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Enhanced Activity of Matrix Metalloproteinase-9 Contributes to the Pathology of Fragile X Syndrome and can be Ameliorated with Minocycline Treatment

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ABSTRACT OF THE DISSERTATION

Enhanced Activity of Matrix Metalloproteinase-9 Contributes to the Pathology of Fragile X Syndrome and can be Ameliorated with Minocycline Treatment

by

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Fragile X syndrome (FXS) is the most common single-gene inherited form of intellectual disability, with behaviors characteristic of autism. FXS is caused by silencing of the Fragile X mental retardation gene (*FMR1*). Individuals with FXS display childhood seizures, hyperactivity, anxiety, developmental delay, attention deficits, and visual-spatial memory impairment, as well as repetitive and perseverative behavior. Many of these behavioral deficits are also associated with mice that lack the *Fmr1* gene (*Fmr1 KO*). Furthermore, *Fmr1 KO* neurons, similar to the human condition, exhibit defects in dendritic spine maturation which may underlie the cognitive and behavioral abnormalities in FXS. Dendritic spines are small protrusions on the surface of the dendrite that receive the majority of excitatory synapses in the brain and changes in their morphology affect synaptic efficacy. Much research has focused on treatment options to improve the deficits

associated with FXS to ultimately improve the quality of life for the individuals afflicted with this syndrome. The basis of my research examined how minocycline improved behavioral deficits, such as a reduction in hyperactivity, anxiety, obsessive compulsive tendencies and susceptibility to audiogenic seizures as well as promoted dendritic spine maturation in the *Fmr1 KO*. Minocycline is a semi-synthetic antibiotic that also has many non-microbial targets, such as inhibiting the enzyme matrix metalloproteinase-9 (MMP-9). MMP-9 has been implicated in many neurological disorders, including multiple sclerosis, cerebral ischemia and mild cognitive impairment. The concurrent focus of my project was on the involvement of MMP-9 in FXS. We discovered that active MMP-9 promoted an immature dendritic spine profile in cultured hippocampal neurons, and that MMP-9 expression and activity were upregulated within the hippocampus of the *Fmr1 KO.* To further expound upon the role of MMP-9, we also analyzed MMP-9's expression levels within the blood and brain of individuals with FXS and found a decrease in relative activity in the blood, but an increase in total expression within postmortem hippocampal and neocortical tissue. Overall, this study elucidated a novel mechanism for MMP-9 that appears to play a causative role in the pathology of FXS as well as provided a new therapeutic option for FXS treatment.

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Abbreviations

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

AMPAR: AMPA receptor

CNS: central nervous system

DIV: days *in vitro*

E: embryonic day

ECM: extracellular matrix

ECS: extracellular space

ERK: extracellular regulated kinase

FAK: focal adhesion kinase

Fmr1 KO: Fragile X mental retardation gene knockout mouse

FMRP/Fmrp: Fragile X mental retardation protein

FXS: Fragile X Syndrome

GSK: glycogen synthase kinase

LTD: long-term depression

LTP: long-term potentiation

L-LTP: late phase LTP

MAPK: mitogen activation protein kinase

Min/Mino: minocycline

MMP: matrix metalloproteinase

Mmp9 KO: matrix metalloproteinase-9 gene knockout mouse

MT-MMP: membrane type matrix metalloproteinase

mTOR: mammalian target of rapamycin

NMDA: N-methyl-D-aspartate

NMDAR: NMDA receptor

P: postnatal day

PNN: perineuronal net

PNS: peripheral nervous system

PSD: postsynaptic density

TIMP: tissue inhibitor of metalloproteinases

WT: wild-type

Chapter 1 – Background

INTRODUCTION

Dendritic Spines

Over a century ago, Santiago Ramón y Cajal first discovered the existence of dendritic spines upon analysis of the surface of Purkinje cell dendrites, and proposed that electrochemical signals may be transduced through those spines (Ramon y Cajal, 1888; Ramon y Cajal, 1899). Since then, it has been well established that dendritic spines are small extensions from the surface of a dendrite that accommodate the postsynaptic sites of most excitatory synapses in the brain and our knowledge of their structure and function has significantly progressed in recent years (reviewed in Sorra and Harris, 2000; Hering and Sheng, 2001; Yuste and Bonhoeffer, 2004; Ethell and Pasquale, 2005). While aspiny interneurons receive both excitatory and inhibitory pre-synaptic inputs directly onto the dendritic shaft, spiny neurons preferentially form excitatory synapses on dendritic spines, which come in a variety of shapes and sizes, ranging from long thin spines with small heads to short stubby- and mushroom-shaped spines with large heads (Fig. 1.1 A-D). Excitatory postsynaptic sites are usually located on spine heads, which are bulbous, biochemical chambers that are separated from the rest of the dendrite by a thin neck (Fig. 1.1 E), giving it the ability to function as a relatively independent synaptic response element. The postsynaptic site is identified as an electron-

dense region known as the postsynaptic density (PSD in Fig. 1.1 E), which populates about 10% of the spine head area and is juxtaposed with the presynaptic terminal containing neurotransmitter-packaged synaptic vesicles (SV in Fig. 1.1 E; Harris and Stevens, 1989; Knott et al., 2006; Zhang et al., 2010). Glutamate, the most common excitatory neurotransmitter in the brain, is released from presynaptic terminals during synaptic transmission to activate specific glutamate receptors located at the postsynaptic site. In addition to glutamate receptors, the PSD contains cell adhesion molecules and various cell surface receptors that act together with scaffolding proteins to link the PSD to filamentous actin (F-actin). Dendritic spines are enriched in F-actin that plays an important role in shaping dendritic spines as well as the trafficking of functional glutamate receptors in and out of the PSD. Some dendritic spines also contain a spine apparatus, which consists of smooth endoplasmic reticulum (SER)-like membrane compartments that serve as Ca^{2+} stores and as a harbor for additional glutamate receptors.

Dendritic spine formation directly coincides with the formation of postsynaptic sites. Before the appearance of dendritic spines, dendrites exhibit long, thin filopodia-like protrusions. As the brain matures, the number of dendritic filopodia declines and the number of stable dendritic spines with mushroom-like and stubby shapes increases (see examples of different spines in Fig. 1.1 C). Although dendritic spines can arise directly from the dendritic shaft (Fiala et al., 1998; Marrs et al., 2001; Kwon and Sabatini, 2011), it is widely accepted that

spines can also form from filopodia, which are perceived as dendritic spine precursors both *in vitro* and *in vivo* (Dailey and Smith, 1996; Ziv and Smith, 1996; Maletic-Savatic et al., 1999; Lendvai et al., 2000; Marrs et al., 2001; Okabe et al., 2001; Trachtenberg et al., 2002; Portera-Cailliau et al., 2003; Ziv and Garner, 2004; Knott et al., 2006). Filopodia are long, headless and transient extensions, which can transform into immature thin spines upon contact with axon terminals (Fig. 1.1 F). It has also been suggested that filopodia may have additional functions, such as involvement in dendritic growth and branching (Niell et al., 2004; Yuste and Bonhoeffer, 2004; Zhang et al., 2010). Filopodia-derived spines typically first appear as immature thin spines with long, thin necks and small head areas (Fig. 1.1 F), which can directly transform into mature mushroom spines (Vaughn, 1989; Ziv and Smith, 1996; Marrs et al., 2001; Knott et al., 2006; Knott and Holtmaat, 2008), or can shrink and completely collapse followed by the formation of mushroom spines from the dendritic shaft (Dailey and Smith, 1996; Fiala et al., 1998; Knott and Holtmaat, 2008). Immature dendritic spines and filopodia are very dynamic and plastic structures that are highly susceptible to changes in synaptic activity (Lang et al., 2004; Matsuzaki et al., 2004; Nimchinsky et al., 2004; Ashby et al., 2006; Zhang et al., 2010).

Dendritic spine morphology undergoes developmental changes leading to spine maturation, which reflects the transformation of immature thin spines into mature spines and coincides with postsynaptic differentiation. Mature spines are primarily characterized as two types: mushroom spines with short thin necks and

large head areas, and stubby spines with thick necks and head areas that are not clearly distinguishable (Fig. 1.1 F). Mature spines are shown to be more stable and highly sensitive to glutamate than immature spines due to a larger PSD and sometimes contain perforated synapses that exhibit discontinuous PSD profiles (Matsuzaki et al., 2001; Murthy et al., 2001; Smith et al., 2003; Nicholson et al., 2006). Perforated synapses are typically much larger, contain a higher number of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and N-methyl-D-aspartate receptors (NMDARs) and are capable of generating large synaptic conductances (Nicholson et al., 2006). Several studies demonstrated a direct correlation between the size of the PSD and the number of glutamate receptors that the spine accommodates (Matsuzaki et al., 2001; Shinohara and Hirase, 2009). Although, there is a large variation in the expression of functional AMPARs among different types of dendritic spines, the size of the PSD was shown to be proportional to the spine head size, and mushroom spines with a larger head volume contained a higher proportion of functional AMPARs in hippocampal neurons (Matsuzaki et al., 2001).

The majority of mature spines are highly stable structures that can last from months to a lifetime, implicating mature spines as the physical manifestation of long-term information storage (Grutzendler et al., 2002; Holtmaat et al., 2005; Zuo et al., 2005; Zuo et al., 2005; Yang et al., 2009; Zhang et al., 2010). However, mature spines remain plastic in the adult brain and can also undergo structural remodeling, such as enlargement, reduction or complete elimination, in

response to various stimuli (Segal, 2005; Yasumatsu et al., 2008; Kasai et al., 2010; Zhang et al., 2010). Mature mushroom-shaped spines can transform back into immature thin spines, revert into filopodia lacking synaptic contact or completely collapse. This structural plasticity of dendritic spines has been shown to correlate with functional plasticity at the level of the individual synapse (Matsuzaki et al., 2004; Okamoto et al., 2004; Zhou et al., 2004; Yasumatsu et al., 2008; Kasai et al., 2010). The induction of long-term potentiation (LTP) or long-term depression (LTD), which may enhance or decrease synapse strength has been shown to trigger changes in dendritic spine morphology. For example, high-frequency stimulation (HFS) typically results in high calcium influx, LTP and the insertion of additional AMPARs into the postsynaptic membrane, which also coincides with the enlargement of existing dendritic spines and formation of new spines (Lang et al., 2004; Matsuzaki et al., 2004; Nagerl et al., 2004; Okamoto et al., 2004; Otmakhov et al., 2004). Induction of LTP in mouse CA1 hippocampal neurons by HFS demonstrated a transient expansion of dendritic spines that was further enhanced with subsequent stimulations (Lang et al., 2004). LTP-induced spine enlargement was dependent on the activation of NMDA- and AMPA-type glutamate receptors, as well as actin reorganization that shifted the equilibrium toward polymerized F-actin (Okamoto et al., 2004). In addition, Gu and colleagues (2010) have recently shown that LTP-induced spine enlargement temporally lags behind AMPAR insertion, suggesting that changes in spine shape may accommodate maintenance of newly inserted AMPARs rather than

induce their insertion into the postsynaptic site (Gu et al., 2010). In contrast, LTD that is usually triggered by persistent, low frequency stimulation (LFS) and low levels of calcium influx, results in AMPAR endocytosis, spine shrinkage and elimination (Nagerl et al., 2004; Okamoto et al., 2004; Zhou et al., 2004; Bastrikova et al., 2008). LFS stimulation of mouse CA1 hippocampal neurons induced synapse loss as well as selective spine retraction that could be reversed with subsequent HFS stimulation (Nagerl et al., 2004; Zhou et al., 2004; Bastrikova et al., 2008). LFS-induced spine shrinkage and retraction were dependent on NMDAR activation (Zhou et al., 2004) and actin depolymerization, which led to changes in dendritic spine morphology and number (Okamoto et al., 2004).

Since dendritic spines are responsible for the majority of postsynaptic excitatory transmissions in the brain it is important for us to further characterize and understand how changes in their morphology affect their function and assess how these changes are related to abnormalities associated with cognitive disorders (Fig. 1.2 A,B).

Fragile X Syndrome

Fragile X Syndrome (FXS) is the most common single-gene inherited form of intellectual disability, with behaviors characteristic of autism. Individuals with FXS display varying spectrums of cognitive impairments which, in the most severe cases, include susceptibility to childhood seizures, manifestations of

hyperactivity, anxiety, developmental delay, attention deficits, and visual-spatial memory impairment, as well as the propensity for obsessive-compulsive tendencies (Musumeci et al., 1999; Hagerman and Hagerman, 2002). Different from other neurodevelopmental disorders, FXS is also characterized by many physical manifestations, such as large ears, long faces, hyperextensible joints and extremely soft skin, all of which are extensions of modified extra-cellular matrix (ECM) regulation (Hagerman and Hagerman, 2002). FXS is caused by excessive CGG repeats in the 5' region of the X-linked, Fragile X Mental Retardation (*FMR1*) gene. Hypermethylation of these repeats causes silencing of the gene which knocks out expression of the encoded protein, FMRP (Khandjian, 1999). In the most severe cases of FXS there is complete silencing of the gene causing a complete knock out of FMRP expression.

Studies of the FMRP mouse homolog, Fmrp, have demonstrated that this protein can localize to post-synaptic sites, bind to ~4% of brain mRNA transcripts and is responsible for regulating the translation of many post-synaptic molecules involved in dendritic spine plasticity (Fig. 1.2; Feng et al., 1997; Brown et al., 1998; Khandjian, 1999; Greenough et al., 2001; Todd et al., 2003). Fmrp regulates translation through the translation initiation complex and contains two KH2 binding domains and one RGG-box, which are domains specifically associated with RNA binding (Sung et al., 2000; Zalfa et al., 2003; Napoli et al., 2008). To study effects of the loss of FMRP, a mouse model was derived in which a portion of the *Fmr1* gene was excised to create a knockout (*Fmr1 KO*),

which demonstrates many of the behavioral and morphological manifestations that are associated with the human condition (Comery et al., 1997; Mineur et al., 2002). From this model, it has been suggested that many of the abnormalities associated with FXS are due to hypersensitivity to group 1 metabotropic glutamate receptor 5 (mGluR5) signaling which leads to increased mGluR5-induced LTD (Fig. 1.2 C; Bear et al., 2008; Dölen and Bear, 2008; Dölen and Bear, 2009; Osterweil et al., 2010).

The mGluR theory of Fragile X is based on inhibitory effects of Fmrp on protein synthesis required for mGluR5-dependent LTD (Bear, 2005). The activation of mGluR5 leads to the synthesis of synaptic proteins through mRNA translation which is typically suppressed by Fmrp (Fig. 1.2 C). Without Fmrp there is little to no regulation of the translation of those mRNAs, which results in enhanced mGluR5-induced LTD and aberrant dendritic spine morphology (Fig. 1.2 B; Bear, 2005). The most salient dendritic spine abnormalities associated with FXS patients are the occurrence of an increased density of dendritic protrusions as well as an increase in the proportion of those protrusions that exhibit immature phenotypes (Fig. 1.2 B; Irwin et al., 2000; Kaufmann and Moser, 2000; Barnes and Milgram, 2002; Fiala et al., 2002).

Exactly what mechanisms and receptor signaling pathways are promoting these aberrant changes in dendritic spine morphology in the absence of Fmrp is still under investigation. Since FXS is also characterized by abnormal physical manifestations in addition to the altered neuronal morphologies, it is

possible that both problems could be linked to alterations of the ECM. A major candidate for investigation would be the family of matrix metalloproteinases (MMPs), which are the primary remodelers of the ECM (Mott and Werb, 2004; Ethell and Ethell, 2007).

Extracellular Matrix and Matrix Metalloproteinases

Extracellular space (ECS) occupies around 20% of the brain (Ruoslahti, 1996; Nicholson and Syková, 1998), but its volume and composition varies depending on the brain region (Brückner et al., 1996; Hausen et al., 1996; Matthews et al., 2002). ECS volume is higher in the hippocampus, striatum and cerebellum as compared to the cerebral cortex (Zhang and Verkman, 2010). ECS is filled with ECM that surrounds neurons and forms a perineuronal net (PNN), which is prominent around inhibitory interneurons, but also found around excitatory neurons. PNNs envelope neuronal cell bodies and proximal dendrites, where they control the three-dimensional organization, growth, movement, and shape of neurons that is important for maintaining structural integrity (Celio et al., 1998). Although synapses in the CNS do not contain basal lamina, ECM components also extend into the synaptic clefts as well as extrasynaptic space surrounding dendritic spines in the brain (Pappas et al., 2002; Chen et al., 2003; Indyk et al., 2003; Lucic et al., 2005).

Each component of the ECM is specialized for different functions such as repulsion or adhesion, and can have varying properties from rigidity to

elasticity (Mecham, 1998; Kleinman et al., 2003). ECM composition ranges from scaffolding proteins such as laminin, fibronectin and tenascin that link various ECM components into a net, to heavily glycosylated proteins called proteoglycans, to matrix metalloproteinases (MMPs) that cleave and remodel the ECM.

MMPs belong to a family of metzincin endopeptidases and were named for their ability to cleave the ECM. 24 mammalian MMPs have been identified so far, which share a highly conserved catalytic domain (Fig. 1.3; reviewed in Mott and Werb, 2004; Ethell and Ethell, 2007; Wilczynski and Kaczmarek, 2008). While most MMPs are secreted proteins, there are six membrane-type MMPs (MT-MMPs) that were also identified (Transmembrane and GPI-linked in Fig. 1.3). Prior to MMP enzymatic activity, MMPs begin in an inactive zymogen form, which contains a pro-domain that occupies the catalytic domain and prevents MMP activation (Fig. 1.3). Therefore, removal of the pro-domain by proteolytic cleavage or displacement is required for MMP activation. Tissue inhibitors of metalloproteinases (TIMPs) are involved in regulation of MMPs and also regulate the activity of ADAMs (A disintegrin and metalloproteinase protein), which have many functions that are similar to MMPs. Within the brain, expression of several MMPs, including MT-MMP-5, MMP-3 and the gelatinases, MMP-2 and MMP-9 (named for their ability to cleave gelatin), was shown to be regulated during development and in response to learning (Ayoub et al., 2005; Ulrich et al., 2005; Meighan et al., 2006; Monea et al., 2006). Both MMP-3 and MMP-9 were

increased in the hippocampus of mice following successful learning in the Morris water maze, which was impaired in the presence of MMP inhibitors (Meighan et al., 2006). With regards to developmental expression, mRNA transcripts of MMP-2, -9, -11, -13, -14, -15, -24 and the tissue inhibitors of metalloproteinases (TIMP)-1, -2, and -3 were all found to be upregulated within the proencephalon and rhombencephalon of 1 week old mice (Ulrich et al., 2005). Within the cerebellar cortex, measures of the mRNA transcript levels of MMP-2 and MMP-9 revealed an initial decrease between postnatal day 3 (P3) to P6, with a relatively constant expression thereafter (Ayoub et al., 2005). Zymography analysis demonstrated that the expression and gelatinase activity of both MMP-2 and MMP-9 persisted longer than their transcripts with downregulation occurring around P9 and subsequent undetectable immunoreactivity after P21. Specific inhibition of MMP-2 and MMP-9 within organotypic cerebellar slices significantly increased the thickness of the external granule layer and decreased the number of migrating granule neurons in the molecular layer, indicating a role for MMP-2 and MMP-9 in postnatal cerebellar morphogenesis. Purification of the synaptosomal fraction as well as fluorescence immunolabeling of cultured rat cortical neurons demonstrated synaptic localization of MT-MMP-5 at 2 and 5 days in vitro (DIV), as well as within the growth cones of both axons and dendrites indicating a role for MT-MMP-5 in promoting the extension of processes and synaptogenesis (Monea et al., 2006).

The regulation of MMPs during development and in response to learning may impact the formation and maintenance of dendritic spines. Analysis of organotypic hippocampal slice cultures and dissociated hippocampal neurons from transgenic rats overexpressing an autoactivating MMP-9 demonstrated that enzymatic activity of MMP-9 promotes longer and thinner dendritic spines in a manner dependent on integrin β1 signaling (Michaluk et al., 2011). On the other hand, brief exposure of CA1 pyramidal neurons to active MMP-9 enhanced synaptic potentiation in acute rat hippocampal slices, leading to expansion of dendritic spines and more mature dendritic spine morphology (Wang et al., 2008). Incubation of these slices with a broad-spectrum MMP inhibitor or with integrin-blocking antibody impaired LTP as well as dendritic spine expansion induced by theta burst stimulation. Several other studies have also shown that MMP-9 can enhance LTP through integrin-dependent regulation of NMDAR activity (Fig. 1.4; Nagy et al., 2006; Michaluk et al., 2009).

These effects of MMPs on the synaptic plasticity and morphology of dendritic spines are suggested to be mediated through the ability of MMP-9 to cleave ECM proteins and to trigger integrin signaling (Fig. 1.4). Specifically, cleavage of laminin and fibronectin by MMP-9 has been proposed to be a mechanism by which MMP-9 mediates its effects on dendritic spine morphology and LTP via integrin-dependent regulation of NMDAR activity (Nagy et al., 2006; Wang et al., 2008). In addition to integrins, MMPs may also influence dendritic spines by regulating the activities of several other synaptic receptors that can be

cleaved by MMPs. Cadherins can be cleaved by several MMPs (Fig. 1.4), such as MMP-9, MMP-12 (Dwivedi et al., 2009) and MT-MMP-5 (Monea et al., 2006), to produce an extracellular fragment which has been implicated in promoting dendritic spine elongation (Togashi et al., 2002). Another cell-surface receptor, β-dystroglycan is found within the PSD of dendritic spines and has been suggested to mediate MMP-9 effects on dendritic spines (Michaluk et al., 2007; Gawlak et al., 2009). Inhibition of $GABA_A$ receptors demonstrated an increase in MMP-9 levels and MMP-9 dependent cleavage of β-dystroglycan within the rat hippocampus (Michaluk et al., 2007). Eph receptors, a family of tyrosine kinases that are implicated in dendritic spine formation and maintenance, and their ligands ephrins can also be cleaved by MMPs (Fig. 1.4; Lin et al., 2008; Inoue et al., 2009). MMP-9 cleavage of the EphB2 receptor enhanced EphB2 signaling and EphB2-mediated growth cone collapse (Lin et al., 2008), whereas MMP-induced cleavage of EphA4 was independent of its stimulation with ephrins-A3 ligand (Inoue et al., 2009). However, it is still unclear whether the ability of MMPs to cleave Ephs and cadherins contributes their effects on dendritic spines. MMPs have clear indications in affecting the normal development of dendritic spines, and changes in their expression and activity have also been implicated in various neurological disorders.

MMPs have been previously characterized for their role in the regulation of the blood-brain barrier (Zlokovic, 2008), and were suggested to underlie the progression of the autoimmune disorder multiple sclerosis (Agrawal et al., 2008),

to facilitate immune access to the CNS in experimental autoimmune encephalomyelitis (Buhler et al., 2009) and to increase inflammation associated with cerebral ischemia (Cunningham et al., 2005). In addition to the effects on the blood brain barrier, recent studies have also implicated MMPs in synapse pathologies associated with brain injuries. Elevation of MMP-3 expression and activity correlated with trauma-induced synaptogenesis following brain injury (Kim et al., 2005; Falo et al., 2006). Moreover, upregulation of MMP-9 has been linked to the fiber sprouting and reactive synaptogenesis associated with kainite-evoked epilepsies (Szklarczyk et al., 2002; Wilczynski et al., 2008) or traumatic brain injury (Wang et al., 2000). Changes in the levels and activity of MMP-9 has also been linked to dendritic spine pruning associated with epilepsies (Wilczynski et al., 2008). Increased MMP-9 activity was reported in patients with mild cognitive impairment and Alzheimer's disease, suggesting MMP-9 may also be responsible for dendritic spine loss seen in these diseases (Bruno et al., 2009). MMPs influences normal physiology of dendritic spines and synapses, and alterations in their patterns of expression and regulation of activity may underlie abnormal dendritic spine development and neuropathologies. The focus of this dissertation is on the connection of MMP-9 to dendritic spine development and morphology and how it may contribute to the causes underlying the morphological abnormalities associated with FXS (Fig. 1.2).

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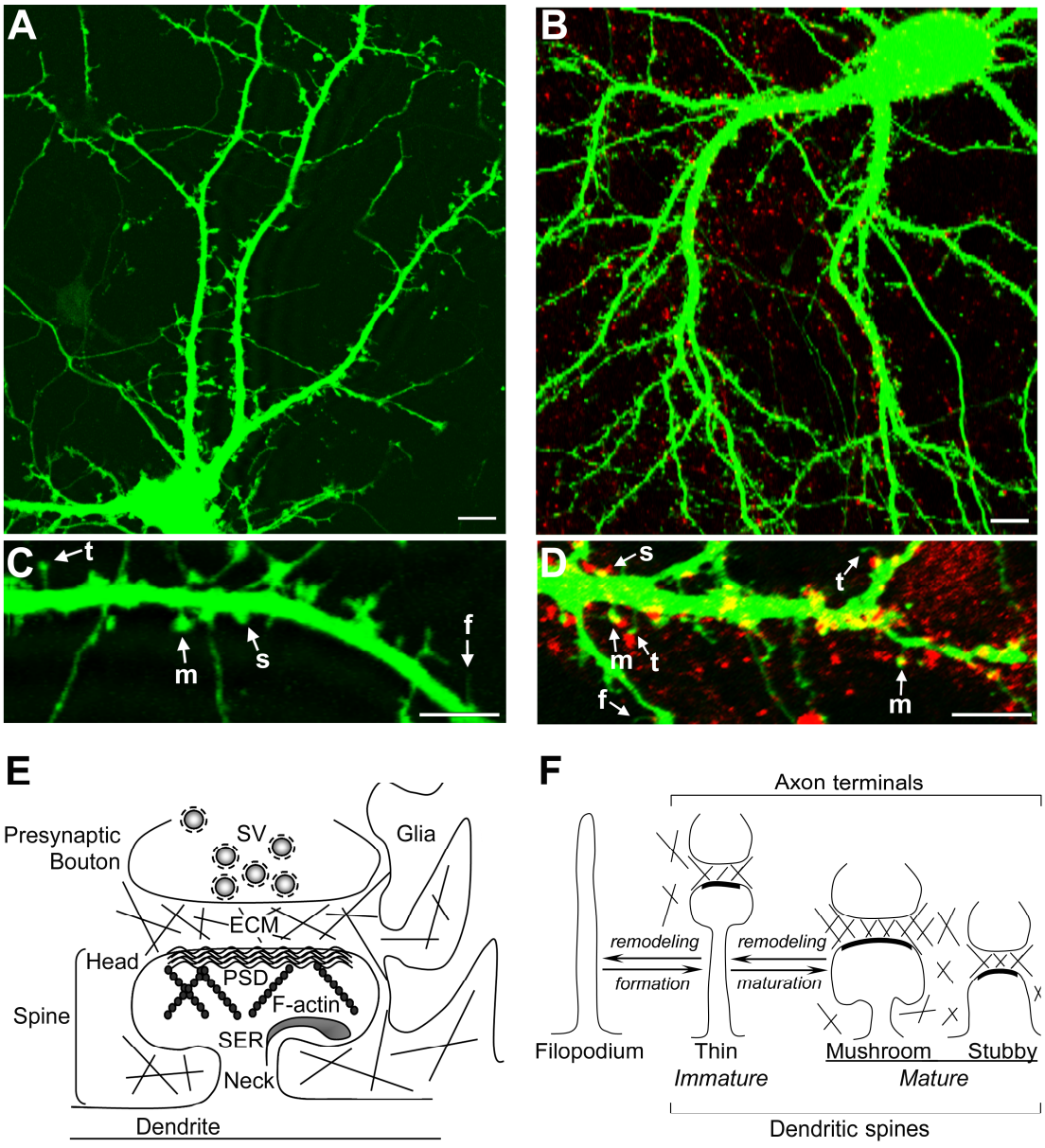
Figure 1.1 Dendritic Spine Ultrastructure and Morphology. (A) GFP-expressing hippocampal neuron from 14DIV hippocampal cultures. (B) GFP-expressing hippocampal neuron (green) with presynaptic boutons labeled by immunostaining against synaptophysin (red). (C) High magnification view of the dendrite shows the typical thin (t), mushroom (m) and stubby (s) spines and dendritic filopodia (f). (D) High magnification view of the dendrite shows GFP-positive spines (green) with presynaptic synaptophysin-positive boutons (red). (E) Schematic illustration showing ultrastructure of dendritic spine with adjacent presynaptic terminal and surrounding glial processes. (F) Schematic illustration of a filopodium and main categories of dendritic spines: thin, mushroom and stubby spines. ECM, extracellular matrix; F-actin, filamentous actin; PSD, postsynaptic density; SER, smooth endoplasmic reticulum; SV, synaptic vesicles. Scale bar in (A) and (C), 10μm; scale bar in (B) and (D), 5μm.

Figure 1.1


A
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Head
PSD
F-actin
Spine
SER
Neck
Dendrite
F
Axon terminals
remodeling
formation
remodeling
maturation
Filopodium
Thin
Immature
Mushroom
Stubby
Mature
Dendritic spines

Figure 1.2 Pathways implicated in FXS dendritic spine development. (A) In the normal dendritic spine, Fmrp regulates translation of synaptic proteins, such as MMP-9, which helps to maintain mature synaptic connections. (B) In the FXS dendritic spine, when Fmrp is absent translation of many synaptic proteins, such as MMP-9, is no longer regulated which can promote an increase in their translation and promote formation of an immature dendritic spine profile. (C) Illustration of the two primary pathways thought to be involved in dendritic spine remodeling downstream of mGluR5 signaling, which is hypersensitive in the *Fmr1 KO*. Highlighted in red are specific proteins (Akt, ERK, GSK3, MMP-9) that have been shown to have alterations in their expression levels within the *Fmr1 KO.* DAG, diacylglycerol; ERK, extracellular-signal regulated kinase; Fmrp, Fragile X mental retardation protein; GSK, glycogen synthase kinase; IP3, inositol triphosphate; MEK, MAPK/extracellular protein kinase kinase; MMP, matrix metalloproteinase; mTOR, mammalian target of rapamycin; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinosityl 3,4,5-triphosphate; PLC, phospholipase C.

Figure 1.2

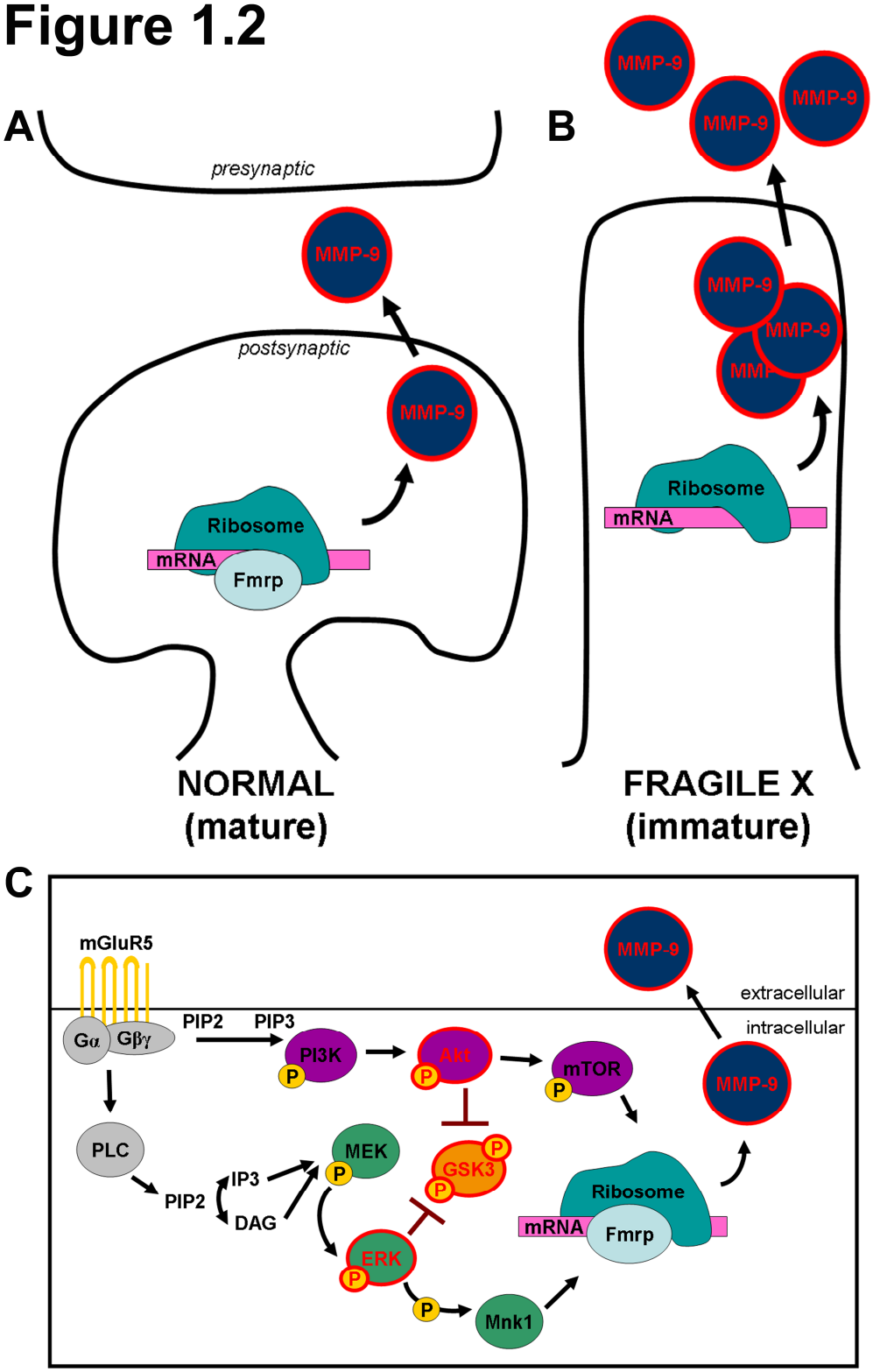

A
presynaptic
MMP-9
postsynaptic
MMP-9
Ribosome
mRNA
Fmrp
NORMAL
(mature)
B
MMP-9
MMP-9
MMP-9
MMP-9
MMP-9
MMP
Ribosome
mRNA
FRAGILE X
(immature)
C
mGluR5
Gα
Gβγ
PIP2
PIP3
PI3K
P
Akt
P
mTOR
P
MMP-9
MMP-9
extracellular
intracellular
PLC
PIP2
IP3
DAG
MEK
P
GSK3
P
P
Ribosome
mRNA
Fmrp
ERK
P
P
Mnk1

Figure 1.3 MMP structure and classification. The transmembrane and GPI linked MMPs are commonly referred to as membrane-type MMPs (MT-MMPs). Alternate names for MMPs 14, 15, 16, 17, 24, and 25 are MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP, MT5-MMP and MT6-MMP, respectively. C, cytoplasmic tail; cat, catalytic domain; cat site, catalytic site; Fn, fibronectin type II domain; furin, furin-susceptible cleavage site; GPI, glycosyl-phosphatidylinositol linker; hemo, hemopexin-like domain; hinge, hinge region; pro, prodomain; TM, transmembrane domain; Zn^{2+}, zinc in the catalytic site. MMP-21 and MMP-23 are not included in the list.

Figure 1.3

Minimal
MMP-7,26

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

Gelatinase
MMP-2,9

Furin-activated
MMP-11,28

Transmembrane
MMP-14,15,16,24

GPI-linked
MMP-17,25

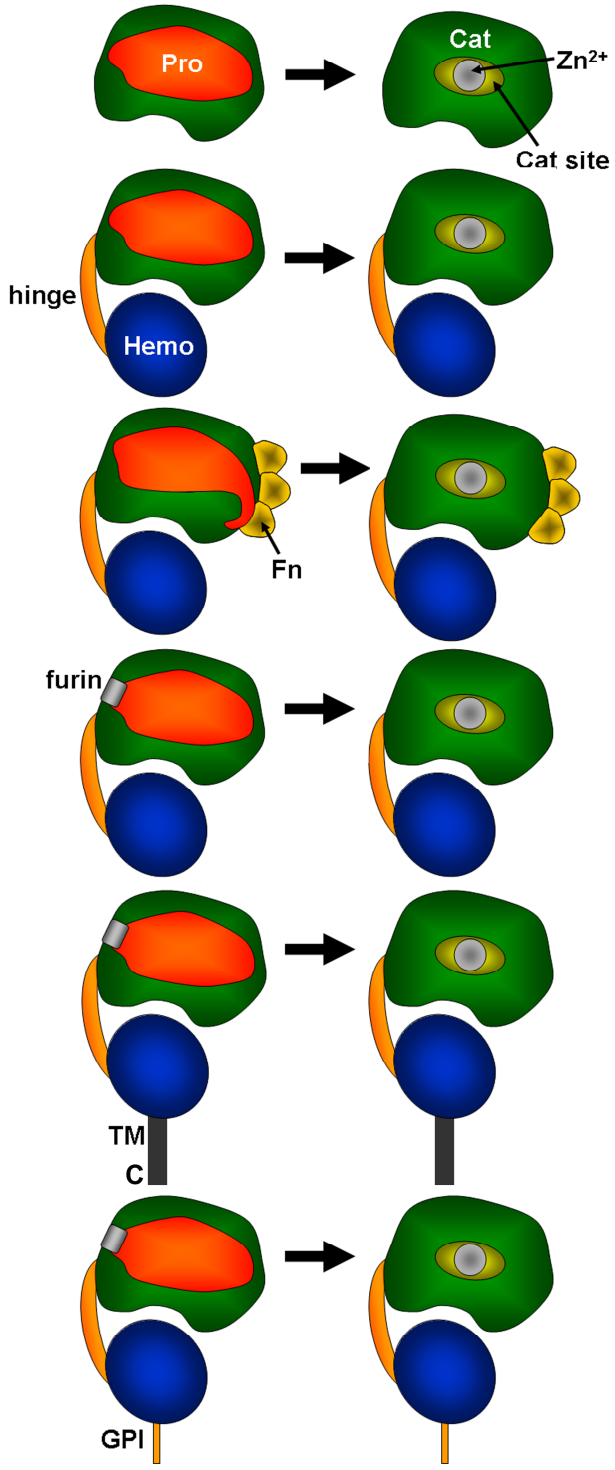
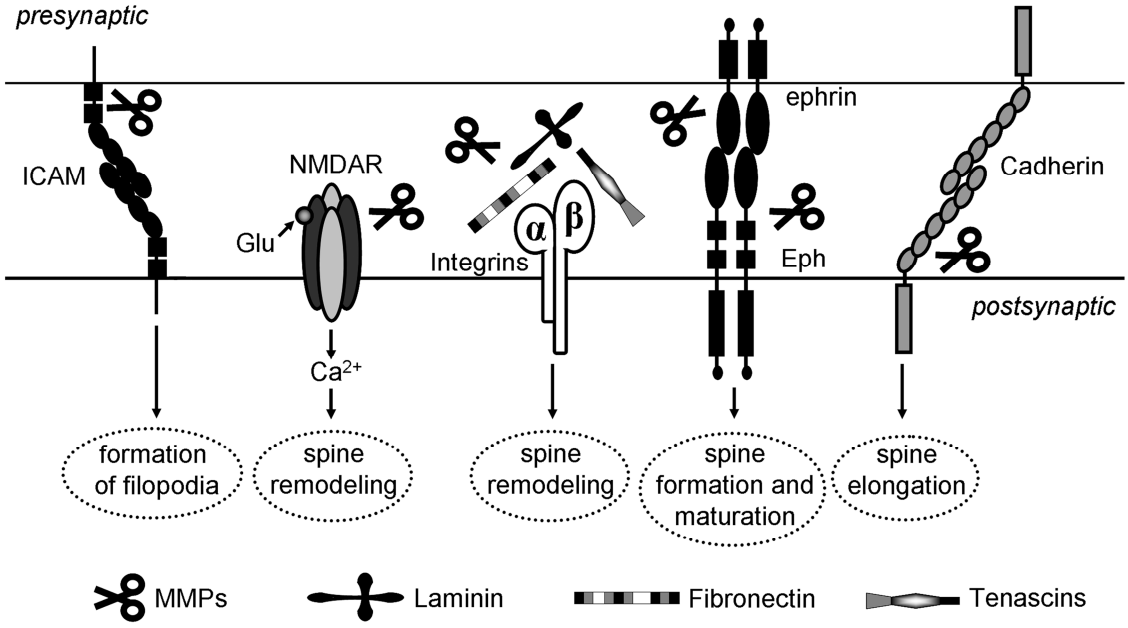


Figure 1.4 MMP cleavage of ECM substrates and cell surface receptors regulates dendritic spines.

Figure 1.4


presynaptic
ICAM
NMDAR
Glu
Ca2+
Integrins
α
β
ephrin
Eph
Cadherin
postsynaptic
formation of filopodia
spine remodeling
spine remodeling
spine formation and maturation
spine elongation
MMPs
Laminin
Fibronectin
Tenascins

Chapter 2 – Matrix Metalloproteinase-9 Contributes to the Abnormal Dendritic Spine Development in the Fragile X Mouse Model

ABSTRACT

Fragile X syndrome (FXS) is the most common single-gene inherited form of intellectual disability, with behaviors characteristic of autism. FXS subjects and Fragile X mental retardation gene knock out (*Fmr1 KO*) mice, an animal model for FXS, have been shown to exhibit defects in dendritic spine maturation that may underlie cognitive and behavioral deficits associated with FXS. Dendritic spines are small protrusions from the surface of dendrites that are responsible for the majority of excitatory transmissions and changes in their morphology can affect their synaptic efficacy. Many extracellular factors can affect the remodeling of dendritic spines. Here we focus on how the secreted, extracellular protein, MMP-9, can affect dendritic spine remodeling in cultured hippocampal neurons as well as contribute to the immature dendritic spine profile in the *Fmr1 KO*. We report that MMP-9 is upregulated in the hippocampus of the *Fmr1 KO* and promotes dendritic spine elongation in WT hippocampal culture similar to cultured *Fmr1 KO* neurons. Specific inhibition of the gelatinases promotes dendritic spine maturation in the *Fmr1 KO*, and cultured *mmp9 KO* hippocampal neurons demonstrates an increase in the size and proportion of mature dendritic spines. Furthermore, the mechanism of MMP-9 promoted spine remodeling may

be mediated through activation of the focal adhesion complex via integrin signaling.

INTRODUCTION

Matrix metalloproteinases (MMPs) are the primary remodelers of the extracellular matrix (ECM) and have been implicated in a variety of neurological conditions. Previous work by Bilousova and colleagues (2006) was the first to demonstrate a direct role for MMPs in affecting the morphology of dendritic spines (Bilousova et al., 2006) which are the primary post-synaptic components of excitatory connections within the brain. Specifically, MMP-7 was shown to promote transformation of mature, mushroom-shaped spines into long, thin filopodia, reminiscent of an immature phenotype. However, MMP-7 is not a common component of the healthy CNS, as it is only found within the CNS at sites of tissue repair and remodeling following pathological disturbances. There have been reports of MMP-7 expression in macrophages from atherosclerotic plaques (Furman et al., 2004), as well as in brain and spinal cord extracts obtained from experimental autoimmune encephalomyelitis-induced mice (Anthony et al., 1997; Clements et al., 1997; Cossins et al., 1997; Hartung and Kieseier, 2000). Based on the effects of MMP-7 and the fact that it is not an intrinsic component of the CNS, we were curious whether other MMPs may play a similar role in dendritic spine remodeling within the normal, healthy CNS. Within the mouse hippocampus it has been demonstrated that both MMP-3 and MMP-9 were increased following successful learning in the Morris water maze,

which was impaired in the presence of MMP inhibitors (Meighan et al., 2006). Furthermore MMP-9, but not its closely related gelatinase family member MMP-2, was shown to be highly expressed in neurons within the adult rat brain, including the hippocampus (Szklarczyk et al., 2002). This information led us to analyze whether MMP-9 had effects on dendritic spine development within the hippocampus and to determine if it may play a causative role in promoting dendritic spine pathologies associated with neurodevelopmental disorders, such as Fragile X Syndrome (FXS). FXS is the most common, single-gene inherited form of intellectual disability with behaviors characteristic of autism (Musumeci et al., 1999; Hagerman and Hagerman, 2002). Unlike other disorders involving cognitive impairment, FXS is also characterized by many physical manifestations, such as large ears, long faces, hyperextensible joints and extremely soft skin, all of which are extensions of modified extra-cellular matrix (ECM) regulation (Hagerman and Hagerman, 2002). It is possible that both the cognitive and physical abnormalities could be attributed to altered ECM remodeling, making this model a particularly likely candidate for the involvement of MMPs.

Since MMPs are secreted into the ECS, it would follow that they would induce intracellular changes, such as those that lead to dendritic spine remodeling, through activation of a receptor signaling pathway. MMPs are known to modify components of the ECM, including laminin and fibronectin, which contain an Arg-Gly-Asp (RGD) motif that can activate integrins (reviewed

in Hynes, 1987; Ruoslahti and Pierschbacher, 1987). Integrins comprise a family of cell surface proteins that are found across many cell types and mediate various cell processes, including cell proliferation, survival, differentiation and migration (reviewed in Ruoslahti, 1991; Giancotti and Ruoslahti, 1999; Hynes, 2002; Milner and Campbell, 2002; Miranti and Brugge, 2002; Parsons, 2003; Ridley et al., 2003; Rolli et al., 2003; Arnaout et al., 2005). Integrins are composed of non-covalently linked α and β subunits, and each αβ pair has its own binding and signaling specificities. Integrins signal through their associations with intracellular signaling proteins, such as focal adhesion kinase (FAK), the non-receptor tyrosine kinase Src, and scaffolding proteins such as Grb2 and paxillin, to mediate their effects on the actin cytoskeleton. Integrins have been implicated in brain development and synaptogenesis (reviewed in Milner and Campbell, 2002), potentiation of N-methyl D-aspartate receptor (NMDAR)-dependent LTP (Chun et al., 2001; Chan et al., 2003; Kramar et al., 2003; Lin et al., 2003) as well as dendritic spine remodeling (Shi and Ethell, 2006). Activation of integrins with RGD-containing peptide was shown to induce the transformation of mature mushroom-shaped spines with large heads into immature thin spines and filopodia in primary hippocampal cultures (Shi and Ethell, 2006). These effects were blocked by function-blocking antibodies against β1 and β3 integrin subunits and by the NMDAR antagonist MK801, suggesting the involvement of NMDARs in integrin induced dendritic spine remodeling. This similarity between the effects of MMPs and integrins on synapses and dendritic

spines make integrins a strong candidate for mediating MMP signaling in dendritic spines.

MATERIALS AND METHODS

Mice

The FVB.Cg-*Mmp9*^{tm1Tvu}/J (*mmp9 KO*) and FVB.129P2-*Fmr1*^{tm1Cgr}/J (*Fmr1 KO*) and FVB.129P2-Pde6b^{+}Tyr$^{c\text{-}ch}$/AntJ controls (WT) were obtained from the Jackson Laboratories. Mice were maintained in an AAALAC accredited facility under 12 hour light/dark cycles. All mouse studies were done within NIH and Institutional Animal Care and Use Committee guidelines.

Hippocampal neuron cultures

Briefly, hippocampal cells were treated with papain (0.5 mg/ml) and DNase (0.6 µg/ml) for 20 min at 37ºC, mechanically dissociated, and then plated on glass coverslips or plastic dishes 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_2/10% O_2 atmosphere at 37ºC. 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).

Treatments *in vitro*

When indicated, hippocampal cultures were treated with 500nM SB-3CT (444285, Calbiochem) overnight. The treatments were performed in conditioned medium under 5% CO_2/10% O_2 atmosphere at 37ºC.

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 synaptophysin using mouse anti-synaptophysin antibody (61 µg/ml; SVP-38; Sigma). The secondary antibodies used were Alexa Fluor 594-conjugated chicken anti-mouse 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 (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 protrusions were grouped into three categories according to their morphology: 1) filopodia-like (long, thin protrusions without head or 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 spines were identified as dendritic protrusions connected with a synaptophysin-positive pre-synaptic terminal. Each experiment was performed at least three times. Statistical analysis was performed using Student's *t*-test.

APMA-mediated activation of MMP-9

Recombinant mouse MMP-9 (CC069, Chemicon-Millipore; 909-MM-010, R&D Systems) was activated with 1 mM APMA (A9563, Sigma) in 50mM Tris-HCl (pH 7.5) containing 150mM NaCl, 5mM $CaCl_2$, 2.5mM $ZnCl_2$, and 0.05% Brij-35 Detergent (activation buffer) at 37ºC for 2 h. Activated MMP-9 was used to treat hippocampal cultures at a concentration of 100ng/ml for 1hr under 5% CO_2/10% O_2 atmosphere at 37ºC.

Immunoprecipitation

14 DIV treated and untreated hippocampal neurons were lysed in ice-cold TBS (25 mM Tris, pH 7.4; 0.15 mM NaCl) containing 1% Triton X-100, 5 mM

EDTA, 0.5mM sodium pervanadate, and protease inhibitor mixture (P8340; Sigma). Cell lysates were cleared by centrifugation at 12,000xg. Supernatant was incubated with 2μL of anti-FAK antibody (06-543; Millipore) and protein A-Agarose beads (P1406; Sigma) for 4 h at 4°C. Bound materials were eluted with SDS-PAGE sample buffer and analyzed via western immunoblotting.

Western immunoblotting

Brain tissues or cultured hippocampal neurons were lysed in ice-cold TBS (25 mM Tris (pH=7.4), 150 mM NaCl) containing 1% Triton X-100, 5 mM EDTA, protease inhibitor cocktail (Sigma), 0.5 mM sodium pervanadate, and cleared by centrifugation at 12,000xg. Proteins were resolved on 8-16% Tris-glycine SDS-PAGE gels (Invitrogen) and immunoblotted with primary antibody against MMP-9 (Chemicon, AB19016), pFAK (Invitrogen, 44-624G), FAK (Millipore, 06-543), or Integrin β3 (Millipore, AB1932), followed by incubation with corresponding secondary HRP-conjugated antibody (Jackson ImmunoResearch) and ECL detection (GE Healthcare, RPN-2132).

Gelatin zymography

Gelatin gel zymography was performed as previously described (Zhang and Gottschall, 1997; Manabe et al., 2005) with minor modifications. Briefly, brain tissues were resuspended in 50 mM Tris-HCl (pH=7.6) buffer containing 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij35, 0.02% Na_3N, 1% Triton X-100, 100 μM PMSF

and PI cocktail (Sigma). The gelatinases, MMP-2 and MMP-9, were pulled down with gelatin agarose (Sigma, G5384) and separated on non-reducing SDS-PAGE gels containing 0.1% gelatin (Invitrogen). After separation, gels were soaked in renaturing buffer (LC2670, Invitrogen) to remove SDS and allow MMPs to refold, regaining most of their enzymatic activity. Following renaturation, gels were incubated in developing buffer (LC2671, Invitrogen) for 3 days, which allowed gelatinases (MMP-2 and MMP-9) in the gel to degrade the gelatin. Gels then were stained with coomassie blue, which uniformly stained the gels due to the gelatin within the gel. Areas of MMP activity were revealed as unstained bands where gelatin was degraded.

RESULTS

Enhanced MMP-9 activity promotes immature dendritic spine morphology in cultured hippocampal neurons. We previously reported that treatment of cultured hippocampal neurons with an active form of MMP-7 induces a dramatic redistribution of F-actin and alters dendritic spine morphology, transforming dendritic spines from mushroom-like into filopodia-like protrusions (Bilousova et al., 2006). Although MMP-7 is not normally expressed in hippocampal neurons, it can process neuronally expressed pro-MMP-9, which is produced in the developing hippocampus, into the fully active form. Subsequently we found that 1hr of treatment with active MMP-9 also induced immature dendritic spine

morphology in 15 DIV hippocampal neuron cultures (Fig. 2.1 A), although the effects were less profound than with MMP-7. While there was not a significant difference in overall protrusion length between untreated and MMP-9 treated cultures (Fig. 2.1 B), there were changes in the distribution of protrusion lengths with fewer short dendritic spines (< 2.0 μm) with large heads (0.5-1.0 μm) and more long dendritic spines (2.0-4.0 μm) with small heads (<0.5 μm) in MMP-9 treated cultures as compared to controls (Fig. 2.1 C,E). MMP-9 treatment also significantly reduced the average size of dendritic spine heads (Fig. 2.1 D). Further analysis revealed that MMP-9 treated neurons exhibited more filopodia and less mushroom-shaped dendritic spines (Fig. 2.1 F). Interestingly, the morphology of immature dendritic spines induced by MMP-9 activity was very similar to the dendritic spines that we observed in *Fmr1 KO* hippocampal neurons. We found that 15DIV hippocampal neurons from *Fmr1 KO* mice exhibit immature dendritic spine profiles with longer spines (Fig. 2.2 A-C), a lower density of mushroom-shaped spines with large heads (Fig. 2.2 D) and excessive filopodia (Fig. 2.2 E) as compared to wild type (WT) hippocampal neurons. This data demonstrates that there is promotion of an immature dendritic spine morphology when excess MMP-9 is present and this profile is similar to what is commonly observed for the *Fmr1 KO*. We next wanted to determine if excessive MMP activity may be contributing to the dendritic spine pathology associated with the *Fmr1 KO*.

MMP-9 expression and activity are up-regulated in the hippocampus of *Fmr1 KO* mice. To determine if enhanced MMP-9 activity contributes to the immature spine profile observed in *Fmr1 KO* neurons, we first evaluated the expression levels of total MMP-9 and its active form by Western blotting and gelatin zymography in P7 hippocampi of *Fmr1 KO* mice. Western blot analysis revealed an upregulation of the active form of MMP-9 within hippocampal lysates of P7 *Fmr1 KO* mice as compared to WT (Fig. 2.3 A,B). Gelatin zymography detected both MMP-9 and MMP-2 activity in P7 hippocampal lysates of WT and *Fmr1 KO* mice (Fig. 2.3 C-E), with a significant upregulation of only MMP-9 levels in the hippocampus of *Fmr1 KO* mice (Fig. 2.3 D). Based on the upregulation of the active form of MMP-9 and the increased activity of MMP-9 within the hippocampus of the *Fmr1 KO* it is likely that MMP-9 is contributing to the immature dendritic spine morphology of the *Fmr1 KO.*

MMP-9 inhibition promotes mature dendritic spines in *Fmr1 KO* neurons. To further characterize the role of MMP-9 in dendritic spine development, as well as how its enhanced activity may be contributing to the dendritic spine abnormalities that are found in *Fmr1 KO* mice, we analyzed how specific inhibition of the gelatinases, MMP-2 and MMP-9, with the MMP-inhibitor SB-3CT affected dendritic spines in hippocampal cultures. 14DIV WT hippocampal cultures treated with SB-3CT revealed no changes in dendritic spine morphology as compared to untreated WT hippocampal neurons. Both treated and untreated

WT neurons showed similar dendritic spine head to length ratio (Fig. 2.4 A,B), as well as proportions of filopodia (Fig. 2.4 C), thin, mushroom and stubby spines (Fig. 2.4 D). Specific inhibition of the gelatinases within the *Fmr1 KO*, however, did promote significant changes. There was an increase in the ratio of mature spines, as demonstrated by a significant increase in the head to length ratio (Fig. 2.4 A,B) and the percent of mushroom spines (Fig. 2.4 D), as well as a significant decrease in the proportion of filopodia (Fig. 2.4 C). These results demonstrate that inhibition of MMP-9 selectively promotes dendritic spine maturation within the *Fmr1 KO* without affecting dendritic spine morphology of WT neurons.

Dendritic spine development is altered in *mmp9 KO* hippocampal neurons.

After analyzing how pharmacological inhibition of MMP-9 affected dendritic spine development we examined dendritic spine morphology when the gene encoding MMP-9 has been removed by comparing 14DIV hippocampal cultures between WT and the *mmp9 KO* (Fig. 2.5 A). In the absence of MMP-9, both dendritic spine maturation and the proportion of mature mushroom spines increase (Fig. 2.5 B and D, respectively) while the proportion of filopodia decreases (Fig. 2.5 C). The results shown here clearly demonstrate an instrumental role for MMP-9 in regulating dendritic spine formation. In the presence of excessive MMP-9 activity there is promotion of an immature dendritic spine profile while absence of MMP-9 increases dendritic spine maturation. This work indicates that MMP-9 is critical for maintaining normal dendritic spine development. However, when this

regulation is altered increased MMP-9 activity appears to contribute to the dendritic spine abnormalities associated with FXS.

MMP-9 alters FAK activity and its association with integrin. Since MMP-9 is a secreted, extracellularly localized protein, there must be a signaling mechanism by which it can mediate its effects on dendritic spine morphology. One primary candidate would be the integrin-family of ECM receptors. MMPs, by definition, are instrumental in cleaving components of the ECM and integrins are characterized as the primary receptors for the ECM (reviewed in Hynes, 1987; Ruoslahti and Pierschbacher, 1987). In addition, several studies have suggested that effects of MMP-9 can be mediated through the activation of integrin signaling (Nagy et al., 2006; Wang et al., 2008). In this study, 14 DIV hippocampal cell cultures were treated with active MMP-9 for 5, 15 or 30 minutes. Cell lysates were prepared from these cultures and immunoprecipitated against FAK. Western blotting revealed a transient increase in the levels of the activated, phosphorylated form of FAK (Fig. 2.6 A,B) and a significant increase in the association of FAK with integrin β3 subunit after 15 minutes of MMP-9 treatment (Fig. 2.6 A,C). These results demonstrate that MMP-9 cleavage of the ECM initiates an integrin signaling cascade that leads to recruitment and activation of FAK, which can promote dendritic spine remodeling (Fig. 2.6 D).

DISCUSSION

This study reveals that enhanced MMP-9 activity can affect dendritic spine morphology specifically by promoting formation of immature, filopodia-like protrusions. Treatment of cultured hippocampal neurons with activated MMP-9 promoted immature dendritic spine morphology, while pharmacological inhibition or genetic ablation of MMP-9 increased dendritic spine maturation. Based on these results, it would appear that tightly regulated MMP-9 activity is instrumental in maintaining normal dendritic spine plasticity which is a crucial mechanism underlying the basis of learning and memory. Several groups have demonstrated a role for MMPs in affecting synaptic plasticity, such as the induction, stabilization or maintenance of LTP and LTD. Broad spectrum MMP inhibition in acute hippocampal slices was shown to impair LTD, as well as induction and stabilization of LTP (Meighan et al., 2007), while MMP-9 has been shown to enhance LTP through integrin-dependent regulation of NMDAR activity (Nagy et al., 2006; Michaluk et al., 2009). Therefore it is not surprising that altering the level of MMP-9 activity could have detrimental consequences and thus may contribute to the aberrant morphological and behavioral abnormalities associated with some cognitive disorders. Changes in MMP-9 levels and activity have been linked to dendritic spine pruning associated with epilepsies (Wilczynski et al., 2008), and MMP-9 activity was increased in patients with mild cognitive impairment and Alzheimer's disease (Bruno et al., 2009). In addition to these studies, our findings indicate that increased MMP-9 expression and activity

may contribute to the abnormal dendritic spine profile associated with Fragile X Syndrome (FXS).

FXS is the most common single-gene inherited form of intellectual impairment and is characterized by an immature dendritic spine profile (Rudelli et al., 1985; Irwin et al., 2000; Kaufmann and Moser, 2000; Fiala et al., 2002). Similar to the human condition, other reports have demonstrated that hippocampal neurons from the FXS mouse model (*Fmr1 KO*) display a higher proportion of long, thin immature dendritic protrusions as compared to controls (Antar et al., 2006). Within the hippocampus of the *Fmr1 KO* we discovered upregulation of MMP-9 activity and also determined that specific inhibition of the gelatinases, MMP-9 and MMP-2, in *Fmr1 KO* cultured hippocampal neurons reversed the immature dendritic spine profile and promoted formation of mature dendritic spines without affecting changes to WT hippocampal neurons. Following this finding it would be very interesting to test in the *Fmr1 KO* the effects that specific MMP inhibitors may have on reversing behavior abnormalities that are associated with FXS and also typical of the mouse model. Unfortunately MMP specific inhibitors lack the ability to permeate the blood-brain barrier making administration cumbersome. However, it is likely that another drug, called minocycline, which also has MMP-9 inhibiting capabilities and can easily cross the blood-brain barrier may provide significant benefits. The use of minocycline in the treatment of the *Fmr1 KO* is analyzed in the next two chapters. In addition to the potential therapeutic benefits that MMP-inhibition may have for

treating FXS, it is also important to determine which mechanisms underlie the MMP-induced effects on dendritic spine remodeling.

Integrins are the primary receptors for the ECM and have been implicated in dendritic spine remodeling (Ruoslahti, 1991), making them likely candidates for promoting MMP-mediated effects on dendritic spines. Recent work by Michaluk and colleagues has demonstrated a connection between MMP-9-induced dendritic spine elongation and integrin signaling (Michaluk et al., 2011). Over-expression of MMP-9 in a transgenic rat model as well as bath application of a recombinant auto-activating MMP-9 mutant in hippocampal cultures promoted an increase in the proportion of long, thin dendritic spines (Michaluk et al., 2011). Pre-incubation of dissociated hippocampal culture with anti-integrin β1 antibody blocked the effect of MMP-9 on dendritic spine elongation. Our analysis has also demonstrated a connection by revealing that addition of activated MMP-9 in culture promoted a transient increase in the levels of the active phosphorylated form of FAK which corresponded with an increase in its association with the integrin β3 subunit.

Activation of FAK leads to actin reorganization which underlies both dendritic spine elongation as well as maturation. The transient activation of FAK that we see here is typical of integrin β3 signaling, but FAK is also activated by EphB receptors (Shi et al., 2009). EphB2 receptor activation of FAK leads to long-lasting dissociation of FAK from integrin β3 as well as promotes dendritic spine maturation (Shi et al., 2009). This suggests competitive inhibition between

EphB receptors and integrins whereby transient FAK activation due to integrins stimulates spine elongation while sustained activation via EphB receptors promotes maturation. Another interesting connection between these competitive pathways is the fact that MMPs can affect EphB receptor signaling in addition to integrins. Eph receptors are a family of receptor tyrosine kinases that, along with their ephrin-ligands, are implicated in dendritic spine formation and maintenance (Lin et al., 2008; Inoue et al., 2009). In connection to MMPs, Lin and colleagues (2008) demonstrated that MMP-9 cleavage of the EphB2 receptor enhanced EphB2 signaling and EphB2-mediated growth cone collapse (Lin et al., 2008). Whether this effect of MMP-9 on EphB2 signaling contributes to normal or pathologic dendritic spine remodeling however, remains to be determined.

In summary, the work we have presented here indicates that enhanced MMP-9 activity promotes formation of immature dendritic protrusions within the *Fmr1 KO* and suggests that MMP-9 may act through integrins to promote FAK-mediated remodeling of the actin cytoskeleton. MMP-9 is an instrumental component in the processes underlying normal synaptic plasticity as well as dendritic spine development and alterations of its activity levels may contribute to abnormal dendritic spine plasticity associated with neurological conditions such as FXS and possibly other forms of cognitive disabilities.

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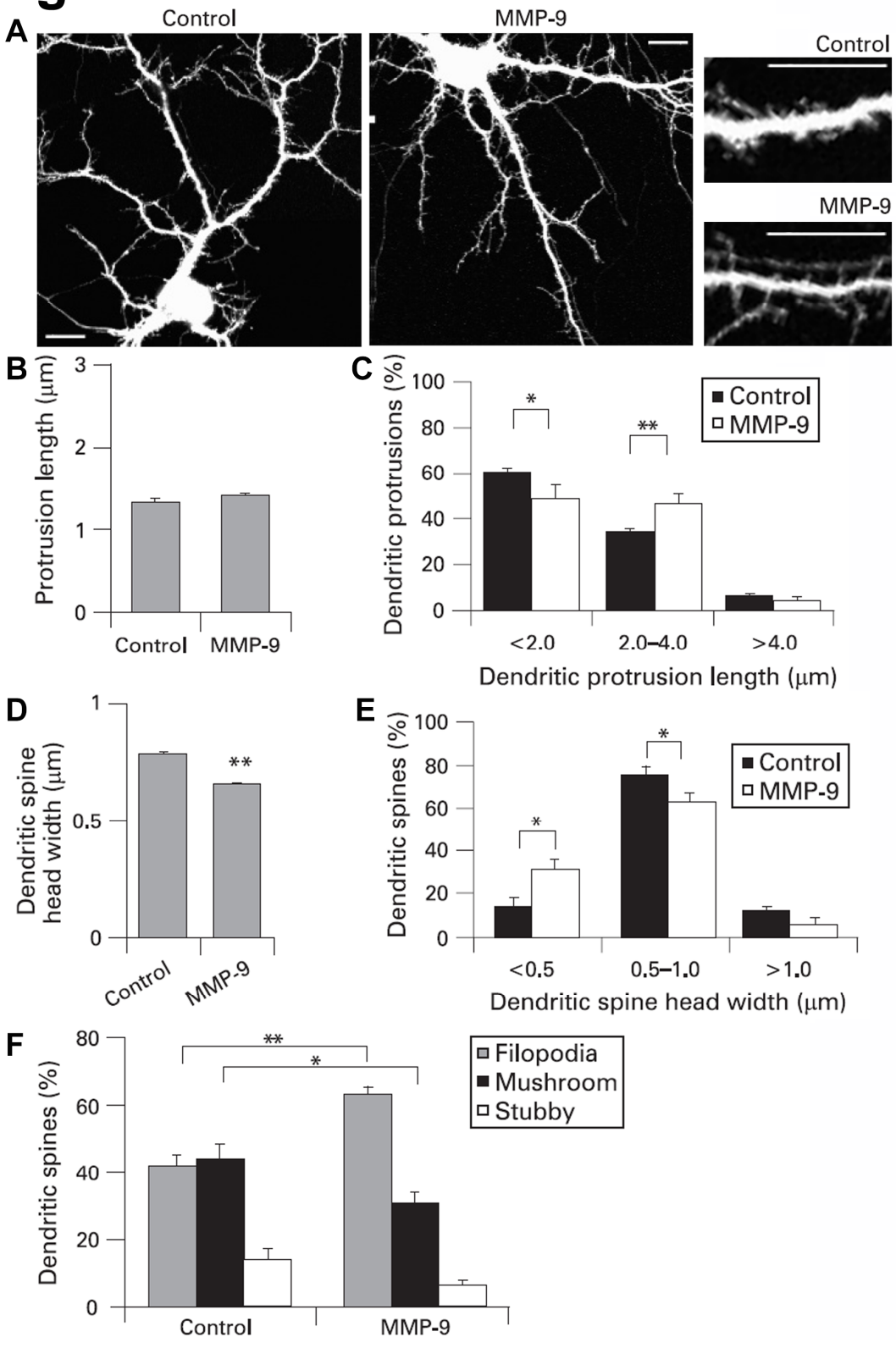
Figure 2.1 MMP-9 induced immature dendritic spine profiles in cultured hippocampal neurons. (A) Confocal images of 14 DIV hippocampal neurons transfected with pEGFP in control untreated or MMP-9 treated cultures. Dendritic morphology was observed with GFP fluorescence. Scale bars, 10 mm. (B, C) Quantification of average dendritic protrusion length (B) and size distribution for dendritic protrusion lengths <2.0 mm, 2.0–4.0 mm and >4.0 mm (C) in control and MMP-9 treated neurons. MMP-9 treated neurons exhibited a higher proportion of long dendritic protrusions (2.0–4.0 mm) than control neurons. Error bars indicate SEM (n=300–500 spines from five neurons per group; *, $p<0.05$; **, $p<0.01$). (D, E) Quantification of average dendritic spine head diameter (D) and size distribution for dendritic spine heads <0.5 mm, 0.5–1.0 mm and >1.0 mm (E) in control and MMP-9 treated neurons. MMP-9 treatment reduced the average size of dendritic spine heads and decreased the proportion of dendritic spines with larger heads. Error bars indicate SEM (n=5 neurons per group; *, $p<0.05$; **, $p<0.01$). (F) Quantitative analysis of the dendritic spine morphology in untreated or MMP-9 treated hippocampal neurons. MMP-9 treated neurons exhibited more filopodia and fewer mushroom shaped dendritic spines. Error bars indicate SEM (n=5 neurons per group; *, $p<0.05$; **, $p<0.01$). The experiments were repeated three times with similar results.

Figure 2.1


A
Control
MMP-9
Control
MMP-9
B
Protrusion length (μm)
3
2
1
0
Control
MMP-9
C
Dendritic protrusions (%)
100
80
60
40
20
0
*
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Control
MMP-9
<2.0
2.0–4.0
>4.0
Dendritic protrusion length (μm)
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Dendritic spine head width (μm)
1
0.5
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Dendritic spines (%)
100
80
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Control
MMP-9
<0.5
0.5–1.0
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Dendritic spine head width (μm)
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Dendritic spines (%)
80
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20
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Filopodia
Mushroom
Stubby
Control
MMP-9

Figure 2.2 Cultured *Fmr1 KO* hippocampal neurons have a characteristically immature dendritic spine phenotype. Confocal images of GFP expressing WT (A) or *Fmr1 KO* (B) hippocampal neurons at 15 DIV. Dendritic morphology was observed with GFP fluorescence (green) and presynaptic boutons were labeled by immunostaining with synaptophysin (red). Scale bars, 10 mm. Quantitative analysis of dendritic spine length (C), size distribution for dendritic spine heads <0.5 μm, 0.5–1.0 μm and >1.0 μm (D) and morphology (E) in 15 DIV hippocampal neurons from WT control and *Fmr1 KO* mice. Error bars indicate SEM (n = 500 dendritic spines from eight neurons per group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). The experiments were repeated three times with similar results.

Figure 2.2

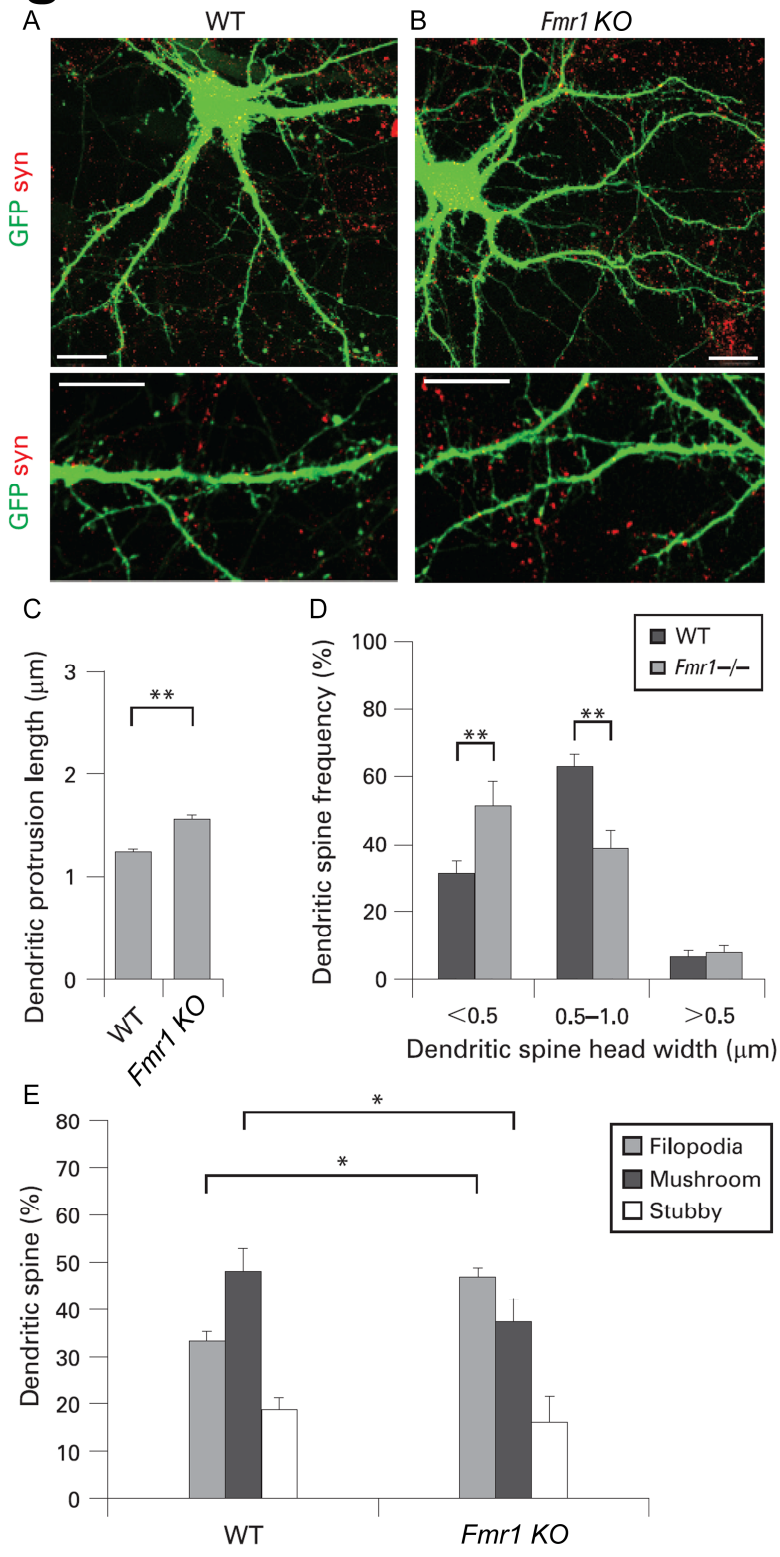

A
WT
B
Fmr1 KO
GFP syn
GFP syn
C
Dendritic protrusion length (μm)
3
2
1
0
**
WT
Fmr1 KO
D
Dendritic spine frequency (%)
100
80
60
40
20
0
WT
Fmr1–/–
**
**
<0.5
0.5–1.0
>0.5
Dendritic spine head width (μm)
E
Dendritic spine (%)
80
70
60
50
40
30
20
10
0
*
*
Filopodia
Mushroom
Stubby
WT
Fmr1 KO

FIGURE 2.3 MMP-9 gelatinase activity was upregulated in the hippocampus of P7 *Fmr1 KO* mice. (A) Detection of pro- and active forms of MMP-9 in hippocampal lysates from P7 brains of *Fmr1 KO* and WT mice by immunoblotting. The levels of pro- and active forms of MMP-9 were quantified by densitometry and normalized to total GAPDH values. The experiments were repeated at least three times and this blot is representative of the findings. (B) Bar graph values indicate the average MMP-9 values and error bars indicate SEM (n=3 male mice per group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). (C) Detection of gelatinase concentrations in hippocampal lysates from P7 brains of *Fmr1 KO* and WT mice by gelatin pull-down followed by gelatin zymography. The gelatin cleaving activity (gelatinase activity) of the gelatinases MMP-9 and MMP-2 in hippocampal cell lysates were quantified by densitometry. (D-E) Bar graph values indicate the average MMP-9 (D) and MMP-2 (E) values, and error bars indicate SEM (n=3 male mice per group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Figure 2.3

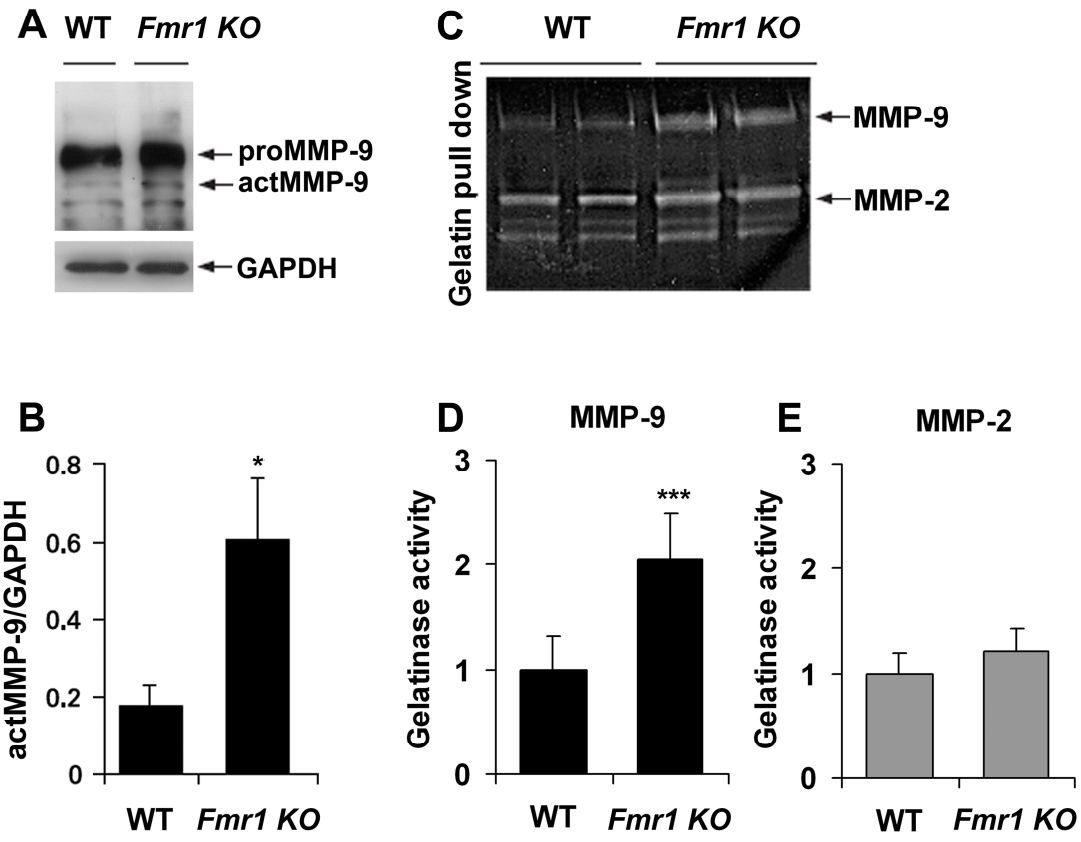

A
WT
Fmr1 KO
proMMP-9
actMMP-9
GAPDH
C
WT
Fmr1 KO
Gelatin pull down
MMP-9
MMP-2
B
actMMP-9/GAPDH
0.8
0.6
0.4
0.2
0
*
WT
Fmr1 KO
D
MMP-9
Gelatinase activity
3
2
1
0
***
WT
Fmr1 KO
E
MMP-2
Gelatinase activity
3
2
1
0
WT
Fmr1 KO

Figure 2.4 Specific inhibition of MMP-9 promoted dendritic spine maturation in *Fmr1 KO* but not WT hippocampal cultures. (A) 14DIV WT and *Fmr1 KO* hippocampal neuronal cultures were untreated or treated overnight with 500nM SB-3CT. Dendritic morphology was observed with GFP fluorescence. Images of untreated and treated WT and *Fmr1 KO* hippocampal neurons. (B-D) Quantification of head area/spine length ratio (B), percentage of filopodia (C), and percentage of thin, mushroom and stubby spines (D) in untreated or treated WT or *Fmr1 KO* neurons. Error bars indicate SEM. (n = 9-15 neurons per group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Statistical analysis was performed using one-way ANOVA followed by Neumann-Keuls post-test for nonpercentage comparisons and z-test for comparisons between percentages. Scale bars are 10μm.

Figure 2.4

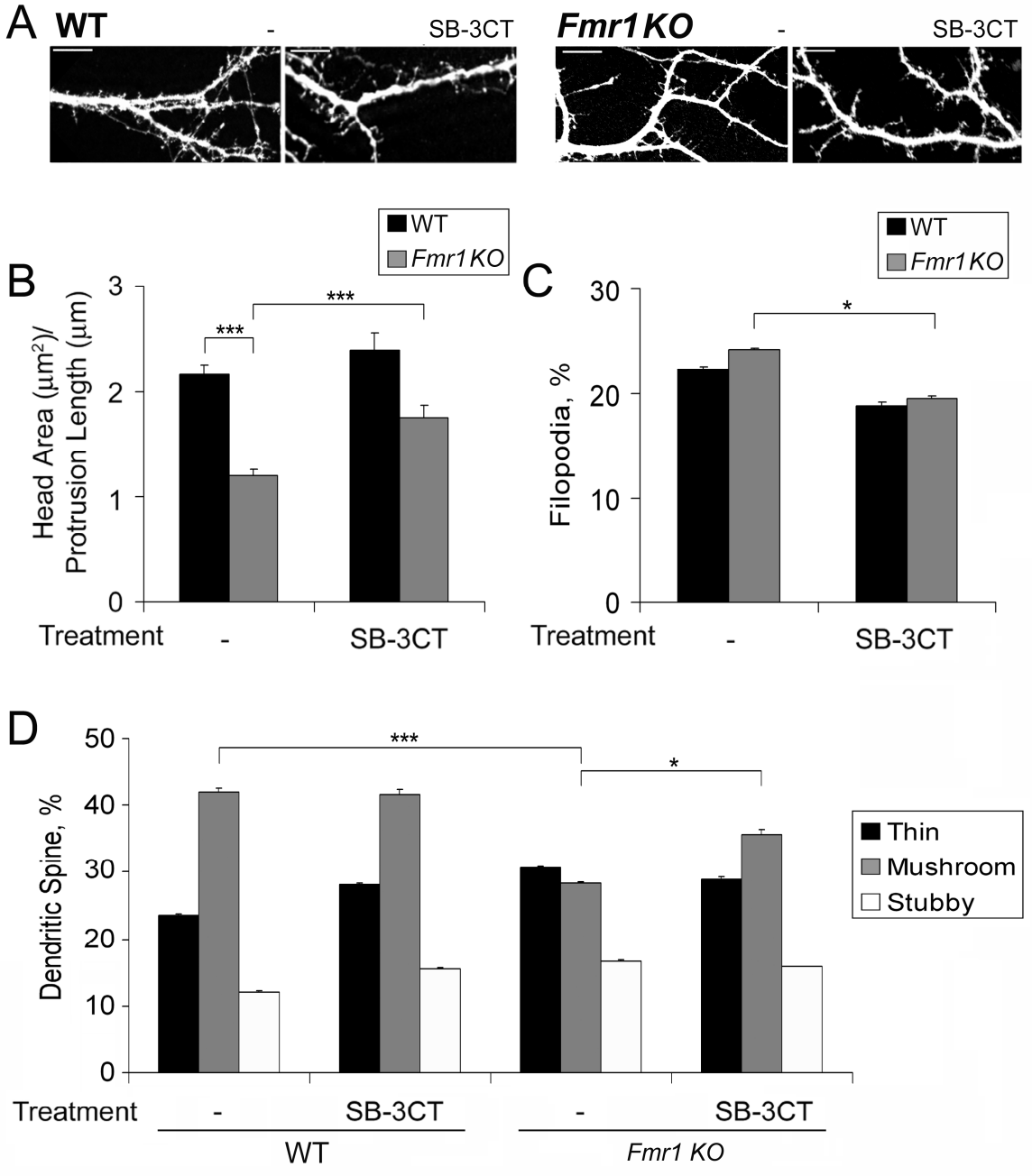

A
WT
-
SB-3CT
Fmr1 KO
-
SB-3CT
B
WT
Fmr1 KO
Head Area (µm²)/ Protrusion Length (µm)
0
1
2
3
***
***
Treatment
-
SB-3CT
C
WT
Fmr1 KO
Filopodia, %
0
10
20
30
*
Treatment
-
SB-3CT
D
Dendritic Spine, %
0
10
20
30
40
50
***
*
Thin
Mushroom
Stubby
Treatment
-
SB-3CT
-
SB-3CT
WT
Fmr1 KO

Figure 2.5 Cultured *mmp9 KO* hippocampal neurons develop more mature spines that WT neurons *in vitro*. (A) Dendritic morphology was observed with GFP fluorescence. Images of 14DIV WT and *mmp9 KO* hippocampal neurons. (B-D) Quantification of head area/spine length ratio (B), percentage of filopodia (C), and percentage of thin, mushroom and stubby spines (D) in WT and *mmp9-/-* neurons. Error bars indicate SEM. (n = 6-15 neurons per group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Statistical analysis was performed using one-way ANOVA followed by Neumann-Keuls post-test for non-percentage comparisons and z-test for comparisons between percentages. Scale bars are 10μm.

Figure 2.5

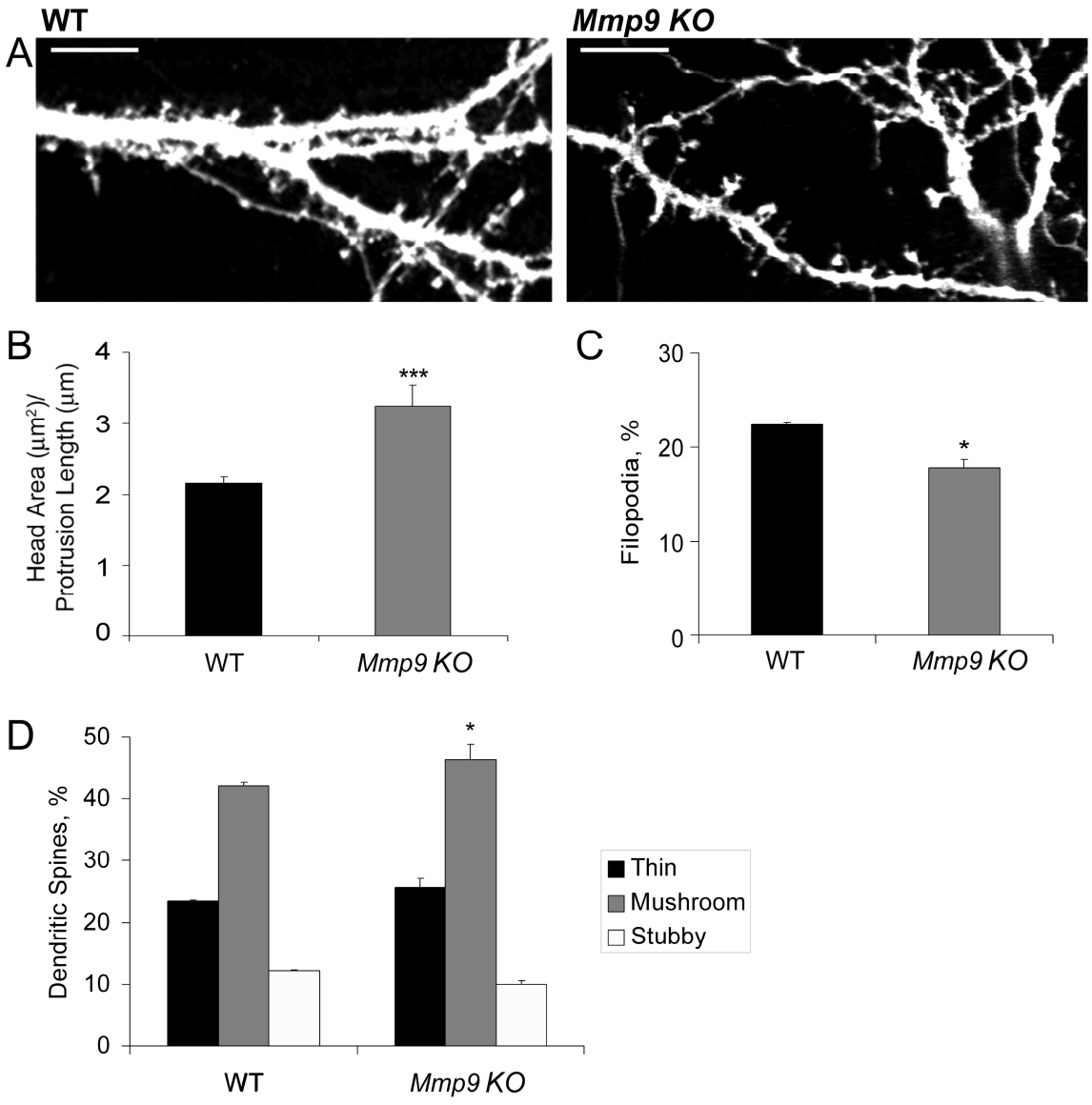
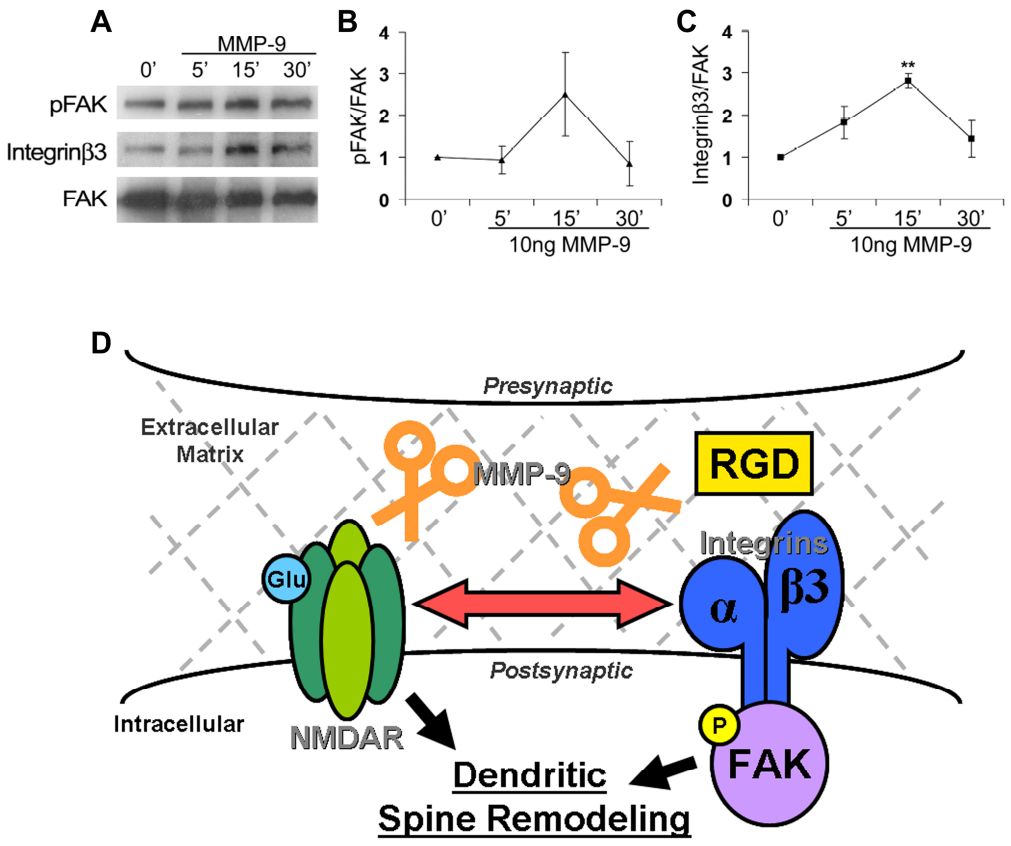

WT
Mmp9 KO
A
B
Head Area (µm²)/
Protrusion Length (µm)
4
3
2
1
0
***
WT
Mmp9 KO
C
Filopodia, %
30
20
10
0
*
WT
Mmp9 KO
D
Dendritic Spines, %
50
40
30
20
10
0
*
WT
Mmp9 KO
Thin
Mushroom
Stubby

Figure 2.6 MMP-9 transiently upregulated FAK phosphorylation and its association with Integrinβ3. (A) 14 DIV hippocampal neurons were treated with 10ng of active MMP-9 for 5, 15 or 30 min in conditioned medium under 5% CO_2/10% O_2 at 37ºC. Immunoprecipitation and Western blot analysis were performed to detect the levels of pFAK and FAK association with integrinβ3 in untreated and MMP-9 treated cultures. (B, C) Graphs show the levels of pFAK (B) and FAK association with integrinβ3 (C) at 0, 5, 15 or 30 min after MMP-9 treatment. (D) A proposed mechanism for MMP-9 mediated dendritic spine remodeling. Statistical differences were resolved using Student's t-test. (n = 2 experiments; **, $p<0.01$).

Figure 2.6


A
MMP-9
0'
5'
15'
30'
pFAK
Integrinβ3
FAK
B
pFAK/FAK
0'
5'
15'
30'
10ng MMP-9
C
Integrinβ3/FAK
**
0'
5'
15'
30'
10ng MMP-9
D
Presynaptic
Extracellular Matrix
MMP-9
RGD
Integrins
α
β3
Glu
Postsynaptic
Intracellular
NMDAR
P
FAK
Dendritic
Spine Remodeling

Chapter 3 – Minocycline Promotes Dendritic Spine Maturation in the Fragile X Mouse Model through Inhibition of Matrix Metalloproteinase-9

ABSTRACT

Fragile X syndrome (FXS) is the most common single-gene inherited form of intellectual disability, with behaviors characteristic of the autistic spectrum. FXS subjects and Fragile X mental retardation gene knock out (*Fmr1 KO*) mice, an animal model for FXS, demonstrate hypersensitivity to Group 1 metabotropic glutamate receptor (mGluR) signaling and have been shown to exhibit defects in dendritic spine maturation which may underlie cognitive and behavioral abnormalities in FXS. Dendritic spines are small protrusions on the surface of the dendrite that receive the majority of excitatory synapses in the brain and changes in their morphology affect synaptic efficacy. Previously we reported that MMP-9 contributes to the immature dendritic spine profile of the *Fmr1 KO* and specific inhibition of MMPs promotes dendritic spine maturation. Since specific MMP inhibitors are not blood-brain barrier permeable it would make them less desirable as potential therapeutic options, so in this chapter we analyze whether treatment with the semi-synthetic tetracycline analog minocycline, which is blood-brain barrier permeable and also serves as an MMP-9 inhibitor, may have similar effects in promoting dendritic spine maturation in *Fmr1 KO* mice. We found that treatment with minocycline promotes dendritic spine maturation in primary cultures of hippocampal neurons as well as in the developing hippocampus of

Fmr1 KO mice and does not affect the relative activation states of other molecular pathways implicated in Fragile X, specifically ERKs, Akts or GSKs, which are downstream of Group 1 mGluR signaling. However, we did find that Group 1 mGluRs can regulate expression of MMP-9. These findings indicate that MMP-9 may be a viable drug target for the treatment of Fragile X Syndrome.

INTRODUCTION

Dendritic spines are small protrusions from the surface of dendrites that are responsible for the majority of post-synaptic excitatory transmissions in the brain (Harris, 1999; Yuste and Bonhoeffer, 2001; Carlisle and Kennedy, 2005; Ethell and Pasquale, 2005), and changes in their morphology have been linked to a broad spectrum of cognitive and developmental disorders, including Fragile X Syndrome (FXS), Rett syndrome and Down syndrome (Rudelli et al., 1985; Kaufmann and Moser, 2000; Fiala et al., 2002). Within many of these disorders is a characterization of long, immature dendritic spines. Previously we demonstrated that MMP-9 promoted dendritic spine elongation similar to the typical dendritic spine phenotype associated with the Fragile X mouse model (*Fmr1 KO*). Furthermore, MMP-9 levels and activity were upregulated within the hippocampus of the *Fmr1 KO*, and specific inhibition of the gelatinases, MMP-2 and MMP-9, promoted formation of short, mature spines with large head areas within the *Fmr1 KO*. The question remained however, as to what potential mechanisms may contribute to this upregulation in the *Fmr1 KO*.

Prior to zymogen formation there is evidence that some MMPs, such as MMP-9, are strongly regulated at the level of translation (Sato and Seiki, 1993; Jiang and Muschel, 2002), which may suggest a role for the Fragile X Mental Retardation Protein (FMRP), the protein that is lacking in Fragile X Syndrome (FXS). Initial microarray studies of FMRP-associated with mRNAs do not report MMP-9 mRNA as a binding partner for FMRP within lymphoblastoids isolated from FXS males or from mouse brain lysates that were coimmunoprecipitated with the mouse homolog, Fmrp (Brown et al., 2001; Bittel et al., 2007). However, those works were looking for global changes in total gene expression which may not reflect region specific regulation, such as within specific areas of the brain, or regulation that involves low levels of mRNA transcripts. Preliminary work by the Kaczmarek lab (2010) has indicated that Fmrp can directly bind and transport MMP-9 mRNA within the dendrites of primary rat hippocampal cultures where it is locally translated following neuronal stimulation (Dziembowska et al., 2010). This translational regulation may also be mediated by upstream extracellular regulated kinase (ERK) activation (Kim et al., 2008). ERK signaling has been implicated in metabotropic glutamate receptor 5 (mGluR5)-mediated mRNA translation that is regulated by Fmrp (Gallagher et al., 2004; Zhao et al., 2004; Dolen and Bear, 2005) and has also been shown to promote MMP-9 gene expression and activity (Iyer et al., 2005). Within FXS, hypersensitivity to Group 1 mGluRs, specifically mGluR5, and enhanced ERK signaling has been characterized as factors contributing to the pathology (Osterweil et al., 2010).

Within this chapter we first analyze how activation and inhibition of Group 1 mGluRs affect expression of MMP-9. Our second goal is to determine if another MMP-9 inhibitor, minocycline, may promote dendritic spine maturation in *Fmr1 KO* mice, since it has the ability to perfuse the blood-brain barrier, unlike specific MMP inhibitors.

Minocycline, a semi-synthetic tetracycline derivative, is a small, lipophilic molecule, with the ability to cross the blood-brain barrier. It was originally developed as an antibiotic over 30-years ago for use in the treatment of acne and rosacea and has been demonstrated to have a good safety record for long-term use (Yong et al., 2004). Minocycline has a low occurrence of serious side-effects, although of note is the permanent yellow discoloration of teeth and black discoloration of some bodily fluids and organs after prolonged treatment (Cheek and Heymann, 1999; Tacon et al., 2008). In addition to inhibiting MMP-9, minocycline has also been demonstrated to have other neuroprotective properties, such as inhibition of both microglial proliferation (Tikka et al., 2001; Zhang et al., 2004) and neuronal apoptosis (Chen et al., 2000). The exact mechanisms of non-microbial minocycline activity are currently under investigation. With regards to MMP-9 inhibition though, it has been suggested that minocycline directly impairs the enzymatic activity of MMP-9 as well as its translation (Brundula et al., 2002). Interestingly, minocycline has also been implicated in affecting the activation states of specific kinases within pathways that are involved in Fmrp-mediated protein translation and are downstream of

mGluR5 signaling, specifically the ERK, Akt and glycogen synthase kinases (GSKs; Peineau et al., 2008; Min et al., 2009; Mines et al., 2010; Sharma et al., 2010; Yuskaitis et al., 2010). For the purpose of this chapter we characterize a role for minocycline in affecting the dendritic spine development of the *Fmr1 KO*, look to determine if MMP-9 inhibition is one of the primary mechanisms of minocycline action within *Fmr1 KO*$^{-}$ mice, and see how Group 1 mGluR signaling affects MMP-9 expression.

MATERIALS AND METHODS

Mice

The FVB.129P2-*Fmr1*^{tm1Cgr}/J (*Fmr1 KO*) and FVB.129P2-Pde6b^{+}Tyr^{c-ch}/AntJ controls (WT) were obtained from the Jackson Laboratories. These mice do not suffer from retinal degeneration due to restoration of the *pde6b* allele. Mice were maintained in an AAALAC accredited facility under 12 hour light/dark cycles. All mouse studies were done within NIH and Institutional Animal Care and Use Committee guidelines.

Minocycline administration in vivo

Minocycline was administered to newborn mice (WT and *Fmr1 KO* mice) by adding it to the mother's drinking water at 30 mg/kg/day every day for 7-28 days. This method has been previously reported to result in high concentrations

of minocycline in the breast milk of lactating mothers (Hunt et al., 1996). Minocycline administration through drinking water has been shown to significantly decrease MMP-9 expression and activity in the brain due to its ability to cross the blood-brain barrier (Lee et al., 2006).

Slices

Coronal 300 μm thick sections were prepared from the brains (prefixed in 4% paraformaldehyde to preserve morphology) of WT and *Fmr1 KO* mice treated with minocycline or control water (total: 12 mice, 3 per condition). Brains were prefixed in 4% paraformaldehyde from 1-4 hours to preserve morphology. Developing hippocampal neurons were labeled in slices with the red fluorescent membrane dye, DiI, using a biolistic particle-mediated gene transfer method (gene gun) as previously described (Wu et al., 2004). This approach allows for efficient delivery of lipophilic dye-coated particles to brain slices and fluorescent labeling of plasma membranes to visualize neuronal morphology. DiI labeled slices were analyzed using a confocal laser scanning microscope (model LSM 510; Carl Zeiss Microimaging, Germany). Serial optical sections of CA1 hippocampal neurons were taken at 0.5 μm intervals within the X-Y plane. Three-dimensional fluorescence images were created by the projection of optical serial sections. Morphometric analysis of dendritic spines and quantification of the numbers and lengths of dendritic spines, as well as sizes of dendritic spine heads, were performed as previously described (Henkemeyer et al., 2003; Shi

and Ethell, 2006). Briefly, 5-10 DiI-labeled CA1 hippocampal neurons were randomly selected for each group, and dendrites in the *stratum radiatum* (CA1) were analyzed. Lengths of dendritic spines and sizes of dendritic spine heads were determined using Adobe photoshop and Image J software. Quantification of the lengths, sizes and types of different spines within and between the groups were quantified using Student's t-test or one-way ANOVAs statistical analyses where appropriate. Following ANOVA, post hoc pair-by-pair differences between groups were resolved with the Newman-Keuls post-test.

Gelatin zymography

Gelatin gel zymography was performed as previously described (Zhang and Gottschall, 1997; Manabe et al., 2005) with minor modifications. Briefly, brain tissues were resuspended in 50 mM Tris-HCl (pH=7.6) buffer containing 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij35, 0.02% Na_3N, 1% Triton X-100, 100 μM PMSF and PI cocktail (Sigma). The gelatinases, MMP-2 and MMP-9, were pulled down with gelatin agarose (Sigma, G5384) and separated on non-reducing SDS-PAGE gels containing 0.1% gelatin (Invitrogen). After separation, gels were soaked in renaturing buffer (LC2670, Invitrogen) to remove SDS and allow MMPs to refold, regaining most of their enzymatic activity. Following renaturation, gels were incubated in developing buffer (LC2671, Invitrogen) for 3 days, which allowed gelatinases (MMP-2 and MMP-9) in the gel to degrade the gelatin. Gels then were stained with coomassie blue, which uniformly stained the gels due to the

gelatin within the gel. Areas of MMP activity were revealed as unstained bands where gelatin was degraded.

Hippocampal neuron cultures

Briefly, hippocampal cells were treated with papain (0.5 mg/ml) and DNase (0.6 µg/ml) for 20 min at 37ºC, mechanically dissociated, and then plated on glass coverslips or plastic dishes 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_2/10% O_2 atmosphere at 37ºC. 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).

Treatments *in vitro*

Hippocampal cultures were treated with 20µM minocycline (155718, MP Biomedicals), 20µM DHPG (50811275, Tocris Bioscience), 100µM MPEP (1212, Tocris Bioscience), or 500nM SB-3CT (444285, Calbiochem) for durations of 20min, 3h or 17h (ON), as indicated. The treatments were performed in conditioned medium under 5% CO_2/10% O_2 atmosphere at 37ºC.

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 synaptophysin using mouse anti-synaptophysin antibody (61 µg/ml; SVP-38; Sigma). The secondary antibodies used were Alexa Fluor 594-conjugated chicken anti-mouse 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 (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 protrusions were grouped into three categories according to their morphology: 1) filopodia-like (long, thin protrusions without head or 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 spines were identified as dendritic protrusions connected with a synaptophysin-positive pre-synaptic terminal. Each experiment was performed at least three times. Statistical analysis was performed using Student's *t*-test.

Western immunoblotting

Brain tissues or cultured hippocampal neurons were lysed in ice-cold TBS (25 mM Tris (pH=7.4), 150 mM NaCl) containing 1% Triton X-100, 5 mM EDTA, protease inhibitor cocktail (Sigma), 0.5 mM pervanadate, and cleared by centrifugation at 12,000xg. Proteins were resolved on 8-16% Tris-glycine SDS-PAGE gels (Invitrogen) and immunoblotted with primary antibody against MMP-9 (Chemicon, AB19016 or R&D Systems AF909) followed by incubation with corresponding secondary HRP-conjugated antibody (Jackson ImmunoResearch) and ECL detection (GE Healthcare, RPN-2132). The blots were re-probed with antibody against GAPDH (RDI-TRK5G4-6C5, Fitzgerald Industries Intl) to confirm equal loading.

Multi-Kinase ELISA Array

Hippocampi from 4 week minocycline-treated (from birth) or age-matched *Fmr1 KO* mice were dissected, lysed and analyzed for relative activation states of various kinases through analysis of their specific phosphorylation levels. The phosphorylation of serine (pS), tyrosine (pY) or threonine (pT) residues of Akt-1

(pS473), Akt-2 (pS474), ERK1/2 (ERK1: pT202, pY204; ERK2: pT185, pY187), p38 MAPK (pT180, pY182), GSK3α (pS21), and GSK3β (pS9) were detected following the protocol for the multi-kinase ELISA array (Symansis, MKA001). Briefly, hippocampi were collected and lysed in a buffer containing 6M urea, 10mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1mM sodium pervanadate, 0.5% Triton X-100 and 1% protease inhibitor cocktail. Lysates were diluted 1:2 for analysis of Akt-1/2, ERK1/2, and GSKα/β and were diluted 1:5 for analysis of p38 MAPK. Total protein concentrations were evaluated in hippocampal lysates using the protocol for the BCA colorimetric protein assay (Pierce, catalog #23235).

RESULTS

MMP-9 is regulated by Group 1 mGluR signaling. Hypersensitivity to Group 1 mGluR signaling is a hallmark of FXS (Bear et al., 2004; Osterweil et al., 2010). Here we report that treatment of 14DIV WT hippocampal cultures with Group 1 mGluR agonist, DHPG, increased MMP-9 levels (Fig. 3.1 A,C) while specific inhibition of mGluR5 with its antagonist, MPEP, caused a significant decrease in MMP-9 levels (Fig. 3.1 B,D). We also demonstrated that minocycline downregulates levels of MMP-9 within 14DIV WT hippocampal cultures (Fig. 3.1 E), and achieves significant levels of decreased MMP-9 expression after only 3 hours of incubation (Fig. 3.1 E) whereas overnight (ON) treatment with MPEP was required to achieve MMP-9 downregulation (Fig. 3.1 B,D). These results

indicate a connection between hypersensitivity to mGluR5 signaling and increased MMP-9 activity found in the *Fmr1 KO* and demonstrate that minocycline may be useful to target MMP-9 within the *Fmr1 KO* to promote decreased MMP-9 expression.

Minocycline promotes dendritic spine maturation in *Fmr1 KO* neurons *in vitro.* Since minocycline can downregulate MMP-9 activity, we wanted to analyze the effects of minocycline on dendritic spine morphology in 14DIV cultured *Fmr1 KO* hippocampal neurons (Fig. 3.2 A). Minocycline treatment promoted the formation of mature dendritic spines, as indicated by the increase in the dendritic protrusion head to length ratio (Fig. 3.2 B) and the increase in the proportion of mushroom spines (Fig. 3.2 D), as well as promoted a decrease in the proportion of filopodia (Fig. 3.2 C). Importantly, minocycline was well tolerated by hippocampal neurons, with no neurotoxicity at concentrations as high as 20 μg/ml (40μM; data not shown). These effects of minocycline in promoting dendritic spine maturation in the *Fmr1 KO* were similar to the effects of SB-3CT, the MMP-2/MMP-9 specific inhibitor, and MPEP, the mGluR5 antagonist on 14DIV *Fmr1 KO* hippocampal neurons. Specifically, SB-3CT and MPEP both caused an increase in the head to length ratio of dendritic protrusions (Fig. 3.2 B), decreased the proportion of filopodia (Fig. 3.2 C) as well as increased the proportion of mature, mushroom spines (Fig. 3.2 D). As many properties of neurons are different *in vitro* and *in vivo*, we also performed *in vivo*

analysis of the effects of minocycline treatment on dendritic spine development in the hippocampus of WT and *Fmr1 KO* mice and found similar effects on promoting dendritic spine maturation (Bilousova et al., 2009). These studies conclusively demonstrate that minocycline treatment can induce dendritic spine maturation in the hippocampus of *Fmr1 KO* mice, correcting aberrant dendritic spine development caused by Fmrp-deficiency.

Minocycline treatment reduces MMP-9 expression and activity within the hippocampus of *Fmr1 KO* mice. To determine if minocycline affects MMP-9 expression and activity *in vivo* we examined the levels of pro and active forms in protein lysates isolated from the hippocampus of untreated or minocycline-treated *Fmr1 KO* mice by immunoblotting with an anti-MMP-9 antibody. Levels of active MMP-9 were higher in P7 hippocampal lysates from *Fmr1 KO* mice, as compared to WT controls, and minocycline treatment was able to significantly reduce levels of MMP-9 in the *Fmr1 KO* hippocampus (Fig. 3.3 A,B). Gel zymography also demonstrated that MMP-9 gelatinase activity was significantly lower in the hippocampus of minocycline treated *Fmr1 KO* mice (Fig. 3.3 C,D). Our findings suggest that minocycline's inhibition of MMP-9 promotes dendritic spine maturation in the hippocampus of *Fmr1 KO* mice.

Minocycline does not effect relative activity of specific kinases implicated as downstream mediators of Group 1 mGluR hypersensitivity. Group 1

mGluR signaling promotes protein translation that is regulated by Fmrp through activation of two primary kinase pathways: the ERK or mitogen activated protein kinase (MAPK) pathway and the phosphoinositol-3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway (Gallagher et al., 2004; Hou and Klann, 2004; Banko et al., 2006; Narayanan et al., 2007). Both of these pathways affect the activation states of GSKs which have also been implicated in FXS (Peineau et al., 2008; Min et al., 2009; Mines et al., 2010). When we analyzed the relative phosphorylated levels of these families of kinases using a multi-kinase ELISA (Symansis, MKA001), within 4-week old *Fmr1 KO* mice, we found upregulation of pAkt-1 and pAkt-2 (Fig. 3.4 A, left), upregulation of pERK1/2 (Fig. 3.4 A, center), and downregulation of serine phosphorylated GSK3α (Fig. 3.4 A, right). Since minocycline has been shown to affect levels of pAkts, pERK 1/2, and serine-phosphorylated GSKs within other models (Pi et al., 2004; Wilkins et al., 2004; Yao et al., 2004; Hashimoto and Ishima, 2010), we wanted to determine if it also had any effects on these kinases within the *Fmr1 KO*. Following 4 weeks of minocycline treatment from birth within the *Fmr1 KO* however, there was no significant change in the relative phosphorylation levels of any of the analyzed kinases (Fig. 3.4 B). Furthermore, we did not see any changes in total protein levels after minocycline treatment (Fig. 3.4 C). These results support the role of minocycline as primarily acting through inhibition of MMP-9 to promote recovery in *Fmr1 KO* mice.

DISCUSSION

Our analyses of MMP-9 within the *Fmr1 KO* indicate that increased expression and activity of MMP-9 contributes to the abnormal synapse development associated with FXS and minocycline serves as an MMP-9 inhibitor to promote dendritic spine maturation *in vivo* and *in vitro*. Abnormal synapse and dendritic spine development is a common feature in FXS and may represent the anatomical and physiological basis for the cognitive and behavioral abnormalities associated with this disorder (Fiala et al., 2002). Several studies have established strong correlations between the size of dendritic spine heads and synaptic strength, wherein filopodia-like spines with smaller heads have fewer AMPA receptors (AMPARs) (Matsuzaki et al., 2001) and are less stable than mushroom-like spines with larger heads (Matsuzaki et al., 2001; Lippman and Dunaevsky, 2005; Matus, 2005; Tada and Sheng, 2006). The development of dendritic spines is abnormal in the brains of subjects with FXS, Down's and Rett syndromes, as they show a preponderance of long, thin filopodia-like spines at ages when control subjects have more mature, mushroom-shaped dendritic spines (Rudelli et al., 1985; Irwin et al., 2000; Kaufmann and Moser, 2000). Our findings demonstrate the beneficial effects of minocycline in the brains of *Fmr1 KO* mice by transforming long, thin spines into mature mushroom-shaped spines similar to specific inhibition of MMPs.

Mechanisms responsible for the preponderance of immature dendritic spines in *Fmr1 KO* mice are unresolved, but may be related to metabotropic

glutamate receptor (mGluR) hypersensitivity. The mGluR theory of FXS posits that Fmrp normally suppresses protein synthesis that attenuates mGluR-dependent long-term depression (LTD). Fmrp-deficiency leads to hypersensitivity to group 1 mGluR signaling, resulting in enhanced LTD (Bear et al., 2004; Osterweil et al., 2010). This theory is supported by the observation that exaggeration of signaling through group 1 mGluRs and increased mGluR-induced LTD are present in the hippocampus of *Fmr1 KO* mice (Huber, 2006). Pharmacological activation of group 1 mGluRs with the specific agonist DHPG also induces dendritic spine elongation (Vanderklish and Edelman, 2002), producing a morphology that is similar to what we have observed in hippocampal neurons treated with MMP-7 or MMP-9. Our studies demonstrate that DHPG also induces MMP-9 expression/activation in cultured hippocampal neurons while MPEP, the mGluR5 specific inhibitor, can downregulate levels of MMP-9, suggesting that MMP-9 activation induced by group 1 mGluR hypersensitivity may contribute to abnormal dendritic spine development in Fmrp-deficient hippocampal neurons. Group 1 mGluR effects on Fmrp-mediated regulation of protein synthesis has been suggested to depend on the activation of two primary signaling pathways: ERK/MAPK pathway and the PI3K-Akt-mTOR pathway (Gallagher et al., 2004; Hou and Klann, 2004; Banko et al., 2006; Hou et al., 2006; Narayanan et al., 2007; Sharma et al., 2010), both of which also affect the activation states of the GSKs (Peineau et al., 2008). Changes in the activation states of ERK, Akts and GSKs have been implicated in the pathology of FXS

(Hou et al., 2006; Min et al., 2009; Mines et al., 2010; Sharma et al., 2010). Interestingly, while minocycline treatment has been shown to alter the activation state of these kinases in other systems (Pi et al., 2004; Wilkins et al., 2004; Yao et al., 2004; Hashimoto and Ishima, 2010), minocycline treatment did not affect the relative activity of these kinases in the hippocampus of *Fmr1 KO* mice. This further supports that theory minocycline is acting through MMP-9 inhibition to promote development of mature dendritic spines in the hippocampus of *Fmr1 KO* mice.

Within the previous chapter we discussed how upregulation of MMP-9 within the *Fmr1 KO* may contribute to the abnormal dendritic spine phenotype. Our findings presented here indicate that, like specific inhibition of MMP-9, minocycline may also have beneficial effects in neurodevelopmental disorders that exhibit elevated brain levels of MMP-9. Consistent with our findings, recent studies by Siller and Broadie (2011) also support our hypothesis and demonstrate that overexpressing TIMP, an endogenous MMP inhibitor in *Drosophila*, and *mmp1* deficiency could both rescue synaptic architecture within the neuromuscular junction of the *dFmr1* null mutant, the Drosophila FXS model. Minocycline treatment also prevented both structural over-elaboration and synaptic developmental defects in a wide range of circuits in *dFmr1* null mutants (Siller and Broadie, 2011).

Since MMP-9 is an extracellularly localized protein that can promote intracellular responses to alter synaptic architecture, it is important to determine

what mechanisms MMP-9 utilizes. MMP-9 could affect dendritic spine morphology by cleaving components of the extracellular matrix (ECM) and/or cell surface proteins that participate in synaptogenesis and dendritic spine maturation (Mott and Werb, 2004; Ethell and Ethell, 2007). For example, MMP-9-mediated cleavage of perineuronal ECM networks may trigger the release of RGD containing peptides that could bind to integrins and facilitate NMDAR activation. We have previously shown that RGD-containing peptides can induce rapid dendritic spine elongation in cultured hippocampal neurons, mediated by NMDAR (Shi and Ethell, 2006). In addition we demonstrated in the previous chapter that MMP-9 promoted a transient increase in levels of integrin β3 that are associated with active, phosphorylated form of FAK. Further, MMP-7 effects on dendritic spines are similar to those of RGD and are also blocked by the NMDAR specific inhibitor MK-801 (Bilousova et al., 2006). While abnormally high MMP-7 or MMP-9 activities interfere with normal physiological functions and induce dendritic spine remodeling, modest levels of MMP-9 have been shown to regulate non-pathological synaptic functions and plasticity in the mature hippocampus through an integrin-dependent mechanism and NMDAR activation (Nagy et al., 2006; Bozdagi et al., 2007). Integrins are known to modulate NMDAR-mediated synaptic currents and play an important role in LTP (Chavis and Westbrook, 2001; Lin et al., 2003; Bernard-Trifilo et al., 2005). Although there is a clear connection between MMP-9 and integrin signaling, other MMP-9 substrates in synapses could also be involved, including pro-BDNF, cadherins

and Eph/ephrins (Ethell and Ethell, 2007). Interestingly, while Fmrp does not affect the expression levels of BDNF or its high-affinity receptor TrkB, BDNF infusion fully restores LTP in slices from *Fmr1 KO* mice, suggesting that modulating BDNF activity may be a potentially useful therapeutic strategy for FXS (Lauterborn et al., 2007). Furthermore, FXS patients may also be affected by MMP dysregulation outside the CNS as they display several characteristics of modified ECM regulation including large ears, long faces, hyperextensible joints and extremely soft skin.

While more work needs to be done to determine how MMP-9 contributes to abnormal dendritic spine development in *Fmr1 KO* mice, these findings establish that specific inhibition of MMP-9 as well as treatment with minocycline can supersede Fmrp-deficiency to achieve normal dendritic spine profiles in *Fmr1 KO* mice. These findings firmly establish the beneficial effects of targeted MMP-9 inhibition in the *Fmr1 KO* mouse model. Our next step is to focus on whether minocycline can promote behavioral recovery within the *Fmr1 KO* and thus have potential as a therapeutic option for the treatment of FXS.

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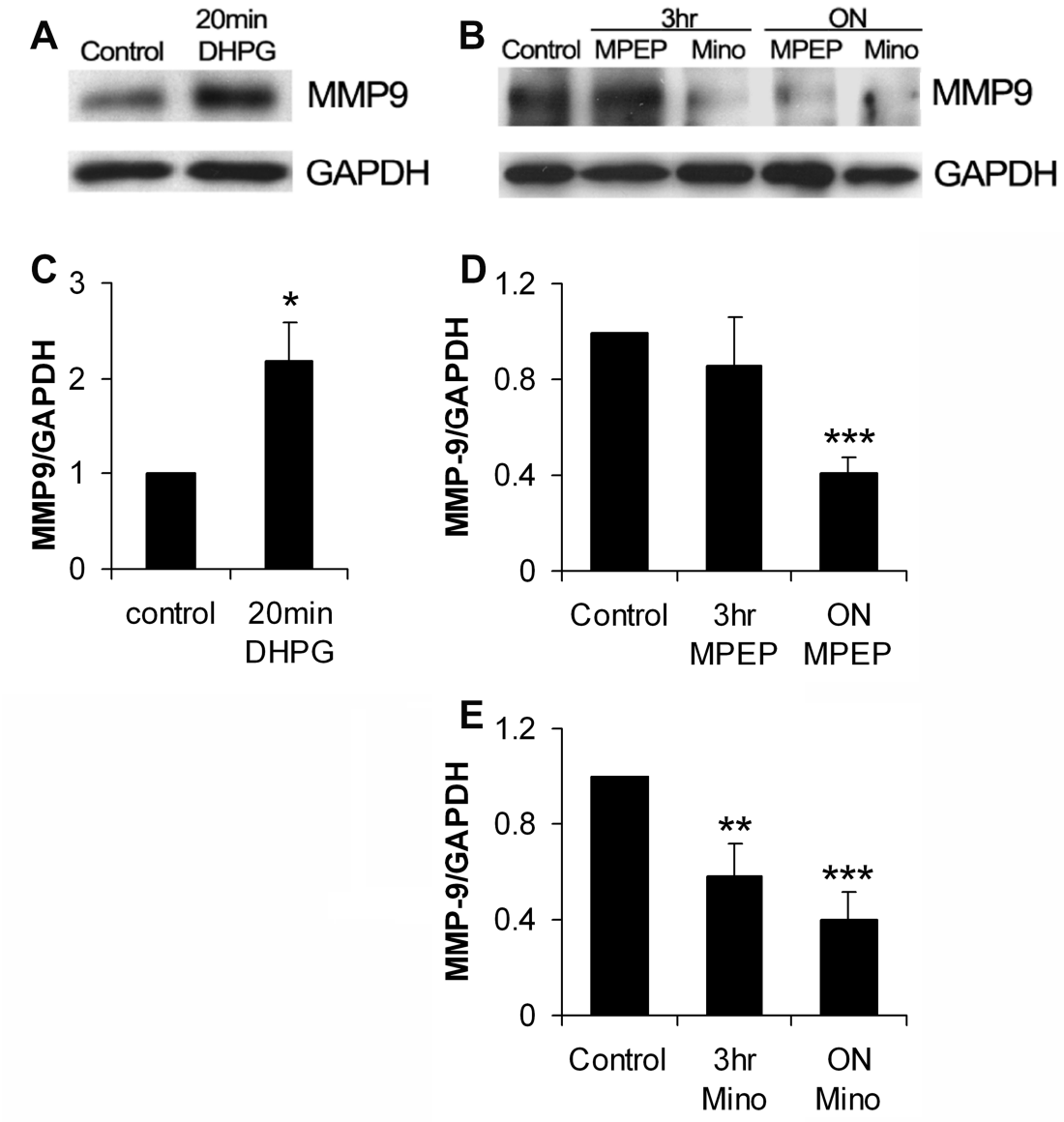
Figure 3.1 Group I mGluR activation upregulates levels of MMP-9 whereas minocycline and mGluR5 antagonist downregulates MMP-9 expression in hippocampal cultures. (A-B) Representative western blots of MMP-9 and GAPDH protein levels in WT 14 DIV hippocampal neurons either untreated or treated with DHPG (A), MPEP or minocycline (B). For the activation of group I mGluRs, hippocampal cultures were treated with 20 μM DHPG (0342, Tocris Cookson) for 20 min with NMDAR antagonist MK-801 and AMPAR antagonist NBQX both at 10 μM. Cultures were also treated with either 100μM MPEP or 20μM minocycline for 3 hours or overnight (ON) as indicated. The treatments were performed in conditioned medium under 5% CO_2/10% O_2 atmosphere at 37ºC. The blots are representative of three independent experiments. (C-E) Comparison of MMP-9 levels quantified by densitometry and normalized to GAPDH levels following DHPG treatment (C), MPEP treatment (D) or minocycline treatment (E). Error bars indicate SEM. (n = 3 experiments per treatment condition; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Figure 3.1


A
Control
20min DHPG
MMP9
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B
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Control
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C
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3
2
1
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control
20min DHPG
D
MMP-9/GAPDH
1.2
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Control
3hr MPEP
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Control
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Figure 3.2 Inhibition of MMP-9 promoted dendritic spine maturation in *Fmr1 KO* hippocampal cultures similar to minocycline and MPEP treatment. (A) 14DIV *Fmr1 KO* hippocampal neuronal cultures were untreated or treated overnight with 500nM SB-3CT , 20μM minocycline or 100μM MPEP. Dendritic morphology was observed with GFP fluorescence. Images of untreated and treated *Fmr1 KO* hippocampal neurons. (B-D) Quantification of head area/spine length ratio (B), percentage of filopodia (C), and percentage of thin, mushroom and stubby spines (D) in untreated or treated *Fmr1 KO* neurons. Error bars indicate SEM. (n = 9-15 neurons per group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Statistical analysis was performed using one-way ANOVA followed by Neumann-Keuls post-test for nonpercentage comparisons and z-test for comparisons between percentages. Scale bars are 10μm.

Figure 3.2

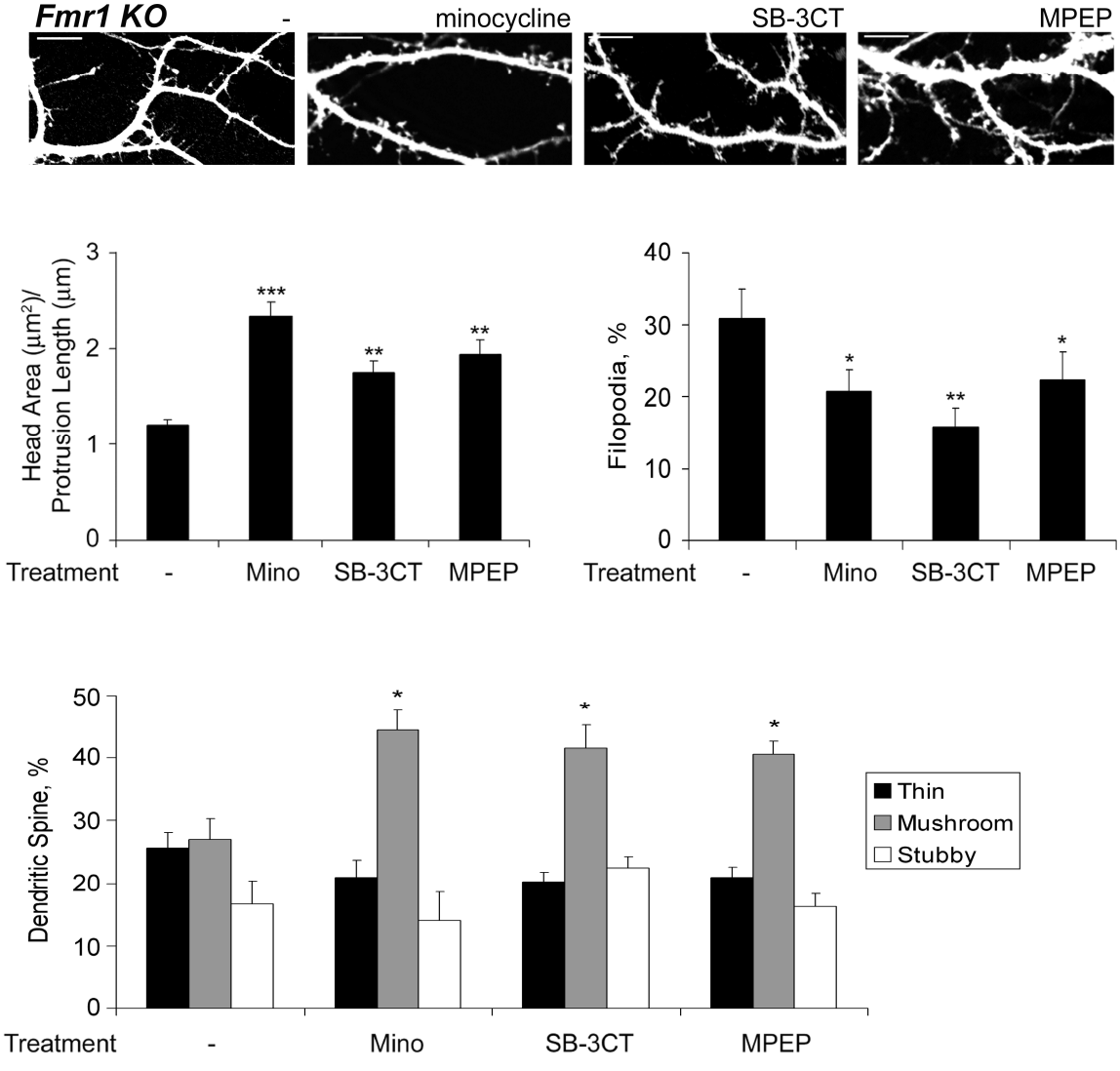

Fmr1 KO
-
minocycline
SB-3CT
MPEP
Head Area (μm²)/ Protrusion Length (μm)
3
2
1
0
***
**
**
Treatment
-
Mino
SB-3CT
MPEP
Filopodia, %
40
30
20
10
0
*
**
*
Treatment
-
Mino
SB-3CT
MPEP
Dendritic Spine, %
50
40
30
20
10
0
*
*
*
Thin
Mushroom
Stubby
Treatment
-
Mino
SB-3CT
MPEP

Figure 3.3 Minocycline treatment reduced MMP-9 levels in the hippocampus of P7 *Fmr1 KO* mice. (A) Detection of pro- and active forms of MMP-9 in hippocampal lysates from P7 brains of untreated or minocycline treated *Fmr1 KO* and WT mice by immunoblotting. The levels of pro- and active forms of MMP-9 were quantified by densitometry and normalized to total GAPDH values. The experiments were repeated at least three times and this blot is representative of the findings. (B) Bar graph values indicate the average MMP-9 values and error bars indicate SEM (n=3 male mice per group; *, $p<0.05$). (C) Detection of gelatinase concentrations in hippocampal lysates from P7 brains of untreated *Fmr1 KO* or minocycline treated *Fmr1 KO* mice by gelatin pull-down followed by gelatin zymography. The activities of gelatinases MMP-9 and MMP-2 in hippocampal cell lysates were quantified by densitometry. (D) Bar graph values indicate the average MMP-9 and MMP-2 values, and error bars indicate SEM (n=3 male mice per group; *, $p<0.05$).

Figure 3.3

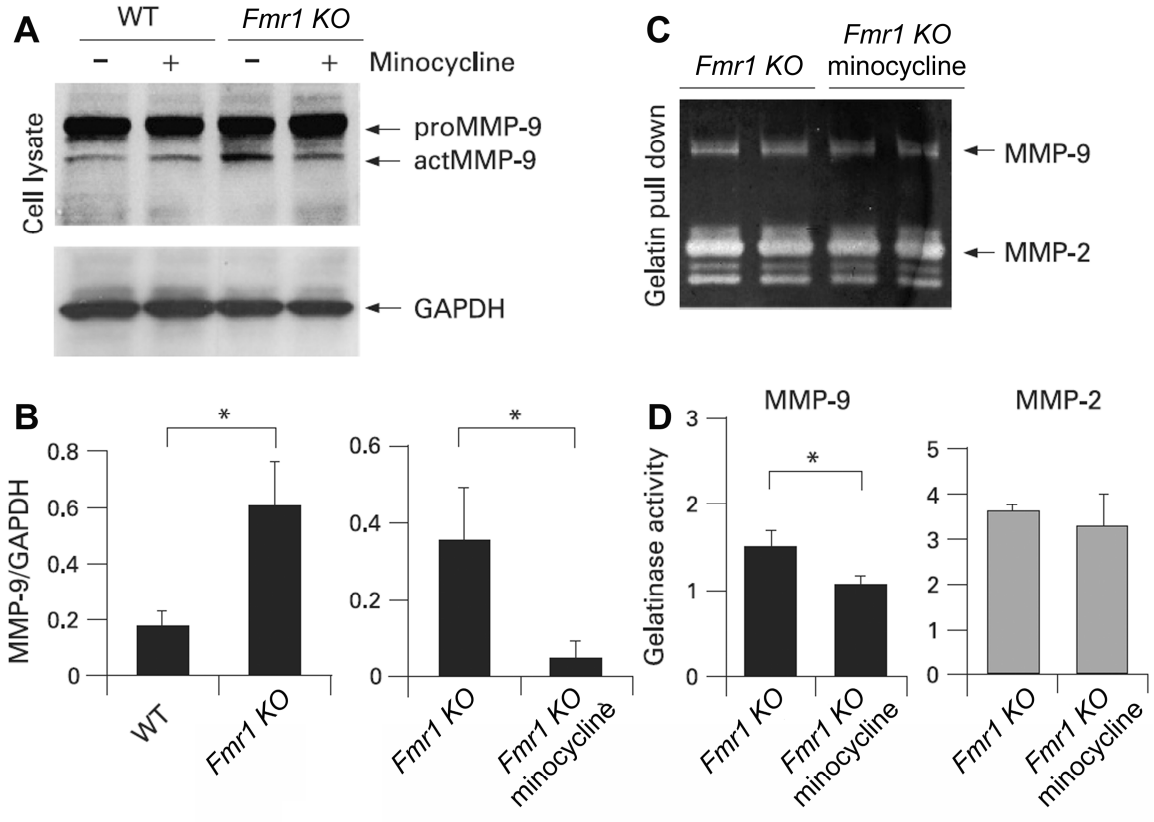
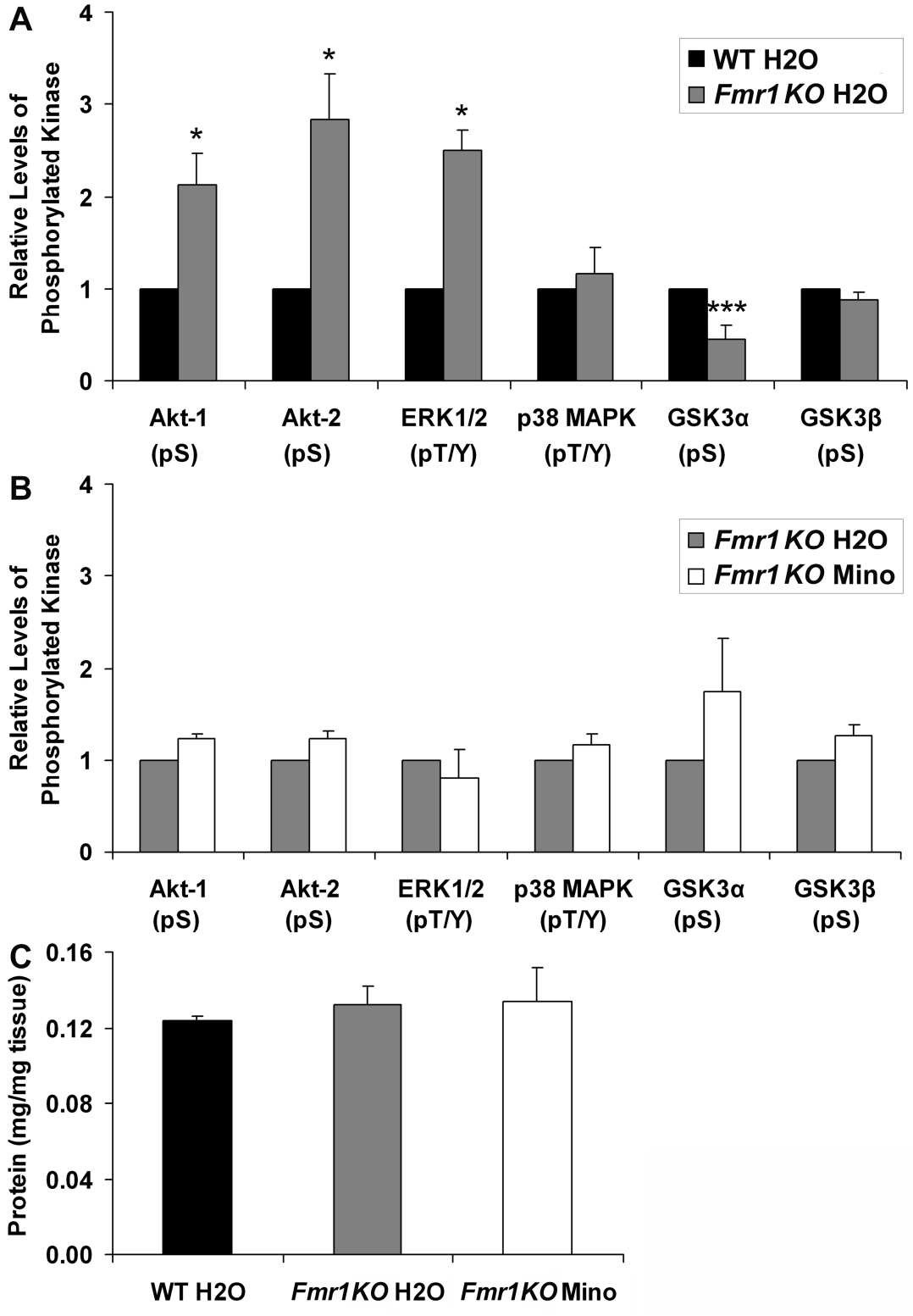

A
WT
Fmr1 KO
– + – +
Minocycline
Cell lysate
proMMP-9
actMMP-9
GAPDH
C
Fmr1 KO
Fmr1 KO minocycline
Gelatin pull down
MMP-9
MMP-2
B
MMP-9/GAPDH
0.8
0.6
0.4
0.2
0
*
WT
Fmr1 KO
0.6
0.4
0.2
0
*
Fmr1 KO
Fmr1 KO minocycline
D
MMP-9
MMP-2
Gelatinase activity
3
2
1
0
*
Fmr1 KO
Fmr1 KO minocycline
5
4
3
2
1
0
Fmr1 KO
Fmr1 KO minocycline

Figure 3.4 Minocycline does not affect relative levels of the phosphorylated forms of Akt1/2, pERK1/2, p38 MAPK, or GSK3α/β within the hippocampus of *Fmr1 KO* mice. (A) Comparison of hippocampal lysates of age-matched untreated WT and untreated *Fmr1 KO* mice for the relative phosphorylated levels of the pS forms of Akt-1, Akt-2, GSK3α, and GSK3β, and the pT/Y forms of ERK1/2 and p38 MAPK. Within the *Fmr1 KO* there was an increase in the relative phosphorylated levels of Akt-1, Akt-2, pERK1/2, a decrease in the relative phosphorylated levels of GSK3α, and no change in the relative phosphorylated levels of p38 MAPK and GSK3β as compared to WT. (B) Comparison of hippocampal lysates of age-matched untreated *Fmr1 KO* and 4 week minocycline treated from birth *Fmr1 KO* mice for relative phosphorylated levels of the pS forms of Akt-1, Akt-2, GSK3α, and GSK3β, and the pT/Y forms of ERK1/2 and p38 MAPK. Minocycline treatment had no effect on the relative phosphorylated levels of any of the kinases within the *Fmr1 KO*. (C) The total protein levels for the hippocampal lysate samples analyzed in A-B. There were no differences in total protein between untreated WT, untreated *Fmr1 KO* and minocycline treated *Fmr1 KO* mice. Untreated *Fmr1 KO* was normalized to untreated WT (A), minocycline treated *Fmr1 KO* was normalized to untreated *Fmr1 KO* (B). Vertical bars indicate SEM. (n = 3 mice per treatment group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Figure 3.4


A
Relative Levels of Phosphorylated Kinase
WT H2O
Fmr1 KO H2O
*
*
*
***
Akt-1 (pS)
Akt-2 (pS)
ERK1/2 (pT/Y)
p38 MAPK (pT/Y)
GSK3α (pS)
GSK3β (pS)
B
Relative Levels of Phosphorylated Kinase
Fmr1 KO H2O
Fmr1 KO Mino
Akt-1 (pS)
Akt-2 (pS)
ERK1/2 (pT/Y)
p38 MAPK (pT/Y)
GSK3α (pS)
GSK3β (pS)
C
Protein (mg/mg tissue)
WT H2O
Fmr1KO H2O
Fmr1KO Mino

Chapter 4 - Behavioral Deficits in the Fragile X Mouse Model are Improved Following Minocycline Treatment

ABSTRACT

Fragile X Syndrome (FXS) is the most common single-gene inherited form of impaired intellectual development, with behaviors at the extreme of the autistic spectrum. Human subjects with FXS display childhood seizures, hyperactivity, anxiety, developmental delay, attention deficits, and visual-spatial memory impairment, as well as a propensity for obsessive-compulsive behavior. Several of these aberrant behaviors and FXS-associated synaptic irregularities are also seen in fragile X mental retardation gene knock-out (*Fmr1 KO*) mice. Most excitatory synapses in the brain occur on small post-synaptic protrusions called dendritic spines, and changes in the morphology of these structures impact learning and memory. Defects in dendritic spine development may underlie at least some of the aberrant behaviors seen in *Fmr1 KO* mice. In the previous chapter we demonstrated that the semi-synthetic tetracycline analog, minocycline, can promote dendritic spine maturation within the *Fmr1 KO* and were thus curious if it could also impact their behavior. In this chapter, we compared the effectiveness of minocycline treatment in young and adult *Fmr1 KO* mice, and determined the dependence of behavioral improvements on the continuous administration of minocycline. We found that 4 or 8 weeks of minocycline treatment significantly reduced anxiety, hyperactivity and obsessive-

compulsive behaviors in both young and adult *Fmr1 KO* mice. Interestingly, the anxiolytic effect of minocycline persisted in young mice 4 weeks post-treatment, but ended when the treatment was stopped in adults. We also show, for the first time, that minocycline treatment can reduce the number and severity of audiogenic seizures in *Fmr1 KO* mice. This report provides further evidence that minocycline effectively counters FXS-associated behaviors in the *Fmr1 KO* mouse model.

INTRODUCTION

Fragile X Syndrome (FXS) is the most common, single gene inherited form of impaired intellectual development, with behaviors that are characteristic of autism. Individuals with FXS display childhood seizures, hyperactivity, anxiety, developmental delay, attention deficits, and visual-spatial memory impairment, as well as a propensity for obsessive-compulsive behaviors (Musumeci et al., 1999; Hagerman and Hagerman, 2002; Hagerman et al., 2010). Hypermethylation of long CGG repeats in the promoter region of the X-linked, Fragile X Mental Retardation (FMR1) gene leads to gene silencing and subsequent FMR1 protein (FMRP) deficiency (Brown et al., 1998; Khandjian, 1999). In mice, the homolog Fmrp has been shown to regulate protein synthesis by binding to and transporting mRNAs involved in the translation of key post-synaptic proteins within dendritic spines and has the ability to stall ribosomal translocation on mRNAs linked to synaptic functions (Khandjian, 1999; Greenough et al., 2001;

Hou et al., 2006; Zalfa et al., 2006; Pfeiffer and Huber, 2009; Darnell et al., 2011).

Dendritic spines are small protrusions from the surface of dendrites that host the majority of post-synaptic excitatory contacts in the brain (Harris, 1999; Yuste and Bonhoeffer, 2001; Carlisle and Kennedy, 2005; Ethell and Pasquale, 2005). Spine morphology is linked with the activity of excitatory synapses and factors that change the shape of dendritic spines impact learning, memory and cognition. Aberrant dendritic spine morphology has also been noted in a broad spectrum of cognitive and developmental disorders, including FXS, Rett syndrome and Down syndrome (Rudelli et al., 1985; Kaufmann and Moser, 2000; Fiala et al., 2002). *Fmr1 KO* mice, an animal model for FXS, exhibit similar dendritic spine abnormalities (Grossman et al., 2006) and display FXS-associated behavioral impairments, such as hyperactivity, anxiety, obsessive-compulsive behavior and a susceptibility to audiogenic seizures (Yan et al., 2005; Dolen et al., 2007). Work shown in the previous chapter demonstrated that minocycline treatment from birth induces dendritic spine maturation in developing *Fmr1 KO* hippocampal neurons. It is quite probable that those morphological changes may be translated to behavioral improvements.

The major issues we wanted to address in the use of minocycline for treating FXS-associated behaviors were: 1) the relative effectiveness of minocycline at different ages, particularly young versus adult; 2) the dependence of behavioral improvements on the continuous administration of minocycline.

That is, which behavioral improvements will revert after the drug is discontinued. To clarify these issues we have measured the effectiveness of different durations of minocycline treatment on several representative behaviors in young and adult *Fmr1 KO* mice.

MATERIALS AND METHODS

Mice

The FVB.129P2-*Fmr1*^{tm1Cgr}/J (*Fmr1 KO*) and FVB.129P2-Pde6b^{+}Tyr^{c-ch}/AntJ controls (WT) were obtained from the Jackson Laboratories. These mice do not suffer from retinal degeneration due to restoration of the pde6b allele. Mice were maintained in an AAALAC accredited facility under 12 hour light/dark cycles. All mouse studies were done according to NIH and Institutional Animal Care and Use Committee guidelines.

In vivo administration

Minocycline was administered to newborn mice (WT and *Fmr1 KO* mice) by adding it to the nursing mother's drinking water at 30 mg/kg/day every day until the pups were weaned. This method has been previously reported to result in high concentrations of minocycline in the breast milk of lactating mothers (Hunt et al., 1996). Minocycline was administered to weaned pups or adult mice directly through their drinking water at 30 mg/kg/day every day for 4 or 8 weeks (Fig. 4.1). Minocycline administration through drinking water has been shown to

significantly decrease MMP-9 expression and activity in the brain due to its ability to cross the blood-brain barrier (Lee et al., 2006).

Young mice

Male WT and *Fmr1 KO* mice were either untreated or treated with minocycline from birth for 4 weeks. 17 WT and 16 *Fmr1 KO* mice were behaviorally tested immediately following 4 weeks of minocycline treatment and prior to weaning (group 1), together with age-matched untreated 16 WT and 23 *Fmr1 KO* mice (group 2). Neither group received minocycline after weaning (4 weeks of treatment). A separate group of 14 WT and 13 *Fmr1 KO* mice continued to receive minocycline for an additional 4 weeks after weaning (8 weeks of treatment, group 3) and were tested at 8 weeks of age, together with age-matched, untreated 17 WT and 18 *Fmr1 KO* mice (group 4). All groups were tested again 4 weeks after treatment was stopped (4+4 weeks and 8+4 weeks, respectively). Age-matched untreated WT and *Fmr1 KO* mice received only water. All minocycline-treated mice were only tested after they had received their full course of treatment. Behavioral assessments at all ages included the open field test, elevated maze and marble burying assay.

Adult mice

8 week old male WT and *Fmr1 KO* mice were tested prior to treatment. 14 WT and 14 *Fmr1 KO* mice were treated with minocycline for 4 weeks (group

1) and tested immediately following treatment together with age-matched, untreated 15 WT and 19 *Fmr1 KO* mice (group 2). 16 WT and 16 *Fmr1 KO* mice were treated with minocycline for 8 weeks (group 3) and tested together with age-matched, untreated 18 WT and 14 *Fmr1 KO* mice (group 4). Both 4 week and 8 week treatment groups were tested again 4 weeks after treatment was stopped (4+4 weeks and 8+4 weeks, respectively). Age-matched untreated WT and *Fmr1 KO* mice received only water. Similar to young mice, adult mice treated with minocycline were only tested after they had received the full course of treatment. Behavioral assessments at all ages included the open field test, elevated maze and marble burying assay.

Open Field Test

Mice were tested for anxiety by evaluating a 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. The open field arena was placed in a room brightly lit with fluorescent lights. One mouse at a time was placed in a corner of the open field and allowed to explore for 5 min 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. This test was always performed prior to the elevated maze. 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. For a diagram of the arena, see Figure 4.2 A. To score locomotor activity the following measures were used: 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). A tendency to travel to the center of an open field (total entries into a large and small center squares) was used as an indicator of anxiety. Average velocity and total line crosses were measured to score locomotor activity. Assessments of the digital recordings were done by blinded observers

Elevated Maze

The elevated maze consisted of 4 arms in a plus configuration. Two opposing arms had 15 cm tall walls (closed arms), and 2 arms were without walls (open arms). The entire maze sat on a stand 1 meter above the floor. Each arm measured 30 cm long and 10 cm wide. For a picture of the elevated maze, see Figure 4.2 B. Mice were allowed to explore the maze for 5 min while being recorded by digital video from above. The tester left the room during testing. The maze was wiped with 2-3% acetic acid, 70% ethanol and water between each test to eliminate odor trails. This test was always done following the open field

test. TopScan Lite software was used to measure the percent of time spent in open arms and velocity. The percent of time spent in open arms was used as an indicator of anxiety (Carobrez and Bertoglio, 2005). The velocity and total arm entries were measured to evaluate overall locomotor activity. Assessments of the digital recordings were done by blinded observers

Marble Burying Test

Following all other behavior tasks, male mice were individually housed in a 28×17.5×12 cm transparent, plastic cage with 3-4 cm of bedding overnight. The next day each animal was tested within that same cage for marble burying activity: a test of anxiety and obsessive-compulsive behavior (Njung'e and Handley, 1991; Pan et al., 2008). Specifically, 15 blue marbles, each 1.4 cm in diameter were evenly spaced within the cage: 4.5 cm apart, 3.5 cm from the long edge and 4.5 cm from the short edge. For a picture of a typical cage set-up, see Figure 4.2 C. Marbles were cleaned with 2-3% acetic acid, then rinsed with water and thoroughly dried between trials. During testing, cages were covered with a clear acrylic sheet. Animals were given 30 min of exposure to the marbles and recorded by digital video from above. Marble burying was assessed as the number of individual marbles that the animal actively buried during the test. Assessments of the digital recordings were done by blinded observers.

Audiogenic Seizure Susceptibility

Male WT and *Fmr1 KO* mice were either untreated or treated with minocycline from birth for 28-30 days. 10 WT and 26 *Fmr1 KO* mice were tested immediately following minocycline treatment and prior to weaning, together with age-matched untreated 12 WT and 22 *Fmr1 KO* mice. All mice were exposed to a high intensity siren generated by alternating 500 msec upward frequency modulated (FM) sweeps (2-6 kHz) and 495 msec downward FM sweeps (6-2 kHz) at an average sound pressure level of 110 dB at 19.5 cm for up to 15 min in an empty, transparent plastic cage with an open grid lid (28x17.5x19.5 cm). The high intensity siren was generated using a custom written program (Batlab, Dr. Don Gans, Kent State University), a Microstar digital signal processing board and Tucker Davis System programmable attenuators (PA5). The sounds were further amplified with a power amplifier (Parasound) and presented through a speaker (Fostex FF165K, Madisound, WI) that was mounted on top of the open grid lid of the plastic cage. The experiment was performed on one cage of mice at a time (maximum of 5 mice per cage) that was placed in a 8x8 sq.ft. sound-proof chamber lined with anechoic foam (Gretch-Ken Industries Inc), with digital video recording from the long side of the cage. A similar method has previously been shown to trigger seizures in *Fmr1 KO* mice of that age (Yan et al., 2004; Yan et al., 2005). Audiogenic seizures were scored by a blinded observer. Periods of wild running and jumping (WRJ), as well as seizures, were scored by the time of

occurrence, length and type: tonic or status epilepticus (SE). Clonic seizures were omitted from scoring since they can be subject to the scorer's interpretation.

Statistical Analysis

For all behavior analyses, one way ANOVA was used, with exceptions noted below. For analysis of baseline adult behavior (0 Week) that resulted in only two groups being compared, Student's T-test was used. For open field, elevated maze and marble burying behavior analyses, following ANOVA post-hoc pair-by-pair differences between groups were resolved with the least significant difference (LSD) using Dunnett's method and Hsu's Multiple Comparison with the Best treatment (MCB). For audiogenic seizure behavior analysis when ANOVA was applicable post-hoc pair-by-pair differences between groups were resolved with the LSD using Dunnett's method and Hsu's MCB treatment (Fig. 4.8 B,C), otherwise differences between groups were resolved using Fisher's exact test, the effects of likelihood chi-square ratio and the odds ratio comparison (Fig. 4.8 A,D).

Blood Analysis

Blood was collected from untreated or minocycline-treated young *Fmr1 KO* mice, coagulated for 15 minutes at room temperature, spun down at 16 x g for 15 minutes at 4^{o}C after which the plasma was saved and used for analysis. Plasma blood samples were diluted 1:4000 and 1:8000 and analyzed for total

levels of IgG following the protocol for the IgG ELISA (Bethyl Laboratories, E99-131).

RESULTS

Behavioral Performance of Young *Fmr1 KO* Mice in the Elevated Plus maze.

Young *Fmr1 KO* mice were tested for anxiety and hyperactivity by measuring time spent in the open arms and total number of entries in the elevated-plus maze (Fig. 4.2 B). Young *Fmr1 KO* mice showed hyperactivity by making significantly more total arm entries in the elevated plus maze than WT mice (Fig. 4.3 A, B), but they spent significantly less time in the open arms than age-matched WT mice (Fig. 4.3 C,D), indicating higher overall anxiety. Both 4 and 8 weeks of treatment with minocycline led to significant improvements in the behavior of young *Fmr1 KO* mice, minocycline-treated mice increased their time spent in open arms and decreased total arm entries, as compared to untreated *Fmr1 KO* mice (4 weeks and 8 weeks in Fig. 4.3 A-D). The effects of minocycline on time spent in open arms and total arm entries were maintained in young *Fmr1 KO* mice 4 weeks after treatments had been stopped (4+4 weeks and 8+4 weeks in Fig. 4.3 A-D). No significant differences were observed between WT and *Fmr1 KO* mice older than 3 months of age in the elevated maze (data not shown), suggesting the elevated maze may not be a reliable indicator of behavior in adult mice. Nonetheless, these findings confirm that minocycline effectively reduces anxiety and hyperactivity in young *Fmr1 KO* mice in the

elevated maze, and demonstrate that these effects can be maintained after treatment is stopped, at least for 4 weeks.

Beneficial Effects of Minocycline on Anxiety, but not Hyperactivity, were Maintained in Young *Fmr1 KO* Mice. The open field test was also used to gauge anxiety and hyperactivity by measuring tendency to travel to the center of an open field and total number of line crosses, respectively (Fig. 4.2 A). Both young and adult *Fmr1 KO* mice demonstrated an increased tendency to travel to the center of an open field and made higher numbers of line crosses, along with higher average velocities than age-matched WT mice (Fig. 4.4, 4.5 and 4.6). After 4 weeks of minocycline treatment, the behavioral performance of treated young *Fmr1 KO* mice was significantly improved, as compared to untreated *Fmr1 KO* mice, and was similar to age-matched WT mice (4 weeks in Fig. 4.4 A-C). Treated young *Fmr1 KO* mice made fewer entries into the small and large center squares (4 weeks in Fig. 4.4 A,B), and showed decreases in total line crosses (4 weeks in Fig. 4.4 C). This effect of minocycline on the number of large center entries was maintained in treated young *Fmr1 KO* mice 4 weeks after treatment had been stopped (4+4 weeks in Fig. 4.4 B); whereas, no significant differences were found between numbers of total line crosses of treated and untreated young *Fmr1 KO* mice (4+4 weeks in Fig. 4.4 C).

The ability of minocycline to reduce the number of large center entries in young mice was also maintained 4 weeks after an 8-week-long treatment had

been stopped (8+4 weeks in Fig. 4.4 D,E), whereas the performance of young *Fmr1 KO* minocycline treated mice was not significantly different from untreated age-matched *Fmr1 KO* mice immediately after completion of the 8-week-long treatment (8 weeks in Fig. 4.4 D-F). This delayed effect may have been due to gastrointestinal side effects of the minocycline in treated mice immediately after 8-week-long treatment, which diminished after 4 weeks without minocycline. Note that minocyline had no significant effects on the behavior of WT animals. These results demonstrate that the anxiolytic effect of minocycline in young *Fmr1 KO* mice was independent of its effect on hyperactivity and was maintained 4 weeks following the course of treatment.

Continuous Minocycline Treatment is Necessary to reduce Hyperactivity in Adult *Fmr1 KO* Mice. Adult *Fmr1 KO* mice also made more center entries, a higher number of line crosses, and showed higher velocity than WT mice (Fig. 4.5, 4.6). Similar to young mice, the behavioral performance of adult *Fmr1 KO* mice in the open field test was significantly improved by all measures after 4 weeks of treatment, becoming comparable to age-matched WT mice (4 weeks in Fig. 4.6 A-C). These effects were even more pronounced after an additional 4 weeks of treatment, for a total 8 weeks of treatment (Fig. 4.6 D-F). However, 4 weeks after minocycline treatment was stopped all behaviors of the treated adult *Fmr1 KO* mice reverted to those of the untreated adult *Fmr1 KO* mice (4+4 week and 8+4 week, Fig. 4.6 A-F). The ability of minocycline to decrease both the

number of center entries and total line crosses in treated adult *Fmr1 KO* mice indicates its effectiveness in reducing the hyperactivity of adult mice. These observations demonstrate that both 4- and 8-week-long minocycline treatments are effective in reducing hyperactivity in adult *Fmr1 KO* mice, but maintaining those beneficial effects requires ongoing minocycline treatment.

Minocycline Reduces Marble Burying Behavior in *Fmr1 KO* Mice. Obsessive compulsive disorder (OCD) is a common trait in human subjects with FXS, and we tested a similar perseverance task in mice with the marble burying assay (Fig. 4.2 C). *Fmr1 KO* mice buried significantly more marbles than WT mice at 4 and 8 weeks of age, and as adults (>2 months; Fig. 4.7 A-D). Young *Fmr1 KO* mice treated with minocycline from birth, buried significantly fewer marbles than untreated *Fmr1 KO* mice at 4 and 8 weeks of age (Fig. 4.7 A,B). A significant reduction in marble burying activity was also observed in adult *Fmr1 KO* mice treated for 4 or 8 weeks (Fig. 4.7 C,D). Although marble burying behavior increased in treated adult *Fmr1 KO* mice when they were re-tested 4 weeks post-treatment (4+4 weeks in Fig. 4.7 C,D), young *Fmr1 KO* mice that were treated with minocycline for 8 weeks from birth still buried significantly fewer marbles than untreated *Fmr1 KO* mice when tested 4 weeks post-treatment (8+4 weeks in Fig. 4.7 B). Findings with this task demonstrate that minocycline reduces obsessive-compulsive-like behavior in both young and adult *Fmr1 KO* mice, but

its lasting effects on perseverance are maintained only in young *Fmr1 KO* mice 4 weeks post-treatment.

Minocycline Reduces Audiogenic Seizures in *Fmr1 KO* Mice. At 4 weeks of age *Fmr1 KO* mice are highly susceptible to audiogenic seizures (Fig. 4.8) that are often fatal (Yan et al., 2004; Yan et al., 2005); whereas, WT mice are not susceptible to audiogenic seizures. Minocycline reduced seizure susceptibility as the number of *Fmr1 KO* mice that never seized significantly increased after minocycline treatment (Fig. 4.8 A). Among the mice that did seize, minocycline treated *Fmr1 KO* mice had significantly fewer seizures per animal (Fig. 4.8 B) along with a significantly shorter total duration of seizures (Fig. 4.8 C), compared to untreated *Fmr1 KO* mice. This reduction in seizure susceptibility was accompanied by better survival of *Fmr1 KO* treated with minocycline, although the difference was not statistically significant (Fig. 4.8 D).

DISCUSSION

Here we report the effectiveness of minocycline treatment in alleviating FXS-like behaviors in young and adult *Fmr1 KO* mice. Further, we have clarified the importance of continuous minocycline administration in maintaining the behavioral benefits in both age groups. The open field test measured anxiety and hyperactivity by assessing the tendency of mice to travel to the center of an open field and by scoring the total number of line crosses, respectively (Spencer et al.,

2011). Minocycline treatment promoted a significant reduction of total center entries and total line crosses in both young and adult *Fmr1 KO* mice, indicating that minocycline is effective in reducing hyperactivity in both young and adult *Fmr1 KO* mice. However, interesting differences emerged in the way minocycline treatment affected anxiety and hyperactivity in young versus adult *Fmr1 KO* animals. The reduced tendency of young *Fmr1 KO* mice to travel to the center of the open field persisted 4 weeks post-treatment, but there was little difference in the total number of line crosses between untreated and treated *Fmr1 KO* mice, indicating that minocycline has an ability to affect anxiety in young *Fmr1 KO* mice independent of hyperactivity. On the other hand, adult *Fmr1 KO* mice required continuous minocycline treatment to maintain reductions in the total number of line crosses and tendency to travel to the center of an open field, as both effects were lost 4 weeks post-treatment. These effects of minocycline on hyperactivity in adult *Fmr1 KO* mice likely contributed to the reduced tendency of these animals to travel to the center of the open field.

The ability of minocycline to reduce anxiety was also observed with young *Fmr1 KO* mice in the elevated-plus maze and was retained 4 weeks post-treatment. We and others have found this test to be a reliable indicator of anxiety and hyperactivity in young animals, but inconclusive for adult animals (Carobrez and Bertoglio, 2005; Qin and Smith, 2008; Bilousova et al., 2009; Romero-Zerbo et al., 2009; Yuskaitis et al., 2010). Similar to the open field test, we found significant differences in total entries and time spent in the open arms between

young *Fmr1 KO* and WT mice, both significantly improved by minocycline. Anxiolytic effects of minocycline in the open field test and elevated plus maze were maintained 4 weeks post-treatment, demonstrating a prolonged effectiveness of minocycline for treating anxiety in young *Fmr1 KO* mice.

These studies also demonstrate the beneficial effects of minocycline on obsessive compulsive behavior in *Fmr1 KO* mice, as indicated by marble burying behavior. While there may be an anxiety driven component, marble burying seems to correlate mostly with repetitive and perseverative behavior, similar to obsessive-compulsive tendencies (Gyertyan, 1995; Thomas et al., 2009). Thomas and colleagues (2009) discovered that mice on the FVB background inherently bury more than other strains, and that inherent burying and digging behavior can be significantly decreased if animals are tested within a familiar environment ("home cage") in which they have already spent ample amounts of time prior to testing. Therefore, we habituated mice to their cage overnight to help decrease the inherent digging behavior and to determine if *Fmr1 KO* mice exhibit increased obsessive-compulsive behavior that might be affected by minocycline treatment. Our results are consistent with increased marble burying within the *Fmr1 KO* mice and demonstrate a significant reduction of the behavior immediately following minocycline treatment in both young and adult animals. Moreover, prolonged administration of minocycline demonstrated the maintenance of the effects in young *Fmr1 KO* mice 4 weeks after treatment had been stopped.

From 2-8 years of age, individuals with FXS are susceptible to seizures when presented with specific auditory stimuli (Musumeci et al., 1999), which is replicated in a similar developmentally-dependent fashion in *Fmr1 KO* mice most prominently around 4 weeks of age (Yan et al., 2004; Yan et al., 2005). Minocycline treatment significantly reduced seizure susceptibility in young *Fmr1 KO* mice by decreasing the duration of seizures and the number of seizures per animal, and by increasing the percent of animals that never seized, along with a higher survival trend.

For all of the behaviors examined in this study we discovered that while the effectiveness of minocycline was most profound in young animals, its benefits were not exclusively restricted to a specific age; adult mice also responded to minocycline treatment, with improvements immediately following treatment, that further support its use in treating adult human subjects with FXS. However, our findings indicate that continuous minocycline treatment is required to maintain behavioral improvements in adult animals. A concern of long-term minocycline treatment in both young and adult subjects is a higher risk of developing autoimmunity, which has been reported to occur in ~1 in 10,000, especially in younger individuals (Elkayam et al., 1999; Lawson et al., 2001). We analyzed levels of total immunoglobulin G (IgG; Bethyl Laboratories, E99-131), the class of antibodies responsible for most antibody-based autoimmunity (Corley, 2004), and found neither differences after 4 weeks of continuous minocycline treatment or 6 weeks after minocycline treatment had been stopped in young *Fmr1 KO*

mice (Fig. 4.9), although a frequency of 1 in 10,000 would not have been detectable in these studies. All together, the findings presented here demonstrate that minocycline treatment helps to reverse some behavioral deficits in *Fmr1 KO* mice and these beneficial effects are maintained in young *Fmr1 KO* mice even after treatment was stopped, indicating there may be a developmental window when minocycline treatment is most effective.

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Figure 4.1 Experimental design correlating age of young and adult mice with their duration of minocycline treatment.

Figure 4.1

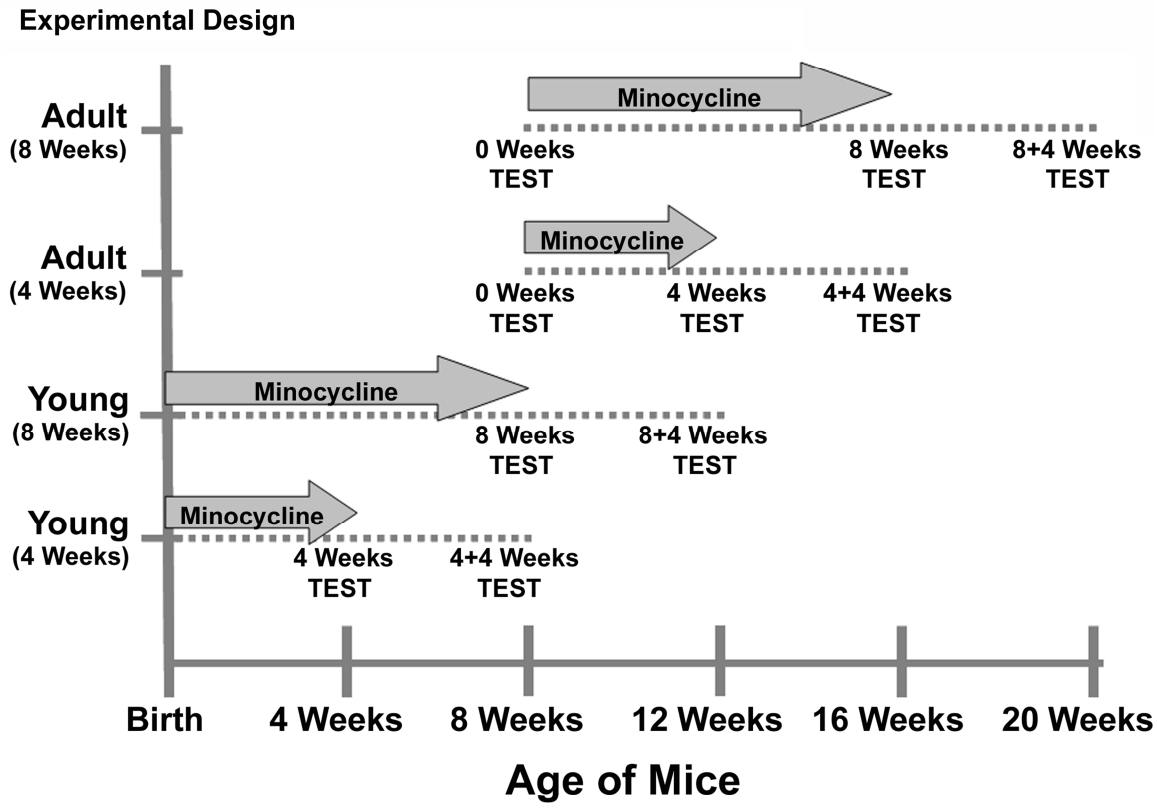
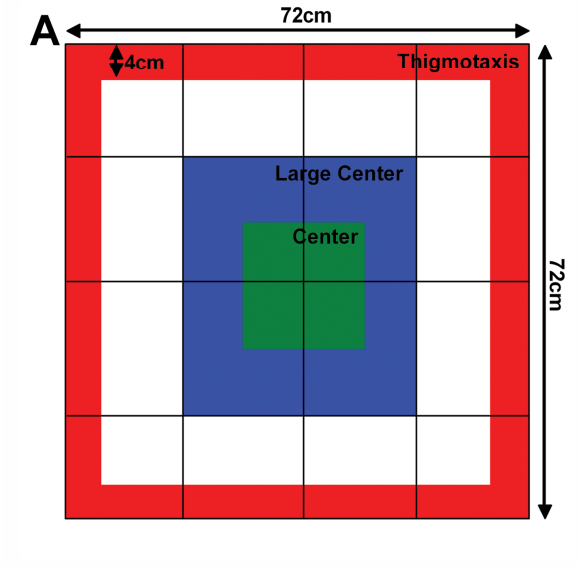
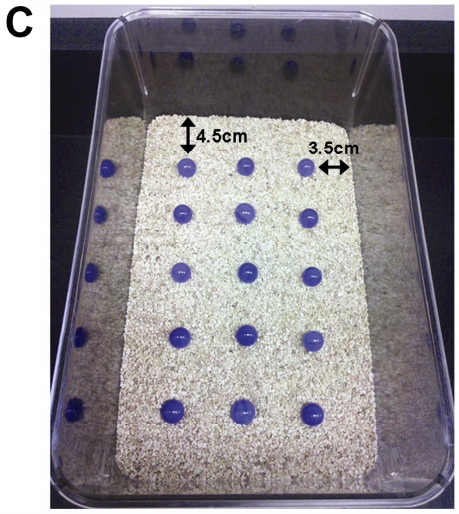

Experimental Design
Adult
(8 Weeks)
Minocycline
0 Weeks
TEST
8 Weeks
TEST
8+4 Weeks
TEST
Adult
(4 Weeks)
Minocycline
0 Weeks
TEST
4 Weeks
TEST
4+4 Weeks
TEST
Young
(8 Weeks)
Minocycline
8 Weeks
TEST
8+4 Weeks
TEST
Young
(4 Weeks)
Minocycline
4 Weeks
TEST
4+4 Weeks
TEST
Birth
4 Weeks
8 Weeks
12 Weeks
16 Weeks
20 Weeks
Age of Mice

Figure 4.2 Diagrams of various mazes used for behavior analysis of young and adult mice. (A) Diagram of the floor outlining the parameters used for open field analysis. (B) Picture of the elevated maze used for behavioral analysis. (C) Picture of a typical cage layout for marble burying analysis.

Figure 4.2


A
72cm
4cm
Thigmotaxis
Large Center
Center
72cm


C
4.5cm
3.5cm

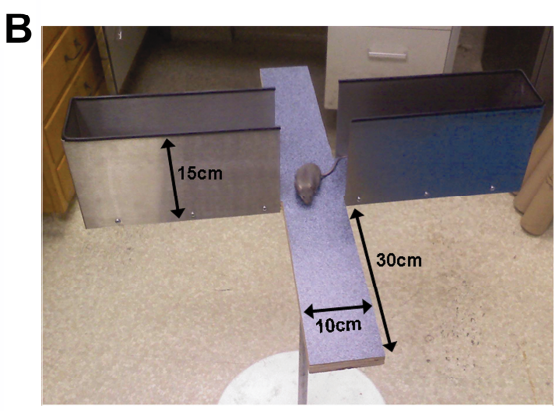

B
15cm
30cm
10cm

Figure 4.3 Behavioral performance of young *Fmr1 KO* mice in the elevated plus maze. (A-D) Graphs illustrating the performance of young wild-type (WT) and *Fmr1 KO* (KO) mice in the elevated maze as measured by the total number of entries (A, B) and the time spent in the open arms per entry (C, D). The left panels represent their performance immediately after 4 weeks (A, C) and 8 weeks (B, D) of minocycline treatment. The right panels show their performance 4 weeks after the respective treatments had stopped (4+4 weeks or 8+4 weeks, A-D). Vertical bars indicate SEM. (n = 13-24 mice per treatment group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ indicate significant differences between untreated WT and *Fmr1 KO* mice; +, $p<0.05$; ++, $p<0.01$; +++, $p<0.001$ indicate significant differences between treated and untreated *Fmr1 KO* mice).

Figure 4.3

Elevated Maze (Young mice)

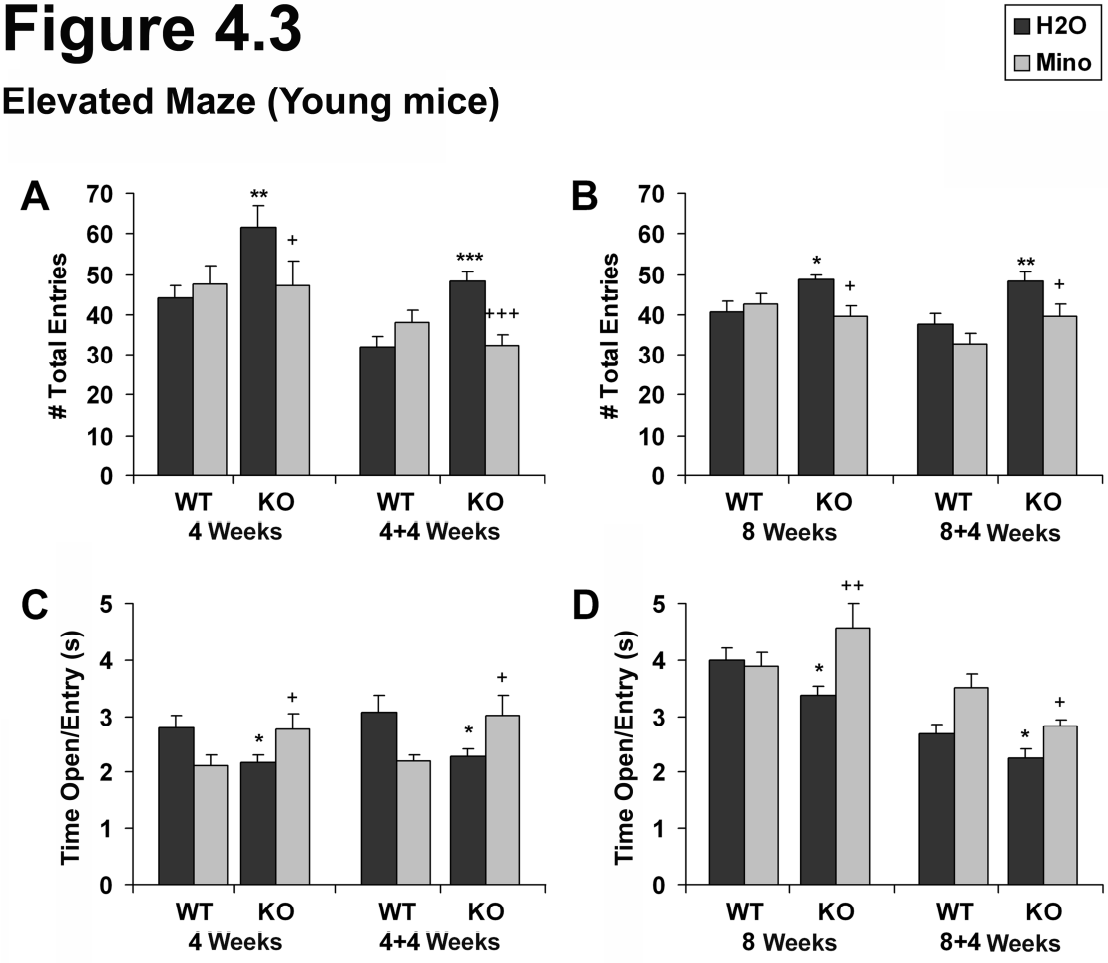


Figure 4.4 Minocycline alters the performance of young mice in the open field test and the effects are maintained 4 weeks post-treatment. (A-F) Graphs illustrating the performance of young wild-type (WT) *Fmr1 KO* (KO) mice in the open field as measured by the number of small center entries (A, D), the number of large center entries (B, E), and the total number of line crosses (C, F). The left panels show their performance after 4 weeks of treatment (4 weeks, A-C) or 8 weeks of treatment (8 weeks, D-F). The right panels show their performance 4 weeks after the treatments had stopped (4+4 weeks, A-C or 8+4 weeks, D-F). Vertical bars indicate SEM (n = 13-23 mice per treatment group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ indicate significant differences between untreated WT and *Fmr1 KO* mice; +, $p<0.05$; ++, $p<0.01$; +++, $p<0.001$ indicate significant differences between treated and untreated *Fmr1 KO* mice).

Figure 4.4

Open Field (Young mice)

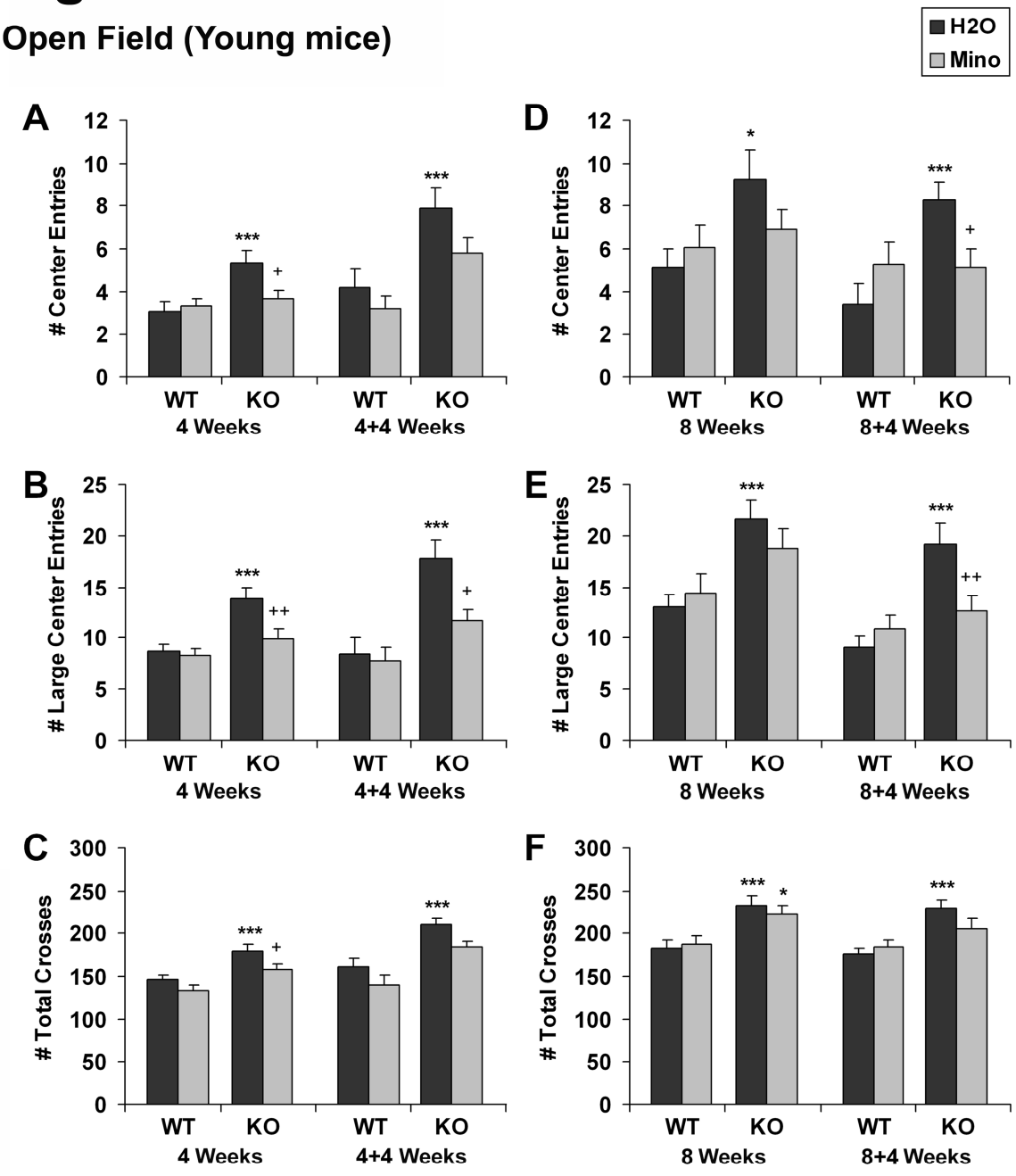


Figure 4.5 Effect of minocycline on velocity of young and adult *Fmr1 KO* mice in the open field test (A-D) Graphs illustrating the overall velocity of young (A, B) and adult (C, D) wild-type (WT) and *Fmr1 KO* (KO) mice in the open field. The left panel show their performance after 4 weeks of treatment (4 weeks, A, C) or 8 weeks of treatment (8 weeks, B, D). The right panel show their performance 4 weeks after their respective treatments had stopped (4+4 weeks, A, C or 8+4 weeks, B, D). Vertical bars indicate SEM (n = 13-23 mice per treatment group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ indicate significant differences between untreated WT and *Fmr1 KO* mice; +, $p<0.05$; ++, $p<0.01$; +++, $p<0.001$ indicate significant differences between treated and untreated *Fmr1 KO* mice).

Figure 4.5

Open Field

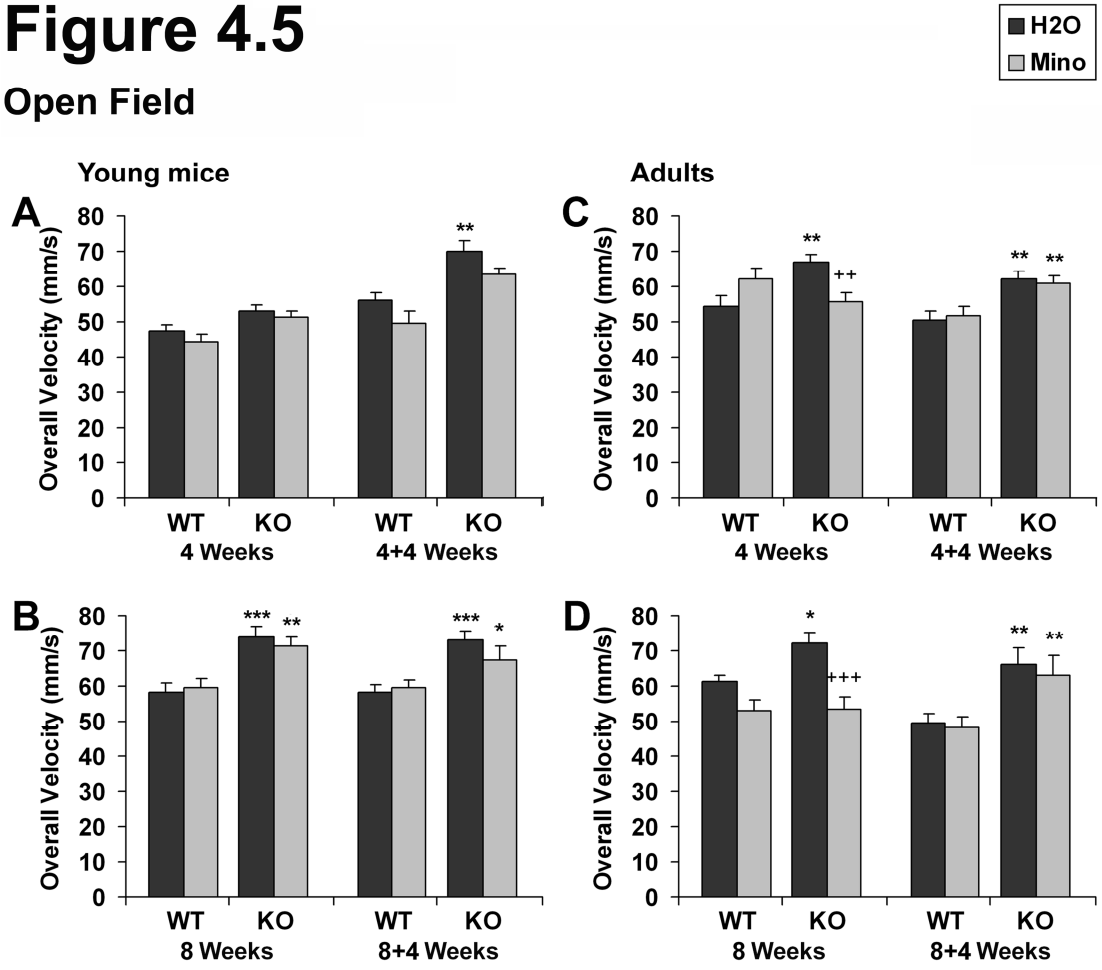


Figure 4.6 Minocycline-treated adult *Fmr1 KO* mice demonstrated reduced hyperactivity and tendency to travel to the center of an open field, but these effects were not maintained 4 weeks post-treatment. (A-F) Graphs illustrating the performance of adult wild-type (WT) and *Fmr1 KO* (KO) mice in the open field as measured by the number of small center entries (A, D), the number of large center entries (B, E), and the total line crosses (C, F). The left panels show their performance after 4 weeks of treatment (4 weeks, A-C) or 8 weeks of treatment (8 weeks, D-F). The right panels show their performance 4 weeks after their treatments had stopped (4+4 weeks, A-C or 8+4 weeks, D-F). Vertical bars indicate SEM (n = 13-23 mice per treatment group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ indicate significant differences between untreated WT and *Fmr1 KO* mice; +, $p<0.05$; ++, $p<0.01$; +++, $p<0.001$ indicate significant differences between treated and untreated *Fmr1 KO* mice.

Figure 4.6

Open Field (Adults)

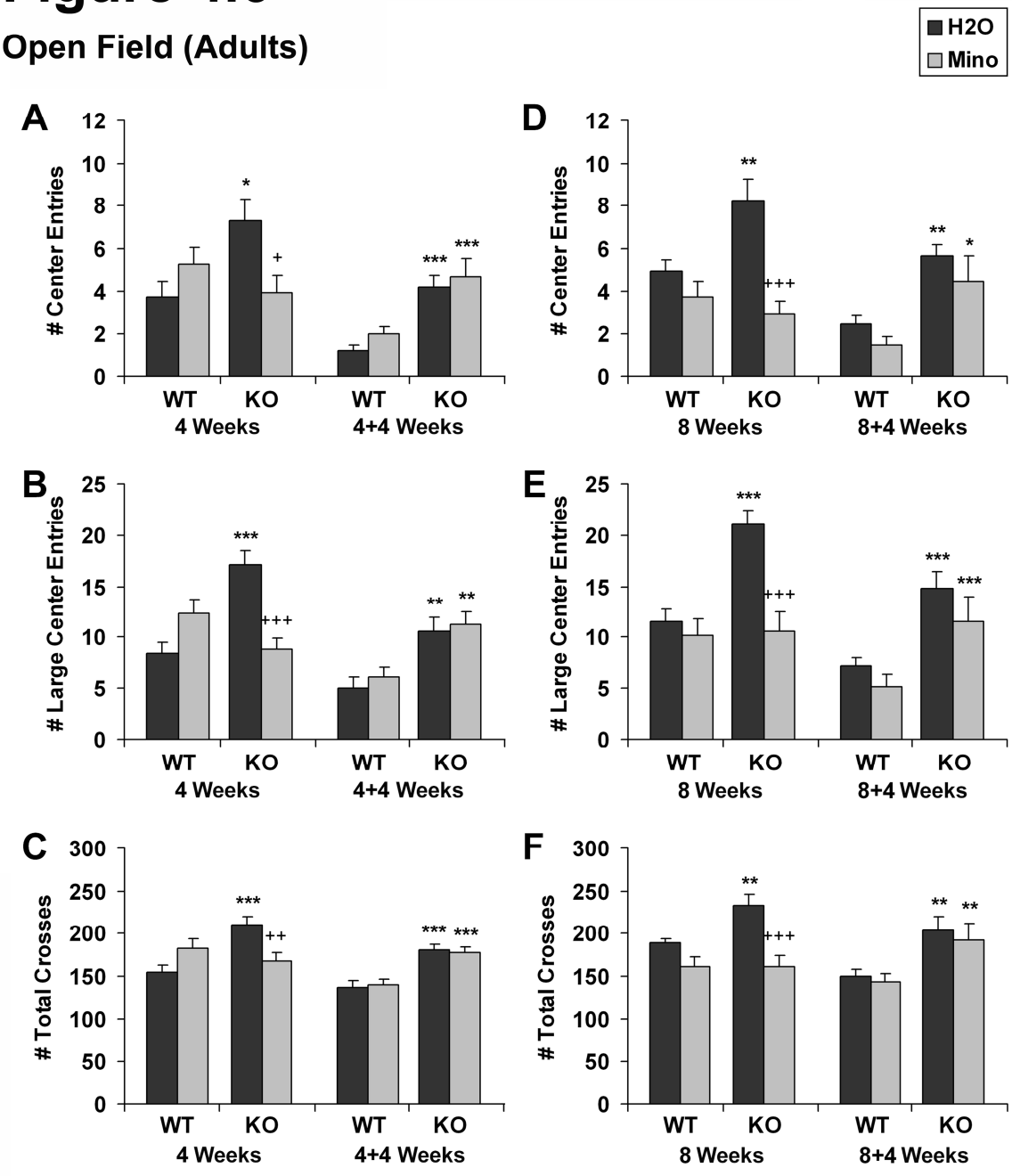


Figure 4.7 Minocycline decreased obsessive-compulsive behavior in young and adult *Fmr1 KO* mice and demonstrated maintenance of the effect. (A-B) Quantitative analysis of the number of marbles buried by wild-type (WT) and *Fmr1 KO* (KO) young mice: after 4 weeks of continuous treatment (4 weeks, A), 4 weeks after the 4-week-long treatment had stopped (4+4 weeks, A), after 8 weeks of continuous treatment (8 weeks, B), and 4 weeks after the 8-week-long treatment had stopped (8+4 weeks, B). (C-D) Quantitative analysis of the number of marbles buried by adult mice: immediately before beginning the treatment (0 weeks, C and D), after 4 weeks of continuous treatment (4 weeks, C), 4 weeks after the 4-week-long treatment had stopped (4+4 weeks, C), after 8 weeks of continuous treatment (8 weeks, D), and 4 weeks after the 8-week-long treatment had stopped (8+4 weeks, D). Vertical bars indicate SEM (n = 13-24 mice per treatment group for young mice, n = 14-23 mice per treatment group for adult mice; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ indicate significant differences between untreated WT and *Fmr1 KO* mice; +, $p<0.05$; ++, $p<0.01$; +++, $p<0.001$ indicate significant differences between treated and untreated *Fmr1 KO* mice).

Figure 4.7

Marble Burying

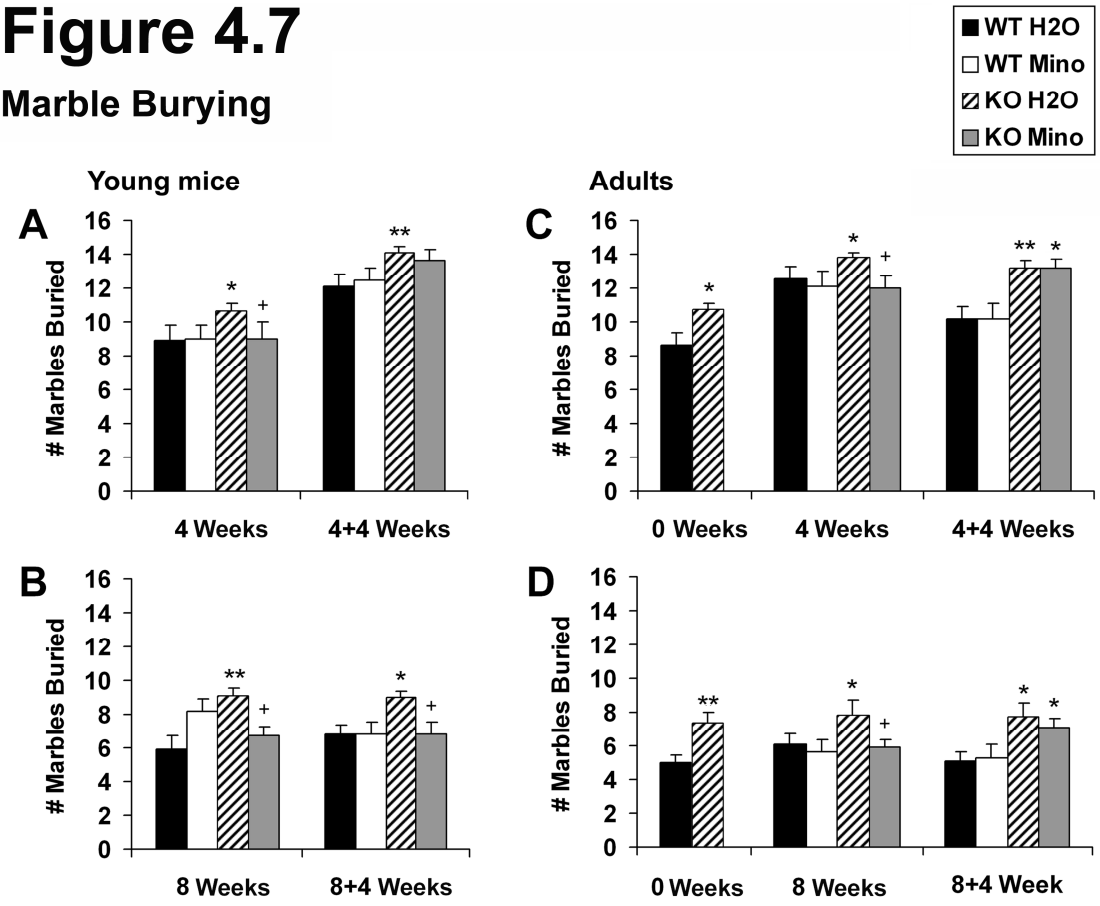


Figure 4.8 Minocycline reduced susceptibility of P28-30 *Fmr1 KO* mice to audiogenic seizures and increased the odds of survival. Comparison of seizure susceptibility in wild-type (WT) and *Fmr1 KO* (KO) mice with and without minocycline treatment. (A) The percent of animals that never seized. (B) The average number of seizures per animal. (C) The total duration of tonic seizures. (D) The percent of animals that survived the test. Minocycline treatment provided a 2-fold increase in the rate of survival in the *Fmr1 KO* mice (D) and caused a 7-fold reduction in the seizure activity of *Fmr1 KO* mice (A). When applicable, statistical analysis was performed using one-way ANOVA following following which post-hoc pair-by-pair differences were resolved using Dunnett's comparisons with control and Hsu's multiple comparisons with the best (B,C). For all other measures, statistical differences were resolved using Fisher's Exact Test, effect of likelihood Chi-square test and the odds ratio comparison (A,D). When present, vertical bars indicate SEM (n = 13-24 mice per treatment group; +, $p<0.05$; ++, $p<0.01$; +++, $p<0.001$ indicate significant differences between treated and untreated *Fmr1 KO* mice).

Figure 4.8

Audiogenic Seizures

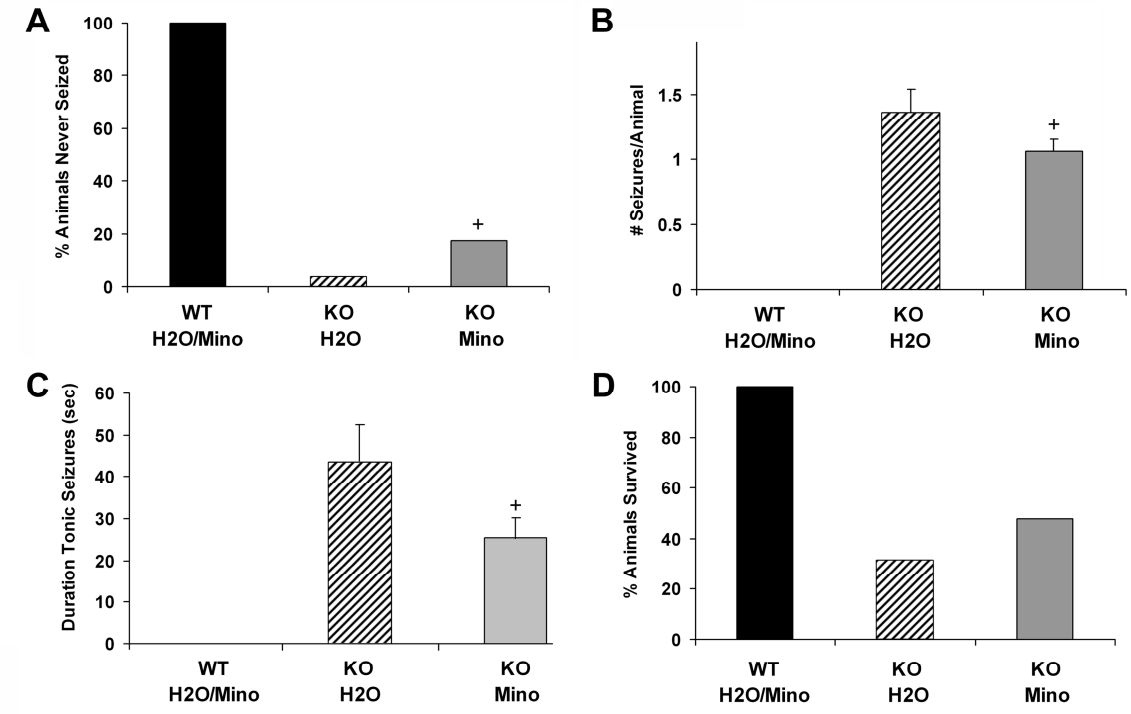
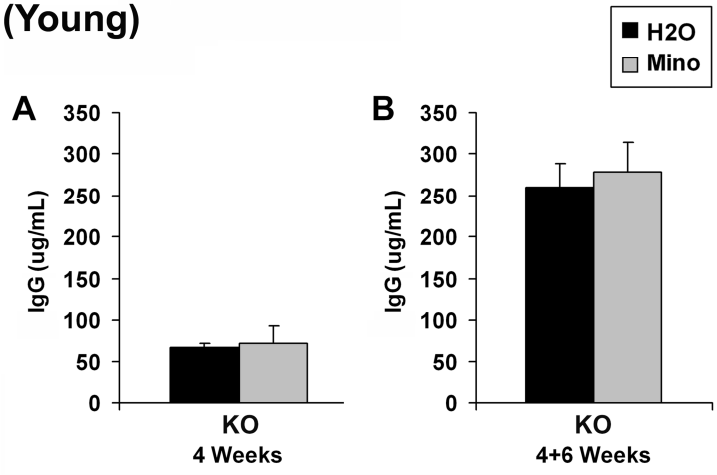


Figure 4.9 Analysis of IgG levels following minocycline treatment in *Fmr1 KO* mice. (A-B) Graph shows total IgG levels in blood samples of untreated and minocycline treated *Fmr1 KO* (KO) mice after 4-week-long treatment from birth (4 weeks, A) and 6 weeks after treatment had stopped (4+6 weeks, B). There was no difference in total IgG between minocycline treated and untreated *Fmr1 KO* mice. Vertical bars indicate SEM (n = 6 mice per treatment group for A, n = 7 mice per treatment group for B; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Figure 4.9

(Young)



Chapter 5 – The Relationship of Matrix Metalloproteinase-9 and its Endogenous Inhibitor to the Human Condition of Fragile X Syndrome

ABSTRACT

Fragile X Syndrome (FXS) is the most common single-gene inherited form of impaired intellectual development, with behavioral deficits characteristic of autism. Our previous work has characterized a role for MMP-9 in contributing to the abnormal dendritic spine development within the FXS mouse model (*Fmr1 KO*). Dendritic spines are the primary, postsynaptic compartments of excitatory synaptic connections. In chapters 2 and 3, we determined that MMP-9 can promote immature dendritic spine morphology and its total levels and activity were upregulated within the hippocampus of the *Fmr1 KO*. Based on this data we were curious whether MMP-9 also contributes to the human condition of FXS. In this study, we analyzed the relative active and total levels of MMP-9 within blood plasma samples from FXS patients as well as postmortem neocortical and hippocampal brain tissue samples. We discovered that the ratio of active to total MMP-9 was significantly decreased within the blood of FXS patients while total levels of MMP-9 were significantly increased within the hippocampus and neocortex of postmortem FXS brain tissue. This is the first evidence that connects MMP-9 to the pathology of the human condition of FXS.

INTRODUCTION

Thus far our work has demonstrated that increased MMP-9 expression and activity within the hippocampus of the *Fmr1 KO*, the Fragile X mouse model, contributes to its abnormal dendritic spine profile. We have also identified minocycline as a potential therapeutic option for the correction of structural and behavioral abnormalities associated with Fragile X Syndrome (FXS). Based on this research, clinical trials have begun utilizing minocycline for the treatment of Fragile X and have shown promising results. An open-label trial conducted in Canada revealed that minocycline provided significant functional benefits to FXS patients and that it was a well-tolerated drug (Paribello et al., 2010). They demonstrated significant behavioral improvements including decreases in perseverative and repetitive behaviors, anxiety, self-injurious activities, abnormal vocalizations, and mood swings (Paribello et al., 2010). Currently a double-blind study is underway that hopes to further characterize how beneficial minocycline may be in treating FXS. Thus far results from that study have indicated improvements in language use, such as the use of more intelligible and expressive language as well as increased verbal output (Utari et al., 2010). If minocycline becomes a valuable therapeutic for the treatment of FXS it would be important to characterize how minocycline is promoting its beneficial effects. Our theory thus far has hypothesized that minocycline is primarily acting through inhibition of MMP-9 to promote morphological and behavioral recovery within *Fmr1 KO* mice. To analyze the role of MMP-9 as it relates to the human

condition, we obtained plasma blood samples from Fragile X patients as well as neocortical and hippocampal postmortem brain samples from individuals who were diagnosed with Fragile X and analyzed them for the relative concentrations and activities of MMP-9, the closely related gelatinase family member MMP-2, as well as the primary endogenous inhibitor of MMP-9, the tissue inhibitor of metalloproteinases-1 (TIMP-1).

MATERIALS AND METHODS

Brain Tissue Samples

Brain tissue samples of human neocortex and hippocampus were obtained from Dr. Cara Westmark from the University of Wisconsin with permission from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. Tissue samples were lysed at 10% w/v in buffer containing 10mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, and 0.5% Triton.

Blood Plasma Samples

Dr. Berry-Kravis and colleagues collected blood plasma samples from Fragile X patients using a previously described method (Jung et al., 1998; Jung, 2005). Briefly, blood was collected in S-Monovette lithium heparin-coated plastic tubes (Sarstedt; Monovette system 03.1628). All tubes were stored at room temp and were centrifuged within 30 min after venipuncture at 1,600 x g for 15 min at

4^{O}C. The supernatants were carefully removed, centrifuged again at 10,000 x g, and stored at -80^{O}C until analysis was performed.

Activity, ELISA and total protein assays

Endogenous and total activities of either MMP-2 or MMP-9 were measured in appropriately diluted plasma blood and tissue lysate samples following the respective protocols for lower endogenous levels using the Amersham MMP Biotrak Activity Assays (RPN2631, RPN2634, GE Healthcare). Total levels of TIMP-1 were measured in appropriately diluted plasma blood and tissue lysates samples following the protocol for the Amersham TIMP-1 Human Biotrak ELISA System (RPN2611, GE Healthcare). Original brain tissue lysate samples were diluted in distilled water and measured for total protein concentrations following the protocol for the BCA colorimetric protein assay (Pierce, 23235). Appropriate dilutions were determined for every trial by running a dilution curve on two randomly selected prepared samples. Data processing was performed following the instructions provided with the protocols. Each assay was performed at least two times and each sample was analyzed in duplicate or triplicate.

RESULTS

Increased MMP-9 total activity, but no change in the levels of TIMP-1, in the postmortem neocortex and hippocampus of individuals with FXS.

Since our previous data has indicated that increased levels of expression and activity of MMP-9 within the hippocampus of the *Fmr1 KO* may be contributing to the pathology, we analyzed postmortem human brain tissue from the neocortex and hippocampus to see if MMP-9 levels were altered in the human condition. Expression of the endogenous inhibitor of MMP-9, TIMP-1, was not significantly changed in the neocortex or the hippocampus (Fig. 5.1 A), whereas levels of MMP-9 were significantly upregulated in both areas, especially within the neocortex of individuals who had FXS (Fig. 5.1 D). Total protein levels were also upregulation within the FXS neocortex but not the FXS hippocampus as compared to controls (Fig. 5.1 B, E). The ratio of total TIMP-1 to total protein levels revealed no differences between FXS and control samples within the neocortex or hippocampus (Fig. 5.1 C), while the proportion of MMP-9 was significantly increased within both areas in FXS samples as compared to controls (Fig. 5.1 F). These results provide evidence that MMP-9 is increased in the brain of FXS individuals similar to what has been observed in the mouse model. Therefore, if the actions of MMP-9 within the human condition are similar to the mouse, it is possible that MMP-9 may contribute to the abnormal dendritic spine deficits found in FXS individuals.

MMP-9, but not MMP-2, is reduced in blood plasma samples from FXS patients while TIMP-1 tends to be increased. Since levels of total MMP-9 were so elevated in the brain, we wanted to determine if MMP-9 expression was

also altered in the blood. Analysis of the ratio of active to total MMP levels revealed a significant reduction of only MMP-9 (Fig. 5.2 F) with no changes in MMP-2 (Fig. 5.2 C) within the plasma blood samples from FXS patients. Furthermore, 9 out of 10 FXS patients demonstrated a significant increase in their levels of TIMP-1 (Fig. 5.2 G) and the ratio of active MMP-9 to TIMP-1 was significantly decreased (Fig. 5.2 I). With respect to MMP-9, the decrease in the relative concentration of active MMP-9 in the blood was in contrast to the total MMP-9 activity found in brain. It is possible that different mechanisms control MMP-9 regulation between the two regions which could cause the dramatic difference in expression levels especially if either of the mechanisms are adversely altered in FXS. Conversely, the body may recognize the pathologic increase of MMP-9 within the brain and is attempting to reduce it. Within the brain the only endogenous MMP-9 inhibitor is TIMP-1, while within the blood there are several others, including α2-macroglobulin and plasminogen activator inhibitor 1, which may lead to the significant decrease of MMP-9 within the blood.

DISCUSSION

After the discovery that hypersensitivity to Group 1 metabotropic glutamate receptors (mGluRs) contributed to the pathology observed in the *Fmr1 KO* (Bear et al., 2004) research began looking at how components of the downstream pathways, such as extracellularly-regulated kinases (ERKs) and the glycogen synthase kinases (GSKs), may be altered due to the hypersensitivity

(Min et al., 2009; Mines et al., 2010; Osterweil et al., 2010; Yuskaitis et al., 2010). Work from previous chapters of this dissertation has demonstrated that MMP-9 levels can be regulated by mGluR5 activity and are upregulated within hippocampus of the *Fmr1 KO*. Application of active MMP-9 to cultured hippocampal neurons can promote a dendritic spine profile characteristic of the *Fmr1 KO*, while specific inhibition of MMPs with the preferential MMP-2 and MMP-9 specific inhibitor, SB-3CT, can promote dendritic spine maturation. Furthermore, MMP-9 inhibition with minocycline can also promote dendritic spine maturation as well as significantly improve behavioral deficits within the *Fmr1 KO* and even have beneficial long-term effects, primarily when administered during development. The developmental window is of particular interest since research has shown that MMPs are regulated during development and in response to learning and memory.

Many MMP and TIMP mRNA transcripts were found to be upregulated within the proencephalon and rhombencephalon of 1 week old mice (Ulrich et al., 2005), including the gelatinases MMP-2 and MMP-9 as well as the primary endogenous inhibitor for MMP-9, TIMP-1. Within the cerebellar cortex, MMP-2 and MMP-9 mRNA transcripts were shown to have an initial decrease between postnatal day 3 (P3) to P6 (Ayoub et al., 2005), while gelatin zymography demonstrated that their expression and activity persisted longer than their transcripts with a decrease occurring around P9 and reaching undetectable levels by P21. With respect to learning and memory, there was an increase in

the levels of MMP-3 and MMP-9 following successful learning in the Morris water maze in the hippocampus of mice (Meighan et al., 2006). With these levels of regulation during development and expression in response to learning, it is not surprising that excessive alterations in the activity or expression of MMPs would cause impairments that could underlie cognitive dysfunctions.

MMPs were classically characterized for the role in regulating the blood-brain barrier (Zlokovic, 2008), and have been suggested to increase inflammation, degrade the neurovascular matrix and promote neuronal apoptosis associated with cerebral ischemia (Lo et al., 2002; Lee et al., 2004; Cunningham et al., 2005; Zhao et al., 2006). In addition to the blood-brain barrier, recent work has indicated a role for MMPs is affecting synapse pathologies associated with epileptogenesis. Following kainite-evoked seizures there is an increase in MMP-9 expression which is thought to promote fiber sprouting and reactive synaptogenesis (Szklarczyk et al., 2002; Wilczynski et al., 2008). Increased MMP-9 activity was also found in patients who suffer from mild cognitive impairment and Alzheimer's disease (Bruno et al., 2009). In addition to these studies, our work has now demonstrated a role for MMP-9 to be involved in the pathology of FXS.

Our analysis of postmortem FXS brain tissue clearly demonstrated an upregulation of MMP-9 in both the neocortex and hippocampus, indicating that MMP-9 is likely contributing to the pathology of the human condition of FXS, similar to the *Fmr1 KO*. Interestingly, the ratio of active MMP-9 was significantly

reduced in plasma blood samples from FXS patients, while there was a trend for the main endogenous MMP-9 inhibitor, TIMP-1, to be increased. This difference between the brain and the blood may be a result of the body attempting to reduce the overexpression of MMP-9. Within the brain the only endogenous MMP-9 inhibitor is TIMP-1, whereas in the blood there are other components that can directly or indirectly inhibit MMPs, such as α2-macroglobulin and plasminogen activator inhibitor 1 (Baker et al., 2002; Visse and Nagase, 2003) which may allow for more effective clearing in the blood as opposed to the brain. Ultimately the work shown here demonstrates the first connection illustrating MMP-9 as a potential contributor to the human condition of FXS.

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Figure 5.1 MMP-9, but not TIMP-1, is significantly upregulated within the neocortex and hippocampus of individuals with FXS. (A-D) Levels of total TIMP-1 (A-B) or total MMP-9 (C-D) within the neocortex (NeoC) and hippocampus (Hipp) of individuals with FXS normalized to mass of lysed tissue sample (A, C) or total protein (B, D). (E) Total protein within the neocortex and hippocampus of individuals with FXS. Statistical differences were resolved using Student's t-test. (Control NeoC = 4 samples, Control Hipp = 4 samples, FXS NeoC = 4 samples, FXS Hipp = 3 samples; *, $p<0.05$).

Figure 5.1

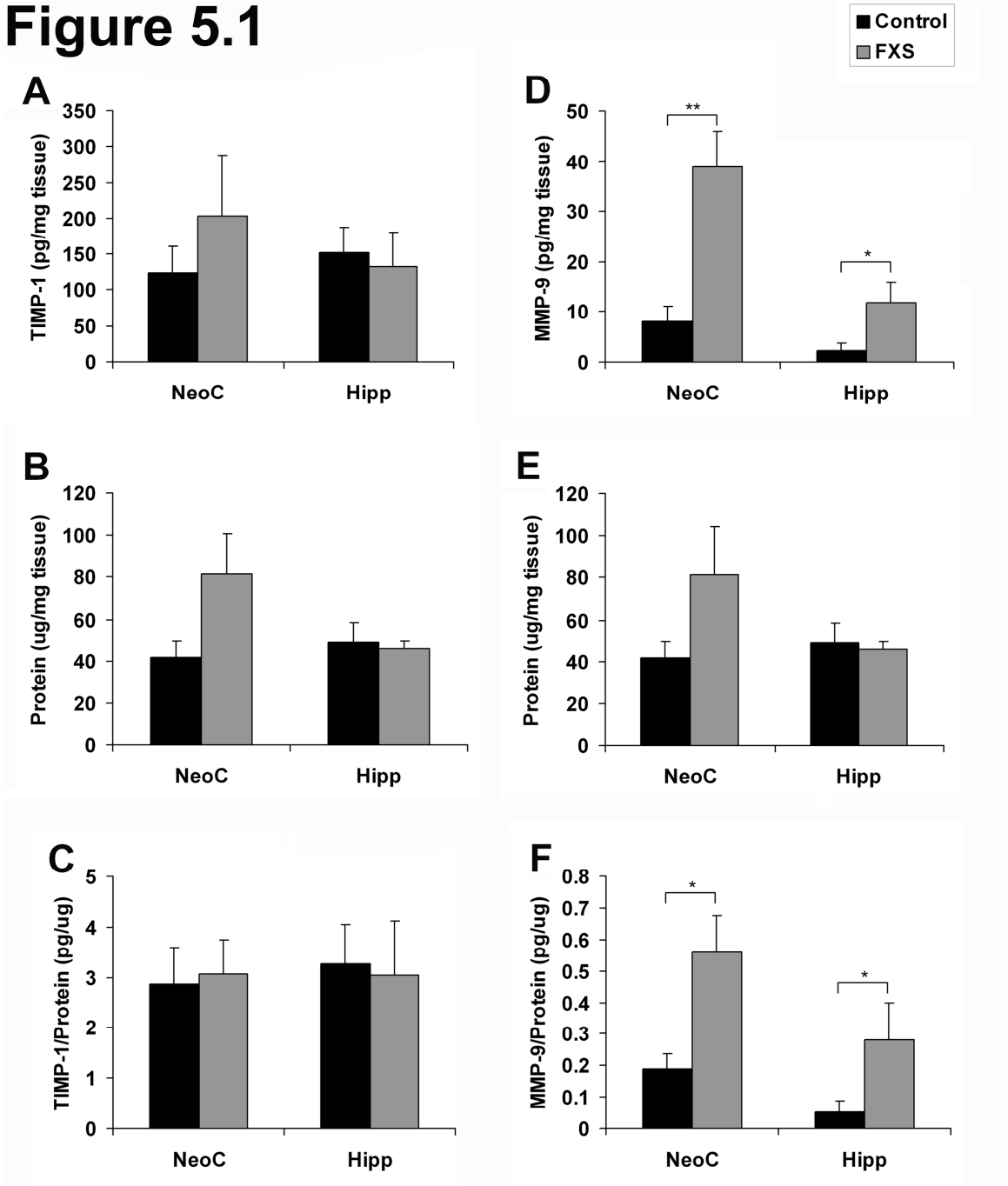
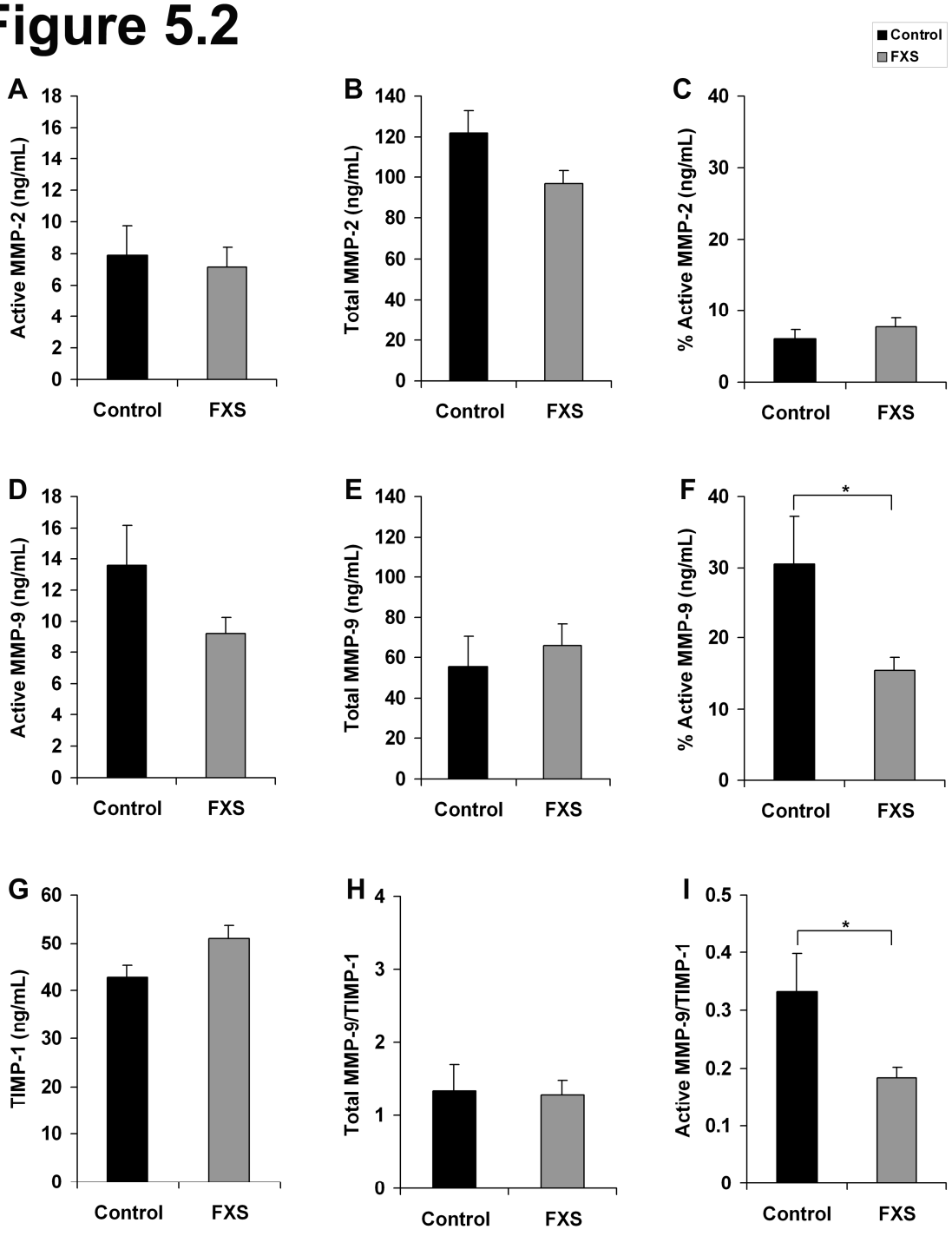

Control
FXS
A
TIMP-1 (pg/mg tissue)
350
300
250
200
150
100
50
0
NeoC
Hipp
D
MMP-9 (pg/mg tissue)
50
40
30
20
10
0
**
*
NeoC
Hipp
B
Protein (ug/mg tissue)
120
100
80
60
40
20
0
NeoC
Hipp
E
Protein (ug/mg tissue)
120
100
80
60
40
20
0
NeoC
Hipp
C
TIMP-1/Protein (pg/ug)
5
4
3
2
1
0
NeoC
Hipp
F
MMP-9/Protein (pg/ug)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
NeoC
Hipp

Figure 5.2 Plasma blood samples from Fragile X patients contain a significantly lower percentage of active MMP-9, and tend to have higher levels of TIMP-1, but show no differences in levels of MMP-2. (A-F) Levels of active MMP-2 (A) and MMP-9 (D), total MMP-2 (B), MMP-9 (E), and TIMP-1 (G), percentage of active MMP-2 and MMP-9 normalized to total MMP-2 (C) and MMP-9 (F), respectively, total MMP-9 normalized to TIMP-1 (H), active MMP-9 normalized to TIMP-1 (I). The percent of active MMP-9 was significantly lower in FXS patients (F), while active MMP-2 levels and percent of active MMP-2 (A, C) were not significantly different. In contrast, 9 out of 10 FXS patients showed significantly higher levels of TIMP-1 (G). Furthermore the ratio of active MMP-9 to TIMP-1 was significantly decreased in FXS patients (I). Statistical differences were resolved using Student's t-test. (Control = 7 patients, FXS = 9 patients; *, $p<0.05$)

Figure 5.2


Control
FXS
A
Active MMP-2 (ng/mL)
0
2
4
6
8
10
12
14
16
18
Control
FXS
B
Total MMP-2 (ng/mL)
0
20
40
60
80
100
120
140
Control
FXS
C
% Active MMP-2 (ng/mL)
0
10
20
30
40
Control
FXS
D
Active MMP-9 (ng/mL)
0
2
4
6
8
10
12
14
16
18
Control
FXS
E
Total MMP-9 (ng/mL)
0
20
40
60
80
100
120
140
Control
FXS
F
*
% Active MMP-9 (ng/mL)
0
10
20
30
40
Control
FXS
G
TIMP-1 (ng/mL)
0
10
20
30
40
50
60
Control
FXS
H
Total MMP-9/TIMP-1
0
1
2
3
4
Control
FXS
I
*
Active MMP-9/TIMP-1
0
0.1
0.2
0.3
0.4
0.5
Control
FXS

Chapter 6 - Conclusions

Dendritic spines are the microscopic, biochemical chambers that form on the surface of dendrites and accommodate the majority of post-synaptic excitatory transmissions within the brain (reviewed in Sorra and Harris, 2000; Hering and Sheng, 2001; Yuste and Bonhoeffer, 2001; Ethell and Pasquale, 2005). Spines are highly plastic, actin-rich structures, even within the adult brain, whose morphology is of paramount importance in determining the maturity and stability of their synaptic connections. Prior to spine formation is the emergence of long, thin, headless extensions known as filopodia which can transform into immature spines upon contact with axon terminals (Dailey and Smith, 1996; Ziv and Smith, 1996; Maletic-Savatic et al., 1999; Lendvai et al., 2000; Marrs et al., 2001; Okabe et al., 2001; Trachtenberg et al., 2002; Portera-Cailliau et al., 2003; Ziv and Garner, 2004; Knott et al., 2006). Immature spines, or thin spines, are typically characterized by long necks and small heads and tend to be more flexible as well as more susceptible to changes induced by synaptic input (Lang et al., 2004; Matsuzaki et al., 2004; Nimchinsky et al., 2004; Ashby et al., 2006; Zhang et al., 2010). Immature spine can transform into more stable mature spines that are typically characterized by either a mushroom shape with a shorter, thinner neck and a larger head or a stubby shape with a head area that has no discernible neck. Dendritic spine heads contain a proportionally sized specialized area known as the post-synaptic density (PSD) which is filled with an

entire host of receptors and signaling proteins responsible for transducing the excitatory neurotransmissions. Changes in the size of the PSD are correlated with changes in the size of spine head and maturity which both affect inherent spine stability (Matsuzaki et al., 2001).

Whereas filopodia and immature spines tend to be transient protrusions, the majority of mature spines are highly stable structures that can last from months to a lifetime (Grutzendler et al., 2002; Holtmaat et al., 2005; Zuo et al., 2005; Zuo et al., 2005; Yang et al., 2009; Zhang et al., 2010). However, mature spines do remain plastic within the adult brain and are susceptible to structural remodeling such as enlargement, reduction or complete elimination in response to a variety of stimulatory events. Spine plasticity occurs in response to normal physiological changes in synaptic activity which underlies learning and memory. The induction of long-term potentiation (LTP) or long-term depression (LTD) can enhance or decrease synaptic strength, respectively, to promote changes in dendritic spine morphology. LTP has been correlated with dendritic spine formation and enlargement (Lang et al., 2004; Matsuzaki et al., 2004; Nagerl et al., 2004; Okamoto et al., 2004; Otmakhov et al., 2004) whereas LTD can result in dendritic spine shrinkage and elimination (Nagerl et al., 2004; Okamoto et al., 2004; Zhou et al., 2004; Bastrikova et al., 2008). Stimulation of LTP or LTD is affected by receptor signaling which is triggered by presynaptic input and can be altered by extracellular influence located within the synaptic cleft via components of the extracellular matrix (ECM).

Surrounding neurons in the brain is a specialized form of ECM known as perineuronal nets (PNNs), which envelopes neuronal cell bodies and proximal dendrites in order to control their three-dimensional organization, growth, movement and shape required to maintain their structural integrity (Celio et al., 1998). There are a variety of components to the ECM which serve different functional roles such as repulsion versus adhesion and can have a variety of properties including rigidity or elasticity (Mecham, 1998; Kleinman et al., 2003). Structural elements of the ECM include scaffolding proteins such as laminin, fibronectin and tenascin which link the ECM into a net, as well as remodeling proteins such as matrix metalloproteinases (MMPs) which cleave and modify the ECM.

MMPs are members of the family of metzincin endopeptidases, and were named for their ability to cleave the ECM. To date there have been 24 MMPs identified which share a highly conserved catalytic domain (reviewed in Mott and Werb, 2004; Ethell and Ethell, 2007; Wilczynski and Kaczmarek, 2008). Most MMPs are secreted and first exist as inactive zymogens which require displacement or removal of their pro-domain to allow access to and functioning of their catalytic site. Within the brain several MMPs are expressed, including the subfamily of gelatinases, MMP-2 and MMP-9, which have been shown to be regulated during development and in response to learning (Ayoub et al., 2005; Ulrich et al., 2005; Meighan et al., 2006). With respect to the hippocampus, the region of primary focus within this work as it is highly implicated in learning and

memory, many studies have shown MMP-9 to be of primary importance. MMP-9 is increased in the hippocampus of mice following successful learning in the Morris water maze (Meighan et al., 2006), and has been recently implicated in promoting changes to dendritic spine morphology through an integrin-dependent mechanism (Wang et al., 2008; Michaluk et al., 2011). Whereas Michaluk and colleagues (2011) have demonstrated a role for MMP-9 to promote formation of long, thin dendritic spines, the Huntley lab (2008) suggests that MMP-9 drives dendritic spine enlargement coordinately with LTP. Our work is more synonymous with Michaluk and colleagues and has demonstrated a role for MMP-9 to promote dendritic spine elongation within cultured hippocampal neurons which is further corroborated by analysis of cultured hippocampal *mmp9*$^{-/-}$ neurons, which have a higher occurrence of mature spines in the absence of MMP-9. Our work indicates a role for MMP-9 in dendritic spine development and may also indicate it as having the potential to play a causative role in abnormal dendritic spine formation. Studies have also shown that changes in MMP-9 levels and activity are linked to dendritic spine pruning associated with cognitive impairments, such as epilepsies (Wilczynski et al., 2008). Furthermore, the spine morphology we see upon analysis of MMP-9 treated neurons is reminiscent of the immature dendritic spine phenotype from many types of intellectual disabilities, including Rett Syndrome, Down's Syndrome and Fragile X Syndrome (FXS; Irwin et al., 2000; Kaufmann and Moser, 2000; Fiala et al., 2002). When we began to study the *Fmr1 KO*, the mouse model for FXS, we discovered an

upregulation of MMP-9 expression and activity within the hippocampus demonstrating a potential link between the aberrant dendritic spine morphology of FXS and MMP-9 activation.

FXS is the most common single-gene inherited form of intellectual disability with behaviors at the extreme of the autistic spectrum. FXS is caused by hypermethylation of CGG repeats within the promoter region of the X-linked, Fragile X mental retardation (*FMR1*) gene, which causes silencing of the encoded protein, FMRP. Individuals afflicted with FXS display a variety of cognitive deficits which, in the most severe cases, include susceptibility to seizures during childhood, propensity for hyperactivity, anxiety, developmental delay, attention deficits, and visual-spatial memory impairments, as well as a tendency for obsessive-compulsive behavior (Musumeci et al., 1999; Hagerman and Hagerman, 2002). Unlike other cognitive impairment disorders, FXS is also characterized by many physical manifestations such as hyperextensible joints, extremely soft skin, large ears, and long faces, all of which could be explained by modified ECM remodeling (Hagerman and Hagerman, 2002), making MMPs likely candidates for study. After the discovery of the upregulation of MMP-9 within the *Fmr1 KO* we further analyzed how the upregulation may be explained. Beginning in 2004, the Bear lab demonstrated that there was hypersensitivity to Group 1 metabotropic glutamate receptors (mGluRs), specifically mGluR5, within the *Fmr1 KO* which could promote increased protein expression in the absence of Fmrp, the mouse homolog of the human FMRP (Bear et al., 2004). Fmrp is

responsible for the translocation of mRNAs as well as the regulation of their translation, including many post-synaptically localized molecules involved in dendritic spine plasticity (Feng et al., 1997; Brown et al., 1998; Khandjian, 1999; Greenough et al., 2001; Todd et al., 2003). Based on this work, we analyzed if Group 1 mGluR activation or inhibition affected MMP-9 regulation. We discovered that activation of Group 1 mGluRs promoted increased expression of MMP-9 while specific inhibition of mGluR5 promoted downregulation. After making this connection we then analyzed if specific inhibition of MMPs could improve dendritic spine morphology within the *Fmr1 KO*. Indeed, we found that specific inhibition of MMPs caused dendritic spine maturation within the *Fmr1 KO* which then led us to wonder if MMP specific inhibitors might have therapeutic implications for FXS. Unfortunately these inhibitors tend to be large molecules that lack the ability to perfuse the blood-brain barrier which would make treatment difficult. However, the semi-synthetic tetracycline-derived antibiotic, minocycline, also acts as an MMP-9 inhibitor and can cross the blood-brain barrier. Upon treatment of *Fmr1 KO* dendritic spines both *in vitro* and *in vivo* we discovered that minocycline promoted dendritic spine maturation as well as decreased expression and activity of MMP-9 within the *Fmr1 KO* hippocampus. We used this promising result to begin behavior analysis in the *Fmr1 KO* and discovered that minocycline treatment was able to decrease hyperactivity, anxiety and the propensity for obsessive-compulsive behavior in both young and adult animals, as well as reduce susceptibility to audiogenic seizures.

Furthermore, 4 weeks after treatment had stopped in the young animals, there was still a significant reduction in their anxiety and obsessive-compulsive behaviors, indicating that minocycline causes some prolonged, beneficial changes when administered during development.

Since minocycline is already an FDA approved drug, and we've shown that it is beneficial for the treatment of FXS-associated behaviors within the mouse model, clinical trial have begun utilizing minocycline to treat FXS (Paribello et al., 2010; Utari et al., 2010). In one study, more than half (54%) of FXS subjects treated with minocyline showed improvements in language use. Those improvements included the use of more intelligible and expressive language, and an overall increase in their verbal output (Utari et al., 2010). Additionally, minocycline treatment improved attention span in 50% and social communication in 44% of FXS subjects. An open-label trial demonstrated that minocycline treatment promoted significant behavioral improvements in 12 of 19 FXS subjects, including attention deficit, perseveration, anxiety, self-injurious behavior, abnormal vocalizations, mood swings, social avoidance, and repetitive behavior (Paribello et al., 2010). These clinical studies demonstrated that minocycline is well tolerated and provides significant functional benefits to individuals afflicted with FXS. Since minocycline appears to be a promising therapeutic option for FXS, and we think the primary action of minocycline in FXS is inhibition of MMP-9, as demonstrated in the mouse model, we were curious whether increased levels of MMP-9 also contributed to the human condition. To

analyze this potential we examined whether the activity and overall expression of MMP-9 were altered within FXS postmortem brain tissue as well as within the blood of FXS patients. We discovered overall MMP-9 expression was significantly increased within the hippocampus and neocortex while the relative activity of MMP-9 was reduced within the blood, which could potentially be a physiological response to the upregulation found within the brain. Overall, this work implicates MMP-9 as a contributor to the pathology of FXS and that MMP-9 inhibition via minocycline treatment can correct the morphological deficits as well as promote behavioral recovery, making minocycline a promising avenue for therapeutic intervention in the treatment of FXS.

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