Neuronal dysfunction in Fragile X spectrum disorders

by

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Dedication

For Mr. Alberto Real
(Mystery Al),
who taught me to be curious,
and how to learn,
and to love science.

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This dissertation work is the result of a very successful collaboration between Drs. Peter Todd and Mike Sutton and me. I was fortunate enough to begin work on these projects at the beginning of the collaboration, and as a result serve as a unique tie between the labs. Peter and Mike have very complimentary approaches to problems, and together make an excellent team. Both Mike and Peter have also gone out of their way for me many times, to ensure I had the most successful graduate career possible. I am eternally grateful for the opportunity to have worked with both of them, and I hope they continue the fruitful collaboration they've begun.

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correctly, and instilled me with many good habits. Christian Althaus is another superb scientist, who can always be counted on for assistance, and good conversation. I have benefit immensely from working with all three of these researchers.

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Chapter 1
Introduction[1]

Abstract

Fragile X spectrum disorders are a family of allelic syndromes caused by expanded trinucletide CGG repeats in the 5' untranslated region (UTR) of the *FMR1* gene on the X chromosome. The full mutation (greater than 200 CGG repeats) causes Fragile X Syndrome (FXS), the most common monogenic cause of autism and intellectual disability. Premutation range repeats (50-200) cause the neurodegenerative disorder Fragile X-associated Tremor/Ataxia Syndrome (FXTAS). As FXS is developmental and is implicated in a host of neuronal phenotypes, and FXTAS is an age-related degenerative disorder characterized by movement symptoms, the field has classically considered the pathogenesis of these two syndromes as separate despite their shared mutation. While the mechanisms of neurodegeneration in FXTAS are likely distinct from those in FXS, recent work suggests younger premutation carriers may have an increased incidence of autistic- and ADHD-like symptoms. Moreover, mouse models of both FXS and Fragile X premutation repeat expansions demonstrate shared molecular, behavioral and physiological phenotypes. Together, these findings suggest that some mechanisms of neuronal dysfunction could be shared between FXS and premutation patients. This report aims to explore the similarities and differences between FXS and FXTAS-based research, and encourage consideration of mechanistic overlap across the Fragile X spectrum.

[1] Portions of this chapter were originally published as: Renoux AJ, Todd PK. 2012. Neurodegeneration the RNA way. Prog Neurobiol. 97(2):173-89.

Fragile X Syndrome (FXS) is the most common known inherited cause of intellectual disability and monogenic autism which affects upwards of 1:4000 boys and 1:7000 girls (Bhakar et al 2012, Hernandez et al 2009, Lozano et al 2014, Nelson et al 2013, Rogers et al 2001, Santoro et al 2012). FXS results from a large expansion of a CGG trinucleotide repeat in the 5' untranslated region (UTR) of the *FMR1* gene on the X chromosome (Fu et al 1991, Oberle et al 1991, Verkerk et al 1991, Yu et al 1991). CGG expansion to greater than 200 repeats induces hypermethylation of the repeat sequence and a neighboring CpG island within the *FMR1* promoter, decreasing transcription of FMR1 mRNA and leading to the absence of the FMR1 protein product, FMRP (Figure 1.1C; (Bell et al 1991, Feng et al 1995, Pieretti et al 1991)).

Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) is a neurodegenerative disorder which is often seen in the maternal grandfathers of children with Fragile X Syndrome (FXS). While initially thought to be asymptomatic, carriers of a "premutation" range repeat of 55-200 CGGs have a distinct clinical and molecular phenotype (Figure 1.1B; (Hagerman 2013, Hagerman et al 2001)). FXTAS patients typically do not have prominent developmental delays early in life as seen in FXS patients, but they acquire a progressive neurodegenerative disorder characterized by action tremor, gait difficulties, neuropsychiatric symptoms, peripheral neuropathy and dementia which usually develop after the age of 50 (Basuta et al 2011, Hagerman 2013, Hagerman et al 2001). Repeat expansions between 40 and 55 are deemed "gray zone" alleles, and recent work indicates these patients may also share some neurological symptoms with premutation carriers who have larger expansions (Hall et al 2009, Kenna et al 2013). Work is ongoing to understand the prevalence of gray zone alleles, and the impact of these intermediate expansions on neurological function (Hall 2014).

As younger premutation patients are identified, studies find increased incidence of autism and ADHD symptoms prior to the development of neurodegeneration (Clifford et al 2007, Farzin et al 2006, Grigsby et al 2006, Grigsby et al 2014, Lozano et al 2014, Wheeler et al 2014). The prevalence of this premutation is estimated to be as high as 1:250-810 males and 1:250-300 females (Dombrowski et al 2002, Lozano et al 2014,

Rousseau et al 1995). However, the frequency of FXTAS is much lower, as a result of incomplete penetrance (Jacquemont et al 2004, Rodriguez-Revenga et al 2009). This effect is associated with age, with 17-47% penetrance in premutation males between 50 and 80 years of age, and upwards of 75% in males over the age of 80 (Jacquemont et al 2004). Females are partially protected as they have an additional X chromosome, and can display different symptoms depending on the ratio of X inactivation (Franke et al 1996, Nolin et al 2003, Wheeler et al 2014). Estimates of penetrance in the female premutation carrier population are therefore also much lower, closer to 16.5% in individuals over the age of 50 (Rodriguez-Revenga et al 2009). Despite the incomplete penetrance of FXTAS, the high estimates of premutation range frequencies mean that understanding the impact of this mutation will affect the relatively large population carrying this mutation.

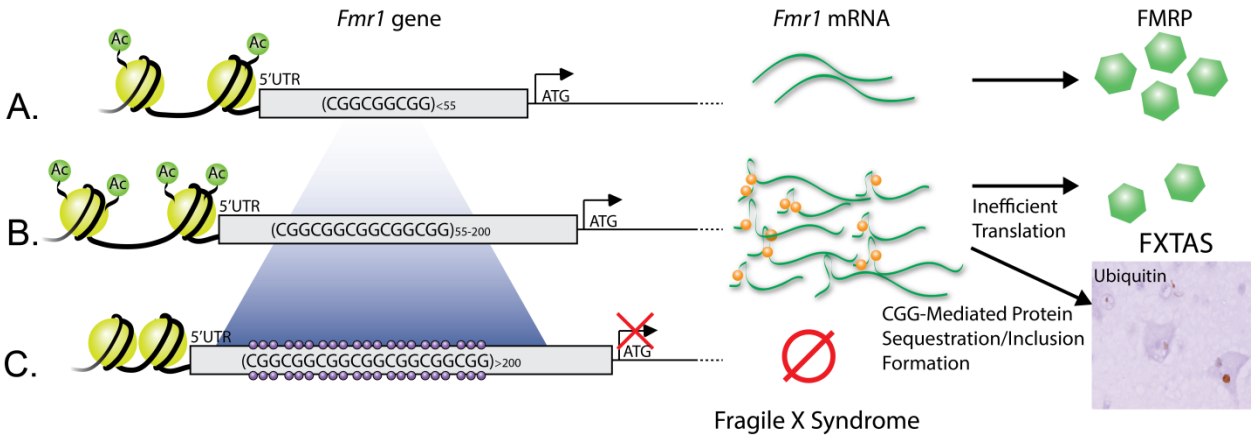

Figure 1.1: Molecular hallmarks of Fragile X family disorders.
(A) The normal 5'UTR of Fmr1 contains <55 (CGG) repeats, which codes for normal Fmr1 mRNA and FMRP levels. (B) In FXTAS these repeats expand to between 55-200, which causes increased mRNA through histone acetylation, and altered local chromatin structure. However, despite this increase in transcription, there is reduced FMRP. FXTAS patients at autopsy exhibit ubiquinated neuronal intranuclear inclusions in multiple brain regions, including the cerebellum. (C) Should the (CGG) repeats expand to greater than 200, the gene is hypermethylated and surrounding histones deacetylated, resulting in no Fmr1 mRNA nor FMRP made, culminating in FXS.

Neurodegeneration in FXTAS

Pathologically, FXTAS patients display widespread neurodegeneration and brain atrophy with intranuclear ubiquitin positive inclusions in neurons and astrocytes (Greco et al 2002, Iwahashi et al 2006). Further, premutation CGG repeat expansions lead paradoxically to increased expression of FMR1 mRNA and decreased expression of FMRP, depending on the size of the repeat expansion and the tissue type sampled (Kenneson et al 2001b, Tassone et al 2000c). The increase in expression of the CGG repeat mRNA is significant, often as high as 5-fold greater in patient derived cell lines or in animal models of the disease. This increased FMR1 RNA expression results from greater *FMR1* transcription and not from an increase in FMR1 mRNA stability associated with the enlarged repeat (Tassone et al 2007, Tassone et al 2000a). Evidence now suggests this augmented transcription is triggered by epigenetic alterations induced by the CGG repeat expansion itself as DNA (Mulvihill et al 2005, Todd et al 2010, Wang et al 1996). Specifically, CGG repeat expansions elicit chromatin changes *in vitro,* in a *drosophila* model of the disease, and in patient derived cell lines. Interestingly, these alterations in local chromatin structure are dynamic and modifiable by genetic and pharmacologic means, suggesting that agents aimed at chromatin remodeling might have therapeutic potential in FXTAS (Todd et al 2010).

The concept that RNA itself acts as a primary toxic species in a neurological disorder was first proposed and established for Myotonic Dystrophy Type 1 (DM1). DM1 is the most common adult onset muscular dystrophy and the third most common overall (Kanadia et al 2003, Liquori et al 2001, Mankodi et al 2000, Philips et al 1998, Wheeler & Thornton 2007). An autosomal dominant disorder, DM1 results from an expanded CTG repeat in the 3'UTR of the *DMPK* gene (Brook et al 1992, Fu et al 1992, Mahadevan et al 1992). Initial evaluations tested whether this repetitive sequence impaired production of the DMPK protein which contained the repeat, or if it may alter the expression of the genes surrounding the locus of the mutation. However, the expanded CTG repeats do not significantly alter DMPK protein expression and altered expression of neighboring genes did not recapitulate key features of the clinical disorder (Filippova et al 2001, Jansen et al 1996, Klesert et al 2000, Reddy et al 1996, Sarkar et al 2000). However, expression of CTG repeats in isolation *was* capable of eliciting cytotoxicity and recapitulating many of the central aspects of the human disease

(Mankodi et al 2000, Taneja et al 1995). In particular, CUG repeat mRNA form nuclear foci that co-localize with multiple members of the muscleblind-like (MBNL) splicing factor family in animal models and in affected tissues from patients (Mankodi et al 2003, Miller et al 2000, Taneja et al 1995). MBNL binds to CUG RNA repeats and redistributes from a diffuse pattern of nuclear staining to punctate foci in both patient tissue samples and when co-expressed with CUG RNA in cell culture models (Mankodi et al 2000, Miller et al 2000, Taneja et al 1995). Based on these findings, it was proposed that expanded repeats of CUG RNA might act to sequester MBNL and perhaps other key proteins as a primary disease mechanism (Mankodi et al 2000, Mankodi et al 2003, Miller et al 2000, Philips et al 1998, Taneja et al 1995). In DM1 tissues, there is mis-splicing of a number of MBNL target messages in a fashion that indicates loss of MBNL function (Mankodi et al 2002, Philips et al 1998, Savkur et al 2001). These include retention of a destabilizing splice variant in the chloride channel CIC1, which leads to myotonia; a splice variant of the glutamate receptor NMDAR1 that impairs proper dendritic targeting of the mRNA; and multiple splicing alterations in the microtubule-associated protein tau that may influence this proteins stability and aggregation properties (Dhaenens et al 2008, Dhaenens et al 2011, Ghanem et al 2009, Itoh et al 2010, Jiang et al 2004, Leroy et al 2006, Mankodi et al 2002, Sergeant et al 2001, Wheeler et al 2007).

However, MBNL1 sequestration is only part of the DM1 story. In addition to causing splicing deficits, studies in patient derived tissues and multiple animal models of the disease demonstrate alterations in CUG Binding Protein 1 (CUGBP1) expression and activation (Philips et al 1998, Timchenko et al 2001). CUGBP1 is also a splicing factor, but it has numerous other roles in RNA processing, transport, and translation within neurons. Although CUGBP1 can bind to CUG RNA, it is not sequestered in RNA foci and instead appears to be activated by the presence of expanded CUG RNA expression. Importantly, overexpression of CUGBP1 recapitulates some critical features of DM1 pathology (Ward et al 2010). Together, these data suggest a concurrent role for MBNL and CUGBP1 in DM1 (Orengo et al 2008, Wang et al 2007a).

RNA-binding protein sequestration is not unique to DM1 and DM2. In total, sequestration of specific RNA binding proteins are proposed as a primary pathogenic

mechanism in at least five different RNA dominant disorders. One recently described example of this is spinocerebellar ataxia Type 10 (SCA10). SCA10 is a rare cerebellar ataxia and epilepsy syndrome that results from an ATTCT repeat expansion in the 3'UTR of the E46L gene (Lin & Ashizawa 2003, Matsuura et al 2000, White et al 2010). In patient derived cells and in transfected cells, this RNA repeat drives formation of nuclear and cytoplasmic foci and cellular apoptosis (White et al 2010). The RNA foci co-localize with the RNA binding protein hnRNP K and strongly bind to it *in vitro* and *in vivo* (White et al 2010). Moreover, central aspects of the cellular RNA toxicity can be recapitulated by siRNA knockdown of hnRNP K (White et al 2010). Future work will need to address whether interactions of AUUCU repeat RNA with hnRNP K are sufficient to explain the full clinical phenotype in animal models and whether overexpression of hnRNP K can suppress the cellular toxicity elicited by the AUUCU repeat RNA (White et al 2010).

Another form of spinocerebellar ataxia has recently been described, SCA31 (Sato et al 2009). SCA31 arises from an inserted TGGAA repeat on chromosome 16. When this repeat is transcribed it forms nuclear inclusions and co-localizes with the serine/arginine-rich splicing factors (SFRS) 1 and 9 (Sato et al 2009). While this finding does not yet directly link SFRS1 and 9 inactivity to the pathology associated with SCA31, it does indicate yet another neurological disorder may be associated with nucleotide repeats capable of mediating neurodegeneration by way of protein sequestration.

FXTAS pathogenesis is hypothesized to result from a primary mRNA toxic gain of function mechanism akin to myotonic dystrophy type 1 (DM1; (Hagerman et al 2001, Todd & Paulson 2010)). FMR1 mRNA but not FMR protein was found in FXTAS patient derived nuclear inclusions (Tassone et al 2004b). Further, ectopic expression of an expanded CGG repeat in the context of a heterologous transcript was sufficient to cause neurodegeneration and intranuclear inclusions in *Drosophila* (Jin et al 2003), transfected cells (Hashem et al 2009), and in murine purkinje cells (Hashem et al 2009). However, unlike DM1, the primary sequestered RNA binding protein in FXTAS remains unclear. A proteomic analysis of the nuclear inclusions in FXTAS revealed a large number of proteins in the aggregates, including the RNA binding protein hnRNPA2/B1,

the nuclear envelope protein lamin A/C and the small heat shock protein αB-crystallin (Iwahashi et al 2006). Importantly, a number of other potential toxic aggregating proteins were not seen in the inclusions, including α-synuclein and tau, however TDP-43 is found in inclusions (He et al 2014). Subsequently, Jin and colleagues identified two CGG repeat RNA binding proteins, Pur α and hnRNPA2/B1, by affinity chromatography (Jin et al 2007). Both are present in FXTAS patient inclusions and co-expression of either of these proteins suppresses CGG repeat mediated toxicity in a *Drosophila* model (Jin et al 2007, Sofola et al 2007). Interestingly, hnRNPA2/B1 binds to CUGBP1 and overexpression of CUGBP1 suppresses the CGG repeat phenotype in *Drosophila* (Sofola et al 2007). However, as the changes in CUGBP1 are in the *opposite* direction of those seen in DM1, the relevance of this finding to FXTAS pathogenesis remains unclear (Sofola et al 2007). In addition to its interaction with CUGBP1, hnRNPA2/B1 overexpression itself can also rescue CGG toxicity (Muslimov et al 2011, Tan et al 2012). Work from our group demonstrated that expression of TDP-43, but not FUS, can rescue CGG-associated phenotypes through interactions with hnRNPA2/B1 (He et al 2014).

TDP-43 is not the only splicing factor involved in models of FXTAS, but splicing in general appears to play a key role in its pathogenesis (Figure 2.1B-C). Sam68 is another RNA binding protein identified within FXTAS human patient brain inclusions (Sellier et al 2010). Sam68 binds to both RNA and DNA and is a known splicing factor which interacts with additional RNA binding proteins (Lukong & Richard 2003, Richard 2010). The absence of Sam68 causes a motor coordination phenotype in knockout mice (Lukong & Richard 2003). In cell culture, Sam68 is recruited to CGG repeat aggregates as an early event in pathogenesis. This sequestration leads to altered splicing of a number of Sam68 target mRNAs, which are also mis-spliced in FXTAS model mice and human patient brain samples (Sellier et al 2010). Moreover, knockdown of Sam68 prevented CGG RNA induced aggregate formation. Sam68 does not bind directly to CGG RNA, but rather associates with DGCR8, a double stranded RNA-binding protein which binds directly to CGG repeats (Sellier et al 2013). DGCR8 normally recruits the miRNA processing protein, DROSHA, to primary miRNA transcripts to be cleaved into precursor miRNAs (Han et al 2004, Landthaler et al 2004,

Lee et al 2003, Wang et al 2007b). When DGCR8 is bound to CGG repeats it then sequesters DROSHA, impairing the production of numerous miRNAs, and contributing to CGG-mediated toxicity (Sellier et al 2013).

Although sequestration of Sam68, DROSHA, and other RNA binding proteins by CGG repeat RNA may be important aspects of FXTAS pathogenesis, other mechanisms likely contribute to neuronal dysfunction and neurodegeneration in this disorder. Recent reports suggest a novel mechanism by which non-coding RNA repeats might lead to toxicity and neurodegeneration. Zu et al first described an aberrant repeat-associated non-AUG (RAN) translation which generates polyglutamine, polyalanine, and polyserine tracts from otherwise non-coding CAG repeat mRNA messages (Zu et al 2011). This was initially identified when transfection of a CAG repeat-containing Ataxin8OS construct lacking the ATG start site failed to prevent translation of the polyglutamine tract. Further experiments using C-terminal epitope tags in differing reading frames demonstrated that this aberrant translation occurred in all three possible reading frames of the CAG repeat. The effect of RAN translation was repeat length-dependent, in that constructs with 15 CAG repeats did not trigger AUG independent translation, but those with 45 or greater did. This RAN translation only occurs when the repeat tract is complementary and forms a hairpin structure. Zu et al further identified staining for these novel proteins in a spinocerebellar ataxia type 8 (SCA8) mouse model, and in human SCA8 cerebellum using an antibody generated against the putative SCA8-GCA frame (polyalanine) peptide. A similar approach demonstrated a novel polyglutamine protein in DM1, presumably as a result of RAN translation from an antisense transcript though the CTG/CAG repeat (Cho et al 2005, Zu et al 2011).

Our group has demonstrated that RAN translation also occurs at expanded CGG repeat contexts, producing peptides in polyglycine and polyalanine reading frames (Todd et al 2013). Expression of CGG repeats in cell culture *Drosophila* causes ubiquitin positive inclusions, which contain the polyglycine peptide generated from RAN translation through the repeat (Todd et al 2013). In the context of the Fmr1 mRNA, a novel FMRpolyG protein is generated in mouse models and FXTAS human brain samples (Todd et al 2013). Similarly, the recently described hexinucleotide GGGGCC

repeat in *C9ORF72* which is the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) also triggers RAN translation and causes aberrant peptide synthesis (Ash et al 2013, DeJesus-Hernandez et al 2011, Mori et al 2013, Renton et al 2011). These findings suggest a novel mechanism for aberrant protein synthesis from repetitive nucleotide sequences in the absence of an obvious open reading frame. While further studies are required to establish whether RAN translation significantly contributes to pathogenesis in the disorders described, the potential extension of this mechanism to other nucleotide repeat disorders could dramatically change our understanding of what drives neurodegeneration in this class of diseases (Figure 1.2E; (Pearson 2011)).

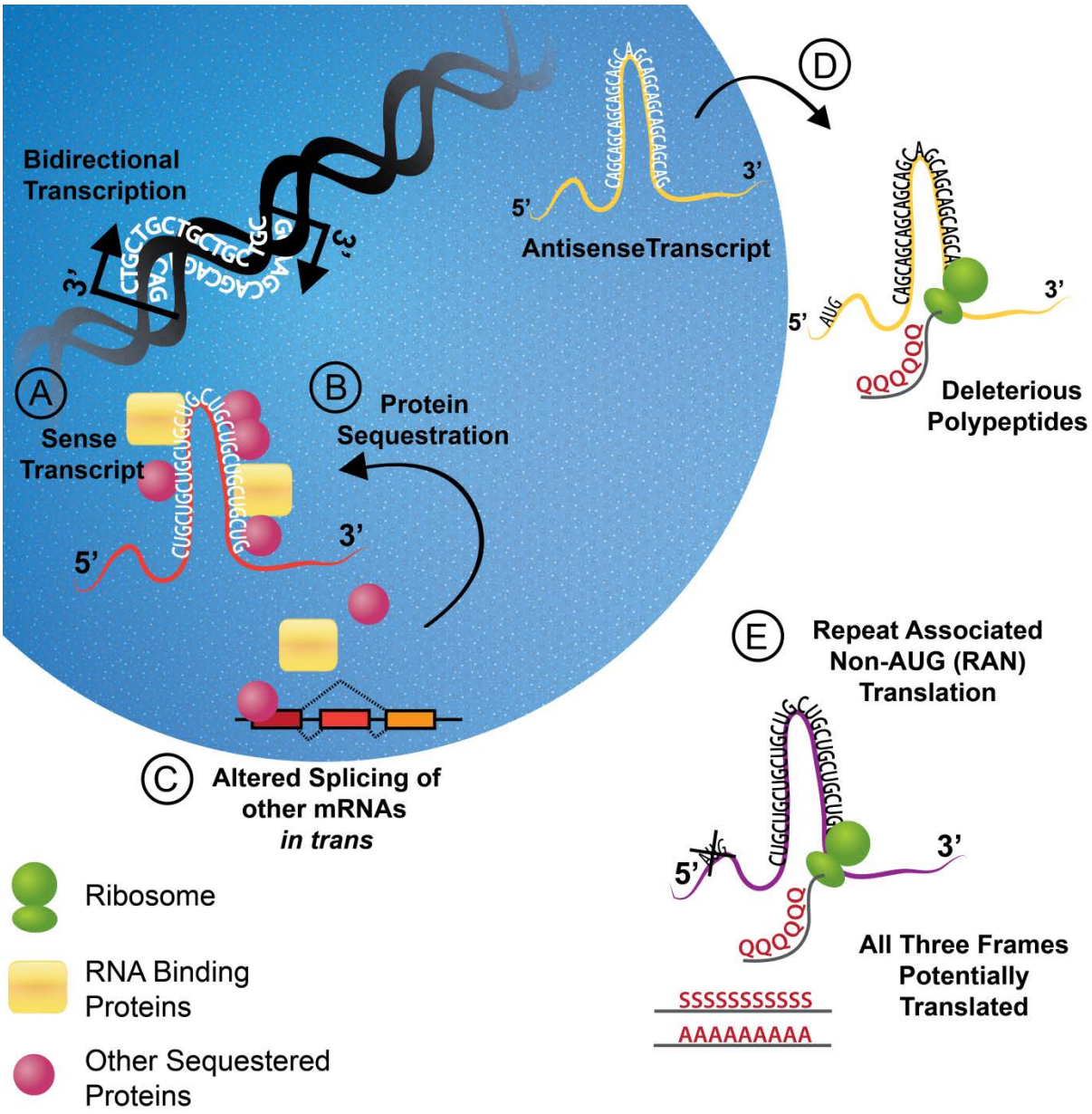

Figure 1.2: Mechanisms underlying RNA mediated neurodegeneration in nucleotide repeat disorders.
(A) Bidirectional transcription at loci with expanded nucleotide repeats leads to the generation of multiple potentially toxic products. (B and C) Sense transcripts of tri-nucleotide CUG (shown here), CAG, or CGG repeats form secondary mRNA hairpin structures that can bind to and sequester RNA-binding proteins. The decreased availability of RNA binding proteins such as splicing factors leads to alterations in the splicing and expression of other mRNAs *in trans*. Transcription factors and other nucleotide associated proteins may also be sequestered with effects on neuronal homeostasis. (D) Antisense transcription through the repeats leads to the generation of other potentially toxic mRNA sequences. These antisense transcripts sometimes contain open reading frames through the repeat, leading to translation of potentially

toxic amino acid homopolymer production (e.g. polyglutamine containing proteins). (E) Homopolymer containing proteins may also be generated through a novel process known as repeat-associated non-AUG initiated (RAN) translation. Shown is the production of a polyglutamine peptide; however, alternative homopolymers in all three potential reading frames may be generated from each transcript.

These mechanisms of protein sequestration and aberrant RAN translation likely both contribute to neuronal toxicity in FXTAS. Protein quality control is also closely linked to both of these phenomena. Both FXTAS model systems and patient samples show evidence of large proteinaceous ubiquitinated intranuclear inclusions, indicating alterations in the ubiquitin proteasome system (UPS) as a result of CGG-mRNA expression (Greco et al 2002, Willemsen et al 2003). In a *drosophila* model of the disease, co-expression of the chaperone heat shock protein 70 (Hsp70) suppresses CGG repeat induced neurodegeneration (Jin et al 2003). Similarly, in patient derived fibroblast cultures and in neuronal cultures from female heterozygous FXTAS model mice, there are significant markers of cellular stress, including elevated expression of multiple heat shock proteins, increased nuclear heterochromatin formation and decreased cellular viability (Chen et al 2009, Garcia-Arocena et al 2010). In HEK cells stably transfected with 120 CGG repeats, gene activation triggers reduced cell viability and an increased sensitivity to drugs that impair protein quality control pathways (Handa et al 2005). In transfected human neuroblastomas containing the *FMR1* 5'UTR with 88 repeats preceding eGFP, there was a similar reduction in viability associated with gene activation and αB-crystallin positive (but ubiquitin negative) intranuclear inclusion formation at 72 hours after gene transfection (Arocena et al 2005). Interestingly, in both patient derived fibroblasts as well as transfected cells, CGG repeat expression leads to alterations in the expression and distribution of Lamin A/C within the nuclear membrane (Arocena et al 2005, Garcia-Arocena et al 2010). Given that Lamin A/C is found as a component of the intranuclear inclusions, the authors proposed that one component of FXTAS pathogenesis could be as a "laminopathy", as occurs in Emory–Dreifuss muscular dystrophy and the premature aging disorder Progeria (Arocena et al 2005, Capell & Collins 2006, Garcia-Arocena et al 2010, Zaremba-Czogalla et al 2011).

Recent work from our group has identified an overlap between the RAN peptides produced in CGG-expressing models and UPS impairment (Oh et al 2015). In

drosophila and cell-based models, expression of CGG-containing mRNAs led to repeat-dependent impairment of the UPS, and degeneration; this effect was suppressed by expression of the chaperone Hsp70 (Oh et al 2015). Enhancing production of the CGG-Fmr1 RAN peptide, FMRpolyG, caused increased UPS impairment, while blocking its translation ameliorated the associated phenotypes (Oh et al 2015). These findings support a model in which production of at least one of the aberrant RAN peptides are linked to toxicity through disruption of UPS function. Work is ongoing to identify similar mechanistic underpinnings in related expanded repeat disorders.

FMRP expression in FXTAS

Although FXTAS is a degenerative disease that affects primarily a geriatric population, a number of recent studies suggest that the CGG premutation also alters neuronal and synaptic development. To understand the relevance of these findings, it is important to appreciate the normal functions of FMRP, the dynamics of FMR1 translation under normal circumstances and the potential effect of the expanded CGG repeat on translational inefficiency. FMRP is an RNA binding protein that regulates dendritic synthesis of proteins in response to synaptic stimulation (Ronesi & Huber 2008, Zukin et al 2009). Interestingly, one of the messages FMRP likely regulates at synapses is Fmr1, which is rapidly translated in response to metabotropic glutamate receptor (mGluR) stimulation (Hou et al 2006, Iliff et al 2013, Todd et al 2003a, Weiler et al 1997). Thus, efficient translation of FMRP at synapses is an important part of the proteins basal function. The CGG repeat in Fmr1 acts to impair translation by interfering with ribosomal scanning through the 5'UTR, preventing appropriate loading of CGG expanded FMR1 mRNA into polyribosomal complexes (Ludwig et al 2011, Ludwig et al 2009, Primerano et al 2002). Although FMRP expression levels in patients with FXTAS have been widely reported as only slightly decreased (Kenneson et al 2001, Primerano et al 2002, Singh et al 2007, Tassone et al 2000c), more recent reports on patient brain tissue indicates a significant decrease in neuronal FMRP levels (Ludwig et al 2014, Pretto et al 2014, Renoux et al 2014a). Given evidence from mice that FMRP expression declines with age (Gaur & Prasad 2014, Iliff et al 2013, Ludwig et

al 2014, Singh et al 2007), a role for FMRP insufficiency in FXTAS, especially in patients with large CGG repeat expansions, deserves further consideration (Hessl et al 2011).

Most of the studies addressing a potential role for FMRP insufficiency have utilized one of two knock-in (KI) mouse models where a portion of the human 5'UTR from a premutation carrier has been inserted into the *Fmr1* mouse locus (Bontekoe et al 2001, Entezam et al 2007, Hashem et al 2009, Peier & Nelson 2002). Both mice, as well as a YAC transgenic model mouse, demonstrate intra-generational instability, elevated Fmr1 mRNA expression and variably decreased FMRP expression at larger premutation repeat lengths (Brouwer et al 2008, Entezam et al 2007, Hashem et al 2009, Iliff et al 2013, Ludwig et al 2014, Peier & Nelson 2002, Willemsen et al 2003).

In the best characterized of the models, generated by a Dutch consortium (Berman & Willemsen 2009, Willemsen et al 2003), CGG KI mice show signs of hippocampal-dependent cognitive impairment at an early age that precedes frank neurodegeneration or inclusion formation (Hunsaker et al 2009). These same mice also show signs of age-dependent cognitive decline in later life as measured by impaired visual-spatial learning and performance on the Morris water maze (Van Dam et al 2005). Interestingly, cultured hippocampal neurons from heterozygous female CGG KI mice demonstrate early developmental defects including delayed dendritic complexity and outgrowth (Chen et al 2010). As a follow up to this study, Cunningham and colleagues identified significant delays in cortical development in CGG KI mice during embryogenesis characterized by migrational defects in the neocortex and altered expression of neuronal lineage markers (Cunningham et al 2010). As these mice have relatively large CGG repeat expansions (~150 CGGs), they express only about 50% of basal FMRP levels in embryonic tissues. Importantly, defects in neural stem cell proliferation, which have been reported in *Fmr1* knockout mouse models (Tervonen et al 2009), were not present in these animals, suggesting the etiology of the developmental defects in the CGG KI mice may be more than just FMRP insufficiency (Cunningham et al 2010).

In the more recently described of the two knock-in models, generated by a group at the NIH, the inefficiency of CGG-Fmr1 translation appears to be greater, for unknown

reasons (Entezam et al 2007, Iliff et al 2013, Qin et al 2011). This translational inefficiency leads to lower FMRP levels for a given CGG repeat size, especially in certain brain regions, though greater basal CGG Fmr1 RNA expression (Entezam et al 2007, Iliff et al 2013, Qin et al 2011). Despite the increased amount of CGG-containing RNA produced, these mice generate few intranuclear inclusions, although they do demonstrate mild cerebellar and cortical neurodegeneration with age (Entezam et al 2007). Differences between cloning strategies to generate these two KI mice resulted in differential RAN translation between models. Specifically, the Dutch CGG KI mice contain more of the human 5'UTR sequence, and undergo RAN translation as a result (Entezam et al 2007, Todd et al 2013, Willemsen et al 2003). The production of a FMRpolyG peptide in the Dutch CGG KI model is likely responsible for the increased inclusion incidence when compared to the NIH KI model (Todd et al 2013).

In a recent study, the NIH CGG KI mice were found to exhibit some features also seen in *Fmr1* KO mice. Most notably, they had decreased dendritic arborization and longer thin spines on histological analysis, along with increased regional brain protein synthesis rates (Qin et al 2011). Taken together, these data suggest that therapeutic strategies aimed selectively at eliminating the toxic CGG repeat mRNA run a significant risk or exacerbating symptoms of FXTAS that may relate to decreased basal or activity dependent FMRP expression (Todd et al 2010).

Neuronal function of FMRP

As FXS is caused by loss of the FMR protein, an extensive body of research into the function of FMRP has amassed. As discussed above, FMRP is an RNA binding protein found associated with polyribosome complexes and target mRNAs (Darnell et al 2005, Darnell et al 2011, Khandjian et al 2004, Stefani et al 2004, Zukin et al 2009). FMRP is phosphorylated basally by S6 kinase, and neuronal activity (specifically of mGluRs) causes FMRP to be dephosphorylated by PP2A (Narayanan et al 2007, Narayanan et al 2008). This dephosphorylation causes FMRP to dissociate from its ribosome complex, leading to a local burst of new protein synthesis (Bassell & Warren 2008, Ceman et al 2003, Waung & Huber 2009). Recent work has gone on to show

that FMRP is ubiquitinated and degraded upon mGluR stimulation (Hou et al 2006, Nalavadi et al 2012). One target transcript associated with FMRP is the Fmr1 mRNA (Ashley et al 1993, Schaeffer et al 2001). As a result, FMRP is newly translated at the synapse with mGluR stimulation (Hou et al 2006, Iliff et al 2013, Todd et al 2003b, Weiler et al 1997).

A large body of work aimed at identifying FMRP-regulated transcripts has revealed a long list of putative mRNA targets. Initial work identified mRNA motifs and sequences associated with FMRP binding regions (Brown et al 2001, Chen et al 2003a, Darnell et al 2005, Darnell et al 2001, Miyashiro et al 2003, Schaeffer et al 2001, Zalfa et al 2003). More recent work made possible with advances in high throughput and deep sequencing has identified over 100,000 potential targets (Ascano et al 2012, Darnell et al 2011). Many transcripts coding for synaptic proteins were identified, indicating that FMRP plays a critical role in the regulation of synaptic composition (Ascano et al 2012, Darnell et al 2001). While the majority of these transcripts have yet to be validated as FMRP targets, several critical transcripts have been evaluated, and found to be altered in Fmr1 KO cells. These transcripts include the immediate early gene Arc, amyloid precursor protein (APP), and the postsynaptic scaffolding protein PSD95 (Ascano et al 2012, Muddashetty et al 2007, Niere et al 2012, Todd et al 2003a, Waung et al 2008, Westmark & Malter 2007).

Synaptic translation of these key proteins is of particular interest as local translation is required for synaptic plasticity (Kelleher et al 2004, Klann & Dever 2004, Malenka & Bear 2004, Richter & Klann 2009, Sutton & Schuman 2006). FMRP's role as a translational regulator indicated that Fmr1 KO animals may have alterations in synaptic plasticity. mGluR-dependent long term depression (LTD) is significantly enhanced in Fmr1 KO hippocampal slices (Huber et al 2002). Interestingly, this enhanced synaptic weakening persisted in the absence of protein synthesis in Fmr1 KO animals (Nosyreva & Huber 2006). The absence of FMRP basally causes an increase in key synaptic proteins independent of mGluR signaling (Bear et al 2004, Todd & Malter 2002). Subsequent work has identified dendritic Arc expression to be increased basally in cultured hippocampal neurons lacking FMRP (Niere et al 2012). This basal increase in proteins required for plasticity likely underlies the enhanced LTD phenotype,

and renders synaptic weakening independent of new protein synthesis (Bear et al 2004, Todd & Malter 2002).

While mGluR-LTD in Fmr1 KO mice is enhanced and protein synthesis independent, another form of synaptic weakening, NMDA-LTD, is unchanged (Huber et al 2002). Several groups have also evaluated the role of FMRP in long term potentiation (LTP); however the results have been inconsistent. There are differences depending on the kind of LTP induction protocol used, and the brain region studied (Chung et al 2012, Godfraind et al 1996, Larson et al 2005, Lauterborn et al 2007, Lee et al 2011, Li et al 2002, Meredith et al 2007, Zhao et al 2005). The stronger high frequency stimulation showed no change in hippocampal LTP, however a similar induction protocol in the cortex found significant impairments in Fmr1 KO mice (Godfraind et al 1996, Li et al 2002). High frequency stimulation also found impaired LTP in the amygdala and anterior cingulate cortex in Fmr1 KO mice (Zhao et al 2005). However, a more subtle theta burst stimulation found impaired LTP in the hippocampus and in the anterior piriform cortex of older Fmr1 KO animals (Larson et al 2005, Lauterborn et al 2007, Lee et al 2011). Spike timing LTP is also altered in the cortex of animals lacking FMRP (Meredith et al 2007).

In addition to Hebbian LTP and LTD, FMRP also plays a role in homeostatic plasticity (HSP). There are two types homeostatic plasticity: global HSP and local HSP. Global HSP is observed after chronic blockade (48 hours) of action potentials in cultured neurons with the voltage-gated sodium channel antagonist tetrodotoxin (TTX). Blocking neurons with TTX produces a compensatory increase in miniature excitatory postsynaptic current (mEPSC) amplitudes due to a transcription-dependent accumulation of GluR2-containing AMPARs (Gainey et al 2009). On the contrary, local HSP is observed by blocking NMDA receptors and action potentials (TTX+APV) in cultured hippocampal neurons (Sutton et al 2006). In turn, this leads to an increase in the amplitude of mEPSCs within 60 minutes (Sutton et al 2006). AMPAR blockade in place of NMDAR blockade can lead to similar results, however this treatment also causes presynaptic changes (Henry et al 2012, Jakawich et al 2010).

Interestingly, FMRP has been shown to play an important role in the mechanism of HSP (Soden & Chen 2010). CA1 pyramidal neurons show increased mEPSC

amplitude following TTX+APV treatment, which was absent in Fmr1 KO neurons (Soden & Chen 2010). Reduced FMRP expression causes a threshold-dependent reduction in surface GluR1-containing AMPARs at baseline (Nakamoto et al 2007). Furthermore, postsynaptic expression of mutant FMRP which cannot bind target transcripts (I304N) in Fmr1 KO neurons reduces the total, surface, and synaptic levels of AMPA receptors, hence suggesting a role for FMRP-regulated protein translation in controlling synaptic AMPAR abundance (Soden & Chen 2010). Remarkably little research has been performed to understand how HSP mechanisms may be impacted in the presence of the Fragile X premutation. While Hebbian mGluR-LTD is altered in premutation model mice, it is not clear whether the impaired activity-dependent FMRP production resulting from the expanded CGG repeat will also impact HSP (Iliff et al 2013).

FMRP also regulates the translation of several key voltage-gated channels. The L-type calcium channel subunit α1D is an FMRP-regulated protein, in addition to the voltage-gated potassium channels Kv4.2 and Kv3.1 (Chen et al 2003a, Gross et al 2011, Lee et al 2011, Strumbos et al 2010). Expression of the AMPA subunit GluR1 is also regulated by FMRP and the related protein FXR2 (Guo et al 2015, Li et al 2002, Muddashetty et al 2007). Besides regulating the translation of proteins required for synaptic function and plasticity, FMRP also directly interacts with several important ion channels. FMRP associates with the regulatory β4 subunit of BK potassium channels, increasing action potential duration in Fmr1 KO neurons (Deng et al 2013). It also interacts with the sodium-activated potassium channel Slack to regulate its open probability (Brown et al 2010). Taken together, these findings suggest a strong role for FMRP in the control of neuronal firing and plastic adaptation properties. It's likely all of these features converge to give way to the altered intellectual status and autistic symptoms seen in FXS patients. As mentioned above, the role of reduced FMRP in premutation patients has not been widely examined for some of these same neuronal properties that have been described in Fmr1 KO animals and FXS human patients.

<u>Hypotheses and addressable questions</u>

FXTAS is a complex disorder, comprised of a toxic RNA gain-of-function, a toxic protein gain of function and alterations in FMRP expression under basal and activity dependent conditions (Hagerman 2013, Nelson et al 2013). Much of the work on this disorder has focused on the mechanisms of CGG-mediated toxicity, including protein sequestration which is linked to splicing and miRNA processing changes, and UPS impairment partially dependent on RAN translation of the repeat (Hagerman 2013, Hagerman & Hagerman 2013, Oh et al 2015, Sellier et al 2013, Sellier et al 2010, Todd et al 2013). However, more recent work has begun to focus on the impact of reduced FMRP in FXTAS models and human patients.

As FMRP is a critical translational regulator involved in many neuronal functions, including neuronal signaling and plasticity, the question of what happens in the premutation population base, which is potentially very large, is of particular interest. The goal of this work is to explore the impact of the premutation in a mouse model of FXTAS and explore the differences and contributions of the increased CGG-mRNA and reduced FMRP levels. Similarly, exploring and identifying key phenotypic overlaps between the Fmr1 KO mouse and the premutation mouse model could have the clinical advantage of applying therapeutics developed for FXS to symptomatic premutation carriers. In addition, understanding how the premutation may affect neuronal function prior to neurodegeneration could help understand the sequence of events that lead up to the age and time-dependent features of neurodegeneration in FXTAS.

There is a significant amount of data now suggesting that the presence of an expanded CGG repeat in the 5'UTR of FMR1 results in functional outcomes that phenotypically overlap with those observed in the absence of FMRP. This data suggests that at least a portion of the clinical phenotype observed in patients with premutation repeat expansions and in premutation model mice are a result of insufficient FMRP.

As expanded CGG repeats in Fmr1 impair the translation of FMRP, I hypothesize that activity dependent production of new FMRP in particular will be blocked in premutation model mice and patients with premutation expansions. This inability to produce new FMRP in response to neuronal activity will alter synaptic function and plasticity, and these alterations in synaptic function will correlate with behavioral

phenotypes in these mice. I expect that these alterations in synaptic and behavioral metrics will overlap with those described in Fmr1 KO mice and FXS patients. I also hypothesize that FMRP target protein expression and its impact on ion channel function will also be altered in CGG KI model and FXTAS patient brain tissues.

To examine the questions and hypotheses above, I will use the CGG KI mouse generated by the NIH group to model animal behavior, slice physiology, and primary neuronal culture. I have taken advantage of the fact that *Fmr1* is on the X chromosome to produce an additional model which generates intra-animal WT and CGG KI neurons for better control in culture experiments. By crossing a transgenic female animal with a GFP transgene knocked into the X chromosome to male premutation CGG KI/Y animals, the heterozygous females will be mosaic and express GFP+/WT Fmr1 neurons, and GFP-/CGG KI Fmr1 neurons by way of X-inactivation.

In all of the cases listed above, an alternative model is that increased CGG-mRNA mediated toxicity is responsible for the phenotypes observed, as opposed to differential expression of FMRP. To evaluate the mechanistic contribution of these two features, I will use WT rat hippocampal neurons and a lentivirus construct to overexpress CGG repeats in the background of normal FMRP levels. Future work will utilize an shRNA vector against Fmr1 to reduce FMRP levels to those seen in CGG KI cells, and explore similar phenotypes observed in CGG KI animals.

Chapter 2
Impaired activity-dependent FMRP translation and enhanced mGluR-dependent LTD in Fragile X premutation mice[2]

Abstract

Fragile X premutation-associated disorders, including Fragile X-associated Tremor Ataxia Syndrome, result from unmethylated CGG repeat expansions in the 5'untranslated region of the FMR1 gene. Premutation sized repeats increase FMR1 transcription but impair rapid translation of the fragile X protein, FMRP, which is absent in Fragile X Syndrome. Normally, FMRP binds to RNA and regulates metabotropic glutamate receptor (mGluR) mediated synaptic translation, allowing for dendritic synthesis of several proteins. FMRP itself is also synthesized at synapses in response to mGluR activation. However, the role of activity-dependent translation of FMRP in synaptic plasticity and Fragile X-premutation associated disorders is unknown. To investigate this question, we utilized a CGG knock-in mouse model of the Fragile X premutation with 120-150 CGG repeats in the mouse Fmr1 5'UTR. These mice exhibit increased Fmr1 mRNA production but impaired FMRP translational efficiency, leading to a modest reduction in basal FMRP expression. Cultured hippocampal neurons and synaptoneurosomes derived from CGG KI mice demonstrate impaired FMRP translation in response to the group I mGluR agonist DHPG. Electrophysiological analysis reveals enhanced mGluR mediated long term depression (mGluR-LTD) at CA3-CA1 synapses in acute hippocampal slices prepared from CGG KI mice relative to wild-type littermates,

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(*Authors contributed equally.)

similar to Fmr1 knockout mice. However, unlike mGluR-LTD in mice completely lacking FMRP, mGluR-LTD in CGG knock-in mice remains dependent on new protein synthesis. These studies demonstrate partially overlapping synaptic plasticity phenotypes in mouse models of FXS and Fragile X premutation disorders and support a role for activity-dependent synthesis of FMRP in enduring forms of synaptic plasticity.

Introduction

Fragile X Syndrome (FXS) is the most common known monogenic cause of autism and intellectual disability, affecting upwards of one in 4000 boys and one in 8000 girls (Hernandez et al 2009, Rogers et al 2001). FXS results from expansion of a CGG microsatellite repeat in the 5' untranslated region of the *FMR1* gene on the X chromosome. In humans, this sequence is normally less than 45 CGG repeats. Expansions to greater than 200 repeats trigger hypermethylation of the repeat and *FMR1* promoter, resulting in transcriptional silencing of the *FMR1* gene and absence of the Fragile X mental retardation protein, FMRP (Bell et al 1991, Kremer et al 1991, Oberle et al 1991, Pieretti et al 1991, Verkerk et al 1991).

FMRP is an RNA binding protein that regulates activity-dependent translation of associated transcripts at the synapse (Bassell & Warren 2008). Mice lacking FMRP (*Fmr1* KO mice) exhibit specific defects in synaptic signaling mediated through group I metabotropic glutamate receptors (mGluRs; (Huber et al 2002)). At CA3-CA1 synapses in the hippocampus, mGluR activation normally leads to a long term depression (LTD) of synaptic efficacy that requires new dendritic protein synthesis (Huber et al 2000, Nosyreva & Huber 2006, Park et al 2008, Shepherd et al 2006). mGluR agonists trigger rapid FMRP dephosphorylation and degradation, which allows synaptic translation of FMRP associated transcripts (Hou et al 2006, Nalavadi et al 2012, Niere et al 2012). In mice lacking FMRP, mGluR LTD is enhanced and no longer requires new protein synthesis, and mGluR agonists fail to trigger translation of FMRP target mRNAs (Huber et al 2002, Muddashetty et al 2007, Niere et al 2012, Nosyreva & Huber 2006, Todd et al 2003a). The absence of FMRP is thought to decouple mGluR 1/5 activity from

protein translation, such that basal dendritic translation of these target mRNAs is increased, but mGluR coupled dendritic translation is lost (Bear et al 2004).

One of the dendritically-localized transcripts whose translation is regulated by mGluR signaling is FMRP itself (Antar et al 2004, Hou et al 2006, Todd et al 2003a, Weiler et al 1997). Although the function of this newly synthesized FMRP is unknown, it has been proposed to act as a brake on local protein production, hence constraining LTD by limiting the new translation of LTD effector proteins (Bear et al 2004, Todd & Malter 2002). A critical prediction of this model is that the magnitude of LTD should be enhanced by diminished mGluR-dependent translation of FMRP. Despite its appeal, and its consistency with studies using the *Fmr1* knockout mouse as an experimental model, this idea has never been directly tested.

"Premutation" expansions at the *FMR1* locus to between 55 and 200 CGG repeats are associated with the age-related neurodegenerative condition Fragile X-associated Tremor Ataxia Syndrome ((FXTAS; (Berry-Kravis et al 2007, Bourgeois et al 2009, Greco et al 2006, Sullivan et al 2005)). This disorder, characterized clinically by gait ataxia, action tremor, dementia and neuropsychiatric symptoms, occurs in ~40% of male premutation carriers over the age of 50 (Jacquemont et al 2004). However, premutation range repeats are relatively common in the population (estimates upwards of 1:813 males and 1:259 females (Jacquemont et al 2007, Seltzer et al 2012)), and have the potential to significantly influence the risk of other human diseases. Recent studies in young premutation carriers demonstrate higher rates of autism and ADHD-like symptoms in the absence of FXTAS symptoms (Clifford et al 2007, Farzin et al 2006, Grigsby et al 2006, Loesch et al 2003a, Loesch et al 2002, Loesch et al 2004, Loesch et al 2007) and FXS phenotypes have been reported in larger premutation and unmethylated full mutation carriers who produce *FMR1* mRNA but inefficiently translate FMRP (Allen et al 2005, Chonchaiya et al 2009, Feng et al 1995, Hagerman et al 1996, Jacquemont et al 2011, Tassone et al 2000a, Tassone et al 2000b).

Unlike full mutation expansions, premutation sized repeats are unmethylated and over-transcribed, leading to a 2-8 fold elevation in production of *FMR1* mRNA (Tassone et al 2007, Tassone et al 2000c, Todd et al 2010). However, the CGG repeat expansion forms a hairpin loop in the 5′UTR of the RNA transcript that impairs

ribosomal scanning and induces significant translational inefficiency (Kaufmann et al 1999, Ludwig et al 2011, Primerano et al 2002, Zumwalt et al 2007). This leads to low-normal or decreased basal FMRP expression in Fragile X-premutation carriers, depending on the repeat size (Kaufmann et al 1999, Tassone et al 2004a). The neurodegeneration seen in FXTAS and other age-related premutation phenotypes are thought to result primarily from an RNA gain-of function mechanism (Hagerman 2012, Li & Jin 2012, Renoux & Todd 2012). In contrast, work in two independently generated *FMR1* premutation mouse models suggests an additional role for FMRP insufficiency in aspects of the premutation phenotype, especially in younger animals who do not yet demonstrate neurodegenerative sequelae (Berman & Willemsen 2009, Chen et al 2010, Cunningham et al 2011, Qin et al 2011). Defects in these mice include alterations in neuronal migration, dendritic branching, synaptic activity in cultured neurons, and behavioral defects including altered performance on measures of anxiety and social interaction (Cao et al 2012, Cunningham et al , Qin et al 2005).

Given the known critical roles for FMRP in synaptic function and the translational inefficiency induced by CGG repeat expansions, we hypothesized that mice with large un-methylated CGG repeat expansions would exhibit a specific defect in their ability to rapidly translate FMRP at synapses. A defect in activity-dependent synthesis of FMRP would allow for analysis of the function of newly produced synaptic FMRP, including its role in long-lasting forms of synaptic plasticity. We therefore evaluated dendritic FMRP synthesis and synaptic function in a premutation mouse model where a CGG repeat expansion has been knocked into the mouse *Fmr1* locus (Entezam et al 2007, Qin et al 2011).

Here, we show that mice with 120-150 CGG repeats in the mouse *Fmr1* 5'UTR have modestly reduced basal FMRP expression despite elevated *Fmr1* mRNA levels, consistent with a robust impairment in translational efficiency. Strikingly, these animals exhibit impaired mGluR-dependent translation of dendritic FMRP. Young CGG KI mice exhibit normal basal synaptic properties, but enhanced mGluR-LTD, as in *Fmr1* KO mice (Huber et al 2002). However, the mechanism underlying this functional alteration is distinct from *Fmr1* KO animals, as mGluR-LTD in CGG KI mice remains dependent on new protein synthesis. Our results provide a link between local FMRP synthesis and

mGluR-dependent synaptic plasticity, and raise the possibility that some aspects of the cognitive defects observed in premutation carriers and unmethylated FXS patients may result from altered activity dependent translation of FMRP.

Results

Reduced FMRP translational efficiency in premutation model mice

To evaluate the neurobiological effects of “premutation” range CGG repeats in the *Fmr1* gene, we utilized a mouse model of the Fragile X premutation which contains ~120-150 CGG repeats knocked-in to the endogenous mouse *Fmr1* 5’UTR (CGG KI, (Entezam et al 2007), Figure 2.1A). Similar to human premutation patients, the expression of *Fmr1* mRNA is significantly increased in cortical tissue (*Fmr1* 2/4 exon junction WT 1 ± 0.27, KI 5.24 ± 0.98 $P<0.05$; *Fmr1* 16/17 exon junction WT 1 ± 0.24, KI 4.46 ± 0.74; $P<0.05$, n=5; Figure 2.1B), as well as hippocampus (*Fmr1* 2/4 exon junction WT 1 ± 0.07, KI 4.04 ± 1.11 $P<0.05$; *Fmr1* 16/17 exon junction WT 1 ± 0.07, KI 4.40 ± 1.99, data not shown) in CGG KI mice at 1 month of age (P28-37) compared to littermate controls (Figure 2.1B and data not shown). Despite this increase in mRNA, FMRP expression is significantly reduced in both CGG KI cortex (P28-37, WT 100 ± 10.09%, KI 37.50 ± 4.37%, $P<0.05$, n=5; Figure 2.1C) and hippocampus (P35-60, WT 100 ± 17.00%, KI 44.93 ± 14.71%, $P<0.05$, n=5; Figure 2.1D) from young animals compared to littermate controls. To determine the relative translational efficiency of *Fmr1* mRNA in cortical tissues, we created a ratio of total FMRP/relative *Fmr1* mRNA from the same animals. Using this analysis, we find that the efficiency of *Fmr1* mRNA translation is dramatically reduced in young CGG KI mice compared to littermate controls (FMRP CTX/*Fmr1* mRNA; WT 100 ± 21.26%, KI 7.60 ± 0.99%, $P<0.05$, n=5; Figure 2.1E).

Consistent with previous reports (Entezam et al 2007, Qin et al 2011), FMRP is also reduced in the cortex of older (6 month old) CGG KI mice (WT 100 ± 17.57%, KI 18.57 ± 2.68%, $P<0.05$, n=3; Figure 2.1C) and, interestingly, as compared to WT littermates, the reduction in FMRP expression is greater in older CGG KI animals than in younger animals (1mo: KI 37.50 ± 4.37%, n=5; 6mo: KI 18.57 ± 2.68%, n=3;

P<0.05). This may reflect either a relatively greater decrease in FMR1 transcription in CGG KI vs WT mice with age or could result from somatic instability that is known to occur in these mice (Lokanga et al 2012, Singh et al 2007b).

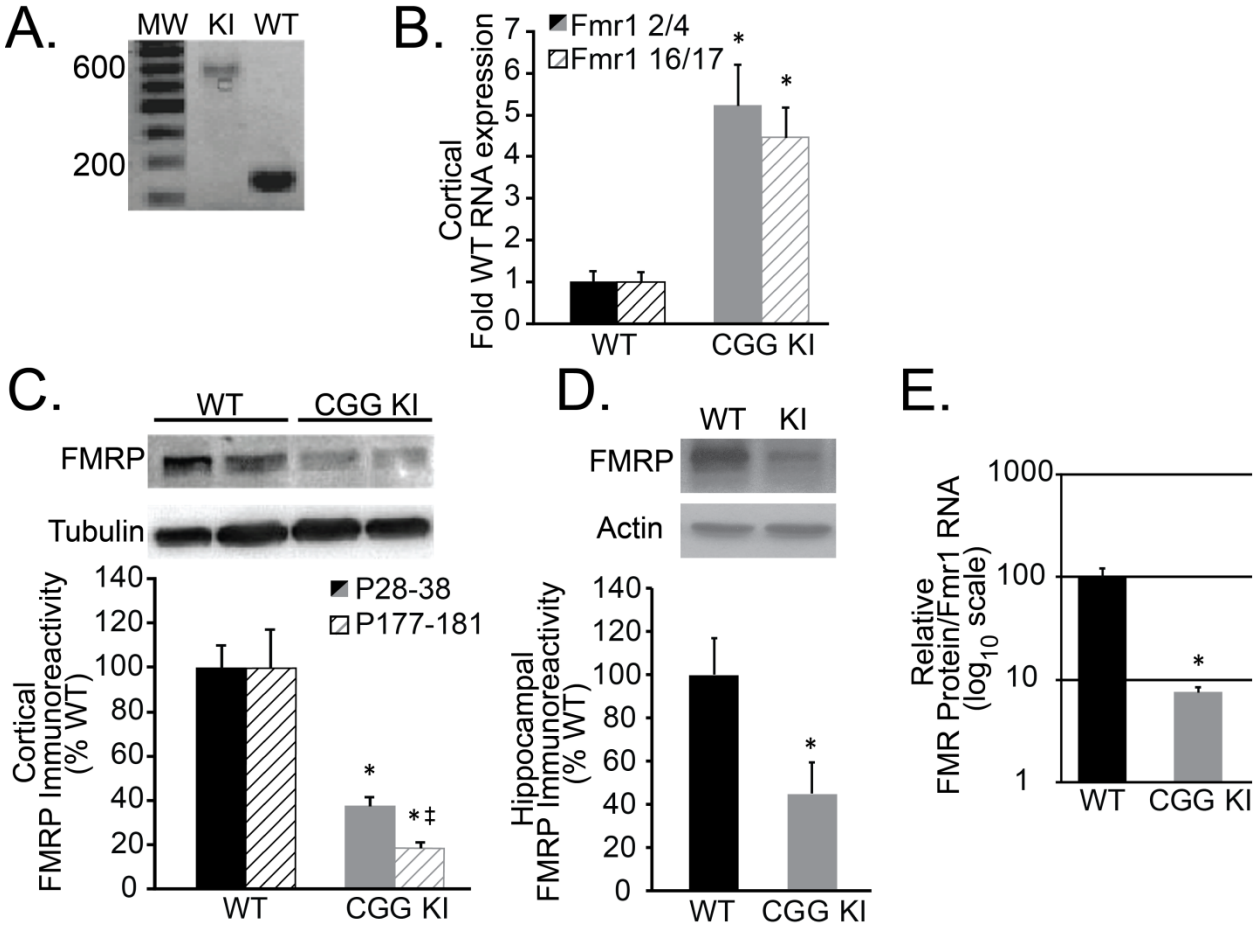


Figure 2.1: Elevated cortical Fmr1 mRNA and decreased FMRP in the fragile X premutation mouse.
(A) PCR genotyping of CGG KI male mice and WT littermates showing the expanded CGG repeat. KI band corresponds to ~120 repeats; WT band corresponds to 8 CGG repeats. (B) *Fmr1* mRNA levels in the cortex of p28-37 fragile X premutation male mice by qPCR using two different sets of primers against *Fmr1*. Bar graph summarizes three experiments, n=5. (C) Representative immunoblot to FMRP (1C3 1:1000) in p28-37 male mouse cortices from the indicated genotypes. Below: summary of three experiments. Mean (±SEM) cortical FMRP in 1 month old (p28-38; n=5) and 6 month old (p177-181; n=3) CGG KI mice is decreased compared to littermate controls. The relative decrease between genotypes is greater in older animals. (D) Representative immunoblot against FMRP (17722 1:1000) in hippocampi of p35 CGG KI animals compared to WT littermate controls. Below: summary of three experiments. Decreased hippocampal FMRP in p35-60 male CGG KI mice compared to WT littermate controls. Summary of three independent experiments; n=5. (E) Translational efficiency of cortical *Fmr1* RNA expressed as the ratio of FMRP to *Fmr1* RNA levels in each individual animal, plotted on $\log_{10}$ scale; n=5. *P<0.05, students t-Test.

Activity-dependent synaptic translation of FMRP is impaired in CGG KI mice

To examine the sub-cellular distribution of FMRP in CGG KI neurons, we generated dissociated hippocampal neurons from CGG KI and WT littermate controls (P1-3). Neurons were probed with antibodies to FMRP on day *in vitro* (DIV) 14-17 (Figure 2.2A), and FMRP expression in somatic and dendritic regions was assessed. FMRP expression was reduced in both the cell soma and proximal dendrite by similar amounts (soma: WT 100 ± 5.74%, KI 49.82 ± 2.69%, $P<0.05$; dendrite: WT 100 ± 4.63%, KI 66.87 ± 2.94%, $P < 0.05$, n=23-24 neurons from 2 animals; Figure 2.2B-D), suggesting that, while FMRP expression is lower, what FMRP is expressed in CGG KI neurons is appropriately distributed.

The reduced efficiency of *Fmr1* mRNA translation in CGG KI mice suggests that rapid, mGluR-dependent synthesis of FMRP might also be disrupted in the CGG KI mice. To address this question, we first examined changes in FMRP expression upon mGluR1/5 simulation in synaptoneurosomes (SNs), a biochemical preparation enriched for synaptic components and often used as a means to examine protein synthesis at isolated synapses (Muddashetty et al 2007). SNs were prepared from neocortex of P14-21 CGG KI mice and their WT littermates. In all experiments, we verified the appropriate enrichment of the synaptic scaffolding protein PSD95 at different stages of SN preparation, and found that the enrichment of PSD95 was similar between WT and CGG KI mice (Figure 2.3A). PSD-95 expression in SNs were similar in WT and CGG KI mice (WT 100±23.8%, CGG KI 99.2±24.8%, n=6). Consistent with our immunocytochemical results (Figure 2.2), the expression of FMRP in unstimulated SNs was reduced in CGG KI, relative to WT mice (% WT, 42.92 ± 21.51%, $P<0.05$, n=5; Figure 2.3B). We next examined changes in FMRP expression in response to mGluR stimulation: SNs were stimulated with the group 1 mGluR agonist, (RS)-3,5-DHPG (100μM) for either 10 or 30 min at 37˚C. Similar to effects seen previously in WT SNs (Weiler et al 1997), DHPG induced significant increases in FMRP at both 10 and 30 min time points relative to controls (10 min: WT 199.77 ± 56.97%; 30 min: WT 202.13 ± 54.83%, $P<0.05$, n=15; Figure 2.3C-D). This increase was dependent on new protein synthesis (% 30 min untreated samples: 30min DHPG: 162.5±32.6%; 30min DHPG+Anisomycin: 124.5±15.1%, n=6, data not shown).

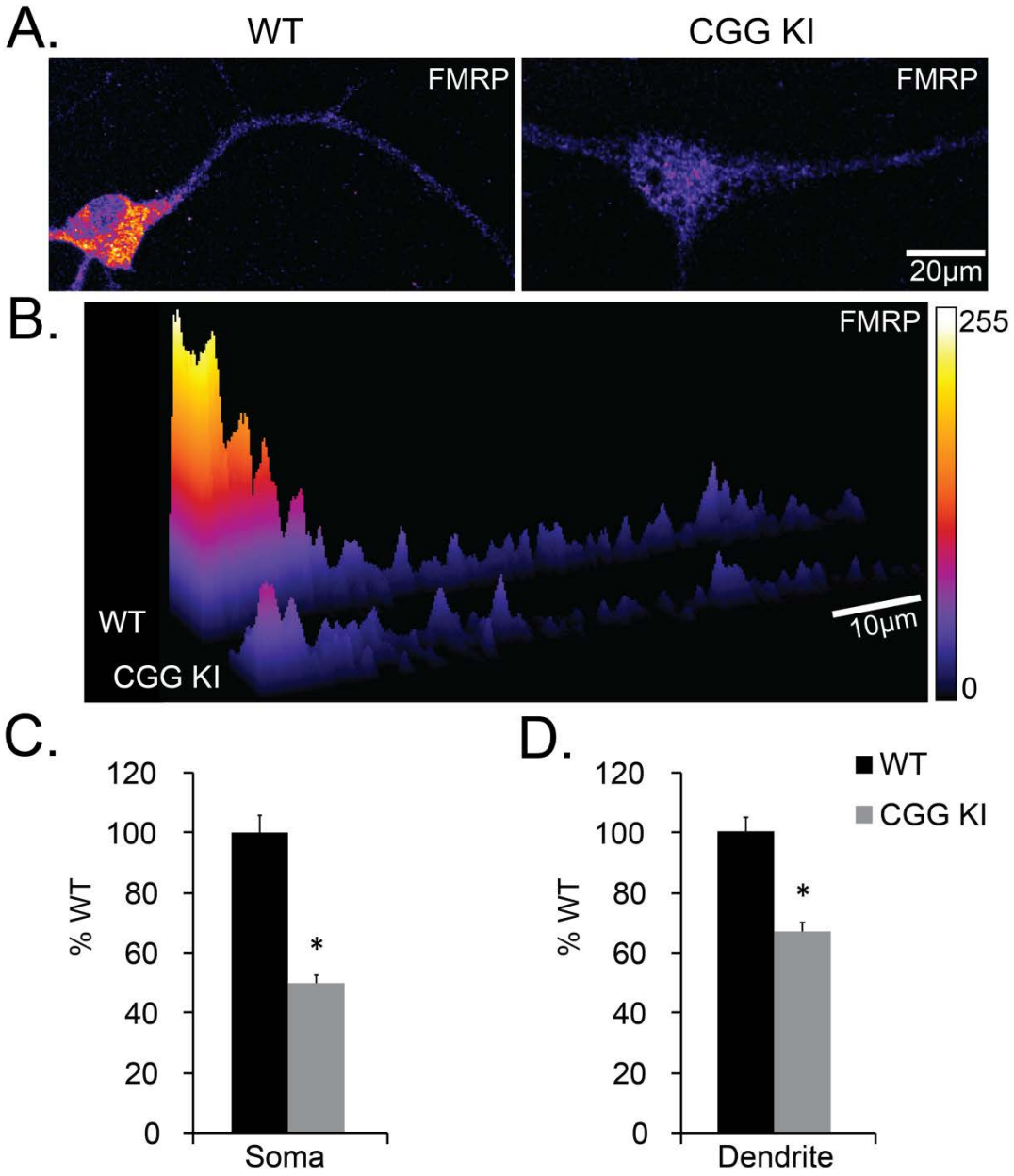


Figure 2.2: Reduced FMRP is distributed throughout dendrites in cultured CGG KI neurons.
(A) DIV 14-17 cultured hippocampal neurons from male P1-3 CGG KI and littermate WT animals stained for FMRP (1C3 1:500). (B) 3D surface plot of relative pixel intensity for the linearized images shown in A demonstrating reduced FMRP expression throughout the soma and dendrite. (C) Total non-zero FMRP fluorescence intensity was quantified in soma, revealing CGG KI neurons have 50% of WT FMRP levels. (D) Summary of fluorescence intensity studies in dendrites (0-40µm), showing reduced FMRP in CGG KI neurons compared to WT neurons; n=23-24 neurons from 2 animals in each group. *P<0.05, students t-Test.

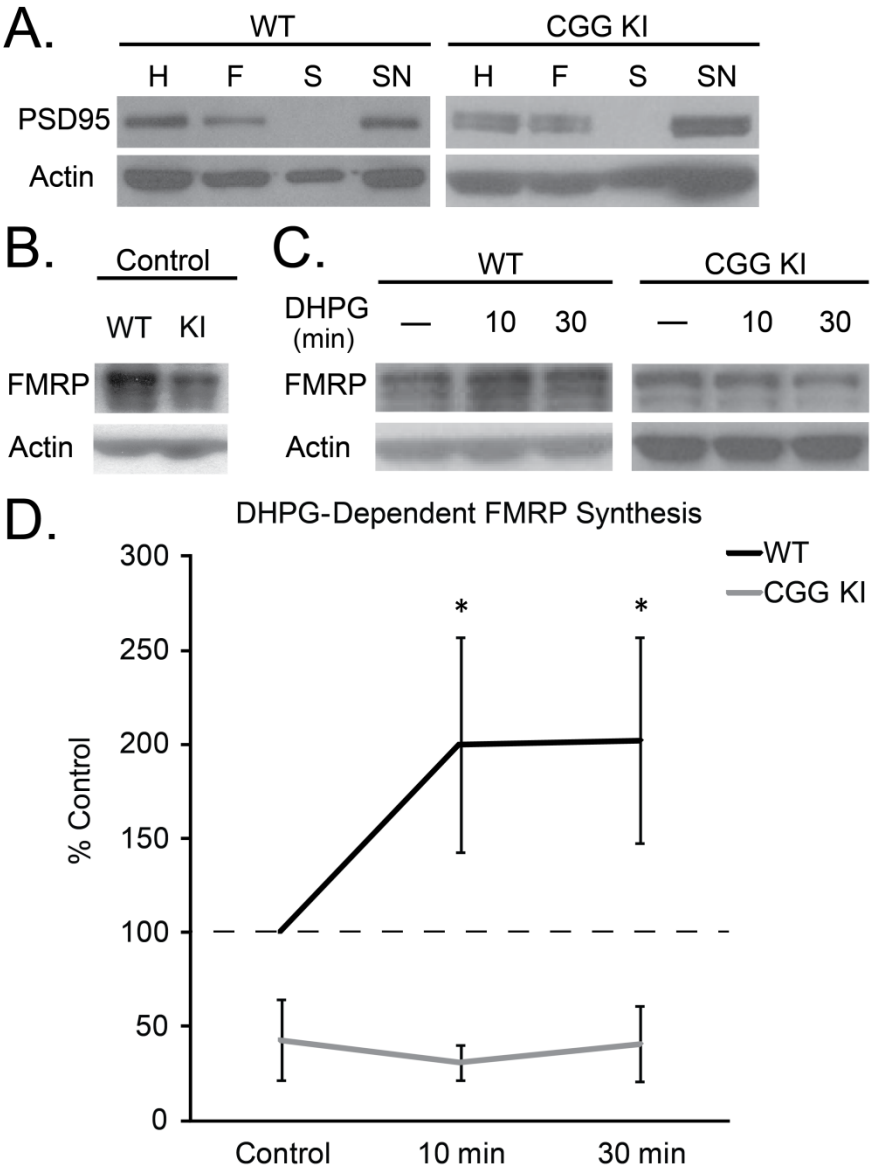


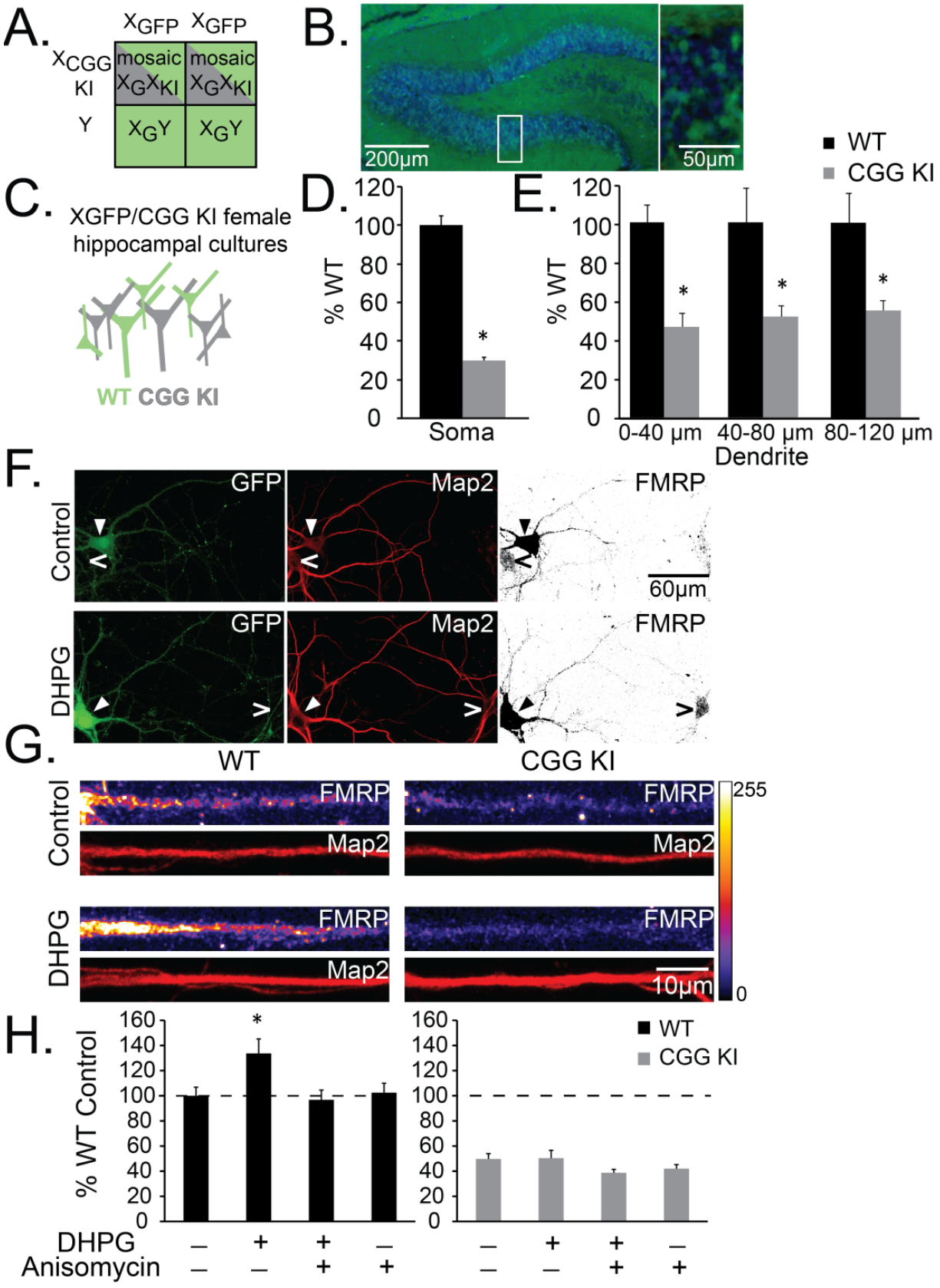
Figure 2.3: CGG KI synaptoneurosomes do not respond to mGluR stimulation. Synaptoneurosomes (SNs) were prepared from WT and CGG KI cortical homogenates. (A) Verification of SN preparation was confirmed by PSD-95 enrichment between initial homogenate (H), filtered sample (F), post-centrifugation supernatant (S), and final synaptoneurosome fraction (SN) in each WT and CGG KI preparation. (B) Representative immunoblot against FMRP (17722 1:1000) in CGG KI SNs compared to littermate WT control. (C) SNs treated with 100μM DHPG for 10 or 30 minutes. Samples were immunoblotted for FMRP (17722 1:1000) and actin (1:5000). (D) Quantification of FMRP immunoreactivity normalized to untreated samples. WT n=15, CGG KI n=5, *P<0.05, Kruskal–Wallis one-way ANOVA.

By contrast, SNs prepared from CGG KI mice did not show changes in FMRP expression in response to DHPG stimulation, consistent with impaired mGluR-dependent translation (Control: KI 42.92 ± 21.51%; 10 min: KI 31.16 ± 9.35%; 30 min: KI 40.84 ± 20.12%; NS, n=5; Figure 2.3C-D).

To further assess mGluR-dependent FMRP translation in CGG KI neurons, we took advantage of mice expressing GFP on the X chromosome to generate hippocampal cultures where neurons harboring the premutation are intermingled with normal length CGG repeat WT neurons (Figure 2.4A-C). This approach allows us to evaluate cell autonomous roles of the premutation by comparing CGG KI neurons with neighboring WT neurons in the same culture, a strategy similar to that used previously for other X-linked mutations (Hanson & Madison 2007, Niere et al 2012). Mice expressing GFP on the X chromosome (Hadjantonakis et al 1998, Kalantry et al 2009) were crossed with CGG KI mice to generate heterozygous XGFP/CGG KI females (Figure 2.4A). This cross generates females possessing one WT X chromosome with a normal copy of *Fmr1* and GFP and one X chromosome with a premutation range CGG repeat knocked-in to the *Fmr1* allele, but no GFP. Due to X-inactivation, roughly half the neurons will inactivate the CGG KI X chromosome and express normal *Fmr1* mRNA along with GFP. The remaining neurons will inactivate the GFP-expressing chromosome and instead express the CGG KI *Fmr1* allele (Hadjantonakis et al 1998). Analysis of dissociated neuronal cultures and histological staining of hippocampi shows roughly equal proportions of GFP+ and GFP- cells in both XGFP/WT and XGFP/KI female mice (Figure 2.4B and data not shown).

We first confirmed the effects of CGG repeat expansions on basal FMRP expression in XGFP/CGG KI cultures. GFP(-)/CGG KI(+) neurons exhibit reduced FMRP immunoreactivity in mixed XGFP/CGG KI cultured networks at DIV14-17 compared to neighboring GFP(+)/FMR1 WT neurons (Figure 2.4C-E). Consistent with studies in non-mosaic neuronal cultures (Figure 2.2), these effects were seen both in the soma (WT 100 ± 4.79%, KI 30.07 ± 1.70%, $P<0.05$, n=14-24 neurons; Figures 2.4D-E) and in both proximal and distal dendritic segments of CGG KI GFP neurons (0-40µm: WT 100 ± 8.65%, KI 46.46 ± 7.03%; 40-80µm: WT 100 ± 17.22, KI 51.47 ± 5.92%; 80-120µm: WT 100 ± 15.05%, KI 55.05 ± 5.36%, $P<0.05$, n=13-23 neurons; Figure 2.4D).

The total amount of FMRP detected decrements with distance from the cell soma in both control and CGG KI neurons. However, the relative difference in expression of basal FMRP between WT and CGG KI neurons is smaller in proximal and distal dendritic compartments than in the cell soma, suggesting that decreases in FMRP reflect a primary failure in translational efficiency rather than a breakdown in FMRP transport into dendrites. We next examined whether the premutation had a cell autonomous effect on mGluR-initiated translation of new FMRP. XGFP/CGG KI cultures were stimulated with DHPG (100μM, 20 minutes) prior to FMRP and Map2 immunostaining. After mGluR activation, WT neurons showed a significant increase in dendritic FMRP immunoreactivity (Control: WT 100 ± 6.85%; DHPG: WT 133.74 ± 11.46%, $P<0.05$; Figure 2.4F-H) and this effect was blocked by pretreatment with the protein synthesis inhibitor anisomycin (40μM, 30 min prior to and throughout DHPG application; Anisomycin+DHPG: WT 96.81 ± 7.75%; Anisomycin: WT 102.53 ± 7.50%; Figure 2.4H). By contrast, DHPG did not alter FMRP expression in CGG KI neurons in the presence or absence of anisomycin (Control: KI 49.70 ± 4.33%; DHPG: KI 50.42 ± 6.20%; Anisomycin+DHPG: KI 38.73 ± 2.74%; Anisomycin: KI 42.04 ± 3.20%; NS; Figure 2.4F-H). These data support the hypothesis that premutation range expanded CGG repeats impair mGluR-dependent synthesis of FMRP in a cell-autonomous fashion.


A.
XGFP XGFP
XCGG KI
Y
mosaic XGXKI
mosaic XGXKI
XGY
XGY
B.
200µm
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XGFP/CGG KI female hippocampal cultures
WT CGG KI
D.
% WT
Soma
E.
WT
CGG KI
% WT
0-40 µm
40-80 µm
80-120 µm
Dendrite
F.
Control
DHPG
GFP
Map2
FMRP
60µm
G.
WT
CGG KI
Control
DHPG
FMRP
Map2
255
0
10µm
H.
% WT Control
WT
CGG KI
DHPG
Anisomycin

Figure 2.4: CGG KI/XGFP heterozygous cultures reveal selective DHPG induction of FMRP in WT neurons.
(A) Breeding scheme used to generate mosaic female mice with one WT (GFP+) X chromosome, and one CGG KI (GFP-) X chromosome. (B) Fluorescent nuclei staining (DAPI 1:10000) in coronal sections from an XGFP/WT female reveal GFP+ and GFP- cells in the hippocampus. (C) Primary hippocampal neurons from mosaic XGFP/CGG KI mice allow both WT (GFP+) and KI (GFP-) neurons in culture. (D) Quantitative analysis on soma from DIV14-17 XGFP/CGG KI neurons stained for Map2 (Sigma 1:1000) and FMRP (17722 1:500). CGG KI (GFP-) soma showed reduced basal FMRP fluorescence compared to WT (GFP-) neurons. (E) Basal FMRP expression is maintained in proximal and distal dendrites of CGG KI mice. WT n=24, CGG KI n=14, *P<0.05, student's t-Test. (F) Cultures were treated with DHPG (100μM for 20 min) prior to FMRP and Map2 staining. (G) Proximal dendrite segments showed selective FMRP immunofluorescence increases in WT (GFP+) neurons, but not in CGG KI (GFP-) neurons. (H) The effects of DHPG are mitigated by pre-treatment with anisomycin (40μM for 30 min) in WT proximal dendrites. There is no effect of DHPG or anisomycin on FMRP expression in the initial segment of CGG KI dendrites. WT n=15-42 neurons from 1-2 animals, CGG KI n=7-25 neurons from 1-2 animals. *P<0.05, one-way ANOVA with Fishers-LSD.

Enhanced mGluR-LTD in hippocampal slices prepared from CGG knock-in mice

Since mGluR-dependent translation is critical for certain forms of synaptic plasticity that are altered in FXS model mice, we next tested whether there was any overlap between the synaptic plasticity phenotypes in *Fmr1* KO mice and CGG KI mice. We first examined basal synaptic properties at CA3-CA1 synapses in acute hippocampal slice preparations from young CGG KI mice with their WT littermates (P31-35). Field EPSPs were evoked by stimulating Schaffer collaterals and recording in stratum radiatum of area CA1. In response to a series of stimulation pulses of increasing intensity, we found that the corresponding increase in fEPSP slope was nearly identical in WT and CGG KI mice (Figure 2.5A). These largely overlapping input/output curves show that CGG KI mice do not exhibit alterations in basal synaptic efficacy relative to WT mice. In addition, we tested whether paired pulse facilitation (PPF), a measure of short term synaptic plasticity and presynaptic function, was altered in CGG KI mice. In response to pairs of stimulation pulses with varying inter-pulse intervals, WT and CGG KI mice exhibited similar robust facilitation of the second synaptic response at all intervals (Figure 2.5B), suggesting that neurotransmitter

release probability is largely similar between the two genotypes. Hence, basal synaptic function is similar between CGG KI mice and their WT littermates.

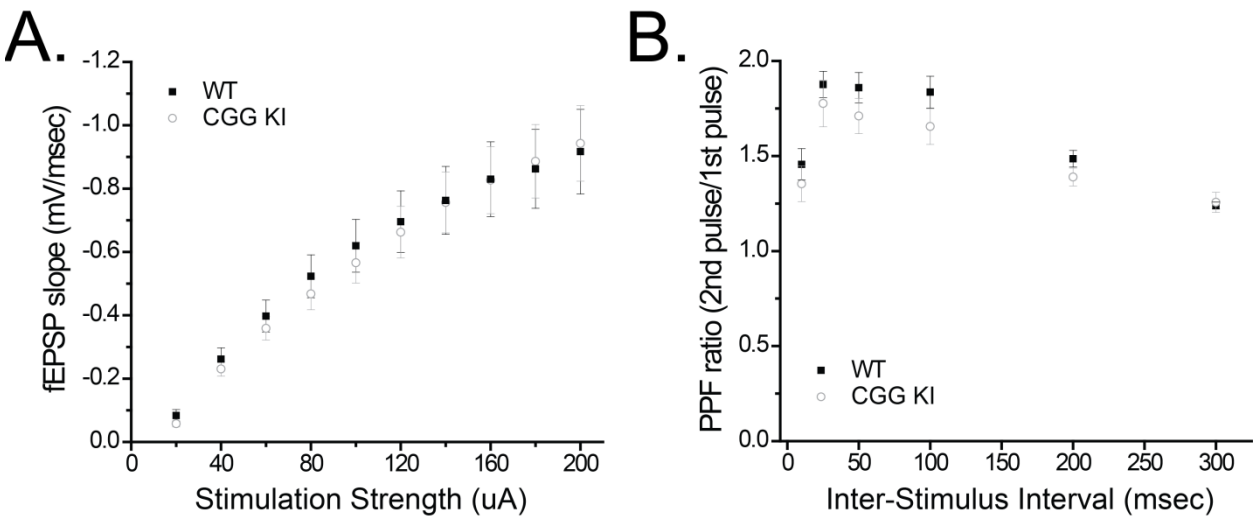

Figure 2.5: Basal syanptic function is unchanged in CGG KI mice.[3]
(A) Hippocampal field EPSPs in response to Schaffer collateral stimulation of increasing strength show a similar input/output response curve in CGG KI animals compared to littermate WT controls. n=19 (WT) and 19 (CGG KI). (B) No difference is detected in paired-pulse facilitation, a measure of basal neurotransmitter release probability, between CGG KI mice and littermate WT mice at any inter-stimulus interval, suggesting that neurotransmitter release probability at CA3-CA1 synapses is not altered by the premutation. n=8 (WT) and 8 (CGG KI).

Our results suggest that premutation range repeats impair FMRP translation even in young mice, raising the question of whether this loss of new FMRP synthesis might mimic aspects of the FXS phenotype. To test this idea, we next examined mGluR-dependent long term depression (LTD) at these CA3-CA1 synapses. After confirming that evoked fEPSPs were stable over time, LTD was induced by brief application of DHPG (100 µM, 10 min; Figure 2.6). As previously described, DHPG treatment induced a sustained depression of fEPSPs in WT slices that persisted well beyond drug application (Figure 2.6). Interestingly, we found that this mGluR-dependent LTD was significantly exaggerated in slices from CGG KI mice (Figure 2.6A), a synaptic phenotype that is similar to *Fmr1* KO mice (Huber et al 2002). These results

[3] Experiments performed by AJ Iliff.

demonstrate that, even during early life, the expanded premutation CGG repeat in the *Fmr1* gene leads to altered hippocampal synaptic plasticity.

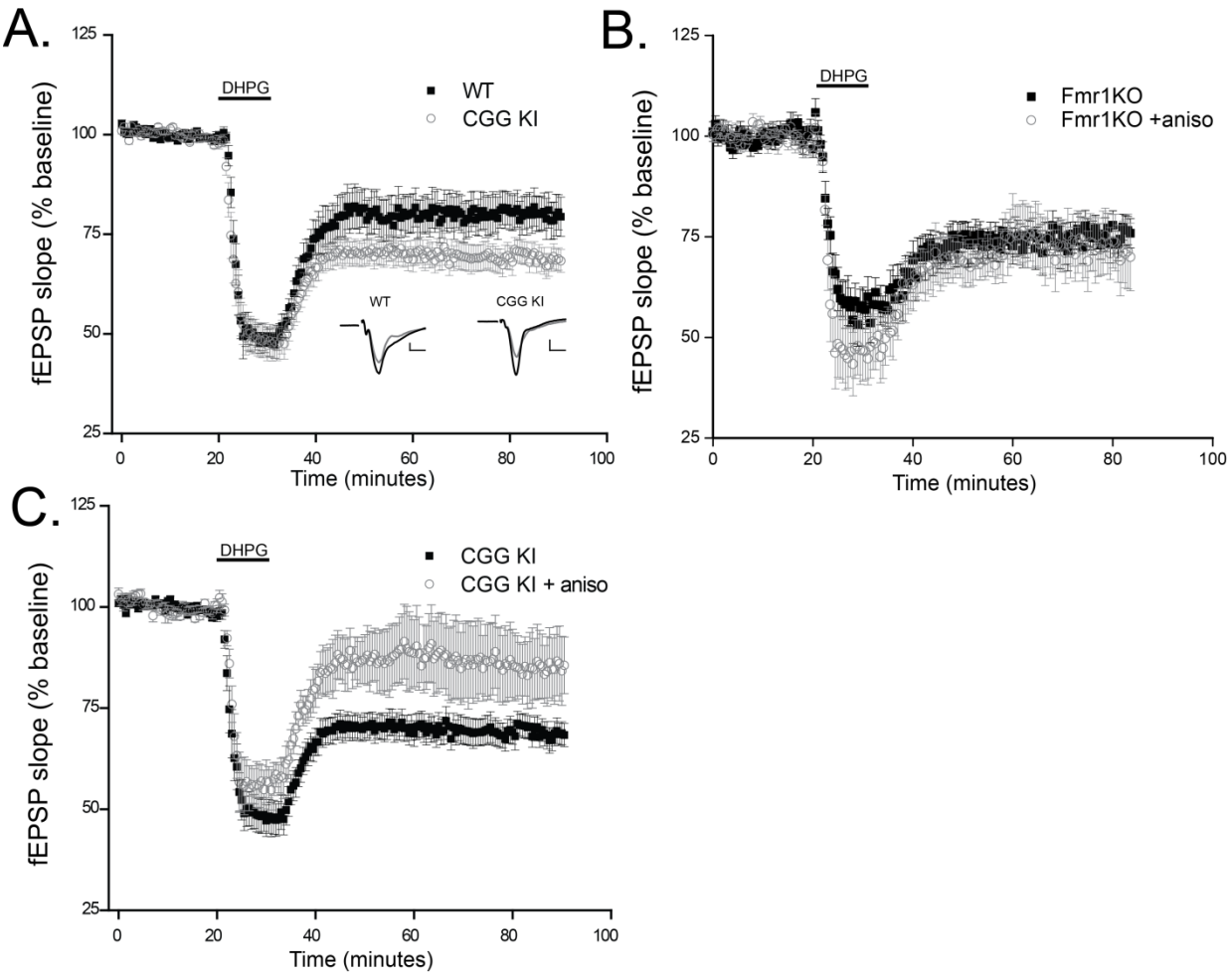

Figure 2.6: Exaggerated mGluR-LTD in CGG KI mice is protein synthesis dependent.[4] (A) Field EPSPs were recorded in CA1 stratum radiatum in response to Schaffer collateral stimulation. Addition of the group 1 mGluR agonist DHPG (100μM; 10 min) induced LTD at CA3-CA1 synapses; this mGluR-LTD was significantly enhanced in CGG KI mice. n=9 (WT) and 13 (CGG KI). Inset: Shown are representative averages of 4 consecutive field potential waveforms from each group during the baseline period and one hour after LTD induction. (B) mGluR LTD in FMR1 KO mice persists in the presence of the protein synthesis inhibitor anisomycin (20μM), as previously reported (Huber et al 2002). n=7 (control) and 8 (aniso). (C) In contrast, the enhanced mGluR-LTD in CGG KI mice remains sensitive to protein synthesis inhibitors. n=13 (control) and 7 (aniso).

[4] Experiments performed by AJ Iliff.

Enhancement of mGluR-LTD in premutation and FXS model mice are mechanistically distinct

Like young CGG KI mice, *Fmr1* KO mice also exhibit enhanced mGluR-LTD (Huber et al 2002). Since this exaggerated mGluR-LTD in FXS model mice is thought to contribute to intellectual disability and/or autistic features in FXS, it was of interest to determine to what extent the exaggerated LTD in each case was due to similar or distinct mechanisms. To explore this issue, we examined whether protein synthesis inhibitors would impair the induction of mGluR-LTD in CGG KI and *Fmr1* KO mice. In WT mice, mGluR-LTD requires rapid dendritic protein synthesis for its induction (Huber et al 2000), whereas mGluR-LTD in *Fmr1* KO mice is completely resistant to protein synthesis inhibitors (Hou et al 2006, Nosyreva & Huber 2006). Consistent with these findings, we found that the magnitude of mGluR-LTD in *Fmr1* KO mice was not affected by blocking protein synthesis with anisomycin (Figure 2.6B). In contrast, the enhanced mGluR-LTD seen in young CGG KI mice was significantly diminished with anisomycin (Figure 2.6C), indicating that mGluR-LTD remains dependent on new protein synthesis in these mice, as in WT mice. Taken together, these results suggest that while young FXS and premutation model mice share the same exaggerated mGluR-LTD phenotype, the mechanism underlying this plasticity is distinct in the two mouse models.

Discussion

The roles of FMRP in both normal and aberrant control of synaptic function have received considerable attention in the past two decades. This effort has been greatly facilitated by work in the *Fmr1* KO mouse, which recapitulates several important features of FXS, and has been instrumental in the rapid development of novel therapeutic approaches (Bhakar et al 2012). In addition, significant advances have been made in our understanding of the molecular consequences of premutation CGG repeat expansions, which enhance *FMR1* transcription but impair FMRP translation and elicit toxicity directly as RNA (Renoux & Todd 2012). By contrast, considerably less is known about the impact of premutation range CGG repeat expansions on neuronal

function. Premutation expansions do not typically lead to overt intellectual disability, but they are increasingly linked to a broad range of important clinical phenotypes in patients, including neuropsychiatric symptoms and autistic features earlier in life (Berry-Kravis et al 2007, Bourgeois et al 2009, Farzin et al 2006). These clinical features are recapitulated in Fragile X premutation model mice, who exhibit altered social interactions and anxiety behaviors compared to littermate controls (Qin et al 2011). We therefore examined neuronal function in young Fragile X premutation model mice, with a specific focus on the impact of the CGG repeat on activity-dependent FMRP translation. Our results demonstrate that premutation model mice exhibit a dramatic decrease in the translational efficiency of *Fmr1* mRNA that impairs rapid, activity-dependent synthesis of FMRP in dendrites. This defect in local FMRP synthesis is associated with exaggerated mGluR-dependent LTD, a phenotype first reported in *Fmr1* KO mice. This shared synaptic phenotype, however, is mechanistically distinct between *Fmr1* KO and premutation model mice, as mGluR-dependent LTD in CGG KI mice remains dependent on new protein synthesis (Figure 2.6B-C). Coupled with data demonstrating altered dendritic spine morphology and development in CGG KI mice (Chen et al 2010, Cunningham et al 2010, Qin et al 2011), our results reveal a shared defect in synaptic plasticity in FXS and premutation model mice and suggest an important role for activity-dependent FMRP synthesis at synapses in regulating the magnitude of synaptic strength.

FMRP is an RNA-binding protein found associated with stalled ribosomes (Darnell et al 2011), where it acts primarily as a translational suppressor (Laggerbauer et al 2001, Li et al 2001, Qin et al 2005). mGluR signaling induces dephosphorylation of FMRP, which then dissociates from polysome-transcript complexes and is rapidly degraded, leading to an activity-dependent burst of translation of FMRP target mRNAs (Figure 2.7A) (Nalavadi et al 2012, Narayanan et al 2007, Narayanan et al 2008). Intriguingly, FMRP also binds and regulates the translation of its own mRNA *in vitro* and FMRP is rapidly synthesized at synapses in response to mGluR activation *in vivo* (Hou et al 2006, Li et al 2001, Siomi et al 1994, Todd et al 2003a, Todd et al 2003b, Weiler et al 1997). The role of FMRP as a translation repressor, and the clear role of certain FMRP targets (e.g., activity-regulated cytoskeletal-associated protein; Arc) as mediators

of mGluR-LTD (Park et al 2008, Waung et al 2008), has bolstered the hypothesis that newly synthesized FMRP functions to provide negative feedback on further local translation, thus constraining the magnitude of LTD after mGluR activation (Figure 2.7A) (Bassell & Warren 2008, Bear et al 2004, Todd & Malter 2002). This notion of newly synthesized FMRP as a "brake" on local translation is consistent with observations that mGluR-LTD and other forms of mGluR mediated plasticity require local protein synthesis in only a brief time window after induction (Huber et al 2000, Merlin et al 1998). In the complete absence of FMRP, mGluR-LTD is enhanced but no longer requires new protein synthesis (Huber et al 2002, Nosyreva & Huber 2006). This has been interpreted as resulting from an uncoupling of mGluR activation and synthesis of critical mGluR-LTD effector proteins (Figure 2.7B) (Niere et al 2012, Park et al 2008, Waung et al 2008). Thus, whereas synaptic levels of Arc and other LTD mediator proteins are low basally in WT neurons and increase as a result of mGluR-dependent synthesis, Arc in FMR1 KO neurons is basally elevated, but is no longer synthesized in response to mGluR activation (Figure 2.7B) (Niere et al 2012).

In CGG KI mice, our results demonstrate that mGluR-LTD is exaggerated as in *Fmr1* KO mice, but that this enhanced mGluR-LTD remains dependent on new protein synthesis, as occurs typically in WT animals (Figure 2.6C). We suggest that this protein-synthesis dependent enhancement of mGluR-LTD occurs because of a specific failure in activity-dependent FMRP production (Figure 2.7C). Although basal FMRP levels are lower in CGG KI mice, FMRP is maintained in both proximal and distal dendritic compartments at levels that are 40-60% of normal, which is above the threshold at which alterations in mGluR triggered AMPA receptor recycling occurs (Nakamoto et al 2007). This suggests that basal synthesis of FMRP, although inefficient, is adequate to achieve suppression of translation of LTD effector proteins in the absence of mGluR activity (Figure 2.7C). However, with mGluR activation, the rapid synthesis of dendritic FMRP is significantly impaired by the CGG repeat expansion. This means that there is inadequate new FMRP produced to halt ongoing translation of FMRP target mRNAs, leading to an over-production of these LTD effector proteins. This overproduction of LTD effector proteins presumably drives the enhanced LTD phenotype, but unlike FMR1 KO cultures, production of these proteins remains coupled

to mGluR activity, as release of FMRP cargo transcripts is still required to initiate the LTD (Figure 2.7C). Within this framework, we propose that new translation of FMRP at synapses is critical for constraining mGluR-LTD, likely through limiting the sustained expression of LTD effectors by repressing their continued synaptic translation. However, some aspects of the effects observed here may also derive from either basal insufficiency of FMRP or from CGG repeat RNA mediated toxic effects. Future experiments will be required to demonstrate altered synthesis of LTD effector proteins in CGG KI mice and to formally exclude contributions from these additional factors on synaptic function in CGG KI mice.

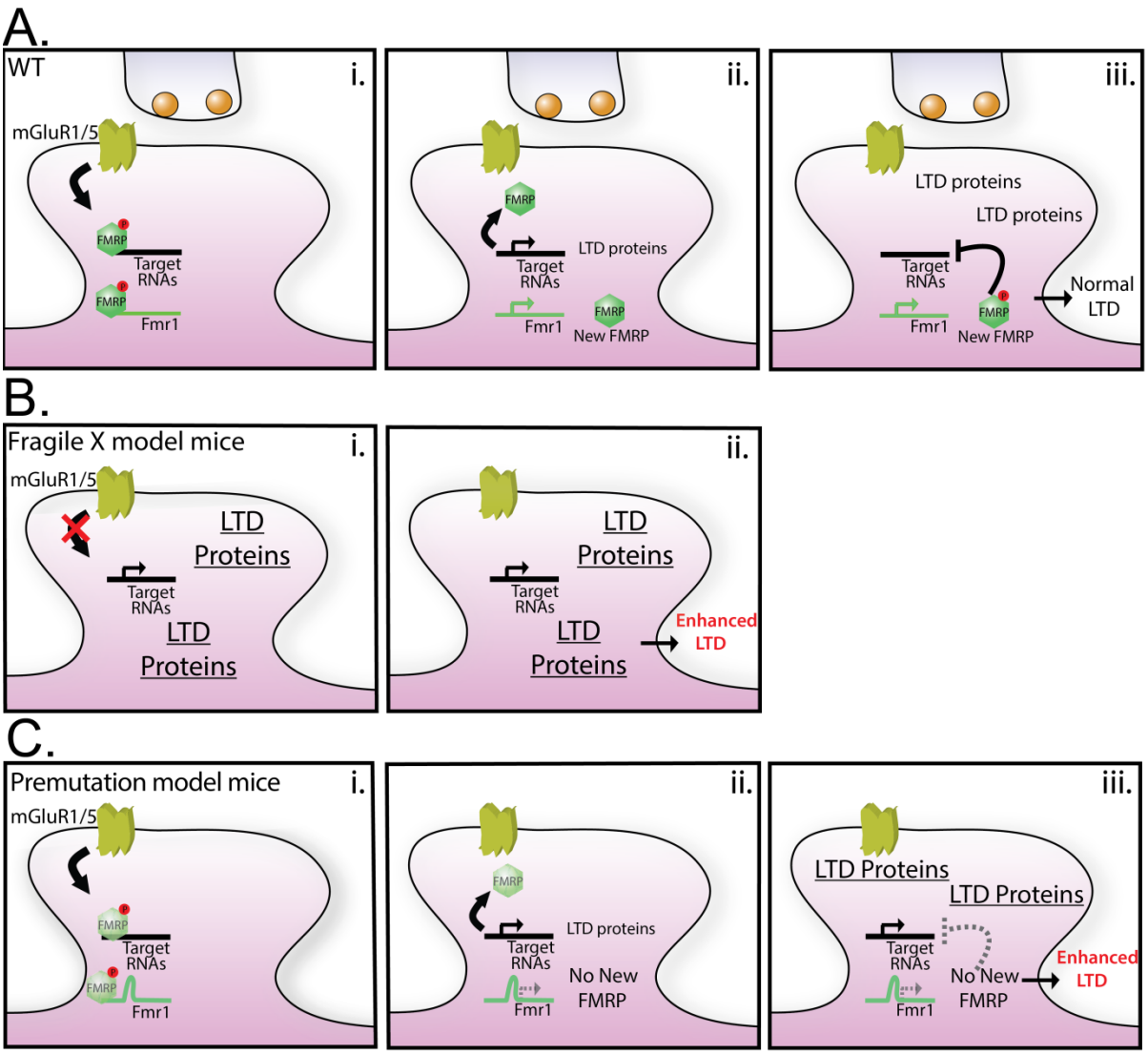

Figure 2.7: A working model of mGluR-LTD in WT, KO and CGG KI mice.
Group I mGluR receptors are critical modulators of synaptic overactivity. (A) Normally, FMRP bound transcripts, including Fmr1 mRNA, exist in stalled polyribosomal complexes at synapses. i) Activation of group I mGluRs triggers internalization of AMPA receptors and the dissociation/clearance of FMRP from target mRNAs. ii) This allows for the rapid translation of proteins required for maintenance of AMPA receptor internalization (LTD proteins), leading to long lasting changes in synaptic strength. In parallel, FMRP is itself synthesized at synapses. iii) This new FMRP acts as a brake on further translation of mRNA targets. The end result is mGluR-LTD that requires a temporally constrained burst of local protein translation after receptor activation. (B) In Fragile X Syndrome model mice, translation of FMRP target transcripts is uncoupled from mGluR signaling. i) This results in a basal increase in production of LTD proteins. Upon mGluR activation, AMPA receptors are internalized normally but the presence of excess basal LTD effector proteins leads to enhancement of mGluR-LTD. As the over-synthesis of LTD effector proteins is not tied to mGluR activation, induction of mGluR-

LTD in FXS model mice does not require new protein synthesis. C) In Fragile X premutation model mice, there is adequate basal expression of FMRP to allow for localization of FMRP with associated transcripts at synapses. i) mGluR activation triggers dissociation of FMRP from these transcripts normally. ii) However, the CGG repeat expansion blocks rapid FMRP synthesis. Without this new FMRP, there is no brake to prevent ongoing synthesis of FMRP target transcripts. iii) The result is over-production of LTD effector proteins and enhanced mGluR-LTD. In contrast to FXS model mice, synaptic protein translation in premutation model mice remains coupled to mGluR activation and the mGluR-LTD is thus dependent on new protein synthesis. This working model makes a number of specific predictions which will be tested in future studies.

In humans, the consequences of premutation range CGG repeats are age-dependent. Of relevance, a recent study examined mGluR-dependent synaptic plasticity in aged animals (10-13 months old), comparing wild-type animals and a different mouse model of the fragile X premutation (Hunsaker et al 2012). They found that aged premutation model mice exhibited weaker immediate synaptic depression following mGluR activation relative to their wild-type counterparts, but the level of sustained synaptic depression was similar across genotypes. By contrast, in younger animals, we find no difference in acute synaptic depression driven by mGluR activation, but a significant increase in the magnitude of enduring synaptic depression following mGluR stimulation. Although Hunsaker et al. (2012) used a different *Fmr1* premutation mouse model than the one employed here, these results raise the interesting possibility that the impact of the *Fmr1* premutation may evolve as a function of age. One possibility is that the effects of enhanced mGluR-LTD on the development of childhood and early adult onset phenotypes in premutation carriers may be dissociable from the development of late-adult-onset FXTAS in premutation carriers, where RNA mediated toxicity and neurodegeneration might be expected to have a greater impact.

In this work, we focused on the features of mGluR-LTD in young premutation model mice, given that exaggerated hippocampal mGluR-LTD in *Fmr1* KO mice is widely considered relevant to the intellectual disability and autistic symptoms seen in FXS. However, it is likely that *Fmr1* premutation repeats may have a broader impact on neural excitability. A recent series of *in vitro* studies demonstrated that neurons cultured from premutation mice develop abnormal firing properties (Cao et al 2012).

These neuronal networks exhibit clustered firing and increased Ca^{2+} oscillations, as well as disruptions in neurotransmitter transport machinery (Cao et al 2012). Neurons derived from induced pluripotent stem cells (iPSCs) generated from premutation carrier fibroblasts exhibit a similar increase in Ca^{2+} dynamics (Liu et al 2012). The authors speculated that the functional deficits arise from an improper excitation/inhibition ratio created by the altered transport of glutamate and GABA. While changes in the ratio of excitation to inhibition would influence Ca^{2+} dynamics and thus the firing properties of neurons, we did not find evidence of altered basal synaptic transmission in our *ex vivo* experiments (Figure 2.5).

Recent clinical evidence highlights potential points of confluence in symptoms found in young premutation carriers with FXS, suggesting that comparisons between FXS and premutation model mice may help to better identify specific behavioral and neurophysiological correlates of disease features. Specifically, work by a number of groups has demonstrated increased rates of autism and ADHD in premutation carriers, as well as neuropsychiatric symptoms, and executive and amygdala dysfunction (Cornish et al 2005, Farzin et al 2006, Hessl et al 2007, Hessl et al 2005, Hessl et al 2011, Hocking et al 2012, Hunter et al 2008, Kogan et al 2008, Loesch et al 2003b). This amygdala dysfunction and structural changes in premutation carriers without FXTAS correlate with lower blood FMRP expression (Hessl et al 2011). Consistent with this, two CGG KI mouse models exhibit numerous behavioral defects that mirror those observed in *Fmr1* KO animals (Hunsaker et al 2012, Hunsaker et al 2009, Qin et al 2011). We find that FXS model mice and *Fmr1* premutation model mice of similar ages share an important synaptic plasticity phenotype. Our data raise the intriguing possibility that neuropsychiatric abnormalities, autism and ADHD-like symptoms in young premutation patients may be linked to the mGluR-dependent plasticity deficits examined in mouse models of these disorders. However, it should be noted that the repeat sizes studied in CGG KI mice here and elsewhere are significantly larger than that seen in the average premutation carrier, as repeats become progressively less stable with expansions above 55 repeats. These findings are therefore more relevant to those rare patients who have greater than 100 CGG repeats or who have an unmethylated full mutation. This model may be particularly relevant to this latter

category as recent data suggesting both that a significant (>30 %) portion of FXS patients exhibit incomplete *FMR1* DNA methylation and produce at least some FMR1 RNA (Jacquemont et al 2011). Importantly, this epigenetic alteration correlates with clinical severity and response to some experimental therapies (Jacquemont et al 2011). As clinical trials proceed in this patient population with agents that either directly or indirectly target the mGluR pathway (Bhakar et al 2012, Hagerman et al 2012, Jacquemont et al 2011), it will be important to understand how mechanistic differences in different mutation states elicit altered mGluR-LTD, and incorporate this knowledge into better practice and drug development.

Materials and Methods

Mice and Cell Culture

Animal use followed NIH guidelines and was in compliance with the University of Michigan Committee on Use and Care of Animals. DNA was extracted from tail biopsies and isolated with DirectPCR lysis reagent (Viagen) and proteinase K (0.2 µg/µl, Roche), incubated overnight at 55˚C. Proteinase K was heat inactivated and DNA samples were genotyped first with primers against the Y chromosome (5'GTGAGAGGCACAAGTTGGC, 5'GTCTTGCCTGTATGTGATGG) to determine the sex of each animal using Platinum® PCR Supermix (Invitrogen). To amplify the knocked-in CGG repeat expansion, we targeted mouse specific *Fmr1* allele (5'AGCCCCGCACTTCCACCACCAGCTCCTCCA, 5'GCTCAGCTCCGTTTCGGTTTCACTTCCGGT) in male hemizygous animals using the Expand High Fidelity PCR System (Roche) supplemented with 2M Betaine (Sigma) and 5% DMSO (Fisher Scientific) as described previously (Tassone et al 2008). As genotyping was performed on tail samples early in life, small expansions in repeat length may have occurred due to somatic instability in older animals (Lokanga et al 2012). Dissociated hippocampal neuron cultures were prepared from postnatal (P1-3) mice as previously described (Jakawich et al 2010). Experiments were performed at 14-17 days *in vitro* (DIV).

Drugs

R,S-3,5-DHPG (Tocris) was prepared fresh each day in sterile water, or artificial cerebrospinal fluid (aCSF, in mM: 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$, and 10 dextrose). Anisomycin (Sigma) was prepared as a 1000x stock in DMSO, stored at -20°C, and diluted to final concentration in aCSF or conditioned media.

Western Blotting

Brain lysate samples were homogenized in RIPA buffer (50mM Tris-HCl, 150mM NaCl, 0.1% SDS, 1% NP-40, 0.5% deoxycholic acid-sodium salt, pH 7.4) containing Complete Mini protease inhibitor cocktail (Roche). Samples were sonicated, centrifuged, and total protein content of the supernatant measured using a DC Protein assay (Bio-Rad). Equal amounts of protein were mixed with 4x Laemmli buffer and boiled for 5 minutes before separation on 10 or 12% polyacrylamide gels. Gels were transferred to PVDF membranes and blocked with Tris-buffered saline containing 0.1% Triton-X (TBST) and 5% nonfat milk for 60 min at RT, and incubated with an antibody against FMRP (Millipore mouse monoclonal 1C3 1:1000 or Abcam rabbit polyclonal 17722, 1:1000) or PSD-95 (Abcam, 6G6-1C9, 1:2000) overnight at 4°C. After washing with TBST, blots were incubated with a corresponding HRP-conjugated secondary antibody (anti-rabbit or anti-mouse 1:5000; Jackson Immunoresearch); this was followed by chemiluminescent detection (Western Lightning Plus-ECL, PerkinElmer). The same blots were reprobed with a mouse monoclonal antibody against β-tubulin (University of Iowa's Developmental Studies Hybridoma Bank E7, 1:5000) or β-actin (Sigma 1:5000) to confirm equal loading. Band intensity was quantified in the linear range with densitometry using NIH ImageJ.

QPCR

Dissected cortex or hippocampi from P28-60 male mice were flash frozen and stored at -80C. RNA was extracted using TRIzol Reagent (Invitrogen), following manufacturer's guidelines. Equal amounts of extracted RNA (1µg) were used to generate cDNA (iScript™ cDNA synthesis kit, Bio-Rad). QPCR was performed using

iQ™ SYBR© Green Supermix (Bio-Rad) and primers against the 2/4 (5'CATGAAGATTCAATAACAGTTGC, 5'CACTTTAGCTAACCACCAACAG) or 16/17 (5'CCGAACAGATAATCGTCCACG, 5'ACGCTGTCTGGCTTTTCCTTC) exons of mouse *Fmr1*, and actin (5'GGCATCCTCACCCTGAAGTA, 5'AGAGGCGTACAGGGATAGCA). Samples were run in triplicate, and *Fmr1* expression data normalized to actin expression for each sample.

Translational efficiency calculation

The translational efficiency ratio was calculated by deriving FMR1 mRNA expression levels determined by qRT PCR from one cortex while total protein lysates were prepared from the contralateral cortex from the same animal. For each animal, cortical FMR1 mRNA expression (relative to actin) was normalized to the mean FMR1 mRNA expression in control cortices. Similarly, cortical FMRP levels were expressed as a ratio to actin expression and then normalized to the mean FMRP expression in control cortices. These numbers were then expressed as a ratio of normalized FMRP expression/normalized FMR1 mRNA expression. Finally, the mean value of this ratio in WT animals was set at 100 and all individual animal values were expressed as a percentage of this number.

Synaptoneurosomes

Synaptoneurosomes (SNs) were prepared from male P14-21 WT and CGG KI mice as described previously (Hollingsworth et al 1985, Muddashetty et al 2007, Scheetz et al 2000). Briefly, cortices were homogenized in 3 ml of homogenization buffer (containing (in mm): 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 1.53 KH_2PO_4, 212.7 glucose, and 1 DTT, pH 7.4), supplemented with Complete Mini protease inhibitor cocktail (Roche) on ice. Samples were passed through a 100 µm nylon mesh filter, followed by two 10 µm nylon mesh filters (Millipore), followed by centrifugation at 1000g for 15 min at 4˚C. The pellets were suspended in 1.1 ml homogenization buffer per cortex. SN preparations were divided into 10 x100µl aliquots for technical duplicates, and pre-warmed for 10 minutes at 37˚C before stimulation with (RS)-3,5-DHPG (Tocris, 100µM). After incubation with DHPG at 37˚C, samples were passed through a 28

gauge needle, and processed for western blotting as above. Expression of all samples was normalized to unstimulated samples maintained at 37ºC for 60 minutes and statistical significance was determined using a Kruskal–Wallis one-way analysis of variance. Similar results were observed when comparisons were done with pre-stimulated samples (i.e., samples from the same SN prep that were never warmed to 37ºC, data not shown).

Immunohistochemistry

Animals were anesthetized with 0.2 mg Ketamine/20 µg Xylazine per kilogram prior to transcardial perfusion (2 ml per minute) with 5-10ml of ice cold sterile PBS and 5-10ml of 4% paraformaldehyde followed by brain dissection. Brains were sunk in 30% sucrose in PBS at 4˚C prior to sectioning with a vibratome at 30 µm. Free-floating sections were stored in cryostorage (30% sucrose, 33.33% ethylene glycol, 0.05 M PB pH 7.4) at -20˚C. Sections were removed from cryostorage by rotating in PBS at 4˚C overnight. Sections were permeabilized in 0.1% Triton X in PBS for 5 minutes, followed by staining with DAPI (1:10000) for 15 minutes at room temperature. Sections were washed 2X with PBS, and mounted on slides in ProLong® Gold Antifade Reagent with DAPI (Invitrogen).

Immunocytochemistry and Microscopy

All experiments were conducted at 37ºC. Neurons were treated with anisomycin (40 µM) or vehicle (DMSO 1:1000) for 30 minutes in conditioned media. Cultures were then stimulated with DHPG (100 µM) for 20 minutes in the presence of anisomycin, or left as controls with vehicle, or with anisomycin alone. After treatment, neurons were fixed with warmed 4% paraformaldehyde (PFA)/4% sucrose in phosphate buffered saline with 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS-MC), permeabilized (0.1% Triton X in PBS-MC, 5 min), blocked with 5% normal goat serum (NGS) in PBS-MC for 1 hour, and labeled with an antibody against FMRP (Millipore 1C3 1:200 or Abcam 17722 1:500). For co-labeling of dendrites, we used antibodies against Map2 (Sigma M4403 1:1000, Millipore AB5622 1:1000) for 60 minutes at RT, or overnight at 4˚C. Secondary

detection was achieved with Alexa 488-, 555-, or 635-conjugated goat anti-rabbit or goat anti-mouse antibodies (1:500 or 1:1000) for 60 min at RT.

All imaging was performed on an inverted Olympus FV1000 laser scanning confocal microscope with identical acquisition parameters for each treatment condition. Image analysis was performed on maximal intensity z-projected images using custom written analysis routines for ImageJ. Statistical analysis utilized a one way ANOVA to detect differences across conditions within genotype. N≈20-40/condition across multiple individual experiments for each genotype.

Electrophysiology

Hippocampal slices were prepared from P35–42 male CGG KI mice and their male wild-type (WT) littermates. Mice were lightly anesthetized with isofluorane before decapitation. Then, the brain and hippocampal lobules were rapidly removed and placed in ice cold artificial cerebrospinal fluid (aCSF, containing in mM: 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$, 10 dextrose, pH 7.4, saturated with 95% O_2, 5% CO_2). Transverse slices (400 µm) of the hippocampus were cut using a tissue chopper (Stoelting, Wood Dale, IL) and CA3 was surgically isolated from CA1 with a scalpel. Slices recovered for 2-5 hours at room temperature in a submersion chamber containing aCSF prior to recording. For recording, hippocampal slices were transferred to a recording chamber and continuously perfused at 32ºC with aCSF at a rate of 1-2 ml/min.

Recording electrodes were pulled from borosilicate capillary glass (G150-4, Warner) and filled with aCSF. The recording pipette was placed in the middle of stratum radiatum of CA1. Synaptic responses were elicited using cluster stimulation electrodes (FHC, Bowdoin, ME) placed in CA1stratum radiatum, lateral to the recording electrode. Current was delivered for 100 µs with an ISO-flex stimulator (AMPI, Jerusalem, Israel). Stable baseline responses were collected every 30 sec (0.033 Hz) by using a stimulation intensity (20–140 µA) yielding ~50% of the maximal synaptic response. The fEPSP signal was amplified 1000 times with a DAM-50 DC differential amplifier (WPI) and filtered at 3 kHz. Recordings were collected at 10 kHz using Clampex 10.2 and analyzed using Clampfit 10.2 (Molecular Devices, Sunnyvale, CA).

For all experiments, the initial slope of each fEPSP was expressed as the percentage of the baseline average. Pooled data represent mean fEPSP slope (+/- SEM). Statistical significance was determined using independent t test, $P<0.05$.

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Chapter 3
Fragile X mental retardation protein expression in Alzheimer's disease[5]

Abstract

The *FMR1* protein product, FMRP, is an mRNA binding protein associated with translational inhibition of target transcripts. One FMRP target is the amyloid precursor protein (APP) mRNA, and APP levels are elevated in *Fmr1* KO mice. Given that elevated APP protein expression can elicit Alzheimer's disease (AD) in patients and model systems, we evaluated whether FMRP expression might be altered in Alzheimer's autopsy brain samples and mouse models compared to controls. In a double transgenic mouse model of AD (APP/PS1), we found no difference in FMRP expression in aged AD model mice compared to littermate controls. FMRP expression was also similar in AD and control patient frontal cortex and cerebellum samples. Fragile X-associated tremor/ataxia syndrome (FXTAS) is an age related neurodegenerative disorder caused by expanded CGG repeats in the 5'UTR of the *FMR1* gene. Patients experience cognitive impairment and dementia in addition to motor symptoms. In parallel studies, we measured FMRP expression in cortex and cerebellum from three FXTAS patients and found reduced expression compared to both controls and Alzheimer's patient brains, consistent with animal models. We also find increased APP levels in cerebellar, but not cortical, samples of FXTAS patients compared to controls. Taken together, these data suggest that a decrease in FMRP expression is unlikely to be a primary contributor to Alzheimer's disease pathogenesis.

[5] Most of this chapter was originally published as: Renoux AJ, Carducci NM, Ahmady AA, Todd PK. 2014. Fragile X mental retardation expression in Alzheimer's disease. Front Genet. 5:360.

Introduction

Fragile X-associated disorders result from intergenerational instability in a CGG microsatellite repeat expansion located in the 5' untranslated region (UTR) of the Fragile X mental retardation (*FMR1)* gene. In the general population, the mean repeat length is 30 CGG repeats with a range from 4 to 55 (Strom et al 2007). When this repeat expands beyond this normal range, it can cause symptoms associated with Fragile X spectrum disorders (Nelson et al 2013, Renoux & Todd 2012). Greater than 200 CGG repeats elicits transcriptional silencing of the *FMR1* locus, with absent or markedly reduced FMR1 mRNA and FMR protein (FMRP) production (Bagni & Oostra 2013). These large expansions result clinically in Fragile X Syndrome (FXS), which is the most common monogenic cause of autism and intellectual disability (Hernandez et al 2009, Rogers et al 2001).

In contrast, repeat expansions in the "premutation" range between 55 and 200 CGG repeats cause a distinct set of human disorders, including Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) and Fragile X-associated Premature Ovarian Insufficiency (FXPOI) (Berry-Kravis et al 2007, Hagerman 2013, Nelson et al 2013). FXTAS is an age-related neurodegenerative disorder characterized by gait difficulties, action tremor, and variably present Parkinsonism, dysautonomia, and dementia (Berry-Kravis et al 2007). Unlike the scenario in FXS, premutation sized CGG repeats elicit enhanced *FMR1* transcription through alterations in the local chromatin structure (Hagerman & Hagerman 2013, Tassone et al 2007, Tassone et al 2000a, Todd et al 2010). However, this increase in FMR1 mRNA is paradoxically associated with a reduction in total and activity-dependent FMRP expression (Entezam et al 2007, Iliff et al 2013, Kenneson et al 2001, Ludwig et al 2014, Pretto et al 2014, Qin et al 2011, Tassone et al 2000b). This decrease in FMRP likely derives from an alteration in FMR1 mRNA translational efficiency, where the repeat forms a hairpin secondary structure that impairs ribosomal scanning (Chen et al 2003b, Feng et al 1995, Kenneson et al 2001, Ludwig et al 2011, Primerano et al 2002). Research into the mechanisms of neurodegeneration in FXTAS have largely focused on how the FMR1 mRNA might elicit

a gain of function toxicity, either through sequestration of RNA binding proteins or through triggered aberrant translation through the repeat of aggregate prone proteins that underlie the intranuclear inclusions observed in patients (Hagerman & Hagerman 2004, Hagerman & Hagerman 2013, Iwahashi et al 2006, Jin et al 2007, Jin et al 2003, Renoux & Todd 2012, Sellier et al 2013, Sellier et al 2010, Sofola et al 2007, Todd et al 2013, Todd & Paulson 2010). However, more recent work has begun to explore whether a reduction in FMRP expression might contribute to aspects of disease pathogenesis in these individuals (Iliff et al 2013, Ludwig et al 2014, Pretto et al 2014, Renoux et al 2014a, von Leden et al 2014).

The absence of FMRP causes the cognitive impairment seen in FXS and may contribute to some of the symptoms observed in FXTAS. A significant body of work has explored the normal functions of FMRP (O'Donnell & Warren 2002, Santoro et al 2012, Wang et al 2012). FMRP is an RNA binding protein found associated with poly-ribosome complexes in soma and synapses (Ascano et al 2012, Chen et al 2014, Darnell et al 2005, Darnell et al 2011, Feng et al 1997, Tamanini et al 1996, Willemsen et al 1996, Zalfa et al 2003). It is normally phosphorylated, and upon metabotropic glutamate receptor (mGluR) activation is dephosphorylated to allow its associated transcripts to be translated (Bear et al 2004, Ceman et al 2003, Muddashetty et al 2007, Nalavadi et al 2012, Narayanan et al 2008, Weiler et al 2004). This allows FMRP to participate in temporal and spatial control of activity-dependent translation. In an effort to understand how reduced levels of FMRP may alter synaptic function, many groups have identified possible FMRP target transcripts. One transcript associated with FMRP is the amyloid precursor protein (APP) mRNA (Lee et al 2010, Westmark & Malter 2007). High-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (HITS-CLIP and PAR-CLIP) analysis on FMRP-associated transcripts identified APP mRNA (Ascano et al 2012, Darnell et al 2011). This interaction appears to play a role in regulating APP translation, as APP synthesis in response to mGluR activation is increased in control mice (Westmark & Malter 2007). Moreover, a mouse model of FXS which lacks FMRP (*Fmr1* KO) exhibit higher basal levels of APP, and of the pathogenic product of APP cleavage, β-amyloid (Aβ), and FXS patients show abnormal Aβ levels in plasma and brain tissues (Westmark & Malter

2007, Westmark et al 2011). Overexpression of APP in *Fmr1* KO mice increases mortality and seizure susceptibility (Westmark et al 2008). Conversely, genetic reduction of APP in the *Fmr1* KO mouse improved seizure activity, anxiety-associated behavior, spine morphology and altered mGluR-dependent long term depression (LTD), indicating a role for Aβ expression levels in FXS pathology (Westmark et al 2011).

As there is evidence indicating FMRP participates in regulating APP production and that FMRP and APP interact genetically, we sought to explore the possibility that reduced FMRP levels may contribute to increased APP and Aβ levels in AD mouse models and spontaneous cases of AD. This is especially relevant given evidence for decreased FMRP expression with age in mouse models (Gaur & Prasad 2014, Iliff et al 2013, Ludwig et al 2014, Singh et al 2007). Impaired FMRP expression in older individuals could lead to increased basal APP translation, increasing the amyloidogenic burden and thus serving as a contributor to AD pathogenesis. We sought to test this hypothesis by measuring cortical FMRP levels by western blot and immunohistochemistry in a double transgenic AD mouse model (APP/PS1; (Haass et al 1995, Prihar et al 1999)). We also measured FMRP immunoreactivity in human cortex and cerebellum from control and confirmed AD samples. Concurrently, we included samples from three FXTAS patients who exhibited reduced FMRP levels. We found similar FMRP expression in AD model mice and AD human samples. We further examined APP expression in FXTAS patient samples, and found a selective increase in cerebellar lysates, but not in frontal cortex or in CGG KI model mice. Taken together, our data suggest that impaired FMRP expression is unlikely to contribute significantly to end-stage Alzheimer's disease pathogenesis.

Results

To accurately evaluate the expression of FMRP in our experiments, we compared two commonly used antibodies for their specificity by western blot and immunohistochemistry. To assess the sensitivity of each antibody, we compared FMRP levels in WT, littermate CGG KI, and *Fmr1* KO mouse cortical lysates (Figure 3.1). Both the 1C3 mouse anti-FMRP (Millipore) and the 17722 rabbit anti-FMRP (Abcam) show

reactivity in the *Fmr1* KO samples (Figure 3.1A-B). The largest band at ~75kD corresponds to FMRP and is absent in *Fmr1* KO lysates with both antibodies. This band was used for all measurements in subsequent figures. However, a smaller band at ~71kD results at least partially from cross-reactivity with the related protein, FXR1(Ceman et al 1999, Tamanini et al 1997), is still reactive with both antibodies tested. Similarly, we examined specificity of the 17722 anti-FMRP antibody in WT, CGG KI and *Fmr1* KO coronal brain sections by immunohistochemistry (WT n=3, CGG KI n=3, *Fmr1* KO n=4; representative images Figure 3.1C-D). Conditions were optimized to minimize DAB reactivity in Fmr1 KO tissue. We reliably observed reduced FMRP levels in CGG KI mice both by western analysis and immunohistochemistry, consistent with previous reports ((Entezam et al 2007, Iliff et al 2013, Kenneson et al 2001, Ludwig et al 2014, Pretto et al 2014, Qin et al 2011, Tassone et al 2000a); Figure 3.1).

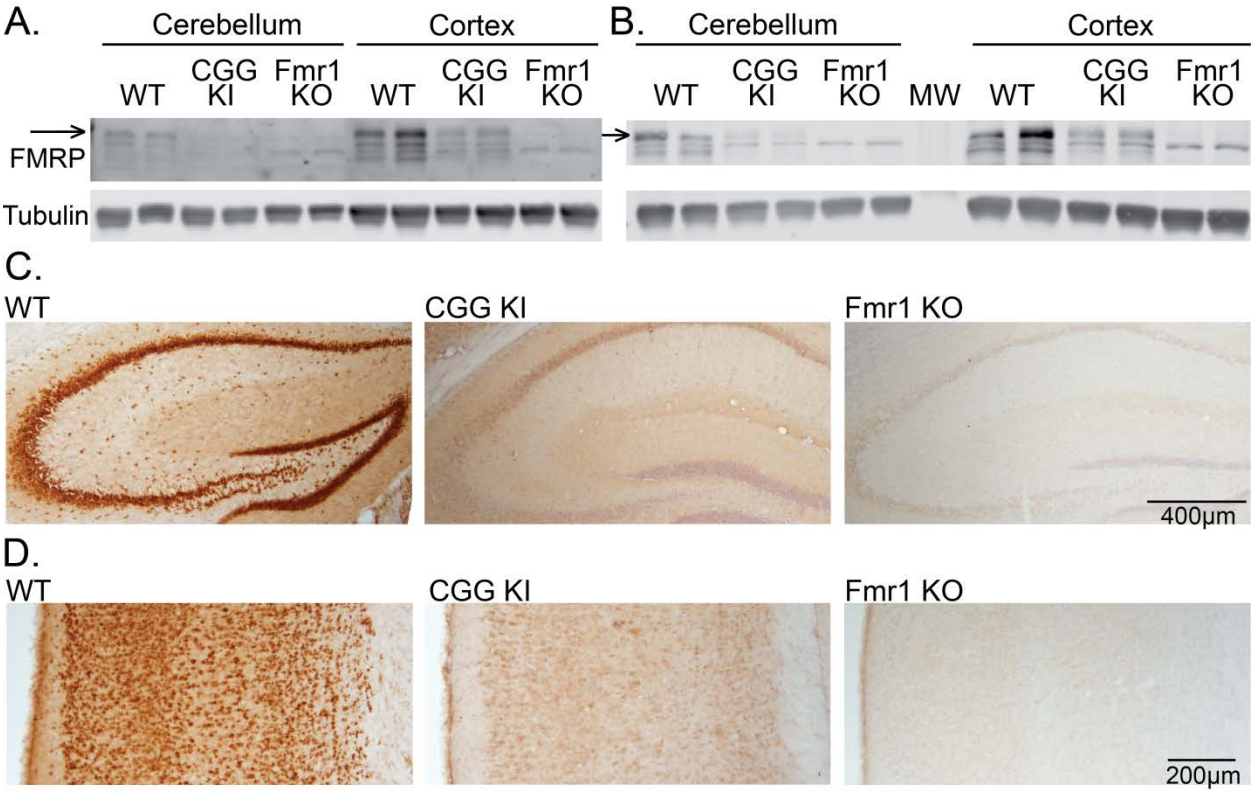

Figure 3.1: FMRP antibody specificity.
(A) Mouse α-FMRP 1C3 (Millipore) 1:250 on cerebellar and combined cerebral cortex and subcortical brain lysates of WT (n=2), premutation model CGG KI (n=2), and Fmr1 KO mice (n=2). Arrow indicates the FMRP-specific band which is absent in Fmr1 KO

lysates. (B) Rabbit α-FMRP 17722 (Abcam) 1:1000 on the same brain lysates as in (A). (C) Immunohistochemistry with the 17722 α-FMRP antibody in WT (n=3), CGG KI (n=3) and Fmr1 KO (n=4) hippocampus. (D) Cortical FMRP expression from identical animals as in (C) stained with the 17722 antibody. Sections were counter-stained with hematoxylin to label nuclei.

In an effort to probe a potential role of altered FMRP expression in Alzheimer's disease pathogenesis, we evaluated FMRP in a double transgenic model of AD which contains an additional copy of the human *APP* gene carrying the familial Swedish (K670N/M671L) missense mutation (Haass et al 1995), and a deletion of exon 9 in the presenilin1 gene (Prihar et al 1999). These double transgenic mice (APP/PS1; n=6: 6 female) and age-matched control littermates (n=8: 4 male, 4 female) were compared at 80-90 weeks of age for combined cortical and subcortical FMRP levels. We performed western blot analysis in triplicate, averaging the percent control FMRP for each animal across blots to minimize error between experiments. We find no difference in FMRP levels in APP/PS1 mice compared to controls (t=0.358, df=12, NS; Figure 3.2A). Cerebellar samples from the same animals demonstrated no significant difference in FMRP expression (t=1.300, df=12, NS; Figure 3.2A). Any contribution of sex was evaluated in the control animals (as the APP/PS1 mice were all female), and FMRP expression was not significantly different between male and female control cortical values (male n=4, female n=4, t=0.717 df=6, NS; data not shown). As several groups have found FMRP levels change with age (Gaur & Prasad 2014, Iliff et al 2013, Ludwig et al 2014, Singh et al 2007), we explored expression in young (8 week old) APP/PS1 mice (WT n=3: 3 female; APP/PS1 n=3: 3 female). We find no difference in cortical FMRP expression at this age (t=0.919, df=4, NS). Cerebellar FMRP expression demonstrated a non-significant increase in AD model mice (t=2.627, df=4, NS; Figure 3.2B). We also compared age-dependent FMRP expression of the 8 week old and the 80 week old animals, and did not find a significant difference in the cortex or the cerebellum (Cortex: 8wk n=3 females; 80wk n=8, 4 males, 4 females; t=0.342, df=9, NS; Cerebellum: 8wk n=3 females; 80wk n=8, 4 males, 4 females; t=1.188, df=9, NS; Figure 3.2C). There was no significant difference between same sex 8 and 80 week old female FMRP expression (Cortex: 8wk female n=3, 80wk female n=4, t=1.034 df=5, NS;

Cerebellum: 8wk female n=3, 80wk female n=4, t=1.136 df=5, NS; data not shown). We went on to probe FMRP levels by immunohistochemistry in 80 week old control and APP/PS1 mice. Comparing cortex, hippocampus, and subcortical regions using the 1C3 α-FMRP antibody, there were no differences detected (WT n=2, APP/PS1 n=3; Figure 3.2D-E).

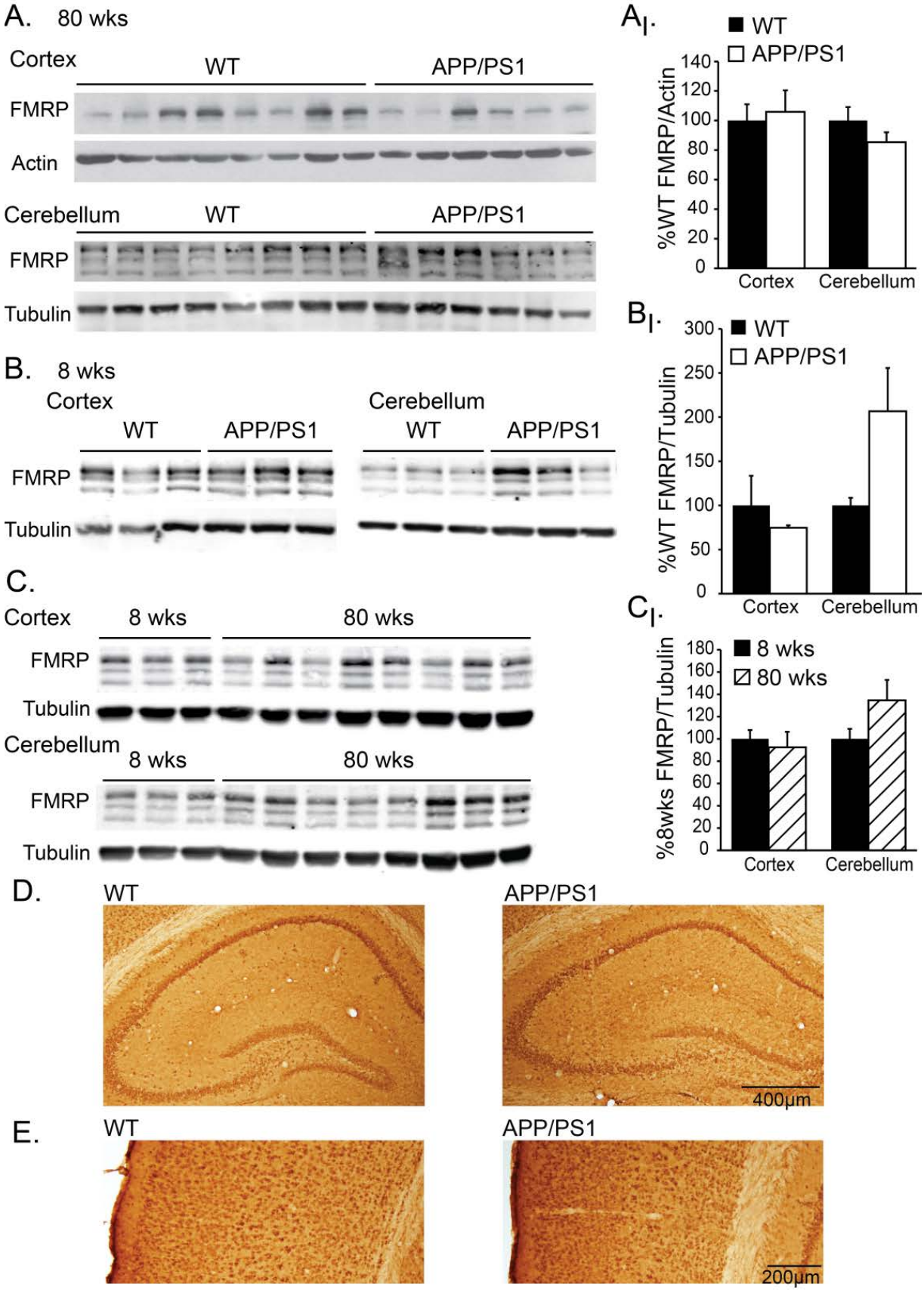
A. 80 wks
Cortex
WT
APP/PS1
FMRP
Actin
Cerebellum
WT
APP/PS1
FMRP
Tubulin
A|.
WT
APP/PS1
%WT FMRP/Actin
140
120
100
80
60
40
20
0
Cortex
Cerebellum
B. 8 wks
Cortex
Cerebellum
WT
APP/PS1
WT
APP/PS1
FMRP
Tubulin
B|.
WT
APP/PS1
%WT FMRP/Tubulin
300
250
200
150
100
50
0
Cortex
Cerebellum
C.
Cortex
8 wks
80 wks
FMRP
Tubulin
Cerebellum
8 wks
80 wks
FMRP
Tubulin
C|.
8 wks
80 wks
%8wks FMRP/Tubulin
180
160
140
120
100
80
60
40
20
0
Cortex
Cerebellum
D.
WT
APP/PS1
400μm
E.
WT
APP/PS1
200μm

Figure 3.2: FMRP expression in AD model APP/PS1 mice.
(A) Cortical and subcortical, and cerebellar lysates from 80 week old APP/PS1 (n=6) and age matched controls (n=8) probed with the 17722 α-FMRP antibody and actin or tubulin. (A1) FMRP/actin values were calculated and normalized to WT levels, and expressed as %WT in the quantification. Results are the summary of values calculated in technical triplicate. (B) 8 week old APP/PS1 (n=3) and WT littermate controls (n=3) were compared for FMRP expression in cortical and subcortical and cerebellar lysates. (B1) FMRP/tubulin values are expressed as %WT, and data are the summary of experiments performed in technical triplicate. (C) 8 week old WT (n=3) and 80 week old WT (n=8) cortical and cerebellar lysates compared for FMRP expression. (C1) FMRP/tubulin values are expressed as percentage of mean 8 week old samples, and data are the summary of experiments performed in duplicate. (D) Hippocampal FMRP expression using the 1C3 α-FMRP antibody in WT and APP/PS1 mice. (E) Cortical FMRP in the same mice as in (D).

As the mouse model we used was genetically modified to mimic some AD phenotypes, it may not recapitulate proximal pathogenic events that contribute to spontaneous cases of AD. In an attempt to better answer the question of a possible role for FMRP in AD development, we obtained frontal cortex and cerebellar autopsy samples from control and AD patients (details included in Table 3.1). As with our murine samples, we performed western blot analysis in technical triplicate to minimize variability in our measurements. Using this technique, we found no difference in FMRP expression level in the frontal cortex ($t=0.2836$, $df=18$, NS; control n=10, AD n=10; Figure 3.3A, C). As two of the control individuals were younger than the majority of the other donors (39 and 47 years old), we performed analysis excluding those values, and found no change in the result ($t=0.3915$, $df=16$, NS). Similarly there was no difference in FMRP expression found in the cerebellar samples analyzed ($t=0.2837$, $df=18$, NS; control n=10, AD n=10; Figure 3.3B-C). Again, excluding the youngest individuals did not impact the finding ($t=0.3618$, $df=16$; NS). As there was a wide range in post-mortem interval (PMI) and age of the samples tested, we compared normalized values against these two variables, in addition to tissue pH (where available), and found a significant impact on FMRP levels with longer PMI (Age: $r = -0.111$, $df=38$, NS; PMI: $r = -0.371$, $df=38$, $P<0.05$; pH: $r = 0.277$, $df=22$, NS; Figure 3.3D, data not shown). We also evaluated the impact of gender in this experiment by comparing the data with a two-way ANOVA, and found no significant difference of FMRP expression in any group in the

cortex (Gender: $F_{(1, 16)} = 0.3187$, NS; Diagnosis: $F_{(1, 16)} = 0.05139$, NS; Interaction: $F_{(1, 16)} = 0.02455$, NS; data not shown). Cerebellar samples similarly showed no significant impact of gender (Gender: $F_{(1, 16)} = 0.1176$, NS; Diagnosis: $F_{(1, 16)} = 0.09488$, NS; Interaction: $F_{(1, 16)} = 0.006407$, NS; data not shown). While these analyses only showed significant contributions of PMI on FMRP levels, the negative trends of decreasing FMRP with age and lower pH suggest that these variables should also be controlled for in future studies.

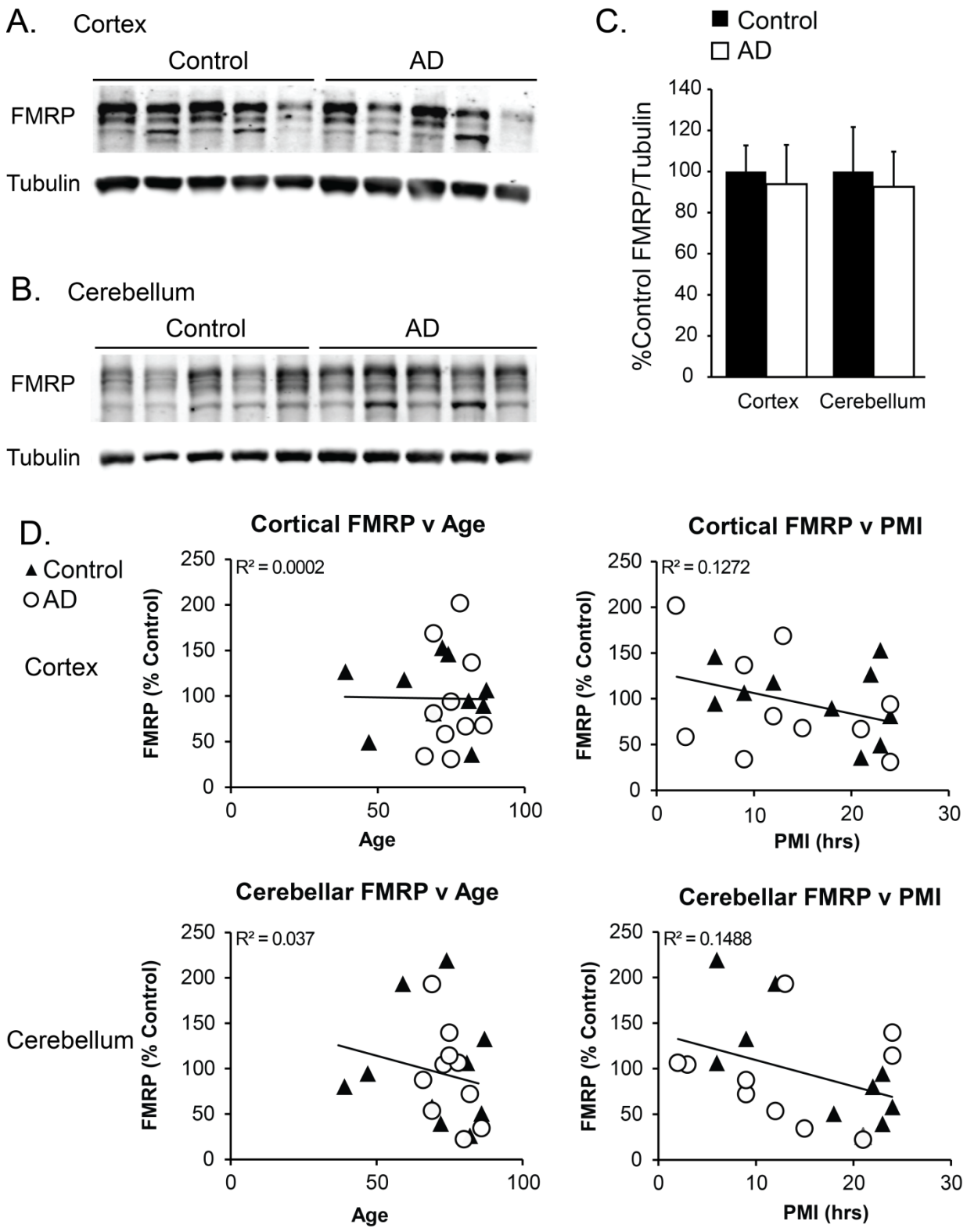

Figure 3.3: FMRP expression in Alzheimer's Disease cortex and cerebellum.
(A) Frontal cortex lysates from control (n=10) and autopsy-confirmed AD patients (n=10) probed with α-FMRP 17722. (B) Cerebellar lysates from the same patients as in (A)

were evaluated concurrently. (C) Quantification of cortical and cerebellar FMRP/tubulin values performed in technical triplicate. (D) Normalized FMRP values were compared to individual patient ages and post-mortem indices. Diagnosis-independent best fit curves display overall trends in FMRP expression.

We obtained samples from FXTAS patient brains, and compared FMRP levels to the controls, finding a decrement in FMRP immunoreactivity in two of the three samples tested, though the number of samples evaluated was not sufficient to discern a significant difference when the two youngest control samples were excluded (Cortex: t=2.161, df=9, NS; Cerebellum: t=1.793, df=9, NS; control n=8, FXTAS n=3; Figure 3.4A-C). Similarly, we compared the age and PMI as a factor which may alter FMRP levels, and found no significant difference of either variable (Age: r = -0.180, df=24, NS; PMI: r = 0.007, df=24, NS; data not shown).

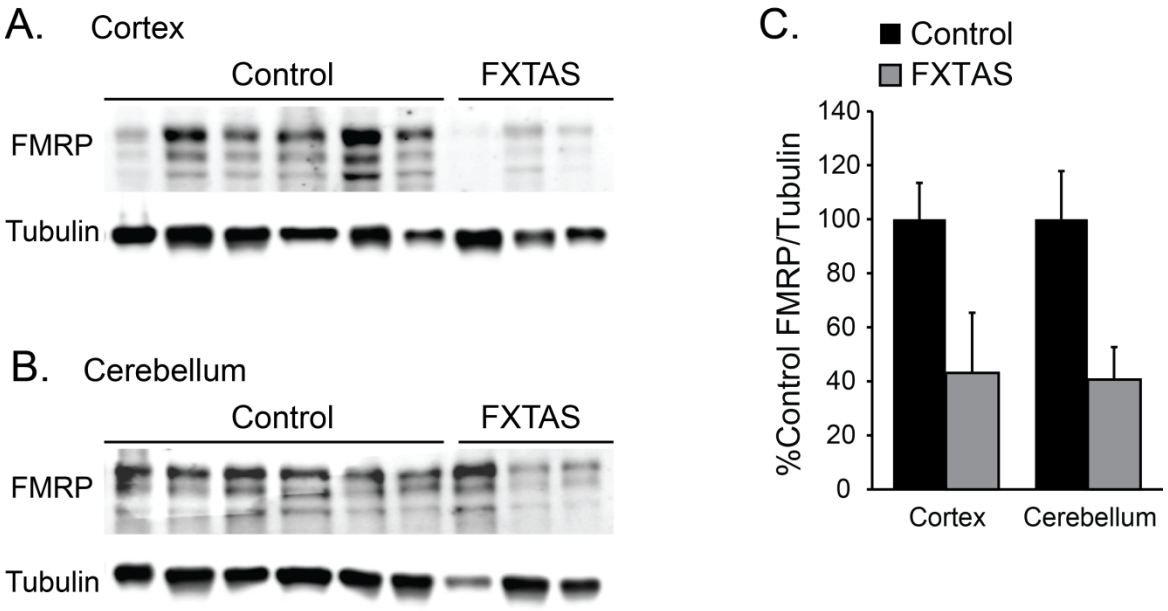

Figure 3.4: Cortical and cerebellar FMRP expression in FXTAS patients.
(A) Frontal cortex lysates from FXTAS patients (n=3) and the same control tissues (n=8) probed with α-FMRP 17722. (B) Cerebellar lysates from the same individuals as in (A). (C) Normalized FMRP expression as a percent of controls, performed in technical triplicate.

To further test the hypothesis that reduced FMRP might enhance APP synthesis, we evaluated total full length APP expression in the same FXTAS patient samples. Despite the small sample size, we did find a selective increase in cerebellar APP

expression in FXTAS samples (t=4.704, df=11, P<0.05; control n=10, FXTAS n=3; Figure 3.5A-C). However, APP levels were unchanged in frontal cortex samples compared to controls (t=0.4603, df=11, NS; control n=10, FXTAS n=3; Figure 3.5A-C). These results were not affected by the inclusion of the two youngest control samples (Cortex: t=0.2636, df=9, NS; Cerebellum: t=4.844, df=9, P<0.05). This finding was not recapitulated in 12 month old CGG KI mice, which showed unchanged levels of APP in both the cortex and cerebellum (Cortex: t=1.877, df=5, NS; Cerebellum: t=1.131, df=5, NS; WT n=3, CGG KI n=4; Figure 3.5D-F). To assess any age-dependent effects of APP expression we compared CGG KI and WT cortical and cerebellar lysates at 2 and 16-18 months of age, and found no significant difference between genotypes (2mo cortex: WT = 100 ± 11.66, CGG KI = 99.86 ± 21.63; t=0.006, df=8, NS; WT n=5, CGG KI n=5; 16-18mo cortex: WT = 100 ± 15.31, CGG KI = 93.09 ± 6.1; t=0.367, df=5, NS; WT n=4, CGG KI n=3; 16-18mo cerebellum: WT = 100 ± 10.84, CGG KI = 75.75 ± 7.70; t=1.690, df=5, NS; WT n=3, CGG KI n=3; data not shown). The C-terminal APP antibody used cannot detect Aβ fragments, so while total APP levels are unchanged, the relative processing or cleavage events may be altered.

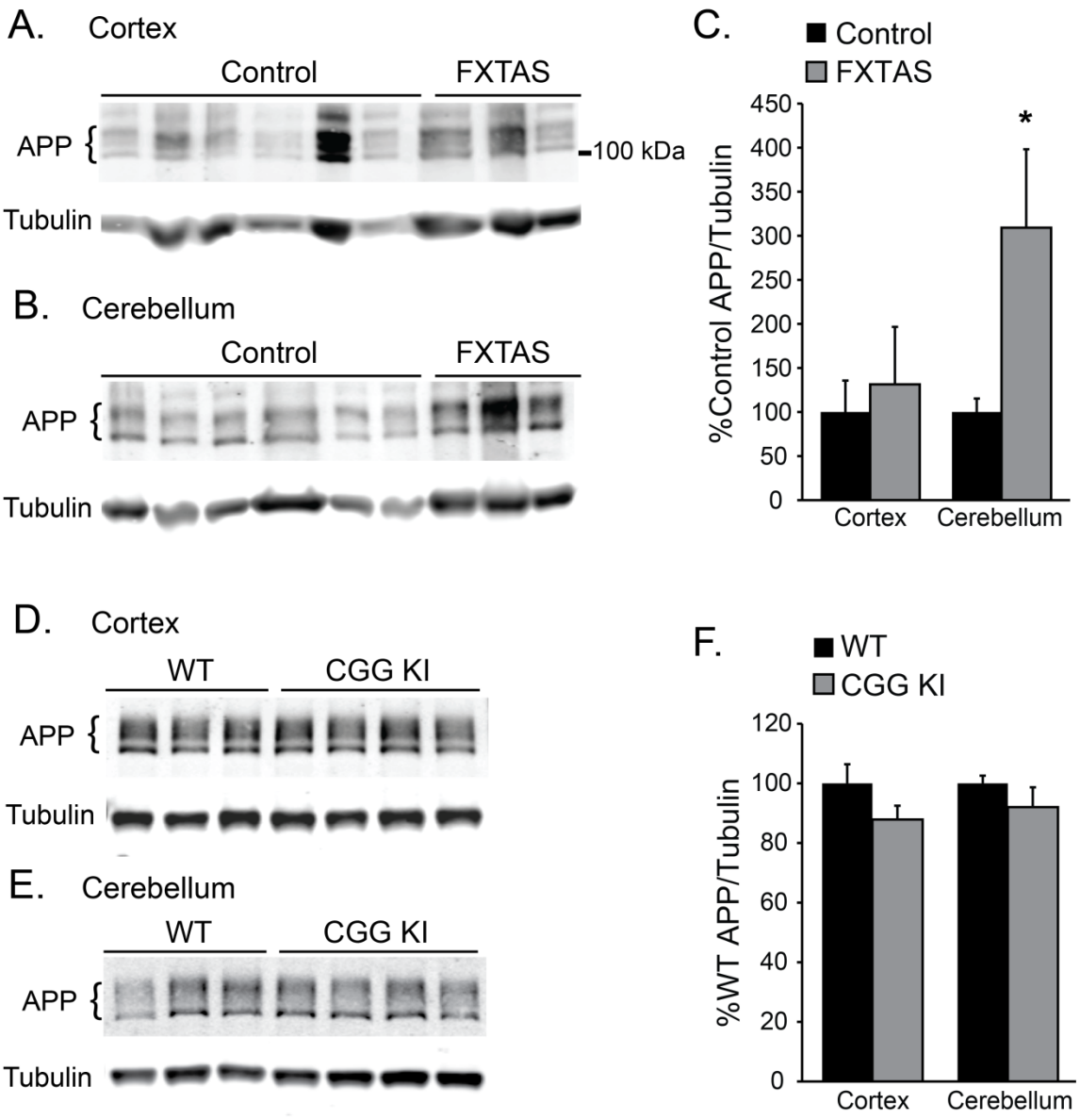

Figure 3.5: APP expression in FXTAS patients and mouse models.
(A) Frontal cortex lysates from control (n=10) and FXTAS patients (n=3) probed with a C-terminal αAPP antibody which detects the three primary isoforms of full length APP (100-125 kDa). (B) Cerebellar lysates from the same individuals as in (A). (C) Normalized APP expression relative to tubulin expressed as a percent of controls, performed in technical triplicate. (D) 12 month old CGG KI (n=4) and WT littermate control (n=3) cortex and subcortical lysates probed with αAPP. (E) Cerebellar lysates from the same animals as in (D). (F) Normalized APP expression in CGG KI mice expressed as a percent of WT controls, performed in technical triplicate. *P<0.05 student's t-test.

Discussion

FMRP is a synaptic mRNA binding protein implicated in Fragile X spectrum disorders. FMRP normally acts to inhibit translation of target transcripts until synaptic transmission and mGluR activation cause its dissociation, allowing for a burst of rapid local translation. One FMRP target is APP, the precursor to the amyloidogenic Aβ peptide (Westmark et al, 2007). APP expression is enhanced in mouse models that lack FMRP (Westmark et al, 2007). FMRP expression is reported to decline with age in mouse models and might follow a similar trajectory in humans (Gaur & Prasad 2014, Ludwig et al 2014, Singh et al 2007). We hypothesized that a natural age-related decline in FMRP might trigger an increase in basal APP synthesis in neurons, leading to enhanced Aβ-42 production and possibly serving as a proximal trigger of AD pathogenesis. Moreover, as AD pathogenesis progresses, we hypothesized that neuronal dysfunction and toxicity might further impair FMRP expression and hence enhance APP translation, creating a feed-forward loop that could drive AD pathogenesis and lower FMRP expression in AD models and patient tissues. To test this hypothesis, we examined FMRP expression in a double transgenic mouse model of AD (APP/PS1), and found no significant difference between AD model and control animals. Furthermore, we tested control and AD patient samples, and found no difference in cortical or cerebellar FMRP expression. In contrast, we observed a decrease in FMRP levels in the two of the three FXTAS patients tested, consistent with published results (Ludwig et al 2014, Pretto et al 2014), and found increased cerebellar levels of APP in these same FXTAS patients, suggesting an impact of lower FMRP on APP expression in humans. Taken together, these data indicate that decreased FMRP expression is not a common finding in AD and suggests that a primary deficiency in FMRP expression is unlikely to play a proximal role in most cases of AD. However, manipulation of FMRP expression and activity retains the potential to influence APP expression and aspects of AD pathogenesis.

FMRP's regulatory function is dependent on phosphorylation by S6K, and dephosphorylation by PP2A (Narayanan et al 2007, Narayanan et al 2008). Recent work has described a function for FMRP degradation upon mGluR activation, and dephosphorylation by PP2A (Nalavadi et al 2012). While the goal of this study was to

examine total FMRP expression levels, alterations in FMRP phosphorylation would impact its regulatory function, and therefore the control of target protein translation. While we did not observe large alterations in FMRP levels, it is possible that relative phosphorylation state could play a role in AD pathogenesis. Further studies comparing phospho-FMRP would be required to examine this possibility.

Our group and others have observed decreases in FMRP expression with age (Gaur & Prasad 2014, Iliff et al 2013, Ludwig et al 2014, Singh et al 2007). However, we observed no significant difference in FMRP levels between 8 and 80 week old WT mice evaluated in this study (Figure 3.2C). The reason for this difference is not immediately clear, although the smaller number of young animals evaluated and the significant variance observed in older animals may explain the discrepancy. Other groups have evaluated various time points ranging from neonate to adult (20 weeks of age; (Gaur & Prasad 2014, Ludwig et al 2014, Singh et al 2007)), and as old as 60-70 weeks of age (Gaur & Prasad 2014, Singh et al 2007). Both groups have observed cerebellar expression to steadily decrease in older animals (Gaur & Prasad 2014, Ludwig et al 2014), though cortical FMRP expression may be more complex during postnatal development into adulthood (Ludwig et al 2014). While we and others have studied animals near 4-6 weeks of age (Gaur & Prasad 2014, Iliff et al 2013, Ludwig et al 2014, Singh et al 2007), the specific 8 week time point utilized here has not been compared directly to old animals previously.

Our study is likely underpowered to detect subtle changes in human FMRP expression. We observed a wide range of FMRP expression levels in both the control and patient samples, consistent with published results (Ludwig et al 2014, Pretto et al 2014). This variance was not completely explained by patient age, tissue pH, or PMI (Figure 3.3), although all of these variables demonstrated a trend towards lower FMRP. This leaves open the possibility that lower basal or activity dependent FMRP expression could still contribute to altered APP expression in a subset of patients or in certain brain regions.

Impaired FMRP expression in FXTAS model mice and patients have been described previously (Entezam et al 2007, Iliff et al 2013, Kenneson et al 2001, Ludwig et al 2014, Pretto et al 2014, Qin et al 2011, Tassone et al 2000a). Similarly, we find

reduced FMRP levels in two of the three FXTAS patient samples tested (Figure 3.4). Symptomatic FXTAS patients can develop cognitive impairment in addition to motor behavior symptoms (Berry-Kravis et al 2007, Leehey 2009). While early studies suggested that pathologic hallmarks of Alzheimer's Disease are rare in FXTAS (Greco et al 2002), recent studies in female premutation carriers with dementia demonstrated plaque and neurofibrillary tangle development consistent with AD pathology (Tassone et al 2012). However, a recent study examining AD patient populations for *FMR1* CGG expansions did not find a significant association (Hall et al 2014).

To test whether FMRP insufficiency might lead to increased APP synthesis in FXTAS, we evaluated APP levels in our patient samples (Figure 3.5). We detected an increase in cerebellar, but not cortical APP expression in FXTAS patients and no differences in a mouse model of FXTAS (Figure 3.5). The C-terminal antibody used for this study does not detect Aβ fragments, so it remains possible that APP processing is altered in FXTAS or CGG premutation models. However, in the context of the published studies noted above, a strong direct relationship between CGG repeats and induction of amyloid pathology appears unlikely.

The group 1 mGluR, mGluR5, has been identified as a receptor of the APP peptide Aβ42 (Hamilton et al 2014, Renner et al 2010, Sokol et al 2011, Um et al 2013). A recent report explored the role of increased mGluR5 signaling in the double transgenic APP/PS1 model of AD (Hamilton et al 2014). Genetic modulation of mGluR5 improved behavioral learning performance, and decreased amyloid plaques found in the APP/PS1 mice (Hamilton et al 2014). Furthermore, cortical FMRP expression was increased in 12 month old APP/PS1 mice compared to WT controls, presumably due to increased mGluR5 signaling, which generates a positive feedback loop leading to increased APP production and cleavage (Hamilton et al 2014). The reason for differing results in our study is not entirely clear, however the animals examined in this report were significantly older (18 months old compared to 12 months old), and the procedures for tissue isolation and lysate generation were different (Hamilton et al 2014). Of note, the increase observed in (Hamilton et al 2014) is consistent with our observations of human AD cerebellar samples which showed increased FMRP expression compared to controls (Figure 3.3).

While reduced FMRP might contribute to neuronal dysfunction in FXTAS patients, it is unlikely to be a primary cause of neurodegeneration in FXTAS. Expression of CGG repeats as RNA in heterologous contexts is sufficient to elicit toxicity in *Drosophila,* cells, and mice (Hagerman 2013, Hagerman & Hagerman 2004, Hashem et al 2009, Iwahashi et al 2006, Jin et al 2007, Jin et al 2003, Renoux & Todd 2012, Sellier et al 2013, Sellier et al 2010, Sofola et al 2007, Todd & Paulson 2010). However, FMRP insufficiency may nonetheless contribute to the cognitive decline observed in mouse models and patients with FXTAS. In addition to the potential differential amyloidogenic burden, reduced FMRP likely impacts synaptic function in premutation mouse models (Hunsaker et al 2012, Iliff et al 2013, von Leden et al 2014). Synaptic dysregulation is thought to precede neurodegeneration, and contribute to the onset of symptoms prior to gross neuronal loss (Dong et al 2009, Milnerwood & Raymond 2010). Delineating the role of FMRP insufficiency in FXTAS thus remains an important objective going forward given the implications for therapeutic development in patients.

In summary, we find no evidence to support a direct link between lower basal FMRP expression and Alzheimer's disease pathogenesis. Future work will be needed to define whether changes in FMRP activity influence Alzheimer's disease development, given the known roles of FMRP in APP processing and neuronal function.

Materials and Methods

Mice

Animal use followed NIH guidelines and was in compliance with the University of Michigan Committee on Use and Care of Animals. *Fmr1* KO (Bakker et al 1994) and CGG KI (Entezam et al 2007) mice were genotyped as described previously (Iliff et al 2013, Renoux et al 2014b). APP/PS1 mouse (Haass et al 1995, Prihar et al 1999) genotypes were confirmed with western analysis for the human APP transgene (clone 6E10 1:2000; Millipore).

Patient Donor Samples

All human tissues were obtained and distributed under oversight by appropriate institution specific review boards. Frontal cortex and cerebellar tissue from 10 control and 10 clinically probable Alzheimer's disease patients were obtained from the University of Michigan Alzheimer's Disease Brain Bank. All AD cases were confirmed at autopsy. Brain tissues from two previously described FXTAS patients (Louis et al 2006, Todd et al 2013) and an additional clinically definite FXTAS patient were used as controls for reduced FMRP expression. CGG repeat size was determined in FXTAS patients by DNA isolation followed by PCR using C and F primers. See Table 3.1 for PMI, age, and sex of each individual.

Table 3.1: Patient donor information.
Diagnosis, age at death, post mortem interval (PMI) in hours for all patients, and FMR1 5'UTR CGG length for FXTAS patients included below. N.D. indicates not determined.

Diagnosis	**Sex**	**Age**	**PMI (hrs)**	**FMR1 Repeats**
Control	F	82	21	n.d.
Control	M	39	22	n.d.
Control	M	47	23	n.d.
Control	M	69	24	n.d.
Control	M	72	23	n.d.
Control	F	86	18	n.d.
Control	F	87	9	n.d.
Control	F	74	6	n.d.
Control	M	59	12	n.d.
Control	M	81	6	n.d.
AD	F	78	2	n.d.
AD	M	69	12	n.d.
AD	F	75	24	n.d.
AD	F	66	9	n.d.
AD	M	82	9	n.d.
AD	M	73	3	n.d.
AD	F	86	15	n.d.

AD	M	80	21	n.d.
AD	F	75	24	n.d.
AD	M	69	13	n.d.
FXTAS	M	78	3.5	90 CGGs
FXTAS	M	74	3	102 CGGs
FXTAS	M	80	6.5	116 blood/180 cheek

Western Blot Analysis

Western blotting was performed as described previously (Iliff et al 2013). Briefly, brain tissue samples (cerebral cortex and subcortical regions from mice, or frontal cortex from human, and cerebellum) were homogenized in RIPA buffer containing Complete Mini protease inhibitor cocktail (Roche). Samples were sonicated and centrifuged, and total protein content of the supernatant measured using a DC Protein assay (Bio-Rad). Equal amounts of protein were mixed with 6X Laemmli buffer and boiled for 5 min before separation on 8% polyacrylamide gels. Gels were transferred and blocked with Tris-buffered saline containing 0.1% Triton-X (TBST) and 5% non-fat milk for 60 min at RT, and incubated with an antibody against FMRP (Millipore mouse monoclonal 1C3 1:1000 or Abcam rabbit polyclonal 17722, 1:1000), or against the C-terminal of APP (Invitrogen 51-2700, 1:500) and β-tubulin (University of Iowa's Developmental Studies Hybridoma Bank E7, 1:5000) overnight at 4°C. Blots were incubated with corresponding fluorescent secondary antibody (1:15000; IRDye® 680RD or 800CW, LI-COR) for 60 min at RT, and imaged with the Odyssey® Imaging System (LI-COR).

Band intensity was quantified in the linear range with densitometry using LI-COR Image Studio™ Software. Experiments were performed in technical triplicate, and FMRP/tubulin or APP/tubulin ratios to two control samples included in every experiment were combined. These ratios were averaged, normalized to control values for each experiment, and expressed as %Control in experiments.

Immunohistochemistry

Antibody control experiments were performed on mice aged 50-75 weeks (n=6 WT, n=5 CGG KI, n=1 *Fmr1* KO), and experimental analysis on 80-90 week old mice (n=2 WT, n=3 APP/PS1) which were anesthetized with 0.2 mg Ketamine/20 µg Xylazine per kilogram prior to transcardial perfusion with 15-20 mL PBS and 15-20 mL 4% PFA in PBS. Brains were dissected, fixed overnight in 4% PFA, and then sunk in 30% sucrose in PBS at 4°C. Brains were sectioned at 30µm and placed in a cryostorage solution of 30% sucrose/33.33% ethylene glycol/0.05 M PBS until needed.

Prior to staining, slices were rotated in PBS overnight at 4°C to remove cryostorage solution, then basic antigen retrieval was performed by placing the slices in 0.01M sodium citrate (pH 8.5) at 80°C for 10 minutes followed by three 5-minute washes in PBS. The slices were then placed in 1% H_2O_2 in Tris to block endogenous peroxidases. Slices were permeabilized in 0.1% Triton-X/.05% BSA in Tris for 30 minutes at room temperature (RT) and blocked in 5% normal goat serum (NGS) for 1 hour at RT. Slices were then incubated overnight in anti-FMRP antibody (1:3500). Following two washes, slices were incubated in horseradish peroxidase-conjugated secondary antibody (1:1000) in 5% NGS in Tris. Prior to peroxidase development, slices were treated with the Vectastain ABC kit (Vector) to increase DAB visibility. Following washes, slices from all genotypes were placed simultaneously in ImmPACT DAB solution (Vector) for 10-15 seconds until they just started to turn brown. Following washes, slices were mounted, allowed to dry overnight then either counterstained in Gill's 1:2 hematoxylin for 45-60 seconds, or immediately dehydrated in an alcohol gradient and mounted.

Microscopy

A slide scanning microscope (Zeiss) was used to image all DAB stained tissue. Fields of view were selected in the hippocampus and cortex to be easily reproducible across multiple sections. Images were taken using the same exposure settings for all genotypes.

Statistical Analysis

All values are expressed as the mean ± standard error of the mean. Western blot immunoreactivity for each sample was measured in technical triplicate, and combined as a ratio of %control for each blot. These combined values were averaged, and compared using a Student's T-test, with significance indicated by a P value <0.05. FMRP expression compared to age, PMI, and pH were evaluated using a Pearson's correlation coefficient on combined cortical and cerebellar FMRP values from either control and AD samples, or combined control and FXTAS cortical and cerebellar values, with significance indicated by $P<0.05$. Comparison of donor gender in FMRP expression was performed using a two-way ANOVA with posthoc Sidak's multiple comparison test.

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Chapter 4
Impaired sensorimotor gating in Fmr1 knock out and Fragile X premutation model mice[6]

Abstract

Fragile X syndrome (FXS) is a common inherited cause of intellectual disability that results from a CGG repeat expansion in the FMR1 gene. Large repeat expansions trigger both transcriptional and translational suppression of Fragile X protein (FMRP) production. Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) is an allelic neurodegenerative disease caused by smaller "pre-mutation" CGG repeat expansions that enhance FMR1 transcription but lead to translational inefficiency and reduced FMRP expression in animal models. Sensorimotor gating as measured by pre-pulse inhibition (PPI) is altered in both FXS patients and Fmr1 knock out (KO) mice. Similarly, FXTAS patients have demonstrated PPI deficits. Recent work suggests there may be overlapping synaptic defects between Fmr1 KO and CGG knock-in premutation mouse models (CGG KI). We therefore sought to interrogate PPI in CGG KI mice. Using a quiet PPI protocol more akin to human testing conditions, and a background noise protocol based on previously published literature, we find that Fmr1 KO animals have significantly impaired PPI. Using the quiet protocol, we find CGG KI mice demonstrate an age-dependent impairment in PPI compared to wild type (WT) controls. Furthermore, we find a significant increase in WT and CGG KI PPI when animals are treated with the mGluR5 antagonist, fenobam. This study describes a novel phenotype

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(*Authors contributed equally.)

in CGG KI mice that can be used in future therapeutic development targeting premutation associated symptoms.

Introduction

Fragile X Syndrome (FXS) is the most common known inherited cause of intellectual disability (Rogers et al 2001). FXS patients exhibit motor developmental delays, executive dysfunction, and 30-50% of patients qualify for a DSM-IV diagnosis of autism (Rogers et al 2001). FXS is caused by expansion of a CGG trinucleotide repeat in the 5' untranslated region (UTR) of the *FMR1* gene on the X chromosome to more than 200 repeats. This large expansion inhibits production of the FMR1 protein product, FMRP, by triggering hypermethylation of the repeat and *FMR1* promoter region, resulting in transcriptional silencing of *FMR1*. When *FMR1* methylation is incomplete and FMR1 mRNA is transcribed, the CGG repeat expansion inhibits FMRP translation, presumably by impairing ribosomal scanning through the repeat in the 5'UTR (Feng et al 1995).

Associated with FXS is the allelic age-related neurodegenerative condition, Fragile X-associated Tremor/Ataxia Syndrome (FXTAS). FXTAS patients display cognitive and neurological signs with age but are typically asymptomatic until age 50. The main features of FXTAS include action tremor and ataxia, with cognitive decline in a significant fraction of patients. FXTAS patients have an intermediate "premutation" repeat expansion between 55 and 200 CGGs (Nelson et al 2013). In premutation carriers, the CGG repeat expansion triggers enhanced transcription, resulting in an increase of FMR1 mRNA (Todd et al 2010). However, as in unmethylated FXS patients, the repeat RNA forms a hairpin loop that impairs FMRP translation (Farzin et al 2006, Iliff et al 2013). This effect is repeat length dependent, such that larger CGG repeats elicit greater impairments in translational efficiency and lower FMRP levels (Ludwig et al 2014, Nelson et al 2013, Pretto et al 2014). Consistent with reduced FMRP levels, younger premutation carriers can develop symptoms more commonly associated with FXS, such as higher rates of autistic and attention deficit hyperactivity disorder (ADHD)-like symptoms (Clifford et al 2007, Farzin et al 2006).

One phenotype observed in both FXS patients and mouse models is sensorimotor gating abnormalities. This is the process by which an acoustic startle response (ASR) is modulated, as measured by pre-pulse inhibition (PPI) (Powell et al 2012). FXS patients show impaired PPI, suggesting a basal failure in sensorimotor gating (Hessl et al 2009). Similarly, mice lacking FMRP (Fmr1 KO) also show disruptions in PPI, although the direction of change is variable (Baker et al 2010, Chen & Toth 2001, de Vrij et al 2008, Frankland et al 2004, Nielsen et al 2002, Paylor et al 2008, Veeraragavan et al 2012).

Our group has recently demonstrated that premutation model mice exhibit deficits in a form of synaptic plasticity (mGluR-LTD) that is also implicated in Fmr1 KO mice (Iliff et al 2013). Additionally, symptomatic FXTAS patients exhibit altered ASR and PPI (Schneider et al 2012). We therefore sought to define whether premutation model mice, which have 120 CGG repeats knocked in to the murine *Fmr1* 5'UTR (CGG KI; (Iliff et al 2013)), exhibit alterations in their ASR or PPI. Establishing a phenotypic readout in premutation model mice that has direct correlation with patient findings would greatly aid in preclinical testing and development of novel therapeutics for FXTAS. Furthermore, identifying areas of phenotypic overlap between CGG KI and Fmr1 KO mice may allow cross-application of therapeutic strategies developed for FXS patients to be applied to premutation carriers.

Here we show that Fmr1 KO mice exhibit reduced PPI using two different protocols. In older CGG KI mice, we see a similar impact on sensorimotor gating, with altered ASR and reduced PPI compared to controls. However this phenotype in premutation model mice is age-dependent, as younger CGG KI mice show normal PPI. We also find significant increases in WT and CGG KI PPI with acute fenobam administration. This study establishes altered PPI as a shared phenotype in CGG KI and Fmr1 KO mice that may be amenable to pharmacologic intervention.

Results

To ensure that hearing function was not affecting our experiments, we evaluated ABRs in the mice we tested. Using three tonal frequencies (12 kHz, 24 kHz, 48 kHz),

there was a significant effect of frequency across all genotypes, consistent with published studies in C57BL/6 mice (Johnson et al 1997). In the younger (3-5 month old) Fmr1 KO and WT littermate controls, we found no difference in thresholds between genotypes (all ABR thresholds are in dB SPL; WT: 12 kHz = 26.3 ± 0.92, 24 kHz = 35 ± 2.39, 48 kHz = 60.1 ± 3.79; Fmr1 KO: 12 kHz = 25 ± 2.19, 24 kHz = 34.2 ± 4.06, 48 kHz = 48.1 ± 8.07; $F_{(1, 54)}$= 2.060, not significant (NS), n=10 for each group; data not shown). Similarly, we found no difference in younger CGG KI mice compared to WT controls (WT: 12 kHz = 26 ± 2.83, 24 kHz = 28 ± 3.62, 48 kHz = 68 ± 8.87; CGG KI: 12 kHz = 37.25 ± 13.59, 24 kHz = 39.75 ± 14.33, 48 kHz = 76 ± 17.30; $F_{(1, 18)}$= 1.624, NS, n=4 for each group; data not shown). In older (7-8 months old) CGG KI and WT littermates, we observed a significant elevation in ABR thresholds for the CGG KI mice compared to WTs at higher frequencies (WT: 12 kHz = 23.14 ± 2.14, 24 kHz = 24.71 ± 4.16, 48 kHz = 46 ± 13.39, n=7; CGG KI: 12 kHz = 27 ± 1.74, 24 kHz = 40.22 ± 5.27, 48 kHz = 77.78 ± 2.54; $F_{(1, 42)}$= 14.99, P<0.05, n=9; interaction between genotype and frequency, $F_{(2, 42)}$= 3.380, P<0.05; data not shown). As ABR profiles were similar across all genotypes at the 12 kHz frequency, we restricted our analysis to either BBN or 12 kHz stimulation.

We first evaluated the ASR and PPI in Fmr1 KO mice and littermate controls. Basal ASR in animals over a range of intensities (60-120 dB SPL) demonstrated no significant difference between Fmr1 KO animals compared to WT (Figure 4.1A; $F_{(1, 108)}$= 0.088, NS, n=7 for each group).

In an effort to better mimic the PPI testing conditions used in patient studies (Hessl et al 2009, Schneider et al 2012), we recorded PPI using an established protocol with minimal background noise (Dolan et al 2012). Using this protocol we found a significant decrease in the %PPI of Fmr1 KO animals compared to WT controls (Figure 4.1B; $F_{(1, 48)}$= 6.864, P<0.05; post-hoc Fisher's LSD at 65 dB t=2.887, P<0.05, n=8). While these results are consistent with studies in FXS patients and some trials in Fmr1 KO mice (de Vrij et al 2008, Frankland et al 2004, Hessl et al 2009), other studies have observed an opposite effect, with enhanced PPI in Fmr1 KO mice (Baker et al 2010, Chen & Toth 2001, Frankland et al 2004, Nielsen et al 2002, Paylor et al 2008, Veeraragavan et al 2012). It is notable that we observed some habituation in both WT

and Fmr1 KO animals tested. However, this effect was not different between genotypes, therefore did not affect the overall results.

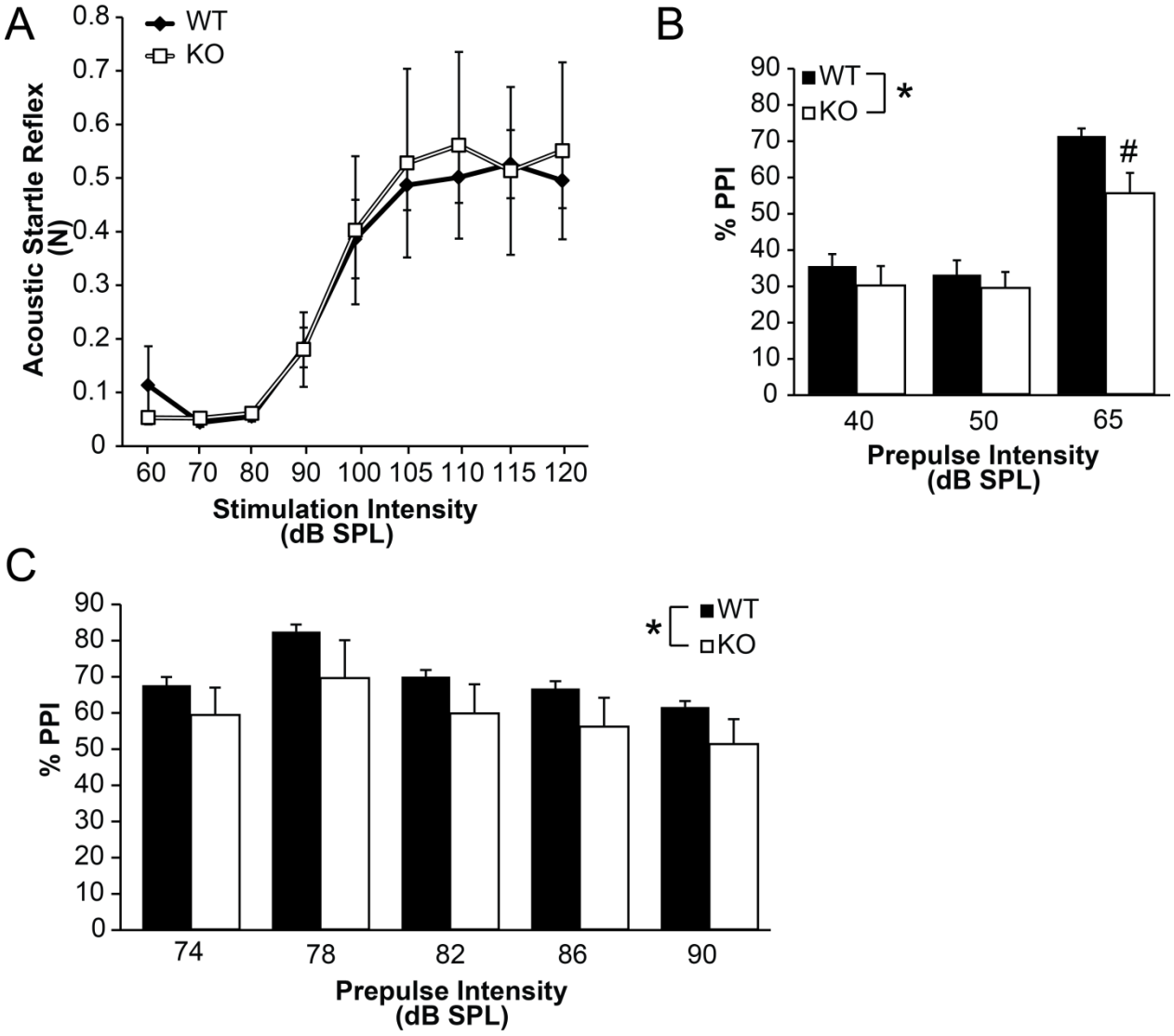

Figure 4.1: Auditory startle response and reduced pre-pule inhibition in Fmr1 KO animals.
(A) Startle response curves reveal that Fmr1 KO mice (n=7) ages 2–3 months have normal responses compared to littermate WTs (n=7). (B) WT mice (n=10) show a significantly higher %PPI than Fmr1 KO mice (n=8) when tested with a 12 kHz, 100 dB startle and 45–65 dB pre-pulses. (C) The same animals as in (B) were tested in a protocol with 70 dB BBN background noise. WT mice continue to show a significantly higher %PPI than KO mice when tested in background noise with a 100 dB BBN startle and 74, 78, 82, 86, and 90 dB BBN pre-pulses. *P<0.05 main effect of genotype by two-way ANOVA; #P<0.05 post hoc Fisher's LSD.

While these results are consistent with studies in FXS patients and some trials in Fmr1 KO mice (de Vrij et al 2008, Frankland et al 2004, Hessl et al 2009), other studies using force generation as a measure of acoustic startle have exhibited an opposite phenotypic effect, with an enhancement of PPI in Fmr1 KO mice (Baker et al 2010, Chen & Toth 2001, Frankland et al 2004, Nielsen et al 2002, Paylor et al 2008, Veeraragavan et al 2012). As we were using a different testing protocol, we assessed whether the observed differences might result from technical alterations in our testing conditions. We therefore created a second protocol to mimic testing conditions reported in published literature examining Fmr1 KO mice that included constitutive background 70 dB SPL BBN sound, with testing of pre-pulses at 74-86 dB (Baker et al 2010, Chen & Toth 2001, Frankland et al 2004, Nielsen et al 2002, Paylor et al 2008, Veeraragavan et al 2012).

Using a new cohort of animals, we assessed both protocols, alternating which animals received each protocol first. Studies using our "silent" protocol demonstrated similar results to our initial evaluations (data not shown). Using our second protocol in the setting of background noise, we observed similar trends to our initial evaluations, with significant effects for pre-pulse intensity and a sustained impairment of PPI in Fmr1 KO mice compared to littermate controls (Figure 4.1B; WT: 74 dB = 67.47 ± 2.25, 78 dB = 82.31 ± 1.92, 82 dB = 69.87 ± 1.81, 86 dB = 66.53 ± 2.03, 90 dB = 61.44 ± 1.66 n=10; Fmr1 KO: 74 dB = 59.25 ± 7.56, 78 dB = 69.41 ± 10.49, 82 dB = 59.65 ± 8.09, 86 dB = 56.03 ± 7.94, 90 dB = 51.17 ± 6.94, $F_{(4, 80)}$= 4.018, P<0.05 for pre-pulse intensity and $F_{(1, 80)}$= 10.51, P<0.05 for genotype, two-way ANOVA. Post-hoc Fisher's LSD was not significantly different for individual prepulse intensities, n=8). This indicates a consistent phenotype across different protocols that mimics the impairment in PPI observed in FXS patients (Frankland et al 2004, Hessl et al 2009).

We next evaluated PPI and ASR in premutation CGG KI mice. We measured basal ASR in younger (2-5 month old) CGG KI and WT littermate controls, and observed a significant reduction in startle amplitude in CGG KI mice (Figure 4.2A; $F_{(1, 112)}$= 9.916, P<0.05, n=10 KI, 6 WT; t=2.164, P<0.05 for 105 dB point by Fisher's LSD). However, we observed no significant difference in PPI between premutation and control animals (Figure 4.2B; $F_{(1, 42)}$= 0.2025, NS, n=10 KI, 6 WT).

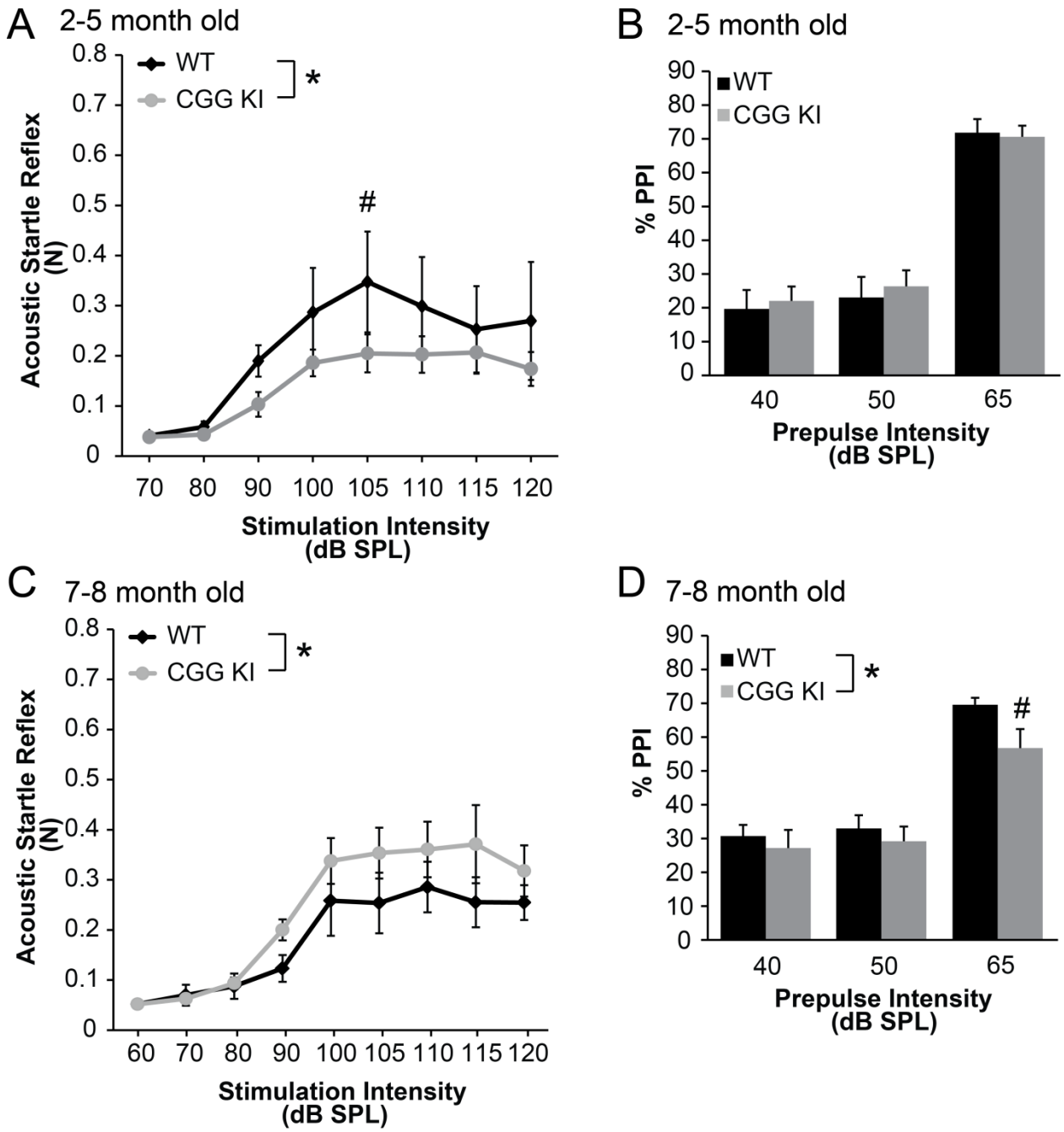

Figure 4.2: Auditory startle response and age-dependent pre-pulse inhibition in CGG KI mice.
(A) Startle response curves show that 2–5 month old CGG KI mice (n=10) have altered ASR compared to littermate controls (n=6). (B) PPI in younger CGG KI mice revealed no difference. (C) Startle response curves in older 7–8 month old CGG KI mice (n=8) have altered ASR compared to WTs (n=4). (D) Older CGG KI mice (n=14) show significantly impaired PPI compared to littermate controls (n=7). *P<0.05 main effect of genotype by two-way ANOVA; #P<0.05 post hoc Fisher's LSD.

In patients, symptomatic male premutation carriers have altered PPI (Schneider et al 2012). We therefore chose to evaluate older male CGG KI and littermate controls (7-8 months old), hypothesizing that they may have age-related alterations in sensorimotor gating. We saw altered basal ASR in aged CGG KI animals compared to WTs (Figure 2C; $F_{(1, 90)}$= 6.958, P<0.05, n=8 KI; no individual point was significant by Fisher's LSD). In contrast to our findings, symptomatic human premutation carriers are reported to have reduced basal startle response (Schneider et al 2012). Our observation may be a hyperacusis-elicited response to age-related hearing impairment in mice as previously described (Ison et al 2007). We found a significant decrease in CGG KI %PPI compared to littermate controls (Figure 2D; $F_{(1, 57)}$= 4.312, P<0.05, n=14 KI, 7 WT; t= 2.285, P<0.05 for 65 dB pre-pulse, post-hoc Fisher's LSD), indicating some time-dependent phenotypic overlap between Fmr1 KO and CGG KI animals.

PPI is a behavior task which has been used previously to compare the effects of possible therapeutic intervention in FXS and Fmr1 KO mouse models (Berry-Kravis et al 2009, de Vrij et al 2008, Hessl et al 2009). In particular, several mGluR5 antagonists have been tested for symptomatic amelioration, such as 2-Methyl-6-(phenylethynyl)pyridine (MPEP; (de Vrij et al 2008)) and fenobam (Berry-Kravis et al 2009, Montana et al 2009). As we identified a behavioral task with phenotypic overlap, we next evaluated the impact of acute fenobam administration in young (3-5 month old) CGG KI and WT animals (Figure 3). Basal ASR was not affected by fenobam in either WT ($F_{(1, 32)}$= 0.2623, NS, n=3) or CGG KI ($F_{(1, 128)}$= 2.130, NS, n=9) groups, however the significant difference between genotypes was maintained in vehicle and fenobam (Figure 3A; Vehicle: $F_{(1, 80)}$= 27.85, P<0.05, n=3 WT, 9 KI; 100 dB t= 2.689, 105 dB t= 3.453, 110 dB t= 2.562, 120 dB t= 2.817, P<0.05 post-hoc Fisher's LSD; Fenobam: $F_{(1, 80)}$= 14.72, P<0.05, n=3 WT, 9 KI; 100 dB t= 2.412, 105 dB t= 2.161, 110 dB t= 2.090, P<0.05 post-hoc Fisher's LSD). PPI was significantly increased with administration of fenobam in both WT ($F_{(1, 6)}$= 11.61, P<0.05; matching subjects $F_{(6, 6)}$= 9.615, P<0.05, n=3; 50 dB t= 3.668, P<0.05 post-hoc Bonferroni) and CGG KI ($F_{(1, 24)}$= 5.782, P<0.05; matching subjects $F_{(24, 24)}$= 5.026, P<0.05, n=9) groups (Figure 3B-C). However, as in Figure 2, PPI between young WT and CGG KI animals was not significantly different

(Figure 3B; Vehicle: $F_{(1, 30)}$= 1.092, NS, n=3 WT, 9 KI; Fenobam: $F_{(1, 30)}$= 0.079, NS, n=3 WT, 9 KI). The overall increase in %PPI of both groups with an mGluR antagonist (Figure 3C) is consistent with previously published results (de Vrij et al 2008). Additional experiments are required to determine if fenobam administration could restore the impaired PPI seen in older CGG KI animals.

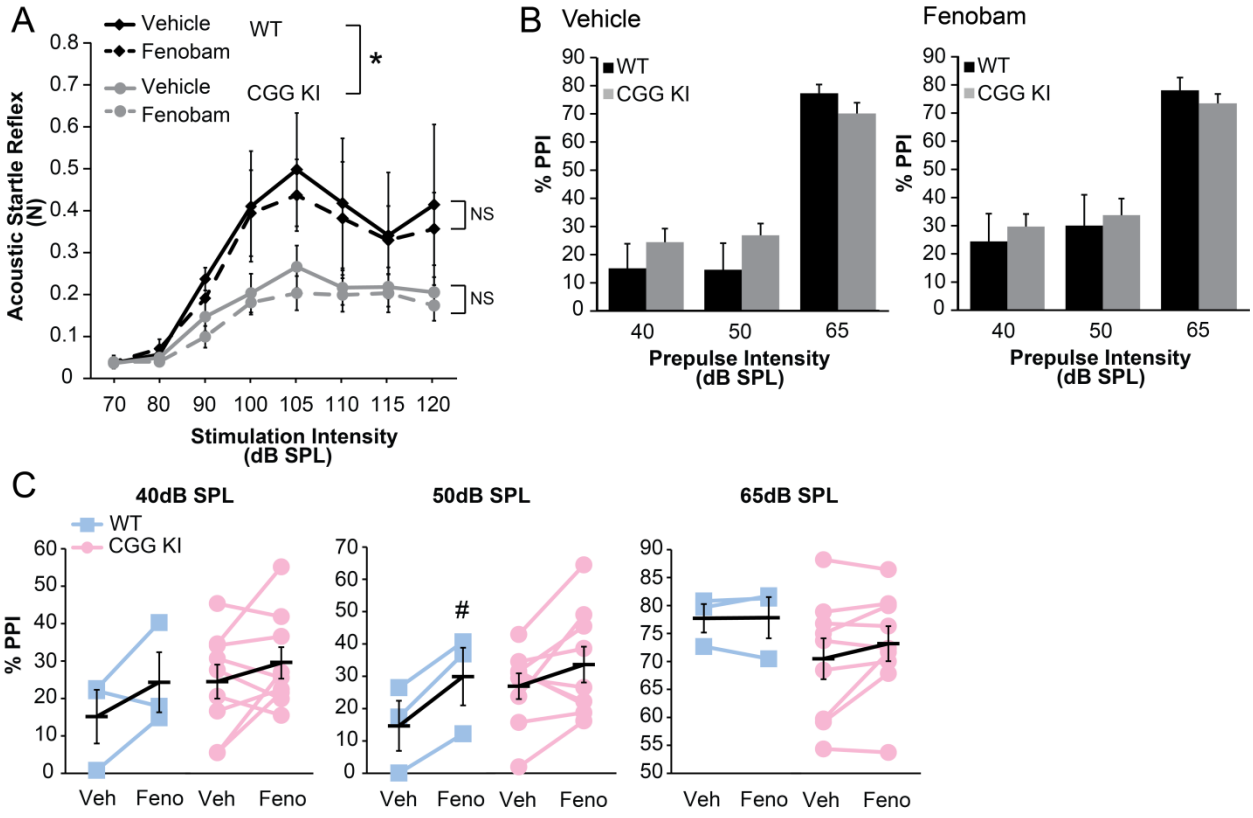

Figure 4.3: Auditory startle response and increased pre-pulse inhibition with fenobam administration.
(A) Startle response curves show that young (3-5 month old) CGG KI mice (n=9) have significantly reduced acoustic startle responses compared to WT littermate controls (n=3). Fenobam (30mg/kg administered 30min prior to testing) does not significantly alter ASRs. (B) There is no significant effect of genotype between vehicle- or fenobam-treated animals (WT n=3, CGG KI n=9). However, there is an overall significant increase in PPI with fenobam across all prepulse intensities in both genotypes. (C) Significantly increased individual animals' PPI with fenobam at each prepulse intensity. *P<0.05 main effect of genotype by two-way ANOVA; #P<0.05 post hoc Bonferroni.

Discussion

In this study we demonstrate a new behavioral phenotype in premutation model mice. Both CGG KI and Fmr1 KO mice exhibit impaired PPI, a measure of sensorimotor gating, which mimics what is seen in Fragile X spectrum disorder patients (Frankland et al 2004, Hessl et al 2009, Schneider et al 2012). We observed impairment in sensorimotor gating as measured by PPI using two different protocols in Fmr1 KO mice. While similar to studies in human FXS patients, this finding differs from previous reports where Fmr1 KO mice exhibit enhanced PPI (Baker et al 2010, Chen & Toth 2001, Frankland et al 2004, Nielsen et al 2002, Paylor et al 2008, Veeraragavan et al 2012). The reason for these differences is unclear, although minor modifications in testing protocols may be responsible. We attempted to control for this feature by creating a similar protocol to published studies, but this did not significantly alter the degree or direction of change in PPI between Fmr1 KO mice and littermate controls (Figure 1). Alternatively, environmental variance or genetic drift in the Fmr1 KO background on C57BL/6 may be a contributing factor. One previous study in Fmr1 KO mice, using eye blink to measure startle similar to human tests, also demonstrated impaired PPI, which is typically observed in patients (de Vrij et al 2008).

The impaired PPI observed in Fmr1 KO and older CGG KI mice indicates an additional behavioral phenotype in common between these models. Recent work demonstrates that these CGG KI mice exhibit a number of features originally thought to be specific to Fmr1 KO animals. Specifically, hyperactivity, decreased anxiety in open field and elevated zero maze assays, and social behavior alterations (Qin et al 2011). However, not all mouse models of FXTAS exhibit these phenotypes (Nelson et al 2013), so the generalizability for these findings to other models remains unknown.

The mechanism for altered PPI in FXTAS patients and CGG KI mice is unclear. These mice do not exhibit significant neurodegeneration within the ages used here, but there could be an age-dependent decline in neuronal function which impacts sensorimotor gating. Of note, the age-dependent hearing loss in C57BL/6 mice is unlikely to be a major contributor to the change in PPI, as age-related deafness typically enhances rather than suppresses PPI at lower frequencies, opposite of what we observe (Willott et al 1994).

An alternative explanation for the observed PPI alterations is the reduced FMRP expression in CGG KI mice. Work by our group and others demonstrate that CGG premutation model mice express reduced levels of basal FMRP and impaired activity-dependent FMRP translation (Iliff et al 2013, Ludwig et al 2014, Qin et al 2011). This defect is coupled to enhanced mGluR-dependent synaptic weakening (Iliff et al 2013), a phenotype also seen in Fmr1 KO mice (Huber et al 2002).

Interestingly, in both Fmr1 KO mice and in FXS patients, alterations in PPI are reversible with mGluR 1/5 antagonists (Berry-Kravis et al 2009, de Vrij et al 2008). We explored the impact of one mGluR5 antagonist, fenobam on PPI in a small group of young CGG KI and WT controls (Figure 3). We observed a significant increase in PPI with acute fenobam administration in both WT and CGG KI animals. Whether older CGG KI mice or premutation patients will benefit similarly from mGluR antagonists will be an important area of future study.

These data, along with behavioral and electrophysiological assessments in premutation model mice, suggest that there are significant areas of pathophysiologic overlap in these models. The progress in treating FXS may also be applicable to premutation carriers and may have important implications for therapeutic development across the Fragile X disorder spectrum.

Materials and Methods

Mice

Mouse handling regulations were followed in accordance to NIMH/NIH Guidelines on the Care and Use of Animals and the University Committee and the Use and Care of Animals at the University of Michigan (UCUCA).

Mice were maintained on a C57BL/6 background. CGG KI mice were obtained from the Usdin laboratory at the NIH (Bethesda, MD (Entezam et al 2007, Iliff et al 2013)). CGG KI and littermates were genotyped as described previously (Iliff et al 2013). Fmr1 KO mice were received from Cara Westmark (University of Wisconsin) and Jim Malter (UT Southwestern). Genotyping was performed as described previously (Consortium 1994).

Prepulse Inhibition

Kinder Scientific Startle Monitor equipment and software was used to measure acoustic startle reflexes (http://www.kinderscientific.com). The testing chamber was fixed on a movement sensor within a small padded chamber (to mute reverberation) housed within a larger sound proof cabinet.

On day one, basal startle response was measured with a startle input-output program run in succession with a 15 minute break between trials. Noise was played for 40ms four times each in a pseudo-random order with no pre-pulse between stimuli. Fmr1 KO cohort n=7 KO, 7 WT; young CGG KI cohort n=10 KI, 6 WT; old CGG KI n=8 KI, 4 WT.

PPI trials began on day two and continued for four testing days. The quiet PPI protocol consisted of two sessions using 100 dB SPL acoustic startle and 50ms pre-pulses of narrow-band or broad band noise at 40, 50, or 65 dB SPL played 100ms before the startle with 5-9 seconds inter-trial intervals played in a pseudo-random order. Fmr1 KO cohort n=8 KO, 10 WT; young CGG KI n=10 KI, 6 WT; old CGG KI n=14 KI, 7 WT. Ambient sound for the quiet protocol was measured at 49.3 dBA SPL, with the majority of spectral energy falling below 1.8 kHz (Quest model 2200 sound level meter). Ambient background sound levels above 1.8 kHz (murine auditory range is 2.5-70 kHz (Taberner & Liberman 2005)) did not exceed 25 dB.

An additional PPI protocol was performed in 70 dB SPL broad band noise (BBN) with bandwidth of 2-20 kHz to manufacture a pseudo-“white noise” as detailed previously (de Vrij et al 2008, Paylor et al 2008). Two sessions of 100 dB SPL startles with BBN pre-pulses of 74, 78, 82, 86, and 90 dB SPL were played in a pseudo-random order. Stimulus durations, inter-trial intervals, inter-stimulus intervals were the same as those in the quiet protocol above.

The Fmr1 KO cohort run in both the quiet and background noise protocols were separated into two groups, one of which received the quiet protocol first followed by the background noise protocol, and vice versa.

%PPI was calculated as follows: 100 − [(response to pre-pulse plus startle stimulus/startle response alone) × 100]. The average %PPI value of each session per mouse was combined for final values compared between genotypes.

Following all testing, the hearing ability of the animals was verified using Auditory Brainstem Response Testing (ABR) as described previously (Karolyi et al 2007).

Drug Treatment

Young (3-5 month old) CGG KI (n=9) and WT (n=3) littermate controls were used to evaluate the impact of the mGluR5 antagonist fenobam (Tocris) on PPI. Fenobam or equal volume of vehicle (dimethyl sulfoxide, DMSO) was injected intraperitoneally at 30mg/kg 30 minutes prior to experimentation.

Half the animals began testing on fenobam, the other half on vehicle (DMSO), and switched after the completion of each set of experiments: 4 trials of the quiet PPI protocol as outlined above. Startle input-output curves were measured prior to and following all PPI testing for each animal treated with fenobam and vehicle.

Statistical Analysis

Values are reported as mean ± SEM. Data were compared by two-way ANOVA comparing genotype and stimulation intensity. A significant effect of stimulation intensity was expected in all experiments. Interactions are noted in the results where applicable; * indicates an overall difference in genotype of $P<0.05$. Significant effects of genotype were followed with a Fisher's LSD or Bonferroni correction for multiple comparisons as post-hoc analysis on individual points (# indicates $P<0.05$).

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DC05188) to KEH and DFD; National Institute on Aging (R01AG028488) to GGM; and by the Veterans Administration (BLRD #1I01BX001689) and NIH (1K08NS069809) to PKT.

Chapter 5
Axonal protein expression and homeostatic plasticity are altered in Fragile X premutation model mice

Abstract

Neuronal FMRP expression is critical for synaptic function, as its absence causes Fragile X Syndrome and cognitive dysfunction in patients. Fragile X premutation patients also suffer cognitive decline with age, along with neurodegeneration and executive dysfunction. Premutation patients and mouse models both show reduced FMRP expression, likely owing to impaired translational efficiency of the CGG-containing Fmr1 mRNA. Our group has previously identified enhanced mGluR-dependent long term depression (LTD) in a CGG knock-in (CGG KI) premutation mouse model. However, the relative contribution of increased CGG-containing mRNA and reduced FMRP expression in this synaptic plasticity phenotype remain unclear. Here we show a selective decrease in the voltage-gated sodium channel Nav1.2 and scaffolding protein ankyrin G in CGG KI axon initial segments. Additionally, CGG KI neurons show alterations in homeostatic plasticity induced by application of the AMPA receptor antagonist, CNQX. This effect may be at least partially dependent on CGG-containing mRNA, as exogenous expression in WT rat neurons show a similar trend after CNQX treatment. Together these data suggest premutation model neurons have altered axonal composition and homeostatic plasticity, both of which indicate network activity may be altered as a result of reduced FMRP and increased CGG-containing mRNA.

Introduction

Protein synthesis in dendrites is important for enduring forms of synaptic plasticity necessary for normal neuronal function, learning, and memory. Conversely, improper control of local translation may contribute to cognitive dysfunction and neuropsychiatric symptoms associated with certain forms of intellectual disability and autism. One protein known to be critical to synaptic function and dendritic protein translation is FMRP. FMRP binds to and inhibits the translation of numerous transcripts at synapses (Ascano et al 2012, Darnell et al 2011, Laggerbauer et al 2001). Activation of metabotropic glutamate receptors (mGluRs) triggers FMRP dissociation and degradation resulting in rapid, dendritically-localized protein synthesis (Nalavadi et al 2012, Narayanan et al 2007, Narayanan et al 2008, Weiler & Greenough 1999, Weiler et al 1997, Weiler et al 2004). However, this pulse of translation is temporally constrained, such that mGluR dependent forms of plasticity require translation for only a few minutes after activation, despite the conferral of long lasting changes in synaptic strength (Huber et al 2000, Merlin et al 1998). Among the many mRNA binding targets of FMRP is the Fmr1 transcript itself, which is rapidly translated at synapses upon mGluR stimulation (Hou et al 2006, Iliff et al 2013, Li et al 2001, Siomi et al 1994, Todd et al 2003a, Todd et al 2003b, Weiler et al 1997). The function and importance of this newly produced FMRP, however, remains unknown (Bear et al 2004).

FMRP plays important functional roles in the pathogenesis of two allelic human diseases. Fragile X Syndrome (FXS), the most common known inherited cause of autism and cognitive impairment (Hernandez et al 2009, Rogers et al 2001). In FXS, a normally small (~30 in average individuals) CGG repeat tract in the 5'UTR of the *FMR1* gene expands to a large repeat (>200) resulting in methylation of the gene, and transcriptional silencing of *FMR1* (Bell et al 1991, Kremer et al 1991, Oberle et al 1991, Pieretti et al 1991, Verkerk et al 1991). This disorder is caused by the absence of *FMR1*'s functional protein product, FMRP. In contrast, Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) is a neurodegenerative disorder associated with dementia, psychiatric symptoms and gait difficulties (Jacquemont et al 2004). It results from premutation-range expanded (CGG) repeats in the 5'UTR of the fragile X gene,

FMR1, which causes a counterintuitive increase in transcription of FMR1 (Tassone et al 2007, Tassone et al 2000a, Tassone et al 2000c, Todd et al 2010). However, the large CGG repeat forms an mRNA hairpin that impairs rapid FMRP translation, leading to lower basal and activity-dependent FMRP expression (Entezam et al 2007, Iliff et al 2013, Ludwig et al 2014, Pretto et al 2014, Qin et al 2011, Renoux et al 2014a).

The absence of FMRP is associated with a variety of neuronal phenotypes in animal models of FXS (Fmr1 KO), including increased cortical activity, epileptogenesis and susceptibility to audiogenic seizures, indicating altered network behavior (Gibson et al 2008, Hays et al 2011, Musumeci et al 2000, Musumeci et al 2007). Both connectivity and network activity are altered in mouse models of the premutation (CGG KI; (Chen et al 2010, Cunningham et al 2011, D'Hulst et al 2009, Qin et al 2011)) and human premutation patient induced pluripotent stem cell (iPSC)-derived neurons (Liu et al 2012). Furthermore, CGG KI model mice show increased mRNA levels of components of the GABAergic system, suggesting altered inhibitory tone (D'Hulst et al 2009). FMRP also regulates the translation of a myriad of proteins involved in excitability and action potential generation, including Kv3.1(Strumbos et al 2010), Kv4.2 (Gross et al 2011, Lee et al 2011), and putatively Nav1.6, Nav1.2, and ankyrin G (Darnell et al 2011). FMRP is also able to alter channel function through direct interaction, as in the case of the potassium BK and Slack channels (Brown et al 2010, Deng et al 2013). Taken together, these findings indicate a potential role for FMRP's involvement in neuronal excitability which may impact disease pathogenesis.

In addition to intrinsic excitability, work by many groups has established that loss of FMRP alters mGluR-dependent synaptic plasticity, and it is thought that such plasticity defects contribute to the cognitive disability and autistic symptoms associated with FXS (Bear et al 2004, Huber et al 2002, Huber et al 2000). Specifically, in Fmr1 KO mice there is an enhancement of mGluR-dependent long-term synaptic depression (LTD) at CA3-CA1 synapses in the hippocampus (Huber et al 2002). While mGluR-LTD is dependent on local dendritic protein synthesis in wild-type (WT) animals, this enhanced LTD in Fmr1 KO animals occurs even in the absence of new protein synthesis (Huber et al 2002). To understand the role of newly synthesized FMRP, our previous work examined the related physiological example of FXTAS (Iliff et al 2013).

In a CGG KI mouse model of the premutation, we identified enhanced mGluR-LTD, which was still dependent on new protein synthesis (Iliff et al 2013). This synaptic phenotype is correlated with impaired activity-dependent FMRP production; however the relative contribution of FMRP haploinsufficiency and increased CGG-containing Fmr1 mRNA remains unknown.

While the function of FMRP in Hebbian mGluR-LTD is relatively well studied, its role in homeostatic plasticity (HSP) is less clear. HSP refers to the ability of neurons to regulate their own excitability relative to the neuronal network. Neurons have a preferred firing pattern, and if there is any deviation from this pattern, neurons can compensate to return to that set point (Davis & Bezprozvanny 2001, Desai et al 1999, Golowasch et al 1999, Marder & Prinz 2002, Turrigiano et al 1994). Theoretical networks that operate purely by Hebbian plasticity rules have been shown to be inherently unstable due to the positive feedback nature of LTP and LTD, as there is no way to control for excessive coincidental presynaptic input (Miller 1996).

Interestingly, FMRP has been shown to play an important role in the mechanism of HSP (Soden & Chen 2010). CA1 pyramidal neurons show increased mEPSC amplitude following TTX+APV treatment, which was absent in Fmr1 KO neurons (Soden & Chen 2010). Reduced FMRP expression causes a threshold-dependent reduction in surface GluR1-containing AMPARs at baseline (Nakamoto et al 2007). Furthermore, postsynaptic expression of mutant FMRP which cannot bind target transcripts (I304N) in Fmr1 KO neurons reduces the total, surface, and synaptic levels of AMPA receptors, hence suggesting a role for FMRP-regulated protein translation in controlling synaptic AMPAR abundance (Soden & Chen 2010). Remarkably little research has been performed to understand how HSP mechanisms may be impacted in the presence of the Fragile X premutation. While Hebbian mGluR-LTD is altered in premutation model mice, it is not clear whether the impaired activity-dependent FMRP production resulting from the expanded CGG repeat will also impact HSP (Iliff et al 2013).

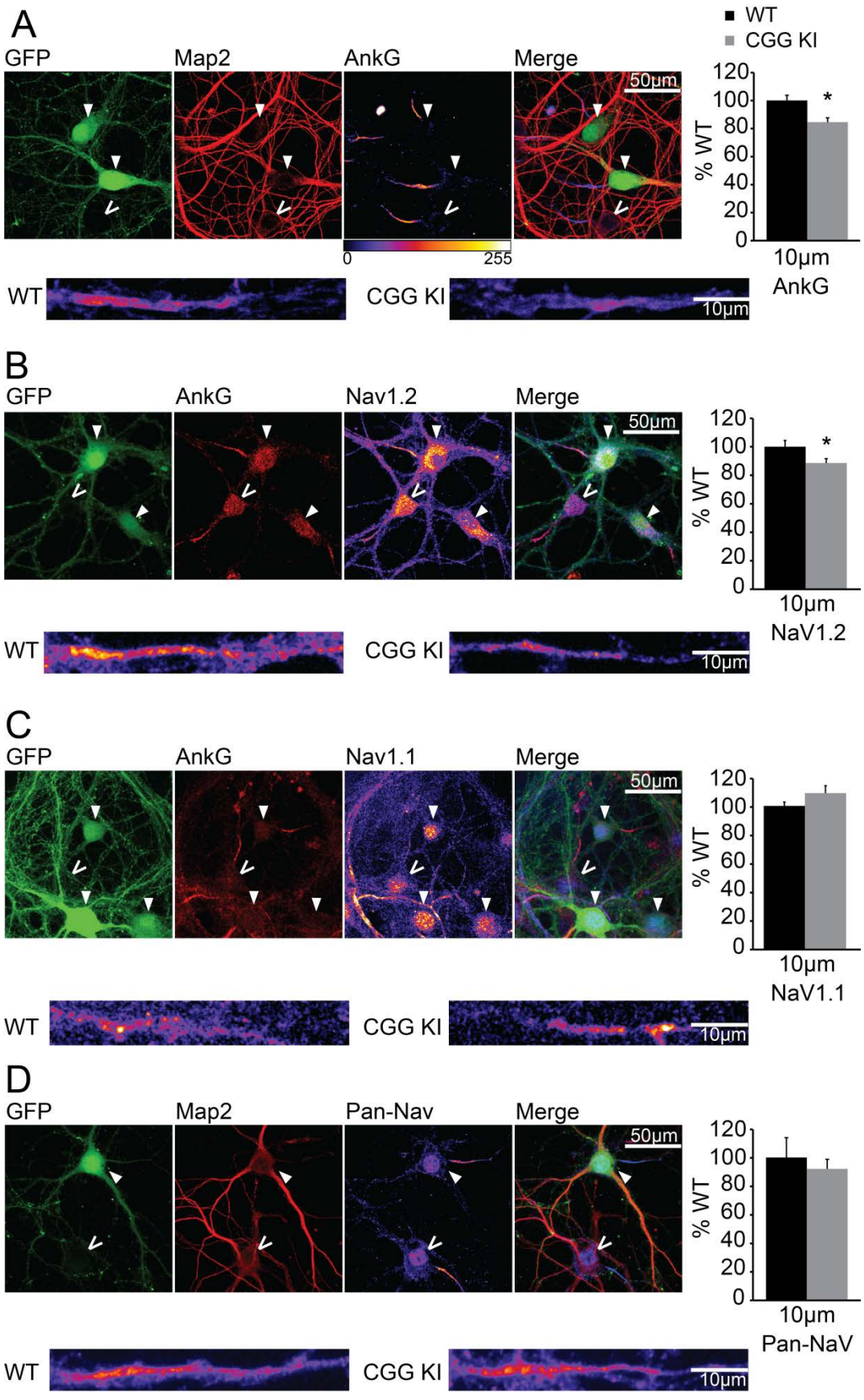
Here, we aimed to explore hippocampal neuronal properties in CGG KI model mice, and compare the relative contribution of FMRP haploinsufficiency and toxic CGG-containing mRNA in this model. We describe altered basal axonal neuronal properties

in cultured hippocampal neurons from CGG KI and Fmr1 KO model mice, in addition to evaluating two forms of synaptic plasticity. Ongoing work will describe the role of CGG-RNA or reduced basal FMRP levels in the generation and maintenance of mGluR-LTD and homeostatic plasticity.

Results

Axonal protein expression and intrinsic excitability

As a recent report identified potential presynaptic mRNAs as targets of FMRP, we began by examining their expression in CGG KI cultured hippocampal neurons (Darnell et al 2011). Primary hippocampal neurons were isolated from XGFP/CGG KI female animals, which allowed GFP-positive WT neurons and GFP-negative CGG KI neurons from the same animal owing to X-inactivation (Hadjantonakis et al 1998, Hanson & Madison 2007, Iliff et al 2013, Kalantry et al 2009, Niere et al 2012). Cultured neurons were fixed and probed with antibodies for ankyrin G (AnkG) and Map2, Nav1.2 and AnkG, Nav1.1 and AnkG, or Pan-Nav and AnkG to identify Map2-negative or AnkG-positive axon initial segments (AIS). We found significant decrements in AnkG and Nav1.2 in the first 10μm of the AIS in CGG KI neurons (AnkG: WT = 100 ± 3.70, CGG KI = 83.77 ± 3.07; $t=3.396$ $df=107$, $P<0.05$; WT n=47, CGG KI n=62; Nav1.2: WT = 100 ± 4.42, CGG KI = 88.57 ± 3.01; $t=2.173$ $df=104$, $P<0.05$; WT n=36, CGG KI n=70; Figure 5.1A-B). However, Nav1.1 was not different in CGG KI AIS (Nav1.1: WT 100 ± 2.64, CGG KI = 108.9 ± 5.24; $t=1.630$ $df=87$, NS; WT n=51, CGG KI n=38), nor was total Nav content altered, as measured by a Pan-Nav antibody (Pan-Nav: WT = 100 ± 13.84, CGG KI = 91.97 ± 6.78; $t=0.579$ $df=29$, NS; WT n=12, CGG KI n=19; Figure 5.1C-D).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Map2
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10µm
% WT
120
100
80
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20
0
10µm
Pan-NaV

Figure 5.1: AIS protein expression in XGFP/CGG KI neurons
Dissociated hippocampal neurons from XGFP/CGG KI female mice stained for axonal proteins at DIV14-17. (A) Ankyrin G expression in Map2-negative projections. AnkG fluorescence intensity in the first 10μm of the AIS are significantly reduced in CGG KI neurons. WT n=47, CGG KI n=62. (B) Nav1.2 expression in AnkG-positive projections show significant reduction in the proximal AIS. WT n=36, CGG KI n=70. (C) Nav1.1 levels in AnkG-positive AIS are unchanged in CGG KI neurons. WT n=51, CGG KI n=38. (D) Total Nav content as measured by a pan-Nav antibody in Map2-negative projections show no overall change in CGG KI neurons. WT n=12, CGG KI n=19. Students t-test, *P<0.05.

We also evaluated these AIS proteins in Fmr1 KO neurons using the same XGFP/Fmr1 KO strategy to generate both WT and KO neurons from the same female animals. We found a similar decrement in Nav1.2 expression in Fmr1 KO AIS (Nav1.2: WT = 100 ± 3.77, Fmr1 KO = 87.83 ± 2.68; t=2.702 df=73, P<0.05; WT n=33, Fmr1 KO n=42; Figure 5.2A), however AnkG expression was unchanged (AnkG: WT = 100 ± 13.10, Fmr1 KO = 104.9 ± 7.51; t=0.335 df=52, NS; WT n=15, Fmr1 KO n=39; Figure 5.2B).

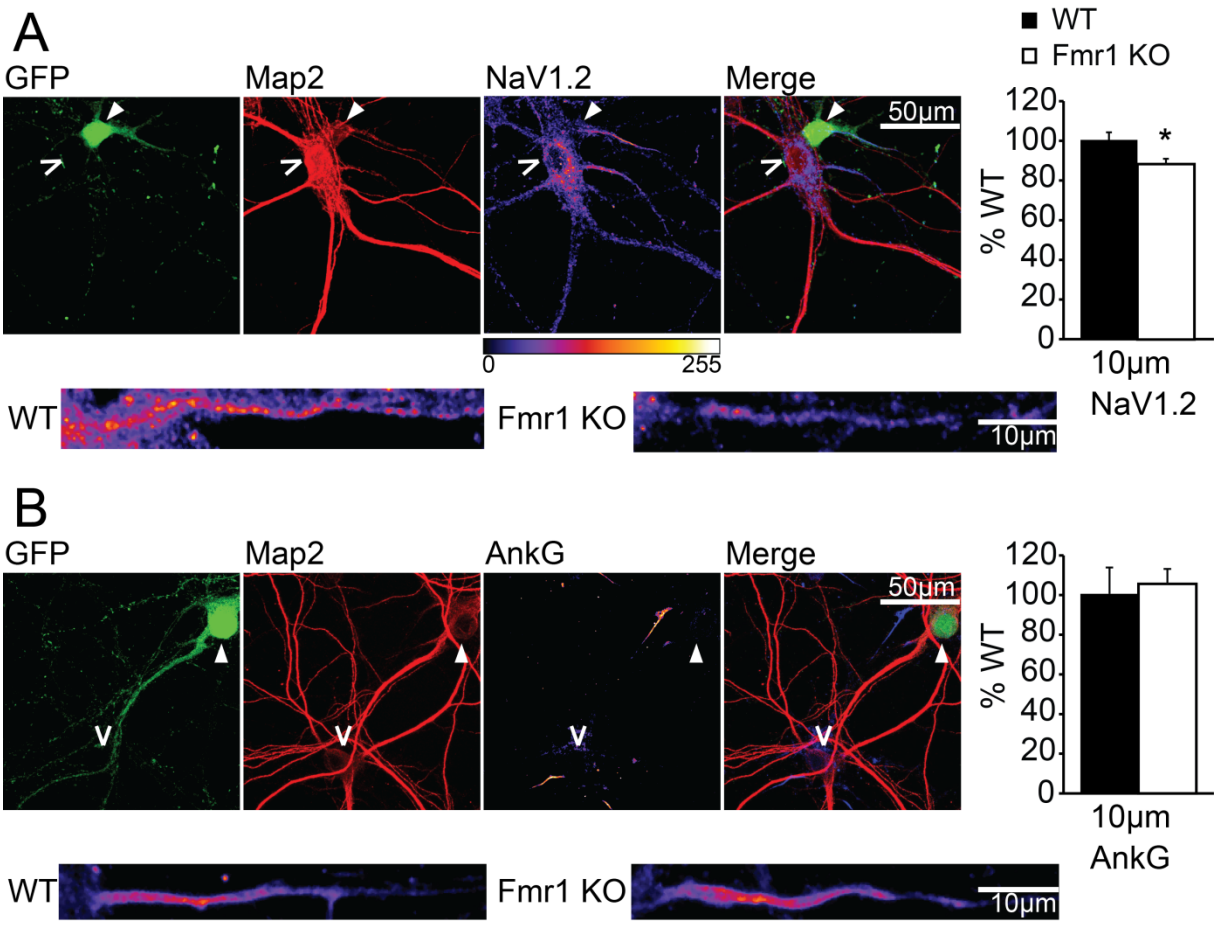
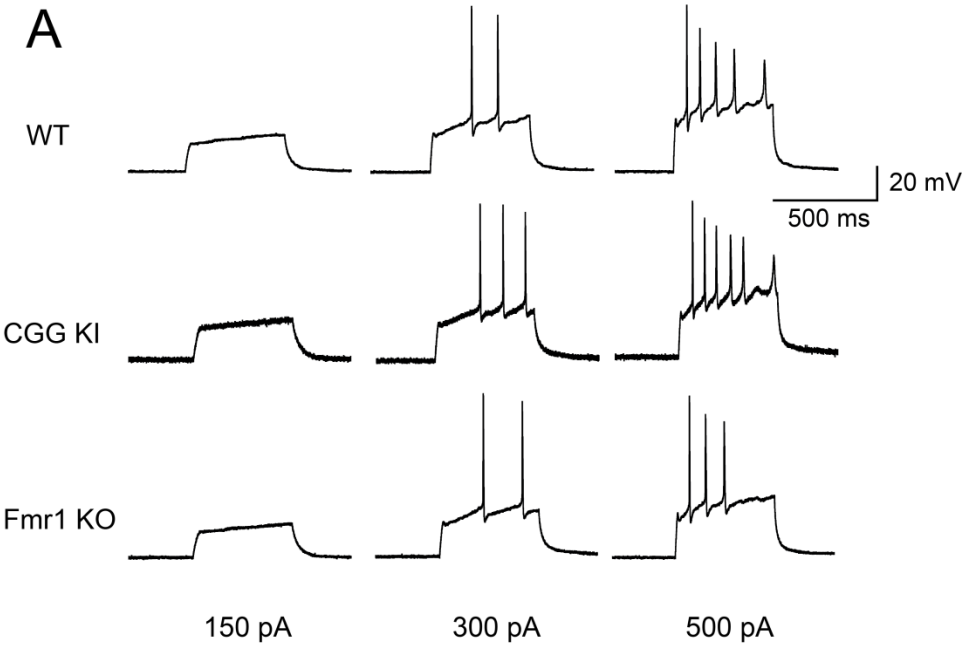


Figure 5.2: Axonal protein expression in XGFP/Fmr1 KO cultures
Dissociated hippocampal neurons from XGFP/Fmr1 KO female mice stained for axonal proteins at DIV14-17. (A) Nav1.2 expression in Map2-negative projections is significantly reduced in the first 10µm of the AIS in Fmr1 KO neurons. WT n=33, Fmr1 KO n=42. (B) Ankyrin G expression is unchanged in Fmr1 KO AIS. WT n=15, Fmr1 KO n=39. Students t-test, *P<0.05.

As we found significant alterations in the composition of critical voltage-gated sodium channels necessary for action potential generation, we evaluated intrinsic excitability in cultured hippocampal neurons from XGFP/CGG KI and XGFP/Fmr1 KO mice. We counted the number of actions potentials fired in response to increasing 500ms depolarizing current injections in pharmacologically isolated neurons (10µM CNQX, 20µM APV, 10µM bicuculline) as a metric of excitability (Figure 5.3). Importantly, input resistance was unchanged between CGG KI and Fmr1 KO neurons compared to their intradish WT controls (XGFP/CGG KI: WT = 176.8 ± 7.84, n=34; CGG KI = 191.2 ± 7.05, n=41; t=1.368 df=73, NS. XGFP/Fmr1 KO: WT = 165.6 ± 8.87,

n=22; Fmr1 KO = 164.3 ± 6.57, n=36; t=0.118 df=56, NS. Figure 5.3B-C). Despite significant alterations in AnkG and Nav expression, neither CGG KI nor Fmr1 KO neurons showed altered action potential generation with increasing current injections steps (XGFP/CGG KI: No significant effect of genotype $F_{(1, 1825)}$=0.322, or interaction $F_{(24, 1825)}$=0.239, NS. Significant effect of current injection $F_{(24, 1825)}$=46.85, P<0.05. WT n=34, CGG KI n=41. XGFP/Fmr1 KO: No significant effect of genotype $F_{(1, 1399)}$=1.988, or interaction $F_{(24, 1399)}$=0.159, NS. Significant effect of current injection $F_{(24, 1399)}$=26.41, P<0.05. WT n=22, Fmr1 KO n=36. Figure 5.3B-C).

These results were surprising following our evaluation of protein composition in the AIS of both these animal models. However, we did not evaluate the other voltage-gated sodium channels also expressed in the AIS, and it is possible these are upregulated to compensate for reduced Nav1.2 and AnkG expression to maintain normal excitability.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Fmr1 KO
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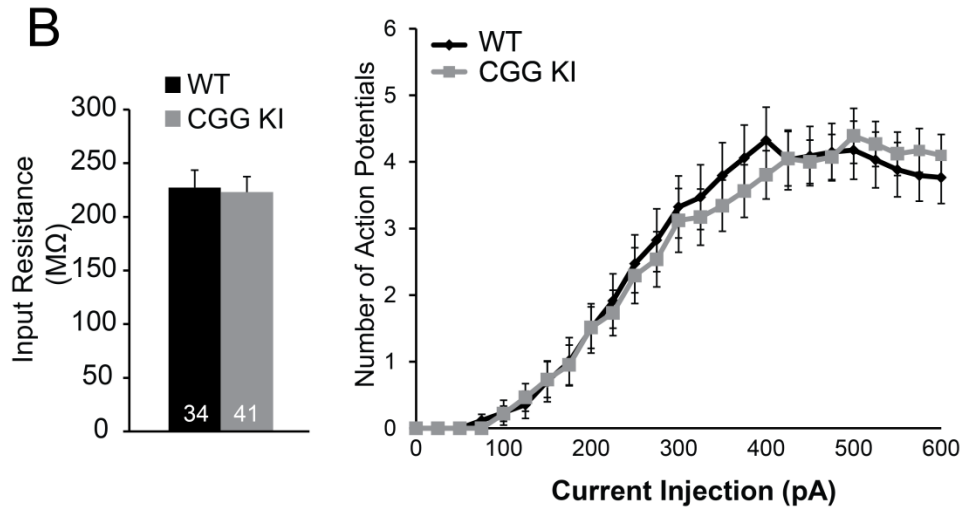
500 pA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CGG KI
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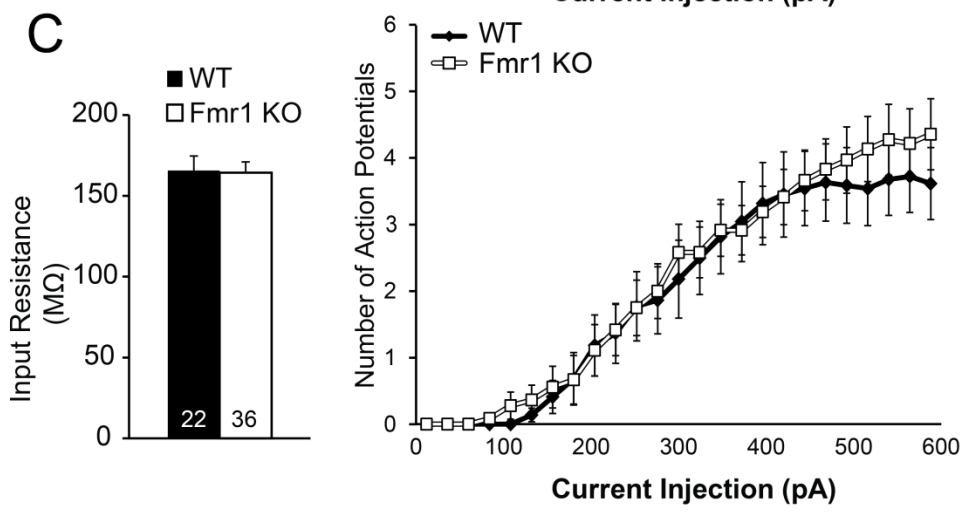
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Fmr1 KO
Input Resistance (MΩ)
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Number of Action Potentials
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0
100
200
300
400
500
600
Current Injection (pA)

Figure 5.3: Intrinsic excitability in CGG KI and Fmr1 KO neurons
Cultured hippocampal neurons from XGFP/CGG KI and XGFP/Fmr1 KO female mice were recorded from between DIV14-21. (A) Example traces of changes in membrane potential with increasing current injection. (B) Input resistance and intrinsic excitability are not significantly altered in CGG KI neurons. WT n=34, CGG KI n=41. (C) Input resistance and intrinsic excitability are not significantly altered in Fmr1 KO neurons. WT n=22, Fmr1 KO n=36. Students t-test, two-way ANOVA.

mGluR-LTD in cultured neurons

Previous work from our group identified enhanced, protein synthesis-dependent mGluR-LTD in acute hippocampal slices from CGG KI animals (Iliff et al 2013). We hypothesized this phenotype was a result of impaired activity-dependent synthesis of new FMRP, which results in increased translation of proteins required for LTD. However, the presence of basal amounts of FMRP regulates basal LTD proteins, allowing the requirement of new protein synthesis (Iliff et al 2013). The relative contribution of the toxic CGG-containing mRNAs in altered synaptic function is not clear, and a model in which genetic manipulation could be combined with mGluR-LTD studies was necessary to explore this question. Expression of various target genes or constructs is attainable in cultured neurons, by ether transfection or viral infection. As a result, we decided to measure mGluR-LTD in cultured neurons by measuring mEPSCs after DHPG stimulation. Basal mEPSC amplitude and frequency were unaltered in CGG KI neurons (Amplitude (pA): WT = 17.21 ± 1.13, n=18; CGG KI = 14.72 ± 0.74, n=13; t=1.684 df=29, NS. Frequency (Hz): WT = 1.00 ± 0.11, n=18; CGG KI = 0.87 ± 0.14, n=13; t=0.7770 df=29, NS; Figure 5.4A-B). Basal amplitude was significantly increased in Fmr1 KO neurons, while the frequency was unchanged compared to WT intradish controls (Amplitude (pA): WT = 15.47 ± 0.86, n=18; Fmr1 KO = 20.43 ± 1.25, n=13; t=3.336 df=32, $P<0.05$. Frequency (Hz): WT = 1.06 ± 0.14, n=18; Fmr1 KO = 0.97 ± 0.07, n=13; t=0.5780 df=32, NS; Figure 5.4A, C).

To induce synaptic depression, cultures were treated with DHPG (100µM) and TTX (1µM) for 10min in conditioned media, and exchanged to recording HBS (1µM TTX, 10µM bicuculline). Time post-DHPG was marked when a cell was recorded from (Figure 5.4D-E). DHPG caused a significant decrease in mEPSC frequency in 15-

60min post-DHPG treated cells (XGFP/CGG KI cultures: Frequency (Hz): WT Control = 1.00 ± 0.11, n=18; WT DHPG = 0.61 ± 0.10, n=8; CGG KI Control = 0.87 ± 0.14, n=13; CGG KI DHPG = 0.74 ± 0.10, n=13. Significant effect of drug $F_{(1, 48)}$=4.344, P<0.05; not significant effect of genotype $F_{(1, 48)}$=0.002, or interaction $F_{(1, 48)}$=1.118. XGFP/KO cultures: Frequency (Hz): WT Control = 1.06 ± 0.14, n=18; WT DHPG = 0.75 ± 0.09, n=14; Fmr1 KO Control = 0.97 ± 0.07, n=16; Fmr1 KO DHPG = 0.76 ± 0.10, n=15. Significant effect of drug $F_{(1, 59)}$=6.213, P<0.05; no significant effect of genotype $F_{(1, 59)}$=0.071, or interaction $F_{(1, 59)}$=0.370. Figure 5.4). This decrease in frequency was not accompanied by a change in mEPSC amplitude, in agreement with previously published findings (Snyder et al 2001, Waung et al 2008, Xiao et al 2001). This reproducible decrease in mEPSC frequency likely corresponds to a complete loss of synaptic AMPA receptors, leading to postsynaptic failures. While DHPG elicited a decrease in mEPSC frequency, this effect was not enhanced in CGG KI nor Fmr1 KO neurons (Figure 5.4). There is the caveat of a floor effect in measuring decreases in the already low mEPSC frequencies in this experiment. Observing a further significant decrement in frequency from WT DHPG levels (near 0.7 Hz) is very difficult and would require a large sample size to reach significance.

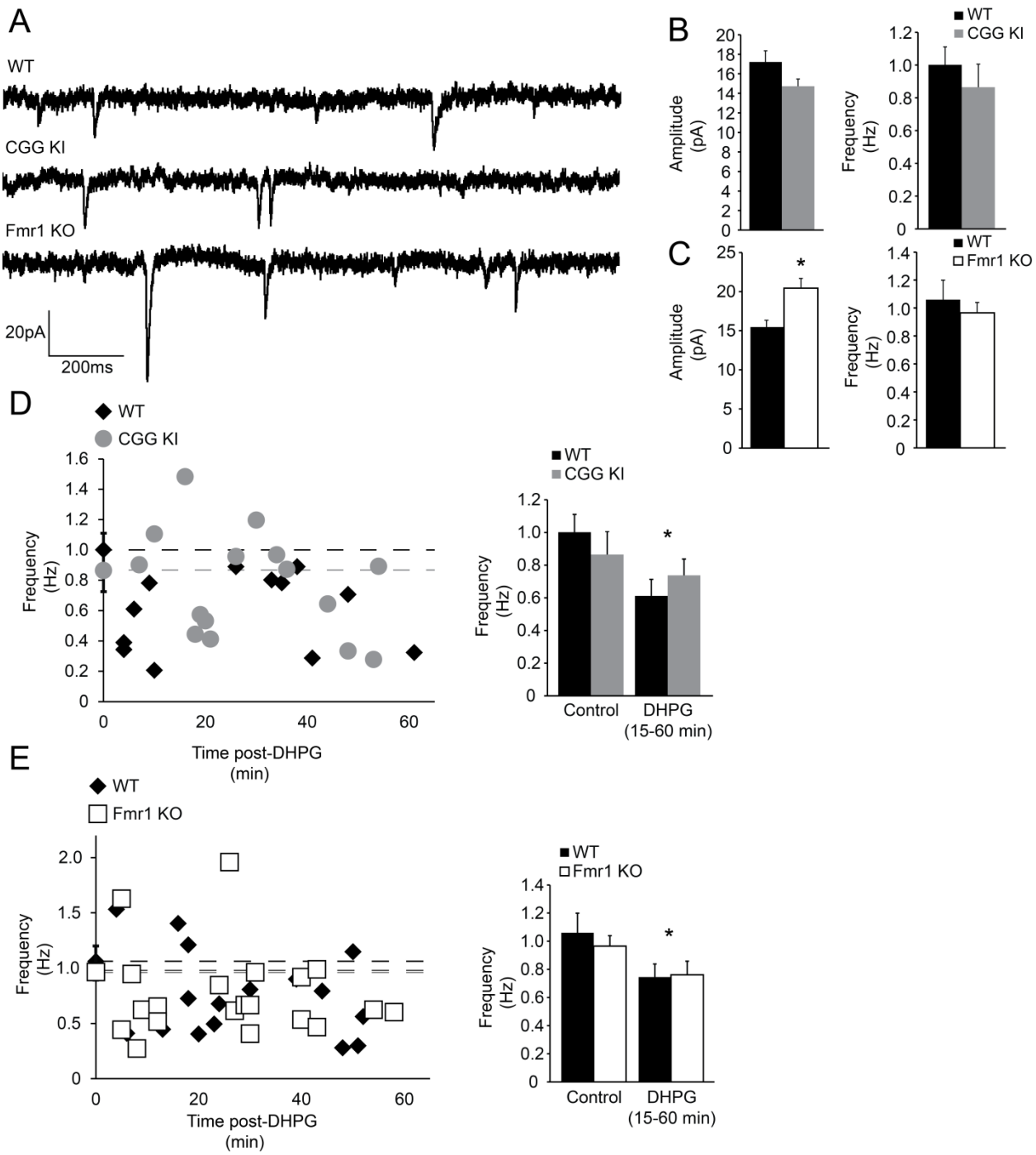

Figure 5.4: mGluR-LTD in cultured neurons
Dissociated hippocampal neurons from XGFP/CGG KI and XGFP/Fmr1 KO female animals were recorded from between DIV15-28. (A) Example traces of mEPSCs from each genotype. (B) Basal amplitude and frequency are normal in CGG KI neurons. WT n=18, CGG KI n=13. (C) mEPSC amplitude is significantly increased in Fmr1 KO neurons compared to WT neurons, but frequency is unchanged. WT n=18, Fmr1 KO n=13. (D) Each neuron recorded from after DHPG (100μM) + TTX (2μM) treatment for 10min is plotted with the time post-DHPG. mESPC frequencies were evaluated

between 15-60min post-DHPG. There was a main effect of DHPG treatment, though no difference between genotypes. WT n=8, CGG KI n=13. (E) All Fmr1 KO neurons and WT controls plotted as a function of time post-DHPG. Binned data between 15 and 60min after DHPG treatment show a significant effect of DHPG, though no significant difference between genotypes. WT n=14, Fmr1 KO n=15. Student's t-test, two-way ANOVA with posthoc Bonferroni, *P<0.05.

Homeostatic plasticity in cultured neurons

mGluR-dependent LTD is but one form of synaptic plasticity. In addition to this metric of Hebbian plasticity, there is also homeostatic plasticity. FMRP also plays a pivotal role in regulating proteins required for homeostatic plasticity, however this finding was only evaluated in Fmr1 KO animals (Soden & Chen 2010). We hypothesized that activity-dependent FMRP production may also play a role in homeostatic plasticity, and that CGG KI animals may demonstrate altered synaptic scaling as a result.

Homeostatic plasticity was induced by treating XGFP/CGG KI cultures with the AMPAR antagonist CNQX (40µM) for 6 hours in conditioned media. Cultures were then live-labeled with an antibody against the AMPAR subunit GluR1 as a metric of surface AMPAR levels. Neurons were co-stained for the synaptic marker, PSD95, and GluR1 fluorescence intensity in PSD95 puncta was analyzed between groups (Figure 5.5). We found a significant difference between WT and CGG KI neurons in their response to AMPAR blockade with CNQX (WT Control = 100 ± 6.84, n=35; WT CNQX = 106 ± 6.94, n=55; CGG KI Control = 163.51 ± 23.9, n=30; KI CNQX = 285.26 ± 50.54, n=46. Significant effect of drug $F_{(1, 162)}$=4.398, P<0.05; significant effect of genotype $F_{(1, 162)}$=15.56, P<0.05; no significant interaction $F_{(1, 162)}$=3.509, NS. Posthoc Bonferroni test revealed a significant difference between WT Control and CGG KI CNQX (t=4.298), Control KI and CNQX KI (t=2.699), and WT CNQX and KI CNQX (t=4.646); Figure 5.5B).

This experiment was repeated with XGFP/Fmr1 KO cultures, and we did not find a significant difference in the response of Fmr1 KO neurons (WT Control = 100 ± 7.37, n=10; WT CNQX = 167 ± 28.67, n=9; Fmr1 KO Control = 116.69 ± 35.67, n=3; KO CNQX = 253.15 ± 156.28, n=3. Significant effect of drug $F_{(1, 21)}$=4.558, P<0.05; no

effect of genotype $F_{(1, 21)}$=1.149, NS; nor interaction $F_{(1, 21)}$=0.522, NS. Posthoc Bonferroni test showed no significant difference between any two groups. Figure 5.5C).

This discrepancy from previously published findings may result from differences in homeostatic plasticity induction protocols. Previous findings evaluated synaptic scaling as a result of TTX+APV treatment in neurons from hippocampal slices (Soden & Chen 2010). The mechanism of synaptic AMPAR insertion varies depending on the neuronal silencing used: chronic TTX treatment to block all network activity, or faster AMPAR blockade (Aoto et al 2008, Henry et al 2012, Jakawich et al 2010, Sutton et al 2006, Wang et al 2011). These data indicate FMRP is playing a role in homeostatic plasticity of chronic network silencing, but not in the more rapid response to AMPAR blockade.

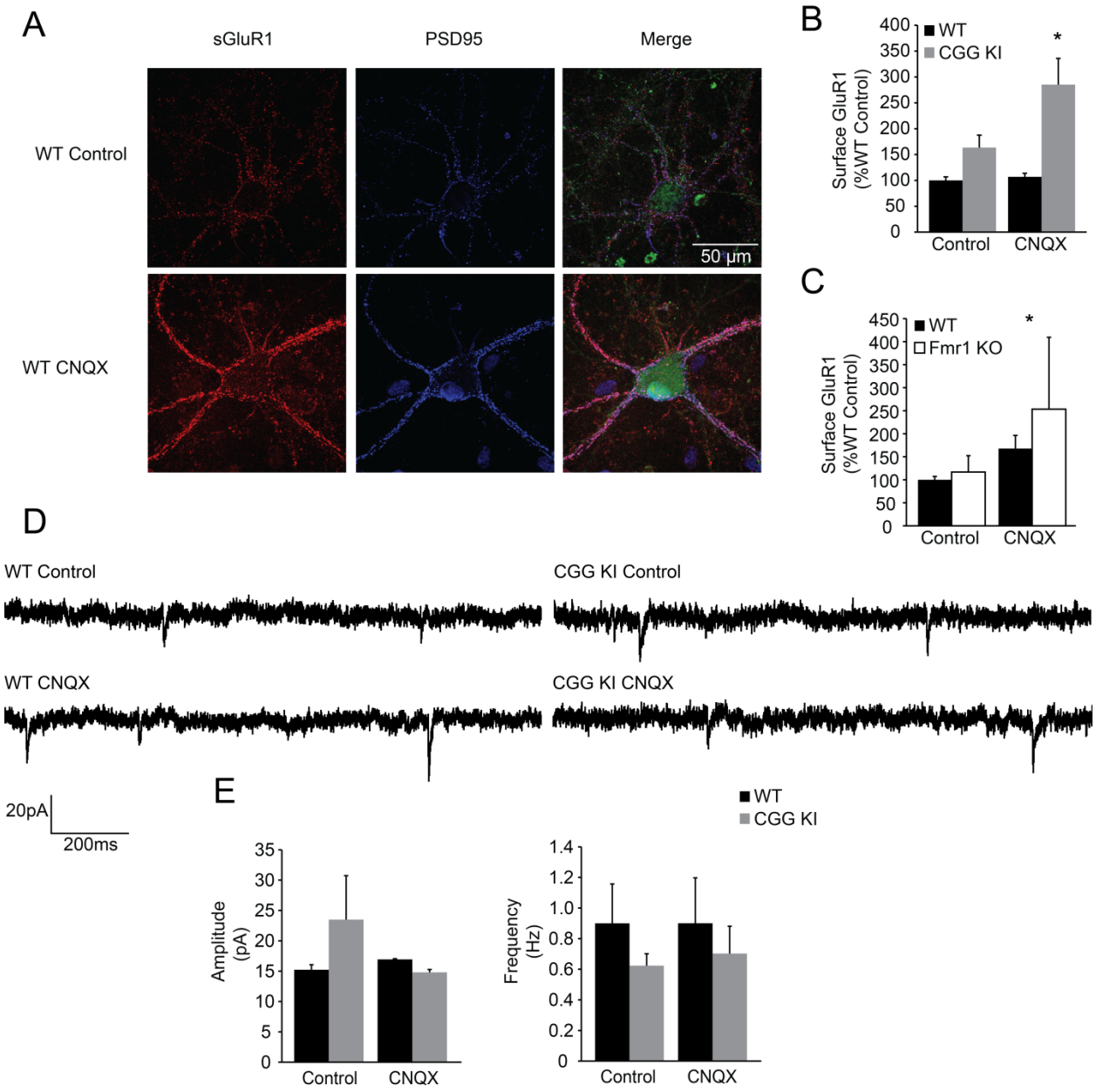

Figure 5.5: Homeostatic plasticity in CGG KI and Fmr1 KO neurons
(A) Cultured hippocampal neurons stained for surface GluR1 levels between DIV14-17 following 6hrs CNQX treatment (40µM). Merged image includes GFP expression of WT neurons. (B) CGG KI neurons express significantly more surface GluR1 compared to WT controls following CNQX treatment. Control WT n=35, CGG KI n=30; CNQX WT n=55, CGG KI n=46. (C) A main effect of CNQX was seen, though no significant difference was found between Fmr1 KO and WT GluR1 levels. Control WT n=10, Fmr1 KO n=3; CNQX WT n=9, Fmr1 KO n=3. (D) mEPSCs were recorded following AMPAR blockade from WT and CGG KI neurons. (E) No significant difference between genotypes was found in either mEPSC amplitude or frequency. Control WT n=3, CGG KI n=3; CNQX WT n=2, CGG KI n=4. Two-way ANOVA, posthoc Bonferroni test, *P<0.05.

As CGG KI neurons demonstrated a significant difference in surface GluR1 levels after CNQX treatment, we hypothesized this would correspond to increases in mEPSC amplitude and frequency. mEPSCs were recorded from XGFP/CGG KI treated immediately following washout of CNQX (40µM, 6hrs). While we did not find a significant difference between genotypes, we found a strong trend towards impaired synaptic strengthening seen in WT neurons (Amplitude (pA): WT Control = 15.23 ± 0.84, n=3; WT CNQX = 16.94 ± 0.10, n=2; CGG KI Control = 23.49 ± 7.24, n=3; CGG KI CNQX = 14.79 ± 0.48, n=4. No significant difference: Interaction $F_{(1, 8)}$=1.897, genotype $F_{(1, 8)}$=0.655, drug $F_{(1, 8)}$=0.856, NS. Frequency (Hz): WT Control = 0.9 ± 0.26, n=3; WT CNQX = 0.9 ± 0.30, n=2; CGG KI Control = 0.62 ± 0.08, n=3; CGG KI CNQX = 0.70 ± 0.18, n=4. No significant difference: Interaction $F_{(1, 8)}$=0.035, genotype $F_{(1, 8)}$=1.282, drug $F_{(1, 8)}$=0.036, NS. Figure 5.5D-E). The number of cells evaluated was small in this study, and future work may reveal a significant alteration in homeostatic plasticity in CGG KI neurons.

Any significant difference in homeostatic scaling as measured by surface GluR1 levels, or mEPSC increases in premutation model neurons could be the result of either reduced FMRP levels, or increased toxic CGG-containing mRNA. In an attempt to isolate the relative contribution of these two features, we designed a lentiviral construct containing 100 CGG repeats in an engineered 5'UTR upstream of GFP (LV-CGG-GFP). WT rat hippocampal cultures were infected with this virus or a control GFP-only lentivirus (LV-GFP), for 2 days prior to induction of synaptic strengthening with CNQX (40µM, 3hrs). GFP-positive neurons were recorded from following CNQX washout. While we find no significant difference in either the amplitude (LV-GFP Control = 15.45 ± 0.89, n=4; LV-GFP CNQX = 16.95 ± 1.74, n=8; LV-CGG-GFP Control = 17.26 ± 1.09, n=8; LV-CGG-GFP CNQX = 16.10 ± 0.75, n=10. No significant difference: Interaction $F_{(1, 26)}$=1.031, genotype $F_{(1, 26)}$=0.135, drug $F_{(1, 26)}$=0.017; Figure 5.6B) or the frequency (LV-GFP Control = 2.53 ± 0.70, n=4; LV-GFP CNQX = 2.45 ± 0.30, n=8; LV-CGG-GFP Control = 2.05 ± 0.27, n=8; LV-CGG-GFP CNQX = 2.03 ± 0.45, n=10. No significant difference: Interaction $F_{(1, 26)}$=0.003, genotype $F_{(1, 26)}$=1.078, drug $F_{(1, 26)}$=0.014; Figure 5.6C), we see a similar trend to that of CGG KI neurons (Figure 5.5D-E). Additional

experiments will be needed to confirm this finding, though these results indicate CGG-containing mRNA expression itself may have an impact on synaptic function.

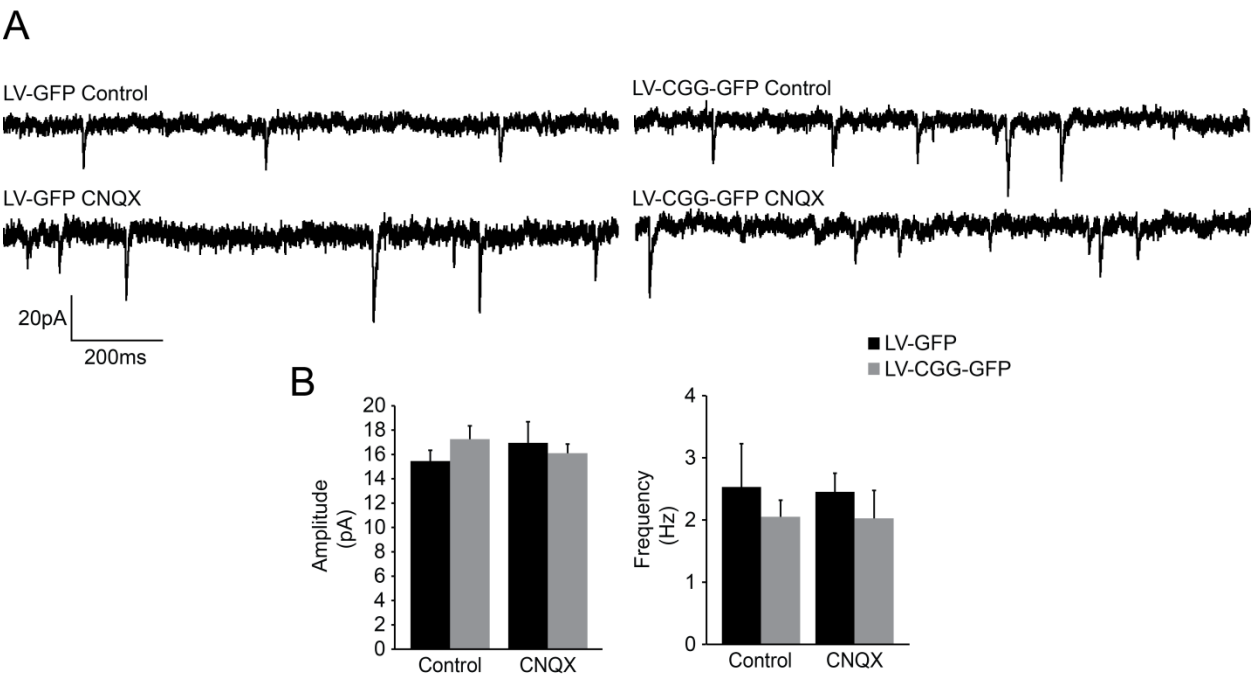

Figure 5.6: Homeostatic plasticity in CGG-expressing neurons
(A) WT rat hippocampal cultures were infected with lentiviral constructs containing 100 CGG repeats upstream of a GFP reporter, or a GFP control on DIV17-19. Homeostatic plasticity was induced using CNQX (40µM, 3hrs), and mEPSCs recorded following washout. (B) No significant change in mEPSC amplitude or frequency was measured between CGG-expressing and control GFP positive neurons. Control LV-GFP n=4, LV-CGG-GFP n=8; CNQX LV-GFP n=8, LV-CGG-GFP n=10. Two-way ANOVA.

Discussion

In this chapter, we explore additional synaptic impacts of the premutation in cultured rodent hippocampal neurons, with the primary aim of evaluating the relative contribution of increased CGG-mRNA expression seen in CGG KI model mice. We find altered axonal protein expression in both the CGG KI and Fmr1 KO neurons, though intrinsic excitability is spared in both models. We go on to measure mGluR-dependent LTD using mEPSCs, and find similar induction of LTD in both CGG KI and Fmr1 KO cells. The lack of differentiation between genotypes is likely a result of already low frequencies in the neurons recorded from, resulting in the inability to detect enhanced

synaptic weakening in the mutants tested. We compare another form of synaptic plasticity, homeostatic scaling, and find altered surface GluR1 levels in CGG KI neurons following CNQX treatment, though preliminary results were not sufficient to detect differences in mEPSC amplitude or frequency. In an attempt to separate the reduced FMRP levels from CGG repeat expression, we compared homeostatic plasticity in WT rat cultures infected with a CGG-containing lentivirus, and find a similar trend in mEPSC amplitude following AMPAR blockade as seen in CGG KI neurons. These findings indicate that both axonal protein expression, and HSP are altered in premutation model neurons, perhaps in part due to increased CGG-containing mRNA levels.

Previous work has shown FMRP localizes presynaptically, and contributes to normal synaptic function (Akins et al 2012, Hanson & Madison 2007, Till et al 2011). Additionally, FMRP is found associated with, or regulating the translation of, a variety of presynaptic and axonal channels, including Slack and BK potassium channels, and N-type calcium channels (Brown et al 2010, Deng et al 2013, Ferron et al 2014, Myrick et al 2015, Zhang et al 2012). Our finding of significantly reduced levels of Nav1.2 and AnkG in CGG KI neurons suggests that premutation models may also have alterations in neuronal excitability. However, we found intrinsic excitability to be unaltered in CGG KI and Fmr1 KO neurons (Figure 5.3). This suggests some level of compensation between Nav1.2 and the other voltage-gated sodium channels, which include Nav1.1, Nav1.3, and Nav1.6 (Catterall et al 2005). We were able to examine Nav1.1 levels, and found normal expression in CGG KI neurons (Figure 5.1). It is possible Nav1.3 or Nav1.6 are upregulated to maintain normal action potential firing despite reduced levels of Nav1.2 and AnkG. It is also noteworthy that intrinsic excitability was measured under basal conditions. It is possible that the altered AIS protein composition would cause changes in action potential generation under different conditions, such as situations of high neuronal activity, or as the cell ages. Future experiments would be needed to address these possibilities.

Our group previously identified enhanced protein synthesis-dependent mGluR-LTD in CGG KI model acute hippocampal slices (Iliff et al 2013). Acute hippocampal slices are not as readily manipulable, so we aimed to study mGluR-LTD in cultured neurons. Several other groups have used this approach previously, though with mixed

results in mouse or rat cells (Niere et al 2012, Snyder et al 2001, Waung et al 2008, Xiao et al 2001). One study examined decreases in mEPSC frequency as a metric of mGluR-LTD in Fmr1 KO mouse neurons, which was enhanced compared to WT controls, though the WT neurons were unchanged after DHPG stimulation (Niere et al 2012). In contrast, our results show significant decrements in mEPSC frequencies in both WT and Fmr1 KO as well as CGG KI neurons after DHPG stimulation (Figure 5.4). However, we did not find a significant enhancement in this metric of synaptic weakening in mutant neurons compared to WT. This difference may be due to differences in experimental solutions or conditions; however our observance of mGluR-dependent depression in WT neurons suggests adequate parameters. The inability to differentiate between Fmr1 KO or CGG KI neurons compared to WT is possibly due to a floor effect in measuring frequencies. The WT mEPSC frequencies drop by roughly 25% (1 Hz to 0.7 Hz; Figure 5.4), and an additional decrease in measurable frequency becomes more difficult to discern. This result suggests that while mGluR-LTD can be measured in culture, it is a difficult system to manipulate to test mechanistic underpinnings of this phenotype.

In addition to mGluR-LTD, we examined homeostatic plasticity in Fmr1 KO mice, and premutation model animals. While we found differences in synaptic scaling using immunocytochemistry for surface GluR1 levels, this did not correspond to significant changes in mEPSCs (Figure 5.5). Recordings of mEPSCs are relatively preliminary, and will need to be repeated to verify that homeostatic plasticity is intact in premutation model mice. While significant differences in mEPSCs cannot be detected, the trend of amplitude changes with CNQX treatment is interesting; basal increase in mEPSC amplitude, and no expected increase with AMPAR blockade (Figure 5.5). FMRP expression is correlated with surface GluR1 levels, wherein less FMRP corresponds to less synaptic GluR1 (Nakamoto et al 2007). Our results do not agree with this finding, though the previous work was performed in WT neurons transfected acutely with a construct to knock down FMRP levels incompletely (Nakamoto et al 2007). It may be that developmental or chronic reduction of FMRP activates a compensatory mechanism to restore AMPAR content at synapses, and perhaps even super inflate the surface AMPAR levels.

In an effort to discern between the basal reduction in FMRP and CGG-containing mRNA in premutation model neurons, we designed a lentiviral construct to exogenously overexpress a CGG transcript. We asked what the consequence on synaptic function of this increased trinucleotide repeat RNA would be assessing mEPSCs following CNQX treatment. While we did not find any significant changes in either amplitude or frequency, the trend in increased basal amplitude which is resistant to AMPAR blockade exists (Figure 5.6). These results are preliminary, and need to be repeated to validate this effect.

In this chapter we have evaluated several key metrics of neuronal function in the absence of FMRP, and in the context of the Fragile X premutation. We identified several shared phenotypes and functional profiles between FXS and premutation model neurons, further indicating potential mechanistic overlap between these two disorders. The hope is that the mechanistic overlap between these disorders will allow for the successful cross-application of emerging therapies for FXS to premutation patients.

Materials and Methods

Mice and Cell Culture

Animal use followed NIH guidelines and was in compliance with the University of Michigan Committee on Use and Care of Animals. DNA was extracted from tail biopsies and isolated with 5mM NaOH incubated for 0.5-1 hr at 90˚C. Samples were neutralized with 0.5M Tris pH 8.0, and DNA samples were genotyped with primers against the Y chromosome (5'GTGAGAGGCACAAGTTGGC, 5'GTCTTGCCTGTATGTGATGG) to determine the sex of each animal using Platinum® PCR Supermix (Invitrogen). Dissociated hippocampal neuron cultures were prepared from postnatal (P1-3) mice, or WT rats (P0-2) as previously described (Jakawich et al 2010). Experiments were performed at 14-17 days *in vitro* (DIV) for immunocytochemistry, or 15-28DIV for electrophysiology.

Drugs

Tetrodotoxin (TTX; Tocris) was solubilized in sterile water (1mM), stored at -20°C, and aliquots diluted as needed in conditioned media or HBS (1µM). Bicuculine (Tocris) was diluted in DMSO (50mM), and stocks stored at -20°C prior to dilution in HBS (10µM). D-(-)-2-Amino-5-phosphonopentanoic acid (APV; Tocris) was solubilized in sterile water (20mM), and stored at -20°C until diluted in HBS (20µM). 6-Cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate (CNQX; Sigma) was diluted in sterile water (10mM), stored at -20°C until use in conditioned media or HBS for intrinsic excitability recordings (10µM), or induction of homeostatic plasticity (40µM). R,S-3,5-DHPG (Tocris) was prepared fresh each day in sterile water (10mM), and diluted in conditioned media as needed (100µM).

Immunocytochemistry and Microscopy

Basal protein expression

All experiments were conducted at 37ºC. Neurons were washed with warmed phosphate buffered saline with 1mM $MgCl_2$ and 0.1mM $CaCl_2$ (PBS-MC), fixed with 4% paraformaldehyde (PFA)/4% sucrose in PBS-MC for 15 min, permeabilized (0.1% Triton X in PBS-MC, 5 min), blocked with 5% normal goat serum (NGS) or 2% bovine serum albumin (BSA) in PBS-MC for 1 hour, and labeled with antibodies against the target of interest: Ankyrin G (NeuroMab 75-146, clone N106/36; 1:500), Nav1.2 (NeuroMab 75-024, clone K69/3; 1:500), Nav1.1 (NeuroMab 75-023, clone K74/71; 1:200), pan-Nav (Sigma S8809; 1:500), Map2 (Millipore AB5622; 1:1000).

Live GluR1 staining

Cultures were treated with CNQX (40µM) for 3 or 6 hours in conditioned media, followed by live labeling with a rabbit polyclonal antibody recognizing surface epitopes of GluR1 (Calbiochem PC246; 1:10) for 15min at 37ºC. Cells were then fixed with 2%PFA/2% sucrose in PBS-MC for 15min at room temp (RT), and blocked in 2% BSA in PBS-MC for 20min. Secondary anti-rabbit Alexa 555-conjugated antibody (Invitrogen, 1:500) was incubated on cultures for 60min at RT. Cells were then permeabilized with 0.1% Triton X in PBS-MC for 5min, and blocked with 2% BSA for 60min at RT. Synapses were labeled with co-staining for PSD95 (Millipore MAB1596,

1:1000) overnight at 4ºC, and labeled with secondary anti-rabbit Alexa 635-conjugated antibody (Invitrogen, 1:1000).

All imaging was performed on an inverted Olympus FV1000 laser scanning confocal microscope. Prior to image collection, the acquisition parameters for each channel were optimized to ensure a dynamic signal range and to ensure no signal bleed-through between detection channels. Identical acquisition parameters were used for each treatment condition in an experiment. Image analysis was performed on maximal intensity z-projected images using custom written analysis routines for ImageJ. For surface GluR1 analysis, 'synaptic' GluR1 was defined as a particle that occupied greater than 10% of the area defined by a PSD95 particle, and the average integrated intensity (total # of non-zero pixels * intensity) of synaptic GluR1 particles was calculated.

Electrophysiology

Whole-cell patch-clamp recordings were made with an Axopatch 200B amplifier from cultured hippocampal neurons bathed in HEPES-buffered saline (HBS; containing, in mM: 119 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 30 Glucose, 10 HEPES, pH 7.4). Whole-cell pipette had resistances ranging from 3-5 MΩ. Intrinsic excitability internal solution contained (in mM): 115 $KMeSO_4$, 15 KCl, 5 NaCl, 0.02 EGTA, 1 $MgCl_2$, 10 Na_2-Phosphocreatine, 4 ATP-Mg, 0.3 GTP-Na, pH 7.2. Cultured neurons with a pyramidal-like morphology were voltage-clamped at –70 mV. Membrane potentials were recorded in current clamp. Input resistance was measured using a hyperpolarizing 25 pA current step. Intrinsic excitability was assessed in pharmacologically isolated neurons using a series of 500 ms current injections to depolarize the soma from resting membrane potential. Action potential properties and intrinsic excitability data were analyzed using Clampfit.

Internal solution for mEPSCs contained (in mM): 100 cesium gluconate, 0.2 EGTA, 5 $MgCl_2$, 2 ATP-Mg, 0.3 GTP-Na, 40 HEPES, pH 7.2. mEPSCs were recorded at −70 mV from neurons with a pyramidal-like morphology in the presence of 1 μM TTX and 10 μM bicuculline and analyzed off-line using Synaptosoft mini analysis software.

Statistical analysis

All values are reported as the mean ± SEM. A student's t-test, or two-way ANOVA with posthoc Bonferroni was used to identify significant differences. A P value of less than 0.05 was considered significant.

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Chapter 6
Conclusions and Future Directions

Summary and significance

Fragile X disorders are caused by a CGG trinucleotide repeat expansion in the 5'UTR of the *FMR1* gene on the X chromosome. Fragile X Syndrome (FXS) is the most common inherited cause of autism and intellectual disability, which is elicited by the absence of the FMR protein. Premutation range repeats cause Fragile X-associated Tremor/Ataxia Syndrome (FXTAS), which is an age-related neurodegenerative disorder characterized by increased CGG-containing FMR1 mRNA, and reduced FMRP. FXTAS is a relatively recently described disorder, but more current work suggests that premutation carriers may share some neurologic features with FXS patients prior to degeneration and the development of FXTAS (reviewed in (Grigsby et al 2014, Wheeler et al 2014)). FMRP is an RNA-binding protein required regulation of synaptic protein translation and mGluR-dependent long term depression (LTD). FMRP regulates its own synaptic translation in response to mGluR stimulation, and this new FMRP is hypothesized to function as a brake on new translation, thus capping production of proteins required for mGluR-LTD. The goal of this work was three fold: test the hypothesis that newly translated FMRP is regulating mGluR-LTD by controlling synaptic translation using a mouse model of the premutation; test for novel behavioral phenotypes in CGG KI mice which may be correlated to translation and plasticity; examine additional forms of synaptic plasticity and neuronal function in CGG KI neurons. Evaluating the synaptic effect of expanded CGG repeats in *Fmr1* is a critical goal, as relatively little is known about what impact the premutation has in this potentially very large carrier population.

There are several important discoveries made in this body of work. We tested the hypothesis that new FMRP functions as a translational brake in the context of the premutation, as the large CGG hairpin prevents mGluR-activated translation of new FMRP. This was an ideal model to test this hypothesis, as the Fmr1 KO model has a distinct phenotype owing to the absence of FMRP to control basal translation of its targets. The premutation model, however, still expresses basal FMRP, though we discovered that the activity-dependent production of new FMRP was blocked in these animals. This allowed us to more accurately ask what happened to synaptic plasticity without new FMRP being translated, and we found that this resulted in a similar enhancement of mGluR-LTD to that seen in Fmr1 KO mice. However, the LTD in CGG KI mice was still dependent on new protein synthesis, as existing FMRP is likely able to regulate basal translation of proteins required for LTD. These data support the hypothesis that the absence of new FMRP leads to increased production of LTD proteins which mediate the enhanced synaptic weakening observed. Additionally, this was one of the first reports to examine synaptic plasticity in a model of the premutation. A similar phenotype to that seen in Fmr1 KO mice suggests there are more similarities between these models, and was a key piece of evidence indicating that premutation carriers may have more in common with FXS patients than considered previously.

We performed preliminary experiments to explore which, if any, LTD proteins might be mediating this synaptic deficit. Examination of Arc, PSD95, and Shank1-3 did not show any major trend of super-induction in CGG KI neurons after mGluR stimulation as we had hypothesized. Arc expression does appear to be differentially regulated in CGG KI neurons, as we found a basal increase in dendritic Arc compared to WT neurons, which was resistant to mGluR stimulation.

An additional FMRP target is the amyloid precursor protein (APP). We examined APP expression levels in CGG KI mice and FXTAS human brain tissues. We found increased APP levels in the cerebellum of human samples, but no change in patient cortical tissue, nor in the mouse model of FXTAS. These data likely speak more to the age-related features of FXTAS, and indicate that while APP expression may not be a primary cause of pathogenesis, work in FXS and Fmr1 KO animals indicate that amyloid processing is a key effector in the absence of FMRP.

As the CGG KI mice have altered synaptic scaling which is correlated to impaired new FMRP synthesis, we hypothesized that there would be a behavioral corollary which may also be shared between Fmr1 KO and CGG KI animals. We found impaired prepulse inhibition (PPI) in CGG KI mice, which was very similar in the degree and direction to that observed in Fmr1 KO mice. This experiment was particularly useful as PPI is altered in human FXS and FXTAS patients in addition to Fmr1 KO mice, and is an easy behavior to test potential therapeutics. Therapeutic development for FXS has grown dramatically in the last several years, and there have been numerous clinical trials testing mechanistic findings discovered in Fmr1 KO models (reviewed in (Gross et al 2015b)). We took advantage of this to test one such potential drug, fenobam, which is an mGluR antagonist. As expected, fenobam treatment increased PPI in both WT and CGG KI animals, however the age of mice tested was prior to the development of a PPI phenotype in CGG KI animals. This proof of principle experiment demonstrates that PPI is an easily performed task which could be used to evaluate other therapeutic targets currently under development, with the benefit of potentially treating premutation symptoms which may be shared with FXS patients.

There is relatively limited research into FMRP's function presynaptically (reviewed in (Wang et al 2012)). Examination of putative FMRP target transcripts suggested that several important components of the axon initial segment may be regulated by FMRP (Ascano et al 2012, Darnell et al 2011). We examined expression of the voltage-gated sodium channels Nav1.1 and Nav1.2, as well as the scaffolding protein ankyrin G in axon initial segments (AIS) of cultured hippocampal neurons. We found significantly reduced levels of Nav1.2 and AnkG in CGG KI neurons, and reduced Nav1.2 in Fmr1 KO neurons. These results suggested that intrinsic excitability may be altered in these cells, however action potential number with step-wise current injection showed normal action potential dynamics. While this result is surprising, it suggests that there is some compensation likely occurring to maintain normal action potential generation. This could occur from expression of other voltage-gated sodium channels, such as Nav1.6. The overall result remains that the protein composition of the AIS in CGG KI neurons is significantly altered, and likely have an impact on neuronal firing under different conditions.

The novel phenotypes and mechanistic insights discovered in this work are still hampered by the question of what role reduced FMRP and increased CGG-mRNA expression is playing. To compare these relative contributions, we needed a synaptic paradigm which was easily testable in cell culture models which are more easily genetically manipulated. To that end, we measured a proxy of mGluR-LTD in cultured neurons by recording mEPSC frequency after DHPG stimulation. Overall, DHPG caused a significant decrease in mEPSC frequency due to synaptic silencing, however the values obtained were relatively low which made detection of enhanced LTD between genotypes very difficult. FMRP also functions in homeostatic plasticity, and we sought to compare the effects of AMPAR blockade on homeostatic potentiation in Fmr1 KO and CGG KI cells. By recording mEPSCs, we found a trend toward impaired synaptic scaling in the CGG KI neurons. Using a lentiviral vector to express expanded CGG repeats in WT rat neurons, we found a similar trend toward attenuated homeostatic potentiation.

This body of work is unique as it attempts to examine the younger premutation model, and find phenotypic overlap with the Fmr1 KO mouse. The Fragile X field has long treated FXS and FXTAS as two distinct disorders, however this work makes a strong case for the consideration of the Fragile X spectrum. Previous work has demonstrated that FMRP expression is inversely linked with CGG repeat number, and it follows that symptoms would also vary based on repeat and FMRP expression (Ludwig et al 2014). This work also indicates that symptomatic premutation carriers may also benefit from treatments developed for FXS patients, as they share several key mechanistic overlaps.

Outstanding questions

Are premutation phenotypes the result of CGG repeat expression or FMRP haploinsufficiency?

The results described in Chapter 5 of this thesis suggest that the CGG mRNA may have an autonomous role in synaptic function, independent of FMRP levels. This

is a very intriguing idea, and indicates that there may be unexplored phenomena underlying the synaptic defects we see in CGG KI model neurons. How these CGG repeats may be eliciting changes in synaptic function is not immediately clear, but several possibilities exist. The repeats may be sequestering proteins required for additional cellular processes, altering cell-wide protein expression, or splice isoforms of various proteins required for the fine modulation of synaptic strength. This possibility seems relatively likely overall, in particular in light of recent data examining CGG-binding proteins, and the downstream effects of their altered behavior in the presence of increased CGG mRNA expression (Sellier et al 2013, Sellier et al 2010). However the relatively short time (2 days) of CGG expression before observing this phenotype indicates a more rapid effect than long protein turnover and modification of alternate pathways required for the above scenario. Alternatively, the CGG mRNA may be directly interacting with synaptic components to modify either signal relays, or the direct recycling of AMPA receptors in this experiment.

Examination of reduced FMRP levels on homeostatic plasticity will also be required to better understand the molecular events happening in premutation model neurons. Using a knockdown approach in WT neurons to reduce FMRP levels to those seen in CGG KI neurons would be an excellent way to compare the impact of basally reduced FMRP. This approach would also mimic the reduced activity-dependent expression of new FMRP if performed acutely while the construct used to knockdown expression was still present and able to block Fmr1 translation. This approach could be used to compare the role of absent FMRP without the complication of additional CGG-mRNA expression.

What impact, if any, is the RAN peptide FMRpolyG having on synaptic function?

The recently discovered phenomenon of repeat associated non-AUG (RAN) translation in FXTAS and CGG-expression cell models raises the possibility that FMRpolyG may also be playing a novel role in synaptic function. There is precedence for other toxic aggregate-prone peptides altering synaptic function in Alzheimer's disease (Aβ peptides; reviewed in (Palop & Mucke 2010)) and Parkinson's disease (α-

synuclein; reviewed in (Cheng et al 2011)). Understanding the impact of this novel phenomenon is still in its early stages, and as a result it's not known where or under what circumstances RAN translation happens. It is very possible RAN translation can occur at synaptic locations, producing FMRpolyG locally at synapses basally, or even under activity-dependent circumstances. It is probable that this RAN peptide may regulate FMRP itself, or interact with any other of the numerous proteins required for normal synaptic function and plasticity.

Identification of endogenous FMRpolyG in the Dutch CGG KI or YAC premutation model neurons would allow the interrogation of conditions and locations of the RAN peptide, which might elucidate its function. Testing the role of FMRpolyG in synaptic function could also be accomplished by exogenously expressing the peptide in WT cultured neurons, and examining synaptic function and plasticity by recording mEPSCs or staining for surface GluR1 levels. It will be important to investigate the impact of FMRpolyG expression in the context of the premutation, isolate of its role in inclusion formation and proteosomal inhibition.

Do CGG KI animals show additional autism/ADHD phenotypes similar to Fmr1 KO mice?

Behavioral tasks which are designed to test autistic-like behavior are important to the FXS field, as any therapeutic intervention which can improve those behaviors would target potentially helpful treatment for FXS patients. Similarly, examining these more social and complex tasks in CGG KI mice would provide a more complete understanding of the impact of the premutation in network and output behaviors. Social preference and nest-building assays are both altered in Fmr1 KO animals (Gross et al 2015a, Oddi et al 2015, Pietropaolo et al 2011, Udagawa et al 2013). These same tasks should be evaluated in CGG KI mice to see if there are more similar phenotypes shared with the Fmr1 KO mice.

Are the phenotypes observed in CGG KI animals age dependent, or change over time?

We found an age-dependent phenotype in CGG KI PPI sensorimotor gating, indicating some of the cellular and biochemical effects observed do not immediately correlate to network and behavior dysfunction. Potentially, these cellular and molecular events must accumulate to some threshold before animal behavior is affected. It is also possible that the network is able to compensate for individual neurons which are functioning differently, and that either single dysfunctional neurons decline enough to alter behavior; alternatively, a greater population of neurons in the network may become dysfunctional and ultimately change behavior. Comparing cell and slice CGG KI phenotypes at different ages could assess these two possibilities. Cell work is hampered by the young age required for healthy cultures. However, whole cell slice work and animals behavior can be performed from animals at various ages. This would also allow comparison of events which may precede FXTAS-associated symptoms. Pairing histological analysis to look for neurodegenerative features such as gliosis and inclusion formation would provide a better understanding of when and where some of these events may be happening.

Appendices

Appendix A: Arc expression in CGG KI hippocampal neurons

Arc/Arg3.1 is an activity-regulated cytoskeletal protein, which is an immediate early gene involved in synaptic plasticity (Bramham et al 2008, Chowdhury et al 2006, Panja et al 2009). Arc is an FMRP target, and dendritic Arc expression is increased basally in Fmr1 KO neurons (Niere et al 2012a).

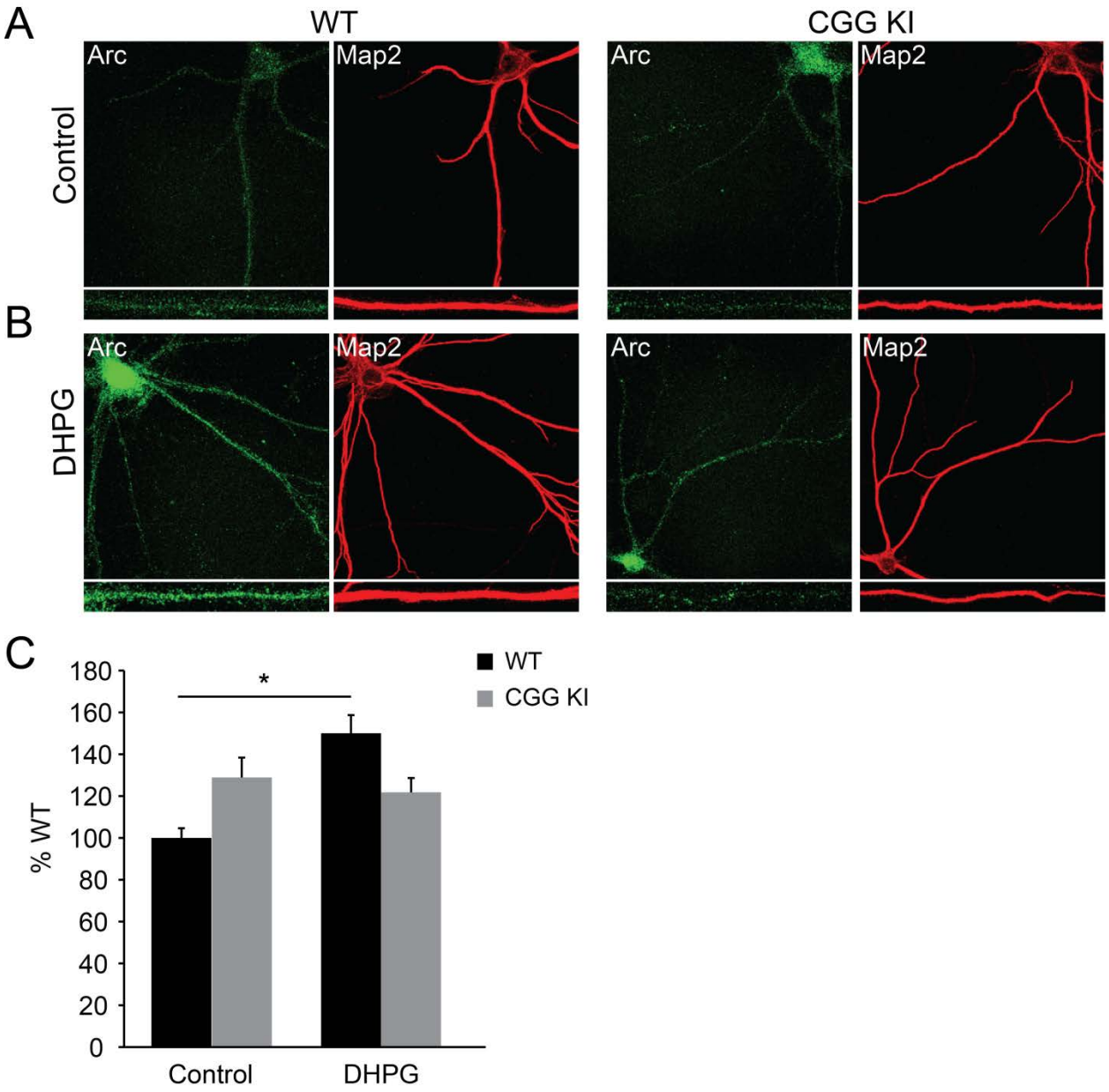

Figure A.1: Arc expression in CGG KI neurons
(A) Primary hippocampal neurons (DIV14-17) from male CGG KI and WT littermate controls. (B) Cultures were treated with 100µM DHPG for 5 min and probed with mouse anti-Arc (Santa Cruz C-7, 1:50 or 1:100) and Map2 (Sigma 1:1000) to label dendrites. (C) The proximal 40µm of dendrites were analyzed and expressed as %WT control. Significant effect of drug ($F_{(1, 524)} = 7.919$, $P<0.05$), and significant interaction ($F_{(1, 524)} = 14.01$, $P<0.05$). Posthoc Bonferroni test showed significant effect of DHPG in WT neurons ($t=4.862$, $P<0.05$), but no change in Arc expression in CGG KI neurons with DHPG treatment ($t=0.6294$, NS). WT control n=138, DHPG n=150; CGG KI control n=117, DHPG n=123.

Appendix B: PSD95 expression in XGFP/CGG KI neurons

PSD95 is an FMRP-regulated synaptic protein (Todd et al 2003a), and its expression is linked to multiple forms of synaptic plasticity (McGee & Bredt 2003, Sheng & Kim 2002).

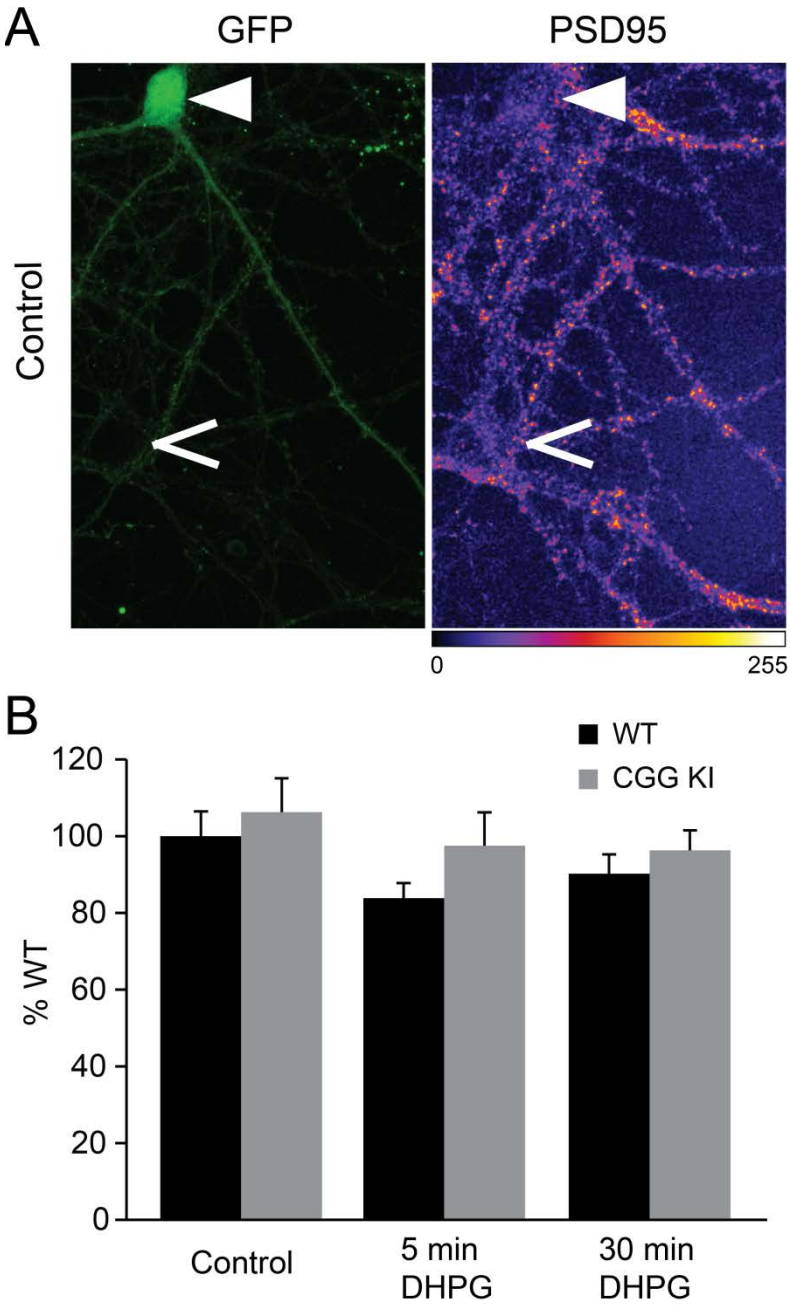

Figure A.2: PSD95 in XGFP/CGG KI neurons
(A) XGFP/CGG KI cultured hippocampal neurons (DIV14-17) stained with mouse anti-PSD95 (Abcam, 1:500). (B) Cultures were treated with 100µM DHPG for 5 or 30 min, and the first 100µm of dendrites analyzed. No significant change by 2-way ANOVA. WT control n=16, 5min DHPG n=17, 30min DHPG n=18; CGG KI control n=11, 5min DHPG n=13, 30min DHPG n=21.

Appendix C: UBE3A expression in CGG KI mice

UBE3A is an E3 ubiquitin ligase which mediates ubiquination of Arc (Greer et al 2010). Ube3a mRNA expression is significantly increased in HEK cells expressing expanded CGG repeats (Handa et al 2005). As basal expression and the induction of Arc synthesis is altered in CGG KI neurons, we wanted to probe UBE3A expression levels.

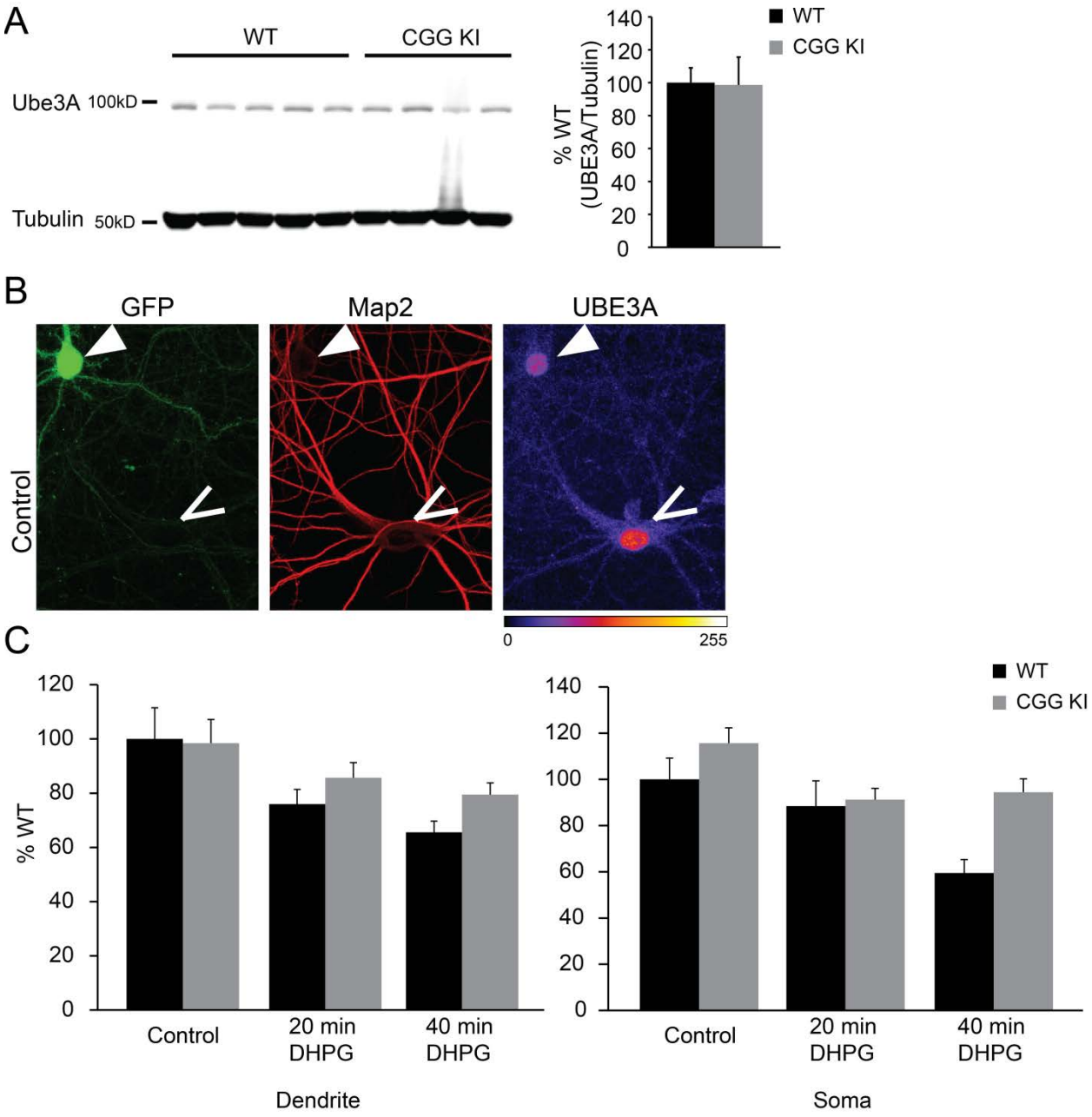

Figure A.3: UBE3A levels in CGG KI mice
(A) WT and littermate CGG KI hippocampal lysates from P58 animals were probed by western blot for UBE3A (Sigma E6AP 1:2000) and Tubulin (Iowa Hybridoma Bank mouse anti-tubulin E7-s, 1:5000). No significant difference in UBE3A expression. WT n=5, CGG KI n=4. (B) XGFP/CGG KI cultured hippocampal neurons (DIV14-17) treated with 100µM DHPG for 20 or 40 minutes and probed for UBE3A (Sigma E6AP, 1:1000) and Map2 (Sigma, 1:1000). (C) Soma and the first 40µm of the dendrite were analyzed. CGG KI neurons showed no significant difference in UBE3A expression. WT control n=6, 20min DHPG n=5, 40min DHPG n=5; CGG KI control n=13, 20min DHPG n=10, 40min DHPG n=14.

Appendix D: Shank 3 expression in CGG KI mice

Shank proteins are scaffolding proteins required for mGluR localization. Mutations in Shank proteins are associated with autism (Guilmatre et al 2014), and Shank3 heterozygous animals phenocopy the protein synthesis-dependent enhanced mGluR-LTD we see in CGG KI mice (Bangash et al 2011). Additionally, all three shank mRNAs are putative targets of FMRP (Darnell et al 2011).

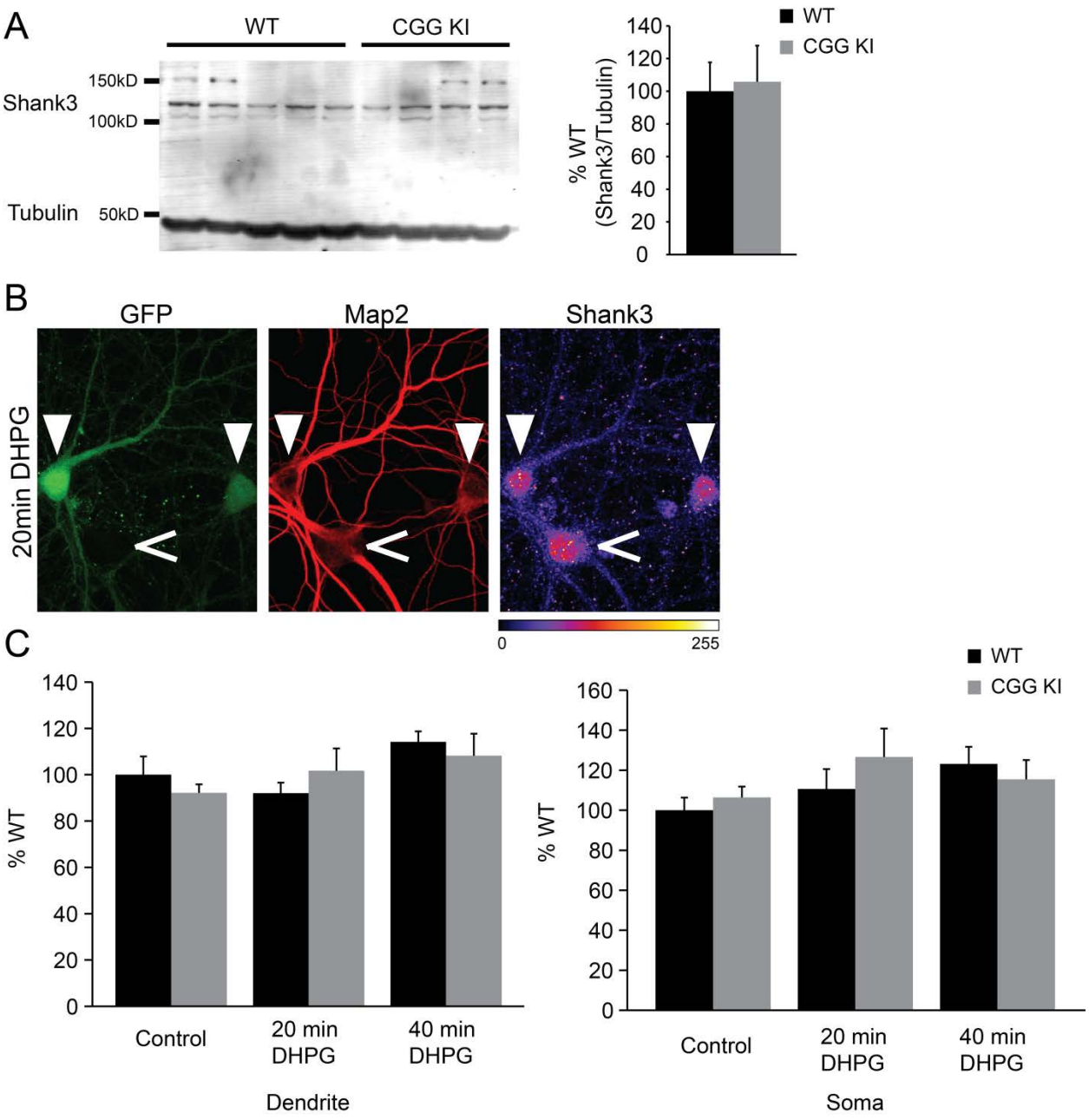

Figure A.4: Shank 3 levels in CGG KI mice
(A) WT and littermate CGG KI hippocampal lysates from P58 animals were probed by western blot for Shank3 (NeuroMab, 1:2000) and Tubulin (Iowa Hybridoma Bank mouse anti-tubulin E7-s, 1:5000). No significant difference in Shank3 expression. WT n=5, CGG KI n=4. (B) XGFP/CGG KI cultured hippocampal neurons (DIV14-17) treated with 100µM DHPG for 20 or 40 minutes and probed for Shank3 (NeuroMab, 1:500) and Map2 (Sigma, 1:1000). (C) Soma and the first 100µm of the dendrite were analyzed and expressed as %WT control values. CGG KI neurons showed no significant difference in Shank3 expression. WT control n=4, 20min DHPG n=5, 40min DHPG n=5; CGG KI control n=9, 20min DHPG n=5, 40min DHPG n=7.

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