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Xiaodi Yao Date

Regulation of A-type potassium channel Kv4.2 expression by FMRP and miR-324-5p

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Doctor of Philosophy

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Abstract

Regulation of A-type potassium channel Kv4.2 expression by FMRP and miR-324-5p

By Xiaodi Yao

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability that is caused by loss of the Fragile X mental retardation protein (FMRP), a RNA binding protein involved in various aspects of mRNA regulation. One prominent characteristic of FXS is high susceptibility to seizure resulting from neuronal hyperexcitability; yet the underlying molecular mechanisms remain elusive. Potassium channels play critical roles in controlling neuronal excitability. Recent findings suggest that FMRP might directly regulate voltage-gated potassium channels. Voltage-gated Kv4.2 potassium channel is the major component mediating the A-type K^+ outward current thereby controlling neuronal excitability in the hippocampus and has been implicated in epilepsy. Thus we investigated the possible involvement of FMRP to regulate Kv4.2 that might explain the high susceptibility to epilepsy in FXS. In this dissertation we uncovered two novel posttranscriptional regulation mechanisms to modulate the expression of Kv4.2. We show that FMRP associates with Kv4.2 mRNA and positively regulates Kv4.2 mRNA translation and protein expression in neurons. Moreover, both total and cell surface protein levels of Kv4.2 are reduced in a mouse model of FXS. Our results suggest that loss of FMRP results in Kv4.2 downregulation which might contribute to excess neuronal excitability in *Fmr1* KO mice and thus imply a potential mechanism underlying FXS-associated epilepsy. In contrast to FMRP, the microRNA (miRNA) miR-324-5p was shown to be an inhibitory regulator for Kv4.2 expression in neurons. Our results show that miR-324-5p targets to the 3'UTR of Kv4.2 mRNA and inhibits Kv4.2 protein expression. Overexpression and inhibition of miR-324-5p lead to reduced and increased levels of endogenous Kv4.2 protein, respectively. In addition, miRNA-324-5p overexpression reduced surface expression of Kv4.2 in neurons. Taken together, our results suggest that miR-324-5p may regulate Kv4.2 expression and functions during neuronal development and plasticity. Collectively, my thesis work provides new insights into the molecular mechanisms in regulating neuronal excitability and eplepitogenesis. Dysregulation of Kv4.2 expression through two mechanisms may be involved in epilepsy. Our findings therefore provide a molecular basis for future studies toward a better understanding the interplay of these mechanisms in the pathogenesis of FXS and other forms of epilepsy.

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Chapter I

General Introduction

Fragile X syndrome and *FMR1* gene

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability with an incident rate of 1 in 4000 males and 1 in 6000 females (O'Donnell and Warren, 2002). Individuals with FXS exhibit mild to severe intellectual disability, which reflects the severity of the disease (Loesch et al., 2004). Besides intellectual disability, affected individuals also display a spectrum of common physical and behavioral phenotypes (Garber et al., 2008). Individuals with FXS often have physical features such as elongated face, prominent ears, high-arched palate, hyperextensible finger joints, double-joint thumbs, single palmar crease, hand calluses, and flat feet (Garber et al., 2008). A predominant phenotype in male patients is macroorchidism which is more significant in postpubertal males (Garber et al., 2008). Behavioral features of FXS patients involves autism, anxiety and learning disability-related executive function deficit, such as poor eye contact, attention deficit, poor topic maintenance and impulsive behavior (Garber et al., 2008). Another prominent feature of FXS patients is high susceptibility to seizures including epilepsy which affects 10 to 20% individuals with FXS (Berry-Kravis, 2002).

Although individuals with FXS display such complex phenotypes, FXS is caused by the loss of function of a single gene, fragile X mental retardation 1(*FMR1*), which encodes the fragile X mental retardation protein (FMRP) (Verkerk et al., 1991). The vast majority cases of fragile x syndrome are caused by the expansion of CGG repeats in the 5' untranslated region (UTR) of *FMR1* gene. The excessive amount of CGG repeats coincides with abnormal methylation pattern of the CpG island in the *FMR1* gene and results in *FMR1* transcriptional silencing, which in turn causes FXS (Coffee et al., 1999;

Pieretti et al., 1991; Verheij et al., 1993). The *FMR1* gene is located on chromosome Xq27.3 and spans about 38Kbp (Krawczun et al., 1985). The variation of the CGG repeats correlates with the disease status of FXS. In unaffected individuals, the 5'UTR of *FMR1* gene contains 4-54 CGG repeats (Fu et al., 1991) (Fig.1-1). An *FMR1* allele with 55 to 200 CGG repeats constitutes a premutation (Fu et al., 1991). Individuals carrying a premutation do not show the typical FXS phenotypes. However, the premutation leads to excessive amount of Fmr1 transcripts (Kenneson et al., 2001; Tassone et al., 2000), which is the cause of the fragile X-associated tremor/ataxia syndrome (FXTAS) and fragile X-associated primary ovarian insufficiency (FXPOI) in premutation carriers (Leehey and Hagerman, 2012; Santoro et al., 2012; Sullivan et al., 2011). CGG repeats in the premutation are very unstable during meiosis and can expand to a full mutation (Sullivan et al., 2005). The risk of expansion to a full mutation is associated with the number of CGG repeats (Sullivan et al., 2005). An *FMR1* allele with CGG repeats greater than 200 represents the full mutation (Fu et al., 1991). A full mutation leads to no FMRP expression and causes FXS. Besides the CGG expansion, other mutations in Fmr1 gene were also found in FXS patients. Deletions of DNA fragments in the promoter or coding region of *FMR1* lead to clinical FXS (Hirst et al., 1995; Meijer et al., 1994; Oberle et al., 1991; Wohrle et al., 1992). These deletions vary from 660bp to less than 250kb. Several point mutations in Fmr1 gene were also reported to link to FXS. In a rare case, a 149C to G conversion in the CpG island induces transcriptional silencing of *FMR1* (Mila et al., 2000). Missense point mutations in the coding region of Fmr1, such as Ile304Asn (De Boulle et al., 1993) and Arg138Gln (Collins et al., 2010), were also found in FXS patients. More recently, autism-associated chromosomal copy number variants were

shown to affect the FMR1 gene (Griswold et al., 2012), further corroborating the importance of FMRP for brain function.

The FMRP Protein

The *FMR1* gene encodes FMRP which is widely but not ubiquitously expressed in tissues with high abundance in the brain and testis (Devys et al., 1993; Hinds et al., 1993). FMRP contains one nuclear localization signal (NLS) in the N-terminus as well as one nuclear export signal (NES) following the KH domains, which allow FMRP to shuttle between the nucleus and the cytoplasm (Eberhart et al., 1996; Feng et al., 1997b) (Fig. 1-2). In the brain, FMRP is primarily expressed in neurons and predominantly localized to the cytoplasm, being found in soma, dendrites and synapses (Devys et al., 1993; Feng et al., 1997b; Weiler et al., 1997). FMRP proteins from different species are evolutionally conserved; for example, human and chicken FMRP exhibits 92% identity(Ashley et al., 1993a). In mammals, FMRP has two other paralogous proteins, Fragile X mental retardation syndrome-related protein 1 and 2 (FXR1 and FXR2); while Drosophila only has one ortholog, dFMRP (Bassell and Warren, 2008). FMRP protein has several splicing isoforms with distinct carboxy termini (Ashley et al., 1993b; Verkerk et al., 1993).The major form of FMRP in mammals contains 632 amino acids and has a molecular weight of 71 kD.

FMRP has several highly conserved functional domains, including three conventional RNA binding domains. Two heterogeneous nuclear ribonucleoprotein (hnRNP) K homology domains (KH1 and KH2) sit in the middle of FMRP protein with 100% amino acid similarity in human, mouse and chicken (Ashley et al., 1993a). Another

RNA binding arginine-glycine-glycine domain (RGG box) locates to the C-terminus of FMRP(Ashley et al., 1993a). The N-terminus of FMRP contains an Agenet domain-like pair, which belongs to the tudor domain family, which also exhibits RNA binding capability (Maurer-Stroh et al., 2003).

FMRP is an RNA binding protein

The presence of multiple RNA binding domains in FMRP reflects its role as a RNA binding protein. As estimated by protein titration studies, FMRP binds to approximate 4% of total mRNAs in mammalian brain (Ashley et al., 1993a). More than 600 FMRP target mRNA candidates have been identified using IP-microarray, yeast three-hybrid and more recently, high-throughput sequencing of RNAs isolated through a cross-linking immunoprecipitation(HITS-CLIP) assay(Brown et al., 2001; Dolzhanskaya et al., 2003; Miyashiro et al., 2003; Zou et al., 2008). These mRNAs encode proteins, which are involved in a wide range of neuronal and synaptic functions, suggesting that FMRP plays important roles in different neuronal processes. Among hundreds of candidate mRNAs, at least 14 mRNAs have been individually tested and were shown to directly interact with FMRP via different FMRP domains (Bassell and Warren, 2008; Santoro et al., 2012).

The precise modalities of how FMRP associates with mRNAs are still controversial. While earlier work showed that FMRP binds to the 3'UTRs of its target mRNAs (Brown et al., 2001), more recent work suggested that binding may also occur via the 5'UTR (Bechara et al., 2009) and the coding regions of the mRNAs (Darnell et al., 2011). The most well studied RNA binding domain in FMRP is the RGG box. An

RNA secondary structure, G quartet, is needed to directly bind to the RGG box in FMRP (Darnell et al., 2001). This G quartet has a consensus sequence of DWGGN(0-2)DWGGN(0-1)DWGGN(0-1)DWGG (D is any nucleotide except C, W is U or A, and N is any nucleotide) (Darnell et al., 2001). The interaction of FMRP RGG box and a G quartet structure was first demonstrated in vitro by an RNA selection assay (Darnell et al., 2001). Since then, several mRNAs, including FMRP, MAP1b, SC1, Sema3F and amyloid precursor protein (APP), have been shown to directly bind to FMRP via this class of RNA secondary structures (Menon et al., 2008; Menon and Mihailescu, 2007; Schaeffer et al., 2001; Westmark and Malter, 2007). However, a G quartet structure is not the only type of RNA elements directly interacting with the FMRP RGG box. For example, although a G quartet consensus sequence is found in the mRNA coding region of postsynaptic density protein-95 (PSD95) mRNA, an important scaffolding protein in the postsynaptic compartment, it is not responsible for the direct interaction with FMRP (Zalfa et al., 2007). Instead, another G-rich sequence in the 3'UTR of PSD-95 mRNA contributes to bind to the C-terminal domain (including the RGG box) of FMRP (Zalfa et al., 2007).

A recent study reported that a secondary structure containing three independent stem-loops connected by short stretches of single-stranded RNA in the 5'UTR of superoxide dismutase 1 (*Sod1*) mRNA can also directly bind to FMRP in order to regulate the translation of *Sod1* (Bechara et al., 2009). This novel RNA structure is called *Sod1* stem loops interacting with FMRP (SoSLIP) and it interacts with FMRP through the C-terminal region including the RGG box and competes for binding with the G quartet structure (Bechara et al., 2009).

The KH2 domain in FMRP has been found to bind to a synthetic RNA called loop-loop pseudo knot or kissing complex secondary RNA structure (Darnell et al., 2005). In an RNA competition assay, the kissing complex structure rather than the G quartet can abolish the association of FMRP with polyribosomes(Darnell et al., 2005) indicating that the KH domain might be important for the interaction between FMRP and ribosomes. However, the kissing complex structure has not been identified in endogenous mRNAs; the reason for this could be that the formation of a kissing complex structure requires the assistance of chaperone molecules. Of note, a FXS causing point mutation I304N is located in KH2 domain (De Boulle et al., 1993). This mutation results in severe FXS phenotypes suggesting its importance for FMRP function. The I304N mutation reduces RNA binding activity of FMRP to poly (U) and poly (G) and disrupts the association of FMRP with polyribosomes (Feng et al., 1997a; Laggerbauer et al., 2001; Siomi et al., 1994).

The N-terminal sequence of FMRP also has RNA binding ability (Maurer-Stroh et al., 2003). This FMRP domain consists of two Agenet tandem repeats which belong to the RNA binding tudor domain family(Maurer-Stroh et al., 2003). It has been reported that BC1 RNA , a non-coding RNA, directly binds to FMRP through a novel RNA binding motif right after this tudor domain in the N-terminus of FMRP and this interaction mediates the association of FMRP with other mRNA targets (Zalfa et al., 2005). However, this proposed mechanism underlying FMRP-RNA interaction remains open. Recent studies from several different labs failed to observe the specific interaction between BC1 RNA and FMRP either *in vivo* 1or *in vitro* and these groups suggested that

BC1 is not required for the association of FMRP and its target mRNAs (Zhong et al., 2010).

Another type of RNA secondary structure, U-rich pentamers, also can interact with FMRP (Chen et al., 2003); however, the corresponding binding domains of FMRP are still not identified. U-rich sequences both in the 3'UTR and coding region were found in some FMRP target mRNAs such as *FMR1* and MAP1B mRNAs (Chen et al., 2003). The U-rich region in the 5'UTR of human achaete-scute homologue-1 (hASH1) mRNA directly interacts with FMRP as demonstrated by a UV-crosslinking assay and mutagenesis analysis (Fahling et al., 2009). Taken together, these studies suggest that FMRP associates with its mRNA targets via a variety of different domains and mechanisms. Future studies may show that these different binding mechanisms determine or contribute to the specific function FMRP has for individual mRNA targets.

Besides interacting with mRNAs, FMRP also has been shown in association with microRNAs. Several microRNAs were detected in the same RNP complex containing FMRP, such as miR-125a and miR-125b, by PCR analysis of immunoprecipitated FMRP-RNP complexes (Edbauer et al., 2010; Muddashetty et al., 2011). However, it remains unknown whether FMRP directly interacts with specific miRNAs or not.

Functions of FMRP in mRNA metabolism and dynamics

Synaptic development and plasticity involves tight control of mRNA posttranscriptional regulation including splicing, transport, stability, localization and translation. RNA binding proteins, such as FMRP, are key regulators in mRNA metabolism and dynamics, and therefore alteration of their function results in a broad

spectrum of human diseases. FXS patients display complex physical and behavioral phenotypes in addition to cognitive disorders, suggesting that FMRP plays critical roles in both neuronal and non-neuronal cells. Thus the elucidation of the cellular and molecular functions of FMRP will provide insight toward the understanding of the pathological mechanisms of FXS and may eventually lead to the discovery of therapeutic treatment strategies. Since FMRP is an RNA binding protein, tremendous efforts have been made to understand the role for FMRP in RNA metabolism and dynamics and underlying molecular mechanisms. Accumulated evidence suggests that FMRP is a multifunctional protein involved in various aspects of mRNA regulation, including translational regulation, mRNA stability control and mRNA transport.

FMRP represses translation

The first piece of evidence in support of a function of FMRP in translational regulation was derived from the observation that FMRP co-sediments with polyribosomes after ultracentrifugation in a linear sucrose gradient in 1996 (Khandjian et al., 1996). Polyribosomes are believed to be the sites of active mRNA translation. Follow-up studies further showed that FMRP associates with translating ribosomes in an RNA-dependent manner in different cell types including both neuronal and non-neuronal cells (Feng et al., 1997a; Khandjian et al., 2004; Stefani et al., 2004). . In addition, as mentioned above, the I304N missense mutation in the KH2 domain of FMRP, which results in a severe type of FXS, disrupts the association of FMRP with elongating polyribosomes (Feng et al., 1997a), indicating that FMRP plays important roles in translational regulation. Interestingly, FMRP bearing I304N missense mutation possesses

RNA binding ability (Feng et al., 1997a). However, recent work form Darnell lab showed I304 mutation has lost its RNA binding ability (Zang et al., 2009).

The role for FMRP as a translational repressor has been well established in various model systems. In rabbit reticulocyte lysate, FMRP inhibits translation of different mRNA substrates, such as MBP mRNA (Li et al., 2001). Removal of the FMRP binding site in the 3'UTR of MBP mRNA, completely abolishes the suppressive effect of FMRP on MBP translation (Laggerbauer et al., 2001; Li et al., 2001). In contrast, the FMRP I304N mutant confers only marginal translational repression on target mRNAs (Laggerbauer et al., 2001). Similarly, FMRP represses mRNA translation in microinjected Xenopus Laevis (Laggerbauer et al., 2001). Taken together, these results support that FMRP is a translation repressor and its interaction with mRNAs and ribosomes is critical for this function.

In recent years, studies on individual FMRP mRNA targets further revealed mechanistic details of FMRP function in translational repression. MAP1b mRNA is one of the most well studied FMRP targets. The Drosophila FMRP ortholog dFMRP was first found in association with microtubule-associated Futsch mRNA, the MAP1B ortholog, and controlled its translation efficiency (Zhang et al., 2001). Futsch mRNA was present in dFMRP-associated protein complexes purified by immunoprecipitation using anti-dFMRP antibodies (Zhang et al., 2001). Furthermore, genetic manipulation of dFMRP expression in Drosophila demonstrated that Futsch protein levels inversely correlate with dFMRP protein levels (Zhang et al., 2001). In the nervous system of *dFmr1* null mutants, Futsch levels are increased (Zhang et al., 2001); whereas in *dFmr1* overexpression lines, Futsch levels are decreased (Zhang et al., 2001). A similar phenomenon was observed in

a mouse model of FXS and in cultured neurons (Lu et al., 2004). Besides MAP1B, FMRP was also shown to repress translation of other targets such as for example Arc/Arg3.1, CaMKIIα (Zalfa et al., 2003) and p110 β, the catalytic subunit of PI3K (Gross et al., 2010; Sharma et al., 2010). In absence of FMRP these proteins are increased.

FMRP was shown to regulate protein levels of its target mRNAs at local cellular compartments, such as dendrites and synapses. Many FMRP associated mRNAs were found localized to dendritic shafts and postsynatic compartments. Examples are mRNAs encoding Arc/Arg3.1 , CamKIIα, PSD-95 and AMPA receptors, GluA1/2 (Muddashetty et al., 2007; Waung et al., 2008). Local protein synthesis is believed to play key roles in various neuronal functions. FMRP was suggested to negatively regulate translation of some of these specific mRNAs locally at synapses. Biochemically purified synaptic fractions, called synaptoneurosomes, are a widely used biochemical model system to study local protein synthesis. Purified synaptoneurosome fractions contain highly enriched synaptic compartments, namely pinched-off, resealed presynapses attached to resealed postsynapes, that maintain normal functions of neurotransmitter release, receptor activation, and various postsynaptic responses including signal transduction and protein synthesis. In synaptoneurosomes prepared from cortices of *Fmr1*knock out (KO) brain, PSD-95 protein is more abundant compared to wide type (WT) preparation (Muddashetty et al., 2007). Furthermore, in Fmr1 KO synaptoneurosomes, PSD-95 mRNAs showed an enhanced association with fractions containing actively translating polysomes indicating exaggerated translation of PSD-95 mRNAs (Muddashetty et al., 2007). Similar to PSD-95, many FMRP target mRNAs also showed elevated protein levels including Arc/Arg3.1, CamKIIα, GluA1/2, p110β and APP in FMRP deficient synaptoneurosomes (Gross et al.,

2010; Muddashetty et al., 2007; Waung et al., 2008; Westmark and Malter, 2007). The increased protein levels locally at synaptoneurosomes from *Fmr1* KO brains are consistent with a role of FMRP as a translation repressor.

Exaggerated translation of FMRP target mRNAs during steady state conditions is believed to underlie the observed dysregulated protein synthesis after synaptic stimulation in *Fmr1* KO mice. Since FMRP represses translation, FMRP is proposed to serve as a brake to control activity-induced translation in neurons (Bear et al., 2004). Loss of FMRP in translational control not only leads to increased protein levels at steady state but also makes this tightly regulated protein expression insensitive to neuronal activation. For example, PSD-95 protein exhibits robust increase after DHPG stimulation in both cultured cortical neurons and synaptoneurosomes from WT mouse brains; however, this increase is abolished in neurons and synaptoneurosomes derived from *Fmr1* KO mice (Muddashetty et al., 2007). Moreover, the distribution of PSD-95 mRNA in polysome fractions is altered in synaptoneurosomes from *Fmr1* KO brains after stimulation compared to WT (Muddashetty et al., 2007). In WT synaptoneurosomes upon DHPG stimulation, PSD-95 mRNA exhibits an apparent shift into active translating polysome fractions as short as 5 minutes after stimulation (Muddashetty et al., 2007). Surprisingly, in *Fmr1* KO synaptoneurosome, this shift is reversed; after DHPG stimulation, less PSD-95 mRNA is incorporated into actively translating polysomes compared to steady state (Muddashetty et al., 2007). These data suggest FMRP controls translation of PSD-95 during neuronal activity and loss of this control leads to disrupted regulation of protein synthesis in response to stimuli. In addition to PSD-95, mGluR activation induces rapid synthesis of proteins encoded by several other FMRP target

mRNAs including Arc3.1/Arg3.1, GluA1/2, CamKIIα, SAPAP3, APP and p110β (Gross et al., 2010; Muddashetty et al., 2007; Narayanan et al., 2008; Waung et al., 2008; Westmark and Malter, 2007). The mGluR-mediated increase in protein levels are eliminated in *Fmr1* KO neurons or synaptoneurosomes. Moreover, the activation of dopamine receptors induces rapid increases in protein levels of PSD-95 and SAPAP3 in the prefrontal cortex (Wang et al., 2010b). In the absence of FMRP, this increase is abolished (Wang et al., 2010b).

In addition to neurotransmitter-induced regulation of protein synthesis, the stimulation of protein synthesis after sensory stimuli was shown to be disrupted in *Fmr1* KO. Kv3.1b mRNA, another mRNA ligand of FMRP, is a voltage-gated potassium channel involved in establishing tonotopic gradients in the brain stem and distribute along the tonotopic gradient (Strumbos et al., 2010). Upon acoustic stimulation, Kv3.1b protein levels are increased and the distribution of Kv3.1 is altered (Strumbos et al., 2010). In *Fmr1* KO, this tonotopic gradient is lost in the medial nucleus of the trapezoid body (MNTB) and the stimulus-induced changes are abolished (Strumbos et al., 2010). Collectively, these data indicate that the loss of FMRP, a negative regulator of translation, results in dysregulated translation in neurons and locally at synapses which fail to respond to neuronal activation.

Possible mechanism of FMRP-mediated translational inhibition

Although the function for FMRP as a translational repressor has been substantially explored in recent years, the exact working mechanism is not fully understood yet and remains controversial. So far several models have been proposed to

explain the mechanisms underlying translational suppression by FMRP. One possible mechanism is that FMRP interferes with the formation of the translational pre-initiation eIF4A-eIF4G-eIF4E (eIF4F) complex. The assembly and recruitment of eIF4A-eIF4G-eIF4E (eIF4F) complex to the m7G cap structure is essential for cap-dependent translation. The presence and subsequent binding of 4E binding proteins (4E-BPs) to eIF4E disrupts the eIF4E-eIF4G interaction to inhibit translation initiation. FMRP directly interacts with the cytoplasmic FMRP-interacting protein (CYFIP1) which was recently demonstrated as a 4E-BP. CYFIP1 directly binds to eIF4E, and overexpression of CYFIP1 inhibits general translation (Napoli et al., 2008; Schenck et al., 2001). Several FMRP target mRNAs, such as MAP1b, CamKIIα, Arc/Arg3.1 and App, have been shown to also associate with CYFIP1 in the brain (Napoli et al., 2008). The association of CYFIP1 with FMRP target mRNAs seems dependent on the presence of FMRP, since in the absence of FMRP, the association of CYFIP1 with FMRP target mRNAs is reduced (Napoli et al., 2008). Moreover, in CYFIP1+/- mouse brain, protein levels of the FMRP target mRNAs MAP1b, APP as well as CaMKIIα are increased. These data suggest that FMRP inhibits translational initiation through CYFIP1 (Napoli et al., 2008). Interestingly, the FMRP-CYFIP1-eIF4E complex is localized to dendritic synapses and is regulated by synaptic stimulation (Napoli et al., 2008), suggesting a potential role for this complex in regulating local protein synthesis. Upon DHPG stimulation, the FMRP-CYFIP1-eIF4E complex is disrupted and a portion of eIF4E is released from this complex (Napoli et al., 2008). Presumably, the disruption of FMRP-CYFIP1-eIF4E complex could result in translation activation. Taken together, these data suggest a model whereby FMRP inhibits the translation of its target mRNAs through its

interaction with CYFIP1 which prevents translation initiation by blocking eIF4E-eIF4G interaction. Synaptic activity leads to the disassembly of FMRP-CYFIP1-eIF4E complex; subsequently, mRNA translation starts.

The identification of FMRP's interaction with microRNA (miRNA) machineries revealed another possible mechanism whereby FMRP suppresses the translation of its target mRNAs. MiRNAs are evolutionally conserved, small (about 20 nucleotides in length), non-coding RNAs that modulate protein expression. One well characterized function for miRNAs is to repress translation of specific target mRNAs. The functional association of FMRP and miRNA pathways was first demonstrated in Drosophila using both biochemical and genetic approaches(Caudy et al., 2002). dFMRP interacts with the RNA-induced silencing complex (RISC) proteins, dAGO1, dAGO2 as well as Dicer (Caudy et al., 2002; Ishizuka et al., 2002; Jin et al., 2004). Similar results were obtained using recombinant human and mouse FMRP. Furthermore, FMRP was shown to associate with specific miRNAs and to influence their functions, which supports the notion that FMRP plays an important role in the microRNA pathway. For example, Drosophila dFMRP associates with bantam miRNA and regulates the fate of germline stem cells through bantam miRNA(Yang et al., 2007). In mammalian cells, several specific miRNAs were found to be associated with FMRP-mRNP complexes (Edbauer et al., 2010; Muddashetty et al., 2011). For example, miR-125b associates with FMRP and overexpression of miR-125b results in reduced GluN2A protein levels and abnormal synaptic morphology (Edbauer et al., 2010). Conversely, loss of FMRP leads to the opposite phenotype and rescues GluN2A protein levels and synaptic abnormality caused by overexpressing miR-

125b (Edbauer et al., 2010). These data suggest a possible functional interplay between miRNAs and FMRP in regulating protein expression.

A recent study of PSD-95 mRNA, an FMRP target mRNA, and miR-125a provided mechanistic insights to the functional cooperation between FMRP and miRNA pathway. Muddashetty et al. showed that miR-125a, a miRNA that is abundant in neurons, regulates the translation of PSD-95 mRNA (Muddashetty et al., 2011), thereby affecting dendritic spine morphology. FMRP is required for the association between PSD-95 mRNA and AGO2 (Muddashetty et al., 2011) , a RISC complex protein. Surprisingly, the inhibitory translational control of FMRP on PSD-95 can be completely removed by using a PSD-95 3'UTR bearing a two-nucleotide mutation, which abolishes the interaction between PSD-95 mRNA and miR-125a (Muddashetty et al., 2011). Collectively, these data suggest that FMRP together with miR-125a and AGO2 form an inhibitory complex that regulates the translation of PSD-95 mRNA. In addition, FMRP may also play a role in miRNA processing. In Drosophila brains, dFMRP was found in association with Dicer, a protein involved in miRNA maturation (Xu et al., 2008). This study also showed that dFMRP specifically interacts with a neuron-specific miRNA, miR-124a, and modulates the proper processing of miR-124a (Xu et al., 2008).

FMRP positively regulates translation

Although FMRP is traditionally viewed as a translation repressor, recent evidence suggests that FMRP also can promote translation. Miyashiro et al. reported that putative FMRP target mRNAs could be both up and down regulated in different brain tissues (Miyashiro et al., 2003). Using microarray analysis, Brown et al. found 251 genes with

the same total RNA levels, but altered translational profiles in fragile X cells. Among these 251 genes, 136 genes show increased association with polyribosomes, while 115 genes exhibited decreased association with polyribosomes in FMRP-deficient cells (Brown et al., 2001). These data suggest that FMRP can be either a translational repressor or activator. Indeed, there are at least two different FMRP target mRNAs, which have been shown to be translationally activated by FMRP. Sod1 protein levels are decreased in *Fmr1* null mice while its mRNA levels are unchanged (Bechara et al., 2009). The SoSLIP motif in the 5'UTR of Sod1 mRNA directly interacts with FMRP, therefore positively modulates the protein expression of Sod1 (Bechara et al., 2009). Mutations in the SoSLIP that block the formation of the three stem-loop structure interferes with the positive regulation of reporter activity by FMRP (Bechara et al., 2009). Interestingly, FMRP interacts with SoSLIP through its C-terminus which contains the RGG box and competes with the binding of G quartet (Bechara et al., 2009). This data suggests that the function of FMRP might depend on its additional binding partners. In different cells or subcellular compartments, the unique environment and/or the presence of additional binding partners provide a possibility that FMRP interacts with different target mRNAs, enabling various functions. Another gene that has been shown to be positively regulated by FMRP is hASH1 (Fahling et al., 2009). The U-rich sequence in the 5'UTR of hASH1 mRNA mediates the direct association with FMRP (Fahling et al., 2009). FMRP increases the translation efficiency depending on the presence of the U-rich structure (Fahling et al., 2009). Collectively, these findings suggest that FMRP can act as both, a translational repressor or activator depending on its target mRNAs.

FMRP regulates mRNA stability

Apart from translational control, FMRP can have additional functions on mRNA metabolism. Work by Muddashetty et al. suggested that FMRP is a translational repressor of PSD-95 mRNA leading to increased and stimulus-insensitive PSD-95 mRNA translation in cortical synaptoneurosomes from *Fmr1* KO mice (Muddashetty et al., 2007). However, another study demonstrated that in the *Fmr1* KO hippocampus, both PSD-95 mRNA and protein levels are decreased (Zalfa et al., 2007), suggesting that FMRP may stabilize PSD-95 mRNA. In the cortex, FMRP is involved in the translational regulation PSD-95 mRNA upon stimulation (Muddashetty et al., 2007). In contrast, in the hippocampus, stimulation results in enhanced PSD-95 mRNA stability, and this effect is abolished in *Fmr1* KO mice (Zalfa et al., 2007). The region-specific differential regulation of PSD-95 mRNA by FMRP argues that FMRP may play different roles for its target mRNAs in different brain regions. Besides PSD-95 mRNA, myelin basic protein mRNA (MBP), another FMRP target mRNA, was also found to have decreased mRNA stability in glia cells in absence of FMRP (Zalfa et al., 2007). However, FMRP can also negatively regulate mRNA stability: FMRP together with the nuclear export factor 2 (NXF2) associates with and destabilizes NXF1 mRNA in neurons (Zhang et al., 2007). FMRP specifically interacts with NXF2 and could be a functional partner of NXF2 (Zhang et al., 2007). NXF2 overexpression results in increased degradation of NXF1 mRNA (Zhang et al., 2007). This effect is abolished when FMRP is absent (Zhang et al., 2007), further suggesting that FMRP is involved in the regulation of NXF1 mRNA stability.

FMRP regulates mRNA transport

In neurons, FMRP is localized to the cell body, dendrites and dendritic spines (Antar et al., 2004; Feng et al., 1997b; Ferrari et al., 2007), as well as growth cones and mature axons (Antar et al., 2006; Centonze et al., 2008). In all of these compartments, FMRP is present in the form of mRNP granules, which can contain mRNAs, other mRNA binding proteins and molecular motors. In the dentate gyrus of the *Fmr1* KO brain, RGS5, a FMRP target mRNA, was found to have reduced abundance in the dendritic region (molecular layer) compared to WT; however, the total mRNA levels of RGS5 were not changed (Miyashiro et al., 2003). These data suggest that FMRP may regulate transport and/or localization of its cargo mRNAs. In fact, FMRP is a component of transporting mRNP granules exhibiting bidirectional movement in neuronal processes. Antar et al reported that depolarization with potassium chloride (KCl) leads to the rapid FMRP localization into dendrites (Antar et al., 2005). Moreover, enhanced green fluorescent protein tagged FMRP (EGFP-FMRP) traffics in dendrites after neuronal activation in live cells (Antar et al., 2004). The localization of FMRP to distal neuronal compartments is microtubule-dependent and mediated by molecular motors through active transport (Antar et al., 2004). Disruption of microtubules reduces the number of transporting FMRP granules upon mGluR activation (Antar et al., 2004). Furthermore, FMRP was found as a direct binding partner of kinesin KIF5 and colocalized with mRNA containing granules in dendrites (Kanai et al., 2004). It is possible that FMRP serves as adaptor proteins for other kinesin motors. Apart from KIF5, FMRP also interacts with KIF3C (Davidovic et al., 2007). The expression of a dominant-negative form of KIF3 impairs the distal transport of FMRP-containing RNA granules (Davidovic et al., 2007).

Taken together, these studies provide evidence that FMRP transports mRNAs in dendrites in a microtubule-dependent manner.

A recent study carried out by Dictenberg et al. revealed more detailed insights of FMRP's role in mRNA transport (Dictenberg et al., 2008). Using live cell imaging, an MS2-GFP reporter fused with the 3' UTR of CaMKIIα mRNA, a known FMRP ligand, showed significantly reduced motility after DHPG stimulation in cultured *Fmr1* KO neurons compared to WT neurons (Dictenberg et al., 2008). Further analysis showed that DHPG stimulation facilitates the association of FMRP and KIF5 (Dictenberg et al., 2008). In *Fmr1* KO neurons, FMRP target mRNAs including CamKIIα, Dag1, Rgs5, SAPAP4 and MAP1b showed reduced association with Kif5 and also reduced dendritic localization in response to DHPG treatment (Dictenberg et al., 2008). Similarly, in Drosophila, mRNA granules containing FMRP target mRNAs exhibit less motility and reduced bidirectional movement in *dFmr1* deficient neurons (Ling et al., 2004).

In addition to the function of FMRP to transport mRNA along dendrites, FMRP was also proposed to act as a nuclear shuttle protein that enters the nucleus to bind its target mRNA and then transport them out of the nucleus. FMRP has both NLS and NES, respectively, and although FMRP was first found to be predominantly cytoplasmic, immunogold studies showed that a small portion of FMRP can be detected in the nucleus (Feng et al., 1997b). In fact, in Xenopus tropicalis embryos and zebrafish embryos, FMRP localization is predominantly nuclear at specific development time points, namely 2 h postfertilization in xenopus and 3 h postfertilization in zebrafish, suggesting a particular FMRP function in the nucleus during development(Blonden et al., 2005; van 't Padje et al., 2005). In mammalian cells, FMRP binds RNA in the nucleus (Kim et al.,

2009). FMRP was shown to interact with the bulk mRNA exporter Tap/NXF1 in an RNA-dependent manner (Kim et al., 2009). Knock down of Tap/NXF1 reduces the nuclear localization of FMRP. Furthermore, FMRP was found to bind to the active transcription units of lampbrush chromosomes in amphibian oocytes, both with overexpressed FMRP and endogenous Xenopus FMRP (Kim et al., 2009). Collectively, these data provide first evidence that FMRP binds mRNA in the nucleus suggesting a possible model in which FMRP enters the nucleus to bind and facilitate the transport of its target mRNAs.

FMRP is involved in mRNA editing

Recent evidence also linked FMRP to RNA editing in nucleus. *Drosophila* adenosine deaminase acting on RNA (dADAR) is an adenosine deaminase which catalyzes the conversion of adenosine to inosine (Bassell, 2011; Bhogal et al., 2011). Inosine is recoginzed as guanosine during translation and the conversion from A to I provides a possibility that translation can incorporate amino acid not directly encoded by the genomic template (Bassell, 2011). dFMRP was found as a dADAR interacting partner in nucleus using biochemical approaches such as co-immunoprecipitation (Bhogal et al., 2011). Genetic studies indicate that FMRP is involved in dADAR-dependent RNA editing. Furthermore, in *dFmr1* mull backgrounds, several dADAR target mRNAs with defined role in synaptic transmission showed altered editing (Bhogal et al., 2011), suggesting that FMRP modulates the RNA editing activity of dADAR.

FMRP functions in protein-protein interactions

Despite the fact that FMRP is an mRNA binding protein, FMRP has functions other than mRNA metabolism and dynamics. At least two proteins that are not implicated in mRNA regulation have been identified to be physically associated with FMRP, and FMRP was shown to directly regulate the distribution or functional properties of these interacting proteins, which consequently affects neuronal functions. For example, FMRP was shown to interact with G protein-coupled receptor kinase 2 (GRK2) and to regulate the distribution GRK2 within neurons (Wang et al., 2008). GRK2 plays a critical role in the dopamine-receptor response, and neurons from *Fmr1* KO prefrontal cortex exhibited reduced GluA1 surface expression and phosphorylation in response to dopamine receptor activation (Wang et al., 2008). Furthermore, Brown et al. found that the C-terminus of Slack protein physically interacts with FMRP (Brown et al., 2010). Slack-B is a sodium-activated potassium channel and controls neuronal firing pattern in many brain regions. Expressing a truncated FMRP (amino acids 1-298) in Xenopus oocytes results in strong channel activity and increased channel openings of Slack (Brown et al., 2010). In line with a function of FMRP to control Slack channel gating, $Ik_{(Na)}$ currents, which are predominantly mediated by Slack, are substantially reduced in the medial nucleus trapezoid body (MNTB) of *Fmr1* KO mice (Brown et al., 2010). In another example, FMRP directly interacts with p21-activated kinase (PAK) which plays a critical role in dendritic spine morphogenesis (Hayashi et al., 2007). Inhibition of PAK ameliorates morphological spine abnormality in *Fmr1* KO neurons (Hayashi et al., 2007). These molecular functions of FMRP are less reported compared to roles of FMRP in RNA dynamics and metabolism. Nevertheless, these studies supplement FMRP's function in multiple layers of regulation.

Roles for FMRP in neuronal and synaptic function

The FMRP protein plays critical roles in regulating the function of a large subset of neuronal mRNAs and proteins. Most identified FMRP target mRNAs were shown to have certain neuronal or synaptic functions. FMRP regulates the translation, stability, transport as well as editing of these target mRNAs to control neuronal development and function. Indeed, neuroanatomical studies showed that dendritic spines in both FXS patients and *Fmr1* KO mice exhibit an immature morphology with long, thin and more abundant dendritic spines than in normal controls (Grossman et al., 2006; Irwin et al., 2001). Both FXS patients and *Fmr1* KO mice display substantial neurological dysregulation including learning defects, behavioral abnormalities, and higher susceptibility to seizures, demonstrating the requirement of FMRP in proper brain functions. Neuronal signaling pathways underlying various brain functions are often selected as potential therapeutic targets to treat neuronal disorders. Thus, the understanding of FMRP function for neuronal activity and how absence of this function generates FXS phenotypes will provide insight for potential clinical treatment of FXS. As outlined below, FMRP plays a major role for the regulation of several different neurotransmitter-dependent cellular signaling pathways.

Metabotropic glutamate receptors (mGluRs) -dependent synaptic plasticity was shown to be dysregulated in FXS. This led to the postulation of the mGluR theory of FXS, which posits that inhibition of mGluR signaling might be a therapeutic target. mGluRs and downstream signaling are implicated in synaptogenesis, dendritic spine morphogenesis and activity dependent synaptic plasticity, such as mGluR dependent long- term depression (LTD). A well established neurological pathway involving FMRP

is mGluR-dependent LTD which requires internalization of a-amino-3-hydroxyl-4-isoxazole propionic acid (AMPA) receptors and synaptic local protein synthesis in response to mGluR activation. *Fmr1* KO cells exhibit overactive AMPA internalization at the basal level and enhanced mGluR-LTD which becomes protein synthesis independent (Huber et al., 2002; Nakamoto et al., 2007). The mGluR theory of FXS proposes that FMRP serves as a translational inhibitor downstream of mGluRs to regulate mGluR signaling (Bear et al., 2004). In the absence of FMRP, the excessive protein synthesis at steady state conditions leads to exaggerated AMPA internalization and LTD. In support of this theory, the FMRP itself was found to be quickly upregulated upon mGluR activation and rapidly degraded subsequently (Hou et al., 2006). Interestingly, blocking the rapid degradation of FMRP abolishes mGluR-dependent LTD (Hou et al., 2006). These data suggest that FMRP acts as a translational brake to stop the robust protein synthesis after mGluR activation. In addition, as discussed in the previous section, a subset of proteins, such as PSD-95, Arc/Arg3.1, GluA1/2, CamKIIα, SAPAP3 and APP, that are critical for mGluR-dependent LTD were found to be dysregulated both under basal conditions and in response to mGluR activation in the absence of FMRP (Muddashetty et al., 2007; Narayanan et al., 2008; Waung et al., 2008; Westmark and Malter, 2007). Finally, the mGluR theory predicts that FXS phenotypes can be corrected by reducing excessive mGluR signaling. In fact, pharmacological and genetic modification of mGluR signaling was shown to correct FXS phenotypes. The application of 2-methyl-6-(phenylethynyl)-pyridine (MPEP), an mGluR5 antagonist, rescues morphological, physiological and behavioral phenotypes in the zebrafish, Drosophila and the mouse model of FXS (de Vrij et al., 2008; McBride et al., 2005; Tucker et al., 2006;

Yan et al., 2005). Of interest, lowering mGluR5 expression in *Fmr1* KO mice by deleting one copy of the mGluR5 encoding gene can successfully rescue disease-related phenotypes including higher susceptibility to seizure and a specific form of memory failure that might reflect the cognitive impairments in human patients (Dolen et al., 2007).

Collectively, these data provide adequate evidence for the mGluR theory of FXS and suggest that the mGluR signaling pathway could be a therapeutic target for FXS. However, the detailed mechanisms of how FMRP regulates mGluR signaling are unknown. Recent work showed that signaling pathways downstream of mGluR and other neurotransmitter receptors are also aberrant in *Fmr1* KO mice. For example, phosphorylation and activity of mammalian target of rapamycin (mTOR), which is required for mGluR-dependent LTD, are increased in the absence of FMRP (Gross et al., 2010; Sharma et al., 2010). Phosphoinositide 3-kinase (PI3K) is a downstream cellular signaling molecule, and a key protein both in mGluR and mTOR signaling. Recent studies showed that the mRNA encoding p110β, the catalytic subunit of PI3K, is a target mRNA of FMRP (Gross et al., 2010; Sharma et al., 2010). In the absence of FMRP, p110β protein levels are upregulated and PI3K activity is increased (Gross et al., 2010). Remarkably, we could show that the PI3K inhibitor LY294002 amends the altered AMPA receptor internalization and abnormal dendritc spine morphology in *Fmr1* KO neurons (Gross et al., 2010; Swanger et al., 2011). This suggests that in the future, in addition to targeting upstream neurotransmitter receptors, intracellular downstream signaling molecules might be promising therapeutic targets, too.

Another signaling pathway that is affected in FXS is the GABAergic system. The GABAergic system is the major inhibitory pathway in the brain and is crucial for the

development and maturation of synapses and neuronal circuitries. The dysregulation of GABAergic system has been implicated in various neuronal development disorders including autism spectrum disorders and epilepsy (Paluszkiewicz et al., 2011). Iontropic GABA-A receptors are the major GABA receptors that mediate the function of synaptically released GABA. FMRP was found to bind to the mRNA encoding the δ subunit of GABA-A receptors and regulate its dendritic expression (D'Hulst et al., 2006). Consequently, in the absence of FMRP, mRNA and protein levels of δ, as well as α, β and γ subunits are reduced in different brain regions such as the cortex and hippocampus (D'Hulst et al., 2006; Gantois et al., 2006). Similarly, the levels of GABA receptor subunits are reduced in a Drosophila FXS model (D'Hulst et al., 2006). Interestingly, mGluR activation induces dendritic localization of the mRNA encoding the δ subunit and this effect is abolished in *Fmr1* KO neurons (Dictenberg et al., 2008). Besides the dysregulation of the GABAergic system at the postsynaptic side of *Fmr1* KO neurons, GABA transmitter levels as well as proteins involved in GABA transport and metabolism are also altered in various brain regions (Paluszkiewicz et al., 2011). Taken together, these data provides the evidence that GABAergic system is weakened in FXS. Remarkably, enhancing GABAergic transmitter in different model systems ameliorates many FXS phenotype. In the Drosophila FXS model, overexpression of three molecules that are implicated in GABAergic inhibitory pathway can correct several *dFmr1* null phenotypes in flies, including mushroom body defects, excess Futsch translation and abnormal courtship behavior (Chang et al., 2008). Furthermore, in an FXS mouse model, different GABA receptor agonists can alleviate FXS behavioral abnormalities including defective cognitive functions and hyperactivity (El Idrissi et al., 2009; Olmos-Serrano et

al., 2011). Together, a general picture provided by these studies supports the hypothesis that the GABAergic inhibitory pathway is also a potential therapeutically target for FXS treatment.

FMRP and epilepsy

In general, the excessive excitatory signaling and impaired inhibitory signaling in FXS suggests that the absence of FMRP may lead to enhanced neuronal signal transmission and hyperexcitability. Indeed several FXS-associated symptoms, such as hyperactivity and hypersensitivity to sensory stimuli could be explained by excessive neuronal activity. Of note, epilepsy affects 10 to 20% of FXS patients with an especially high incident rate in boys (Berry-Kravis, 2002). The onset of seizure is usually after 2 years of age and rarely after 9 (Berry-Kravis, 2002). The most common form of seizures in children with Fragile x are complex partial seizures, although other type of seizures have also have been reported such as simple partial febrile convulsions and generalize tonic-clonic seizures (Berry-Kravis, 2002). Increased seizure susceptibility can also be recapitulated in the Fragile X mouse model. *Fmr1* KO mice exhibit significantly higher susceptibility to audiogenic seizures compared to their WT littermates and neurons from *Fmr1* KO are hyperexcitable (Chen and Toth, 2001; Chuang et al., 2005; Musumeci et al., 2000). Lentiviral-mediated reintroduction of FMRP in *Fmr1* KO mice reduced audiogenic seizure susceptibility (Musumeci et al., 2007). However, it is still unclear why the loss of FMRP leads to higher risk of seizures.

Recent studies have provided first line of evidence that the excitatory system in Fmr1 KO is dysregulated. FMRP may regulate neuronal voltage gated ion channels,

which in turn modulate neuronal excitability. The excessive neuronal excitation could be the cause of seizure susceptibility. FMRP binds to the C-terminus of the sodium-activated potassium channel Slack-B and regulates the channel activity by controlling gating of Slack (Brown et al., 2010). In addition, FMRP interacts with mRNA of another potassium channel Kv3.1b and regulates the distribution and activity-dependent protein synthesis of Kv3.1b (Strumbos et al., 2010). Both Slack and Kv3.1b contribute to neuronal firing pattern in the brain stem, namely the MNTB suggesting an FMRP function in modulating neuronal excitability. However, no clear evidence has linked these potassium channels to epilepsy both in human and animal models.

Kv4.2 potassium channel, a possible link between FMRP and epilepsy

The A-type voltage-gated potassium channel Kv4.2 is the major ion channel regulating neuronal excitability in the hippocampus. Kv4.2 has been linked to epilepsy in different rodent models and human patients, and is believed to be a candidate protein for seizure susceptibility.

Voltage-gated Kv4-family potassium channels are the main components mediating the A-type K^+ outward current, which rapidly hyperpolarizes cells in response to depolarization, thereby diminishing the back-propagation of action potentials into dendrites (Birnbaum et al., 2004). By regulating the A-type K^+ current, Kv4 family members control neuronal excitability, which in turn regulates synaptic plasticity. The activation of Kv4 channels results in the A-type K^+ current that attenuates neuronal excitability; conversely, the inactivation of Kv4 channels leads to increased neuronal excitability.

The mammalian Kv4 family consists of three individual members: Kv4.1, Kv4.2 and Kv4.3 (Birnbaum et al., 2004). Kv4 members are highly homologous within the transmenbrane domains, but have divergent amino and carboxyl termini (Birnbaum et al., 2004).

Kv4 family members are highly expressed in the brain, heart and smooth muscles. At the cellular level, particularly in hippocampal neurons, Kv4.2 is highly expressed in both the soma and dendrites with a gradually increased density from the soma to distal dendrites (Hoffman et al., 1997; Sheng et al., 1992). Remarkably, genetic ablation of Kv4.2 protein leads to nearly-complete elimination of the A-type K^+ current in CA1 hippocampal dendrites suggesting that Kv4.2 is the major channel composing A-type K^+ currents in the hippocampus (Chen et al., 2006).

Interestingly, Kv4.2 protein is also localized to dendritic spines. A microscopic analysis of Kv4.2 tagged with the enhanced green fluorescence protein (EGFP) at the C-terminus shown that Kv4.2-EGFP proteins were enriched in dendritic spines of cultured hippocampal neurons (Kim et al., 2005). This observation is in line with a previous finding that Kv4.2 is particularly concentrated at the synapses of hypothalamic neurons compared to adjacent dendritic shafts (Alonso and Widmer, 1997). The synaptic localization of Kv4.2 was further confirmed by electron microscopy studies showing that endogenous Kv4.2 is localized to dendritic spines of hippocampal neurons (Kim et al., 2007). This highly organized subcellular localization of Kv4.2 implicates its crucial role at the synapse that may underlie neuronal plasticity.

The role of Kv4.2 for synaptic function

In CA1 hippocampal neurons, as a result of the increased Kv4.2 protein density along the dendrites, the strength (or amplitude) of A-type K^+ currents is also elevated accordingly (Hoffman et al., 1997). Consequently, the enhanced A-type K+ current dampens back-propagation of action potentials with distance from the soma. By controlling back-propagation potentials, Kv4.2 regulates synaptic plasticity since back-propagation potentials are critical for the induction of long-term potentiation (LTP), a long-lasting increase in synaptic strength (Kim and Hoffman, 2008). In line with this Kv4.2 function, studies showed that the inhibition of Kv4 channels using a selective Kv channel blocker, heteropodatoxin-3 (HpTX3), reduces the threshold for LTP induction (Ramakers and Storm, 2002). In addition, Kv4.2 KO neurons also show a reduced threshold for LTP induction (Chen et al., 2006). Taken together, these data indicate Kv4.2 regulates synaptic plasticity by modulating back-propagation of action potentials.

The role of Kv4.2 in epilepsy

A hallmark of epilepsy is dysregulated neuronal excitability. Since Kv4.2 plays critical functions in regulating neuronal excitability, it is hypothesized that abnormal function or loss of Kv4.2 may lead to epileptic phenotypes. The first link between Kv4.2 and epilepsy is provided by the studies of Kv4.2 mRNA expression in several animal models of epilepsy. In a convulsant drug pentylenetetrazole (Metrazole)-induced seizure model, levels of Kv4.2 mRNAs are downregulated in the dentate gyrus of hippocampus as early as 3 hours post the onset of seizure (Tsaur et al., 1992). Interestingly, this downregulation is reversible as after 12 hours Kv4.2 mRNA returns to normal level (Tsaur et al., 1992). Similarly in a kainic acid-induced seizure model, both Kv4.2 mRNA and protein levels are rapidly decreased after seizure (Francis et al., 1997). Additionally

in a model of cortical malformation that results in epilepsy, both Kv4.2 mRNAs and protein expression are dramatically reduced in hyperexcitable heterotopic neurons (Castro et al., 2001). Moreover, hippocampal neurons from a rat model of human temporal lobe epilepsy exhibit attenuated A-type currents and active and persistent backpropagation action potentials (Bernard et al., 2004). The Kv4.2 channels are markedly decreased in this model due to the loss of Kv4.2 mRNAs and increased phosphorylation which results in rapid Kv4.2 protein degradation (Bernard et al., 2004). Not only changes in Kv4.2 levels but also in Kv4.2 localization are found in epilepsy models. In the rat model of human temporal lobe epilepsy, Kv4.2 channels in the molecular layer of the dentate gyrus are concentrated in just the outer two-thirds, instead of uniformly distributed across the molecular layer (Monaghan et al., 2008). Yet, how this altered Kv4.2 distribution contributes to epilepsy remains unclear. Collectively, these findings implicate the enhanced dendritic excitability caused by decreased Kv4.2 channel levels may underlie the genesis and/or the pathology of epilepsy.

The function of Kv4.2 in epileptogenesis is further supported by studies in human patients and a Kv4.2 KO mouse model. The reduced of A-type current in patients was first found in neurons from lesion-associated temporal lobe epilepsy (Beck et al., 1997). However, potential direct causes of Kv4.2 malfunctions leading to epilepsy have not been identified until recently. Singh et al. discovered a mutation of Kv4.2 gene that results in a truncated form of Kv4.2 lacking 44 amino acids in the carboxy terminal in a temporal lobe epilepsy patient (Singh et al., 2006). Cells transfected with this truncated form of kv4.2 display dampened A-type K^+ currents (Singh et al., 2006), suggesting the functional significance of the mutation and implying that malfunctions of Kv4.2 may be a

cause for epilepsy. A sutdy conducted in Kv4.2 KO mice has been able to recapitulate certain phenomena observed in human patients. For example, Kv4.2 KO mice also show increased susceptibility to convulsant stimulation (Barnwell et al., 2009), suggesting Kv4.2 deficiency may contribute to aberrant hyperexcitability and epileptogenesis.

Similar to the functional loss of the Kv4.2 channel, silencing or mutations of FMRP that lead to the loss of FMRP also result in neuronal hyperexcitability and /or epilepsy. Thus, we hypothesize that Kv4.2 potassium channels and FMRP may function in the same pathway regulating neuronal excitability and epilepsy. Interestingly, in addition to Kv4.2 protein, Kv4.2 mRNA is also localized to distal dendrites of cultured hippocampal neurons and its dendritic localization is determined by specific RNA elements within the 3'UTR of Kv4.2 mRNA (Jo et al., 2010). The dendritic localization of Kv4.2 proteins and mRNAs suggests a possible mechanism, in which the localization of Kv4.2 mRNA and its subsequent local translation may influence the function of Kv4.2 at synapses and in dendrites. The distribution and localization of Kv4.2 mRNAs and proteins is highly organized which opens the possibility that regulation of Kv4.2 mRNAs by RNA binding proteins may contribute to the regulation of Kv4.2 functions. Given the essential role for FMRP in regulating dendritic mRNA regulation, we hypothesized that Kv4.2 mRNA may be an FMRP ligand and FMRP may regulate the expression and functions of Kv4.2 to control neuronal excitability.

Thesis objectives

The overall objective of my thesis is to elucidate novel posttranscriptional mechanisms that control Kv4.2 expression, which would have important implications for

understanding the high susceptibility to epilepsy in FXS. The aberrant function of potassium channel Kv3.1 and Slack in the mouse model of FXS suggests that FMRP, an RNA binding protein, may control neuronal excitability through regulating potassium channels. The voltage-gated potassium channel Kv4.2 whose mRNA is localized to dendrites is the main component controlling neuronal excitability in the hippocampus. Thus, we hypothesized that Kv4.2 mRNA may be an mRNA target of FMRP and the posttranscriptional regulation of Kv4.2 protein expression by FMRP and /or other factors plays critical roles in neuronal excitability and epileptogenesis. In this dissertation, my research has uncovered two posttranscriptional mechanisms that are involved in the regulation of Kv4.2 potassium channel expression. First, I investigated the role of FMRP in regulating Kv4.2 protein expression and translation. Second, I identified novel miRNAs targeting Kv4.2 mRNA and investigate their functions in regulating the expression of Kv4.2. These findings provide insights into novel molecular mechanisms that underlie the regulation of neuronal excitability through Kv4.2 potassium channel. Results presented in this dissertation bring forth a molecular basis for future studies toward a better understanding of the pathogenesis of epilepsy, particularly in the context of FXS.

Figures and legends

Figure 1 -1

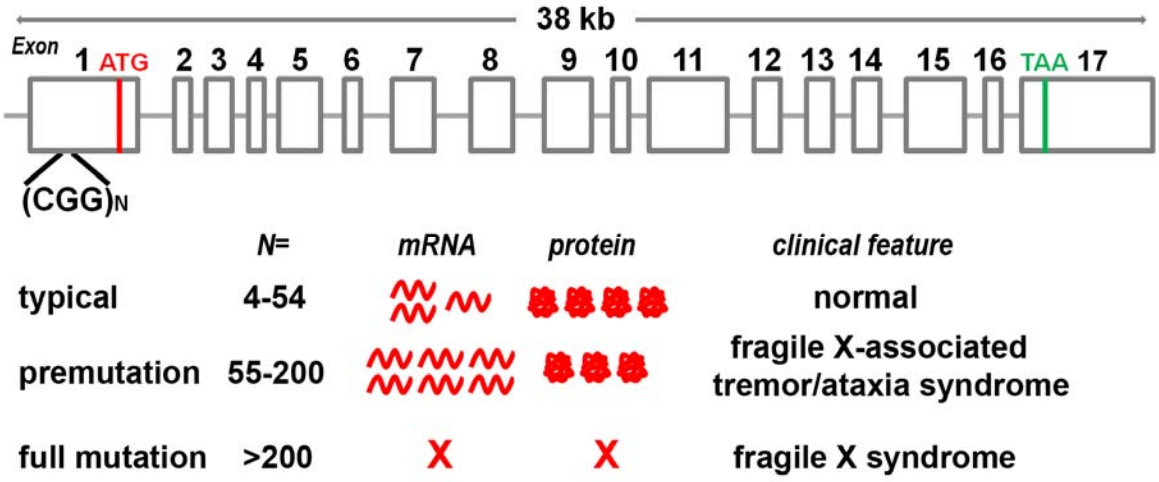

Figure.1-1. A schematic view of the *FMR1* gene. The *FMR1* gene spans 38 kb on the X chromosome and contains 17 Exons. The CGG repeats are located in the first exon of *FMR1* gene which encodes the 5′ UTR of Fmr1 mRNA. FMR1 CGG repeats are classified to three groups, typical (or normal), permutations and full mutation according to the number of repeats. The disease pathology in premutation is different from full mutation. Excess mRNA transcripts in premutation carriers lead to less FMRP protein and cause fragile X-associated tremor/ataxia syndrome. Only the absence of FMRP in individuals with full mutations results in Fragile X syndrome.

Figure 1-2

Figure. 1-2. A schematic of FMRP protein. FMRP protein contains three well-characterized RNA binding domains: KH1, KH2 and RGG box. FMRP protein possesses one nuclear localization signal (NLS) and one nuclear export signal (NES), which together enable FMRP shuttling between the nucleus and the cytoplasm.

Chapter II

Fragile X Mental Retardation Protein Regulates Protein Expression and mRNA Translation of the Potassium Channel Kv4.2

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*C.G. and X.Y. contributed equally to this work.

Abstract

A prominent characteristic of the inherited intellectual impairment disease fragile X syndrome (FXS) is neuronal hyperexcitability, resulting in a variety of symptoms, such as hyperactivity, increased sensitivity to sensory stimuli, and a high incidence of epileptic seizures. These symptoms account for a significant part of the disease pattern, but the underlying molecular mechanisms of neuronal hyperexcitability in FXS remain poorly understood. FXS is caused by loss of expression of fragile X mental retardation protein (FMRP), which regulates synaptic protein synthesis and is a key player to limit signaling pathways downstream of metabotropic glutamate receptors 1/5 (mGlu1/5). Recent findings suggest that FMRP might also directly regulate voltage-gated potassium channels. Here, we show that total and plasma membrane protein levels of Kv4.2, the major potassium channel regulating hippocampal neuronal excitability, are reduced in the brain of an FXS mouse model. Antagonizing mGlu5 activity with 2-methyl-6-(phenylethynyl)-pyridine (MPEP) partially rescues reduced surface Kv4.2 levels in *Fmr1* knock-out (KO) mice, suggesting that excess mGlu1/5 signal activity contributes to Kv4.2 dysregulation. As an additional mechanism, we show that FMRP is a positive regulator of Kv4.2 mRNA translation and protein expression and associates with Kv4.2 mRNA *in vivo* and *in vitro*. Our results suggest that absence of FMRP-mediated positive control of Kv4.2 mRNA translation, protein expression, and plasma membrane levels might contribute to excess neuronal excitability in *Fmr1* KO mice, and thus imply a potential mechanism underlying FXS-associated epilepsy.

Introduction

Patients suffering from fragile X syndrome (FXS), the most common inherited form of intellectual disability, show multiple signs of altered neuronal excitability. These include hyperactivity, attention deficit disorders, hypersensitivity to sensory stimuli, anxiety and in 20% of all cases development of epileptic seizures (Hagerman et al., 2009). Many of these symptoms are recapitulated in *Fmr1* knock-out (KO) mice, and electrophysiological experiments have demonstrated that *Fmr1* KO neurons are hyperexcitable (Chuang et al., 2005). However, the precise mechanisms leading to increased excitability of neurons in the absence of the fragile X mental retardation protein (FMRP) are unknown.

A possible mechanism underlying the heightened neuronal excitability in FXS might be direct regulation of neuronal ion channels by FMRP. Recently, evidence has been growing that FMRP might be involved in the function of potassium channels: FMRP was shown to regulate two different potassium channels, Kv3.1b and Slack, leading to altered potassium signaling of those channels in *Fmr1* KO mice (Brown et al., 2010; Strumbos et al., 2010). However, neither of these potassium channels has been linked to epilepsy in humans or animal models, leaving the underlying mechanism of hyperexcitability in FXS elusive.

The major ion channel regulating neuronal excitability in the hippocampus is the A-type potassium channel Kv4.2, which decreases back-propagating action potentials in dendrites (Birnbaum et al., 2004; Chen et al., 2006). In several different animal models for epilepsy, seizures were shown to regulate hippocampal Kv4.2 protein phosphorylation, localization and levels (Francis et al., 1997; Lugo et al., 2008). Of note, a truncated

mutation of Kv4.2 leads to temporal lobe epilepsy in humans (Singh et al., 2006), and Kv4.2 KO mice have a higher sensitivity to convulsant stimuli (Barnwell et al., 2009). Thus, a functional lack of either Kv4.2 channels or FMRP results in hyperexcitability and/or epilepsy. Based on these findings, we hypothesized that Kv4.2, a key player to dampen neuronal firing, might be dysregulated in FXS.

Here, we show that total, dendritic and cell surface expression of Kv4.2 are reduced in *Fmr1* KO brains. The metabotropic glutamate receptor 5 (mGlu5) antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) partially rescues reduced surface levels in *Fmr1* KO hippocampal slices, suggesting that excess mGlu receptor-signaling can modulate Kv4.2 dysregulation in FXS, which might be directly caused by loss of FMRP-mediated control. We show that FMRP associates with Kv4.2 mRNA, and that absence of FMRP decreases Kv4.2 mRNA translation and protein expression *in vivo* and *in vitro*, whereas FMRP overexpression increases protein levels of Kv4.2 3′-untranslated region (UTR) reporters. Our results suggest that reduced Kv4.2 protein expression in *Fmr1* KO is not just a secondary effect of dysregulated neuronal transmission in FXS, but a direct effect of the absence of FMRP-mediated positive control of Kv4.2 mRNA translation. Loss of FMRP-mediated Kv4.2 mRNA regulation might thus contribute to the neuronal hyperexcitability and epileptic seizure phenotype observed in FXS.

Materials and Methods

Animals and quantitative real-time primers

Male *Fmr1* knock-out mice in C57BL/6J background and male wild-type (WT) littermates were used at 3 weeks (immunostainings, immunoprecipitation and biotinylation assays) and 8 weeks of age (Western blot analysis of cortical lysates). Small interfering RNAs (siRNAs) to knockdown FMRP in cells, and quantitative real-time (qRT)-PCR primers for GFP (GenBank accession number U55763), GluN1 [National Center for Biotechnology Information (NCBI) accession number NM_008169], and PSD95 (postsynaptic density-95 protein) (NCBI accession number NM_007864) have been described previously (Gross et al., 2010). qRT-PCR primers for Kv4.2 were as follows: Kv4.2 forward, gttctatggttgggctgtg; Kv4.2 reverse, gtggctctaactgtatctatg (NCBI accession number NM_019697).

Fluorescent immunostainings on cells and brain sections

Brains from transcardially perfused (4% paraformaldehyde) mice were postfixed overnight, cryoprotected in 30% sucrose, and flash-frozen. Fluorescent immunostainings were performed on WT and *Fmr1* KO brain sections (postnatal day 21) applying antigen retrieval methods, as described previously (Christie et al., 2009; Gabel et al., 2004), with the following modifications: the 10- to 12-μm-thick sections were mounted on microscope slides for processing, and all buffers were based on TBS (100 mm Tris-HCl, pH 7.4, 150 mm NaCl). Permeabilization was performed with 0.5% Triton X-100 for 20 min, and primary antibodies [Kv4.2, 1:200; Kv1.2, 1:500; Kv3.4, 1:1000 (UC Davis/NIH NeuroMab Facility); Kv4.2, 1:250 (Santa Cruz Biotechnology); GluN1, 1:200 (Millipore)] were incubated overnight at room temperature. Brain sections were imaged as *z*-stacks

using a Zeiss LSM 510 Meta confocal microscope, deconvolved (Autoquant X, Media Cybernetics) and quantified using Imaris Software (Bitplane). Immunofluorescence on cells was performed as described previously (Antar et al., 2004). Cultured hippocampal neurons were fixed at 17 days *in vitro* (DIV), and primary antibody (Kv4.2, 1:2000, UC Davis/NIH NeuroMab Facility) was incubated at room temperature for 1 h. Cells were imaged and analyzed as described previously (Antar et al., 2004).

Western blot analysis

Western blot analysis and quantification was performed as described previously (Gross et al., 2010). Antibodies for Kv4.2 were obtained from Millipore, 1:2000; for GluN1 from BD Pharmingen, 1:1000; for β-tubulin from Sigma, 1:500,000; and for green fluorescent protein (GFP) from Clontech, 1:1000.

Biotinylation assays

Surface biotinylation of hippocampal slices was performed as described previously (Nosyreva and Huber, 2005), with the following modifications: Hippocampi were dissected into ice-cold artificial CSF (ACSF) (Chuang et al., 2005), and immediately cut into 350-μm-thick slices. Slices were equilibrated in prewarmed ACSF for 20 min (37°C, 5%CO_2), treated with 50 μm MPEP or vehicle for 40 min, followed by biotinylation for 15 min with 2 mm EZ-Link sulfo-NHS-Biotin (Thermo Scientific) on ice, quenched for 10 min with 100 mm glycine, and washed once with ice-cold TBS before lysis. Cell surface biotinylation assay was performed as described previously (Ehlers, 2000) with the following modifications: high-density cultured cortical neurons were used at 17–21 DIV, and cells were incubated with 1.0 mg/ml EZ-Link sulfo-NHS-Biotin for 30 min.

Quantitative coimmuoprecipitation *in vivo* and *in vitro*

FMRP-specific coimmunoprecipitations and quantitative real-time PCR were performed as described previously (Muddashetty et al., 2007).

Polysomal gradients on synaptoneurosomes

Preparation and analysis of polysomal gradients from cortical synaptoneurosomes, and cell lysates were performed as described previously (Gross et al., 2010; Muddashetty et al., 2007)uddashetty et al., 2007; Gross et al., 2010).

Statistics

All statistical analyses were performed in SPSS 17.0 and PASW Statistics 18. Data were tested for normality and homogeneity of variances, and appropriate tests were used as indicated for each figure.

Results

Decreased dendritic and membrane surface levels of the potassium channel Kv4.2 in *Fmr1* KO mice

The molecular mechanisms underlying hyperexcitability of *Fmr1* KO neurons or increased susceptibility to epileptic seizures in FXS patients have not been characterized. Here, we applied a candidate-based approach to investigate whether Kv4.2, the major ion channel regulating neuronal excitability in hippocampal dendrites, is dysregulated in the absence of FMRP and thus might cause hyperexcitability in FXS. Quantification of fluorescent immunostainings in dendritic areas of the dentate gyrus and CA1 in hippocampal sections (Fig. 2-1*A*) and in dendrites of cultured hippocampal neurons (Fig. 2-1*D*) demonstrated that dendritic Kv4.2 protein levels are significantly reduced in *Fmr1* KO mice. In contrast, other Kv channels, such as Kv1.2 (Fig. 2-1*B*) and Kv3.4 (data not shown), as well as the NMDA receptor subunit GluN1 (Fig. 2-1*C*) were unchanged in *Fmr1* KO hippocampus. A similar reduction of Kv4.2 protein could also be detected in total cortical homogenates by Western blot analysis (Fig. 2-2*A*). Analysis of surface proteins in cultured cortical neurons (Fig. 2-2*B*), and in hippocampal slices (Fig. 2-2*C*) demonstrated that not only total protein levels, but also surface levels of Kv4.2 were reduced in the absence of FMRP. This implies that loss of total Kv4.2 protein was not functionally compensated by altered ratios of surface/internalized channels. Furthermore, reduced surface ion channel levels were not a general phenotype of FXS neurons, as GluN1 membrane levels were not significantly different in either hippocampal slices, or cortical neurons. Pretreatment of hippocampal slices with the mGlu5 antagonist MPEP (50 μm, 40 min) partially increased, but not fully restored Kv4.2 plasma membrane levels

in *Fmr1* KO (Fig. 2-2*C*; 60% of WT before, 80% of WT after MPEP treatment). Of note, our results represent the first evidence for mGlu1/5-mediated regulation of potassium channels at the cell surface of hippocampal neurons, and suggest that excess mGlu1/5 signaling may contribute to reduced Kv4.2 expression in *Fmr1* KO mice. However, we could not detect full rescue of Kv4.2 surface levels to wild-type levels with the same MPEP dose that previously had been shown to rescue several FXS-associated phenotypes (Chuang et al., 2005; Gross et al., 2010; Nakamoto et al., 2007). While we cannot exclude that higher MPEP doses would have led to a complete rescue, we sought to test the hypothesis that additional mechanisms besides dysregulated mGlu5 signaling might account for reduced Kv4.2 total and surface expression in *Fmr1* KO hippocampus.

Reduced association of Kv4.2 mRNA with translating polysomes in *Fmr1* KO brain

FMRP has been shown to regulate mRNA translation of specific targets, as well as general protein synthesis (Bassell and Warren, 2008). To reveal a potential mechanism underlying reduced Kv4.2 protein levels, we therefore analyzed the levels and polysomal association of Kv4.2 mRNA in the absence of FMRP using qRT-PCR and sucrose gradient sedimentation profiling. Whereas total mRNA levels were unchanged in *Fmr1* KO brain (Fig. 2-3*A*), the relative amount of Kv4.2 mRNA associated with puromycin-sensitive (i.e., actively translating) polysomes was reduced in cortical synaptic fractions (Fig. 2-3*B*,*C*), as well as in hippocampal lysates (data not shown). These results suggest that decreased levels of Kv4.2 protein are due to reduced translation of Kv4.2 mRNA in the FMRP-deficient brain. This is an unusual observation, as FMRP was shown to be a negative regulator of global translation in the brain and in synaptic fractions (Dolen et al., 2007; Gross et al., 2010). Furthermore, the great majority of FMRP target mRNAs was

shown to be translated excessively in the absence of FMRP (Gross et al., 2010; Muddashetty et al., 2007; Zalfa et al., 2003). To analyze whether Kv4.2 mRNA might be one of the "unconventional" target mRNAs of FMRP that show reduced translation and protein levels in the FXS mouse model (Bechara et al., 2009; Fahling et al., 2009), we next examined whether Kv4.2 mRNA associates with FMRP in brain cortex. Using Kv4.2-specific quantitative real-time PCR analyses of FMRP-coimmunoprecipitates, we demonstrated that FMRP associates with Kv4.2 mRNA in mouse cortex (Fig. 2-3*D*,*E*). A previous study showed protein-protein interactions between FMRP and the potassium channel Slack (Brown et al., 2010), however we could not detect any association of FMRP with Kv4.2 protein (data not shown).

The 5′- and 3′-UTRs of Kv4.2 mRNA mediate association with and translational regulation by FMRP

Using recombinant GFP-constructs, we demonstrated that both the Kv4.2 5′- and 3′-UTRs were sufficient to mediate association with FMRP (Fig. 2-4*A*), suggesting a cooperative regulation of Kv4.2 mRNA by FMRP via several parts of the Kv4.2 mRNA sequence. In line with this assumption, analysis of Kv4.2 3′-UTR association with several FMRP deletion constructs suggests that the association of FMRP with Kv4.2 mRNA does not seem to be mediated by a single mRNA binding domain, but rather requires multiple domains of FMRP (Fig. 2-4*B*). The protein expression of the FMRP deletion constructs was similar for all constructs, as tested by Western blot (data not shown). Analysis of polysomal association and protein expression of GFP-constructs containing either the Kv4.2 5′-UTR or 3′-UTR further supported the hypothesis that FMRP-regulation of Kv4.2 mRNA occurs via both 5′- and 3′-UTRs. For GFP mRNA fused to either Kv4.2

UTRs, siRNA-mediated FMRP knockdown in cultured cell lines led to decreased polysomal association of mRNA (Fig. 2-4*C–E*), and reduced protein levels (Fig. 2-4*F*). Of note, reduced polysomal association was observed after siRNA-mediated knockdown of FMRP in HEK293T cells, which do not express mGlu1/5 (Gross et al., 2010). This strongly corroborates our hypothesis that there is an additional FMRP-dependent mechanism, apart from aberrant mGlu1/5 signaling, which leads to reduced Kv4.2 expression in *Fmr1* KO mice. Overexpression of FMRP significantly increased the protein expression of recombinant constructs containing the Kv4.2 3′-UTR, but not for a construct just containing the 5′-UTR (Fig. 2-4*G*). Our results imply that FMRP is a positive regulator of Kv4.2 mRNA translation and protein expression, which is mediated by UTR sequences. Absence of FMRP leads to reduced Kv4.2 mRNA translation, protein expression and surface levels, which might partially underlie neuronal hyperexcitability in FXS.

Discussion

Apart from moderate to severe intellectual disability, fragile X patients can have several other symptoms of neuronal dysfunctions, such as hyperactivity, anxiety, self-injurious behavior and epilepsy. While strong evidence supports the hypothesis that loss of FMRP-mediated regulation of neuronal mRNA translation leads to impaired synaptic plasticity and intellectual disability, the mechanisms underlying other symptoms observed in FXS are poorly understood. Our study provides initial insight into a potential molecular mechanism leading to excess neuronal excitability in FXS, which might underlie hyperactivity, sensory hypersensitivity and seizures. Here, we demonstrate that FMRP regulates translation and protein expression of the A-type potassium channel Kv4.2. Functional deletion of Kv4.2 has been shown to cause temporal lobe epilepsy in humans (Singh et al., 2006), and to increase seizure susceptibility in a mouse model (Barnwell et al., 2009). Based on these previous findings and our own results we hypothesize that Kv4.2 dysregulation in *Fmr1* KO mice might represent a mechanistic link between FXS and epilepsy. Furthermore, our observation that MPEP increases Kv4.2 membrane levels in *Fmr1* KO provides evidence for an mGlu1/5- and FMRP-dependent regulation of surface expression of a potassium channel in dendrites, similar to what was reported for the AMPA receptor GluA1 (Nakamoto et al., 2007). In the future, it will be interesting to investigate whether this partial increase of Kv4.2 cell surface levels after MPEP-treatment represents a possible molecular mechanism underlying the MPEP-mediated rescue of audiogenic seizures in *Fmr1* KO mice (Yan et al., 2005).

Apart from neuronal hyperexcitability and FXS-associated epilepsy, dysregulated Kv4.2 expression might contribute to other symptoms of FXS, e.g., self-injurious

behavior. Studies in an FXS mouse model have shown that nociception is defective in the absence of FMRP (Price et al., 2007), and Kv4.2 has been demonstrated to play an important role for mGlu1/5-mediated nociception in the spinal cord (Hu et al., 2007), yet a link between FMRP and Kv4.2 has not been shown previously. Future studies will have to analyze whether dysregulated Kv4.2 expression in the spinal cord might contribute to occurrence of self-injuries in FXS patients.

Our results demonstrate that Kv4.2 mRNA is an unusual FMRP target mRNA as absence of FMRP led to reduced translation and protein expression of Kv4.2. The vast majority of reports finds FMRP to be a translational inhibitor (Dolen et al., 2007; Gross et al., 2010; Muddashetty et al., 2007; Strumbos et al., 2010; Zalfa et al., 2003), but at least two other mRNAs, superoxide dismutase 1(SOD1) and human achaete-scute homolog-1 (hAsh), have been suggested to be positively regulated by FMRP (Bechara et al., 2009; Fahling et al., 2009). Our results suggest that Kv4.2 mRNA regulation by FMRP is mediated via a cooperative effect of large parts of the Kv4.2 mRNA that include the 3′-UTR, and association with several FMRP RNA binding domains, suggesting a different mechanism than proposed for SOD1, which was shown to bind to FMRP via a 64 nt stem-loop structure. However, our data neither supports nor rules out a direct interaction of FMRP with Kv4.2 mRNA, and future studies will have to further analyze the modalities of FMRP association with and translational regulation of Kv4.2 mRNA.

Together, the results of our study add to the variety of functions FMRP can have on specific potassium channels in different brain regions: FMRP alters gating properties of Slack via direct protein interaction (Brown et al., 2010) and negatively regulates protein expression of Kv3.1b via association with its mRNA (Strumbos et al., 2010) in

the brainstem, affecting the function of these potassium channels in the auditory system. In contrast, here we show that FMRP associates with and positively regulates the translation of Kv4.2 mRNA in the hippocampus and cortex. Our study contributes to the emerging picture of FMRP as a diverse regulator of neuronal potassium conductance, and encourages further studies to investigate potassium channel modulators as potential treatment for epilepsy and other symptoms in fragile X syndrome.

Acknowledges

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Figures and Legends

Figure 2-1

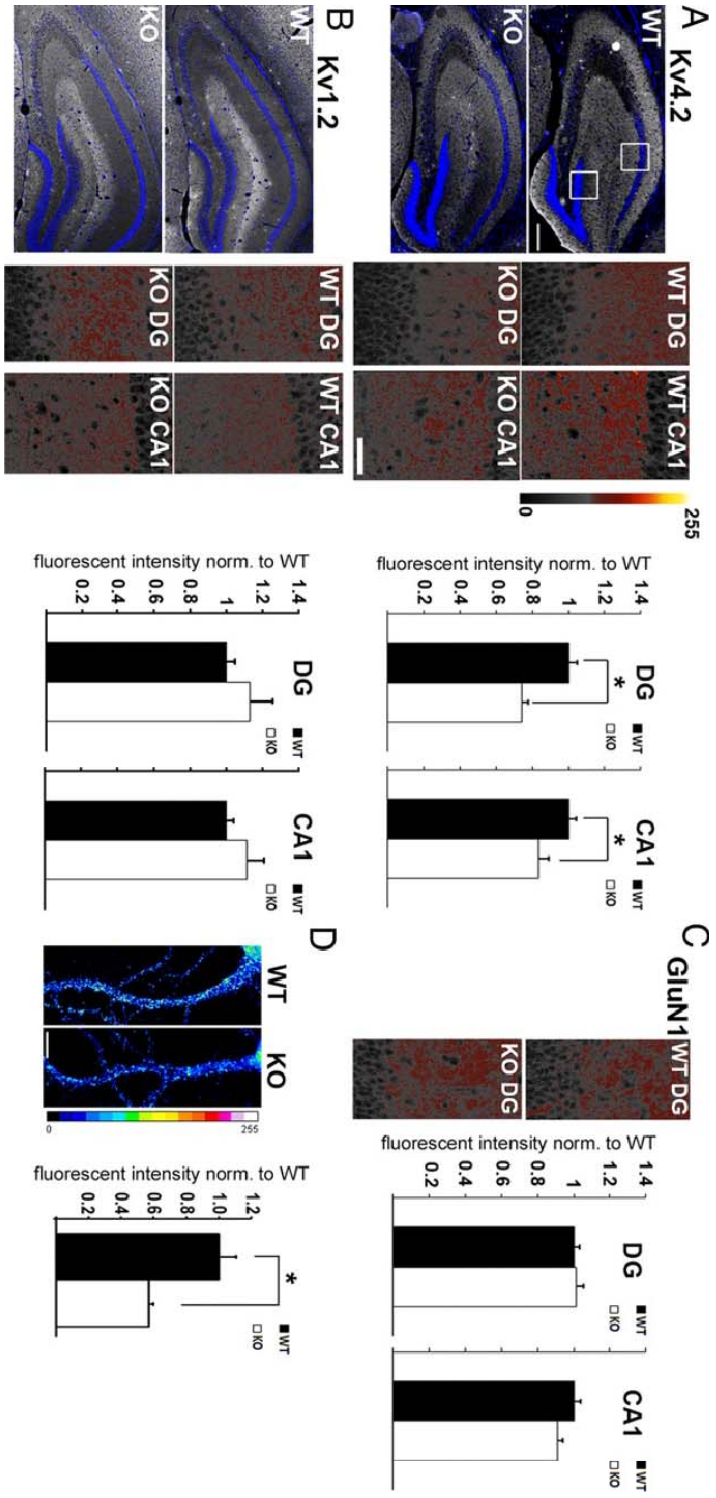

Figure 2-1. Kv4.2 protein levels are reduced in *Fmr1* KO hippocampal dendrites. A–C, Quantitative analysis of fluorescent immunohistochemical stainings in dendritic layers from dentate gyrus (DG) and CA1 shows significantly reduced dendritic levels of Kv4.2 (A) (DG: n_{WT}=27, n_{KO}=25, *p=0.001, Mann–Whitney U test; CA1: n_{WT}=27, n_{KO}=25, *p=0.034, independent t test), whereas Kv1.2 (B) (DG: n_{WT}=21, n_{KO}=18, p=0.547; CA1: n_{WT}=22, n_{KO}=22, p=0.461, independent t tests), and GluN1 (C) (DG: n_{WT}=14, n_{KO}=14, p=0.943; CA1: n_{WT}=16, n_{KO}=16, p=0.394, independent t tests) are not significantly different. Example images of the entire hippocampus (including 4′,6-diamidino-2-phenylindole (DAPI) staining to visualize cell nuclei), the CA1 region, and the DG for Kv4.2 and Kv1.2 are shown on the left; for GluN1, example images from DG are shown. High-magnification images were pseudocolored with the intensity map shown in A. Scale bar, 200 μm. D, Quantitative immunofluorescence analysis in dendrites of 17 DIV cultured hippocampal neurons shows that Kv4.2 protein levels are significantly reduced in KO (n=30, *p=0.0007, independent t test). Immunofluorescent images were pseudocolored with a 16-color intensity map (shown on the right). Scale bar, 10 μm. All error bars represent SEM. (Panel A-C contributed by Dr.Christina Gross)

Figure 2-2

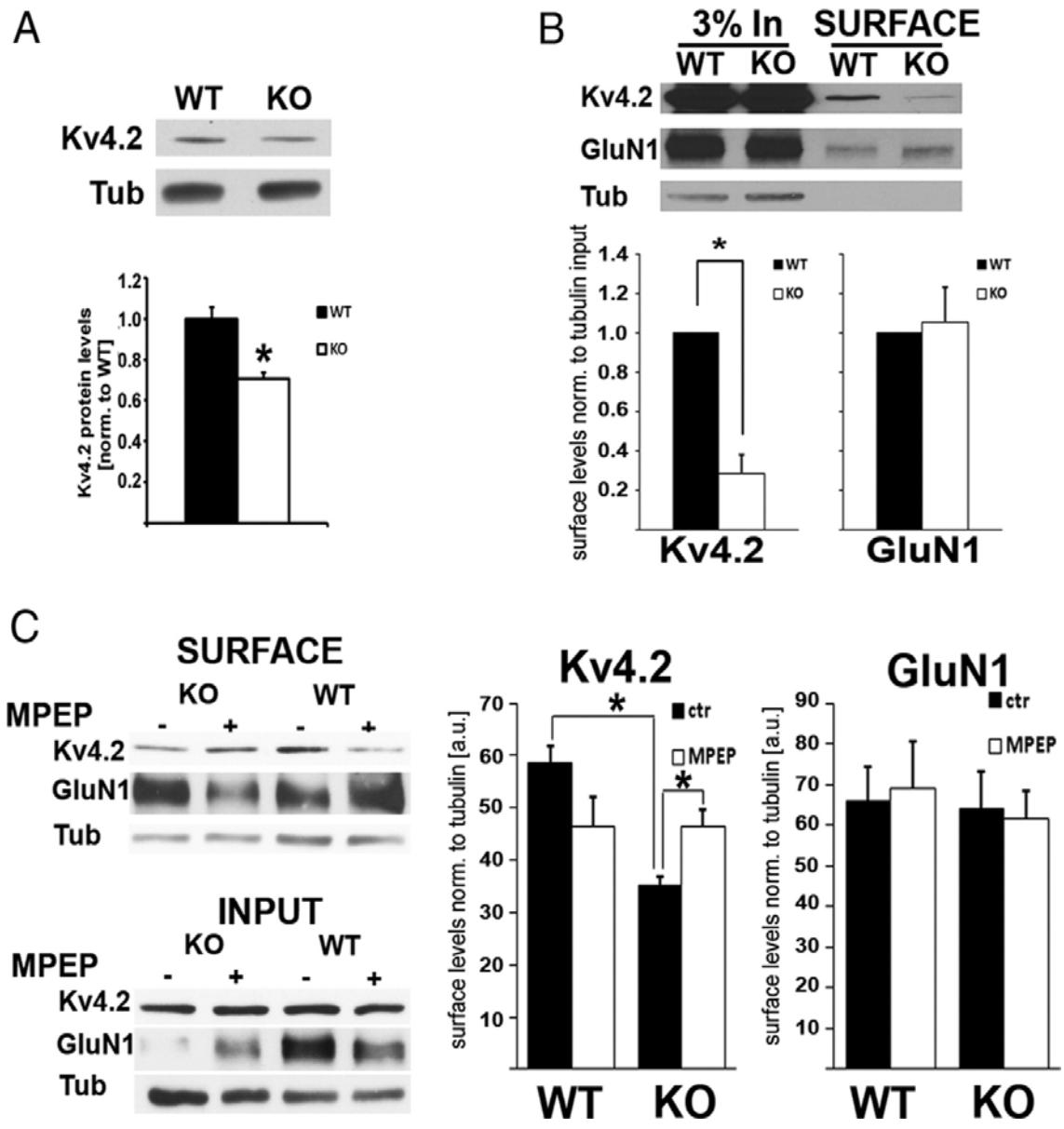
A
WT
KO
Kv4.2
Tub
Kv4.2 protein levels [norm. to WT]
1.2
1.0
0.8
0.6
0.4
0.2
WT
KO
*
B
3% In
SURFACE
WT
KO
WT
KO
Kv4.2
GluN1
Tub
surface levels norm. to tubulin input
1.4
1.2
1.0
0.8
0.6
0.4
0.2
*
WT
KO
WT
KO
Kv4.2
GluN1
C
SURFACE
KO
WT
MPEP
- + - +
Kv4.2
GluN1
Tub
INPUT
KO
WT
MPEP
- + - +
Kv4.2
GluN1
Tub
Kv4.2
surface levels norm. to tubulin [a.u.]
70
60
50
40
30
20
10
*
*
ctr
MPEP
WT
KO
GluN1
surface levels norm. to tubulin [a.u.]
90
80
70
60
50
40
30
20
10
ctr
MPEP
WT
KO

Figure 2-2. Reduced levels of Kv4.2 at neuronal cell surfaces in *Fmr1* KO mice can be partially rescued by antagonizing mGlu5. (A), Western blot analysis of total Kv4.2 protein shows reduced Kv4.2 levels in KO cortex (n=5 each WT and KO, 2 independent litters, *p=0.001, paired t test). (B), Western blot analysis of surface biotinylation assays demonstrates reduced Kv4.2 surface levels in cultured cortical neurons (n=4, *p=0.03, paired t test), whereas GluN1 surface levels are unchanged (n=4, p=0.694, paired t test). Representative Western blots are shown above. Surface protein levels were normalized to β-tubulin input. (C), In hippocampal slices from *Fmr1* KO, Kv4.2, but not GluN1, surface levels are significantly reduced and can be partially rescued by pretreatment with the mGlu5 antagonist MPEP (50 μm, 40 min) [Kv4.2: n=6, two-way ANOVA; treatment: p=0.976, genotype: p=0.01, interaction genotype–treatment: p=0.01, Games-Howell *post hoc* analyses (unequal variances assumed): $p_{wtctr\text{-}wtMPEP}$=0.467, *$p_{wtctr\text{-}koctr} < 0.001$, *$p_{koctr\text{-}koMPEP}$=0.036, $p_{wtctr\text{-}koMPEP}$=0.053; GluN1: n=6, two-way ANOVA; treatment: p=0.936, genotype: p=0.500, interaction genotype–treatment: p=0.679). GluN1 and Kv4.2 surface levels were normalized to β-tubulin; representative Western blots are shown on the left. All error bars represent SEM. (Panel C contributed by Dr.Christina Gross)

Figure 2-3

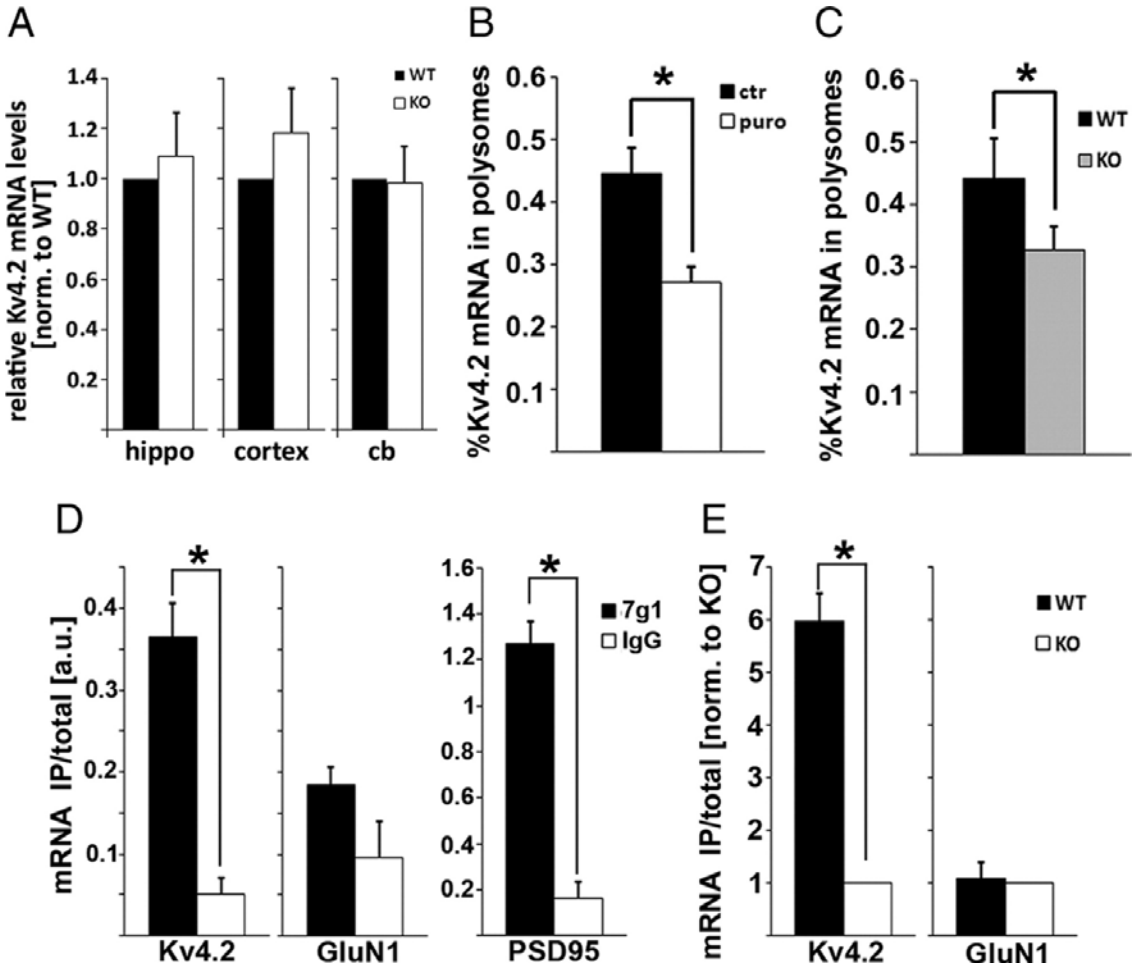
A
relative Kv4.2 mRNA levels [norm. to WT]
1.4
1.2
1.0
0.8
0.6
0.4
0.2
WT
KO
hippo
cortex
cb
B
%Kv4.2 mRNA in polysomes
0.6
0.5
0.4
0.3
0.2
0.1
*
ctr
puro
C
%Kv4.2 mRNA in polysomes
0.6
0.5
0.4
0.3
0.2
0.1
*
WT
KO
D
mRNA IP/total [a.u.]
0.4
0.3
0.2
0.1
*
Kv4.2
GluN1
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
*
7g1
IgG
PSD95
E
mRNA IP/total [norm. to KO]
7
6
5
4
3
2
1
*
WT
KO
Kv4.2
GluN1

Figure 2-3. Kv4.2 mRNA translation is reduced in synaptic fractions from *Fmr1* KO cortices. (A), qRT-PCR analysis of Kv4.2 mRNA levels in different brain regions: hippocampus (hippo) (n=4, p=0.648, paired t test), cortex (n=4, p=0.372, paired t test), and cerebellum (cb) (n=4, p=0.926, paired t test) shows no significant difference between WT and KO. mRNA levels were normalized to tubulin mRNA. (B), Polysome association of Kv4.2 mRNA in cortical synaptoneurosomes is puromycin sensitive, suggesting that Kv4.2 mRNA in these fractions is actively translated (n=4, *p=0.026, paired t test). (C), Association of Kv4.2 mRNA with puromycin-sensitive fractions is significantly reduced in *Fmr1* KO (n=6, *p=0.04, paired t test). (D, E), Kv4.2 mRNA associates with FMRP *in vivo* in forebrain. (D), qRT-PCR analysis of FMRP-specific coimmunoprecipitations from WT brain shows that Kv4.2 mRNA and the positive control PSD95 are significantly enriched compared with IgG control, whereas the negative control GluN1 shows no significant enrichment (n=3; Kv4.2, *p=0.040; GluN1, p=0.147; PSD95, *p=0.007; paired t tests). (E), Specific enrichment of Kv4.2 mRNA, but not GluN1 mRNA, was also detected in FMRP-specific immunoprecipitations from WT and *Fmr1* KO lysates (n=3; Kv4.2, *p=0.015; GluN1, p=0.84; paired t tests). (Panel B-E contributed by Dr.Christina Gross)

Figure 2-4

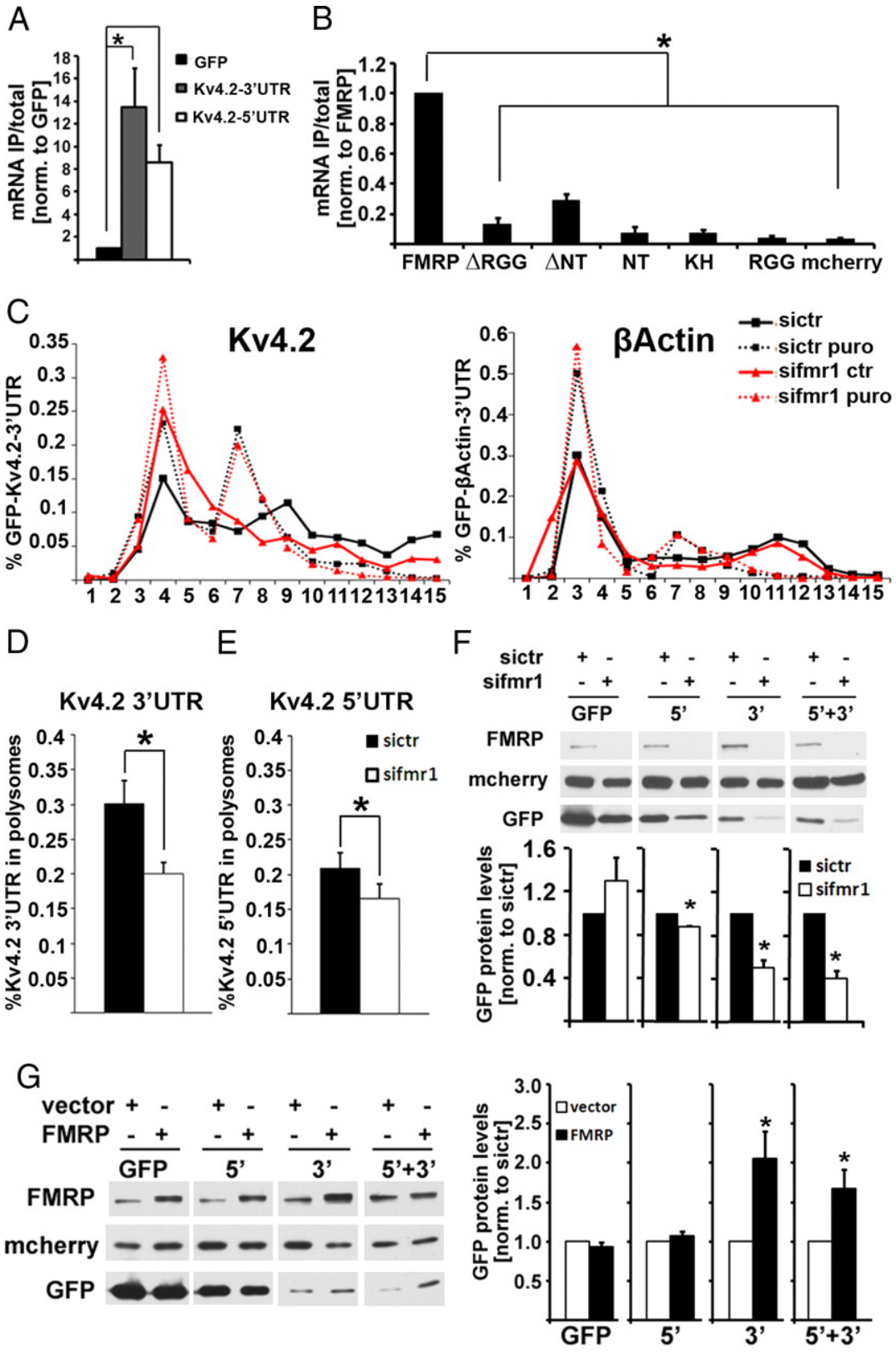
A
mRNA IP/total [norm. to GFP]
GFP
Kv4.2-3'UTR
Kv4.2-5'UTR
B
mRNA IP/total [norm. to FMRP]
FMRP
ΔRGG
ΔNT
NT
KH
RGG mcherry
C
Kv4.2
% GFP-Kv4.2-3'UTR
βActin
% GFP-βActin-3'UTR
sictr
sictr puro
sifmr1 ctr
sifmr1 puro
D
Kv4.2 3'UTR
%Kv4.2 3'UTR in polysomes
E
Kv4.2 5'UTR
%Kv4.2 5'UTR in polysomes
sictr
sifmr1
F
sictr
sifmr1
GFP
5'
3'
5'+3'
FMRP
mcherry
GFP
GFP protein levels [norm. to sictr]
G
vector
FMRP
GFP protein levels [norm. to sictr]

Figure 2-4. FMRP regulation of Kv4.2 mRNA is mediated by both 5′- and 3′-UTRs of Kv4.2 mRNA. (A), The association of both GFP-Kv4.2 3′-UTR and Kv4.2 5′-UTR-GFP mRNAs with mcherry-FMRP protein is significantly higher than that with GFP mRNA alone (n=4, one-way ANOVA with least significant difference *post hoc* tests: $*p_{\text{GFP 3′-UTR}}$=0.007, $*p_{\text{GFP 5′-UTR}}$=0.049). (B), mcherry-FMRP shows significantly higher affinity to the 3′-UTR of Kv4.2 mRNA than does mcherry-FMRP lacking either the RGG box (ΔRGG) or the N terminus (ΔNT), or mcherry-fusion proteins containing either of the three known FMRP RNA-binding domains [N terminus (NT), KH-domain, or RGG-box, respectively] (n=6, one-way ANOVA, $*p < 0.0001$; Tukey's honestly significant difference *post hoc* comparisons, $*p \leq 0.05$). (C), Association of recombinant GFP-Kv4.2 3′-UTR (16 h expression) with puromycin-sensitive polysome fractions is reduced in HEK293T cells after 48 h of siRNA-mediated depletion of FMRP (left), whereas the β-actin 3′-UTR is unchanged (right). (D), Quantitative analysis shows a significant reduction of Kv4.2 3′-UTR in heavy polysomes from FMRP-knockdown cells (n=4, $*p$=0.0195, paired t test). Similar results were seen for a luciferase reporter, and in N2A cells (data not shown). (E), Likewise, Kv4.2 5′-UTR was reduced in polysomes from FMRP-depleted HEK293T cells, but to a lesser extent compared with the 3′-UTR (n=3, $*p$=0.031, paired t test; 3′-UTR, 33%; 5′-UTR, 21% reduction). ***F***, GFP protein levels are reduced when fused to either Kv4.2 5′-UTR (n=3, $*p$=0.003, paired t test), Kv4.2 3′-UTR (n=3, $*p$=0.021, paired t test), or both UTRs (n=3, $*p$=0.017, paired t test) in FMRP-knockdown cells compared with control knockdown cells; however, a GFP construct without any UTRs (n=3, p=0.297, paired t test) shows no significant expression difference (72 h of siRNA treatment, 16 h coexpression of GFP-constructs with mcherry).

Representative Western blot images are shown above; mcherry was used as transfection control. ***G***, Overexpression of FMRP leads to a significant increase of GFP protein levels fused to both UTRs or the Kv4.2 3′-UTR (*n*=7; GFP, *p*=0.301; 5′, *p*=0.303; 3′, **p*=0.023; 5′ + 3′, **p*=0 .033, paired *t* test). Representative western blots are shown on the left. All error bars represent SEM. (Panel C-E contributed by Dr.Christina Gross)

Chapter III

MiR-324-5p Regulates the Expression of the Potassium Channel Kv4.2

Introduction

Kv4.2 is the major potassium channel in the hippocampus that mediates the A type K^+ current, which dampens the back propagation of action potentials, and therefore controls neuronal excitability (Birnbaum et al., 2004). A-type K^+ currents in hippocampal neurons from Kv4.2 knockout (KO) mice is specifically and completely abolished, supporting the assumption that Kv4.2 is the major potassium channel leading to these currents (Chen et al., 2006). Kv4.2 was also shown to be an important regulator of synaptic plasticity since back-propagating action potentials are critical for the induction of long-term potentiation (LTP), a long-lasting increase in synaptic strength (Kim and Hoffman, 2008). Heteropodatoxin-3 (HpTX3), a selective Kv4 channel blocker, reduces the threshold for LTP induction (Ramakers and Storm, 2002). In addition, neurons from Kv4.2 KO mice also exhibit a reduced threshold for LTP induction (Chen et al., 2006).

Recent studies suggest that Kv4.2 may be potentially involved in various neurological disorders, such as epilepsy, autism spectrum disorders and Alzheimer's disease. A truncated form of Kv4.2 lacking 44 amino acids in the carboxy-terminus was found in a patient with temporal lobe epilepsy (Singh et al., 2006) and cells transfected with this truncated form of Kv4.2 display attenuated A-type K^+ currents (Singh et al., 2006), suggesting the functional significance of this Kv4.2 mutation and implying that malfunctions of Kv4.2 may be a cause for epilepsy. Moreover, a Kv4.2 auxiliary protein, dipeptidyl-peptidase-like protein 6 (DPP6), is essential for the proper function of Kv4.2 (Sun et al., 2011). Of note, human mutations in DPP6 have been correlated with susceptibility to autism spectrum disorder (Marshall et al., 2008), suggesting a possible

involvement of Kv4.2 in this neuropsychiatric disorder. Furthermore, soluble forms of the Alzheimer's disease associated amyloid beta protein (Aβ) can increase the A-type K^+ current in neurons and are important for the modulation of Kv4.2 expression in central neurons (Plant et al., 2006). The physiological significance of Kv4.2 in synaptic plasticity and its implications in human diseases underscore the importance of understanding mechanisms underlying the regulation of Kv4.2 expression and function.

Previous studies have shown that post-translational regulation of Kv4.2, such as phosphorylation plays a critical role in controlling the function and membrane trafficking of Kv4.2 in hippocampal neurons (Kim and Hoffman, 2008). Interestingly, emerging evidence suggests that the expression and function of Kv4.2 undergoes posttranscriptional regulation. The protein expression of Kv4.2 is regulated by Fragile X mental retardation protein in a Kv4.2 mRNA-dependent mechanism (chapter 2 and (Lee et al., 2011)). In addition, Kv4.2 mRNA is localized to neuronal dendrites through a targeting RNA element within the 3'UTR of Kv4.2 mRNA (Jo et al., 2010). However, whether there are other mRNA regulatory machineries also involved in the post-transcriptional regulation of Kv4.2 remains elusive.

MicroRNAs (miRNAs) are evolutionary conserved small non-coding RNAs (21-28 nucleotides) that can modulate protein expression post-transcriptionally (Fabian et al., 2010). Emerging evidence draws attentions to the important function of miRNAs for synaptic plasticity (Olde Loohuis et al., 2012) and neurological disorders such as Alzheimer's disease (Delay et al., 2012). A recent study reported for the first time that a miRNA can function as a negative regulator for the expression of ion channels. Saba et al showed that miR-181a modulates the total and cell surface levels of alpha-amino-3-

hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-R) subunit GluA2 (Saba et al., 2012). In addition, overexpression of miR-181a reduces spine formation, and miniature excitatory postsynaptic current (mEPSC) frequency in hippocampal neurons, suggesting that miR-181a can regulate synaptic function (Saba et al., 2012).However, so far no miRNA has been identified that regulates potassium channels in the brain, and might thus be involved in the control of neuronal excitability. Interestingly, several miRNAs including miR-146a, let-7e, miR-23a/b are differentially expressed in humans with temporal lobe epilepsy (TLE) as well as in rat models of TLE, implying the potential involvement of these miRNAs in epileptogenesis (Aronica et al., 2010; Song et al., 2011). However, the specific target mRNAs, through which these miRNAs might mediate their function in epileptogenesis are unknown.

Here, we report that miR-324-5p regulates the expression of Kv4.2, a potassium channel implicated in epilepsy. We identified Kv4.2 as a putative mRNA target of miR-324-5p using computational approaches. We could show that both overexpression and inhibition of miR-324-5p can manipulate the expression of a luciferase reporter containing the 3'UTR of Kv4.2. A mutation that presumably disrupts the association of miR-324-5p with the 3'UTR of Kv4.2 mRNA abolished the effect of miR-324-5p on Kv4.2 3'UTR reporter expression. We further show that overexpression and inhibition of miR-324-5p in neurons leads to decreased or increased total levels of endogenous Kv4.2 protein, respectively. Importantly, overexpression of miR-324-5p also reduces cell surface levels of Kv4.2. Kv4.2 functions at the cell membrane, suggesting that miR-324-5p may regulate Kv4.2-mediated A-type currents. Our results suggest that miR-324-5p regulates the total and cell surface expression of Kv4.2, which may potentially influence

Kv4.2 function and have an impact on neuronal excitability. To our knowledge, this is the first description of a microRNA regulating the expression of a potassium channel in the brain, which plays an important role for neuronal function and synaptic plasticity.

Materials and Methods

DNA constructs, miRNA inhibitors and Lentivirus packaging

The firefly luciferase (ffluc)-Kv4.2-3'UTR reporter was constructed by inserting the full length rat 3'UTR of Kv4.2 mRNA into a ffluc expressing plasmid which made by replacing the EGFP coding sequence in PEGFPC1 vector(Clotech.) with firefly luciferase coding sequence. The ffluc-Kv4.2-3'UTR-MUT was generated by site-directed mutagenesis of the rat Kv4.2 3'UTR using overlapping primers (5'ACTAG TGT**CG** ATCCC CATGA GAAAC ACTGC AGT 3' and 5' TCATG GGGAT **CG**ACA CTAGT AAAAT CTAAA GTGTTC 3') with a two-nucleotide mutation. GFP and miRNA co-expression constructs for control (ctrl), miR-130a, miR-186 and miR-324-5p were purchased from GeneCopoeia. LNA antisense-RNA oligonucleotides (Ambion) were used for antagonizing microRNAs. To generate miRNA expressing lentiviral particles, GFP and miRNA coding sequences were inserted into the downstream CMV promoter of a lentiviral transfer plasmid (pFUGW). Lentiviral particle were produced in HEK293 cells by the Viral Vector Core of the Emory Neuroscience NINDS Core facility and purified by ultracentrifugation.

Primary neuronal culture

Cerebral cortices and hippocampi were dissected from E17 mouse embryos (C57BL/6J, *Jackson Laboratories*). Dissociated neurons were plated on poly-lysine (Sigma P1254, 0.02mg/ml)-coated dishes or acid-treated glass coverslips. Neurons were incubated in minimal essential medium with 10%FBS (Sigma) for 2 h, then low density cultures on coverslips were inverted on to dishes containing secondary glia cells in Neurobasal Medium (Invitrogen) with B27 supplements (Invitrogen) and Glutamax

(Invitrogen); while high density cultures were only changed medium (Kaech and Banker, 2006).

Secondary Cell lines

Neuro2A cells were maintained in DMEM (Hyclone) with 10% FBS (Sigma).

Transfection

Lipofectamine 2000 (Invitrogen) was used to transfect DNA plasmids and LNA-RNA into Neuro2A cells. Magnetofection using Neuromag (OZBioscience) was used to transfect miRNA inhibitors into primary cultured neurons according to according to manufacturer's instructions.

Fluorescence in situ hybridization for miRNAs on brain sections and cells.

Mature hippocampal neurons or brains from p21 mice were used for in situ hybridization as described previously (Muddashetty et al., 2011)

Western blot analysis

Western blot analysis was performed as described before (Gross et al., 2010). Rabbit anti-Kv4.2 antibodies were used at 1:1000 (Millipore); mouse anti-GluN1 antibodies were used at 1:1000 (BD Pharmingen); and mouse anti α-tubulin (Sigma) antibodies wer used at 1:1,000, 000. Specific signals were detected with horseradish-peroxidase-coupled secondary antibodies (GE Healthcare) and Enhanced ChemiLuminescence (Thermo Scientific).

Biotinylation assay

High density cortical neurons were used at 14-18 DIV to perform biotinylation assay. Cells were incubated with 1.0 mg/ml EZ-Link sulfo-NHS-Biotin (Thermo Scientific) in 1X PBS (Hyclone) for 30 min at 4°C. Cell lysates were prepared and

biotinylated proteins were pulled down as decribed previously (Ehlers, 2000). Western blot analysis was used to assess the specific protein levels (Gross et al., 2011).

Dual-luciferase assay

Neuro2A cells were used to perform the luciferase assays and luciferase activity was measured by dual luciferase assay using Renilla and Firefly luciferase according to the manufacturer's protocol (Promega).

Statistics.

All statistical analyses were performed in SPSS 17.0 and PASW Statistics 18. Data were tested for normality and homogeneity of variances, and appropriate tests were used as indicated for each figure

Results

Kv4.2 mRNA is a putative target of miR-324-5p

In order to identify miRNAs that might target Kv4.2 mRNA, we first used computational approaches that predict putative miRNAs based on known consensus sequences between miRNAs and their target mRNAs. We choose three highly cited algorithms (Targetscan 6.1, miRanda and PicTar) to search for miRNAs that potentially target to Kv4.2 miRNA. Among several miRNAs that were found by all algorithms and could theoretically bind to the 3'UTR of Kv4.2 mRNA, we selected three miRNAs: miR-130a, miR-186 and miR-324-5p, to test for their potential to influence Kv4.2 expression in our experimental settings. The reasons why we selected these three miRNAs are: 1) these miRNAs are conserved throughout species; 2) the putative target sites of these miRNAs are also conserved among species; 3) these miRNAs show high complementarities with Kv4.2 mRNAs.

We first tested whether these miRNAs can possibly influence the protein expression of Kv4.2. For this purpose, we employed a Dual-luciferase assay to investigate the possible function of these three miRNAs in regulating the expression of reporters containing the 3'UTR of Kv4.2 mRNA as indicated by relative luciferase activities. The firefly-luciferase reporter, ffluc-Kv4.2-3'UTR, was constructed by cloning the 3'UTR of Kv4.2 behind the firefly luciferase coding sequence. Precursor miRNA expression constructs from GeneCopoeia were used to overexpress the specific miRNAs or a scrambled control. We found that in neuro2A cells, only the ovexpression of miR-324-5p had a significant inhibitory effect on the activities of firefly lucifrease expressed

from the ffluc-Kv4.2-3'UTR (21.1%±4.0%), but not miR-130a or miR-186 (Fig. 3-1A). Our RNA alignment analysis shows that miR-324-5p has 8 perfect matches with a putative target site in the 3'UTR of Kv4.2 mRNA within the seed region (Fig. 3-1B).

To further validate the ability of miR-324-5p to regulate ffluc-Kv4.2-3'UTR reporter expression, we used (LNA^{TM}, Exiqon)-antisense RNA oligonucleotides (anti-miR-324-5p) to antagonize endogenous miR-324-5p and examined the subsequent effect on the expression of firefly luciferase from the ffluc-Kv4.2-3'UTR reporter. Anti-miRNA oligonucleotides bind to and sequester endogenous miRNAs thereby preventing the association with their target mRNAs. Our result showed that in neuro2A cells, anti-miR-324-5p can successfully increase the activity of ffluc-Kv4.2-3'UTR by 60% (±2%) compared to the scrambled control. Collectively, these results suggest the Kv4.2 mRNA is a target of miR-324-5p, and that miR-324-5p may negatively regulate Kv4.2 expression and function. Therefore, we only pursued miR-324-5p for further studies. Interestingly, miR-324-5p has been implicated in epilepsy and synaptic plasticity in previous studies (Schaefer et al., 2010; Song et al., 2011), two processes also involving Kv4.2, suggesting a functional link between miR-324-5p and Kv4.2.

A mutation in the putative target region located in 3'UTR of Kv4.2 mRNA abolished the inhibitory effect of miR-324-5p on ffluc-Kv4.2-3'UTR reporter expression

Although our experiments show that both overexpression and inhibition of miR-324-5p can influence the expression of a ffluc-Kv4.2-3'UTR reporter, this might not be caused by a direct effect of miR-324-5p on Kv4.2 expression. miR-324-5p could possibly

regulate the expression of other mRNAs, and in turn modulating Kv4.2 expression. Thus, we employed a mutagenesis analysis to determine whether the inhibitory effect of miR-324-5p is mediated directly by the putative target region in the 3'UTR of Kv4.2. Accordingly, we designed a reporter construct, ffluc-Kv4.2-3'UTR-MUT, bearing a two nucleotide-mutation in the putative target region of 3'UTR of Kv4.2 (Fig. 3-2A). This point mutation would disrupt the predicted base pairing between miR-324-5p and 3'UTR of Kv4.2. Therefore, the inhibitory effect of miR-324-5p should be eliminated. Indeed, overexpression of miR-324-5p had no significant effect on the luciferase activity of ffluc-Kv4.2-3'UTR-MUT (Fig. 3-2B), suggesting that the target region in 3'UTR is a cis-acting inhibitory element. In line with this observation, ffluc-Kv4.2-3'UTR-MUT exhibits higher luciferase activity than ffluc-Kv4.2-3'UTR (33.0%±8.6%), suggesting that the mutated Kv4.2 construct cannot be regulated by endogenous miR-324-5p (Fig. 3-2C). Overall, these data suggest that the putative target region of miRNA-324-5p within the Kv4.2 3'UTR is responsible for the inhibitory function of miR-324-5p on the Kv4.2 reporter constructs, corroborating our hypothesis that miR324-5p directly affects Kv4.2 expression.

miR-324-5p is localized to neurons

Kv4.2 is highly expressed in the brain and is the predominant potassium channel that composes A-type currents in the hippocampus (Chen et al., 2006). Given the potential function of miR-324-5p for Kv4.2 expression, we hypothesized that miR-324-5p might regulate Kv4.2 in vivo in the brain. We therefore assessed the expression and localization of miR-324-5p in neurons. Fluorescence in situ hybridization (FISH) using digoxigenin labeled LNA^{TM} probes was performed to investigate the neuronal

localization of miR-324-5p in both brain tissues and cultured hippocampal neurons. miR-324-5p is localized to both cell bodies and dendrites in the hippocampus of the brain (Fig. 3-3A-C). Correspondingly, miR-324-5p shows expression in 14 day in vitro (DIV) cultured hippocampal neurons (Fig. 3-3D). Overall, these data demonstrate that miR-324-5p is expressed in neurons and support the hypothesis that miR-324-5p regulates Kv4.2 expression *in vivo* in the brain.

miR-324-5p regulates endogenous Kv4.2 protein levels in primary neurons

To investigate whether miR-324-5p modulates the expression of endogenous Kv4.2 protein, we overexpressed or inhibited miR-324-5p in high-density cultured neurons. Lentiviruses expressing both GFP and miR-324-5p or a scrambled control were used to achieve efficient overexpression of miRNAs in neurons. Six days after transduction, more than 80% neurons were GFP positive (data not shown). We then assessed the Kv4.2 protein levels by quantitative western blot analysis. Total Kv4.2 levels were significantly decreased (-25.9%±7.9%) (Fig. 3-4A), after six days overexpression of miR-324-5p. Conversely, transfection with anti-miR-324-5p LNA oligonucleotides led to a significant increase of Kv4.2 protein levels (45.9%±18.0%). Taken together, these results suggest that Kv4.2 mRNA is a genuine target of miR-324-5p that represses Kv4.2 expression.

miR-324-5p regulates the cell surface expression of Kv4.2

The surface expression and active trafficking of Kv4.2 is critical for its synaptic functions (Shah et al., 2010). Kv4.2 protein is internalized and its surface levels are decreased during various synaptic stimulations such as long term potentiation (LTP) and

NMDA activation (Kim et al., 2007; Shah et al., 2010). To investigate whether miR-324-5p affects the function of Kv4.2, we therefore assessed the impact of miR-324-5p overexpression on Kv4.2 cell surface expression in cultured neurons. Finally, cell surface Kv4.2 levels were revealed by Kv4.2-specific quantitative western blot analysis. Samples were collected at six days after transduction with lentiviruses expressing miR-324-5p or control. Remarkably, overexpression of miR-324-5p resulted in a significant reduction in the surface Kv4.2 levels (-58.1%±14.2%) (Fig. 3-4C). In contrast, expression of a scrambled control miRNA had no effect (Fig. 3-4C). Overall, our results show that miR-324-5p regulates cell surface levels of Kv4.2 and suggest that miR-324-5p influences Kv4.2 function.

Discussion

The voltage-gated potassium channel Kv4.2 is a key regulator of neuronal excitability thereby contributing to synaptic plasticity. The expression of proteins involved in synaptic plasticity is tightly controlled by posttranslational and posttranscriptional modifications (Flavell and Greenberg, 2008; Wang et al., 2010a). Multiple forms of posttranslational regulation of Kv4.2 have been identified to regulate both steady state and activity-dependent function of Kv4.2 (Birnbaum et al., 2004). For example, phosphorylation of Kv4.2 by different protein kinases, such as PKA and PKC controls the cell surface, dendritic localization and activity-dependent trafficking of Kv4.2 (Birnbaum et al., 2004; Hammond et al., 2008; Varga et al., 2004). However, the mechanisms that regulate the mRNA translation and protein expression of Kv4.2 are poorly explored.

MicroRNAs are important regulators of mRNA translation and protein expression, they are abundantly expressed in the brain, and were shown to play a major role for proper neuronal function (Schratt, 2009). To analyze the mechanisms of Kv4.2 translational control, we therefore investigated the role of miRNAs in regulating Kv4.2 protein expression. miRNAs bind to their target through base-pair complementary mechanism, thus we used computational approaches to predict potential miRNA candidates. Based on computational prediction and the results from luciferase reporter assays (Fig. 3-1), we selected miR-324-5p to further study its function on regulating Kv4.2 expression. The expression of a luciferase reporter containing 3'UTR of Kv4.2 could be influenced by overexpression or inhibition of miR-324-5p (Fig. 3-1). Furthermore, miR-324-5p regulates endogenous Kv4.2 protein expression (Fig. 3-4A and

B) in cultured cortical neurons. In order to demonstrate the miR-324-5p putative target site in the 3'UTR of Kv4.2 is directly responsible for mediating the effect of miR-324-5p, we introduced a two-nucleotide mutation within the target site to interrupt the base-pair complementary. Luciferase assays showed that miR-324-5p did not have any effect on the mutant reporter with a disabled microRNA binding site (Fig. 3-2). Collectively, our results suggest that miR-324-5p directly regulates the expression of Kv4.2 in a posttranscriptional way. Cell surface Kv4.2 levels are directly linked to its function as a potassium channel, particularly the amplitude of A-type currents (Shah et al., 2010). We found that overexpression of miR-324-5p significantly reduced the surface expression of Kv4.2 (Fig. 3-4C), suggesting that miR-324-5p regulates the channel properties of Kv4.2. In the future, it will be interesting to measure the amplitude of A-type currents after manipulation of miR-324-5p expression. This will provide direct evidence to support miR-324-5p's role in regulating Kv4.2 function.

Here, we show the neuronal expression of miR-3245-p and provide first evidence suggesting the dendritic localization of miR-324-5p in brain tissues and cultured neurons using (Fig. 3-3). Interestingly, Kv4.2 mRNA is also localized to distal dendrites of cultured hippocampal neurons and its dendritic localization is determined by specific RNA elements within the 3'UTR of Kv4.2 mRNA (Jo et al., 2010). Local protein synthesis is believed to play critical roles in neuronal development and plasticity (Liu-Yesucevitz et al., 2011). A recent study showed that Kv4.2 could be locally translated in dendrites (Lee et al., 2011). The dendritic localization of both Kv4.2 mRNA and miR-324-5p raises the possibility that miR-324-5p could modulate Kv4.2 expression locally in dendrites and at synapses.

Recent studies suggest a role of miR-324-5p in synaptic plasticity, epilepsy and Alzheimer's disease (Mallick and Ghosh, 2011; Schaefer et al., 2010; Song et al., 2011). For instance, in a chronic model of temporal lobe epilepsy, the expression of miR-324-5p was found to be down-regulated 60 days after the induction of status epilepticus (Song et al., 2011), suggesting its function in epilepsy (Song et al., 2011). The functional overlap of Kv4.2 and miR-324-5p in neuronal excitability and certain diseases indicates that these two molecules might be involved in the same molecular pathway. Our results that miR-324-5p represses the expression of Kv4.2 furthermore suggest that manipulation of miR-324-5p expression could be used as a molecular tool to regulate Kv4.2 expression. Such a molecular tool might serve as a potential therapeutic strategy for diseases that are associated with impaired Kv4.2 function, such as epilepsy, Fragile X syndrome, other autism spectrum disorders, and Alzheimer's disease. It will be worthwhile to further study the changes of both Kv4.2 and miR-324-5p and the impact of miR-324-5p manipulation on the function of Kv4.2 in these disease models. Such studies will reveal more detailed molecular mechanisms underlying these neurological diseases and might help to design future therapies. Of note, microRNAs have been used as therapeutic targets in cancer research already: for example, overexpression of specific microRNAs was shown to reduce tumor growth in mouse models (Esquela-Kerscher et al., 2008; Liu et al., 2011; Trang et al., 2011)

Of note, Kv4.2 is not only abundant in brain but also highly expressed and plays important functions in the heart and muscles (Birnbaum et al., 2004). It will be interesting to know whether miR-324-5p is also expressed in the heart and muscles or whether the mechanism described here is a neuronal specific regulation of Kv4.2.

miRNAs repress protein synthesis through different mechanisms including translational inhibition and mRNA degradation (Fabian et al., 2010). However, it is unclear whether miR-324-5p represses the translation of Kv4.2 mRNAs or destabilizes Kv4.2 mRNA. Future effort to distinguish these two possibilities will provide insight into the detailed mechanism underlying the inhibition of miR-324-5p on Kv4.2 expression.

Taken together, our work shows that the specific microRNA miR-324-5p regulates the total and cell surface expression of Kv4.2 potassium channel, which might have important implications for neuronal plasticity and neurological diseases. In addition to the previously shown posttranslational regulation of Kv4.2, our work provides evidence for posttranscriptional modulation of Kv4.2 in neurons, and adds a layer of complexity to Kv4.2 regulation during neuronal activity.

Figures and legends

Figure 3-1

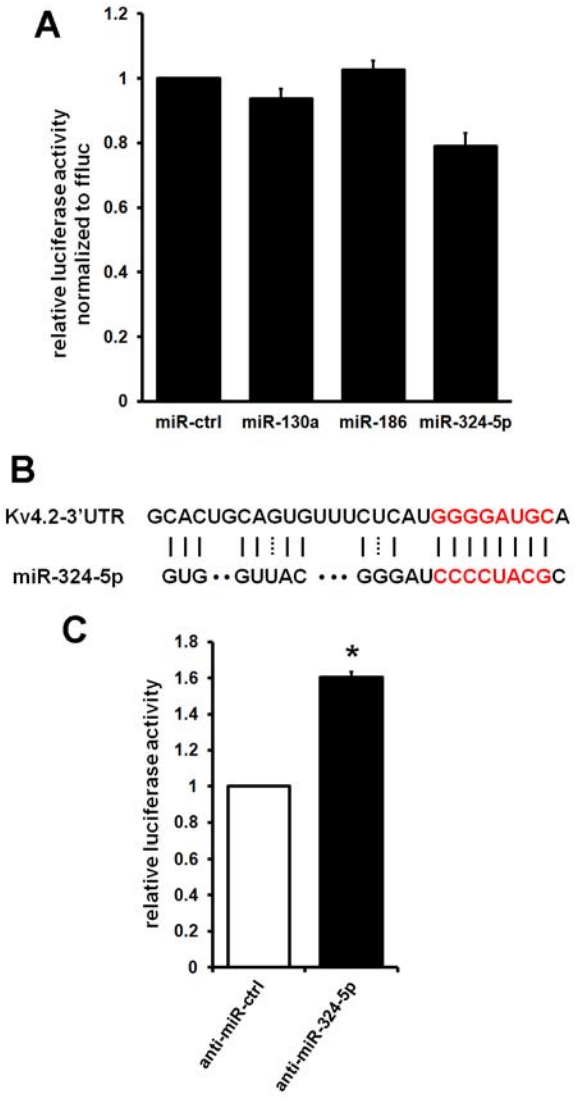

Figure 3-1. Kv4.2 mRNA is a putative target of miR-324-5p. (A) Overexpression of miR-324-5p reduces the relative luciferase activity of ffluc-Kv4.2-3'UTR. ffluc-Kv4.2-3'UTR reporter constructs were transfected into neuro2A cells together with plasmids expressing miR-ctrl, miR-130a, miR-186 and miR-324-5p, respectively. 24 hours after transfection, samples were collected to assess the luciferase activity.(n=3, one-way ANOVA, p=0.00001; post hoc Tukey HSD, $p_{ctrl-130a}$=0.167 ,$p_{ctrl-186}$=0.452 , $p_{ctrl-324-5p}$= 0.034) (B) Predicted miR-324-5p binding site in the rat 3'UTR of Kv4.2 mRNA. Red nucleotides indicate the seed region. (C) Inhibition of miR-324-5p increases relative luciferase activity of ffluc-Kv4.2-3'UTR. (LNA)-antisense RNA oligonucleotides were transfected with ffluc-Kv4.2-3'UTR into neuro2A cells. (n=3, *p=0.0002, paired t-test). All error bars represent SEM.

Figure 3-2

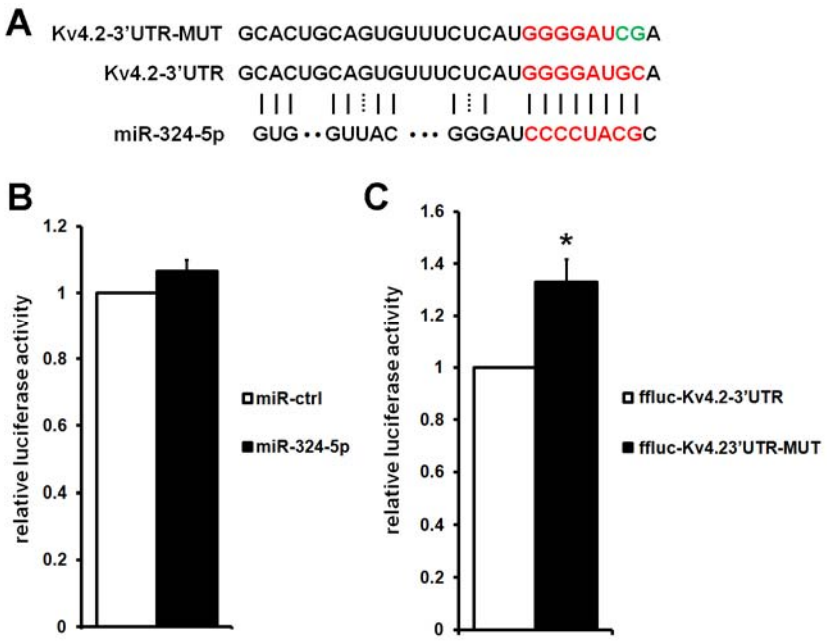
A
Kv4.2-3'UTR-MUT GCACUGCAGUGUUUCUCAUGGGGAUCGA
Kv4.2-3'UTR GCACUGCAGUGUUUCUCAUGGGGAUGCA
miR-324-5p GUG • • GUUAC • • • GGGAUCCCCUACGC
B
relative luciferase activity
1.2
1
0.8
0.6
0.4
0.2
0
miR-ctrl
miR-324-5p
C
relative luciferase activity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
ffluc-Kv4.2-3'UTR
ffluc-Kv4.23'UTR-MUT

Figure 3- 2. Point mutation in the seed region of Kv4.2 mRNA abolishes the function of miR-324-5p Kv4.2 mRNA. (A) The 3'UTR of a Kv4.2 mRNA (full length rat 3'UTR) was mutated in the seed region by site-directed mutagenesis to generate ffluc-Kv4.2-3'UTR-MUT reporter. Mutated bases are shown in green. (B) miR-324-5p overexpression has no effect on the ffluc-Kv4.2-3'UTR-MUT reporter. (n=4, p=0.145, paired t-test) (C) The relative luciferase activity of ffluc-Kv4.2-3'UTR-MUT is higher than ffluc-Kv4.2-3'UTR. (n=4, *p=0.031, paired t-test) All error bars represent SEM.

Figure 3-3

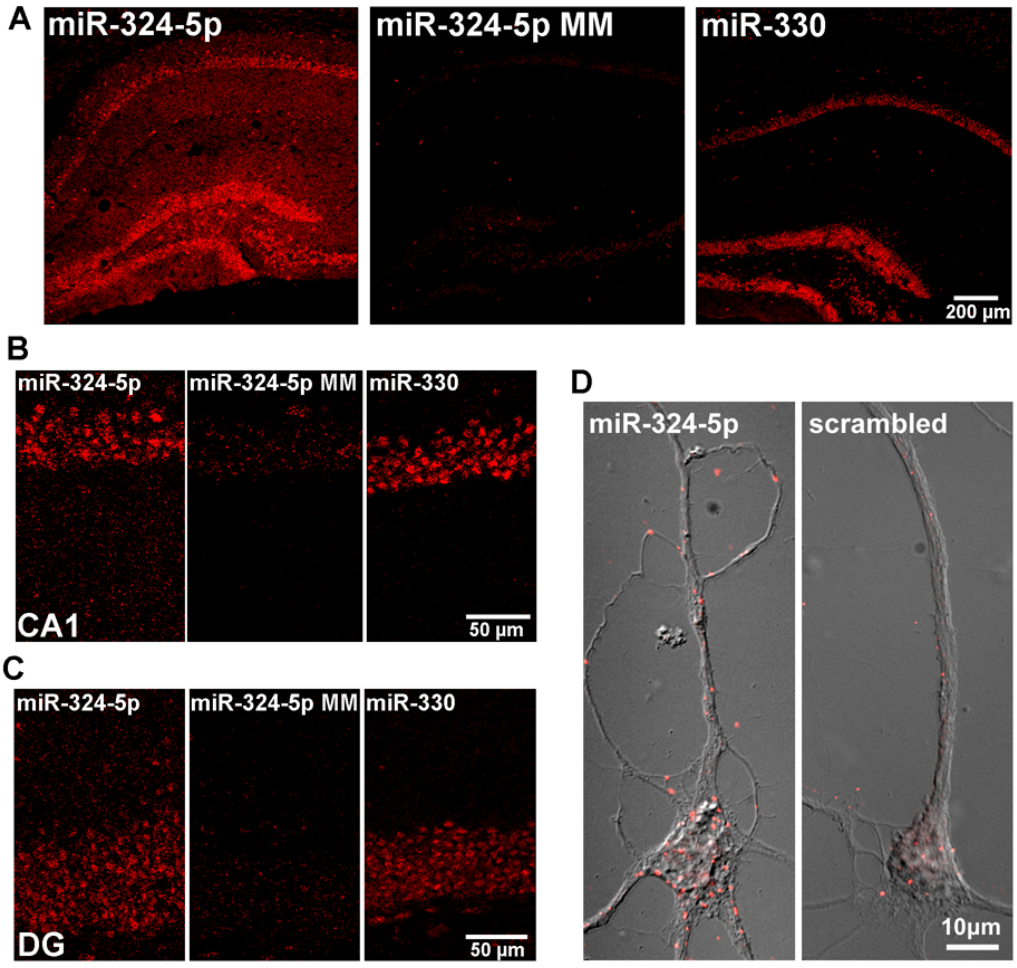
A
miR-324-5p
miR-324-5p MM
miR-330
200 µm
B
miR-324-5p
miR-324-5p MM
miR-330
CA1
50 µm
C
miR-324-5p
miR-324-5p MM
miR-330
DG
50 µm
D
miR-324-5p
scrambled
10µm

Figure 3-3. miR-324-5p is expressed in the hippocampus. (A-C) Fluorescent in situ hybridization (FISH) shows strong expression of miR-324-5p in the mouse hippocampus. The signal is markedly reduced using a FISH probe harboring two nucleotide mutations within the miR-324-5p sequence (*miR-324-5p MM*). miR-324-5p can be detected in cell body layers (pyramidal and granule cell layers), as well as in dendritic layers (stratum radiatum and molecular layer). In contrast, miR-330, a microRNA that is predominantly restraint to the cell body (Kye et al., 2007), can only be detected in the cell body layers, further suggesting that miR-324-5p is present in dendrites. A shows overview images of the hippocampus, B and C show enlarged images of the CA1 and the dentate gyrus (*DG*), respectively. (D) FISH shows miR-324-5p localization in the cell body and dendrites of a cultured mouse hippocampal neuron. Much less granules were detected by FISH using a scrambled LNA probe. (Panel A-C contributed by Christina Gross)

Figure 3-4

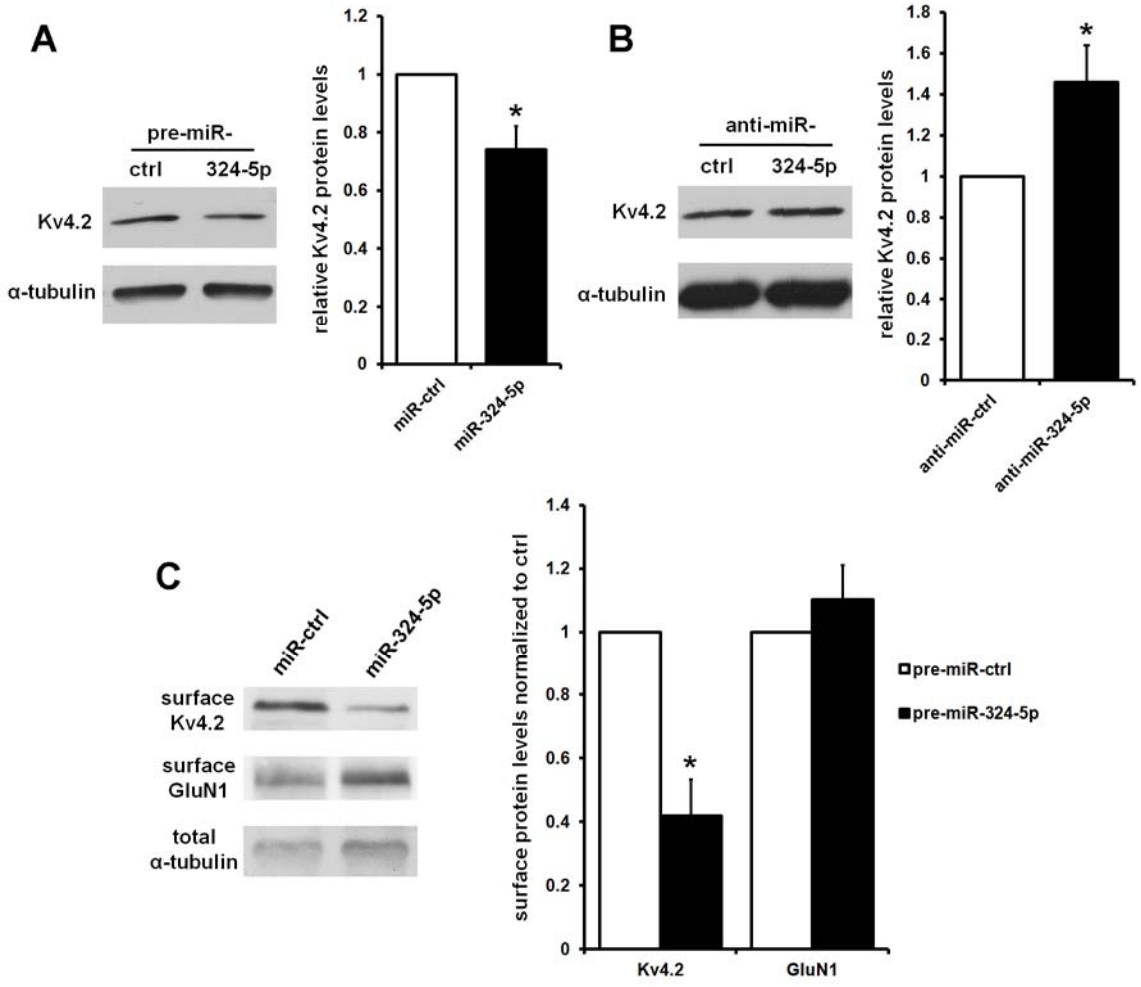
A
pre-miR-
ctrl
324-5p
Kv4.2
α-tubulin
relative Kv4.2 protein levels
1.2
1
0.8
0.6
0.4
0.2
0
*
miR-ctrl
miR-324-5p
B
anti-miR-
ctrl
324-5p
Kv4.2
α-tubulin
relative Kv4.2 protein levels
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
anti-miR-ctrl
anti-miR-324-5p
C
miR-ctrl
miR-324-5p
surface Kv4.2
surface GluN1
total α-tubulin
surface protein levels normalized to ctrl
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Kv4.2
GluN1
pre-miR-ctrl
pre-miR-324-5p

Figure 3-4. miR-324-5p regulates the total and cell surface levels of Kv4.2. (A) Overexpression of miR-324-5p leads to decreased levels of total Kv4.2 protein. High density cortical neurons were transduced with viruses expressing either miR-324-5p or a scrambled control at 10 DIV. Samples were harvested at 16 DIV and western blot were used to analysis protein levels. (n=3, *p=0.047, paired t-test). (B) Antagonizing miR-324-5p increases total Kv4.2 levels. (LNA)-antisense RNA oligonucleotides were expressed in high density cortical neurons for 2 days. (n=4, *p=0.043, paired t-test) (C) Overexpression of miR-324-5p reduces the surface levels of Kv4.2 protein (n=4, *p=0.014, paired t-test); whereas GluN1 surface levels are unchanged (n=4, P=0.413, paired t-test). Samples were harvested at 16 DIV, 6 days after transduction with viruses expressing either miR-324-5p or the scrambled control. Surface protein levels were normalized to α-tubulin input. Representative western blots are shown on the left. All error bars represent SEM.

Chapter IV

Summary and Future Directions

The objective of this dissertation was to elucidate novel posttranscriptional mechanisms in the regulation of Kv4.2 expression which may provide insight into the mechanism underlying high susceptibility to seizure in individuals with epilepsy. A prominent characteristic of FXS, the common inherited intellectual disability caused by the loss of FMRP, is high susceptibility to seizure with 10-20% patients are affected (Berry-Kravis, 2002). *Fmr1* KO mice also exhibit significantly higher susceptibility to audiogenic seizures and neurons from *Fmr1* KO are hyperexcitable (Chen and Toth, 2001; Chuang et al., 2005; Musumeci et al., 2000). The hyperexcitability could be due to excessive neuronal excitation or attenuated neuronal inhibition. Previous studies show that impaired GABAergic inhibitory pathway. The expression of subunits of GABA-A receptors, GABA transmitter levels and proteins involved in GABA transport and metabolism are altered in various brain regions in *Fmr1* KO (Paluszkiewicz et al., 2011). However, the mechanisms underlying weakened GABAergic pathways in FXS are unknown. Potassium channels are crucial for neuronal excitability. Recent studies suggest that FMRP might regulate neuronal excitability through potassium channels since FMRP was shown to regulate potassium channel Kv3.1 and Slack (Brown et al., 2010; Strumbos et al., 2010). However, no evidence has linked these two potassium channels to epilepsy. The potassium channel Kv4.2 that composes A-type K^+ currents may provide a potential link between FMRP and the high susceptibility to seizures in FXS. Kv4.2 is the major potassium channel that contributes to neuronal excitability in hippocampus (Birnbaum et al., 2004). Interestingly, both Kv4.2 proteins and mRNAs are localized to dendrites (Jo et al., 2010) and the mRNA was suggested to be translated locally (Lee et al., 2011). These findings suggest the hypothesis that FMRP, a RNA binding protein shown to regulate

protein synthesis locally at synapses, may regulate the protein expression of Kv4.2 to modulate neuronal excitability.

In this dissertation, we demonstrated that FMRP associates with Kv4.2 mRNA and positively regulates the translation of Kv4.2 mRNA. Our results show that total, dendritic and cell surface expression of Kv4.2 are reduced in *Fmr1* KO mouse brains. Furthermore, the mGluR5 antagonist MPEP can partially rescue the reduced surface levels of Kv4.2 in Fmr1 KO hippocampal slices. Our results suggest that the aberrant Kv4.2 mRNA regulation in the absence of FMRP might contribute to the neuronal hyperexcitability and epileptic seizure phenotypes observed in FXS (Gross et al., 2011).

Recently, another group has also reported a role of FMRP to regulate Kv4.2 (Lee et al., 2011), however, there were some surprising differences. Most notably there work suggests that FMRP negatively regulates the translation of Kv4.2 which is opposite from our findings. The differences between the studies might be due to different mouse strains, which are known to modulate phenotypes in Fmr1 KO. There are also differences in the developmental stage and type of neuron culture systems. Firstly, we used C57BL/6J background mice (Gross et al., 2011); while Lee et al. used FVB background mice (Lee et al., 2011). Second, it is well established that Kv4.2 expression is regulated by activity (Kim and Hoffman, 2008), and the neuronal activity depends on culture conditions, age of cultures/mice and even housing conditions of mice (Spencer et al., 2011; Yan et al., 2004). Third, there were differences in assays themselves. We examined the role of FMRP on Kv4.2 expression in cells (Gross et al 2011); while Lee et al. only examined a reticulocyte lysate with the presence of bacterially expressed recombinant FMRP. FMRP posttranslational modifications which are important for the function of FMRP

(Muddashetty et al., 2011; Narayanan et al., 2007) are not present in bacterially expressed FMRP incubated in the reticulocyte lysate. In contrast, we manipulated the endogenous FMRP levels with siRNA or overexpression in eukaryotic cell lines, which represents the endogenous condition of FMRP. To further clarify the role of FMRP in Kv4.2 expression and function, future work could measure the A-type currents in the hippocampus of *Fmr1* KO. Kv4.2 potassium channels are the major contributors of A-type currents in the hippocampus. By comparing the amplitude of A-type currents in WT and *Fmr1* KO, we will be able to further elucidate the role of FMRP on Kv4.2 function. In the future, it would be interesting to investigate the possible alteration in Kv4.2 expression using human postmortem brain tissues.

In addition to FMRP, we identified another posttranscriptional regulator, the microRNA miR-324-5p, which also regulates the expression of Kv4.2. miR-324-5p targets to the 3'UTR of Kv4.2 mRNA and inhibits the protein expression of Kv4.2. Mutations within the miRNA target site of the Kv4.2 3'UTR abolished the function of miR-324-5p on Kv4.2 expression. Our results suggest that miR-324-5p may regulate Kv4.2 expression and functions during neuronal development and plasticity. My thesis work provides new insights into the mechanisms regulating neuronal excitability. Dysregulation of these mechanisms might be involved in epileptogenesis, e.g. in FXS. Our findings therefore provide a molecular basis for future studies toward a better understanding the pathogenesis of FXS and other forms of epilepsy.

Aberrant Kv4.2 expression in the absence of FMRP suggests that Kv4.2 could be a potential therapeutic target to treat FXS, particularly for the neuronal hyperexcitability and epileptic phenotypes in individuals with FXS. Our finding that a specific miRNA,

miR-324-5p regulates Kv4.2 might provide the basis for a potential therapeutic strategy. It would be interesting to investigate the possible effect of miR-324 -5p antagonists in FMRP deficient animal models. Our results show that FMRP deficient neurons exhibits reduced Kv4.2 expression (total, dendritic and cell surface expression); in contrast, miR-324-5p inhibition led to increased Kv4.2 expression. Therefore, downregulating the expression of miR-324-5p or inhibiting miR-324-5p might be potential strategies to restore the protein levels of Kv4.2 in FXS. Of note , miRNA overexpression or inhibition by anti-miR compounds has been successfully used in animal models to reduce certain disease symptoms (van Rooij et al., 2012). Additionally, an anti-miR-122 compound is in clinic trials to treat Hepatitis C Virus infection (van Rooij et al., 2012). Our work thus may motivate future RNA-based therapies to modulate Kv4.2 expression in epilepsy and FXS-associated epilepsy.

Studies have shown that FMRP biochemically and genetically interacts with miRNA pathways. Specific miRNAs have been identified in the same RNP complexes with FMRP to regulate gene expression in neurons. For example, miR-125b works with FMRP to repress the expression of GluN2A, thereby influencing dendritic spine morphology (Edbauer et al., 2010). Moreover, our lab has shown that miR-125a and FMRP cooperate on the 3'UTR of PSD-95 mRNA to inhibit the translation of PSD-95 mRNA at synapses. The release of this inhibition in response to synaptic stimulation provides the reversibility in regulating PSD-95 expression (Muddashetty et al., 2011). The involvement of both, FMRP and miR-324-5p in the regulation of Kv4.2 mRNA translation suggests a possible functional interaction between FMRP and miR-324-5p to regulate Kv4.2 expression. Furthermore, all three components, FMRP, Kv4.2 and miR-

324-5p are implicated in neuronal plasticity and epilepsy. FXS patients exhibit high susceptibility to seizures (Berry-Kravis, 2002). A truncated form of Kv4.2 lacking 44 amino acids in the carboxy terminus was found in a patient with temporal lobe epilepsy (Singh et al., 2006). In a chronic model of temporal lobe epilepsy, the expression of miR-324-5p was found down-regulated 60 days after the induction of status epilepticus (Song et al., 2011). These findings provide a genetic basis for the possibility that FMRP and miR-324-5p may play roles in the same pathway to regulate Kv4.2 expression and eplipetogenesis. However, the molecular mechanisms are likely to be different to the ones mentioned above. We show that in the case of Kv4.2, FMRP acts as a translational activator, and thereby counteracts the inhibitory function of miR-324-5p.

Future work is needed to test this above mentioned hypothesis. First, it would be essential to investigate possible molecular interactions between FMRP and miRNA machineries on Kv4.2 mRNA. A straightforward experiment would be to investigate the association of miR-324-5p with FMRP-RNP complex. The evidences that miR-324-5p associates with Ago2 in the striatum (Schaefer et al., 2010) and FMRP interacts with Ago2 (Muddashetty et al., 2011) support the hypothesis that miR-324-5p and FMRP may be in the same RNP complex. It is also interesting to analyze the Ago2 association of Kv4.2 and miR-324-5p in *Fmr1* KO. If this association is altered in *Fmr1* KO that means FMRP and miR-324-5p act in the same pathway. In the future, we would also like to investigate the possible direct interaction between FMRP and Kv4.2 mRNA, and to determine the binding site(s) in Kv4.2 mRNA. Results from these studies would bring insights into the molecular mechanism whereby FMRP regulates Kv4.2 expression.

Previous studies suggest a model that FMRP facilitates the function of miRNAs (Edbauer et al., 2010; Muddashetty et al., 2011). However, if FMRP indeed functions together with miR-324-5p in regulating Kv4.2 expression, then our results would rather support a different model, in which FMRP represses the function of miR-324-5p. The functions of RNA binding proteins (RBPs) in miRNAs pathways have been widely studied (Agami, 2010; Chang and Hla, 2011). In addition to modulating the biogenesis of miRNAs, RBPs can also facilitate or inhibit the interaction between miRNAs and their target mRNAs to influence miRNA activities. Thus two possible models are proposed to illustrate the molecular mechanisms and possible interrelationships underlying the function of FMRP in repressing miR-324-5p activities (Fig.4-1). One possible model is that FMRP could direct compete with the binding site of miR-324-5p on the 3'UTR of Kv4.2 mRNA thereby inhibiting the function of miR-324-5p. Such a model has been demonstrated for heterogeneous nuclear ribonucleoprotein L (HNRNPL) which relieves miR-297 or miR-299-mediated repression of vascular endothelial growth factor (VEGF) mRNA through its competitive binding to the CA-rich sites in the 3′ UTR of VEGF mRNA (Jafarifar et al., 2011). To test this model, the binding site of FMRP within the Kv4.2 mRNA would need to be determined to look for possible overlaps with miR-324-5p binding site. In the second possible model, FMRP may bind to the Kv4.2 mRNA outside of the miR-324-5p target region and this interaction results in conformational changes of Kv4.2 mRNA which will release miR-324-5p from Kv4.2 mRNA. For example, miR-221 and miR-222 regulates the expression of p27. The binding of phosphorylated RNA binding protein pumilio-1 to the 3'UTR of p27 mRNA induces local RNA structural changes to favor the association with miR-221 and miR-222 (Kedde

et al., 2010). Theoretically, mRNA conformational changes may also disrupt the binding of a miRNA to its target mRNAs. Besides FMRP and miR-324-5p act in the same pathway, they also can function independently. Experiments to investigate the association of Kv4.2 mRNA with miR-324-5p or Ago2 in Fmr1 KO would address this question. If Fmr1 KO exhibits same association as WT, FMRP and miR-324-5p act independently. This proposed future study may provide new knowledge about the functions of FMRP and underlying working mechanisms in translational regulation. In summary, this thesis work has revealed two new posttranscriptional mechanism to regulate Kv4.2 expression and will motivate further work aimed at understanding the prevalence of epilepsy in FXS. In addition, this thesis will motivate new ideas of microRNA based theapeutics and intervention strategies for epilepsy and FXS.

Figures and legends

Figure 4-1

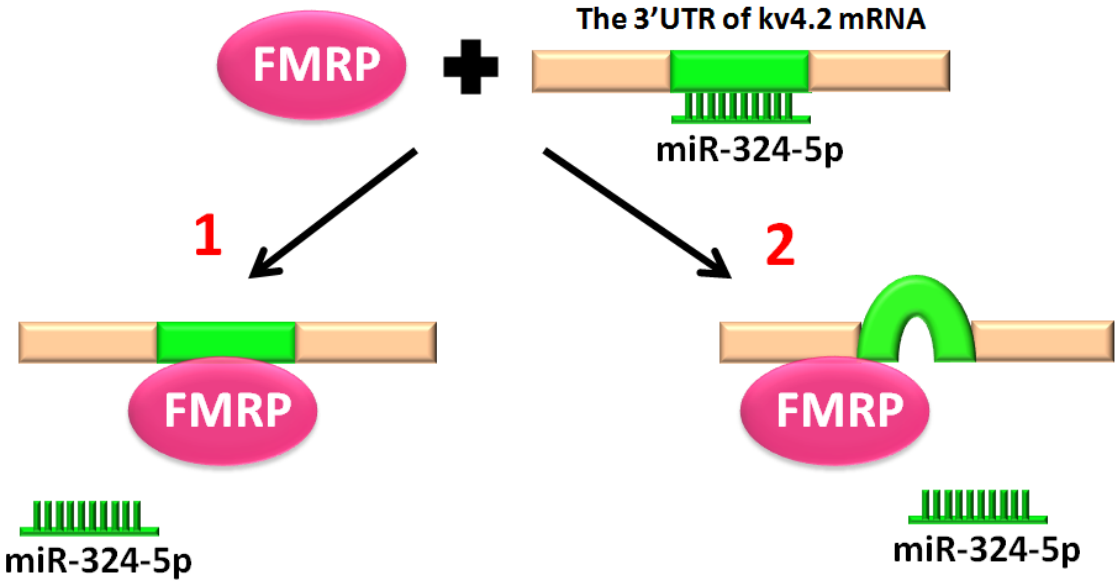

Figure 4-1. Proposed models for the cooperation of FMRP and miR-324-5p in the regulation of Kv4.2 expression. Model1: the binding of FMRP to the 3'UTR of Kv4.2 mRNA may compete with miR-324-5p at their shared binding site. Model 2: the binding of FMRP may lead to conformational changes of the miR-324-5p binding site, which disrupts the binding of miR-324-5p.

Chapter V

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