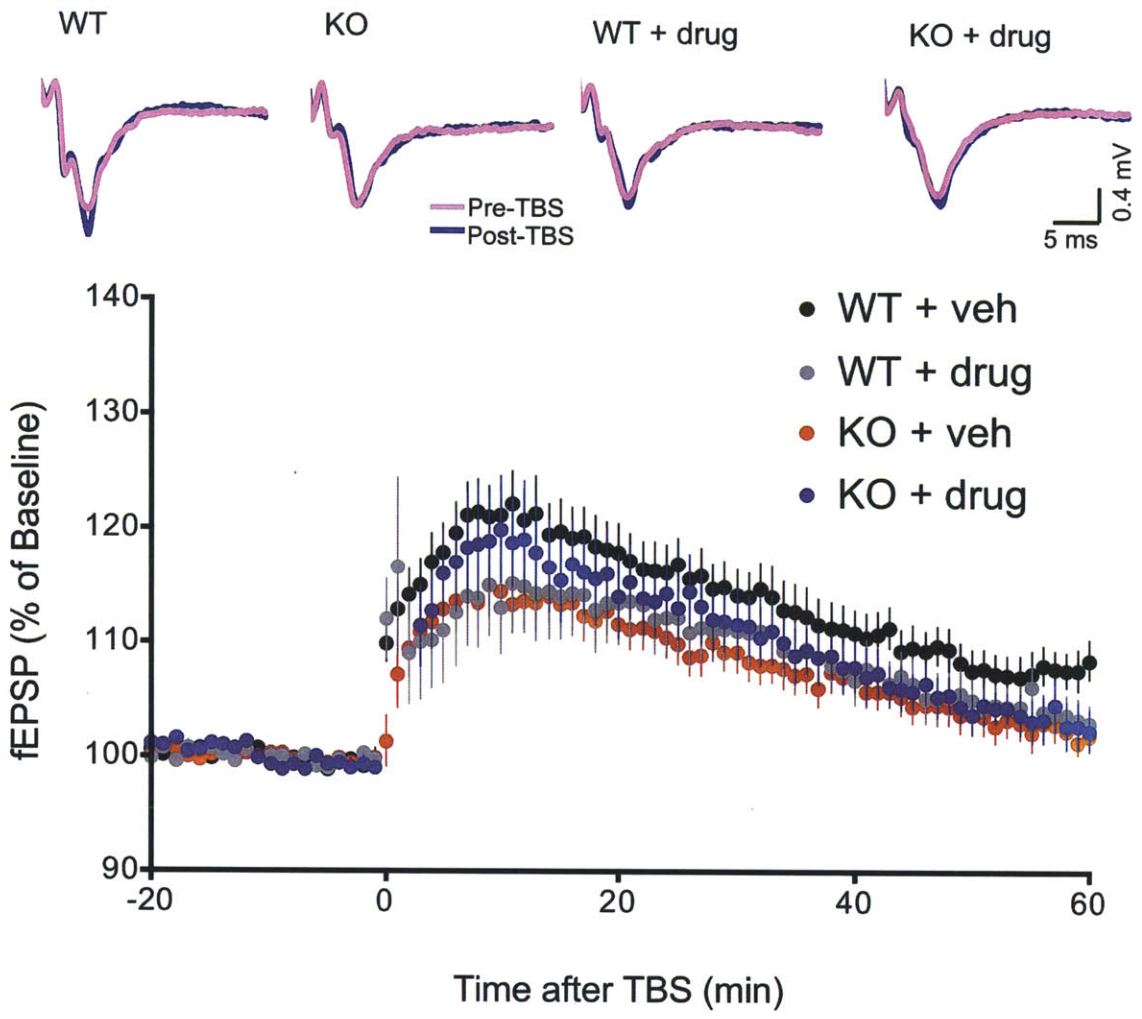

Figure 6-6: **Cortical LTP in FRAX486 Treated Slices Is Indistinguishable from Both Normal LTP in B6 WT and Reduced LTP in B6 *Fmr1* KO Mice.** Extracellular recordings in the temporal cortex layer II/III demonstrate reduced LTP in B6 *fmr1* KO mice when compared to WT controls. Incubation of WT slices in FRAX486 for 2 h prior to recording has no significant impact on LTP, though there is a trend toward a reduction in LTP. FRAX486 treatment of *fmr1* KO slices produces LTP that is similar to WT + veh responses and also similar to KO + veh responses. In addition, there is a trend towards a *fmr1* KO specific effect of FRAX486 on short-term potentiation (i.e. in the first 15 min poststimulation) though it does not reach significance.

FVB WT + veh, n = 16 slices, 12 mice; *fmr1* KO + veh, n = 14 slices, 9 mice; WT + drug, n = 13 slices, 8 mice; *fmr1* KO + drug, n = 10 slices, 7 mice. Mean values and SEM are shown (two-way ANOVA for responses at last minute of recording: Interaction, p = 0.0972; Genotype, p = 0.0436; Drug treatment, p = 0.1418; Bonferroni posttests show a significant effect of genotype on veh but not drug treated slices).

Next we tested whether treatment with a small molecule inhibitor of PAK could rescue the LTP deficit in *fmr1* KO slices. Our results indicate that a 2 h incubation in FRAX486 produced LTP in *fmr1* slices that was indistinguishable from LTP in both WT and *fmr1* KO slices treated with vehicle control. That is, FRAX486 treated *fmr1* KO slices showed LTP that was similar to vehicle treated WT slices, and thereby passed the test of rescuing the LTP phenotype. However, the impact of drug on *fmr1* KO was not large enough to make KO + drug significantly different from KO + veh (two-way ANOVA with repeated measures for minutes 51 - 60 after TBS: KO + drug vs. KO + veh, $p = 0.6663$). In addition, while FRAX486 treatment did not significantly alter LTP in WT slices, there was a trend towards decreased LTP in WT slices incubated in drug. Additional experiments should be conducted to increase the number of slices treated with drug and increase the statistical power of the data.

6.3.3 FRAX486 May Increase Initial Response to TBS in B6 *Fmr1* KO.

Another trend appeared in these experiments: In the first 10 minutes following TBS, the amplitude of responses in FRAX486 treated *fmr1* KO slices showed a trend more similar to WT + veh than *fmr1* KO + veh (two-way ANOVA for responses at minute 10 of recording: Interaction, $p = 0.0580$; Genotype, $p = 0.09720$; Drug treatment, $p = 0.7079$). Neither genotype nor treatment alone is sufficient to explain the result. There was a trend approaching significance of the combined effect of the interaction of genotype and treatment, leaving the reader to decide whether it is important ($p = 0.058$). This trend in the early time points after TBS, demonstrated that treatment almost had a different effect on *fmr1* KO than WT.

6.4 Discussion

A novel PAK inhibitor rescues behavioral abormalities in the mouse model of FXS. In addition, FRAX486 rescues the dendritic spine phenotype on pyramidal neurons in

the cortex of *fmr1* KO mice. We hypothesized that the structural deficit in dendritic spines may be accompanied by a functional deficit in basal synaptic transmission and/or synaptic plasticity. Therefore, we assayed cortical LTP in *fmr1* KO mice. Here we show that the mouse line used for all previous experiments – the *fmr1* KO on the FVB mouse strain – does not have a basal transmission or LTP phenotype in layer II/III neurons of the temporal cortex. Then we demonstrated that normal synaptic function was not affected by FRAX486.

6.4.1 *Fmr1* KO Mice on the FVB Background Do Not Exhibit a Cortical Physiology Phenotype.

In this chapter, we first demonstrate that the input-output ratio of synaptic activity is unchanged in FRAX486 treated cortical slices in both WT and *fmr1* KO mice *in vitro*. This finding suggests that AMPA receptor-mediated basal synaptic transmission was normal in FRAX486 treated temporal cortex. Then we proceeded to test the effect of this kinase inhibitor on synaptic plasticity. We were unable to detect an LTP phenotype in *fmr1* KO slices on the genetic background used for all previous experiments. That is, synaptic plasticity was normal in 5 - 6 week old and 15 - 16 week old *fmr1* KO mice of the FVB background. While a couple of studies have reported an LTP deficit in *fmr1* KO mice on the FVB background, and multiple studies have reported a deficit on the B6 background, there is no standard protocol for these LTP studies. To our knowledge, none of these findings has been reported with an identical protocol in more than one laboratory. In this way, it is not surprising that under the specific circumstances of our protocol, a phenotype was not observed. If we conducted additional LTP experiments with different aged mice, brain regions, and protocols, we might find the right conditions to observe a phenotype in *fmr1* FVB mice. Given that a primary goal was to examine if FRAX486 can rescue multiple *fmr1* KO phenotypes – either independently or through a single, concerted mechanism – we also used a model in which there is a cortical LTP deficit: B6 mice.

6.4.2 Synaptic Plasticity in the Temporal Cortex Is Not Affected by a 2 Hour Incubation with FRAX486.

Overall, a 2 hour incubation with FRAX486 has a minimal effect on cortical synaptic plasticity. There may be a few reasons why this result is inconsistent with our findings in the genetic rescue experiments with the *dnPAK* transgenic mouse. First, 2 hours may not be a sufficient amount of time for significant rescue of the dendritic spine density and morphology phenotype. It may take longer for cytoskeletal dynamics to decrease spine density and/or support the maturation of spines required for full expression of potentiation.

Longer Incubation Periods May Be Required for the Full Effect of FRAX486 on Cortical Slices.

Results of preliminary experiments in which slices were incubated in FRAX486 for 4 hours prior to TBS suggest that a longer incubation may be sufficient to rescue the deficit (data not shown). Unfortunately, given the limitations of using a slice preparation, neither vehicle nor drug treated slices were as healthy after so many hours, and therefore the results were quite variable. In the future, mice could be treated with FRAX486 *in vivo* for days or weeks, then slices could be removed for LTP experiments.

6.4.3 Additional Targets of FRAX486 May Complicate the Interpretation of Results.

Secondly, FRAX486 may be having an effect on more than one kinase. For example, studies have shown that the phosphorylation of ERK is required for the induction and maintenance of LTP in the hippocampus (Kelleher et al. 2004)(Xin et al. 2006). Since MEK is inhibited by FRAX486, ERK activation may be decreased in treated slices. In this way, ERK inhibition may be furthering the LTP phenotype while PAK inhibition is acting to rescue the deficit. These opposite actions may cancel each other out.

6.4.4 Future Studies May Focus on the Hippocampus or Other Cortical Regions.

Finally, the temporal cortex may not be the ideal location for LTP studies to test the effect of drugs on amelioration of phenotypes in the *fmr1* KO mouse. Future experiments on other brain regions, perhaps even regions thought to be involved in hyperactivity and seizure, may yield a stronger LTP rescue. Alternatively, FRAX486 could be tested for its ability to reverse the enhanced mGluR-LTD consistently observed in the hippocampus.

6.4.5 Targeting of Group I PAKs with a Specific Genetic Strategy.

Strikingly, in *fmr1* KO mice, cortical LTP abnormalities are opposite to those in *dnPAK* TG mice (Hayashi et al. 2004). Similarly, cortical spine morphology phenotypes observed in FXS humans and the mouse model of the disease are opposite to those seen in the *dnPAK* TG mice. In our earlier PNAS publication we demonstrated that abnormal cortical LTP and spine morphology are fully restored by expression of *dnPAK* in the adult *fmr1* KO mouse (Hayashi et al. 2007)(Figure 6-2). In the previous chapter of this thesis, we demonstrate that we can replicate the spine density rescue with a small molecule PAK inhibitor, FRAX486. Here we investigated whether acute exposure to FRAX486 is also sufficient to rescue a LTP phenotype in cortical slices from a mouse model of FXS. We discovered that a 2 hour incubation in FRAX486 is not sufficient to impact plasticity in the temporal cortex. The discrepancy may be related to the time course of PAK inhibition. In the genetic rescue strategy, PAK activity was decreased by ~59% in the cortex for more than 3 weeks prior to experimentation. In addition, the previous studies inhibited PAK *in vivo*, while the data in this chapter was generated following brief incubation *in vitro*. Future studies, involving *in vivo* administration of FRAX486 prior to *in vitro* physiology experiments, will be required to determine whether a small molecule PAK inhibitor can replicate the correction of a cortical LTP deficit as observed in the genetic rescue

strategy.

6.4.6 Conclusion.

Taken in the context of all previous chapters in this thesis, our electrophysiology data suggest that restoration of cortical LTP is not the mechanism of rescue of behavioral symptoms. Amelioration of seizures, hyperactivity, lack of habituation, and stereotyped behaviors is more likely linked to, or even caused by, the correction of the dendritic spine phenotype in *fmr1* KO mice.

6.5 Materials and Methods

6.5.1 Animals.

Adult male *fmr1* KO mice were on the C57BL/6 background or the FVB.129P2 background. To generate experimental subjects, females heterozygeous for *fmr1* KO were crossed to WT males (Jackson Labs) to produce *fmr1* KO males and WT male littermates. Animals were breed at MIT, weaned, and housed in groups of 2 to 5 per cage, on a 12 h light/dark cycle, and with food and water freely available. At 3 - 8 weeks of age, they were shipped to Seoul National University in Korea and housed in the facility of Dr. Se-Young Choi. Experiments were performed on 5 - 6 week and 15 - 16 week old FVB mice and 8 - 9 week old B6 mice according to protocols approved by the Massachusetts Institute of Technology Committee on Animal Care and in compliance with National Institutes of Health guidelines.

6.5.2 Electrophysiology.

Coronal brain slices containing temporal cortex were prepared and incubated in room temperature, oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid containing 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM dextrose, and 2 μM FRAX486 or DMSO vehicle. FRAX486 was first dissolved in DMSO to make a 10 mM stock solution, and then further diluted

in this ACSF bath solution for the final concentration of 2 μM. DMSO alone was used as a control (final concentration 0.02%). Slices were incubated in FRAX486 or vehicle for 2 h prior to the electrophysiology experiment. Then slices were transferred to a recording chamber and submerged in the same solution at 30°C. The stimulating electrode was placed in cortical layer IV and the recording electrode was placed in layer II/III (as developed in Mark Bears laboratory by Alfredo Kirkwood and described in (Kirkwood et al. 1996) and modified for the temporal cortex in Alcino Silvas laboratory and detailed in (Frankland et al. 2001). Cortical long-term potentiation (LTP) was induced by theta-burst stimulation (TBS), consisting of eight brief bursts of stimuli (each with four pulses at 100 Hz) delivered every 200 ms. Stimulation consisted of four trains (each with 32 pulses at 10 Hz) delivered at 10 s intervals. The evoked population excitatory synaptic potential was quantified as the amplitude of the extracellular field potential, since in the cortex, the initial slope is contaminated by the presynaptic fiber volley. Clampex and Clampfit software was used to acquire and analyze the data. Mean values and SEM were expressed as percentage of the pre-TBS baseline responses (at least 20 min of stable responses, that is within 5% of the mean baseline). Unhealthy slices were identified by responses that fell more than 5% below baseline. LTP induction was assessed by comparing recorded responses after TBS. Statistical significance was calculated using two-way ANOVA and the Bonferroni post test in the statistical program GraphPad Prism.

Chapter 7

Conclusion

7.1 Relationship to Prior Studies.

This thesis work builds upon discoveries made in our laboratory about the role of PAK in the adult forebrain (Hayashi et al. 2004). Through the creation of a transgenic mouse that expresses *dnPAK* in specific brain regions after development, Hayashi et al. demonstrated that PAK is a critical regulator of dendritic spine density and morphology in pyramidal neurons of the mouse cortex. Dysregulation of PAK resulted in impairments in the consolidation of long-term memories, presumably due to its effects on actin dynamics and structural plasticity. Interestingly, the spine phenotype observed in the mouse was opposite to that reported in mice and humans with FXS. This led to the theory that PAK and FMRP may antagonize one another in a healthy brain, and that in the absence of FMRP this balance may be disrupted. Therefore we predicted that downregulating PAK activity in the brain of *fmr1* KO mice, even late in life, would be sufficient to reverse phenotypes associated with FXS. Our earlier study tested this prediction using a genetic rescue strategy (Hayashi et al. 2007). While this was a valuable proof of principle, it is not an effective strategy for human therapeutics. The work described in this thesis aims to translate our genetic rescue strategy into a therapeutic intervention appropriate for patients with FXS, idiopathic autism, and intellectual disability.

At the start of this thesis project, there was no potent small molecule PAK in-

hibitor for *in vivo* experiments. We began by testing commercially available putative PAK inhibitors – OSU-03012 and emodin – but discovered that they were poor PAK inhibitors, and in the case of emodin, did not cross the blood-brain barrier. Through a collaboration with Dr. Sergio Duron of Afraxis – the company which licenses our patent for the use of PAK inhibitors for the treatment of neurodevelopmental disorders – we screened thousands of compounds for their ability to bind to and inhibit group I PAKs. The best compound to come out of this screen as of 2010 was the small molecule FRAX486. This thesis work represents the first study to test the effect of FRAX486 *in vivo*.

Ninety-eight percent of small molecule drugs do not penetrate our target tissue: the brain. Therefore, our first *in vivo* experiments tested concentrations of FRAX486 in the brain at multiple time points following subcutaneous administration. Not only did FRAX486 cross the blood-brain barrier, but it remained in the brain at clinically relevant concentrations for up to 24 hours. Due to these excellent pharmacokinetic properties, we set forth to test the effect of our novel PAK inhibitor on FXS phenotypes in mice.

A critical early step in our experimental strategy was to determine whether FRAX486 could reverse the dendritic spine phenotype in *fmr1* KO mice. Once it was established that FRAX486 rescues the spine density abnormality in the mouse model of FXS, we could proceed to test the hypothesis that a therapeutic strategy that corrects the spine phenotype may also correct behavioral phenotypes. While one cannot test whether the rescue of spine abnormalities is directly and causatively responsible for the behavioral rescue, accumulating evidence in the human literature suggests a causative relationship. That is, in autism patients, the severity of the spine phenotype correlates with the severity of the cognitive deficit (Hutsler and Zhang 2010). In this way, we predicted that rescuing spine phenotypes may lead to a correction in symptoms.

The *dnPAK* mouse was engineering on a B6 background strain, and therefore the rescue experiments conducted in our previous study were conducted in B6 *fmr1* KO mice. We did not have this limitation in the present study. Therefore we had the

HUMANS	MICE		
FXS Symptom	**Behavioral Assay**	**Phenotype in *fmr1* KO?**	**Rescued by FRAX486?**
Seizures	**Sound-induced seizures**	**Yes**	**Yes**
Hyperactivity	**Increased locomotion**	**Yes**	**Yes**
Attention deficit	**Habituation to open field**	**Yes**	**Yes**
Restrictive and repetitive behaviors	**Bouts of stereotyped grooming or circling**	**Yes**	**Yes**
	Repetitive digging in marble burying task	**No**	NA
	Perseveration and cognitive inflexibility in 8-arm radial maze	**No**	NA
Anxiety	(Hypo)anxiety: Increased time in center of an open field	**No**	NA
Intellectual disability	Memory deficit in trace fear conditioning	**No**	NA
Imparied social interactions	Abnormal social interactions in 3 chamber assay	**No**	NA
	Decreased social dominance in tube test	**No**	NA

Figure 7-1: **FRAX486 Rescues All Observed Behavioral Phenotypes.**

flexibility to switch to a genetic background with a more pronounced neurological and behavioral phenotype: FVB. *Fmr1* mice on the FVB background are more seizure prone and hyperactive than their B6 counterparts (Yan et al. 2005)(Chen and Toth 2001). While this had a behavioral advantage, we did not predict that there would also be a significant disadvantage to this mouse line: less pronounced dendritic spine density phenotype and no deficit in cortical LTP. Luckily, while the *fmr1* KO mouse displayed a spine density phenotype in fewer dendritic segments on the FVB strain than the B6 strain, it was sufficient to test whether FRAX486 could rescue this phenotype. This was not the case with cortical LTP, where no deficit was present. Based on our findings in the *fmr1; dnPAK* double mutant, we predict that a small molecule PAK inhibitor like FRAX486 would reverse a functional synaptic deficit if one existed.

For the first time, we were able to inhibit PAK activity in the seizure-prone FVB *fmr1* KO mice. This is an important assay, as seizures are present in both humans and mice with FXS and are potentially debilitating (Musumeci et al. 1999)(Chen and Toth 2001)(Yan et al. 2004). FRAX486 rescued the AGS phenotype in a dose-dependent fashion. For the first time, we were able to test the impact of PAK inhibition on the habituation phenotype, another behavioral abnormality specific to the FVB background strain. However, we were not able to test whether the compound could reverse a cognitive phenotype, as unlike in the earlier study, we were unable to observe a trace fear conditioning deficit in *fmr1* KO mice. Similarly, while we developed assays to test for a variety of behavioral phenotypes in *fmr1* KO based on predictions from the human condition, FVB *fmr1* KO mice performed normally (see Figure 7-1 for a summary of assays I performed on these mice). More specifically, *fmr1* KO mice do not display restrictive or repetitive behaviors in a marble burying task nor in the eight-arm radial maze. Additionally, *fmr1* KO mice do not exhibit abnormal social behaviors in a social interaction and social novelty test nor in a social dominance test (three-chamber social interaction task and tube test, respectively). In summary, while many behavioral assays were not useful for testing the therapeutic potential of a drug, FRAX486 corrected deficits in all assays in which *fmr1* KO mice

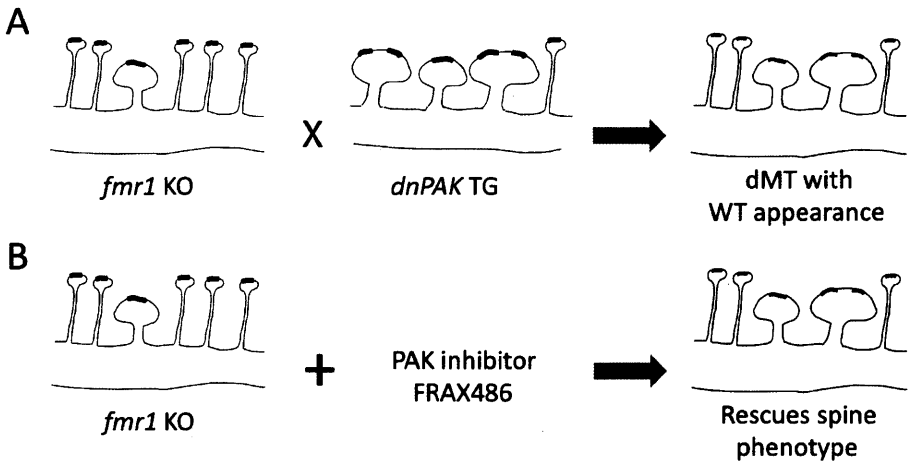

Figure 7-2: **Inhibition of PAK Reverse the Spine Phenotype.** Using either a genetic (A) or pharmacological (B) strategy, inhibition of PAK is sufficient to correct the dendritic spine phenotype in neurons in the mouse model of FXS. Importantly, it is this cellular rescue that we hypothesize is critical to the reversal of behavioral and neurological symptoms in *fmr1* KO mice.

displayed a phenotype. In this way, the results of genetic rescue in B6 *fmr1* KO mice and pharmacological rescue in FVB *fmr1* KO mice compliment one another in support of the discovery that inhibition of PAK corrects phenotypes in the mouse model of FXS.

7.2 Mechanism of Rescue.

Our hypothesis-driven therapeutic strategy focuses on correction of abnormal dendritic spines in diseases like FXS, intellectual disability, and disorders on the autism spectrum. In this way, we inhibited PAK in order to reverse the dendritic spine deficit, as we hypothesized that this will ameliorate symptoms of FXS. A cartoon in Figure 7-2 represents the changes in spine density and morphology that result from inhibition of PAK in *fmr1* KO mice.

The precise molecular mechanism of rescue is beyond the scope of this thesis. Based on what is known about signaling in the *fmr1* KO mouse, we put forth three

models of how PAK and FMRP may interact to antagonize one another in healthy neurons. First, FMRP may antagonize PAK (Figure 7-3A). As FMRP is known to be a translation repressor, it may act through downregulation of the synthesis of PAK or other members of the PAK signaling pathway. Experiments in murine fibroblasts suggest that FMRP binds to Rac1 mRNA and interferes with Rac1-induced remodeling of the actin cytoskeleton (Castets et al. 2005). Alternatively, FMRP could alter the localization of PAK within dendrites. Studies in hippocampal neurons observed a marked increase in PAK3 levels in the PSD of spines in *fmr1* KO mice (Chen et al. 2010). This would position PAK to respond to incoming signals with efficiency and magnitude. In this way, PAK may be overactive in response to synaptic activity in *fmr1* KO mice. Therefore, inhibition of PAK would counteract this to return PAK signaling to healthy levels.

Second, there is evidence that FMRP normally acts to modulate the cytoskeleton. FMRP binds to mRNA for the microtubule stabilization protein MAP1B and its *Drosophila* homolog Futsch (Menon et al. 2008)(Zhang et al. 2001). Through this action of FMRP, and PAK's well established role as a regulator of actin, these pathways may converge to antagonize cytoskeletal dynamics (Figure 7-3B).

Finally, the mGluR theory – the predominant theory of FXS – is based on the observation of abnormal mGluR signaling in *fmr1* KO mice (Huber et al. 2002)(Bear et al. 2004). In healthy neurons, FMRP appears to act as a break on mGluR signaling, and therefore in its absence there is excessive signaling downstream of group I mGluRs. The precise mechanism of how the loss of FMRP relates to mGluRs is unknown, but it is known to produce excessive levels of basal protein synthesis. ERK1/2 is downstream of mGluR signaling and regulates protein synthesis. The translation machinery is hypersensitive to ERK1/2 signaling in neurons from *fmr1* KO mice (Osterweil et al. 2010). Therefore a therapeutic strategy than downregulates ERK1/2 signaling may be sufficient to reverse the runaway protein synthesis that is observed *in vitro* and *in vivo* in neurons lacking expression of *fmr1* (Osterweil et al. 2010)(Qin et al. 2005) (Figure 7-3C). The ERK1/2 pathways is also regulated by PAK through phosphorylation of MEK (Frost et al. 1997). In this way, inhibition

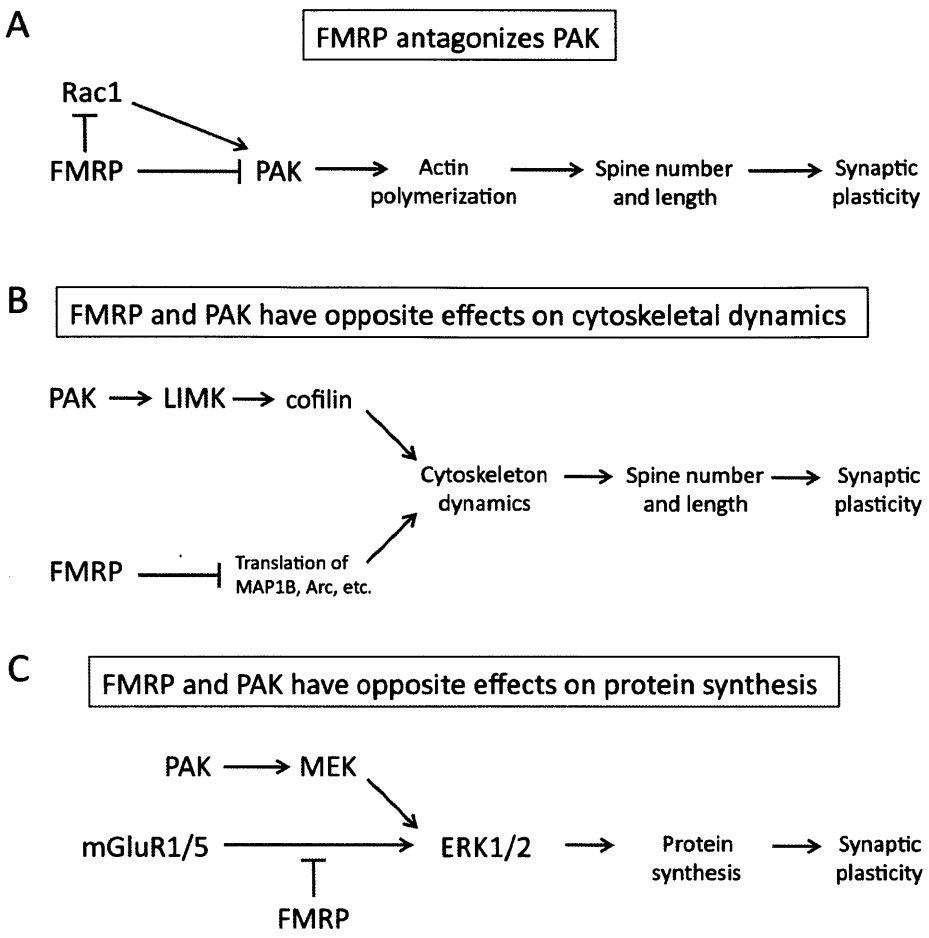

Figure 7-3: **Models for the Interaction of PAK and FMRP that Underlies the Molecular Mechanism of Rescue.**

of PAK may be sufficient to compensate for the absence of FMRP in *fmr1* KO mice and result in levels of protein synthesis similar to those observed in WT mice.

7.3 Alternatives to the PAK Hypothesis of FXS.

The advantage of a genetic rescue strategy that utilizes a fragment of an endogenous protein to act as a dominant negative is specificity. Therefore from our previously published work with *dnPAK*, we can conclude that inhibition of PAK kinase activity is sufficient to correct morphological, functional, and behavioral phenotypes in the

mouse model of FXS. In taking the next step and venturing into the drug discovery arena, we have sacrificed some of that specificity. In this way, we cannot say with confidence that inhibition of PAK and only PAK is responsible for the alleviation of FXS-like symptoms including seizures, hyperactivity, and restrictive and repetitive behaviors. These phenotypes may be impacted by the inhibition of another member of the actin remodeling pathway (LIMK) or other regulators of MEK/ERK signaling (MEK, B-Raf, Ret, ErbB4, or Src). As the MEK/ERK pathway regulates protein synthesis, and excess levels of protein synthesis are reported in *fmr1* KO mice, inhibition of MEK, B-Raf, Ret, ErbB4, or Src may be responsible or partially responsible for the observed effect.

Importantly, whatever the molecular mechanism of rescue may be, it results in an important cellular rescue: reversal of excessive numbers of dendritic spines. In this way, we can say with certainty that a rescue strategy that corrects the dendritic spine phenotype also corrects seizures, hyperactivity, loss of habituation to a novel environment, and repetitive behaviors.

7.4 Implications and Future Directions.

Future experiments may be conducted to determine the precise molecular mechanism of FRAX486 activity *in vivo*.

Which target(s) of FRAX486 are inhibited *in vivo*?

To determine the kinase inhibitory profile of FRAX486 *in vivo*, the phosphorylation state of specific proteins in brain tissue of mice treated with FRAX486 may be analyzed and compared to that of untreated mice. More specifically, Western blots may be run to probe for the phosphorylation state of substrates of PAK and LIMK – in particular cofilin – to determine whether basal activity of the actin remodeling pathway is modulated by FRAX486. Complementary to this, Western blots for substrates of MEK, B-Raf, Ret, ErbB4, and Src – regulators of the MEK/ERK signaling pathway – will answer questions about whether these pathways may be responsible,

or partially responsible, for the observed effects.

Does FRAX486 reverse the protein synthesis phenotype in *fmr1* KO mice?

If MEK/ERK signaling is abnormal, perhaps FRAX486 rescues the excessive protein synthesis phenotype consistently reported in *fmr1* KO mice. *In vitro* and *in vivo* experiments may determine whether basal levels of protein synthesis are affected by FRAX486, and therefore whether the final model presented in Figure 7-3 is a likely contributor to the rescue effects. To start, the *in vitro* metabolic labeling approach of Dr. Mark Bear's laboratory may be utilized to determine whether *in vitro* treatment of brain slices with FRAX486 is sufficient to decrease protein synthesis in *fmr1* KO or WT slices (Auerbach and Bear 2010). If FRAX486 decreases translation in slices, then the *in vivo* quantitative autoradiographic L-[1-14C]leucine method of Dr. Carolyn Smith's laboratory may be employed to investigate whether FRAX486 acts, at least in part, by reversing the elevated protein synthesis in the mouse model of FXS (Qin et al. 2005). It is possible that in order to treat the maximum number of symptoms in *fmr1* KO mice and FXS humans, the most effective strategy will target both dendritic spines and translation. These experiments, in combination with the discoveries presented for the first time in this thesis, will determine whether FRAX486 is a drug with these therapeutically valuable dual actions.

Does FRAX486 impact spine shape?

Both spine density and spine morphology abnormalities have been reported in humans with FXS, autism, and intellectual disability (Irwin et al. 2001)(Hinton et al. 1991)(Rudelli et al. 1985)(Hutsler and Zhang 2010). As spine density, but not spine morphology, was quantified in this thesis work, we do not yet know whether FRAX486 effects spine length or volume in *fmr1* KO mice or WT mice. This would be interesting to know, especially since FRAX486 modulated behavior in both genotypes. The drug treated brain samples from our experiments may be reanalyzed to determine whether our kinase inhibitor regulates spine morphology, and if so, whether it affects only *fmr1* KO mice – as was observed with spine density – or also WT littermates.

Do other PAK inhibitors rescue spines, seizures, hyperactivity, and repetitive behaviors in *fmr1* KO mice?

Next, we would like to determine whether the pharmacological rescue reported in this thesis work is specific to the chemical compound FRAX486 or inhibition of the kinase PAK. To do so, we propose testing the next generation of PAK inhibitors – molecules that are more specific for PAK and/or have different inhibitory profiles than FRAX486 – in the same battery of experiments. Afraxis is generating these small molecule inhibitors and moving forward with morphological assays. Then, the most promising compounds may be tested for their ability to rescue seizures, hyperactivity, and repetitive behaviors.

Do PAK inhibitors alleviate symptoms in people with FXS, idiopathic autism, and/or intellectual disability?

The work described in this thesis demonstrates that a small molecule PAK inhibitor rescues FXS phenotypes in a mouse model of autism. This is an essential step in the drug discovery process, however it is not enough to stop there. The goal of this Ph.D. work was to test a pharmacological strategy with the potential to treat humans with FXS or idiopathic autism and/or intellectual disability. Now the challenge is to apply the knowledge gained here to the development of targeted treatments for humans with neurodevelopmental disorders. Future experiments will involve clinical trials of PAK inhibitors in humans with FXS and related disorders.

Chapter 8

Appendix

Pub. No.: WO/2008/063933 **International Application No.:** PCT/US2007/084325
Publication Date: 29.05.2008 **International Filing Date:** 09.11.2007

IPC: ***A61K 31/454*** (2006.01), ***A61K 31/395*** (2006.01), ***A61P 25/00*** (2006.01)

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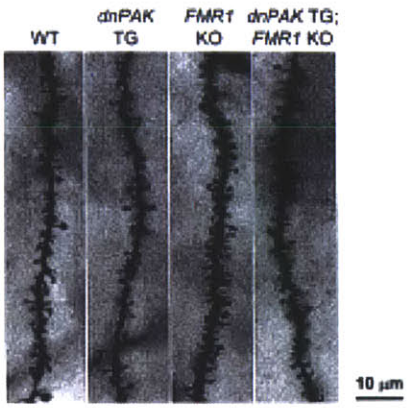
Priority Data: 60/858,108 10.11.2006 US

Title **(EN)** PAK MODULATORS
(FR) MODULATEURS DE PAK

Abstract:



(EN) The present invention provides methods for treating fragile X syndrome and/or other neurodevelopmental disorders by administering p21 -activated kinase (PAK) modulators to a patient suffering from, susceptible to, and/or exhibiting one or more symptoms of FXS and/or other neurodevelopmental disorders. The present invention provides PAK modulators and pharmaceutical compositions comprising PAK modulators. The present invention further provides methods for identifying and/or characterizing PAK modulators.

Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
African Regional Intellectual Property Org. (ARIPO) (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW)
Eurasian Patent Organization (EAPO) (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM)
European Patent Office (EPO) (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR)
African Intellectual Property Organization (OAPI) (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Figure 8-1: **PAK Modulators Patent.**

Disclosure Statement:

Bridget Dolan and Susumu Tonegawa have a financial interest in Afraxis, Inc.

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