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Genetic Removal of Matrix Metalloproteinase 9 Rescues the Symptoms of Fragile X Syndrome in a Mouse Model

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Genetic Removal of Matrix Metalloproteinase 9 Rescues the Symptoms of Fragile X Syndrome in a Mouse Model

A Dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

by

Harpreet Kaur Sidhu

December 2013

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Dedication

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ABSTRACT OF THE DISSERTATION

Genetic Removal of Matrix Metalloproteinase 9 Rescues the Symptoms of Fragile X Syndrome in a Mouse Model

by

Harpreet Kaur Sidhu

Doctor of Philosophy, Graduate Program in Neuroscience
University of California, Riverside, December 2013
Drs. Iryna M. Ethell and Douglas W. Ethell, Co-Chairpersons

Fragile X syndrome (FXS), the most common single gene cause of inherited intellectual disability, is caused by a trinucleotide CGG repeat expansion in the 5’ untranslated region of the Fragile X Mental Retardation gene (FMR1), which results in promoter hypermethylation and gene silencing. This loss or reduction of the gene product expression, Fragile X Mental Retardation Protein (FMRP), results in the translational dysregulation of specific target mRNAs. Patients with FXS and Fragile X mental retardation gene knockout (ko) mice, an animal model for FXS, exhibit defects in dendritic spine maturation that may underlie cognitive and behavioral abnormalities in FXS, which tend to be at the extreme of the autistic spectrum. Dendritic spines are small protrusions on the surface of dendrites that receive the majority of excitatory synapses in the brain, and changes in their morphology affect synaptic strength. Previous studies have shown that minocycline promotes dendritic spine maturation in primary cultures of hippocampal neurons and in the developing hippocampus of Fmr1 ko mice, which is accompanied by improvements in behavioral performance. Here I confirm the
phenotypes previously reported for the Fmr1 ko mouse and investigate a possible link between matrix metalloproteinase-9 (MMP-9) and FMRP by characterizing double Fmr1/Mmp9 ko mice. I show that the deletion of mmp-9 in Fmr1 ko mice rescues the spine development both in vitro and in vivo, and that this deletion also returns mGluR-dependent LTD to the normal levels in Fmr1 ko mice. LTP however remains affected. I also investigated if Fmr1 ko mice exhibit certain behavioral problems, and whether a deletion of mmp-9 can ameliorate these behavioral deficits. The Fmr1 ko mice exhibit increased anxiety and hyperactivity in the open field and defects in social novelty discrimination, most of which are also improved by the removal of mmp-9. Further, I also examined the role of MMP-9 activity in physical traits that are observed in FXS, such as macroorchidism and found that those physical traits are also ameliorated by the genetic removal of mmp-9. Additionally, previous studies have shown the rates of basal protein synthesis to be higher in Fmr1 ko mice. I confirm this and show that the deletion of mmp-9 reduces both the basal levels of protein synthesis and the levels of intracellular signaling as the levels of phosphorylation of Akt, mTOR and eIF4e are reduced to wild type (wt) levels. The levels of MMP-9 and MMP-2 are also upregulated in the hippocampi of the Fmr1 ko mice and since there no differences in the mRNA levels of these two transcripts between Fmr1 ko and wt animals, this upregulation must be due to a translational dysregulation following the loss of FMRP. The fact that most of the FXS phenotypes are reversed by the genetic removal of mmp-9, strongly suggests that increased MMP-9 protein levels underlie these phenotypes and validates the use of specific MMP-9 inhibitors as possible therapeutic avenues for FXS.
Table of Contents

Chapter 1

Introduction...........................................................................................................1
References............................................................................................................31
Figures................................................................................................................47

Chapter 2 – Genetic deletion of matrix metalloproteinase-9 rescues the neurological
deficits associated with Fragile X syndrome in the mouse model.

Abstract..........................................................................................................55
Introduction......................................................................................................56
Materials and Methods..................................................................................62
Results..............................................................................................................69
Discussion........................................................................................................73
References.......................................................................................................79
Figures.............................................................................................................90

Chapter 3 – The genetic deletion of matrix metalloproteinase-9 rescues the behavioral
and physical phenotypes seen in the Fragile X mouse model.

Abstract........................................................................................................101
Introduction....................................................................................................102
Materials and Methods................................................................................104
List of Figures

Figure 1.1 A schematic representation of the Fragile X Mental Retardation 1 (Fmr1) gene………………………………………………………………47

Figure 1.2 A schematic representation of the Fragile X Mental Retardation Protein (FMRP)…………………………………………………………...49

Figure 1.3 A schematic representation of dendritic spine morphology and ultrastructure……………………………………………………………..51

Figure 1.4 A schematic representation of the domain structure of the 24 mammalian matrix metalloproteinases (MMPs)……………………………………………………53

Figure 2.1 Gelatinase levels are upregulated in the hippocampi of Fmr1 KO mice and brain samples of FXS human subjects……………………………………..90

Figure 2.2 Genetic deletion of Mmp-9 promotes formation of mature dendritic spines in the Fmr1 ko hippocampal neurons in vitro………………………………….92

Figure 2.3 Hippocampal neurons develop normal mature dendritic spines in the double Fmr1/Mmp-9 ko mice in vivo…………………………………………….94

Figure 2.4 Genetic deletion of Mmp-9 ameliorates enhanced DHPG-dependent LTD in the hippocampal slices of Fmr1 ko mice…………………………………..96
Figure 3.1 Diagram of the various chambers used for behavior testing………………121

Figure 3.2 Increased anxiety commonly reported for the Fmr1 ko mice is ameliorated in the Db1 ko mice……………………………………………………………………123

Figure 3.3 Increased anxiety in the Fmr1 ko mice is not apparent once the mice are acclimatized to the testing environment……………………………………125

Figure 3.4 The abnormal social interaction behavior exhibited by Fmr1 ko mice is rescued by the dbl ko……………………………………………………..127

Figure 3.5 The macroorchidism phenotype is improved by the genetic removal of Mmp-9 in the dbl ko mice…………………………………………………………129

Figure 4.1 Elevated basal protein synthesis in Fmr1 ko mice is reduced by the deletion of Mmp-9……………………………………………………………………155

Figure 4.2 Increased Akt, mTOR and eIF4e phosphorylation in Fmr1 ko mice is reduced by Mmp-9 deletion……………………………………………………157

Figure 4.3 Reduction in the mRNA levels of some MMPs in the hippocampus of double ko animals…………………………………………………………….159

Figure 4.4 No significant differences in the mRNA levels of most MMPs in the ko animals………………………………………………………………………161

Figure 4.5 No significant differences in the mRNA levels of the membrane bound MMPs in the ko animals…………………………………………………163
Figure 4.6 No significant differences in the mRNA levels of all 4 TIMPs in the hippocampi or cortices of ko animals……………………………………...165

Figure 4.7 Schematic of converging pathways resulting from a variety of cell surface receptors that may lead to increased MMP-9 levels and changes in cytoskeletal and dendritic spine dynamics…………………………………………………………167
List of Tables

**Table 2.1** List and sequence of Mmp-2, Mmp-9 and Gapdh primers used in qRT-PCR screen……………………………………………………………………………………………………………….99

**Table 4.1** List and sequence of primers used in qRT-PCR screen………………………………169
Abbreviations

4E-BP: eukaryotic translation initiation factor 4E-binding protein 1

Abeta/ Aβ: peptide of 36–43 amino acids that is processed from the amyloid precursor protein

ADAM: a disintegrin and metalloprotease protein

ADHD: attention deficit hyperactivity disorder

Akt: serine/threonine-specific protein kinase or Protein Kinase B

ALCAM: activated leukocyte cell adhesion molecules

AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR: AMPA receptor

APP: amyloid precursor protein

APPα: N-terminal fragment released upon the proteolytic processing of APP by α-secretases

Arc: activity-regulated cytoskeleton-associated protein

Arp 2/3: actin related proteins 2/3

BDNF: brain derived neurotrophic factor

BTB: brain testis barrier

CA1: Cornu Ammonis 1 region of the hippocampus

CGG: cytosine guanine guanine nucleotides

CNS: central nervous system

COMP: cartilage oligomeric matrix protein
CSPG: Chondroitin sulfate proteoglycans
CTAP: connective tissue activating peptide
CTEP: 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine; mGluR5 antagonist
CYFIP1: cytoplasmic FMR1 interacting protein 1
DAG: diacylglycerol
Dbl: double
dfmr: drosophila Fmr1 null mutant
dfmr/mmp: drosophila Fmr1 and Mmp null mutant
DHPG: (S)-3,5-Dihydroxyphenylglycine
Dil: 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate
DIV: days in vitro
DNA: deoxyribonucleic acid
DSPG: Dermatan Sulfate Proteoglycan
E15: embryonic day 15
ECM: extracellular matrix
eIF4e: eukaryotic translation initiation factor 4E
EphB R: ephrin B receptor
EPSP: excitatory postsynaptic potential
ER: endoplasmic reticulum
Erk: Extracellular signal-regulated kinase
F-actin: filamentous actin
FGFR: fibroblast growth factor receptor 1

*FMR1*: Fragile X Mental Retardation gene 1

*Fmr1/Mmp9*: *Fmr1/Mmp9* gene double ko

FMRP: Fragile X Mental Retardation Protein

FN: fibronectin

FV: Fiber volley

FXPOI: Fragile X-associated premature ovarian insufficiency

FXS: Fragile X Syndrome

FXTAS: Fragile X-associated Tremor-Ataxia Syndrome

G-actin: globular actin

GABA: γ-Aminobutyric acid

GAD65: glutamic acid decarboxylase 65

GFP: green fluorescence protein

GPCR: G protein coupled receptor

GPI: glycoposphatidylinositol

Gσ: G protein subunit q

HFS: high frequency stimulation

HSPG: heparin sulfated proteoglycans

ICAM-5: intercellular adhesion molecule 5

IGF-BP: insulin-like growth factor binding protein

IP3: inositol 1,4,5-trisphosphate

IL: interleukin
KA: kainic acid
KH: K homology domain
ko: knockout
LTD: long term depression
LTP: long term potentiation
MCP: monocyte chemoattractant proteins
mGluR: metabotropic glutamate receptor
miRNA: micro RNA
Mnk1: MAP kinase-interacting serine/threonine-protein kinase 1
MMP: matrix metalloproteinase
Mmp-9: matrix metalloproteinase 9 gene
MPEP: 2-Methyl-6-(phenylethynyl)pyridine, mGluR5 antagonist
mRNA: messenger RNA
MT: microtubules
MT-MMP: membrane bound MMP
mTOR: mammalian target of rapamycin
NAP: neutrophil activating peptide
NMDA: N-methyl-D-aspartate receptor
NMDAR: NMDA receptor
NES: nuclear export signal
NLS: nuclear localization signal
OCD: obsessive compulsive disorder
P1: postnatal day 1
PCR: polymerase chain reaction
PF: platelet factor
PI3K: Phosphatidylinositol 3-kinase
PIP2: phosphatidylinositol 4,5-bisphosphate
PKC: protein kinase C
PLC: phospholipase Cγ
PNS: peripheral nervous system
Psd: postsynaptic density
RGG: arginine glycine glycine motif
RGD: arginylglycylaspartic acid peptide
RNA: ribonucleic acid
SDF: stromal cell derived factor
SER: sarcoplasmic endoplasmic reticulum
SPAR: Spine-Associated Rap GTPase-activating protein
SV: synaptic vesicles
TACE: tumor necrosis factor-α converting enzyme
TIMP: tissue inhibitor of MMPs
TGF β1: transforming growth factor β1
TM: transmembrane
TNFα: tumor necrosis factor-α
tTG: transglutaminase
UTR: untranslated region
vGLUT1: Vesicular glutamate transporter 1
VEGF: Vascular endothelial growth factor
WT: wildtype
X_p: p arm of X chromosome
X_q: q arm of X chromosome
Chapter 1 – Introduction

Fragile X syndrome (FXS) is the most common single gene cause of inherited intellectual disability and the most common known inherited causes of autism in the United States. It affects about 1 in every 4000 males and 1 in every 6000 females across all socioeconomic and ethnic backgrounds (Rogers et al., 2001; Kau et al., 2004; Clifford et al., 2007; Loesch et al., 2007; Hagerman et al., 2009; Clapp et al., 2001; Garber et al., 2008; Kooy, 2003). Individuals with FXS display a range of symptoms from mild learning problems to more severe cognitive disabilities including language deficits and behavioral dysfunctions. Many FXS subjects display obsessive-compulsive disorder (OCD) behaviors such as hand-flapping, autistic behaviors, and attention deficit and hyperactivity disorder (ADHD). Beyond cognitive and behavioral abnormalities, FXS patients also display characteristic physical traits that include long faces, large protruding ears, macroorchidism, flat feet, hyper-extensible joints, and soft fragile skin. Approximately 25% of patients suffer childhood seizures that improve with age (Clapp et al., 2001; Garber et al., 2008).

Causes of FXS

FXS is caused by expansion of a triplet CGG repeat in the 5’ untranslated region of the Fragile X Mental Retardation (FMR1) gene, located on the X chromosome at q27.1 (Clapp et al., 2001; Bourgeois et al., 2007; Kooy et al., 2000). Most of the human population has <55 repeats at this locus, and expansions from 55-200 CGG repeats are
considered premutation, which are not usually associated with developmental abnormalities early in life, though ~50% of male carriers (Garber et al., 2008; Garcia-Arocena et al., 2010; Jacquemont et al., 2004) and ~8% of female carriers (Coffey et al., 2008) develop Fragile X-associated Tremor-Ataxia Syndrome (FXTAS) after the age of 50. Roughly 20% of female carriers are also prone to Fragile X-associated premature ovarian insufficiency (FXPOI) before the age of 40 (Garber et al., 2008). Subjects with FXS typically display >200 CGG repeats in the \textit{FMR1} promoter (Fig. 1.1). These long repeats lead to hyper-methylation of the region and a decrease or complete silencing of the \textit{FMR1} promoter, causing a deficiency of the FMR protein product, FMRP (Luo et al., 2010; Kooy et al., 2000; Oostra and Willemsen, 2009). As males have only one X chromosome, boys are more severely affected by FXS mutations; whereas females have a second copy of the \textit{FMR1} gene and random inactivation of the X chromosome leads to a mosaic pattern of FMRP expression. Alternate forms of mosaicism can also arise from variations in repeat size or the extent of promoter methylation in different cells that varies from tissue to tissue (Garber et al., 2008).

\textbf{FMRP}

The protein product of \textit{FMR1}, FMRP, has 5 functional domains: 1) Two KH domains that bind RNA, 2) an RGG box that has been implicated in RNA binding specificity, 3) a nuclear localization signal (NLS), 4) a nuclear export signal (NES), and 5) two coiled coils that are important for protein-protein interactions (Oostra and Willemsen, 2009; Kooy et al., 2000; Kooy, 2003; Blackwell et al., 2010; Bassell and
Warren, 2008) (Fig. 1.2). FMRP, has an RNA binding site that facilitates the formation of ribonucleoprotein complexes with target specific mRNAs, proteins, and polyribosomes; it is unclear if FMRP binds to miRNA. FMRP is highly expressed in neurons where it localizes to the cytoplasm, though it has been reported to shuttle in and out of the nucleus, having both a nuclear localization signal (NLS) and a nuclear export signal (NES). This shuttling is thought to be important for its role in transporting target mRNAs to specific neuronal sub-compartments, such as dendritic spines (Willemsen et. al., 2004). A high proportion of FMRP is associated with ribosomes in the cytoplasm of dendrites and at the base of dendritic spines (Luo et. al., 2010; Willemsen et. al., 2004). FMRP interacts with purine-rich G-quartet motifs or U-rich motifs in target mRNAs (Brown et. al., 2001; John et. al., 2004; Denman, 2003; Chen et. al., 2003; Darnell et. al., 2011). In the normal condition, FMRP is thought to bind to its target mRNAs and regulate both their transport to appropriate cellular locations and also regulate their translation, and it inhibits this translation by stalling the ribosomes on its target mRNAs until it receives an intra- or extra-cellular signal to allow the translation to proceed (Darnell et. al., 2011). In the case of the full mutation, the loss of FMRP and misregulated translation of its target mRNAs is thought to be the underlying cause of the phenotypes seen. Outside the brain, FMRP is highly expressed in the testes and loss of FMRP in male patients causes macroorchidism.
FMRP-deficiency and dendritic spine morphology

Learning and memory deficits in FXS are likely due to the FMRP-deficiency effects on the development and maturation of dendritic spines (Clapp et. al., 2001; Luo et. al., 2010; Willemsen et. al., 2004). Dendritic spines are small protrusions on the surface of dendrites that are post-synaptic contact sites for the majority of the excitatory glutamatergic synapses in the brain (Harris, 1999). Post-mortem brain tissue from FXS subjects show delayed dendritic spine maturation, with a preponderance of long thin, immature filopodial like spines compared to the mature, stubby spines seen in brain tissue from controls. Similar dendritic spine profiles to FXS subjects are also seen in the Fmr1 knockout (KO) mice, considered a mouse model for FXS (Braun and Segal, 2000; Irwin et. al., 2000). Although these mice are complete KOs for the Fmr1 gene, and hence do not completely replicate the human condition of more variable expression due to the unpredictability of CGG repeat expansions, they do reproducibly exhibit phenotypes that are similar to the human condition including dendritic spine abnormalities, anxiety, susceptibility to audiogenic seizures, macroorchidism, and learning and spatial memory deficiencies (Braun and Segal, 2000; Kooy, 2003; Cruz-Martin et. al., 2010; Comery et. al., 1997; Bassell and Gross, 2008; Bernardet and Crusio, 2006; Levenga et. al., 2011; Bilousova et. al., 2008).

Dendritic Spines

Santiago Ramon y Cajal first observed small protrusions on the surface of dendrites of Purkinje cells over a hundred years ago using a silver impregnation method
developed by Camillo Golgi (Ramon y Cajal, 1888, 1899). Since then improvements in imaging and visualization techniques have made it easier to study these dendritic spines and establish that these spines are the postsynaptic sites of excitatory synapses (Hering and Sheng, 2001). Dendritic spines are present on many different neuronal populations in the brain and among the most characterized are the spiny pyramidal neurons of the hippocampus, a population that is affected in FXS. Dendritic spines can be classified on the basis of their morphology and 4 major types of spines have been characterized from the immature filopodia and thin spines which have long necks and very small if any, spine heads, to the more mature stubby and mushroom spines which have larger spine heads and shorter necks (Fig. 1.3) (Ethell and Pasquale, 2005). A wide variety of these different spine types are found in various brain regions (Yuste and Bonhoeffer, 2004). Dendritic spines are commonly formed early in development around the time when synaptogenesis occurs, and undergo pruning and remodeling over the course of development. These dendritic spines are dynamic structures that retain the ability to change their morphology even in the adult brain. During development, spines evolve from a more immature morphology to a more mature spine morphology concomitantly with an increase in the density of synaptic input along the dendrite (Yuste and Bonhoeffer, 2004). This development of dendritic spines has also been shown to be impaired in neurodevelopmental disorders like FXS. Dendritic spines serve as independent biochemical compartments that can function semi-independently from the dendritic shaft, having their own cell surface receptors, translational machinery and calcium stores. Cytoskeletal composition of the spine consists primarily of actin
filaments, specifically the β and γ actin isoforms, and have very little microtubules which are concentrated in the dendritic shaft (Cohen et. al., 1985; Wyszynski et. al., 1997). The spine head contains an electron dense structure known as the postsynaptic density (PSD), which can be perforated or continuous, and this structure opposes the active zone of the presynaptic terminal (Fig. 1.3) (Li and Sheng, 2003). This structure can vary in size, which is often proportional to that of the spine head (Harris et. al., 1992; Tashiro and Yuste, 2003). It contains many cell surface receptors and ion channels that form a complex with intracellular signaling effector molecules and scaffolding proteins allowing for the transduction of extracellular signals to intracellular actin filaments to affect changes in spine morphology (Kaecch et. al., 1997).

Actin is present as a pool of monomeric G-actin in spines and these monomers polymerize to form F-actin filaments, which impart a characteristic morphology to that spine (Rao and Craig, 2000; Halpain, 2000). The actin filaments in the spine neck and core of the head organize into longitudinal bundles whereas they form a fine meshwork at the periphery of the head (Fifkova and Delay, 1982). Turnover and rearrangements of these actin filaments results in changes in spine morphology (Trachtenberg et. al., 2002; Pollard, 2003). For example, actin binding proteins that promote reorganization of actin or increased branching like neurabin I, SPAR and arp2/3 respectively result in larger spine heads while proteins like cofilin which depolymerizes actin result in smaller spine heads (Ethell and Pasquale, 2005; Shi et al., 2009; Pontrello et al., 2012). Spine morphology is regulated by synaptic activity through neurotransmitter receptors including the glutamate receptors (Portera-Cailliau et. al., 2003; Matus et. al., 2000;
Passafaro et. al., 2003; Fiala et. al., 2002), but can also be controlled through trans-synaptic (Kossel et. al., 1997) or extracellular signals mediated by cell surface receptors like the EphB receptors and ephrin-B ligand interactions (Dalva et. al., 2000; Ethell et. al., 2001; Henkemeyer et. al., 2003; Moeller et al, 2006; Shi et al., 2009), neureligins and neurexins (Chih et. al., 2004b), cadherins (Togashi et. al., 2002), integrins (Chavis and Westbrook, 2001; Shi and Ethell, 2006), syndecans (Yamaguchi, 2002), and, the receptors for extracellular matrix proteins (Mataga et. al., 2004; Oray et. al., 2004). Dendritic spines are surrounded by extracellular matrix proteins, which form perineuronal nets around the soma and dendrites of neurons (Celio, 1999; Yamaguchi, 2000). These extracellular matrix proteins are processed or activated by a host of different proteins including the matrix metalloproteinase (MMP) superfamily of proteins, which were first discovered for their ability to cleave ECM components, and the products then go on to affect dendritic morphology through cell surface receptors and intracellular signaling cascades (Dityatev and Schacvhner, 2003; Szepesi et. al., 2013; Dziembowska et. al., 2012; Shi and Ethell, 2006). One of the ECM substrates cleaved by a MMP protein, MMP9, is laminin (Doucet and Overall, 2010), which binds to integrins and promotes dendritic spine maturation and could trigger synapse remodeling (Shi and Ethell, 2006). Other groups have also shown the MMPs, specifically MMP-9 to be involved in the regulation of synaptic plasticity through integrin signaling pathways (Nagy et. al., 2006; Meighan et. al., 2006). Other MMPs like MMP-7 have also been shown to influence spine morphology, specifically promoting mature to more immature-filopodial spine transitions (Wlodarczyk et. al., 2011; Bilousova et. al., 2006).
**Group 1 metabotropic glutamate receptors (mGluRs) and their role in FXS.**

Metabotropic-GluRs are a family of glutamate receptors that bind to the amino acid neurotransmitter glutamate. Unlike ionotrophic glutamate receptors, such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, mGluR do not allow ion passage, but instead they are seven transmembrane domain G-protein coupled receptors (GPCR) that trigger intracellular signaling cascades through specific G protein subunits that associate with them (Bonsi et. al., 2005; Platt, 2007). These intracellular signaling cascades modulate the activity of NMDA receptors (NMDAR) and other proteins that mediate pre- or post-synaptic responses (Bonsi et. al., 2005; Platt, 2007; Chu and Hablitz, 2000; Gabriel et. al., 2012; Endoh, 2004). mGluRs have multiple functions in both the peripheral and central nervous systems (PNS and CNS) where they have been implicated in learning, memory, anxiety, and pain perception (Ohashi et. al., 2002). In the CNS, mGluRs can be found at both pre- and post-synaptic sites in different regions of the brain, including the hippocampus and cerebral cortex (Chu and Hablitz, 2000).

There are 8 different types of mGluRs, numbered 1 through 8, which are categorized into 3 different groups (I-III) based on their structure and physiological activity (Bonsi et. al., 2005; Chu and Hablitz, 2000; Endoh, 2004; Ohashi et. al., 2002; Hinoi et.al., 2001). mGluR type I includes mGluR1 and mGluR5 and are mainly found in post-synaptic terminals; whereas, group II and III mGluRs are primarily pre-synaptic (Skeberdis et. al., 2001). Group I mGluRs are associated with the Gq G protein subunit, which is a heterotrimeric protein that activates phospholipase C (PLC) to initiate an
internal signaling cascade. PLC then hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to yield diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG further activates protein kinase C (PKC) while IP3 causes the release of intracellular calcium stores from the endoplasmic reticulum by binding to IP3-receptors on the ER. These signaling pathways go on to modulate a wide variety of other proteins that affect cellular physiology and behavior (Bonsi et. al., 2005, Chu and Hablitz, 2000; Endoh, 2004).

Many different signaling pathways and receptors appear to be deregulated in FXS, including group I mGluRs. Fmr1 ko mice are more sensitive to modulators of group 1 metabotropic glutamate receptor signaling than wt mice (Bear et. al., 2004). Normally, mGluR signaling blocks the translational repression of FMRP from its target mRNAs, allowing for the translation of the protein products encoded, which can cause α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor internalization and long term depression (LTD) - a decrease of synaptic strength. In the absence of FMRP, those proteins are expressed at a higher basal level and more AMPA receptors are internalized even before the glutamate signaling arises, thus lengthening long term depression (LTD) (Bear et. al., 2004; Hou et. al., 2006; Oostra and Willemsen, 2009). mGluR antagonists such as MPEP, Fenobam, and CTEP have been shown to attenuate some of FXS phenotypes in Fmr1 ko mice and in human patients (Yan et. al, 2005; Levenga et. al., 2010; Michalon et. al., 2012). Others have shown that Fmr1 ko mice have altered GABA receptor subunit composition and lower GABAergic inputs to certain hippocampal circuits (Brenman, 2009; D’Hulst et. al., 2009), so that GABA receptor agonists, such as Baclofen and Nipecotic acid partially rescue the FXS phenotypes (Levenga et. al.,
2010; Pacey et. al., 2009). Previous work done in our laboratory has implicated matrix metalloprotease-9 as causing some of FXS-associated symptoms. We found that MMP-9 activity is higher in Fmr1 ko mice than in the wt mice; further, minocycline treatment reduced the MMP9 activity levels in Fmr1 ko mice, ameliorated the dendritic spine phenotype, and improved behavioral abnormalities seen in Fmr1 ko mice. When cultures of mature hippocampal neurons from wt mice were treated with MMP-9 protein they developed long, immature filopodial like spines that mimicked the immature spine phenotype seen in Fmr1 ko hippocampal cultures (Bilousova et. al., 2008). This same relationship between Fmr1 and MMP-9 has also been reported in the drosophila model of FXS where overexpression of TIMP1 (the endogenous regulator of MMPs) or a mmp null mutation rescued all nervous system defects seen in the dfmr null flies, suggesting a genetic interaction between these two genes and/or their protein products (Siller and Broadie, 2011). Other groups have also shown MMP-9 involvement in synaptic plasticity and regulation of NMDAR currents mediated through integrin activity (Nagy et. al., 2006; Meighan et. al., 2006) suggesting all these components that are defective in FXS may be linked.

**Matrix metalloproteinases in FXS**

Matrix metalloproteinases are zinc dependent endopeptidases that are part of a larger superfamily of proteases known as the metzincin proteases. Other members of this superfamily include the adamalysins (ADAMs), serralysins, and astacins, all of which share a highly conserved catalytic domain consisting of 3 conserved histidine residues in
a zinc-binding domain arranged as HExxHxxGxxH. These 4 multi-gene families are
grouped by sequence similarities between members of each family. To date, 24
mammalian MMPs have been identified and each with a variety of substrate preferences,
though some substrates overlap among the MMPs, and can be sub-divided further into
collagenases, gelatinases, stromelysins, matrilysins and ‘other’ MMPs based on major
substrate specificity. All but 6 of the MMPs are secreted into the extracellular space, with
the remainder being tethered to the plasma membrane via a transmembrane domain (Fig.
1.4) (MMP-14, -15, -16 and -24 or the MT1-, MT2-, MT3- and MT5-MMPs) or a
glycophosphatidylinositol (GPI) link (MMP-17 and -25 or MT4- and MT6-MMPs)
(Agrawal et. al., 2008; Ethell and Ethell, 2007; Milward et. al., 2007; Flannery et. al.,
2006; McCawley and Matrisian, 2001).

**MMP structure and activation**

All MMPs share a similar 80 amino acid long pro-domain at their N terminus that
contains a cysteine residue that binds to zinc in the catalytic domain, keeping the MMP
inactive. Full MMP activity is achieved by cleavage of this pro-domain, which disrupts
this cysteine-zinc interaction (also known as a ‘cysteine switch’). Most MMPs are
activated through proteolytic processing of this pro-domain after being released
extracellularly; which can be mediated by other active MMPs or other serine proteases;
for instance, MMP-2 is cleaved and activated by MMP-3. Activation may also be
achieved by chemical modification of methionine and cysteine residues in the catalytic
domain or prodomain by oxidation from reactive oxygen species. However, some MMPs
have furin motifs that allow for intracellular activation prior to appearance on the surface of the cells, including all the membrane bound MMPs (MT-MMPs), MMP-11, MMP-23 and MMP-28. The majority of the MMPs except MMP-7, -23 and -26 also have C-terminal hemopexin-like domains (comprising 4 modules, I – IV) that allow for interaction with endogenous tissue inhibitors of metalloproteinases (TIMPs). The gelatinases, MMP-2 and -9, have three additional cysteine rich fibronectin type-2 repeats in their catalytic domains (Fig. 1.4) (Ethell and Ethell, 2007; Visse and Nagase, 2012; McCawley and Matrisian, 2001).

**MMP Inhibitors**

MMP activity is modulated by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs). TIMPs are small proteins consisting of 2 domains held together by 3 disulfide bonds (Ethell and Ethell, 2007; Maskos, 2005; Rivera et. al., 2010). There are 4 mammalian TIMPs (TIMPs 1-4) that bind to different subsets of MMPs, typically in a 1:1 ratio. TIMP-MMP interactions can be inhibitory by preventing activation of the cysteine switch, but they can also facilitate activation, as is the case with TIMP-1 and TIMP-2, which activate MMP-9 and MMP-2 respectively.

TIMP-1 has a wide distribution in the body, but it is expressed at much lower levels than any other TIMP. The highest levels of TIMP-1 are found in the uterus and heart, but it is nearly undetectable in the brain and testes. TIMP-1 binds to the hemopexin C-domain region of MMP9 and forms an inhibitory trimolecular complex that also includes MMP3. Notably, TIMP-1 does not inhibit any MT-MMPs or any ADAMs.
TIMP1 protein (28.5 kD) is encoded by the *TIMP1* gene, which is located on the X chromosome at Xp11.23-11.4; interestingly *TIMP1* does not contain a TATA box. TIMP-1 is primarily regulated at the translation level (Gardner and Ghorpade, 2003; Gomez et. al., 1997; Nagase et. al., 1999; Fager and Jaworski, 2000; Rivera et. al., 2002).

TIMP-2 (21 kD) is also widely expressed but unlike TIMP-1, it is highly expressed in the brain, especially in the meninges and choroid plexus. The *TIMP2* gene, is located on chromosome 17, 17q2.2-2.5, in humans, and the promoter includes a TATA box. TIMP-2 can inhibit all MT-MMPs, but paradoxically it activates MMP2 and through a trimolecular complex with MT1-MMP and pro-MMP2, by displacing TIMP4 from the hemopexin module III and IV junction of MMP2 (Gomez et. al., 1997; Brew et. al., 2000).

TIMP-3 is also widely expressed with moderate localization in the brain. The *TIMP3* gene is located on human chromosome 22, 22q12.1-13.2, within an intron of the *SYNAPSIN-2* gene, like TIMPs-2 and 4, and includes a TATA box. The 24-27 kD TIMP3 protein is highly glycosylated and associates with the extracellular matrix. Similar to TIMP2, TIMP3 inhibits all the MT-MMPs, as well as Tumor Necrosis Factor cleaving enzyme TACE/ADAM-17. Further, TIMP3 binds the C-terminal hemopexin domain of MMP2, though it does not form the same trimolecular complex as does TIMP-2, but instead it inhibits MMP2.

TIMP-4 (24 kD) has the most restricted expression pattern of any TIMPs and is highly expressed in the brain. The human *TIMP4* gene is located on chromosome 3 at 3p25, within a different synapsin-2 intron, and lacks a TATA box. As with TIMP-2 and -
3, it also inhibits the MT-MMPs, does not inhibit any known ADAMs. TIMP4 binds to the hemopexin domain of MMP2 and prevents activation, though with a lower affinity than TIMP2, which can displace TIMP4 and activate MMP2 as discussed above.

**MMP tissue distribution and regulation**

MMPs are widely expressed all over the body, with some of the MMPs displaying developmental regulation, including MMP-2, -9, -11, -12 during neurodevelopment. Some MMPs are expressed constitutively (MMP-3 and -7 in the developing nervous system) and many are also limited to specific tissue types. For example, MMP-2, -3 and -9 are the most highly expressed MMPs in the brain and while MMP-2 and -9 are expressed only by neurons and astrocytes, MMP-3 is also expressed by the microglia in the brain. MMP-7 is another MMP that is usually not found in the brain except in cases of inflammation when circulating macrophages infiltrate the brain or at sites of tissue remodeling (Buhler et. al., 2009; Ethell et. al., 2002). Their expression levels also change in response to neurological disease (Ethell and Ethell, 2007; Visse and Nagase, 2012).

**MMP substrates and functions**

MMPs were initially discovered for their ability to cleave extracellular matrix proteins but they can also process some non-extracellular matrix substrates like bioactive molecules, chemokines and cell surface receptors. MMPs are required for a number of cellular processes including migration, tissue remodeling, angiogenesis, apoptosis, host immune responses, differentiation and even cell proliferation but at the same time have
also been implicated in pathological states like cancer and neurodegenerative diseases (Szklarczyk et. al., 2008; Ethell and Ethell, 2007; Agrawal et. al., 2008; Rivera et. al., 2010; McCawley and Matrisian, 2001). Some MMPs also appear to have roles in hippocampal dependent learning and memory, dendritic spine development, synaptic plasticity and synaptogenesis. MMP-3 and 9 have been shown to be important in spatial learning (Wright et. al., 2006; Nagy et. al., 2007) and also remodeling in the dentate gyrus following kainite-induced excitotoxicity (Dziembowska and Wlodarczyk, 2012; Ethell and Ethell, 2007; Rivera et. al., 2010). Conversely MMP-9 has also been shown to be upregulated during audiogenic seizures and involved in the establishment of aberrant synaptic connections following KA induced neuronal death (Takacs et. al., 2010). Other studies have demonstrated a role for MMP9 in spine morphology where MMP-9 activity is linked to a more immature spine phenotype (Bilousova et. al., 2009; Dziembowska and Wlodarczyk, 2012; Wang et. al., 2008; Michaluk et. al., 2011). MMP-9 also has a role in long term potentiation (LTP), which is essential for long term synaptic plasticity, and these effects are mediated by NMDAR activity (Nagy et. al., 2006; Meighan et. al., 2006). Spine morphology is also affected by the activity of MMP-7 (Bilousova et. al., 2006).

**The collagenases**

There are 4 known collagenases, collagenases 1-4, also known as MMP-1, -8, -13 and -18, respectively. All 4 collagenase genes are located in a gene cluster on chromosome 11, in humans. The collagenases have a simple domain structure consisting
of a C-terminal pro-domain with a cysteine-thiol group, a zinc-containing catalytic domain, and a hemopexin-like domain separated from the catalytic domain by a hinge domain. The \textit{Mmp-1} gene, previously known as tissue collagenase or interstitial collagenase, is on chromosome 11 at q22-q23. The 27 kD gene product, MMP-1, can cleave a number of native collagen fibers, with the greatest affinity for type III collagen followed by types I, II, VII and X, in order. In addition to collagen, MMP-1 also cleaves gelatin, aggregans, link proteins, entacins, tenascins and perlecan proteins in the extracellular matrix. Besides extracellular matrix substrates, MMP-1 also cleaves some bioactive substrates, including chemokines important to immune responses such as monocyte chemoattractant proteins (MCPs)-1, -3 and -4, which regulate the migration and infiltration of macrophages and monocytes during innate immune responses. MMP1 also acts on pro-1L-1\beta, 1L-1\beta, stromal cell derived factor (SDF) and pro-tumor necrosis factor-\alpha (pro-TNF-a) to activate or inactivate them. Its actions on insulin-like growth factor binding proteins (IGF-BPs)-2 and -3 can act to either enhance or attenuate insulin signaling in a context-dependent manner, with respect to which tissue and substrate. This MMP also acts on \(\alpha_1\)-antichymotrypsin, \(\alpha_2\)-macroglobulin, and \(\alpha_1\)-proteinase inhibitor. Besides these bioactive molecules, MMP-1 can also cleave and activate pro-MMPs, including itself and pro-MMP-2 (McQuibban et. al., 2000, 2001, 2002; Hazuda et. al., 1990; Ito et. al., 1996; Rajah et. al., 1996; Fowlkes et. al., 1994; Whitelock et. al., 1996; Gearing et. al., 1994).

MMP-8, previously known as neutrophil collagenase, is a 53 kD protein that is expressed in the connective tissues of most mammals. Unlike other MMPs, MMP-8 is not
secreted to the extracellular space as a pro-protein that is activated by serine proteinases in extracellular spaces. Instead, MMP-8 is stored in its inactive pro-form within the secondary granules of neutrophils, where it can be activated by autolytic cleavage and secreted to degrade ECM and allow effector cells to migrate into the site of damage during inflammation. The \textit{Mmp-8} gene is on chromosome 11 at q21-q22. MMP-8 also cleaves different collagenase types with different affinities with the highest being for type I followed by types II, III, VII and X in decreasing order. Other extracellular matrix substrates of MMP-8 include the tenascins, aggrecans, gelatin and entacin proteins. Like MMP-1, MMP-8 can also hydrolyze MCP-1, IGF-BPs, pro-TNF-\(\alpha\), \(\alpha\)-2-macroglobulin and \(\alpha\)-1-proteinase inhibitor. In addition, it also acts on L-selection and can cleave to activate pro-MMP-8 and further inactivate active-MMP-8 (McQuibban et. al., 2002).

The \textit{Mmp-13} gene is localized to chromosome 11 at q22.3. This protein is secreted in its inactive pro-form and the pro-domain is cleaved by extracellular proteinases to activate it. It also cleaves collagen fibers in the extracellular matrix with decreasing affinity for different fiber in the following order: type II, III, I, VII and lastly X. The hemopexin-like domain is involved in MMP-13 mediated cleavage of collagen. It can also cleave other substrates like gelatin, tenascins, aggrecans and entacins. Its bioactive substrates include \(\alpha\)-1-antichymotrypsin, \(\alpha\)-2-macroglobulin, pro-TNF-\(\alpha\), MCP-3 and SDF. It can also cleave and activate pro-MMPs- 9 and -13. Further, it also inactivates MMP-13. MMP-13 is highly expressed during embryonic development, specifically in the skeleton where it is essential for bone mineralization through its actions on collagen reorganization. It is also highly expressed in diseases like rheumatoid arthritis,
osteoarthritis and in some human cancers (McQuibban et. al., 2001, 2002; Overall et. al., 2002; Johansson et. al., 2000).

MMP-18 is a Xenopus collagenase with no known human or mouse orthologs to date. It cleaves gelatin and collagen types I, II and III with decreasing affinity in this order (Stolow et. al., 2002).

The Gelatinases

There are 2 known mammalian gelatinases, gelatinases A and B, also known as MMP-2 and MMP-9, respectively. The gelatinases have the same basic structure as the collagenases with the addition of 3 fibronectin (FN) type II domains in the catalytic domain. The major ECM substrate of the gelatinases is gelatin, thus the early nomenclature, but like the collagenases they can also hydrolyze other ECM components and bioactive substrates. MMP-2 and MMP-9 are the most highly expressed MMPs in human and mouse brains.

MMP-2, previously called the 72-kD gelatinase or the 72-kD type-IV collagenase, is encoded by the Mmp-2 locus on chromosome 16 at q13. In addition to gelatin, active MMP-2 also cleaves native collagens of type-I, -IV, -V, -VII, -X and -XI (in decreasing order of specificity), elastin, fibronectin, laminin-5, brevicans, aggrecans, neurocans, vitronectin, decorin and BM-40 in the extracellular matrix. In addition, MMP-2 hydrolyzes some molecules to release biologically active molecules including pro-transforming growth factor (TGF)-β1, pro-TNF-α, MCP-3, pro-Interleukin (IL)-1β, SDF, IGFBP-3 and -5, fibroblast growth factor receptor 1 (FGFR1) and IGFBP. MMP-2 also
acts on big endothelin-1, substance P, galectin-3, α1-proteinase inhibitor, α2-macroglobulin and pregnancy zone protein. Moreover, MMP-2 cleaves the pro-domains of MMP-1, -2 and -13 to activate these MMPs, though it can also cleave the active forms to inactivate them. (Wenstrup et. al., 1996; Gearing et. al., 1994; McQuibban et. al., 2000, 2002; Yu and Stamenkovic, 2000; Schonbeck et. al., 1998; Fowlkes et. al., 1995; Levi et. al., 1996; Imai et. al., 1997; Ochieng et. al., 1994).

MMP-2 activation requires the formation of a trimolecular complex involving MT1-MMP, TIMP-2 and pro-MMP2. Inactive pro-MMP-2 is usually secreted into the extracellular space by the cell. MT1-MMP and TIMP-2 form a complex at the surface of the cell to recruit pro-MMP-2 molecules from the extracellular space to the cell surface where active MT1-MMP and autocatalytic cleavage by pro-MMP-2 yield an active MMP-2 enzyme. This activation process is promoted by other molecules in the plasma membrane like the clustering of integrin chains and also activated leukocyte cell adhesion molecules (ALCAMs). Active MMP-2 is involved in a number of normal biological processes including embryonic development, being highly involved also in neuronal development, tissue remodeling for instance in cases of damage, the inflammatory response, it regulates vascularization and also, it is involved in the breakdown of the endometrial lining during menstruation. It has however also been implicated in a number of pathological conditions including cancer and arthritis (Wenstrup et. al., 1996; Gearing et. al., 1994; McQuibban et. al., 2000, 2002; Yu and Stamenkovic, 2000; Schonbeck et. al., 1998; Fowlkes et. al., 1995; Levi et. al., 1996; Imai et. al., 1997; Ochieng et. al., 1994).
MMP-9 is sometimes called the 92-kDa gelatinase. The gene encoding this proteinase is found chromosome 20 at q11.2-13.1. Like MMP-2, the major extracellular matrix substrate of MMP-9 is gelatin, but it also degrades native collagen types I, IV, V, VII (in decreasing order of specificity), elastin, fibronectin, laminin, aggrecans, link proteins and vitronectin (McQuibban et. al., 2001; Van den Steen, 2000). Its biological substrates include stromal cell derived factor (SDF) which is inactivated, connective tissue activating peptide (CTAP)-III/ neutrophil activating peptide (NAP)-2, platelet factor (PF)-4, GROα which are all degraded, it activates pro-IL-8, pro-TNF-α, pro-TGF-β1 and pro-IL-1β, it releases the cell surface bound IL-2Rα, causes shedding of FGFR1, and cleaves plasminogen, galectin-3, α2-macroglobulin and a1-proteinase inhibitor. It can also cleave and activate pro-MMPs -2, -9 and -13 and inactivate the active forms of these same MMPs and also vascular endothelial growth factor (VEGF).

Besides self-activation, pro-MMP-9 can also be cleaved and activated by MMP-3, -7, -26 and MT1-MMP. Active MMP-9 is involved in embryonic development, is critical for neuronal development, is involved in the development of myelination through its processing of IGF-BPs and has roles in reproduction and tissue remodeling. Some groups have shown MMP-9 to be involved in LTP of synapses and in the surface trafficking of the NR1-NMDAR through an integrin-β1 dependent pathway. Some studies have implicated it in the mobilization of hematopoietic precursor cells from the bone marrow and it appears to be involved in remodeling that is seen in tumors. High expression levels of MMP-9 have also been implicated in pathological conditions like arthritis, cancer, intracerebral hemorrhage, ischemia and some cardiac disorders. Additionally, the loss of
MMP-9 has been linked to hippocampal dependent learning and memory deficits (McQuibban et. al., 2001; Yu and Stamenkovic, 2000; Schonbeck et. al., 1998; Ochieng et. al., 1994; Van den Steen, 2000; Sheu et. al., 2001; Patterson and Sang, 1997; Bergers et. al., 2000; Gu et. al., 2005; Szklarczyk et. al., 2008; Michaluk et. al., 2009; Nagy et. al., 2006; Dziembowska and Wlodarczyk, 2012).

**The transmembrane MMPs**

A total of 6 membrane-tethered mammalian MMPs (MT-MMPs) have been discovered so far. These are MT1-MMP through MT6-MMP, also known as MMP-14, -15, -16, -17, -24 and -25, respectively. These MMPs have the same basic structure as collagenases with additional domains. The MT1-MMP, MT2-MMP, MT-3-MMP, and MP5-MMP each have a transmembrane domain in place of the hemopexin domain found in MMP1, which anchors them to outer membrane of the cell. MT4-MMP and MT6-MMP are anchored to the plasma membrane of the cell by a glycosyl-phosphatidylinositol (GPI) link at the same location. Each of the MT-MMPs has a furin-susceptibility cleavage site (R-X-R/K-R) in the N-terminal pro-domain. This motif allows for these MMPs to be cleaved and activated intracellularly by furin endopeptidases, prior to presentation on the cell’s outer membrane.

*MT1-Mmp* is encoded by a gene localized to chromosome 14q11-q12. It has a number of extracellular matrix and bioactive substrates including collagen types I, II and III, gelatin, fibronectin, vitronectin, aggregans, MCP-3, SDF, CD44, transglutaminase (tTG), α2-macroglobulin and α1-proteinase inhibitor. It can activate and inactivate MMP-
2 and -13. Elevated levels of MT1-MMP have been found in primary tumors and the active protein has been implicated to play a role in angiogenesis and glial cell invasion in malignant gliomas, possibly through the activation of MMP-2 (McQuibban et. al., 2001, 2002; Kajita et. al., 2001; Belkin et. al., 2001; d’Ortho et. al., 1997).

The gene encoding MT2-Mmp is located on chromosome 16 q13-q21. MT2-MMP displays more substrate specificity towards matrix proteins, being able to cleave only the proteoglycans in the matrix. It can however also cleave pro-TNF-a to release the active soluble form of TNF-α, and it cleaves the cell surface bound tTG to release it into the extracellular space. MT2-MMP can also cleave pro-MMP2 (d’Ortho et. al., 1997; Morrison et. al., 2001; Belkin et. al., 2001).

The MT3-Mmp encoding gene is found at chromosome 8q21. This protein is expressed highly in tissues that are undergoing remodeling and is developmentally regulated with highest levels being expressed in fetal tissues, especially in the brain. It specifically hydrolyzes type III collagen and fibronectin proteins in the extracellular matrix. Similar to MT2-MMP, it can cleave tTG, pro-TNF-a and pro-MMP-2 (d’Ortho et. al., 1997; Belkin et. al., 2001).

The MT4-Mmp gene is localized at chromosome 12 q24.3. The active MT4-MMP protein has been shown to be expressed in a variety of tissues such as the colon, ovaries, testis, leukocytes and the brain where it appears to be involved in neurogenesis. It has specificity towards the gelatin and fibrinogen components of the extracellular matrix and additionally cleaves pro-MMP-2 and pro-TNF-α (Wang et. al., 1999; English et. al., 2000).
*MT5-Mmp* is encoded by a gene located at chromosome 20 q11.2. It, like the other MT-MMPs, can cleave pro-MMP-2. It also cleaves fibronectins, proteoglycans and gelatin components of the extracellular matrix (Wang and Pei, 1999; Llano et. al., 1999; Pei et. al., 1999a).

*MT6-Mmp* is also known as leukolysin and is encoded by a gene that is found at chromosome 16 p13.3. It can cleave a variety of extracellular matrix proteins like type IV collagen, gelatin, fibronectin, proteoglycans, specifically DSPG and CSPG, laminin-1 and fibrinogen. In addition, it also inactivates the α1-proteinase inhibitor and activates MMPs-2 and -9 (Velasco et. al., 2000; English et. al., 2001).

**The stromelysins**

3 mammalian stromelysins have been discovered as yet; namely stromelysins 1 through 3, also known as MMPs -3, -10 and -11 respectively. However, all 3 stromelysins do not have the exact same structure. MMPs -3 and -10 have a simple structure akin to that of the collagenases while MMP-11 has an additional furin-susceptibility cleavage site in the N-terminal pro-domain. MMPs -3 and -10 are secreted in their pro-form and are activated extracellularly by peptidases. MMP-11 on the other hand is cleaved by a furin endopeptidase and is activated intracellularly before being secreted by the cell since it has a furin cleavage domain.

The *Mmp3* gene is located on chromosome 11 at q23 and it codes for a protein that has a large number of both bioactive and extracellular matrix substrates. Its matrix substrates include the link proteins, aggrecan, laminin, fibronectin, gelatin, entacin,
perlecan, elastin, decorin, tenascin, vitronectin and it cleaves the non triple helical regions of collagen types II, III, IV, V, IX, X and XI. It also hydrolyzes some biologically active compounds the likes of MCPs 1 through 4, SDF, pro-TNFα, pro-IL-1β, L-selectin, plasminogen, E-cadherin, IGF-BP3, pro-heparin binding-epidermal like growth factor (HB-EGF), α1-proteinase inhibitor, α2-macroglobulin and α1-antichymotrypsin. It also acts to activate other MMPs including itself and MMPs-1, -7, -8, -9 and -13 and it can also inactivate these same MMPs. MMP-3 is the other MMP that is most highly expressed in the brain where it is expressed by both astrocytes and neurons and also by microglia albeit at a lower level. It is known to be released by apoptotic neurons and acts to activate microglia to release other chemokines. In the brain, it has been implicated in hippocampal dependent associative learning, appears to be required for spatial memory acquisition, seems to be involved in hippocampal CA1 region late phase LTP and has also been implicated in NMDAR mediated shedding of intercellular adhesion molecule 5 (ICAM-5) from cortical neurons in vitro. Other studies have shown MMP-3 to be important for tissue remodeling especially in wound repair, to be involved in the initiation of tumors, the progression of atherosclerosis, high MMP3 levels to be correlated with acute myocardial infarction and low levels with congenital malformations like cleft palate and also progressive coronary atherosclerosis (McQuibban et. al., 2001, 2002; Suzuki et. al., 1997; Lijnen et. al., 1998; Whitelock et. al., 1996; Wright et. al., 2006; Ethell and Ethell, 2007; Ito et. al., 2007; Conant et. al., 2010).

The Mmp-10 gene is located on chromosome 11 at the q22.3-q23 locus. The resultant protein degrades the gelatin types I, III, IV and V, fibronectin and proteoglycan
components of the extracellular matrix. It also cleaves to activate MMPs -1, -8 and -10. MMP-10 has been implicated in a form of lung cancer where the cancer stem-like cells secrete MMP-10 and use it as a growth factor like agent to both maintain the ‘stem cell’ colony and also to drive the initiation and spread of the malignant metastases (Chandler et. al., 1997, Nagase and Woessner, 1999; Fosang et. al., 199; Sirum and Brinckerhoff, 1990).

The gene encoding Mmp-11 is found on chromosome 22 at q11.2 and the gene product is a protein that is secreted in its active form into the extracellular matrix. Active MMP-11 can degrade some extracellular matrix components, including fibronectin, aggrecans and laminin, but does so inefficiently. However, MMP11 has a strong substrate specificity for α1-proteinase inhibitor which is inactivated. It also cleaves IGFBP-1 to release active IGF and it cleaves α2-macroblobulin. Elevated levels of MMP-11 have been implicated in some breast cancers, gastric cancers and melanomas (Manes et. al., 1997).

**The Matrilysins**

There are a total of 2 mammalian matrilysins, matrilysin and matrilysin-2, also known as MMPs -7 and -26 respectively. The matrilysins have the most basic domain structure out of all the MMPs, having only 2 domains, the pro-domain and the catalytic domain. Both these MMPs are secreted by the cell in their inactive pro-forms and are cleaved extracellularly.
MMP-7 or PUMP-1 is encoded by a gene located on chromosome 11, at the locus q21-q22. The active MMP-7 protein has the ability to cleave a wide variety of extracellular matrix components and bioactive substrates. It degrades the non helical segments of native collagen types IV, V, IX, X, XI, gelatin, fibronectin, laminin, aggrecan, entactin, tenascin, vitronectin and fibrinogen. It also cleaves to activate pro-a-defensin, the cell surface tethered FasL and pro-TNFα. It also cleaves E-cadherin, β4 integrin, plasminogen to yield angiostatin fragments, decorin, α2-macroglobulin and α1-proteinase inhibitor. Further, it also cleaves and activates pro-MMPs -1, -2, -7 and -9. MMP-7 is expressed by a number of cell types including microglia and Paneth cells and is also expressed in the uterus tissues. It has important roles in wound healing and the regulation of defensins, important immune regulatory proteins, in the intestinal mucosa. Levels of MMP-7 have been reported to be elevated in primary tumors and have been correlated with increased invasiveness of metastases. MMP-7 appears to be required by cancer cells for early tumor growth and progression (Imai et. al., 1997; Patterson and Sang, 1997; Shirafuji et. al., 1999; Noe et. al., 2001; George et. al., 1999; Agnihotri et. al., 2001; Lochter et. al., 1997; Von Bredow et. al., 1997; Pozzi et. al., 2000; Szklarczyk et. al., 2008).

*Mmp-26* or the endometase gene can be found on chromosome 11 at p15. Active MMP-26 cleaves the fibronectin, fibrinogen, type IV collagen and gelatin components of the extracellular matrix. In addition, it also cleaves IGFBP-1 to release active IGF, inactivates a1-proteinase inhibitor and cleaves pro-MMP-9 to activate it. MMP-26 is expressed in a variety of cell types like the normal intestine and endometrial cells. In the
developing fetus, it is highly expressed in the placenta and in fetal nucleated red blood cells. MMP-26 levels appear to be increased in keratinocytes during times of wound repair and in the early stages of skin cancer. Many studies have also shown MMP-26 levels to be elevated in cancer cells with an epithelial origin like breast or prostate cancer (Park et. al., 2000; Uria and Lopez-Otin, 2000; de Coignac et. al., 2000).

Other MMPs

There are several other MMPs that do not fit into the above categories. These are MMPs -12, -19, -20, -21, -23, -27 and -28. All of these MMPs have the same simple structure as the collagenases except MMP-28, which has an additional furin-susceptible cleavage site.

MMP-12 is also known as metalloelastase or the macrophage elastase and the gene encoding this protein is located on chromosome 11, q22.2-q22.3. Active MMP-12 cleaves the laminin, fibrinogen, proteoglycan, fibronectin and elastin components of the extracellular matrix. It can also cleave plasminogen, pro-TNFα and α1-proteinase inhibitor. MMP-12 expression peaks around 4-5 weeks postnatally around the same time as the peak of myelination. MMP-12 has also been shown to regulate oligodendrocyte maturation and myelination and is constitutively expressed in the central nervous system (Cornelius et. al., 1998; Chandler et. al., 1996; Rivera et. al., 2010; Milward et. al., 2007).

MMP-19 or RASI or stromelysin-4 is encoded by the gene found on chromosome 12q14. It has no known bioactive substrates but it can cleave collagen type IV, gelatin, laminin, fibronectin, tenascin, entacin, aggrecan, fibrinogen and cartilage oligomeric
matrix protein (COMP). MMP-19 is expressed by human epidermal and endothelial cells and has important roles in angiogenesis, adhesion and cellular proliferation and migration (Stracke et al., 2000a,b).

The enamelysin or MMP-20 gene is located on chromosome 11 q22.3. Active MMP-20 is able to cleave aggrecans and COMP in the extracellular matrix. It also cleaves amelogenin, which is the major component of dental enamel matrix and thus is thought to play a major role in tooth enamel formation. It has additionally been implicated in abnormal enamel formation (Nagase and Woessner, 1999).

The MMP-21 gene is located on chromosome 10 q26.13 and the active protein cleaves α1-antitrypsin. It has been implicated in having roles in embryogenesis especially in neurogenesis and also leukocyte development and survival. It may also be involved in the tumor progression of certain cancers (Brinckerhoff and Matrisian, 2002).

MMP-23 is encoded by a gene that is part of the duplicated region of chromosome 1 p36.3. Active MMP-23 cleaves gelatin and it is expressed predominantly in the ovaries, testis and prostate. It has been suggested to have a role in reproductive processes (Pei, 1999b; Lopez-Otin et al., 2012).

The gene encoding MMP-27 is found on chromosome 11 q24. This protein however has no defined substrates reported.

MMP-28 or epilysin is encoded by a gene located at chromosome 17 q11.2. This is known to degrade casein and has a wide range of expression in both healthy adult and fetal tissue suggesting a possible role in tissue homeostasis. MMP-28 is also expressed in
a variety of cancer cells and has been implicated in osteoarthritis (Lohi et. al., 2001; Illman et. al., 2001; Klawitter et. al., 2011).

Members of the MMP family can cleave an extensive array of proteins not only intracellularly but also extracellularly. Most known for their actions on extracellular matrix proteins, MMPs have been implicated in many processes in the health and diseased CNS. MMPs are expressed during development in the healthy CNS and are known to regulate neurogenesis and morphogenesis. Their expression is maintained in the adult CNS and they play important roles in learning and memory, synaptic plasticity, the maintenance of normal physiology and also reparative roles in cases of injury or other types of nervous system insults. However, misregulated expression of MMPs can also lead to pathology and increased MMP levels have been implicated in neurological disorders like Multiple Sclerosis and in other diseases like cancer. Our previous research has also implicated increased MMP-9 levels and activity to be the underlying cause of FXS, specifically in promoting a more immature spine phenotype and also affecting some behavior in the Fmr1 ko mice. Here, I investigated if Mmp-9 deficiency would rescue some of FXS-associated deficits in the Fmr1 ko mice. I looked at not only the neurological symptoms commonly reported, but also at the behavioral defects and characteristic physical traits common to FXS. I also investigated whether the levels of other MMPs are affected by the loss of MMP-9, and if they may compensate for this loss, due to overlapping substrate specificities and expression profiles. Finally, I also examined FXS-associated downstream signal effector molecules to determine which
signaling cascades mediate the effects of MMPs and how they are regulated in *Mmp9/Fmr1* KO mice.
REFERENCES


Figure 1.1. A schematic representation of the Fragile X Mental Retardation 1 (Fmr1) gene. The CGG repeats lie in the 5’ untranslated region (UTR) of the Fmr1 gene. In normal individuals, CGG repeats number between 5-55 repeats and the CpG island remains hypomethylated. In fully affected subjects however, there is an expansion in the number of CGG repeats that number greater than 200. This causes the CpG island and promoter region to become hypermethylated resulting in gene silencing.
Figure 1.1

[Diagram showing the comparison between normal and FXS genetic structures, highlighting the differences in CGG repeats and promoter regions.]
Figure 1.2. A schematic representation of the Fragile X Mental Retardation Protein (FMRP). The major protein domains present in, and essential for its function include the nuclear localization signal (NLS), K homology 1 and 2 domains (KH 1 and KH 2 respectively), a nuclear export signal (NES) and an arginine/glycine/glycine box (RGG).
Figure 1.2
Figure 1.3. A schematic representation of dendritic spine morphology and ultrastructure. (A) A schematic representation of the different possible morphologies of dendritic spines. (B) Schematic representation of dendritic spine ultrastructure. Depicted here is a mushroom spine making a synaptic contact with an axon. SV – synaptic vesicles, Psd – postsynaptic density, F-actin – filamentous actin strands, SER – sarcoplasmic endoplasmic reticulum, MT – microtubule fibers.
Figure 1.3

A  Types of spines

Filopodia  Thin  Stubby  Mushroom

B  Spine Ultrastructure

Axon
Synaptic cleft
Psd
F-actin
SER
Spine head
Spine neck
Dendrite
MT
Figure 1.4. A schematic representation of the domain structure of the 24 mammalian matrix metalloproteinases (MMPs). Pro – the pro domain, SH – the cysteine-thiol group, catalytic – the catalytic domain, Zn – the zinc ion in the catalytic pocket, hinge – the hinge region, hemopexin – the hemopexin-like domain, FN II – the fibronectin type II repeats, CLD- collagen like domain, furin – the furin susceptible cleavage site in the pro domain, TM – the transmembrane domain, GPI – glycosyl-phosphatidylinositol linker region. MMP-23 is not included here as it does not share domain similarities to the other MMPs. It has much shorter pro and catalytic domains and lacks any FN II, hemopexin, TM or GPI domains.
Figure 1.4

MMP-2, -9 (Gelatinases)

MMP-1, -3, -8, -10, -12, -13, -18, -19, -20, -27

MMP-7, -26

MMP-11, -21, -28

MT1-MMP, MT2-MMP, MT3-MMP, MT5 MMP

MT4-MMP, MT6-MMP
Chapter 2 - Genetic deletion of matrix metalloproteinase-9 rescues the neurological deficits associated with Fragile X syndrome in the mouse model.

ABSTRACT

Fragile X syndrome (FXS) is the most common single-gene inherited form of mental retardation, with behaviors at the extreme of the autistic spectrum. Patients with FXS and Fragile X mental retardation gene knock out (Fmr1 ko) mice, an animal model for FXS, exhibit defects in dendritic spine maturation that may underlie cognitive and behavioral abnormalities in FXS. Dendritic spines are small protrusions on the surface of dendrites that receive the majority of excitatory synapses in the brain, and changes in their morphology affect synaptic strength. Previous studies have shown that minocycline promotes dendritic spine maturation in primary cultures of hippocampal neurons and in the developing hippocampus of Fmr1 ko mice, which is accompanied by improvements in behavioral performance. These studies suggest that the beneficial effects of minocycline may relate to its inhibitory action on the expression and activity of matrix metalloproteinase-9 (MMP-9), an extracellular protease that is upregulated in the hippocampus of Fmr1 ko mice. I found that the levels of MMP-2 and MMP-9 were higher in the hippocampus of adult Fmr1 ko mice, but their mRNA levels were not altered, suggesting a role for FMRP in the regulation of the translation of these MMPs. To determine if excess MMP-9 is responsible for dendritic spine abnormalities in Fmr1 ko mice I generated Fmr1/mmp9 double ko mice. Here, I report that the genetic deletion
of $Mmp9$ rescued dendritic spine maturation in cultured $Fmr1$ ko neurons, similar to minocycline and MMP-9-specific inhibitors. Moreover, the development of mature spines was accelerated in $Fmr1/mmp9$ double ko neurons in vivo. MMP-9 deficient neurons that expressed FMRP also developed a higher proportion of mature spines with large heads earlier than both $Fmr1$ ko and wt mice in vivo. The genetic deletion of $mmp9$ also reduced mGluR-dependent LTD in the hippocampus of $Fmr1$ ko mice to wt levels. $Mmp-9$ deletion also affected NMDAR-dependent LTP, which was impaired in the hippocampus of $Fmr1/Mmp-9$ double ko mice, confirming the role of activity-dependent regulation of MMP-9 activity in synaptic plasticity.

**INTRODUCTION**

Abnormal spine development observed in FXS humans and $Fmr1$ ko mice most likely underlies FXS-associated neurological deficits (Clapp et. al., 2001; Luo et. al., 2010; Willemsen et. al., 2004). Dendritic spines are small protrusions on the surface of dendrites that are post-synaptic contact sites for the majority of the excitatory glutamatergic synapses in the brain (Harris, 1999). Post-mortem brain tissue from FXS subjects showed abnormal dendritic spine morphology, with a preponderance of long thin, immature filopodial like spines compared to the mature, stubby spines seen in brain tissue from controls. Similar dendritic spine profiles are also seen in the $Fmr1$ knockout (ko) mice, a mouse model for FXS (Braun and Segal, 2000; Irwin et. al., 2000; Bilousova et. al.; 2009). Dendritic spines can have a variety of different morphologies ranging from
thin immature filopodial spines to the more mature mushroom type spine and the morphology of spines changes during development (Arellano et. al., 2007; Rochefort and Konnerth, 2012; Yoshihara et. al., 2009). During a period of active synaptogenesis typically during postnatal day (P) 1 to P12, there is an increase in the number of thin spines along the length of the dendrites. These thin spines develop into mature spines with larger heads and shorter necks. This process is regulated by synaptic activity and experience dependent learning (Rochefort and Konnerth, 2012; Nimchinsky et. al., 2001; Bohlen and Halbach, 2009). Spines can form from the filopodia that extend from the dendrites or arise directly from the dendritic shaft (Nimchinsky et. al., 2001; Bohlen and Halbach, 2009). The morphology of the spines determines the synaptic strength of a particular synapse as the spine head size is directly proportional to the number of postsynaptic receptors like the AMPA receptors, the area of the postsynaptic density, and, also to the pool of neurotransmitters available for release in the opposing presynaptic terminal (Arellano et. al., 2007; Schikorski and Stevens, 1999; Nusser et. al., 1998; Dobrunz and Stevens, 1997) The mature spines like the mushroom spines have more AMPARs and a larger PSD area and form more stable, stronger synapses while the more immature spines like the thin spines are known as ‘silent’ spines as they have less AMPARs, instead they have NMDARs which require activation before a signal can be propagated and hence form weaker synapses (Rochefort and Konnerth, 2012; Kasai et. al., 2003). Spines serve as separate biochemical compartments that can function independently from the dendritic shaft. They compartmentalize calcium ions, have the ability to isolate synaptic inputs and have the ability to potentiate large amplitude calcium
signals, which are the basis of most types of synaptic plasticity like LTP (Arellano et. al., 2007; Nimchinsky et. al., 2001).

Several different signaling pathways and receptors that are dysregulated in FXS are also known to influence dendritic spine maturation and plasticity. Activation of group 1 metabotropic glutamate receptors (mGluRs) with its agonist DHPG was shown to induce an immature spine morphology and increase number of immature thin spines (Vanderklish and Edelman, 2002; Cruz-Martin et. al., 2012). Enhanced activity of mGluR5 receptor was implicated in some FXS-associated deficits (Bear et. al., 2004). Normally, mGluR signaling blocks the translational repression of FMRP from its target mRNAs, allowing for the translation of the protein products encoded, which can cause α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor internalization and long term depression (LTD) - a decrease of synaptic strength. In the absence of FMRP, those proteins are expressed at a higher basal level and more AMPA receptors are internalized even before glutamate signaling arises, thus lengthening LTD (Bear et. al., 2004; Hou et. al., 2006; Oostra and Willemsen, 2009). mGluR antagonists such as MPEP, Fenobam, and CTEP have been shown to attenuate some of the FXS phenotypes in Fmr1 ko mice and in human patients (Yan et. al, 2005; Levenga et. al., 2010; Michalon et. al., 2012). Others have shown that Fmr1 ko mice have altered GABA receptor subunit composition and lower GABAergic inputs to certain hippocampal circuits (Brenman, 2009; D’Hulst et. al., 2009), so that GABA receptor agonists, such as Baclofen and Nipecotinic acid partially rescue the FXS phenotypes (Levenga et. al., 2010; Pacey et. al., 2009).
Increased excitation has been shown to affect spine morphology, specifically to induce maturation of spines, that is, to induce enlargement of the head sizes and shortening of spine necks. Increased excitation also results in increased synaptogenesis so the density of spines also increases (Yuste and Bonhoeffer, 2001; Toni et. al., 1999; Muller et. al., 2000; Engert and Bonhoeffer, 1999). Increased excitation has been shown to not only affect the number and morphologies of spines, but also the protein levels and activity of MMP-9 (Nagy et. al., 2006; Dozdagi et. al., 2007; Meighan et. al., 2007; Okulski et. al., 2007), and these changes in spine dynamics that are seen might be mediated through the actions of this MMP on the extracellular synaptic adhesion proteins surrounding the shaft and spines. This upregulation of MMP-9 has been shown to be mediated by NMDAR signaling and is protein synthesis dependent (might be affected even at basal levels in the Fmr1 ko mice); and MMP-9 might mediate these effects on synaptic function through the cell surface integrin receptors (Nagy et. al., 2006). It is likely that the changes in spines seen in response to increased excitation are mediated by MMP-9 activity since increased MMP-9 activity has also been implicated in remodeling that accompanies kainic- acid induced epileptogenesis in the hippocampus (Zhang et. al., 1998; Szklarczyk et. al., 2002).

Previous work done in our laboratory has implicated matrix metalloprotease-9 (MMP-9) in abnormal spine development in FXS (Bilousova et al., 2009). We found that MMP-9 activity is higher in Fmr1 ko mice than in wild-type; further, minocycline treatment reduced MMP-9 activity in Fmr1 ko mice, ameliorated the dendritic spine phenotype, and improved behavioral abnormalities seen in Fmr1 kos. When cultures of
mature hippocampal neurons from wild-type mice were treated with MMP-9 protein they
developed long, immature filopodial like spines that mimicked the immature spine
precursors seen in Fmr1 ko hippocampal cultures (Bilousova et. al., 2008). This same
relationship between Fmr1 and MMP-9 has also been reported in the drosophila model of
FXS where overexpression of TIMP1 (the endogenous regulator of MMPs) or a
defmr/mmp null mutant rescued all nervous system defects seen in the fmr null flies,
suggesting a genetic interaction between these two genes and/or their protein products
(Siller and Broadie, 2011). Other groups have also shown MMP-9 involvement in
synaptic plasticity and regulation of NMDAR currents mediated through integrin activity
(Nagy et. al., 2006; Meighan et. al., 2006) suggesting all these components that are
defective in FXS may be linked.

Dendritic spines are surrounded by extracellular matrix proteins, which form
perineuronal nets around the soma and dendrites of neurons (Celio, 1999; Yamaguchi,
2000). These extracellular matrix proteins are processed or activated by a host of
different proteins including the matrix metalloprotease (MMP) superfamily of proteins,
which were first discovered for their ability to cleave ECM components, and the products
then go on to affect dendritic morphology through cell surface receptors and intracellular
signaling cascades (Dityatev and Schacvhner, 2003; Szepesi et. al., 2013; Dziembowska
et. al., 2012; Shi et. al., 2006). One of the ECM substrates cleaved by MMP-9 is laminin
(Doucet & Overall, 2010), which binds to integrins and promotes dendritic spine
maturation and could trigger synapse remodeling (Shi and Ethell, 2006). Other groups
have also shown the MMPs, specifically MMP-9 to be involved in the regulation of
synaptic plasticity through integrin signaling pathways (Nagy et al., 2006; Meighan et al., 2006). Other MMPs like MMP-7 have also been shown to influence spine morphology, specifically promoting mature to more immature-filopodial spine transitions (Wlodarczyk et al., 2011; Bilousova et al., 2006).

Some MMPs also appear to have roles in hippocampal dependent learning. MMPs 3 and 9 have been shown to be important in spatial learning (Meighan et al., 2006; Nagy et al., 2007; Wright et al., 2007) while MMP-9 appears to have a role in LTP, which is essential for long term synaptic plasticity, and these effects are mediated by NMDAR activity (Nagy et al., 2006; Meighan et al., 2006; Nagy et al., 2006).

Although these MMPs are highly expressed in the nervous system, only MMP-9 is a constituent of the healthy CNS with MMP-3 and MMP-7 being expressed by microglia and macrophages during inflammation reactions or in response to neurological disease (Ethell et al., 2002; Buhler et al., 2010; Ethell and Ethell, 2007; Dziembowska and Wlodarczyk, 2012; Sbai et al., 2008; Visse and Nagase, 2003).

To examine whether enhanced MMP-9 activity contributes to abnormal development of dendritic spines and impaired synaptic plasticity in hippocampus of Fmr1 ko mice, I characterized the morphologic and functional effects of genetic deletion of Mmp-9 in Fmr1 ko mice. I compared dendritic spine development in the hippocampal neurons of dbl ko, Fmr1 ko, Mmp-9 ko and wt mice using both in vitro and in vivo approaches. I also investigated the induction and maintenance of LTD and LTP in these mice. My results confirmed the preponderance of immature dendritic spines in the adult hippocampus of Fmr1 ko mice due to abnormal spine development; but the genetic
deletion of *Mmp-9* enhanced the formation of mature dendritic spines with large heads in FMRP-deficient (i.e. dbl ko) neurons similar to wt neurons both *in vitro* and *in vivo*. There were no differences in the number of synapses between the *Fmr1 ko* and wt neurons, but the number of filopodia increased in *Fmr1 ko* neurons as compared to wt or dbl ko neurons. *Fmr1 ko* mice also exhibited enhanced mGluR5-dependent LTD in the hippocampal slices, similar to previously reported results, but the dbl ko mice exhibited normal mGluR5-dependent LTD, suggesting the mGluR hypersensitivity is also influenced by MMP9 in the *Fmr1 ko* mice. MMP-9 deletion also affected NMDAR-dependent LTP, which was impaired in the hippocampus of *Fmr1/Mmp-9* double ko mice, confirming the role of activity-dependent regulation of MMP-9 activity in synaptic plasticity.

**MATERIALS AND METHODS**

**Ethics statement**

All animal care protocols and procedures were approved by the UC Riverside Animal Care & Use Program, which is accredited by AAALAC International, and animal welfare assurance number A3439-01 is on file with the Office of Laboratory Animal Welfare (OLAW).
Mice

The FVB.Cg-Mmp9tm1Tvu/J and FVB.129P2-Fmr1tm1Cgr/J (Fmr1 ko) and FVB.129P2-Pde6b"Tyr<sup>c-ch</sup>/AntJ controls (wt) were obtained from the Jackson Laboratories. The FVB.Cg-Mmp9tm1Tvu/J mice were backcrossed, in-house, with the Fmr1 ko and wt mice to generate Fmr1/mmp9 double knockout mice and mmp9 ko mice, respectively. These mice do not suffer from retinal degeneration due to restoration of the pde6b allele and do not develop blindness, making them a suitable model for behavioral analysis. All genotypes were confirmed by PCR analysis of genomic DNA isolated from mouse tails. Mice were maintained in an AAALAC accredited facility under 12 hour light/dark cycles, and fed standard mouse chow. All mouse studies were done within NIH and Institutional Animal Care and Use Committee guidelines.

Hippocampal neuron cultures

Cultures of hippocampal neurons were prepared from embryonic day 15 (E15) or E16 pups. Briefly, hippocampal cells were treated with papain (0.5 mg/ml) and DNase (0.6 μg/ml) for 20 min at 37°C, mechanically dissociated, and then plated on glass coverslips that had been pre-coated with poly-DL ornithine (0.5 mg/ml in borate buffer) and laminin (5 μg/ml in PBS). The hippocampal cells were cultured in Neurobasal medium with 25 μM glutamine, 1% penicillin-streptomycin, and B27 supplement (Invitrogen, Carlsbad, CA), under 5% CO<sub>2</sub>/10% O<sub>2</sub> atmosphere at 37°C. Hippocampal neurons were transfected with pEGFP plasmid at 10 days in vitro (DIV) using a calcium phosphate method, as previously described (Shi and Ethell, 2006).
Immunostaining and image analysis

Cultured hippocampal neurons were fixed in 2% paraformaldehyde in PBS, permeabilized in 0.01% Triton X-100 in PBS, and then blocked in PBS containing 5% normal goat serum and 1% BSA. Dendritic spines and filopodia were visualized by GFP fluorescence. Presynaptic boutons were labeled by immunostaining for the presynaptic vesicle marker synapsin using rabbit anti-synapsin I antibody (10 μg/ml; AB1543P; Millipore), the excitatory synapse marker vesicular glutamate transporter 1 (vGLUT1) using rabbit anti-vGLUT1 (1μg/4μl; 482400; Invitrogen) and the inhibitory synapse marker glutamate decarboxylase 65 (GAD65), mouse anti-GAD65 (10 μg/ml; 559931; BD Pharmingen). Postsynaptic sites were identified by immunostaining against PSD-95 using rabbit anti-Psd95 (CP35, Calbiochem). Secondary antibodies used were Alexa Fluor 594-conjugated goat anti-mouse IgG (4 μg/ml; Molecular Probes), Alexa Fluor 647-conjugated donkey anti-rabbit IgG (4 μg/ml; Molecular Probes) or Alexa Fluor 488-conjugated donkey anti-rabbit IgG (4 μg/ml; Molecular Probes). Fluorescent images were taken using a confocal laser-scanning microscope (model LSM 510; Carl Zeiss MicroImaging, Germany) with 63X water Fluor objective. Image analysis was performed using Image-J software as previously described (Bilousova et al., 2006). Briefly, experimental and control samples were encoded for blind analysis. In each experiment 2-3 coverslips were analyzed for each condition. At least ten GFP-transfected spiny pyramidal neurons were randomly imaged in each group. Primary and secondary dendrites were selected for morphometric analysis of dendritic spines using LSM Image Browser (Carl Zeiss). Densities of dendritic protrusions were determined as the number...
of protrusions per 10 μm of dendrite. Lengths of dendritic protrusions were measured from protrusion tip to dendritic shaft. Dendritic spines were identified as dendritic protrusions connected with a synapsin-positive presynaptic terminal and were grouped into three categories according to their morphology: 1) thin (long, thin protrusions with a small head); 2) mushroom (short protrusions with large heads and thin neck) and 3) stubby (short protrusions with thick neck and no head). Dendritic protrusions lacking opposing pre-synaptic boutons were considered to be filopodia. Each experiment was performed at least three times. Statistical analysis was performed using one-way ANOVA following which post-hoc pair-by-pair differences were resolved with the Tukey-Kramer method.

**Brain Slices**

Age-matched male mice at ages P8, P14, P21 and P60 (adult) were anesthetized with isoflurane and perfused with cold PBS followed by 4% paraformaldehyde (PFA). Brains were extracted and postfixed in 4% PFA at 4 °C for 2-4 hours before being sectioned coronally into 100-μm slices on a vibratome. Sections were then biolistically labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) using a gene gun and incubated in PBS for 3 days at 4 °C before being mounted onto slides in PBS and sealed with Cytoseal 60 (Fisher). Pyramidal neurons in CA1 were imaged by confocal microscopy (model LSM 510; Carl Zeiss MicroImaging) using a series of high-resolution optical sections (1,024 × 1,024-pixel format) that were captured for each neuron with a 63× water-immersion objective (1.2 numerical aperture), with 1× zoom at 1-μm step
intervals (z-stack). All images were acquired under identical conditions. Each z-stack was collapsed into a single image by projection (Zeiss LSM Image software), converted to a tiff file, and analyzed using Image J Software. Apical dendrites were selected for dendritic spine analyses. Sections analyzed for puncta number were labeled by immunostaining for synapsin I, vGlut1, GAD65 and PSD95 as described above, imaged using confocal microscopy in z-stacks and each image in the series was thresholded to identical levels (120 and above included) and puncta were analyzed using Image J Software. At least 3 animals were analyzed per group and at least 20 neurons were imaged per animal.

**Gelatin Zymography**

Gelatin gel zymography was performed as previously described (Zhang and Gottschall, 1997; Manabe et al., 2005) with minor modifications. Briefly, hippocampal tissues isolated from age matched male mice (n=4 mice per group) were resuspended in 50 mM Tris-HCl (pH=7.6) buffer containing 150 mM NaCl, 5 mM CaCl₂, 0.05% Brij35, 0.02% Na₃N, 1% Triton X-100, 100 μM PMSF and PI cocktail (Sigma). The gelatinases, MMP-2 and MMP-9, were pulled down with gelatin agarose beads (Sigma, G5384) and separated on non-reducing SDS-PAGE gels containing 0.1% gelatin (Invitrogen). Following separation, gels were soaked in 1X renaturing buffer (LC2670, Invitrogen) to remove all traces of SDS and allow the MMPs to refold thus regaining most of their enzymatic activity. Following renaturation, gels were incubated in 1X developing buffer (LC2671, Invitrogen) for 96 hours, which allowed the gelatinases (MMP-2 and MMP-9)
to degrade gelatin in the gel. Gels were then stained with coomassie blue overnight to uniformly stain the gels after which destaining revealed areas of MMP activity as unstained bands. Levels of MMP-2 and MMP-9 proteins were quantified by densitometry. Statistical analysis was performed using one-way ANOVA following which post-hoc pair-by-pair differences were resolved with the Tukey-Kramer method.

**Quantitative RT-PCR**

Hippocampal and cortical tissues were dissected from mice of age P8, P14 and P21 (n=3-5 mice per group per time point). Total RNA from each tissue sample was prepared using Trizol (Life Technologies) according to the manufacturer’s instructions and cDNA was synthesized using SuperScript VILO cDNA Synthesis Kit (Life Technologies) as recommended by the manufacturer. To examine mRNA expression of all the mammalian MMPs, TIMPs and some ADAMs, specific forward and reverse primers as listed in supplementary table 1 were used. All samples were normalized to the expression of GAPDH (see table 2.1 for primer sequence). Each reaction mixture contained 1× Power SYBR Green PCR Master Mix (Life Technologies) and all the reactions were run in triplicate. The PCR amplification protocol was as follows: initial DNA Polymerase activation at 95°C for 10 min, followed by 40 cycles with denaturation at 95°C for 15 s, and annealing + extension at 60°C for 1 min. Amplification was performed in a StepOne Real Time PCR System (96-well format) (Life Technologies) and analyzed by normalizing the expression of each gene to GAPDH within each tissue sample. Statistical
analysis was performed using One-way ANOVA followed by pair-wise comparison using the Bonferroni post-hoc test.

**Electrophysiology**

Mice at P21-28 (6 animals, 10-17 slices per group) were anesthetized by isofluorane inhalation and decapitated. Brains were removed from the skull and immediately immersed in chilled cutting solution containing 110mM sucrose, 60 mM NaCl, 28 mM NaHCO₃, 1.25 mM NaH₂PO₄, 3 mM KCl, 7 mM MgSO₄, 0.5 mM CaCl₂, 5 mM glucose, and 0.6 mM ascorbic acid, bubbled with 95% O₂/5% CO₂. Transverse hippocampal slices (400 μm thick) were prepared using a Vibratome (Leica VT1200S) and were allowed to recover in the cutting solution for 30 min at room temperature followed by an additional hour in artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 25 mM NaHCO₃, 1 mM NaHPO₄, 4.4 mM KCl, 1.2 mM MgSO₄, 2mM CaCl₂ and 10mM glucose with constant bubbling of 95% O₂/5% CO₂ gas. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded in the *stratum radiatum* of CA1 with glass electrodes (5 MΩ) filled with ACSF. Stimuli were delivered to the comissural/Schaffer collateral afferents with a concentric bipolar electrode positioned parallel to the recording electrode 200 μm away. Input/output curves were generated by stepping the stimulation amplitude from 0 to 100 μA. The basal stimulation intensity for our experiments was set at 50% of the maximal evoked fEPSP amplitude and recordings were done at 30°C. Input-output curves were generated from field responses to single pulse stimulation of 0-100 μA (stimulus increased in 10 μA steps). Fiber volley (FV) amplitudes and 20-80% fEPSP
slopes were calculated for each slice and mean values for each genotype were grouped by stimulus intensity. For mGluR LTD, after 10 minutes of stable baseline recording, 100 µM DHPG was washed into the slice for 10 minutes before being replaced with ACSF and LTD was sampled at 30 second intervals for 60 minutes post DHPG treatment. Initial fEPSP slopes were calculated as 20-80% of the field recordings obtained and depression was calculated by dividing the average slope of post-induction responses by the average slope of preinduction baseline responses. For LTP experiments, slices were prepared as described above from mice at 2-3 months of age (6 animals, 11-15 slices per group). After 10 minutes of stable baseline recording, LTP was induced with a train of 5 theta bursts, each containing four pulses at 100 Hz with an interburst interval of 200ms (as described in Lauterborn et. al., 2007). LTP was sampled at 30 second intervals for 60 minutes post stimulation and potentiation was calculated by dividing the average slope of post-induction responses by the average slope of pre-induction baseline responses. Statistical analysis was performed using one-way ANOVA following which post-hoc pair-by-pair differences were resolved with the Tukey-Kramer method.

RESULTS

**Mmp-9 levels are higher in the hippocampus of Fmr1 ko mice.**

Our previous studies showed developmental regulation of MMP-9, with higher levels in the hippocampus of Fmr1 ko mice at postnatal day (P) 8 and P14 (Bilousova et al., 2009). I found that this up-regulation of MMP-9 levels was also maintained in the adult Fmr1 ko
hippocampus (Fig. 2.1 A). Gelatin zymography detects total levels of both MMP-9 and MMP-2 since both these proteases share the same major substrate, gelatin. Gelatin zymography showed a significant increase in protein levels of both gelatinases, MMP-9 and MMP-2, in the adult hippocampus of Fmr1 ko mice, compared to wild-type (wt) mice (p=0.0001 and p=0.0176, Fig. 1A). However, while the protein levels of Mmp-9 and Mmp-2 were higher in the brains of Fmr1 ko mice, mRNA levels for these gelatinases were not altered (Fig. 2.1 B), suggesting post-transcriptional dysregulation of Mmp-9 and MMP-2 in the hippocampus of Fmr1 ko mice.

**Mmp-9 deletion corrected spine abnormalities and induced spine maturation in Fmr1/Mmp-9 ko hippocampal neurons in vitro**

I investigated the importance of MMP-9 to the dendritic spine abnormalities in Fmr1 ko mice by generating double (dbl) ko mice that were deficient for Fmr1 and Mmp-9. As previously reported (Bilousova et al., 2009), Fmr1 ko neurons develop a higher proportion of immature spines with smaller heads and more filopodia in vitro (p<0.0001, Fig. 2.2 B, p=0.0030, Fig. 2.2 D). Contrastingly, Mmp9-deficient neurons had the opposite phenotype and exhibited significantly shorter spines than wt mice (p=0.0031, Fig. 2.2 C). I also found a significant enlargement of spine heads, a decrease in the number of filopodia, and a higher proportion of mature mushroom-shaped spines in double Fmr1/Mmp-9 (dbl) ko hippocampal neurons as compared to Fmr1 ko neurons (p<0.0001, Fig. 2B; p=0.0030, Fig. 2.2 D and p=0.0042, Fig. 2.2 E). Dendritic spines in dbl ko hippocampal neurons were not significantly different from wt neurons (Fig. 2.2 A-
E). While, dbl ko neurons developed more mature spines with larger heads and less filopodia than Fmr1 ko neurons, the overall number of pre-synaptic inputs did not change (p=0.8638, Fig. 2.2 F). It appears that the increase in the number of mushroom-shaped spines is concurrent with a decrease in the number of thin spines (Fig. 2.2 E), suggesting that Mmp-9 deletion promotes spine maturation in dbl ko neurons. These observations establish that higher levels of MMP-9 contribute to dendritic spine abnormalities in Fmr1 ko neurons, since they are corrected by the genetic deletion of Mmp-9.

**Genetic deletion of Mmp-9 rescues Fmr1 ko-associated dendritic spine abnormalities in adult hippocampus.**

To determine if Mmp-9 deficiency corrects dendritic spine defects resulting from FMRP loss in vivo, I analyzed spine numbers and morphology in wt, Fmr1 ko, Mmp9 ko and dbl ko mice at P8, P14, P21 and adults (Fig. 2.3 A-D). I found a significant reduction in the size of spine heads, an increase in spine lengths and density in CA1 hippocampal neurons of both developing and adult Fmr1 ko mice (Fig. 2.3 B-D). On the contrary, Mmp-9 deletion triggered earlier maturation of spines with significantly larger spine heads in Mmp9 ko neurons at P8 and P14 (p=0.0001 and p=0.0025, Fig. 3B). However, P8 neurons lacking both Fmr1 and Mmp9 (dbl ko neurons) exhibited normal spines that were not significantly different from wt neurons (Fig. 2.3 B, C). Dendritic spines in dbl ko neurons were significantly shorter, had larger heads and were fewer in numbers than in Fmr1 ko neurons at P8 (p=0.0012, p=0.0037 and p<0.0001, Fig. 2.3 B, C, D), but the spine heads in dbl ko neurons were smaller than in Mmp9 ko neurons (p<0.0003).
Although the spine maturation was accelerated in dbl ko neurons at P8 it slowed down at P21. Mature dendritic spines continue to develop after that age and by 2 months dbl ko neurons exhibited dendritic spine profiles that were similar to wt neurons and more mature than in *Fmr1* ko neurons. Adult dbl ko neurons exhibited spines that had larger heads, were significantly shorter and fewer in numbers than adult *Fmr1* ko neurons (p=0.0001, p=0.0449 and p=0.0244, Fig. 2.3 B, C, D). Taken together, these results indicate that Mmp9 regulation is involved in the development and maintenance of mature dendritic spines *in vitro* and *in vivo*, and that enhanced MMP-9 activity is responsible for the development of immature dendritic spines in *Fmr1* ko neurons.

**Genetic deletion of Mmp-9 ameliorates enhanced mGluR5-dependent LTD in the hippocampal slices of double Mmp-9/Fmr1 ko mice**

I examined LTD in hippocampal slices from wt, *Fmr1* ko, *Mmp9* ko and dbl ko mice by inducing it with the bath application of the mGluR5 agonist DHPG for 10 min. A depression of the field excitatory postsynaptic potential (fEPSP) was found in all four groups, but was significantly greater in *Fmr1* ko mice (down to 30%), upon DHPG treatment, compared to wt (down to 40%) and *Mmp9* ko (40%). The dbl ko mice displayed less depression with the fEPSPs only decreasing to 60% of baseline. Hence, the induction of LTD was enhanced in the *Fmr1* ko mice compared to wt and even more greatly enhanced compared to the dbl ko mice. In comparison, the dbl ko mice displayed less depression than the wt mice while the *Mmp9* ko mice did not vary from wt (Fig. 2.4 B). Maintenance of this LTD was also different in these four groups, being significantly
greater in Fmr1 ko mice (58%), 60 min after DHPG washout, compared to wt (79%), Mmp9 ko (86%) and dbl ko mice (83%, P<0.0001, Fig. 2.4 C). There were no differences in input/output (I/O) curves between the four groups, indicating that basal pre- and post-synaptic responses are not altered by Mmp-9 deficiency (Fig. 2.3 A). While Mmp-9 ko exhibited mGluR5-induced LTD that was similar to wt mice, LTP induced by four 1-s duration 100-Hz tetani of Schaffer collaterals was impaired in hippocampal slices of both Fmr1 ko and Mmp-9 ko mice as compared to wt mice (Fig. 2.4 D, E). Upon stimulation, the wt mice displayed fEPSPs that were potentiated to 250% of the baseline values but in Fmr1 and Mmp-9 ko mice, this potentiation was affected and only increased to about 170%. This induction was even more severely affected in the dbl ko mice where it was only potentiated to about 140% (Fig. 2.4 D). The maintenance of LTP was also affected in the ko mice with a potentiation of 143% found in wt slices 60 min after high frequency stimulation, but only a 115% potentiation detected in dbl ko (p=0.0267), 113% in Fmr1 ko (p=0.0131) and 133% in Mmp-9 ko (Fig. 2.4 E).

Altogether these results indicate that Mmp-9 impacts mechanisms that underlie mGluR5-induced LTD, since genetic deletion of Mmp-9 prevents the deficit in Fmr1 ko mice; however, the deletion of Mmp-9 is unable to rescue the LTP deficit seen in Fmr1 ko mice.

DISCUSSION

Here I provide evidence that MMP-9 plays a dominant role in some neurological FXS-associated defects, namely aberrant dendritic spine maturation and enhanced
mGluR5-dependent LTD. Others have previously suggested that MMP-9 plays a role in these deficits based on pharmacological studies in Fmr1 ko mice (Bilousova et al., 2009) and in dfmr null flies (Siller and Broadie, 2011); here I used genetics to confirm the importance of MMP-9 in FXS with mmp-9/Fmr1 dbl ko mice. Dbl ko mice showed a rescue of many Fmr1 ko-associated features in the brain. These results are consistent with previous work showing that inhibiting MMP-9 with minocycline promotes dendritic spine maturation and improves FXS-associated behavior in the Fmr1 ko mice and FXS subjects (Bilousova et al., 2006, 2009; Ethell & Ethell, 2007; Paribello et al., 2010; Leigh et al., 2013; Dansie et al., 2013). Fmr1 ko mice have higher levels of MMP-9 protein and activity in the hippocampus, which is reduced by minocycline treatment. In this study, I demonstrate that the genetic disruption of mmp-9 rescues Fmr1 ko associated defects in Fmr1/mmp9 dbl ko mice. In sharp contrast to Fmr1 ko mice, dbl ko mice showed normal dendritic spine maturation both in vitro and in vivo at early postnatal and adult ages.

Hippocampal brain slices from Fmr1 ko mice displayed enhanced mGluR5-dependent LTD, which has been suggested to underlie cognitive and behavioral defects in Fmr1 ko mice and FXS subjects (Huber et. al., 2002; Hou et. al., 2006; Bhattacharya et. al.,2012; Michalon et. al.; 2012). Fmr1 ko mice are known to be hypersensitive to mGluR signaling and the increased expression of type I mGluR's in these animals leads to more AMPA receptors being internalized prior to glutamate signaling, thereby lengthening LTD (Bear et. al., 2004; Oostra et. al., 2009; Hou et. al., 2006). Hyperactivation of type I mGluR receptors contributes to this enhanced LTD response as receptor antagonists, including MPEP, Fenobam and CTEP, block this increase and alleviate some FXS-
associated behaviors in *Fmr1* ko mice and FXS patients (Yan et. al., 2005; Michalon et. al., 2012; Levenga et. al., 2010). In this study I also observed enhanced mGluR5-dependent LTD in the hippocampus of *Fmr1* ko mice, as previously reported by others. However, genetic disruption of *mmp-9* ameliorated the LTD defects in dbl ko mice, demonstrating that higher levels of MMP-9 contribute to the FXS-associated mGluR5 hypersensitivity and behaviors. These findings establish that changes in *mmp-9* play a critical role in the development of metabotropic glutamate synaptic receptor hyperactivity that has been linked to enhanced LTD.

Higher MMP-9 levels might affect synaptic activity in a number of ways (Ethell & Ethell, 2007). MMPs were originally discovered for their ability to cleave ECM components, many of which have been shown to participate in dendritic spine development and plasticity (Szepesi et. al., 2013; Dziembowska et. al., 2013; Shi et. al., 2006). Higher MMP levels and activity can affect the integrity of these ECM components, which has been shown to be detrimental to neuronal function in several neurologic diseases (Platt et. al., 2003; Giannelli et. al., 2002; Howell and Gottschall, 2012) and can have adverse effect on synaptic functions and the stability of neuronal circuits (Takacs et. al., 2009; Nagy et. al., 2006; Howell and Gottschall, 2012). Prominent among the ECM substrates cleaved by MMP-9 is laminin (Doucet & Overall, 2010), which binds to integrin to support dendritic spine maturation (Shi and Ethell, 2006). The release of RGD-containing peptide from laminin could enhance integrin signaling in peri-synaptic areas that may destabilize mature spines and trigger synapse remodeling (Shi and Ethell, 2006). Indeed, several studies have shown that MMP-9 can regulate
synaptic plasticity and NMDAR currents by modifying integrin signaling (Nagy et. al., 2006; Meighan et. al., 2006). MMP-9 can also cleave several cell surface receptors, including ephrin-B ligands and EphB receptors, which are expressed at synaptic sites and regulate synapse formation and maintenance (Ethell and Ethell, 2007; Georgakopoulos et. al., 2006; Lin et. al., 2008; Navaratna et. al., 2013; Wang et. al., 2008; Mizoguchi et. al., 2011, 2013). Both integrins and EphB receptors are powerful regulators of the actin cytoskeleton and can mediate the effects of MMP-9 on dendritic spines (Wang et. al., 2008).

Dendritic spine size directly correlates with the size of postsynaptic density (Harris et. al., 1992; Tashiro and Yuste, 2003). Immature thin spines with small heads, which are abundant in FXS, express less AMPAR in the postsynaptic density (Matsuzaki et. al., 2001). Both enhanced MMP-9 activity and loss of FMRP have been shown to induce immature spine phenotypes, while the inhibition of MMP-9 activity increases the proportion of mature mushroom spines in Fmr1 ko neurons (Bilousova et. al., 2009; Michaluk et. al., 2011; Dziembowska et. al., 2012). Enhanced mGluR5 activity was also shown to contribute to the immature spine phenotype, which can be reversed by mGluR5 antagonists (Bassell and Gross, 2008). Synaptic plasticity is also dependent on AMPAR levels at postsynaptic spines, which are regulated by cytoskeletal changes and protein synthesis. For example, increased translation of Arc leads to increased internalization of AMPARs in Fmr1 ko mice (Bagni et. al., 2012) and may underlie enhanced mGluR5 LTD and impaired LTP in Fmr1 ko mice (Huber et. al., 2002; Lauterborn et. al., 2007). Higher levels of MMP-9 may affect the AMPAR trafficking during LTD by regulating
actin cytoskeleton through the integrin signaling. Such cytoskeletal changes, mediated by integrin or EphB receptor signaling cascades, may also underlie the spine morphology changes seen in Fmr1 ko mice.

LTP is modified in the hippocampus of both Fmr1 ko mice (Lauterborn et. al., 2007) and Mmp-9 ko mice, so it was not surprising to see that LTP was also impaired in dbl mutant mice. This result suggests that LTP defects in Fmr1 ko mice are not strictly due to elevated MMP-9 protein, and instead may be due to functional deficiencies in activity-dependent regulation of Mmp-9. Enhancement of basal MMP-9 activity may contribute to synaptic abnormalities in an array of neurological conditions, including FXS, and a disruption of activity-dependent regulation of MMP-9 can also affect LTP induction and maintenance. LTP, like LTD, is critically dependent on cytoskeletal changes that regulate AMPAR levels at the postsynaptic spines and protein synthesis, which are affected by both FMRP and MMP-9. The increased internalization of AMPARs seen in Fmr1 ko mice (Bagni et. al., 2012), which might be a result of increased MMP-9 activity, could also affect the induction of and maintenance of LTP in these animals.

In summary, here I show that the genetic deletion of Mmp-9 is sufficient to prevent abnormal dendritic spine development and correct mGluR5-dependent LTD in the Fmr1 ko mice. The increased Mmp-9 levels and activity seen in Fmr1 ko mice and FXS patients may act on extracellular matrix proteins to affect the ability of the dendritic spines to stay plastic through its cleavage activity on these ECM proteins. This activity could also affect the development time course of the spines and their morphology. The
products of this cleavage could affect the post-synaptic terminals by binding to a number of cell surface receptors found in the PSD, which may have overlapping intracellular signaling cascades with the mGluRs. In the following chapters I investigate if this deletion can also ameliorate the behavioral and physical deficits associated with FXS. I also look at possible mechanisms through which MMP-9 acts to produce the changes we see in Fmr1 ko mice and FXS subjects.
REFERENCES


Figure 2.1. Gelatinase levels are upregulated in the hippocampi of Fmr1 ko mice. (A) Detection of MMP-9 and MMP-2 levels in the hippocampi of wt, Fmr1 ko, Mmp-9 ko and Dbl ko adult mice. The levels of the gelatinases were detected by gelatin zymography, quantified by densitometry and normalized to total protein concentration. Bar graphs show average MMP-9 and MMP-2 levels and the error bars indicate SEM (n = 4 mice per group). Statistical analysis was performed using one-way ANOVA followed by Tukey’s multiple-comparison posttest (**p < 0.01, ***p < 0.001). (B) MMP-9 and MMP-2 mRNA levels were detected by quantitative real-time PCR in P7, P14 and P21 hippocampi of wt, Fmr1 ko, Mmp9 ko and Dbl ko mice. The bar graphs represent the MMP mRNA levels normalized to GAPDH levels for each sample and the error bars indicate SEM (n=3-5 mice per group). Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s multiple-comparison posttest (*p < 0.05, **p < 0.05, ***p < 0.001)
Figure 2.1
Figure 2.2. Genetic deletion of Mmp-9 promotes formation of mature dendritic spines in the Fmr1 ko hippocampal neurons in vitro. (A) Confocal images showing the dendrites of 14 days in vitro (div) hippocampal neurons from wt, Fmr1 ko, Mmp-9 ko and Dbl ko mice. Dendritic morphology was visualized by GFP fluorescence. The arrowheads denote mature mushroom spines while the arrows denote immature thin spines. Scale bars, 10 µm. (B-E) Quantitative analysis of spine head areas (B), spine lengths (C), density (D) and the proportion of thin, mushroom and stubby spines in wt, Fmr1 ko, Mmp-9 ko and Dbl ko hippocampal neurons. Primary and secondary dendrites were selected for all dendritic spine analyses. All bar graphs show average values and the error bars indicate SEM (n= 400-700 dendritic spines from 8-12 neurons per group). Statistical analysis was performed using one-way ANOVA followed by Tukey’s multiple-comparison posttest (*p < 0.05, **p < 0.01, ***p < 0.001). (F) Pre-synaptic boutons were identified by immunostaining against synapsin 1 and GAD65, quantifying the density and average size of each type of puncta. Bar graphs represent average values and the error bars indicate SEM (n= 8-12 neurons per group). Statistical analysis was performed using one-way ANOVA followed by Tukey’s multiple-comparison posttest. No significant differences were found between the groups.
Figure 2.2

A. Dendritic spine analysis

wt  Fmr1 ko  Mmp-9 ko  Db1 ko

14 div hippocampal neurons in vitro

B. Average spine head area, μm²

C. Average spine length, μm

D. Fibopodia / 100 μm dendrite

E. Dendritic spines, %

F. Immunodetection of pre-synaptic boutons

Average puncta per 100 μm²

synapsin 1  GAD65

Average puncta dia. μm

synapsin 1  GAD65
Figure 2.3. Hippocampal neurons develop normal mature dendritic spines in the double *Fmr1/Mmp-9* ko mice *in vivo* early in post natal development and at adult ages. (A) Confocal images of DiI labelled dendrites of wt, *Fmr1* ko, *Mmp-9* ko and *Db1* ko hippocampal neurons from CA1 stratum radiatum at P8, P14, P21 and 2 months. The arrowheads denote mature mushroom spines and the arrows show the immature thin spines. Scale bars, 10 µm. (B-D). Quantitative analysis of the average spine head areas (B), spine lengths (C) and density (D) in CA1 hippocampal neurons of wt, *Fmr1* ko, *Mmp-9* ko and *Db1* ko mice at P8, P14, P21 and 2 months. Only apical dendrites were selected for dendritic spine analyses. Error bars indicate SEM (n= 3 mice per group). Statistical analysis was performed using one-way ANOVA followed by Tukey’s multiple-comparison posttest (*p < 0.05, **p < 0.01, ***p < 0.001).
Figure 2.4. Genetic deletion of Mmp-9 ameliorates enhanced DHPG-dependent LTD in the hippocampal slices of Fmr1 ko mice. (A) Input/output curve as an indicator of basal synaptic transmission in the hippocampal slices of wt, Fmr1 ko, Mmp-9 ko and Dbl ko mice. Graph shows mean fEPSP slopes and FV amplitudes generated from field responses to single pulse stimulation (from 0-100µA, in 10µA steps) for wt, Fmr1 ko, Mmp-9 ko and Dbl ko mice. The error bars represent SEM (n= 13-30 mice per group). Statistical analysis was performed using one-way ANOVA followed by Tukey’s multiple-comparison posttest. No significant differences were found between the groups.

(B, C) LTD of Schaffer collateral-CA1 synapses was evoked by application of 100 µM DHPG for 10 minutes in hippocampal slices from wt, Fmr1 ko, Mmp-9 ko and Dbl ko mice. Field EPSPs were recorded for 10 min prior to the induction to establish a baseline and 60 min after DHPG removal at 0.5 min interval. The fEPSPs were normalized to baseline. (B) The graph shows mean and SEM values (n= 11-17 slices from 6 mice per group). Inset: Overlaid representative fEPSP traces collected during baseline (1), immediately following DHPG treatment (2) and 60 minutes post-DHPG treatment (3) for wt, Fmr1 ko and Dbl ko mice. Calibration: 50 mV, 2 ms. (C) Bar graph shows mean normalized fEPSP values for 65-80 minutes. Error bars indicate SEM. (D, E) LTP of Schaffer collateral-CA1 synapses was evoked by a five theta burst stimulation train in hippocampal slices from wt, Fmr1 ko, Mmp-9 ko and Dbl ko mice. (D) The graph represents mean ± SEM values (n= 11-17 slices from 6 mice per group). Inset: Overlaid representative fEPSP traces collected during baseline (1), 10 minutes post-HFS (2) and 60 minutes post-HFS stimulation (3) for wt, Fmr1 ko and Dbl ko mice. Calibration: 50
mV, 2 ms. (E) Bar graph shows mean normalized fEPSP values for 55-70 minutes. Error bars indicate SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey’s multiple-comparison posttest (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
**Figure 2.4**

**A** Electrophysiology

- wt
- *Fmr1* ko
- *Mmp-9* ko
- *Dbl* ko

**B**

- DHPG

- wt
- *Fmr1* ko
- *Mmp-9* ko
- *Dbl* ko

**C** 65-80 min

- wt
- *Fmr1* ko
- *Mmp-9* ko
- *Dbl* ko

**D** HFS

- wt
- *Fmr1* ko
- *Mmp-9* ko
- *Dbl* ko

**E** 55-70 min

- wt
- *Fmr1* ko
- *Mmp-9* ko
- *Dbl* ko
Table 2.1. List and sequence of *Mmp-2*, *Mmp-9* and *Gapdh* primers used in qRT-PCR screen.
### Table 2.1

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</tr>
<tr>
<td>MMP2 R</td>
<td>CAGGTTATCAGGGATGCGATTCC</td>
</tr>
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<td>MMP9 F</td>
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Chapter 3 – The genetic deletion of matrix metalloproteinase-9 rescues the behavioral and physical phenotypes seen in the fragile X mouse model.

ABSTRACT

Fragile X syndrome, the most common single gene cause of inherited intellectual disability, is caused by a trinucleotide CGG repeat expansion in the 5’ untranslated region of the Fragile X Mental Retardation (FMR1) gene, which results in promoter hypermethylation and gene silencing. This loss or reduction of the gene product expression, the Fragile X Mental Retardation Protein (FMRP), results in the translational dysregulation. Previous research has suggested that increased MMP-9 levels may be responsible for abnormal dendritic spine development in FXS and underlie FXS-associated behaviors. I previously showed that the deletion of mmp-9 in Fmr1 ko mice rescues the spine development both in vitro and in vivo, and that this deletion also returns mGluR-dependent LTD to the normal levels in Fmr1 ko mice. Here, I investigated if Fmr1 ko mice exhibit behavioral problems similar to those seen in FXS subjects and whether a deletion of mmp-9 can ameliorate these behavioral deficits. Further, I also investigated the role of MMP-9 activity in physical traits that are observed in FXS, such as macroorchidism.
INTRODUCTION

Fragile X syndrome (FXS) is the most common single gene cause of inherited intellectual disability and the most common known inherited causes of autism in the United States (Rogers et. al.,2001; Kau et. al.,2004; Clifford et. al.,2007; Loesch et. al.,2007; Hagerman et. al.,2009; Clapp et. al.,2001; NFXF; Garber et. al.,2008; Kooy,2003). Individuals with FXS display a range of symptoms from mild learning problems to more severe cognitive disabilities including language deficits and behavioral dysfunctions. Many FXS subjects display obsessive-compulsive disorder (OCD) behaviors such as hand-flapping, autistic behaviors, attention deficit and hyperactivity disorder (ADHD), visual-spatial memory impairment and developmental delays (Hagerman and Hagerman, 2002; Hagerman et. al., 2002; Musumeci et. al., 1999). Beyond cognitive and behavioral abnormalities, FXS patients also display characteristic physical traits that include long faces, large protruding ears, macroorchidism, flat feet, hyper-extensible joints, and soft fragile skin. Approximately 25% of patients suffer childhood seizures that improve with age (Clapp et. al.,2001; NFXF; Garber at. al., 2008). The Fmr1 ko mice used to study FXS also display similar behavioral impairments.

Previous work done in our laboratory suggests that increased MMP-9 activity underlies these spine deficits in Fmr1 ko mice, since reducing the levels of MMP-9 with a tetracycline analog, minocycline, rescued the spine phenotype of FXS in treated hippocampal neuronal cultures in vitro (Bilousova et. al., 2009). These findings served as an impetus for successful human clinical trials (Paribello et. al., 2010; Utari et. al., 2010; Leigh et. al.,2013; Dziembowska et. al., 2013; Schneider et. al., 2013), and follow up
studies also showed that the same MMP-9 inhibitor, minocycline, was able to improve the behavioral deficits seen in the Fmr1 ko mice (Dansie et. al., 2013; Rotschafer et. al., 2012); In the aforementioned clinical trials, minocycline treatment was reported to improve the attention span of the FXS subjects, improve their language use with greater vocalization, mood swings, attention deficits, social avoidance, repetitive behaviors, improved social interaction and communication and reduced anxiety amongst other behaviors, with few if any, side effects (Paribello et. al., 2010; Utari et. al., 2010; Leigh et. al.,2013; Dziembowska et. al., 2013; Schneider et. al., 2013) suggesting MMP-9 inhibitors to be a potential therapeutic agent for FXS since they are not only effective but well tolerated. This same relationship between Fmr1 and MMP-9 has also been reported in the drosophila model of FXS where overexpression of TIMP1 (the endogenous regulator of MMPs) or a dfmr/mmp null mutant rescued all nervous system defects seen in the fmr null flies, suggesting a genetic interaction between these two genes and/or their protein products (Siller and Broadie, 2011).

In the previous chapter I showed that the genetic deletion of MMP-9 is able to rescue the dendritic spine deficits seen in Fmr1 ko mice and to normalize mGluR-dependent LTD in these mice. Here, I investigated if knocking out the MMP-9 gene has similar beneficial effects on the behavioral performance of the Fmr1 ko mice. Specifically, I looked at the sociability of these mice using the social interaction preference test and measured their anxiety levels and hyperactivity using the open field test. Again, I confirmed that enhanced MMP9 levels contribute to these deficits in the Fmr1 ko mice as the Fmr1/mmp9 double ko mice display behaviors akin to those of wt
mice. I also examined the effects of *Mmp-9* deletion on FXS-associated physical trait, macroorchidism (i.e. enlarged testicles), and found that lack of the *mmp-9* gene also rescued this phenotype, indicating that MMP-9 is involved in not only the neurological aspects of FXS, but also the characteristic physical traits commonly associated with this condition.

**MATERIALS AND METHODS**

**Ethics statement**

All animal care protocols and procedures were approved by the UC Riverside Animal Care & Use Program, which is accredited by AAALAC International, and animal welfare assurance number A3439-01 is on file with the Office of Laboratory Animal Welfare (OLAW).

**Mice**

The FVB.Cg-*Mmp9*tm1Twu/J and FVB.129P2-*Fmr1*tm1Cgr/J (*Fmr1* ko) and FVB.129P2-Pde6b*+TyrCch*/AntJ controls (wt) were obtained from the Jackson Laboratories. The FVB.Cg-*Mmp9*tm1Twu/J mice were backcrossed, in-house, with the *Fmr1* ko and wt mice to generate *Fmr1/mmp9* double knockout mice and *mmp-9* ko mice, respectively. These mice do not suffer from retinal degeneration due to restoration of the pde6b allele and do not develop blindness, making them a suitable model for behavioral analysis. All genotypes were confirmed by PCR analysis of genomic DNA isolated from mouse tails.
Mice were maintained in an AAALAC accredited facility under 12 hour light/dark cycles, and fed standard mouse chow. All mouse studies were done within NIH and Institutional Animal Care and Use Committee guidelines.

**Open Field Test**

Anxiety was tested in P60 mice (12-17 mice per group) by quantifying their tendency to travel to the center of an open field (Yan et al., 2004; Yan et al., 2005). A 72x72 cm open field arena with 50 cm high walls was constructed from opaque acrylic sheets with a clear acrylic sheet for the bottom with a grid placed underneath it for scoring purposes (Fig 3.1 A). The open field arena was placed in a brightly lit room and one mouse at a time was placed in a corner of the open field and allowed to explore for 20 minutes while being recorded with digital video from above. The tester left the room during testing. The floor was cleaned with 2-3% acetic acid, 70% ethanol, and water between tests to eliminate odor trails. The mice were tested between the hours of 8:00 am to 1:00 pm and had not undergone any other behavioral tests prior to this testing paradigm. Locomotor activity was scored as described previously with some modifications (Brown et al., 1999; Yan et al., 2005) using TopScan Lite software (Clever Sys., Inc, Reston, VA 20190). The arena was subdivided into a 4x4 grid of squares with a separate square of equal size in the middle. A line 4 cm from each wall was added to measure thigmotaxis. Total horizontal and vertical line crosses, average velocity, total entries into large and small center squares, time spent in the large and small center squares, velocity within the large and small center squares, and time spent along the walls (thigmotaxis) were scored by the
program and a tendency to travel to the center (total entries into large and small center
squares) was used as an indicator of anxiety (as reported previously in Yan et. al., 2004;
Yan et. al., 2005; Dansie et. al., 2013). Average velocity and total line crosses were
measured to score locomotor activity. The analysis was done in 5 minute bins for the total
20 minute exploration duration. Assessments of the digital recordings were done by
blinded observers. Statistical analysis was performed using one-way ANOVA following
which post-hoc pair-by-pair differences were resolved with the Tukey-Kramer method.

Social interactions test

Sociability and social memory were studied using a three-chamber paradigm as described
previously (Kaidanovich-Beilin et. al., 2011). Briefly, a rectangular box contained three
adjacent chambers 19 cm x 45 cm each, with 30 cm high walls and a bottom constructed
from clear plexiglass. The three-chambers were separated by dividing walls made from
clear plexiglass with openings between the middle chamber and each side chamber (Fig.
3.1 B). Removable doors over these openings permitted chamber isolation or free access
to all chambers. All testing was done in a brightly lit room (650 lux), between 9 am and 6
pm. Prior to testing, mice were housed five mice per cage in a room with a 12 hr
light/dark cycle with ad libitum access to food and water. The cages were transferred to
the behavioral room 30 minutes before the first trial began. The test mouse was placed in
the central chamber with no access to the left and right chambers and allowed to
habituate to the test chamber for 5 minutes before testing began. In session 1, another
mouse (stranger 1) was placed in a wire cup-like container in one of the side chambers.
The opposite side had an empty cup of the same design. The doors between the chambers were removed and the test mouse was allowed to explore all the three chambers freely for 10 minutes, while being video recorded from above. The following parameters were monitored: the duration and number of direct contacts between the test mouse and the stranger mouse or the empty cup, the duration and number of other behaviors exhibited by the subject mouse such as self-grooming, walking and freezing, as well as the number and duration of entries into each chamber. In session 2, a new mouse (stranger 2) was placed in the empty wire cup in the second side chamber. Stranger 1, now familiar mouse remained in the first side chamber. The test mouse was allowed to freely explore all three chambers for another 10 minutes and the same parameters were monitored. Placement of stranger 1 in the left or right side of the chamber was randomly altered between trials. Each testing session lasted 10 minutes and the session was recorded with digital video from above, with the tester leaving the room for the duration of the trial. The floor of the chamber was cleaned with 2-3% acetic acid, 70% ethanol, and water between tests to eliminate odor trails. Both stranger mice were genotype matched to the subject mouse. Assessments of the digital recordings were done by blinded observers. Statistical analysis was performed using one-way ANOVA following which post-hoc pair-by-pair differences were resolved with the Tukey-Kramer method.

**Testes Measurements**

Testes were dissected from male P30 mice into a 1X PBS solution. 6 mice were used for each genotype yielding a total of 12 testes per group. The weight of each testis was
measured to 3 decimal places using a scale. The volume was calculated based on the amount of water each testis displaced from an Eppendorf tube filled with 1 ml of PBS. Finally, pictures of testes were analyzed with Image J Software to calculate the area of each. Each measurement was repeated at least three times for every individual testis. Statistical analysis was performed using One-way ANOVA followed by pair-wise comparison using the Bonferroni post-hoc test.

RESULTS

Behavioral performance in the open field test is improved in double Mmp-9/Fmr1 ko mice

To assess anxiety and locomotor activity, I used the open field test (Fig. 3.1 A) and determined the tendency of mice to travel to the center of the open field, total line crosses, and overall velocity. I found that the Fmr1 ko mice displayed an increased tendency to travel to the center of the open field and spent significantly more time in the center than wt mice (p= 0.0009, Fig. 3.2 A). The Fmr1 ko mice also spent less time in thigmotaxis, or close to the wall of the cage (Fig. 3.2 C). Although Fmr1 ko mice were more hyperactive and had higher overall velocities and made more line crosses than wt mice (p=0.0392 and p= 0.0089 respectively, Fig. 3.2D, E), Fmr1 ko mice also spent more time in the center per entry than wt mice (p= 0.0043, Fig 3.2 B). Mmp-9 deletion reduced the tendency of mice to travel to the center of the open field. Dbl ko mice spent significantly less time in the center of the open field and time per center entry than Fmr1
ko mice (p= 0.0156 and p= 0.0029, Fig. 3.2 A, B). However, dbl ko mice still had higher overall velocities and made more total line crosses than wt mice (p=0.0332 and p= 0.0159 respectively, Fig. 3.2 D, E). All the animals were tested for a duration of 20 minutes and this increased anxiety and locomotor activity was only seen for the first 10 minutes of testing (Fig 3.2). Once the mice acclimatized to the testing cage, after 10 minutes, this behavior was not apparent and the \textit{Fmr1} ko mice displayed similar tendencies to wt animals in terms of the number of entries into the center and the time spent in thigmotaxis. These mice also made fewer line crosses in the last 10 minutes (not significantly different from wt mice) (Fig. 3.3).

**Social interaction deficits in the \textit{Fmr1} ko mice are ameliorated by the removal of \textit{mmp-9}**

An important feature of FXS is reduced socialization. To determine if MMP-9 contributes to reduced socialization in \textit{Fmr1} ko mice, I tested dbl ko, \textit{Fmr1} ko, \textit{Mmp-9} ko and wt mice in a social novelty test. Mice were placed in an enclosure that contained two smaller cages for two 10-min sessions (Fig. 3.1 B). In session 1, an unfamiliar mouse (stranger 1, S1) was placed in one of the smaller cages. All mice spent significantly more time near the stranger mouse cage than an empty cage (Fig. 3.3 A), but \textit{Fmr1} ko mice spent significantly more time in the empty cage than dbl ko mice (p= 0.0233, Fig. 3.4 A). In the second session, another mouse (stranger 2, S2) was placed in the previously empty cage, so the tested mouse can choose to interact with the first familiar mouse (S1) or a new mouse (S2). Wt, \textit{Mmp-9} ko, and dbl ko mice spent significantly more time with the
new mouse (S2) than the familiar mouse (S1) (p<0.0001, Fig. 3.4 B). However, \textit{Fmr1} ko mice spent the same amount of time with both S1 and S2 mice, suggesting they could not or would not discriminate between them. Importantly, the loss of \textit{Mmp-9} in \textit{Fmr1} ko mice (dbl ko) corrected this defect.

\textbf{\textit{Fmr1} ko animals display macroorchidism that is ameliorated by \textit{Mmp-9} deficiency}

To determine whether high levels of MMP-9 activity might contribute to the characteristic physical traits associated with FXS, I examined macroorchidism in \textit{Fmr1} ko, \textit{Mmp-9} ko, dbl ko and wt mice. Testes from \textit{Fmr1} ko mice appeared visibly larger than wt mice (Fig. 3.5A). Measurements of weight and volume also showed that \textit{Fmr1} ko testes were significantly larger than wt or \textit{Mmp-9} ko (p<0.0001, Fig. 3.5 B, C). Remarkably, testes from dbl ko mice were also significantly smaller than \textit{Fmr1} ko, and not significantly different than wt or \textit{Mmp-9} ko (p<0.0001, Fig. 3.5 B, C).

\textbf{DISCUSSION}

Previous studies from our group and others have suggested that high levels of MMP-9 protein and activity underlie many of the deficits seen in FXS, both in human subjects and in animal models used to study this disorder (Bilousova et. al., 2009; Siller and Brodie, 2011; Paribello et. al., 2010; Utari et al., 2010; Dziembowska et. al., 2013;). These studies laid the foundation for MMP-9 inhibitor use in human clinical trials. One such compound that has been successful in recent human trials is minocycline, an
antibiotic tetracycline analog that inhibits MMP-9, among its other actions. In addition to the neurological and cognitive deficits discussed earlier, FXS patients also suffer from a wide range of behavioral and emotional problems that include difficulty with social interactions, speech impairment, anxiety, hyperactivity, aggression, agitation, and reduced attention spans among others. Recent clinical trials of minocycline for treating FXS have been successful by drastically improving these symptoms. These trials reported major improvements in language use, increased speech coherency, improved attention spans, less irritability, decreased anxiety, increased social communication and a general improvement in global behavioral scores (Paribello et. al., 2010; Utari et. al., 2010; Leigh et. al., 2013; Schneider et. al., 2013). However, since minocycline has a broad range of action, it was still unclear if the improvements in behavior seen in these studies is truly a result of its actions on MMP-9.

Here, I first confirm that our Fmr1 ko mice also show some of these behavioral deficiencies seen in FXS subjects, and further, I show improvement of those behaviors in Fmr1 ko mice that also lack the mmp-9 gene. Fmr1 ko mice exhibited increased anxiety, as evidenced by their tendency to travel to the center, and spend more time per entry in the center, of the open field chamber. This anxiety was absent in dbl ko, which showed a tendency to travel to the center that was not significantly different from wt mice. Fmr1 ko mice also displayed hyperactivity, with higher overall velocity for the duration of the open field test and increased number of line crosses made during the testing period. However the dbl ko mice remained hyperactive with higher overall velocity and increased line crosses than wt mice. Fmr1 ko mice also displayed deficits in social
behavior, including a failing to discriminate social novelty. In the social interaction test, $Fmr1$ ko mice displayed normal sociability during the first session when given a choice between a stranger mouse and an empty cage, but failed to discriminate between the first stranger mouse and a second (novel) mouse in the second session. The dbl ko mice did not show this defect and instead displayed normal social preference in both first and second sessions. These interactions were not different from wt mice. This work establishes that increased MMP-9 protein and activity underlie not only spine deficits associated with FXS, but also behavioral deficits; and behavioral improvements seen with minocycline use most likely result from its inhibitory action on MMP-9.

Some studies done on autistic children have shown that these children have difficulties interacting with and interpreting strangers as humans (Robins et. al., 2004). Other studies have also suggested that autistic people view other people as objects (Hobson, 2002). The $Fmr1$ ko mice may also be behaving in a similar fashion and during the second session of the social interaction test, the $Fmr1$ ko mice may be objectifying both stranger mice and hence not be able to distinguish between them or may show equal preference towards a similar ‘object’.

Besides the cognitive and behavioral problems, FXS subjects also display characteristic physical traits, including flat feet, long faces, and macroorchidism (Clapp et. al., 2001; NFXF; Garber et. al., 2008). In this study, I showed the involvement of increased MMP-9 in the development of macroorchidism. $Fmr1$ ko mice had much larger testes, by weight and volume, than wt mice. $Mmp-9$ deficiency ameliorated this macroorchidism phenotype in $Fmr1$ ko mice; indeed, dbl ko mice had significantly
smaller testes than *Fmr1 ko* mice. Slegtenhorst-Eegdeman et al (1997) reported higher Sertoli cell proliferation in *Fmr1 ko* mice. These Sertoli cells support the proliferation and differentiation of germ cells. It stands to reason that an increase in the number of Sertoli cells will also result in an increase in the number of germ cells, which may lead to the macroorchidism seen in these mice. During spermatogenesis, developing spermatocytes cross the blood-testis barrier (BTB), which consists of tight junctions formed by these Sertoli cells and this barrier lies adjacent to the basement membrane, a specialized ECM, which also undergoes restructuring during this migration process (Siu and Cheng, 2004). This basement membrane is rich in MMP-9 substrates, such as laminin, type IV collagen and heparin sulfated proteoglycans (HSPG). MMP-9 and TIMP-1 are produced by the Sertoli cells and are known to cleave type IV collagen and increase the permeability of the BTB to allow the migration of the developing spermatocytes (Siu and Cheng, 2004, 2003; Robinson et. al., 2001; Fritz et. al., 1993; Mruk et. al., 1997; Wong et. al., 2000). Outside of the brain FMRP is also highly expressed in the testis where it regulates the translation of its target mRNAs. Similar to the hippocampus - where MMP-9 levels are upregulated in the absence of FMRP - the loss of FMRP in testes of *Fmr1 ko* mice might lead to increased cleavage and permeability of basement membrane in the testis and increase the proliferation of Sertoli cells and further germ cells, which could combine to increase testes size (macroorchidism). The increased MMP-9 levels might also directly influence the proliferative capacity of the Sertoli and/or germ cells as MMPs do also play a role in cell proliferation.
Some groups have proposed that increased sensitivity of mGluRs underlies the deficits seen in FXS, both in human subjects and in Fmr1 ko mice (Bear et. al., 2004) and reducing mGluR hyperactivity or the activity of downstream signaling pathways associated with mGluRs has ameliorated some of cognitive and behavioral deficits in Fmr1 ko mice. Nonetheless, reductions in mGluR signaling have no impact on macroorchidism, indicating that while mGluR signaling might play a role in this disorder, it is not the sole cause of all the symptoms (Bear et. al., 2004; Bassell and Gross, 2008; Michalon et. al., 2012). Metabotropic GluR signaling may play a greater role in the brain but less of one in the connective tissue and hence therapies targeting these receptors may not completely improve all aspects of this disorder. mGluRs could also be acting downstream of other molecules or the increased mGluR signaling seen might be due to increased active MMP-9. It remains to be seen how all molecules implicated in FXS interact to cause these symptoms; however, since a reduction of MMP-9 can improves both cognitive and behavioral deficits and physical traits of FXS, MMP-9 is the best target for therapeutic development.

Minocycline has been successful in recent clinical trials involving FXS subjects and seems to be a good drug choice since it is well tolerated and has been prescribed to treat acne in adults (Seukeran and Eady, 1997; Goulden and Glass, 1996). The studies involving minocycline reported only minor side effects with occasional mild gastrointestinal problems, headaches or diarrhea (Paribello et. al., 2010; Utari et. al., 2010) so it would be well tolerated by a majority of subjects; however, there have been reports of a small minority of children are susceptible to developing minocycline-induced
autoimmunity and those children may go on to develop chronic symptoms that could be potentially fatal (Farver, 1997; El-Hallack and Giani, 2008). Taken together with the fact that minocycline is a broad acting antibiotic, care should be taken to minimize the long-term usage of this drug in FXS and instead more research should be done to identify new drugs acting more specifically on MMP-9, without antibiotic activity.
REFERENCES


Changes in the expression of junctional and nonjunctional complex component genes when inter-Sertoli tight junctions are formed in vitro. J. Androl.; 21: 227–237.


Figure 3.1. **Diagram of the various chambers used for behavior testing.** (A) Picture of the floor showing the parameters used for open field testing. (B) Diagram showing the chamber used for social interaction behavior testing. The leftmost and rightmost chambers contain either an empty cage and a mouse like depicted in session 1, or a familiar and novel mouse as shown in session 2. The chambers containing either cage were randomized during each trial. The subject mouse to be tested is placed in the central chamber as shown here and allowed to freely explore all three chambers in 10 minute intervals.
Figure 3.1

A  Open Field

B  Social Interaction Behavior

Session 1

Stranger 1

Empty

45 cm

19 cm

Session 2

Stranger 1

Stranger 2

19 cm

Thigmotaxis

Center
Figure 3.2. Increased anxiety commonly reported for the \textit{Fmr1} ko mice is \textbf{ameliorated in the Dbl ko mice}. The open field test was performed to measure anxiety and hyperactivity in wt, \textit{Fmr1} ko, \textit{Mmp-9} ko and Dbl ko mice. Mice were tested for a duration of 20 minutes and performance tabulated in 5 minute bins. This figure shows the behavior for the first 10 minutes of testing. The bar graphs show (A) the time that the mice spent in the center of the open field, (B) the amount of time spent in the center per entry, (C) the time the mice spent in thigmotaxis, (D) the overall velocity and (E) the total number of line crosses. The bar graphs represent average values and the error bars indicate SEM (n= 11-17 mice per group). Statistical analysis was performed using one-way ANOVA followed by Hsu’s MCB post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001, ns - non significant).
Figure 3.2
Figure 3.3. Increased anxiety in the *Fmr1* ko mice is not apparent once the mice are acclimatized to the testing environment. The open field test was performed to measure anxiety and hyperactivity in wt, *Fmr1* ko, *Mmp-9* ko and Dbl ko mice. Mice were tested for a duration of 20 minutes and performance tabulated in 5 minute bins. This figure shows the behavior for the last 10 minutes of testing. The bar graphs show (A) the time that the mice spent in the center of the open field, (B) the amount of time spent in the center per entry, (C) the time the mice spent in thigmotaxis, (D) the overall velocity and (E) the total number of line crosses. The bar graphs represent average values and the error bars indicate SEM (n= 11-17 mice per group). Statistical analysis was performed using one-way ANOVA followed by Hsu’s MCB post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001, ns - non significant).
Figure 3.3
Figure 3.4. The abnormal social interaction behavior exhibited by $Fmr1$ ko mice is rescued by the dbl ko. To measure social interaction behavior I investigated the time that the mice spent with a stranger 1 (S1) compared to an empty cage (-) during session 1 and the time that mice spent with a novel stranger 2 (S2) compared to now familiar stranger 1 (S1) during session 2. C represents the empty central chamber. The bar graphs represent average values and the error bars indicate SEM (n= 13-30 mice per group). Statistical analysis was performed using one-way ANOVA followed by Hsu’s MCB post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001, ns - non significant).
Figure 3.4

A  
session 1

B  
session 2
Figure 3.5. The macroorchidism phenotype is improved by the genetic removal of *Mmp-9 in the Dbl ko mice*. (A) Images showing representative testes isolated from wt, *Fmr1* ko, *Mmp-9* ko and Dbl ko month old mice. (B-C) Quantitative analysis of the mean testis volume (B) and mean testis weight (C). Error bars indicate SEM (n=12 testes from 6 mice per group). Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s multiple-comparison posttest (***p < 0.001).
Figure 3.5

A

B

C

Testis volume, mm$^3$

Testis weight, g

wt  Fmr1 ko  Mmp-9 ko  Dbl ko

wt  Fmr1 ko  Mmp-9 ko  Dbl ko
Chapter 4 – Changes in basal protein synthesis levels and signaling cascades in the \( Fmr1 \) ko mice.

ABSTRACT

Fragile X syndrome, the most common single gene cause of inherited intellectual disability, is caused by a trinucleotide CGG repeat expansion in the 5’ untranslated region of the \( \text{Fragile X Mental Retardation (FMR1)} \) gene, which results in promoter hypermethylation and gene silencing. This loss or reduction of the gene product expression, the Fragile X Mental Retardation Protein (FMRP), results in the translational dysregulation of specific mRNAs. Previous research has suggested that increased MMP-9 levels may be responsible for abnormal dendritic spine development in FXS and underlie FXS-associated behaviors. In previous chapters I showed that the deletion of \( mmp-9 \) in \( Fmr1 \) ko mice rescues the spine development both in vitro and in vivo, and that this deletion also returns mGluR-dependent LTD to the normal levels in \( Fmr1 \) ko mice. I also showed that \( Fmr1 \) ko mice exhibit behavioral problems similar to those seen in FXS subjects and a deletion of \( mmp-9 \) ameliorates these behavioral deficits. Further, I demonstrated a role for MMP-9 activity in physical traits that are observed in FXS, such as macroorchidism. Here, I investigated a possible mechanism of MMP-9 action in neurons by examining the effects of \( Mmp-9 \) deletion on Akt/mTOR pathway, which is shown to increase in FXS, but is normalized to the normal levels in double \( Mmp-9/Fmr1 \)
ko mice. I also explored the role of FMRP in the regulation of mRNA expression of other MMPs.

INTRODUCTION

Fragile X syndrome is a neurodevelopmental disorder that is caused by a functional loss of FMRP, the gene product encoded by the Fmr1 gene (D’Hulst and Kooy, 2009). Subjects with FXS suffer from a wide range of cognitive and behavioral problems including learning difficulties, developmental delay, ADHD and increased susceptibility to audiogenic seizures (D’Hulst and Kooy, 2009). FMRP is an mRNA binding protein that regulates the translation of its target mRNAs, many of which have been shown to have synaptic functions or be involved in neuronal development (Darnell et. al., 2011; Bassell and Warren, 2008; Louhivouri et. al., 2011). Translation of these target mRNA is dependent, amongst other factors, on the phosphorylated state of FMRP. Phosphorylated FMRP is found on stalled ribosomes while dephosphorylated FMRP has been shown to be associated with actively translating ribosomes (Ceman et. al., 2003; Narayanan et. al., 2008). Hence, the loss of FMRP in Fmr1 ko mice results in the loss of this translational regulation of specific mRNAs and increased basal protein synthesis. Indeed, many groups have shown there to be an increase in the basal protein synthesis in these mice (Osterweil et. al., 2010). Some groups have also proposed that this increased protein synthesis is due to a hypersensitivity to mGluR signaling in these mice and increased mGluR signaling may contribute to the cognitive and learning impairments
seen in these mice (Bear et. al., 2004; Osterweil et. al., 2010; Maccarrone et. al., 2010; Bassell and Gross, 2008).

Many different intracellular signaling pathways and cell surface receptors appear to be deregulated in FXS, including the group I mGluRs. *Fmr1* ko mice are more sensitive to modulators of group 1 metabotropic glutamate receptor signaling (Bear et. al., 2004). Normally, mGluR signaling blocks the translational repression of FMRP from its target mRNAs, allowing for the translation of the protein products encoded, which can cause α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor internalization and long term depression (LTD)--a decrease of synaptic strength. In the absence of FMRP, those proteins are expressed at a higher basal level due to enhanced protein translation and more AMPA receptors are internalized even before the glutamate signaling arises, thus lengthening long term depression (Bear et. al., 2004; Hou et. al., 2006; Oostra and Willemsen, 2009). mGluR antagonists such as MPEP, Fenobam, and CTEP have been shown to attenuate some of FXS phenotypes in *Fmr1* ko mice and in human patients (Yan et. al, 2005; Levenga et. al., 2010; Michalon et. al., 2012). These effects are most likely mediated through downregulation of PI3K/Akt/mTOR pathway that is shown to be enhanced in FXS and can contribute to increased protein synthesis (Gross et. al., 2010; Sharma et. al., 2010). Moreover, increased Erk phosphorylation is seen in the cortex but not the hippocampus of *Fmr1* ko mice and is thought to increase S6 kinase phosphorylation throught Rsk (Zukin et. al., unpublished). The regulation of the pathway through the insulin receptor has also been shown to rescue some FXS-associated deficits (Zhao et. al., 2004; Stern, 2011; Scott et. al., 1998). Others have shown that *Fmr1*
ko mice have altered GABA receptor subunit composition and lower GABAergic inputs to certain hippocampal circuits (Brenman, 2009; D’Hulst et. al., 2009), so that GABA receptor agonists, such as Baclofen and Nipecotic acid partially rescue the FXS phenotypes (Levenga et. al., 2010; Pacey et. al., 2009). Previous work done in our laboratory has suggested that higher levels of matrix metalloprotease-9 (MMP-9) may contribute to FXS-associated symptoms (Bilousova et al., 2009). We found that MMP-9 activity is higher in Fmr1 ko mice than in the wild-type mice; further, minocycline treatment reduced the MMP-9 activity levels in Fmr1 ko mice, ameliorated the dendritic spine phenotype, and improved behavioral abnormalities seen in Fmr1 kos. When cultures of mature hippocampal neurons from wild-type mice were treated with MMP-9 protein they developed long, immature filopodial like spines that mimicked the immature spine phenotype seen in Fmr1 ko hippocampal cultures (Bilousova et. al., 2009). This same relationship between Fmr1 and Mmp-9 has also been reported in the drosophila model of FXS where overexpression of TIMP1 (the endogenous regulator of MMPs) or a mmp null mutation rescued all nervous system defects seen in the dfmr null flies, suggesting a genetic interaction between these two genes and/or their protein products (Siller and Broadie, 2011). Other groups have also shown MMP-9 involvement in synaptic plasticity and regulation of NMDAR currents mediated through integrin activity (Nagy et. al., 2006; Meighan et. al., 2006).

Here, I investigated the role of Akt/mTOR and Erk pathways in FXS and whether it is regulated by MMP-9. For this, I have examined the phosphorylated and total levels of Akt, mTOR and Erk in mmp-9 ko and double mmp-9/Fmr1 ko mice. I also evaluated
the effects of mmp-9 deletion on protein synthesis in Fmr1 ko mice. Lastly, I examined if the expression of other MMPs is regulated by FMRP by determining mRNA levels in mmp-9 ko and mmp-9/Fmr1 ko mice.

MATERIALS AND METHODS

Ethics statement
All animal care protocols and procedures were approved by the UC Riverside Animal Care & Use Program, which is accredited by AAALAC International, and animal welfare assurance number A3439-01 is on file with the Office of Laboratory Animal Welfare (OLAW).

Mice
The FVB.Cg-Mmp9tm1Tvu/J and FVB.129P2-Fmr1tm1Cgr/J (Fmr1 ko) and FVB.129P2-Pde6b^Tyr^E-ch/AntJ controls (wt) were obtained from the Jackson Laboratories. The FVB.Cg-Mmp9tm1Tvu/J mice were backcrossed, in-house, with the Fmr1 ko and wt mice to generate Fmr1/mmp9 double knockout mice and mmp-9 ko mice, respectively. These mice do not suffer from retinal degeneration due to restoration of the pde6b allele and do not develop blindness, making them a suitable model for behavioral analysis. All genotypes were confirmed by PCR analysis of genomic DNA isolated from mouse tails. Mice were maintained in an AAALAC accredited facility under 12 hour light/dark
cycles, and fed standard mouse chow. All mouse studies were done within NIH and Institutional Animal Care and Use Committee guidelines.

**Quantitative RT-PCR**

Hippocampal and cortical tissues were dissected from mice of age P8, P14 and P21 (n=3-5 mice per group per time point). Total RNA from each tissue sample was prepared using Trizol (Life Technologies) according to the manufacturer’s instructions and cDNA was synthesized using SuperScript VILO cDNA Synthesis Kit (Life Technologies) as recommended by the manufacturer. To examine mRNA expression of all the mammalian MMPs, TIMPs and some ADAMs, specific forward and reverse primers as listed in supplementary table 1 were used. All samples were normalized to the expression of GAPDH (see table 2.1 for primer sequence). Each reaction mixture contained 1× Power SYBR Green PCR Master Mix (Life Technologies) and all the reactions were run in triplicate. The PCR amplification protocol was as follows: initial DNA Polymerase activation at 95°C for 10 min, followed by 40 cycles with denaturation at 95°C for 15 s, and annealing + extension at 60°C for 1 min. Amplification was performed in a StepOne Real Time PCR System (96-well format) (Life Technologies) and analyzed by normalizing the expression of each gene to GAPDH within each tissue sample. Statistical analysis was performed using One-way ANOVA followed by pair-wise comparison using the Bonferroni post-hoc test.
**Immunoblotting**

The hippocampus was removed from each mouse (n= 4 mice per group), cooled in PBS, and homogenized in cold RIPA buffer (50 mM Tris-HCl (pH=7.4), 150 mM NaCl, 1 mM EDTA (pH=8.0), 1% Triton X-100, 0.1% SDS and 0.5% 21-hydroxyprogesterone) containing protease inhibitor cocktail (Sigma) and 0.5 mM sodium pervanadate. The samples were rotated at 4°C for at least 1 hour to allow for complete cell lysis and then cleared by centrifugation at 13200 rpm for 15 minutes at 4°C. Supernatants were isolated and boiled in reducing sample buffer (Laemmli 2X concentrate, Sigma, S3401), and separated on 8-16% Tris-glycine SDS-PAGE precast gels (Life Technologies, EC6045BOX). Proteins were transferred onto Protran BA 85 Nitrocellulose membrane (GE Healthcare) and blocked for 1 hour at room temperature in 5% skim milk (BioRad, #170-6404). Primary antibody incubations were done overnight at 4°C with antibodies diluted in TBS/0.1% Tween-20/5% BSA. Primary antibodies used were: rabbit α-eIF4e (C46H6) (#2067), rabbit α-phospho-eIF4e(Ser 209) (#9741), rabbit α-mTOR(7C10) (#2983), rabbit α-phospho-mTOR(Ser 2481) (#2974), rabbit α-Akt (#9272), rabbit α-phospho-Akt(Ser 473) (#9271), rabbit α-p44/p42 MAPK (#9102), rabbit α-phospho-p44/p42 MAPK(Thr202/Tyr204) (#9101) and mouse α-GAPDH – 1:1000 (Fitzgerald, #10R-G1099). All primary antibodies were from Cell Signaling Technologies and used at a dilution of 1:1000, unless stated otherwise. Blots were washed 3 x 10 minutes with TBS/0.1% tween-20 and incubated with the appropriate HRP-conjugated secondary antibodies for an hour at room temperature in a TBS/0.1% tween-20/5% skim milk buffer solution. The secondary antibodies used were α-rabbit-HRP at 1:5000, α-mouse-HRP at
1:5000 (GE Healthcare). After secondary antibody incubations, blots were washed 3 x 10 minutes in TBS/0.1% tween-20 and developed with ECL Detection reagent (Thermo Scientific, #80196). For re-probing, membrane blots were washed in stripping buffer (2% SDS, 100 mM β-mercaptoethanol, 50 mM Tris-HCl, pH=6.8) for 30 minutes at 56°C, then rinsed repeatedly with TBS/0.1% tween-20, finally blocked with 5% skim milk, and then re-probed. Developed films were then scanned and protein levels quantified by comparing band density values obtained using ImageJ. Two samples per group were run per blot and P/T ratios for different samples were normalized to averaged P/T ratios of wt samples. Statistical analysis was performed using one-way ANOVA with post-hoc pair-by-pair differences resolved using Hsu’s MCB method.

**Puromycin Protein Synthesis Assay**

3 month old mice (3 animals per group) were anesthetized by isofluorane inhalation. Brains were removed from the skull and immediately immersed in chilled cutting solution containing 110mM sucrose, 60 mM NaCl, 28 mM NaHCO₃, 1.25 mM NaH₂PO₄, 3 mM KCl, 7 mM MgSO₄, 0.5 mM CaCl₂, 5 mM glucose, and 0.6 mM ascorbic acid, bubbled with 95% O₂/5% CO₂. Transverse hippocampal slices (400 μm thick) were prepared using a Vibratome (Leica VT1200S) and were allowed to recover for an hour at room temperature in artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 25 mM NaHCO₃, 1 mM NaHPO₄, 4.4 mM KCl, 1.2 mM MgSO₄, 2mM CaCl₂ and 10mM glucose with constant bubbling of 95% O2/5% CO2 gas. Proteins were labeled using an adaptation of the SUnSEt protocol (Schmidt et. al., 2009). Briefly, 10µg/ml puromycin
(Sigma, P8833) was added to the slices and the slices were incubated for 2 hours with constant bubbling of 95% O₂/5% CO₂ to allow puromycin to be incorporated into newly synthesized proteins. The slices were then transferred to oxygenated ACSF in three successive washes of 2 minutes with constant bubbling of 95% O₂/5% CO₂. Following the washes, the slices were flash frozen on dry ice and the hippocampi from each slice was microdissected and protein lysates were prepared and blotted as described above. Puromycin labeled proteins were identified using a mouse monoclonal antibody 12D10 – 1:5000 (Millipore, MABE343). Protein synthesis levels were quantified by comparing the total lane signals for each sample from 250 to 10kDa and subtracting the signal from an unlabeled protein lane. Statistical analysis was performed using the student’s t-test.

RESULTS

**Mmp-9 deletion reduces protein synthesis in the hippocampus of Fmr1 ko mice.**

To investigate if the loss of Mmp-9 affects the basal protein synthesis levels in the hippocampus of Fmr1 ko mice I measured the protein synthesis by quantifying the amount of puromycin incorporated into newly synthesized proteins. I observed about a 10% increase in the levels of protein synthesis in Fmr1 ko hippocampi as compared to wt hippocampi (p=0.027411). However, this increase in the protein synthesis was reduced by the deletion of Mmp-9 in the hippocampus of double Mmp-9/Fmr1 ko mice which had protein synthesis levels no different from wt mice. Interestingly, a 10% increase in basal levels of protein synthesis was also seen in the Mmp-9 ko mice (p=0.007701).
**Mmp-9 deficiency reduces Akt, mTOR and eIF4e phosphorylation in Fmr1 ko mice.**

To investigate the intracellular signaling cascades that might mediate the effects of enhanced MMP-9 activity in Fmr1 ko neurons, I examined the effects of Mmp-9 loss on activity of mTOR, eIF4e and Akt in dbl ko mice by assessing phosphorylation levels of these proteins (Fig. 4.2). I observed an increase in the levels of the phosphorylated (i.e. active) forms of all three proteins in the hippocampus of adult Fmr1 ko mice as compared to wt mice. There was a 50% increase in the p-mTOR/mTOR ratio in the Fmr1 ko as compared to wt (p=0.0216), but the Mmp-9 deletion in Fmr1 ko (dbl ko) returned the p-mTOR levels back to wt levels. The phospho-mTOR levels in dbl ko were significantly lower than in Fmr1 ko (p=0.0393) (Fig. 4.2 B). This same effect was also seen for peIF4E/eIF4E ratios with a 50% higher level of phosphorylated eIF4E in the Fmr1 ko as compared to wt and a 30% lower level in dbl ko as compared to Fmr1 ko (p=0.0281 and p=0.0495 respectively) (Fig. 4.2 C). Fmr1 ko mice also exhibited a 30% increase in pAkt/Akt ratio in comparison to wt (p=0.0069) and dbl ko mice (p=0.0047) (Fig 4.2 D), suggesting that Akt activation may mediate the effects of MMP-9 on mTOR and eIF4E phosphorylation levels.

**Developmental regulation of MMPs in the hippocampus and cortex of Fmr1 ko and mmp-9 ko mice**

I examined the mRNA transcript levels of all the 24 mammalian MMPs and the 4 TIMPs at three developmental time points to see if the levels of any of the other MMPs were also affected in these ko animals. Significant differences were found in the levels of some
MMPs specifically at the P8 age (Fig. 4.3 A, B). The MMPs that stood out in this screen include the MMPs- 3, 7, 8, 21 and 27. There were no significant changes in the mRNA levels of any of the MMPs investigated at the ages of P14 and P21 (Fig. 4.3 C-F, Fig 4.4-4.6).

First, there was no expression of the mmp-9 transcript in the Mmp-9 ko and dbl ko mice at any age confirming the genetic deletion of Mmp-9 in these mice. The levels of the mmp-9 transcript however did not very significantly between the wt and Fmr1 ko animals in either the hippocampus or the cortex (Fig. 4.3). There was also no significant difference in the levels of the mmp-2 transcript in all animals tested in both brain regions. There were however changes in the levels of MMPs that are shown to regulate activities of MMP-2 and MMP-9. mRNA levels of MMPs- 3, 7 and 8 were slightly lower in the Fmr1 ko mice than in wt mice, but significantly higher in the Mmp-9 ko mice. These levels decreased to levels that were not significantly different from wt animals in the dbl ko mice suggesting a role for FMRP in the regulation of these transcripts. This trend was only true for the hippocampus (Fig. 4.3 A). In the cortex (Fig. 4.3 B), the mRNA levels of these same MMPs were higher in the Mmp-9 ko mice, and the increase was also observed in the dbl ko animals suggesting different roles for FMRP-mediated transcriptional regulation of Mmp expression in the hippocampus and cortex. A similar pattern was seen for the levels of MMP-21, which was shown to be involved in immune responses in the brain, and MMP-27 in the cortex, which were high both in the Mmp-9 and dbl ko animals (Fig. 4.3 B). No significant differences were seen in the mRNA levels of any of the TIMPs at any age (Fig. 4.6).
DISCUSSION

In this study I show that the mRNA levels of some of the MMPs are affected in the single ko animals but these levels are rescued to normal levels in the dbl ko, at least in the hippocampus. This screen also showed some of the MMPs, including MMP-3 and MMP-7, that have the ability to regulate the activities of gelatinases to be upregulated in the *Mmp-9* ko suggesting some sort of compensatory mechanism might be at play to try to compensate for the lack of MMP-9 protein in these animals. FMRP seems to be involved in the transcriptional regulation of the levels of these MMPs in the hippocampus, but not cortex. Since the levels of FMRP are different in these two brain regions; being higher in the hippocampus, it suggests that FMRP might be responsible for the translational regulation of these MMPs in the hippocampus. However, we cannot definitively conclude there are changes in the protein levels of these gelatinase regulators as changes in mRNA levels might not necessarily translate into changes in protein levels. In this screen itself, we do not see changes in the mRNA transcript levels of *Mmp-9* and *Mmp-2* between wt and *Fmr1* ko animals but we did see an increase in both protein levels in the *Fmr1* ko hippocampi (see chapter 2). Hence, further work is needed to show if the protein levels of these other MMPs might be dysregulated in these animals although this data does suggest the involvement of FMRP in the translational regulation of at least MMPs 2 and 9 in the hippocampus.

FMRP is involved in not only regulating the translation of its target mRNAs but also in regulating global protein synthesis as previously reported by others (Qin et. al., 2005; Bhattacharya et. al., 2012; Gross and Bassell, 2011). I also see an increase in the
overall protein synthesis in the hippocampus of Fmr1 ko mice. FMRP was shown to be involved in the formation of complexes that regulate translation and can regulate translation indirectly by stalling ribosomes. Previous findings have shown mTOR signaling to be increased in Fmr1 ko mice and that this enhanced signaling leads to the increased activity of the eukaryotic initiation factor complex 4F (eIF4F) which is required for translation to occur (Sharma et. al., 2010). MAP kinase signaling was also implicated in the increase protein translation in FXS (Osterweil et. al., 2010). These pathways can be mediated by MMP activity either directly or indirectly through its cleavage of extracellular matrix proteins, which can bind and activate cell surface integrins at the synapse causing the transduction of intracellular signaling casades (Pankov et. al. 2003).

Here, I also report an increase in the phosphorylation and activation of the intracellular cascade effector molecules, Akt, mTOR and eIF4E in Fmr1 ko mice which is reduced by the depletion of mmp-9. The increased activation of these molecules likely underlies pathologies underlying FXS since the depletion of mmp-9 reduces the activation of these cascades and also ameliorates most of the symptoms/phenotypes of FXS. Interestingly, eIF4E that is regulated by MMP-9 activity may be also responsible for increased MMP-9 levels in Fmr1 ko mice (chapter 2). In nasopharyngeal carcinomas, the overexpression of eIF4E promotes carcinoma growth and cell cycle progression through the increased translation of mmp9 (Wu et. al., 2013). In FXS, the absence of FMRP is known to result in increased levels of eIF4e (Bagni et. al., 2012) and this may be the pathway affecting the increased MMP-9 levels seen in FXS. These increased MMP-9 levels may then affect the dendritic spine changes seen in FXS through the
cleavage of the intracellular adhesion molecule-5 (ICAM-5) or via their actions on the integrins (Niedringhaus et al., 2012; Tian et al., 2007; Conant et al., 2010; Michaluk et al., 2011, 2009; Nagy et al., 2006). Extracellular matrix components have been shown previously to play a role in synaptic plasticity through their cell surface receptors integrins (Szepesi et al., 2013; Dziembowska et al., 2012; Shi et al., 2006). The ECM and integrins can be affected by other MMPs, which have similar substrates as MMP-9 or other MMPs that regulate the activity of MMP-9. The levels of these MMPs like MMP-3 for instance could be upregulated to compensate for the lack of MMP-9 and may signal back to the cytoskeleton in a similar fashion to allow for synaptic plasticity. Since many of the gelatinase regulators can activate MMP-9, a loss of MMP-9 may result in the increased expression of these other MMPs in a compensatory fashion to make up for this decrease in activity. Here, I showed using a qRT-PCR screen that some of the other mmp transcript levels are upregulated in the mmp9 ko mice. However, these levels are returned back to more normal levels in the dbl ko mice.

FMRP has been shown to associate with and transport mmp-9 mRNA to the dendrites in FMRP containing RNA granules where it is then translated upon neuronal stimulation (Janusz et al., 2013). In the Fmr1 ko mice, a loss of transcriptional regulation of mmp-9 due to the absence of FMRP may result in increased levels of MMP-9 protein, which then could signal to the ECM to affect dendritic spine morphology changes and other signaling cascades. In the absence of FMRP, there is also increased translation of Arc which leads to increased internalization of AMPARs (Bagni et al., 2012). Some groups have shown Fmr1 ko mice to be more sensitive to group 1 metabotropic glutamate
receptor (mGluR) signaling. Normally, mGluR signaling would lift the translational repression of FMRP from its target mRNAs allowing the specific encoded protein to be expressed, some of which cause AMPA receptor internalization and long term depression (LTD). In the absence of FMRP, these proteins are expressed at higher basal level and more AMPA receptors are internalized even before the glutamate signaling arises thus lengthening long term depression (Bear et. al., 2004; Oostra et. al., 2009; Hou et. al., 2006).

In wt mice, mGluR5-dependent LTD in the CA1 region requires rapid protein synthesis. Disrupting mGluR- Homer interactions prevents mGluR mediated protein signaling by inhibiting the PI3K- Akt- mTOR pathway which prevents the translation of Elongation factor 1α and subsequently blocks mGluR-dependent LTD in wt animals (Ronesi and Huber, 2005; Hou and Klann, 2004). There is no effect however on the ERK pathway. In Fmr1 ko mice, mGluR-dependent LTD is enhanced and is protein synthesis independent (Huber et. al., 2002; Hou et.al., 2006; Todd et. al., 2003) although Homer-mGluR association is known to be decreased (Giuffrida et. al., 2005). The mGluR mediated activation of PI3K and mTOR pathways is also lost in Fmr1 ko mice (Ronesi and Huber, 2005) as the mGluRs are associated with more Homer1α isoform, the shorter isoforms which are unable to dimerize and propagate intracellular signaling cascades through the PI3K pathway (Ronesi et al., 2012); but, the downstream targets of mTOR like 4E-BP can be phosphorylated by the ERK- Mnk1 pathway. Since the Erk pathway is unaffected by the disruption of Homer from mGluRs, the increase in protein synthesis
seen in the ko animals might be mediated by the ERK pathway and possibly mediated by receptors other than the mGluRs.

On the other hand, levels of phosphorylated mTOR have been reported to be higher in *Fmr1* ko mice and this has been shown to control the initiation of cap-dependent translation (Sharma et al., 2010). This increase in mTOR phosphorylation could be due to BDNF signaling through its receptor, TrkB, which shares similar intracellular signaling pathways with the mGluR5. BDNF protein levels are known to be higher in the hippocampi of *Fmr1* ko mice and the TrkB mRNA is a known target of FMRP (Castren and Castren, 2013; Louhivouri et. al., 2011; Uutela et. al., 2012). A number of MMPs are known to cleave and activate neurotrophins and MMP9 in particular has been shown to cleave pro-BDNF (Hwang et. al., 2005; Yang et. al., 2009; Mizoguchi et. al., 2011). The increased levels of MMP-9 may function in a positive feedback loop in the ko mice to cleave pro-BDNF to mature BDNF which can then bind to its receptor, TrkB, and activate an intracellular signaling cascade that promotes the translation of more MMP-9 among other proteins through both the ERK and PI3K-Akt-mTOR pathways (Santos et. al., 2010).

In addition, a FMRP binding protein, cytoplasmic FMRP interacting protein 1 (CYFIP1) binds directly to eIF4e and another protein BC1 increases the binding affinity of FMRP for this complex (Napoli et. al., 2008; De Rubeis et. al., 2013). Together FMRP and CYFIP1 form a translation inhibitory complex in dendritic spines and this repression is regulated in an activity dependent manner by BDNF mediated signaling (De rubeis et. al., 2013; Schenck et. al., 2001; Napoli et. al., 2008). BDNF signaling induces
conformational changes in CYFIP1 through Rac1 which causes CYFIP1 to dissociate from eIF4e and allows for the synthesis of specific target proteins including Arc which then goes on to promote AMPAR internalization. The conformational change in CYFIP1 also allows it to affect changes in actin polymerization and subsequently spine morphology (De Rubeis et. al., 2013).

APP is known to facilitate the formation of synapses in the developing brain and the APP mRNA is one of the target mRNAs of FMRP (Westmark and Malter, 2007). Some of the phenotypes seen in Fmr1 ko mice have also been attributed to increased Abeta (Aβ) levels and some of these have been ameliorated by the genetic modulation of amyloid precursor protein (APP) or Aβ (Westmark et. al., 2010). Recent studies have shown MMP-9 overexpressing mice to have increased dendritic spine densities akin to that seen in Fmr1 ko mice and also increased soluble APPα (the N-terminal fragment released upon the proteolytic processing of APP by α-secretases) and these phenotypes were tied to the α-secretase-like activity of MMP-9 (Fragkouli et. al., 2012). This activity has also been shown for MMP-9 in vitro (Fragkouli et. al., 2011; Talamagas et. al., 2007) and it could be another mechanism by which the increased levels of MMP-9 in the Fmr1 ko cause the neurological deficits seen.

In summary, I show here that the intracellular signaling cascades mediated by mTOR and Akt are affected in Fmr1 ko mice and these eventually go on to phosphorylate eIF4e which regulate protein translation. This increase in signaling is however reduced by the deletion of mmp-9. Although many other groups have suggested that this increased signaling is mediated through the mGluRs, these signaling pathways are common to a
number of different cell surface receptors like TrkB and integrins (Fig. 4.7) that might be mediating this increase in intracellular signaling due to the actions of MMP-9 on the BDNF and ECM.
REFERENCES


Figure 4.1. Elevated basal protein synthesis in *Fmr1* ko mice is reduced by the deletion of *Mmp-9*. We measure the basal levels of protein synthesis in all four mouse genotypes by quantifying the amount of puromycin that is incorporated into newly synthesized proteins over a given period of time. (A) shows a representative blot of lysates obtained from the hippocampi of the mice treated with puromycin. (B) The bar graph represents average levels of incorporated puromycin normalized to that of wt samples. Statistical analysis was performed using student’s t-test (*p < 0.05, **p < 0.01, ***p < 0.001).
Figure 4.1

[Bar graph showing relative protein synthesis for different conditions, with labels: Dbl KO, Mmp-9 KO, Fmr1 KO, WT.]
Figure 4.2. Increased Akt, mTOR and eIF4e phosphorylation in Fmr1 ko mice is reduced by *Mmp-9* deletion. Western blots were used to quantify the phosphorylated and total levels of specific proteins. Representative blots are shown in (A). The bar graphs show (B) phospho/total ratios of mTOR in the hippocampi of the different mouse genotypes, (C) phospho/total ratios of eIF4e and (D) phospho/total ratios of Akt. The bar graphs represent average values and the error bars indicate SEM (n= 4 mice per group). Statistical analysis was performed using one-way ANOVA followed by Hsu’s MCB post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).
Figure 4.2
Figure 4.3. Reduction in the mRNA levels of some MMPs in the hippocampus of double ko animals. qRT-PCR was used to quantify mRNA levels of MMPs-2, -3, -7, -8, -9, -12, and -21 in the hippocampi (A, C, E) and cortex (B, D, F) of mice at the ages of P8 (A, B), P14 (C, D) and P21 (E, F). All RNA levels were normalized to the GAPDH levels in each sample. The bar graphs represent average values and the error bars indicate SEM (n= 3-5 mice per group). Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001). The white bars represent wt animals, grey bars- Fmr1 ko mice, black bars- Mmp-9 ko mice and stripped bars- dbl ko mice.
Figure 4.3
**Figure 4.4. No significant differences in the mRNA levels of some MMPs in the ko animals.** qRT-PCR was used to quantify mRNA levels of MMPs-10, -11, -19, -20, -23, -27, and -28 in the hippocampi (A, C, E) and cortex (B, D, F) of mice at the ages of P8 (A, B), P14 (C, D) and P21 (E, F). All RNA levels were normalized the the GAPDH levels in each sample. The bar graphs represent average values and the error bars indicate SEM (n= 3-5 mice per group). Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001). The white bars represent wt animals, grey bars- Fmr1 ko mice, black bars- Mmp-9 ko mice and stripped bars- dbl ko mice.
Figure 4.4
Figure 4.5. No significant differences in the mRNA levels of the membrane bound MMPs in the ko animals. qRT-PCR was used to quantify mRNA levels of MT- MMPs -1 to -6 in the hippocampi (A, C, E) and cortex (B, D, F) of mice at the ages of P8 (A, B), P14 (C, D) and P21 (E, F). All RNA levels were normalized the the GAPDH levels in each sample. The bar graphs represent average values and the error bars indicate SEM (n= 3-5 mice per group). Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001). The white bars represent wt animals, grey bars- Fmr1 ko mice, black bars- Mmp-9 ko mice and stripped bars- dbl ko mice.
Figure 4.6. No significant differences in the mRNA levels of all 4 TIMPs in the hippocampi or cortices of ko animals. qRT-PCR was used to quantify mRNA levels of MMPs-10, -11, -19, -20, -23, -27, and -28 in the hippocampi (A, C, E) and cortex (B, D, F) of mice at the ages of P8 (A, B), P14 (C, D) and P21 (E, F). All RNA levels were normalized the the GAPDH levels in each sample. The bar graphs represent average values and the error bars indicate SEM (n= 3-5 mice per group). Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001). The white bars represent wt animals, grey bars- Fmr1 ko mice, black bars- Mmp-9 ko mice and stripped bars- dbl ko mice.
Figure 4.6
Figure 4.7. Schmatic showing convergent signaling pathways resulting from various cell surface receptors that might mediate the increase in MMP-9 levels in Fmr1 ko mice. These pathways affect not only protein translation but also cytoskeleton reorganization which leads to changes in dendritic spine dynamics and morphology.
Figure 4.7
Table 4.1. List and sequence of primers used in qRT-PCR screen.
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<th>Primer</th>
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MMP28_F  CAGCAAGCTGGGATGTAGTGAA
MMP28_R  GAACCAAAAGAGAGGCCTGACA
TIMP1_F  TGATTTCGCCCGCAACTC
TIMP1_R  GGGCTGCACAGTGGAGAATAA
TIMP2_F  CCGTGTCGCAATTGAAAGGC
TIMP2_R  GCTGTTCGAGGAAAGGATGT
TIMP3_F  CCAACATCTTTTCCATCTTATCCA
TIMP3_R  CCATGTAAGGAGGGATGATTAC
TIMP4_F  CCACCATCTCTGTGCAACTACATTG
TIMP4_R  GGCTCTCCCTCTGCAACCAA
ADAMTS10_F  GCCACGGCCTTTGACCTATT
ADAMTS10_R  CCATCGGTATTGATGCAACAA
ADAMTS12_F  GACAGAAGGAGGATCTGTTACG
ADAMTS12_R  CCCTATATCCCTGACCTTTT
ADAMTS15_F  GGATAGCGAAAAGGAGGTCTGACA
ADAMTS15_R  CCCTAGGGCAGTGCATGATC
ADAMTS17_F  GGCTGGCGCATCTTATCTG
ADAMTS17_R  GGCTGGCGCATCTTATCTG
ADAMTS19_F  ACCAGAGCCACAGATTCGAAA
ADAMTS19_R  CTGTCATCATCCACCTTGTTTGA
GAPDH_F  CCACAGTCCATGCCACAC
GAPDH_R  CACCACCCTGGTGCTGAGCC
Dendritic spines are small protrusions on the surface of dendrites that are post-synaptic contact sites for the majority of the excitatory glutamatergic synapses in the brain (Harris, 1999). Dendritic spines are present on many different neuronal populations in the brain and among the most characterized are the spiny pyramidal neurons of the hippocampus, a population that is affected in FXS. Dendritic spines can be classified on the basis of their morphology and 4 major types of spines have been characterized from the immature filopodia and thin spines which have long necks and very small if any, spine heads, to the more mature stubby and mushroom spines which have larger spine heads and shorter necks (Ethell and Pasquale, 2005). A wide variety of these different spine types may be found in a variety of brain regions (Yuste and Bonhoeffer, 2004). These dendritic spines are dynamic structures that retain the ability to change their morphology even in the adult brain. During development, spines evolve from a more immature morphology (filopodia, thin) to a more mature spine (mushroom, stubby) morphology concomitantly with an increase in the density of spines and synapses along the dendrite (Yuste and Bonhoeffer, 2004). This development of dendritic spines has also been shown to be impaired in neurodevelopmental disorders like FXS. Dendritic spines serve as independent biochemical compartments that can function semi-independently from the dendritic shaft, having their own cell surface receptors for signal transduction, their own translational machinery and calcium stores. These spines are composed primarily of actin filaments, specifically the $\beta$ and $\gamma$ isoforms which are expressed in
neurons, and have very little microtubules which are concentrated in the dendritic shaft (Cohen et. al., 1985; Wyszynski et. al., 1997). The spine head contains an electron dense structure known as the postsynaptic density, which can be perforated or continuous, and this structure opposes the active zone of the presynaptic terminal (Li and Sheng, 2003). PSD size is often proportional to that of the spine head (Harris et. al., 1992; Tashiro and Yuste, 2003). It contains many cell surface receptors and ion channels that form a complex with intracellular signaling effector molecules and scaffolding proteins allowing for the transduction of extracellular signals to the intracellular actin filaments to maintain or change spine shape (Kaech et. al., 1997).

Spine morphology can be regulated by the turnover and rearrangements of the actin filaments which are present predominantly in the spines (Trachtenberg et. al., 2002; Pollard, 2003), by synaptic transmission through the neurotransmitter receptors including the glutamate receptors (Portera-Cailliau et. al., 2003; Matus et. al., 2000; Passafaro et. al., 2003; Fiala et. al., 2002), or trans-synaptic (Kossel et. al., 1997) and extracellular signals mediated by other cell surface receptors like the EphB receptors and ephrin-B ligand interactions (Dalva et. al., 2000; Ethell et. al., 2001; Henkemeyer et. al., 2003; Moeller et al, 2006; Shi et al., 2009), neuroligins and neurexins (Chih et. al., 2004b), cadherins (Togashi et. al., 2002), integrins (Chavis and Westbrook, 2001; Shi and Ethell, 2006), syndecans (Yamaguchi, 2002), and, the receptors for extracellular matrix proteins (Mataga et. al., 2004; Oray et. al., 2004). Dendritic spines are surrounded by extracellular matrix proteins, which form perineuronal nets around the soma and dendrites of neurons (Celio, 1999; Yamaguchi, 2000). These extracellular matrix proteins are processed or
activated by a host of different proteins including the matrix metalloproteinase (MMP) superfamily of proteins, and the products then go on to affect dendritic morphology through cell surface receptors and intracellular signaling cascades (Dityatev and Schacvhner, 2003; Szepesi et. al., 2013; Dziembowska et. al., 2012; Shi and Ethell, 2006). One of the ECM substrates cleaved by a MMP protein, MMP-9, is laminin (Doucet and Overall, 2010), which binds to integrins and promotes dendritic spine maturation and could trigger synapse remodeling (Shi and Ethell, 2006). Other groups have also shown the MMPs, specifically MMP-9 to be involved in the regulation of synaptic plasticity through integrin signaling pathways (Nagy et. al., 2006; Meighan et. al., 2006). Other MMPs like MMP-7 have also been shown to influence spine morphology, specifically promoting mature to more immature-filopodial spine transitions (Wlodarczyk et. al., 2011; Bilousova et. al., 2006). Some MMPs also appear to have roles in hippocampal dependent learning. MMPs 3 and 9 have been shown to be important in spatial learning (Meighan et. al., 2006; Nagy et. al., 2007; Wright et. al., 2007) while MMP-9 appears to have a role in long term potentiation (LTP), which is essential for long term synaptic plasticity, and these effects are mediated by NMDAR activity (Nagy et. al., 2006; Meighan et. al., 2006). Although these MMPs are highly expressed in the nervous system, only MMP-9 is a constituent of the healthy CNS with MMP-3 and MMP-7 being expressed by microglia and macrophages during inflammation or in response to changes induced by neurological disease (Ethell and Ethell, 2007; Buhler et. al., 2010; Dziembowska and Wlodarczyk, 2012; Sbai et. al., 2008; Visse and Nagase, 2003).
Fragile X syndrome (FXS) is the most common single gene cause of inherited intellectual disability and it is caused by a CGG repeat expansion (to greater than 200 repeats) in the 5’ untranslated region of the \textit{Fmr1} gene which results in promoter hypermethylation and subsequently gene silencing (Verkerk et. al., 1991). Individuals with FXS display a range of symptoms from mild learning problems to more severe cognitive disabilities including language deficits and behavioral dysfunctions; display obsessive-compulsive disorder (OCD) behaviors such as hand-flapping, autistic behaviors, and attention deficit and hyperactivity disorder (ADHD). Beyond cognitive and behavioral abnormalities, FXS patients also display characteristic physical traits that include long faces, large protruding ears, macroorchidism, flat feet, hyper-extensible joints, and soft fragile skin. Approximately 25% of patients suffer childhood seizures that improve with age (Clapp et. al., 2001; Garber at. al., 2008). The resultant lack of \textit{FMR1} gene product, FMRP, is thought to underlie the pathology of FXS (Luo et. al., 2010; Oostra and Willemsen, 2009). In the normal condition, FMRP is thought to bind to its target mRNAs and regulate both their transport to their appropriate cellular locations and also regulate their translation and it inhibits this translation by stalling the ribosomes on its target mRNAs until it receives an intra- or extra-cellular signal to allow the translation to proceed (Darnell et. al., 2011). In FXS, the loss of FMRP results in misregulated translation of its target mRNAs which is thought to underlie the learning and memory deficits seen in the brain due to its effects on the development of dendritic spines and also to the development of macroorchidism since it is highly expressed in neurons and in the testis. Post-mortem brain tissue from FXS subjects showed delayed dendritic spine
maturation, with a preponderance of long thin, immature filopodial like spines compared to the mature, mushroom and stubby spines seen in brain tissue from controls. Similar dendritic spine profiles to FXS subjects are also seen in the Fmr1 ko mice, considered a mouse model for FXS (Braun and Segal, 2000; Irwin et. al., 2000) since they reproducibly exhibit phenotypes that are similar to the human condition including dendritic spine abnormalities, anxiety, susceptibility to audiogenic seizures, macroorchidism, and learning and spatial memory deficiencies (Braun and Segal, 2000; Kooy, 2003; Cruz-Martin et. al., 2010; Comery et. al., 1997; Bassell and Gross, 2008; Bernardet and Crusio, 2006; Levenga et. al., 2011; Bilousova et. al., 2008).

Several different signaling pathways and receptors that are dysregulated in FXS are also known to influence dendritic spine maturation and plasticity. Activation of group 1 metabotropic glutamate receptors (mGluRs) with its agonist DHPG was shown to induce an immature spine morphology and increase number of immature thin spines (Vanderklish and Edelman, 2002; Cruz-Martin et. al., 2012). Enhanced activity of mGluR5 receptor was implicated in some FXS-associated deficits (Bear et. al., 2004). Normally, mGluR signaling blocks the translational repression of FMRP from its target mRNAs, allowing for the translation of the protein products encoded, which can cause α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor internalization and long term depression (LTD)--a decrease of synaptic strength. In the absence of FMRP, those proteins are expressed at a higher basal level and more AMPA receptors are internalized even before the glutamate signaling arises, thus lengthening long term depression (Bear et. al., 2004; Hou et. al., 2006; Oostra and Willemsen, 2009). mGluR
antagonists such as MPEP, Fenobam, and CTEP have been shown to attenuate some of the FXS phenotypes in Fmr1 ko mice and in human patients (Yan et. al, 2005; Levenga et. al., 2010; Michalon et. al., 2012). Others have shown that Fmr1 ko mice have altered GABA receptor subunit composition and lower GABAergic inputs to certain hippocampal circuits (Brenman, 2009; D’Hulst et. al., 2009), so that GABA receptor agonists, such as Baclofen and Nipecotinic acid partially rescue the FXS phenotypes (Levenga et. al., 2010; Pacey et. al., 2009).

Synaptic activity has been shown to affect spine morphology. For example, LTP is known to induce maturation of spines, that is, to induce enlargement of the head sizes and shortening of spine necks whereas LTD can trigger spine shrinkage and loss. Synaptic activity can also trigger new synaptogenesis and regulate spine density (Yuste and Bonhoeffer, 2001; Toni et. al., 1999; Muller et. al., 2000; Engert and Bonhoeffer, 1999). Enhanced synaptic activity has been shown to regulate the protein levels and activity of MMP-9 (Nagy et. al., 2006; Meighan et. al., 2007; Okulski et. al., 2007), which can affect spine morphology through the extracellular matrix surrounding the shaft and spines. This upregulation of MMP-9 was shown to be mediated by NMDAR signaling and is protein synthesis dependent (Nagy et. al., 2006). MMP-9 may modulate synaptic functions through the cell surface integrin receptors (Nagy et. al., 2006). It is possible that the changes in spines seen in FXS are also mediated by MMP-9 activity since increased MMP-9 activity has also been implicated in the remodeling that accompanies kainic- acid induced epileptogenesis in the hippocampus (Zhang et. al., 1998; Szklarczyk et. al., 2002).
Previous work done in our laboratory has implicated matrix metalloprotease-9 in abnormal spine development in FXS (Bilousova et al., 2009). We found that MMP-9 activity is higher in Fmr1 ko mice than in the wild-types; further, minocycline treatment reduced the MMP-9 activity levels in Fmr1 ko mice, ameliorated the dendritic spine phenotype, and improved behavioral abnormalities seen in Fmr1 ko mice. When cultures of mature hippocampal neurons from wt mice were treated with MMP-9 protein they developed long, immature filopodial like spines that mimicked the immature spine precursors seen in Fmr1 ko hippocampal cultures (Bilousova et. al., 2008). This same relationship between Fmr1 and MMP-9 has also been reported in the drosophila model of FXS where overexpression of TIMP1 (the endogenous regulator of MMPs) or a dfmr/mmp null mutant rescued all nervous system defects seen in the fmr null flies, suggesting a genetic interaction between these two genes and/or their protein products (Siller and Broadie, 2011). Other groups have also shown MMP-9 involvement in synaptic plasticity and regulation of NMDAR currents mediated through integrin activity (Nagy et. al., 2006; Meighan et. al., 2006) suggesting all these components that are defective in FXS may be linked.

In this study, I confirmed all the previously reported phenotypes of the Fmr1 ko mice showing that they exhibit a more immature spine profile both in vivo and in vitro, upregulation of MMP-9 and MMP-2 protein levels in the hippocampi of these animals although there was no change in the mRNA levels of both, enhanced mGluR dependent LTD, deficiency in LTP, increased anxiety and hyperactivity, reduced social interaction behavior or ability to distinguish between social novelty, macroorchidism, increased
basal protein synthesis and increase in the phosphorylation and activation of intracellular signaling effector molecules including mTOR, eIF4e and Akt compared to wt animals. I also show that depleting Mmp-9 is able to rescue many of these phenotypes. The spine profiles of the double Fmr1/mmp9 ko animals is similar to that of wt mice both in vivo and in vitro, they exhibit normal levels of MMP-2 in the hippocampus, show LTD comparable to wt mice in response to DHPG treatment although they remain deficient in LTP, display reduced anxiety, do not display social behavioral deficits, do not display macroorchidism, and, have reduced levels of phosphorylated mTOR, eIF4e and Akt. Published results show that FMRP regulates the translation of the mmp-9 and mmp-2 mRNAs, at least in the hippocampus (Janusz et. al., 2013). Our results demonstrate that the increased MMP-9 activity underlies many of the phenotypes seen in FXS.

Some groups have proposed that increased sensitivity of mGluRs underlies the deficits seen in FXS, both in human subjects and in Fmr1 ko mice (Bear et. al., 2004) and reducing mGluR activity or the activity of downstream signaling pathways associated with mGluRs has ameliorated some of cognitive and behavioral deficits in Fmr1 ko mice. Nonetheless, reductions in mGluR signaling have no impact on macroorchidism, indicating that while mGluR signaling might play a role in this disorder, it is not the sole cause of all the symptoms (Bear et. al., 2004; Bassell and Gross, 2008; Michalon et. al., 2012). Metabotropic GluR signaling may contribute to neurological deficits in FXS but not in the connective tissues and hence therapies targeting these receptors may not completely improve all aspects of this disorder. mGluRs could also be acting downstream of other molecules or the increased mGluR signaling seen might be due to increased
active MMP-9. In addition, the effector molecules activated in the Fmr1 ko mice like mTOR and eIF4e or Akt can also be regulated by downstream signaling cascades of other receptors and by different pathways. For instance, this increase in mTOR phosphorylation could be due to BDNF signaling through its receptor, Trk B, which shares similar intracellular signaling pathways with the mGluRs. BDNF protein levels are known to be higher in the hippocampi of Fmr1 ko mice and the Trk B mRNA is a known target of FMRP (Castren and Castren, 2013; Louhivouri et. al., 2011; Uutela et. al., 2012). A number of MMPs are known to cleave and activate neurotrophins and MMP-9 in particular has been shown to cleave pro-BDNF (Hwang et. al., 2005; Yang et. al., 2009; Mizoguchi et. al., 2011). The increased levels of MMP-9 may function in a positive feedback loop in the ko mice to cleave pro-BDNF to mature BDNF which can then bind to its receptor, Trk B, and activate an intracellular signaling cascade that promotes the translation of more MMP9 among other proteins through both the ERK and PI3K- Akt- mTOR pathways (Santos et. al., 2010). In addition, a FMRP binding protein, cytoplasmic FMRP interacting protein 1 (CYFIP1) binds directly to eIF4e and another protein BC1 increases the binding affinity of FMRP for this complex (Napoli et. al., 2008; De Rubeis et. al., 2013). Together FMRP and CYFIP1 form a translation inhibitory complex in dendritic spines and this repression is regulated in an activity dependent manner by BDNF mediated signaling (De rubeis et. al., 2013; Schenck et. al., 2001; Napoli et. al., 2008). BDNF signaling induces conformational changes in CYFIP1 through Rac1 which causes CYFIP1 to dissociate from eIF4e and allows for the synthesis of specific target proteins including Arc which then goes on to promote AMPAR internalization. The
conformational change in CYFIP1 also allows it to affect changes in actin polymerization and subsequently spine morphology (De Rubeis et. al., 2013).

Since many MMP-9 substrates occur in and around CNS synapses (Ethell and Ethell, 2007), integrins and Eph receptors are the most likely candidates to mediate MMP-9 effects in Fmr1 ko neurons. Both integrins and EphB receptors are known to signal through the recruitment and activation of FAK and the Src family of non-receptor tyrosine kinases, and the PI3K/Akt cascade (Chen et. al., 2009; Guo et. al., 2004; Legate et. al., 2009; Moeller et al., 2006; Maddigan et. al., 2011). Moreover β1 integrin has been shown to negatively regulate the activity of protein pyrophosphatase2a (PP2A), which de-phosphorylates and inactivates Akt (Fornano et. al., 2000; Pankov et. al., 2003). The PI3K-Akt-mTOR pathway was previously implicated in FXS and shown to regulate protein synthesis through Elongation factor 1α (Hou and Klann, 2004; Ronesi and Huber, 2005).

APP is known to facilitate the formation of synapses in the developing brain and the APP mRNA is one of the target mRNAs of FMRP (Westmark and Malter, 2007). Some of the phenotypes seen in Fmr1 ko mice have also been attributed to increased Abeta (Aβ) levels and some of these have been ameliorated by the genetic modulation of amyloid precursor protein (APP) or Aβ (Westmark et. al., 2010). Recent studies have shown MMP-9 overexpressing mice to have increased dendritic spine densities akin to that seen in Fmr1 ko mice and also increased soluble APPα (the N-terminal fragment released upon the proteolytic processing of APP by α-secretases) and these phenotypes were tied to the α-secretase-like activity of MMP-9 (Fragkouli et. al., 2012). This activity
has also been shown for MMP-9 in vitro (Fragkouli et. al., 2011; Talamagas et. al., 2007) and it could be another mechanism by which the increased levels of MMP-9 in the Fmr1 ko cause the neurological deficits seen.

Here I used genetics to validate the importance of MMP-9 in FXS and I show that enhanced MMP-9 activity is a central mediator of neural and non-neural defects associated with FXS. The lack of Mmp-9 in Fmr1 ko mice (i.e. dbl ko) ameliorated neurological and behavioral deficits associated with FMRP deficiency, as well as macroorchidism. It remains to be seen how all the molecules implicated in FXS interact to cause these symptoms; however, since a reduction of MMP-9 can improves both cognitive and behavioral deficits and physical traits of FXS, MMP-9 is the best target for therapeutic development. One such compound that has been successful in recent human trials is minocycline, an antibiotic tetracycline analog that inhibits MMP-9, among its other actions. Minocycline seems to be a good drug choice since it is well tolerated and has been prescribed to treat acne in adults and is hence FDA approved (Seukeran and Eady, 1997; Goulden and Glass, 1996). It was also shown to improve a wide range of symptoms in FXS subjects including major improvements in language use, increased speech coherency, improved attention spans, less irritability, decreased anxiety, increased social communication and a general improvement in global behavioral scores (Paribello et. al., 2010; Utari et. al., 2010; Leigh et. al.,2013; Schneider et. al., 2013). The studies involving minocycline reported only minor side effects with occasional mild gastrointestinal problems, headaches or diarrhea (Paribello et. al., 2010; Utari et. al., 2010) so it would be well tolerated by a majority of subjects; however, there have been
reports of a small minority of children are susceptible to developing minocycline-induced autoimmunity and those children may go on to develop chronic symptoms that could be potentially fatal (Farver, 1997; El-Hallack and Giani, 2008). Taken together with the fact that minocycline is a broad acting antibiotic, care should be taken to minimize the long-term usage of this drug in FXS and instead more research should be done to identify new drugs acting more specifically on MMP-9, without antibiotic activity.
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