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Molecular Mechanisms Regulating Local Protein Translation During Homeostatic Synaptic Plasticity

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Molecular Mechanisms Regulating Local Protein Translation During Homeostatic Synaptic Plasticity

By

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Abstract

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Precise control of protein translation in neurons, particularly translation occurring in dendrites near synaptic sites, is critical for the proper regulation of synaptic strength. The most direct way to affect the strength of glutamatergic synapses is to alter the abundance of AMPA-type glutamate receptors (AMPARs). Here I demonstrate the critical role that two separate translational regulators play in controlling the translation of the AMPAR subunit GluR1, both under steady-state conditions and during synaptic plasticity.

Homeostatic synaptic plasticity adjusts the strength of synapses during global changes in neural activity, thereby stabilizing the overall activity of neural networks. Suppression of synaptic activity increases synaptic strength by inducing synthesis of retinoic acid (RA), which activates postsynaptic synthesis and insertion of AMPARs. Here, I show that the Fragile X Mental Retardation Protein (FMRP), an RNA-binding protein that regulates dendritic protein synthesis, is essential for increases in synaptic strength induced by RA or by blockade of neural activity in the mouse hippocampus. Although activity-dependent RA synthesis is maintained in *Fmr1* knockout neurons, RA-dependent dendritic translation of GluR1-type AMPARs is impaired. Intriguingly, FMRP is only required for the form of homeostatic plasticity which is dependent on both RA signaling and local protein synthesis. Expression of FMRP in knockout neurons reduced the total, surface, and synaptic levels of AMPARs, implying a role for FMRP in regulating AMPAR abundance. Critically, postsynaptic expression in knockout neurons of wild-type FMRP, but not two different mutant forms of the protein, was able to fully restore synaptic scaling.

microRNAs (miRNAs) are small RNA molecules which bind to the untranslated regions of mRNAs and inhibit translation. Using a bioinformatics approach, I identified a pair of miRNAs, miR-96 and miR-182, which bind specifically to a known sequence in the GluR1 mRNA and prevent its translation. When overexpressed, these miRNAs reduce total and extrasynaptic levels of GluR1 protein in neurons, and prevent the induction of homeostatic plasticity by activity blockade. Both miR-96 and miR-182 are expressed in cortex throughout

postnatal development, although we were unable to detect activity-dependent changes in the abundance of either miRNA. Attempted knockdown of both miRNAs revealed no significant effect on the abundance of GluR1 or the ability of neurons to undergo homeostatic plasticity.

Taken together, these data offer significant insight into the regulation of local translation of glutamate receptors at the synapse, particularly during specific forms of synaptic plasticity. These results also suggest that some of the symptoms of Fragile-X syndrome may be attributed to defects in the induction of homeostatic plasticity.

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Chapter 1
INTRODUCTION

Tight control over the genes and proteins expressed in each particular cell type is what allows a neuron to be distinguished in structure and function from a skin cell or a blood cell or a muscle cell. Neuronal cells have a complex and polarized morphology, and thus must maintain control over not only which proteins are expressed in each cell, but also over *where* in the cell those proteins are localized. Additionally, a typical neuron receives input at thousands of distinct synaptic sites, and the structure and strength of each of these connections is under constant adjustment in response to global and local patterns of activity. The precise regulation of which proteins are present in what amounts at any particular synapse at a given time is an overwhelmingly complex operation, and it is dependent on an incredibly wide range of cellular processes, including gene transcription, mRNA processing and trafficking, and the translation, modification, trafficking, and degradation of proteins. Each step in this process can be regulated by countless intersecting and overlapping signaling cascades, and potential permutations are endless.

Though all of these processes merit attention from researchers, when investigating protein regulation at the synapse one obvious place to start is to examine the local translation of new proteins in dendrites. Components of the translation machinery are found in dendrites (Tiedge and Brosius, 1996; Gardiol et al., 1999), and specific mRNAs are trafficked into dendrites both constitutively and in an activity-dependent manner (Steward et al., 1998; Grooms et al., 2006; Poon et al., 2006). Translation in dendrites is uniquely regulated compared to general somatic translation, and this regulation is altered in an activity-dependent manner (Sutton and Schuman, 2005; Wang et al., 2010). Activity-dependent control of local translation in dendrites is an especially intriguing process, as it provides an obvious mechanism for regulation of synaptic strength during synaptic plasticity.

Local translation during synaptic plasticity

Dynamic regulation of synaptic strength is the critical process that allows for the formation and storage of new memories in the brain. Plasticity at synapses can be separated into two major categories: Hebbian plasticity and homeostatic plasticity. Hebbian plasticity, which includes both long-term potentiation (LTP) and long-term depression (LTD), is an input-specific process. The synaptic connections between a presynaptic and a postsynaptic neuron can be strengthened or weakened, depending on the coordinated patterns of activity in the two cells, without affecting neighboring synapses formed on the same postsynaptic dendrite. This allows for the fine tuning of specific circuits in the brain and the encoding of new information and memories.

By contrast, homeostatic plasticity is a process whereby neurons modify their overall firing properties in order to maintain the stability of neural networks. Repeated rounds of Hebbian plasticity can push a given neuron towards the limits of its capacity to accurately encode and relay information, and can lead to either excessive excitability or overly silenced synapses (Davis and Bezprozvanny, 2001; Turrigiano and Nelson, 2004). Homeostatic plasticity

adjusts neuronal firing properties in order to ensure a relatively constant level of overall network activity, while at the same time maintaining the information encoded during LTP or LTD.

One form of homeostatic plasticity which is of particular interest is called synaptic scaling. During synaptic scaling the numbers of neurotransmitter receptors at each synapse in a neuron are adjusted (synapses are either "scaled up" or "scaled down") in order to compensate for a loss or excess of activity. Critically, the number of receptors added to or removed from each synapse is proportional to the initial strength of that synapse, so that the relative weight of each synapse, and thus the information encoded by Hebbian plasticity, is maintained (Turrigiano, 2008).

Though Hebbian plasticity and homeostatic plasticity achieve separate goals, both are critical for proper functioning of the nervous system, and many parallels exist between the execution of the two processes. Notably, during both LTP and synaptic up-scaling, new AMPA-type glutamate receptors are inserted into the synapse (Turrigiano et al., 1998; Sutton et al., 2006; Derkach et al., 2007). More specifically, local dendritic translation of the GluR1 AMPA receptor subunit and synaptic insertion of GluR1 homotetrameric receptors is critical for certain forms of both Hebbian and homeostatic plasticity (Liu and Cull-Candy, 2000; Sutton et al., 2006; Aoto et al., 2008). Insertion of homomeric GluR1 receptors affects synaptic signaling not only by increasing overall synaptic strength, but also because GluR1 homomers are permeable to calcium, unlike receptors which contain the GluR2 AMPA receptor subunit (Swanson et al., 1997). Calcium is a key activator of many critical intracellular signaling cascades, and thus altered synaptic calcium signaling can have profound effects on the cell. Therefore, local translation of GluR1 during synaptic plasticity is an effective way for neurons to adjust their synaptic strength, and is an absolutely crucial process in the regulation of cell signaling.

Homeostatic plasticity provides an excellent context in which to study local dendritic translation. However, this local translation is a key mechanism for only some forms of synaptic scaling. Synaptic up-scaling can be induced in cultured neurons by blocking neuronal activity with one or more drugs, including tetrodotoxin (TTX), which blocks voltage-gated sodium channels and prevents action potential firing (Turrigiano et al., 1998; Shepherd et al., 2006; Stellwagen and Malenka, 2006; Ibata et al., 2008); APV, which blocks NMDA-type glutamate receptors (Ju et al., 2004; Sutton et al., 2006; Aoto et al., 2008); and NBQX, which blocks AMPA-type glutamate receptors (Thiagarajan et al., 2005). Each pharmacological treatment scales up synapses, but each acts via a distinct molecular mechanism. Long-term treatment with TTX alone leads to the transcription-dependent insertion of heteromeric, calcium-impermeable receptors containing both GluR1 and GluR2 (Turrigiano et al., 1998; Gainey et al., 2009). A number of key molecular players in this process have been identified, including TNFα (Stellwagen and Malenka, 2006), CaMKIV (Ibata et al., 2008), and Arc (Shepherd et al., 2006), though a unified picture of how these proteins work together remains elusive. NBQX treatment, by contrast, enhances both AMPA receptor number and presynaptic release probability (Thiagarajan et al., 2005). And experiments by Sutton et al. (2006) showed that activity blockade with a combination of TTX and APV treatment leads to the local translation of GluR1 AMPA receptor subunit in dendrites, and the insertion of new GluR1 homomeric receptors at the synapse. Research in our lab has verified this phenomenon and uncovered some pieces of the molecular signaling cascade that underlie this form of synaptic scaling.

RA-mediated synaptic scaling

In a series of recent studies (Aoto et al., 2008; Maghsoodi et al., 2008; Poon and Chen, 2008) we revealed a critical role for all-trans retinoic acid (RA) in regulating TTX and APV-induced synaptic scaling. Activity blockade with TTX and APV leads to synthesis of RA in neurons. RA can bind to retinoic acid receptor alpha (RARα), which acts as both a transcription factor in the nucleus and as a translational regulator in dendrites. RARα binds specifically to a known sequence found in the 5' untranslated region (UTR) of a subset of mRNAs, and this binding inhibits translation. RA binding to RARα induces a conformational change in RARα, causing it to release its bound mRNAs and allowing for translation to proceed.

Interestingly, one of the mRNAs recognized by RARα is the mRNA encoding GluR1. Direct RA treatment in neurons mimics the effects of TTX+APV activity blockade, leading to the local translation of GluR1 protein in dendrites, the insertion of GluR1 receptors at the synapse, and a subsequent increase in synaptic strength. Synaptic scaling can be prevented either by blocking RA synthesis or by knocking down RARα, indicating that both of these components are required for this form of plasticity.

Regulators of translation

Though we have identified RA and RARα as key players in regulating the local translation of GluR1 receptors during synaptic scaling, much remains unknown about what other factors may affect the translation of this critical protein. An mRNA can contain multiple cis-acting sequence elements that contribute to its trafficking and translational regulation. These sequences are often found in the 5' or 3' UTRs of the mRNA, and are recognized by trans-acting factors. Trans factors, including proteins and small RNA molecules, bind mRNAs and affect their trafficking, stability, and translational state. For example, the 3'UTR of the mRNA encoding the dendritic protein MAP2 contains both a dendritic targeting element sequence, which is bound by the MAP2-RNA trans-acting (MARTA) family of proteins, and a cytoplasmic polyadenylation element (CPE), which is bound by CPE binding protein (CPEB) (Rehbein et al., 2000; Rehbein et al., 2002; Huang et al., 2003). Both elements contribute to proper localization and translational control of MAP2 mRNA. Tight control of the trafficking and translation of GluR1 mRNA has also been demonstrated, but the specific cis and trans elements which regulate GluR1 remain elusive.

Translation is a complex process which can be regulated at any one of a number of steps. However, because translation initiation is most often the rate-limiting step, it is a frequent target of regulation. Initiation of protein translation is facilitated by the binding of the eIF4F protein complex to the 5' cap of an mRNA. Poly(A) binding protein (PABP) links the 3' poly(A) tail of the mRNA to its 5' end by interacting directly with eIF4E, one of the components in the eIF4F complex. This circularized mRNA is then bound by the 43S preinitiation complex, which contains the 40S ribosomal subunit. This complex scans the 5'UTR of the mRNA until it encounters a start codon, at which point the 60S ribosomal subunit joins the complex and elongation can proceed.

Activity-dependent regulation of the dendritic translation of a given mRNA can occur via a number of different pathways. First, signaling cascades that are triggered at the synapse may modulate recruitment of mRNAs to dendritic or synaptic sites by acting on RNA-binding proteins or cytoskeletal motors. Other RNA-binding proteins, whose RNA-binding affinity can be affected by phosphorylation, may act to either recruit or block the binding of members of the translation initiation complex. mRNAs themselves can be modified in the dendrites, commonly

through the extension of a poly(A) tail. For example, NMDA receptor stimulation triggers phosphorylation of CPEB, which binds to a CPE sequence in the 3'UTR of the CaMKIIα mRNA, triggering polyadenylation and subsequent translation (Huang et al., 2002). In addition to modifications of specific mRNAs or mRNA binding proteins, components of the translational machinery can also be regulated, commonly by phosphorylation. BDNF stimulation, for example, activates the mammalian target of rapamycin (mTOR) signaling cascade, causing phosphorylation of eIF4E-binding proteins, decreasing their affinity for eIF4E and promoting translation (Takei et al., 2004).

The Fragile-X Mental Retardation Protein

One established regulator of translation is the Fragile-X mental retardation protein (FMRP). In human patients, an expansion of CGG repeats in the 5'UTR of the *FMR1* gene, which encodes FMRP, leads to methylation of genomic DNA and silencing of transcription (Oberle et al., 1991). The loss of FMRP expression causes Fragile-X syndrome, the most common inherited form of mental retardation, affecting 1 in 2500 individuals (Hagerman, 2008). Fragile-X patients experience developmental delay, cognitive, speech, and motor impairments, anxiety, social avoidance, hyperactivity, and mood instability (Chonchaiya et al., 2009). Approximately 30% of Fragile-X patients will also be diagnosed with autism (Chonchaiya et al., 2009).

The cellular function of FMRP has been extensively studied, primarily in the context of an *Fmr1* knockout mouse. The mouse model exhibits many behavioral symptoms that mimic the phenotype of Fragile-X patients, including defects in learning, social interactions, perseverance, anxiety, hyperactivity, and responses to sensory stimuli (Mineur et al., 2002; Bernardet and Crusio, 2006). In addition, these knockout mice have impairments in certain forms of LTP (Li et al., 2002; Larson et al., 2005) and enhanced metabotropic glutamate receptor (mGluR)-dependent LTD (Huber et al., 2002).

A large body of evidence implicates FMRP as a regulator of translation. FMRP can bind directly to RNAs through one of three RNA binding domains: two KH domains and an RGG box. The KH domains recognize RNAs possessing a characteristic "kissing complex" structure (Darnell et al., 2005b), while the RGG box recognizes mRNAs with a G-quartet motif (Darnell et al., 2001). FMRP is predicted to bind to up to 4% of mRNAs expressed in the brain (Brown et al., 2001), and a number of screening methods have been employed to identify specific targets (Sung et al., 2000; Brown et al., 2001; Chen et al., 2003; Miyashiro et al., 2003; Zou et al., 2008). FMRP can act as a translational repressor *in vitro* (Laggerbauer et al., 2001), and *in vivo* studies have found elevated translation in *Fmr1* knockout animals (Dolen et al., 2007). A general theme has emerged of both elevated basal translation and dysregulated activity-dependent translation of specific transcripts in the absence of FMRP (Bassell and Warren, 2008).

FMRP has been strongly linked to mGluRs, and one prevailing theory attributes many of the symptoms of Fragile-X syndrome to excessive signaling downstream of mGluRs in the absence of FMRP (Bear et al., 2004). Activation of group I mGluRs leads to a form of LTD which is dependent on local translation, but this plasticity is exaggerated in the absence of FMRP and is no longer blocked by translation inhibitors (Huber et al., 2002). Many Fragile-X phenotypes can be at least partially corrected by the introduction of drugs which block mGluRs or by a reduction in the number of mGluRs expressed (Yan et al., 2005; Dolen et al., 2007; de Vrij et al., 2008).

The specific mechanism by which FMRP regulates mRNA translation is not yet entirely clear. FMRP typically binds its mRNA targets in the 3'UTR, and one known FMRP interacting protein, CYFIP, can bind to eIF4E and may interfere with eIF4F complex formation (Napoli et al., 2008). Interestingly, FMRP can be associated both with translationally repressed messenger ribonucleoprotein (mRNP) particles, as well as with actively translating polyribosomes (Corbin et al., 1997; Zalfa et al., 2003). Phosphorylation at position S499 may regulate the transition of FMRP-bound mRNAs between translationally active and translationally stalled states, with phosphorylation causing inhibition of translation (Ceman et al., 2003).

microRNAs

Another emerging player in the field of translational regulation is a class of small RNA molecules called microRNAs (miRNAs). miRNAs are small (approximately 22 nucleotides) RNA molecules which bind with imperfect base pairing to mRNA targets and inhibit their translation. Encoded either within intergenic regions of the genome or within the introns of protein-coding genes, the initial primary miRNA transcripts are processed by a series of cleavage steps, including final cleavage by an enzyme called Dicer, in order to generate a short mature miRNA. Mature miRNAs are loaded into an RNA-induced silencing complex (RISC), which is comprised of members from the Argonaute (AGO) family of proteins, among others. The RISC is recruited to an mRNA by the interaction between its loaded miRNA and a complementary miRNA recognition element (MRE) sequence, usually located in the 3'UTR of the mRNA. Because miRNAs bind MREs with imperfect base pairing, a given miRNA has the potential to regulate a vast network of mRNAs, and prediction of miRNA targets can be difficult (Tang, 2005).

The precise mechanism by which miRNAs repress protein translation has yet to be defined. Most evidence points to an inhibition of translation independent of mRNA cleavage, though some miRNAs may affect the stability of their mRNA targets (Pillai et al., 2007). Whether miRNAs and the RISC interfere before or after translation initiation is also under debate. Both a 5' cap and poly(A) tail are required on an mRNA in many cases for miRNA-mediated repression (Humphreys et al., 2005), and one proposed mechanism is that AGO interacts directly with the 5' cap to prevent recruitment of eIF4E (Kiriakidou et al., 2007).

The list of evidence describing the contribution of miRNAs to regulation of neuronal function in general and local translation in particular continues to grow. miRNAs which regulate large networks of genes in neurons and play a critical role in cell fate decisions have been identified (Sempere et al., 2004; Krichevsky et al., 2006), and a role for miRNAs in activity-dependent synaptic plasticity have also been uncovered. Dicer is localized to the post-synaptic density (PSD), and it can be activated in a calcium-dependent manner (Lugli et al., 2005). In *Drosophila*, another member of the miRNA machinery called Armitage is not only localized to the synapse, but is locally degraded in response to neuronal activation. This degradation is essential for the formation of new long-term memories (Ashraf et al., 2006). More specifically, a number of individual miRNAs can regulate dendritic morphology and spine size in response to changes in activity (Edbauer et al.; Schratt et al., 2006; Siegel et al., 2009).

Intriguingly, a connection has emerged between FMRP and miRNAs. FMRP binds to miRNAs as well as to Dicer and AGO, and in *Drosophila* AGO1 is required for proper FMRP function (Jin et al., 2004). *In vitro*, FMRP can accept miRNAs after cleavage by Dicer and facilitate their binding to mRNA targets (Plante et al., 2006), and several specific miRNAs which bind directly to FMRP have been identified (Edbauer et al., 2010). Whether FMRP is a

constitutive member of the RISC remains to be seen, but it is feasible that at least some of FMRP's interactions with and repression of mRNAs are mediated through miRNA binding.

Contents of the dissertation

Though both FMRP and miRNAs are critical factors in controlling translation and regulating synaptic plasticity, whether either of these systems is directly involved in controlling the translation of AMPA receptors is unknown. In this dissertation, I will describe my efforts to uncover the molecular mechanisms regulating local translation of the AMPA receptor subunit GluR1, particularly in the context of RA-mediated synaptic scaling.

Chapter 2 will describe the materials and methods used to conduct the experiments.

Chapter 3 will present my results regarding the role of FMRP in synaptic scaling. I established that FMRP is required for RA-induced local translation and the subsequent increase in synaptic strength that occurs in response to activity blockade with TTX+APV. FMRP is not required for RA synthesis, nor is it required for RA-independent forms of homeostatic plasticity, but it is absolutely necessary for induction of the form of synaptic scaling which is expressed via local synthesis of GluR1 receptors. Using different mutant versions of FMRP, I showed that FMRP plays a role in the regulation of baseline AMPA receptor abundance, and that the ability of FMRP to both repress and permit translation is critical for its activity during synaptic scaling.

Chapter 4 will detail my search for miRNAs with the potential to regulate GluR1 translation. I identified a set of co-regulated miRNAs, miR-96 and miR-182, which downregulate GluR1 when overexpressed in neurons. These miRNAs decrease both surface and total GluR1 levels, and prevent induction of synaptic scaling when overexpressed. I used an artificial reporter to demonstrate that this regulation depends on a specific predicted miRNA binding site in the GluR1 3'UTR. However, though both miR-96 and miR-182 are expressed in the brain throughout postnatal development, detection of activity-dependent changes in expression remained elusive, and knockdown of these miRNAs was unable to reveal a significant phenotype.

Chapter 5 will present conclusions, perspectives, remaining questions, and future directions.

Chapter 2
MATERIALS AND METHODS

DNA constructs

The 3xRARE-EGFP reporter construct is as described (Aoto et al., 2008). Briefly, three copies of the retinoic acid response element were placed upstream of a TK promoter driving EGFP. All FMRP constructs used were the full length isoform 1 (Ashley et al., 1993). For CoIP experiments, FMRP was tagged with FLAG at the N terminus, RARα with Myc at the N terminus, and FXR1 with Myc at the C terminus. The lentiviral transfer vector JHUG was derived by Jason Aoto from the original L307 vector. The IRES sequence downstream of a ubiquitin promoter in L307 was deleted and replaced with a multiple cloning site followed by the EGFP coding sequence. Mouse FMRP and FMRP(I304N) coding sequences were then inserted into the MCS. The RGG box [amino acids RRGDGRRRGGGGRGQGGRGRGGGFKGN, as described by Darnell et al., (2005a)] was removed using PCR deletion.

For expression of miRs, the pFantastic vector was created by replacing the Neo cassette of the pCI-Neo vector (Promega) with a PGK promoter driving expression of EGFP (amplified from the pSuper vector, Oligoengine). Approximately 300 base pairs of the primary sequence surrounding each miR was amplified from rat genomic DNA and inserted into the multiple cloning site of pFantastic. To construct the mCherry reporter, the EGFP coding sequence in pEGFP-N1 was replaced by mCherry, with an added destabilizing motif at the 3' end of the protein (AGCCATG GCTTCCCGCC GGAGGTGGAG GAGCAGGATG ATGGCACGCT GCCCATGTCT TGTGCCCAGG AGAGCGGGAT GGACCGTCAC CCTGCAGCCT GTGCTTCTGC TAGGATCAAT GTGTAG). The 3'UTR of GluR1 was inserted downstream of the mCherry stop codon. For the Δbinding site reporter, the entire miR-96/182 binding site (AGCCCATCCCAAGCCCTTCAGTGCCAA) was eliminated using PCR deletion.

Design and construction of the miRNA sponge was as described by (Ebert et al., 2007). Briefly, a modified version of the pEGFP-N1 vector was constructed by using PCR to add a destabilizing motif (see above) to the 3' end of the EGFP sequence. Four copies each of binding sites for miR-96 and miR-182 were inserted downstream of dsEGFP using annealed oligonucleotides inserted between successive pairs of restriction enzyme cut sites. Binding sites were designed to anneal perfectly to the miRNA with the exception of a bulge at miRNA positions 9-12, in order to prevent cleavage of the target. The miR-96 binding site was 5'-TGTGAGTTCTGTGTTGCCAAA-3', and the miR-182 binding site was 5'-GCAAAAATGTTGCGTGCCAAA-3'.

Antibodies

The following mouse monoclonal primary antibodies were used in this study: actin, FMRP, GluR1 N terminus, GluR2, GFP, and RARα (Millipore), PSD95 (Affinity Bioreagents), NR1 (BD Pharmingen), Arc (Santa Cruz), Flag (Sigma), Myc (Roche). The following rabbit polyclonal primary antibodies were used: GluR1 (Millipore), EF2 and Phospho-EF2 (Thr56) (Cell Signaling), Stargazin and Myc (Abcam), MAP1b 750 (a generous gift from Dr. Itzhak Fischer). The following goat polyclonal secondary antibodies were used: anti-rabbit cy2, anti-

mouse cy2, anti-rabbit cy3, anti-mouse cy3, anti-rabbit horseradish peroxidase (HRP) and anti-mouse HRP (all from Jackson ImmunoResearch).

Drugs and Chemicals

The following drugs and chemicals were purchased from Sigma Aldrich: all-trans retinoic acid, actinomycin D, cycloheximide, picrotoxin, philanthotoxin-433, and 4-(diethylamino)-benzaldehyde (DEAB). Tetrodotoxin was purchased from Tocris Biosciences, and D-APV from Fisher.

Mice

Wild-type and *Fmr1* knockout mice in the FVB background were obtained from Jackson Labs (Bar Harbor, Maine).

Cell Cultures and Drug Treatment:

Primary hippocampal cultures were prepared from mice at postnatal day 0 or 1, or from rats at embryonic day 22, and maintained in serum-free Neurobasal medium supplemented with B-27 and Glutamax (GIBCO) for 2 weeks *in vitro* (Nam and Chen, 2005). Hippocampal slice cultures were prepared from P6 or P7 animals and maintained in Neurobasal-A medium supplemented with horse serum (Hyclone), insulin (Sigma), and Glutamax (Aoto et al., 2008). Stock solutions of all-trans RA in DMSO were freshly made immediately before treatment, and the final concentration of DMSO in culture media was 0.05% or lower. Twenty-four-hour treatment of 1 μM TTX and 100 μM APV was used to induce synaptic scaling in dissociated cultures, and 36-hr treatment of 10 μM TTX and 1 mM APV was used to induce synaptic scaling in slice cultures. 4 hour treatment of 2 μM RA was used to induce synaptic scaling in slice cultures, and 30 minute treatment of 1 μM RA followed by 1 hour of washout was used to induce synaptic scaling in dissociated cultures. Where indicated, 100 μM cycloheximide or 0.5 μg/mL Actinomycin-D was applied for 30 minutes prior to RA treatment, and remained in the media during RA treatment. To induce RA-independent synaptic scaling, 48-hour treatment of 1 μM TTX in dissociated culture or 60-hour treatment of 10 μM TTX in slice culture was used. 10 μM DEAB was applied where indicated.

Acute Slices

For analysis of activity-regulated miRNA expression, acute 350 μm thick coronal slices of cortex were made using a tissue chopper from rats at age P21. Slices recovered at room temperature for one hour in external recording solution (see above), continually bubbled with CO_2 and O_2. Slices were treated for three minutes with either 90 mM KCl or 100 μM glutamate, or mock treatment, and then returned to the recovery solution for the indicated time before RNA isolation.

RARE assay

Dissociated cultures used for RARE imaging were transfected using Lipofectamine 2000 (Invitrogen) with a protocol described previously (Aoto et al., 2008), and were fixed with 4% paraformaldehyde (15 min, room temperature) and washed with PBS before mounting. Images were acquired and quantified as described previously (Nam and Chen, 2005) using an Olympus FV1000 BX61WI laser-scanning confocal microscope.

Lentivirus Production and Infection of Slices and Dissociated Neurons

Lentivirus was produced and purified as previously described (Aoto et al., 2008). Briefly, human embryonic kidney 293T cells were transfected using calcium phosphate with the transfer vector and three helper plasmids. After 48 hr, supernatants were pooled, spun at 25,000 rpm through a sucrose cushion for 1.5 hr and resuspended in PBS. Virus was injected into the CA1 region of slices on the day of cutting. For imaging, slices at 6-7 DIV were fixed overnight in 4% paraformaldehyde at 4°C. Slices were washed in PBS, mounted, and imaged as described above. To infect dissociated cells, purified virus was applied to the culture media overnight and washed out the following day. Neurons were infected at DIV 7 and lysates harvested at DIV 13 to mimic the expression time seen in slice cultures.

Electrophysiology

Patch-clamp recordings from the CA1 region of slice cultures were made at room temperature from 5-7 DIV slices with a 4–6 MOhm patch pipette filled with an internal solution containing (in mM) 140 CsCl, 2 $MgCl_2$, 5 EGTA, 10 HEPES, 0.3 Na_3-GTP, 4 Na_2-ATP (pH 7.35). Slices were continuously superfused with external solution (in mM, 120 NaCl, 26 $NaHCO_3$, 2.5 KCl, 11 glucose, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.0 NaH_2PO_4). Tetrodotoxin (1 μM) and picrotoxin (100 μM) were included in the external saline, along with 5 μM philanthotoxin where indicated. Cells were held at -60 mV. Miniature responses were analyzed with Mini Analysis Program (Synaptosoft).

Outside-out patches were formed by pulling the electrode away from the cell after a whole cell patch was formed. Glutamate (1 mM) and cyclothiazide (100 μM) in external recording solution were perfused directly onto the patch for a duration of two seconds.

Surface Biotinylation Assay

Cultured hippocampal cells were washed with cold PBS/$Mg^{2+/}Ca^{2+}$, and surface proteins were biotinylated with 1 mg/ml Ez-link sulfo-NHS-SS-biotin (Pierce) in PBS/$Mg^{2+/}Ca^{2+}$ for 25 min on ice. Cells were washed with 0.1 M glycine in ice-cold PBS/$Mg^{2+/}Ca^{2+}$ to stop further biotinylation of the surface proteins. After additional washes with ice-cold PBS, cells were collected and solubilized in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.25% Na-deoxycholate, 1% NP-40, 1 mM EDTA, 0.1% SDS, protease inhibitor cocktail). Lysates were centrifuged to remove cell debris and nuclei at 14,000 rpm for 20 minutes and supernatants were rotated with Ultralink-immobilized streptavidin beads (Fisher) for 2 hours at 4ºC to bind biotinylated proteins. Beads were then pelleted and washed four times with lysis buffer. Biotinylated surface proteins were eluted with denaturing buffer at 65°C. Surface-expressed AMPA receptors were detected by western blot analysis.

Synaptoneurosome Preparation

Whole hippocampi or cultured hippocampal slices were gently homogenized in a solution containing 33% sucrose, 10 mM HEPES, 0.5 mM EGTA (pH 7.4), and protease inhibitors. Nuclei and other debris were pelleted at 2000 x g for 5 min at 4°C and the supernatant filtered through three layers of 100 μm pore nylon mesh (Millipore) and a 5 μm pore PVDF syringe filter (Millipore). The filtrate was then centrifuged for 10 min at 10,000 x g at 4°C, and the supernatant removed. The synaptoneurosome-containing pellet was then resuspended in lysis buffer (see above).

Spine Morphology Assay

Neurons were transfected as described above at DIV12-13 with pSUPER, a plasmid that expresses high levels of EGFP. Cells were then treated, fixed, and imaged as described above. 2-3 secondary branches per cell were analyzed for spine density and spine length using Matlab software; length was determined by measuring the distance from the dendritic shaft to the spine tip.

qPCR

RNA from cultured slices or synaptoneurosomes was isolated using the Aurum Total RNA Mini Kit (Bio-Rad). Equal amounts of RNA from each sample were reverse transcribed using SuperScriptII transcriptase and random hexamer primers (Invitrogen) according to manufacturer's protocol. qPCR was performed using Sybr Green supermix (Bio-Rad) on an iQ5 thermal cycler (Bio-Rad). Ct values obtained from triplicate technical replicates for each sample were averaged, and relative abundance was determined using a dilution curve. Expression levels for all genes were normalized to GAPDH. GluR1, GluR2, and GAPDH primer sequences were adapted for mouse from those used by (Dijk et al., 2004). All primers were tested for specificity and efficiency using melting curve and dilution curve analysis. Primer sequences are available in table 2.1.

RT-PCR of microRNAs

RNA was collected from animal tissues or treated slices using Trizol (Invitrogen), according to manufacturers instructions. Stem-loop RT-PCR for mature miRs was performed as described by (Varkonyi-Gasic et al., 2007). Briefly, reverse transcription was performed using SuperScriptIII (Invitrogen) and a stem-loop primer specific for each miR. Pulsed RT temperature cycling was used: 30 min at 16°C, followed by 60 cycles at 30°C for 30 s, 42°C for 30 s and 50°C for 1 s. End-point PCR was performed with miR specific forward primers and a universal reverse primer under the following cycling conditions: 94°C for 2 min, followed by 20-40 cycles of 94°C for 15 s and 60°C for 1 min. Products were visualized on 3% agarose gels. Primer sequences are presented in table 2.2.

^{35}S Metabolic Labeling and Immunoprecipitation

Culture media was removed from dissociated neurons at 13-14 DIV and cells were washed twice and maintained in DMEM lacking methionine and cysteine (GIBCO). RA or DMSO was added along with EasyTag Express^{35}S protein labeling mix (Perkin Elmer). After two hours media was removed and cells were washed twice with ice cold PBS. Immunoprecipitation was performed as described by(Muddashetty et al., 2007). Briefly, cells were lysed with rotating at 4° in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate). Debris was pelleted with centrifugation, and supernatants were rotated for 1 hour at 4° with the appropriate antibody. Protein G beads (Invitrogen) were added and rotated with lysates overnight at 4°. Beads were washed one time in lysis buffer, three times in wash buffer 2 (50mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.1% NP40, 0.05% Na-deoxycholate), three times in wash buffer 3 (50 mM Tris-HCl, pH 7.4, 0.1% NP40, 0.05% Na-deoxycholate), and in cold PBS. Samples were eluted in SDS sample buffer, loaded on polyacrylamide gels, and transferred to PVDF membranes. The appropriate sized band (identified by Western blot) was cut out from the membrane and analyzed for incorporated radioactivity using liquid scintillation counting. Background cpm (determined by analyzing a similar-sized band cut out

Table 2.1. Primer sequences used for qPCR

Gene	Forward primer	Reverse primer
GluR1	CGAGTTCTGCTACAAATCCCG	TGTCCGTATGGCTTCATTGATG
GluR2	TTGAGTTCTGTTACAAGTCAAGGGC	AGGAAGATGGGTTAATATTCTGTGCA
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGA

Table 2.2. Primer sequences used for RT-PCR of miRNAs

miRNA	**Stem loop (RT) primer**
96	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCAAA
182	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGGTGT
134	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCCTC
miRNA	**Forward primer**
96	GCGGCTTTGGCACTAGCACATT
182	CTAGCTTTGGCAATGGTAGAACTC
134	GCTCGTGTGACTGGTTGACCA
Reverse primer (universal)	GTGCAGGGTCCGAGGT

from an unstained region of each lane) were subtracted from the AMPAR cpm values. Duplicate technical replicates were averaged for each sample.

Co-IP

HEK293T cells were transfected (using calcium phosphate) with equal amounts of each construct, as indicated. 24 hours after transfection, cells were washed and resuspended in PBS, pelleted, and lysed with rotating for 30 minutes at 4º (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.5% NP40, protease inhibitors). Samples were spun to pellet nuclei, and lysate was pre-cleared with Protein-G beads (Invitrogen). Cleared lysates were rotated at 4º with antibody for 4 hours, then with beads overnight. Beads were washed 3 times in lysis buffer, and bound protein was eluted in SDS sample buffer.

Immunocytochemistry

9-11 DIV neurons were transfected with Lipofectamine 2000 as described above. Immunocytochemistry was performed on cultures fixed with 4% paraformaldehyde (15min, room temperature) and washed with PBS containing 0.3% Triton X100 before blocking and incubation with primary and secondary antibodies in PBS containing Triton and 2% normal goat serum. Signal intensity in transfected cells was normalized to the signal intensity in neighboring untransfected cells, in order to account for non-uniform staining across the coverslip.

3'UTR Assay

The full-length or Δbinding site mCherry UTR reporters were transfected along with the indicated miRNAs (in the pFantastic vector) into HEK293T cells using HEKfectin (Bio-Rad) according to manufacturers instructions. Twenty-four hours after transfection, cells were fixed and imaged as described above. Laser powers were set to ensure no saturation of signal intensity in either the red or green channel. mCherry signal intensity for each cell was normalized to EGFP intensity from the same cell in order to control for variations in transfection efficiency.

miRNA sponge HEK cell assay

Either dsEGFP or dsEGFP-sponge UTR constructs were transfected in HEK293T cells along with the indicated miRNAs. Twenty-four hours after transfection, cells were resuspended and lysed (50mM Tris pH 7.5, 150mM NaCl, 5mM EDTA, 1%
Triton X-100, protease inhibitors). Equal amounts of protein were analyzed by SDS-PAGE and immunoblotting for GFP.

Statistical Analysis

Single-factor ANOVA was used for statistical analysis unless otherwise stated. Values are presented as mean ± SEM in the figures.

Chapter 3
Fragile-X protein FMRP is required for RA-induced homeostatic plasticity

INTRODUCTION

Homeostatic synaptic plasticity, working in concert with Hebbian-type synaptic plasticity, refines neuronal connectivity during development and contributes to network stability (Davis and Bezprozvanny, 2001; Turrigiano and Nelson, 2004; Kaneko et al., 2008). One well-studied form of homeostatic plasticity, called synaptic scaling, is induced by long-term blockade of neuronal firing and synaptic transmission, and is manifest as new translation and insertion of AMPA-type glutamate receptors (AMPARs) (Ju et al., 2004; Thiagarajan et al., 2005; Sutton et al., 2006; Aoto et al., 2008).

Our lab recently reported a critical role for all-trans retinoic acid (RA) in the induction of the synaptic scaling form of homeostatic plasticity (Aoto et al., 2008). Inhibition of action potential firing with TTX, along with blockade of NMDA receptors with APV, stimulates synthesis of RA in neurons. RA alone is both necessary and sufficient to induce local translation and synaptic scaling, placing RA into a key role in regulating synaptic strength (Aoto et al., 2008). The effect of RA is mediated by dendritically localized retinoic acid receptor RARα, which inhibits protein translation through direct binding to target mRNAs containing a specific target sequence (Poon and Chen, 2008). Addition of RA reverses the RARα-dependent repression of translation of target mRNAs (Maghsoodi et al., 2008; Poon and Chen, 2008), and acute knockdown of RARα completely blocks synaptic scaling (Aoto et al., 2008). One of the RARα targets is the mRNA encoding GluR1, an AMPA receptor subunit (Poon and Chen, 2008). TTX+APV treatment or direct RA application leads to the local translation of GluR1 receptors in dendrites and the insertion of GluR1 homotetramers at the synapse, increasing synaptic strength (Aoto et al., 2008).

FMRP, encoded by the *Fmr1* gene, is another dendritically localized RNA-binding protein. Absence of FMRP in human patients causes Fragile-X syndrome, the most common inherited form of mental retardation. FMRP knockout mice exhibit normal baseline synaptic transmission, but have altered spine morphology (Comery et al., 1997; Irwin et al., 2000), impairments in certain forms of LTP (Li et al., 2002; Larson et al., 2005), and exaggerated mGluR-dependent LTD (Huber et al., 2002).

FMRP is associated both with translationally repressed messenger ribonucleoprotein particles (mRNPs) and with actively translating polyribosomes (Corbin et al., 1997; Zalfa et al., 2003), and is believed to specifically bind to mRNAs and regulate their translation (Laggerbauer et al., 2001; Li et al., 2001; Bassell and Warren, 2008). FMRP has two KH domains, which bind to RNAs with a specific "kissing complex" structure, and one RGG box, which binds to G-quartet RNAs (Darnell et al., 2001; Darnell et al., 2005b). Debate continues regarding which domain is more critical for RNA binding, and each domain may interact with a distinct subset of mRNAs. Consistent with a proposed role for FMRP as a translational repressor, dysregulated translation and elevated basal protein synthesis are found in *Fmr1* knockout neurons (Dolen et

al., 2007; Muddashetty et al., 2007). However, whether FMRP is involved in translational regulation during homeostatic plasticity is unknown.

The search for mRNAs which bind directly to FMRP has yielded a number of validated targets that may contribute to synaptic plasticity, including Map1b, CaMKII, and Arc (Brown et al., 2001; Hou et al., 2006; Zalfa et al., 2007; Park et al., 2008). Notably, a recent report identified mRNAs encoding the synaptic proteins Shank1, SAPAP1-3, PSD-95 and the NMDA-type glutamate receptor subunits NR1 and NR2B as being associated with FMRP (Schuett et al., 2009), indicating a likely role for FMRP in regulating synaptic strength. The contribution of FMRP to regulation of AMPA receptors, however, remains unclear. Though elevated GluR1 and GluR2 mRNA levels have been found in the polyribosomes of *Fmr1* knockout mice (Muddashetty et al., 2007), no direct binding between FMRP and any AMPA receptor mRNA has been demonstrated.

Here we report that FMRP is required postsynaptically for the form of synaptic scaling that is mediated by RA. While RA synthesis is normal in *Fmr1* knockout neurons, RA-induced local translation of specific mRNAs is impaired. As a consequence, activity blockade or RA treatment fails to increase synaptic strength in the absence of FMRP. Intriguingly, the requirement for FMRP is specific to the form of synaptic scaling which is expressed by local translation of GluR1 receptors. Reintroduction of FMRP into knockout neurons reduces AMPA receptor abundance, and an intact RGG box is required for this effect. Finally, FMRP must be able to enter actively translating polyribosomes in order for synaptic scaling to occur. These data reveal an unanticipated role for FMRP in homeostatic synaptic plasticity and the translational control of AMPA receptors.

A version of this chapter was published in the Journal of Neuroscience.

RESULTS

FMRP is required for TTX+APV-induced synaptic scaling

To directly investigate a possible role for FMRP in homeostatic plasticity, we examined the effect of activity blockade on synaptic transmission in cultured hippocampal slices from *Fmr1* knockout mice. One effective measure of synaptic strength is miniature EPSC (mEPSC) events. mEPSCs are the currents caused by the spontaneous release of single vesicles of neurotransmitter from a presynaptic cell. The average size of the mEPSCs is an indication of how many receptors are on the postsynaptic side, and the frequency of events is reflective of both the number of synapses formed on a postsynaptic cell and the probability of vesicle release at those synapses.

While 24 hours of TTX+APV is sufficient to induce homeostatic plasticity in dissociated neurons, 36 hours of treatment is required for robust scaling in slice culture (Aoto et al., 2008). TTX+APV treatment increased the amplitude of mEPSC events in slices obtained from wild-type mice (Figure 3.1A-C). By contrast, TTX+APV had no effect on mEPSCs in slices from *Fmr1* knockout mice (Figure 3.1A-C), indicating that loss of FMRP causes a defect in synaptic scaling. Consistent with previous reports (Braun and Segal, 2000), the baseline amplitude and frequency of mEPSC events was not different between wild-type and knockout slices (Figure 3.1C, D). Neither genotype showed a change in the frequency of mEPSCs after treatment (Figure 3.1D).

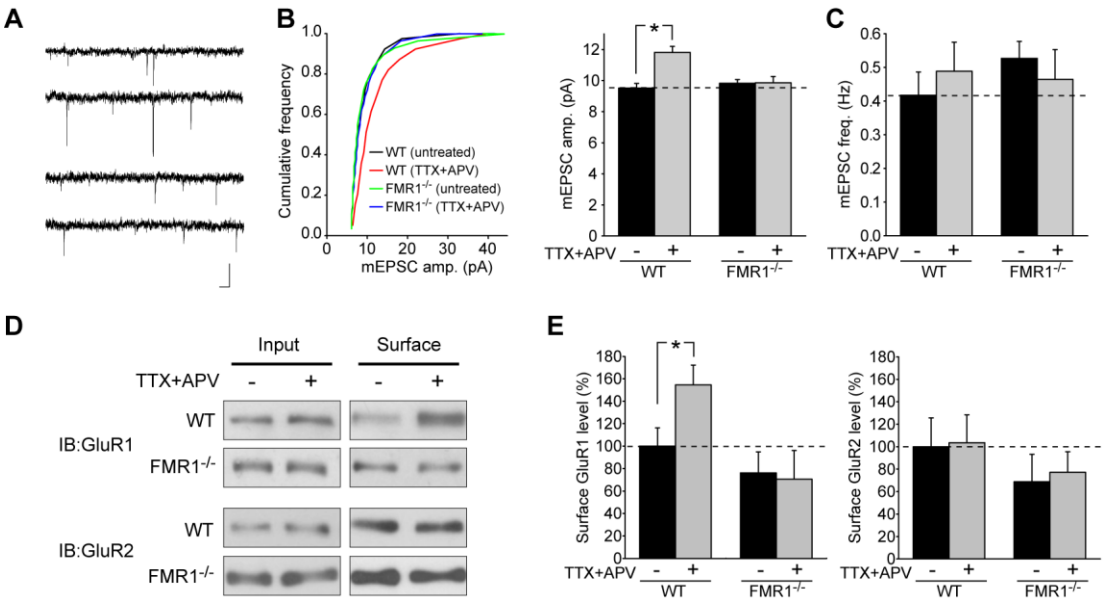

Figure 3.1. FMRP is required for TTX+APV-induced synaptic scaling. (A) Representative mEPSC traces from wild-type and *Fmr1* knockout (untreated and TTX+APV treated) neurons in hippocampal slice culture. Scale bar: 10 pA, 40 ms. **(B)** Cumulative distribution of mEPSC amplitudes from WT and KO neurons treated with 36 hours TTX+APV ($p < 0.001$, Kolmogorov-Smirnov test). **(C)** Quantification of average mEPSC amplitude (n = 28-34; ***, $p<0.001$). **(D)** Quantification of average mEPSC frequency (n= 28-34, $p>0.5$). **(E)** Representative blots for biotinylation of surface AMPARs in primary cultured neurons after 24 hours TTX+APV treatment. **(F)** Quantification of **E**. Surface band intensity was normalized to input, and all groups were compared to WT untreated (n = 4-6; *, $p<0.05$). In all figures, error bars represent SEM.

During synaptic scaling induced by TTX+APV, the AMPA receptor subunit GluR1 is synthesized locally in dendrites, and homomeric GluR1 AMPA receptors are inserted into the synaptic membrane, thereby increasing the strength of the synapse (Ju et al., 2004; Sutton et al., 2006; Aoto et al., 2008). Consistent with this process, activity blockade with TTX+APV caused a significant increase in the levels of surface GluR1, but not GluR2 protein, in wild-type neurons (Figure 3.1E, F). However, TTX+APV treatment failed to increase the surface levels of either GluR1 or GluR2 protein in neurons from *Fmr1* knockout mice (Figure 3.1E, F). This result corroborates the impairment in homeostatic plasticity seen with electrophysiology, and indicates that FMRP is required for synaptic scaling upstream of the insertion of new GluR1 receptors into the plasma membrane. Dendritic GluR1 and GluR2 mRNA levels are normal in *Fmr1* knockout neurons (Muddashetty et al., 2007), and the basal levels of GluR1 and GluR2 protein in both whole hippocampal lysate and synaptoneurosomes were not different between wild-type and knockout mice (Figure 3.2A-C). In addition, we saw no difference in the levels of RARα protein (Figure 3.2A-C), which is also required for synaptic scaling (Aoto et al., 2008).

RA synthesis is normal in *Fmr1* knockout neurons

Because RA synthesis is both necessary and sufficient for synaptic scaling (Aoto et al., 2008), we tested whether impaired synaptic scaling in *Fmr1* knockout neurons is due to altered RA synthesis, using a genetic reporter system (Aoto et al., 2008). Dissociated hippocampal neurons from wild-type or *Fmr1* knockout mice were transfected with a plasmid containing multiple copies of a retinoic acid response element (RARE) driving transcription of GFP (Figure 3.3A). RARα is not only a translational regulator, but also a transcription factor that binds to RARE sequences in the presence of RA and promotes transcription of GFP from the reporter plasmid. Thus, the GFP intensity in transfected neurons serves as a readout of RA levels in those neurons. Using this system, we found that TTX+APV treatment caused a significant increase in the intensity of GFP fluorescence in both wild-type and knockout neurons (Figure 3.3B, C), demonstrating that FMRP is not required for the stimulation of RA synthesis in response to activity blockade.

RA-dependent scaling requires FMRP and new protein translation

Since RA synthesis is maintained in *Fmr1* knockout neurons, we wondered whether direct application of RA is still capable of up-scaling synaptic strength in these neurons. In cultured hippocampal slices from wild-type mice, 4 hours of RA treatment caused a significant increase in mEPSC amplitude without affecting event frequency (Figure 3.4A-C). The increase in mEPSC amplitude in wild-type neurons was fully reversed by philanthotoxin-433 (PhTx), a blocker of GluR2-lacking AMPA receptors (Figure 3.4B), indicating that the increase in synaptic strength after RA treatment is caused by insertion of homomeric GluR1 receptors at the synapse. In addition, RA-induced up-scaling in wild-type slices was unaffected by transcription blockers, but required *de novo* translation of pre-existing mRNAs (Figure 3.4D). This is similar to previous findings demonstrating increased local translation of specific proteins during TTX+APV or RA-mediated synaptic scaling (Aoto et al., 2008). Importantly, RA treatment did not increase synaptic strength in slices from *Fmr1* knockout mice (Figure 3.4A, B). Thus, FMRP is required for synaptic scaling induced by TTX+APV or RA, and acts downstream of RA.

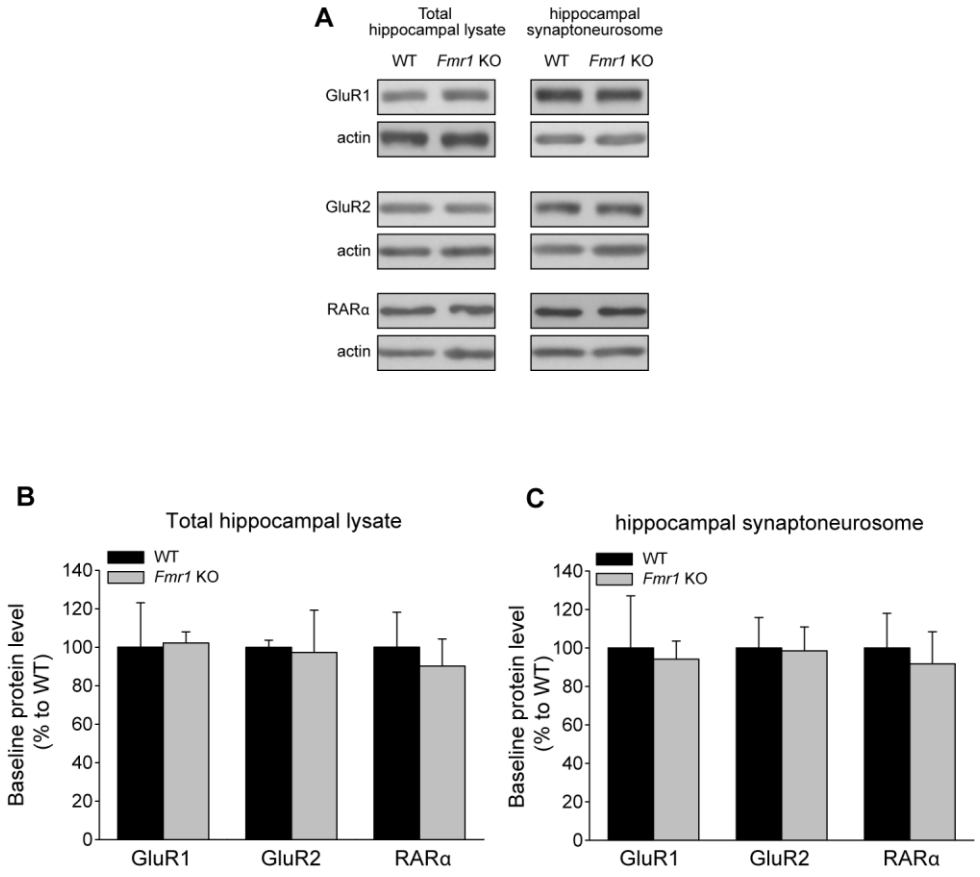

Figure 3.2. Baseline AMPAR and RARα levels are unaltered in *Fmr1* knockout animals **(A)** Representative blots and **(B-C)** quantification of protein levels from whole cell lysate or the SNS fraction from hippocampi of P14 mice. Band intensities were normalized to actin (n = 3, $p>0.5$).

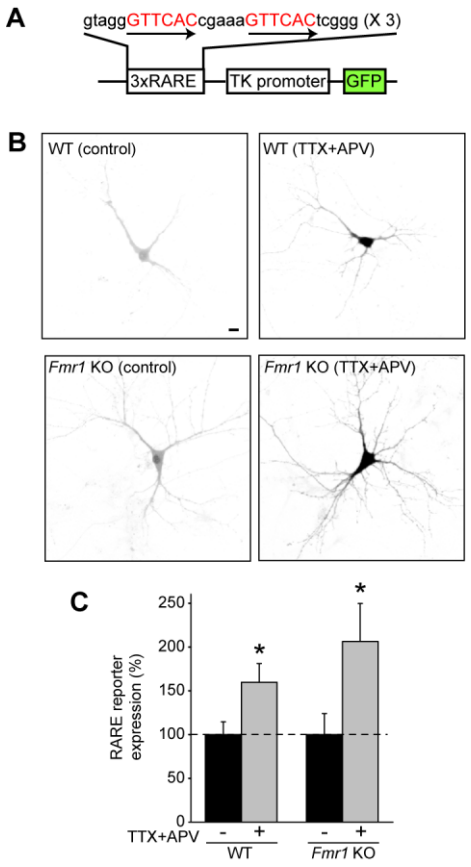

Figure 3.3. FMRP is not required for RA synthesis. (A) Schematic of the 3xDR5-RARE-GFP reporter construct. **(B)** Representative images of RARE-GFP reporter expression in WT and KO neurons with and without 24 hours TTX+APV treatment. Scale bar, 10 μm. **(C)** Quantification of **B** (n = 16-18; *, $p<0.05$).

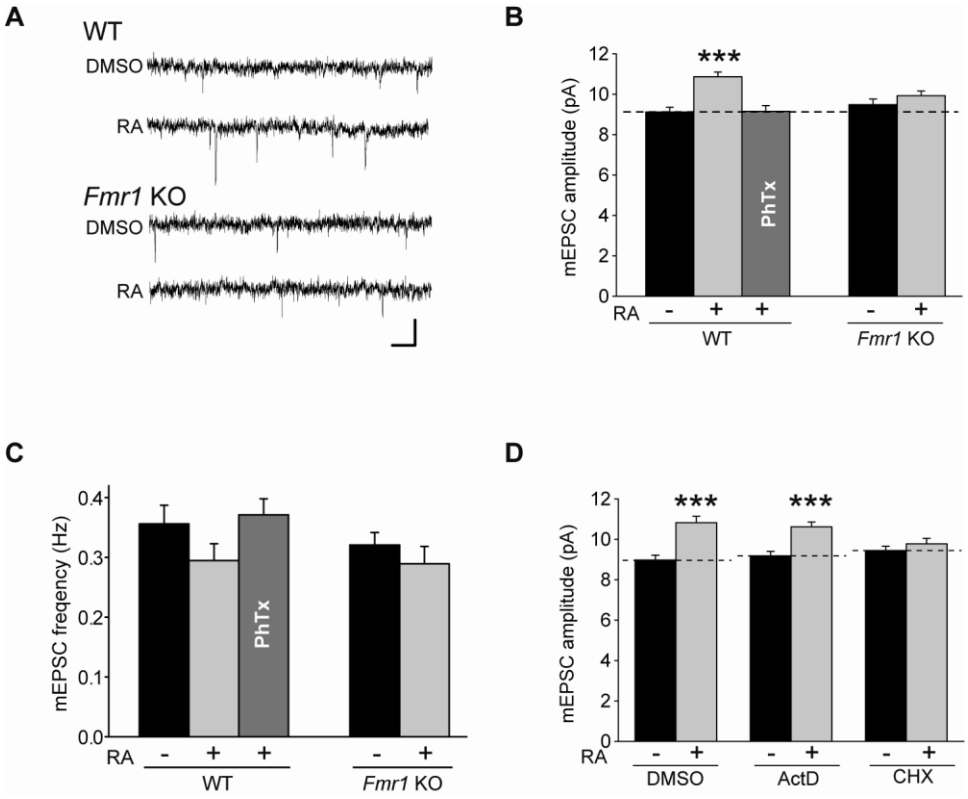

Figure 3.4. FMRP is specifically required for RA-induced local translation-dependent synaptic scaling. (A) Representative traces and **(B)** quantification of mEPSC amplitude in WT and KO neurons after four hours DMSO or RA treatment (n = 31-33; ***, $p<0.001$). Philanthotoxin-433 was used to block GluR2-lacking AMPA receptor-mediated responses in WT-RA group (n = 22). Scale bar: 10 pA, 40 ms. **(C)** Quantification of mEPSC frequency (n = 31-33, $p>0.1$). **(D)** Effect of transcription inhibitor Actinomycin D and translation inhibitor cycloheximide on RA-induced synaptic scaling in WT neurons (n = 22-27; ***, $p<0.001$).

RA treatment does not affect synaptic AMPAR mRNA levels or spine morphology

FMRP is known to play a role in the activity-dependent dendritic trafficking of specific mRNAs (Dictenberg et al., 2008). We wondered whether RA induces the movement of mRNAs towards synapses, and if so, whether FMRP is required for this process. Synaptoneurosomes were collected from wild-type or knockout cultured hippocampal slices treated with DMSO or RA, and total RNA was isolated from these preparations. qPCR showed no differences in AMPAR mRNA levels between wild-type and knockout synaptoneurosomes (Figure 3.5). Also, no effect of RA on mRNA levels was found in either genotype (Figure 3.5). This implies that AMPAR mRNA trafficking does not play a significant role in synaptic scaling, and that FMRP has no obvious effect on the synaptic localization of these mRNAs.

Neurons from FMRP knockout mice have altered dendritic spine morphology, showing an increased spine length and a larger proportion of immature spines (Comery et al., 1997) (Nimchinsky et al., 2001; Grossman et al., 2006). Because changes in spine morphology are known to accompany changes in synapse strength (Muller et al., 2000; Matsuzaki et al., 2004), we asked whether TTX+APV or RA treatment affects spine morphology, and whether this might account for impaired homeostatic plasticity in FMRP knockout animals. Analysis of GFP-expressing wild-type and knockout neurons treated with TTX+APV or RA found no changes in spine density between genotypes or between treatments (Figure 3.6A, B), confirming our physiology results showing no change in mEPSC frequency during homeostatic plasticity (Figures 3.1D and 3.4C). Consistent with the literature, we found an increased average spine length in knockout neurons compared to wild-type, but neither TTX+APV nor RA treatment had any effect on spine length in either genotype (Figure 3.6A, C). Though this does not rule out that subtle spine shape changes may occur during synaptic scaling, we find no obvious link between the FMRP knockout altered spine phenotype and the inability of these neurons to increase their synaptic strength after TTX+APV or RA treatment.

RA and FMRP are not involved in the slow transcription-dependent form of synaptic scaling

Activity blockade with TTX and APV, applied for 24 hours in dissociated neurons or 36 hours in slice culture, is only one of several manipulations that can be used to induce homeostatic plasticity. A similar magnitude of increase in mEPSC amplitude is seen after long term treatment with TTX only [48 hours in dissociated neurons (Turrigiano et al., 1998) or 60 hours in slice culture (our results)]. Different from the rapid GluR1-dependent up-scaling induced by TTX+APV, up-scaling produced by TTX alone is mediated by an increase in GluR1/GluR2 heteromeric receptors, and is transcription-dependent (Wierenga et al., 2005; Ibata et al., 2008). We wondered whether FMRP is also necessary for this slower, transcription-dependent scaling induced by TTX alone. First, we confirmed that in our hands 36 hours of TTX alone treatment in wild-type slice cultures is insufficient to induce synaptic scaling (Figure 3.7A, B). Intriguingly, however, we found that long-term (60 hour) TTX treatment induced synaptic up-scaling of mEPSC amplitudes even in the absence of FMRP (Figure 3.7C, D). The increase in synaptic strength was not reversed by philanthotoxin, confirming that the change in mEPSC amplitude is caused by the insertion of GluR2-containing receptors (Figure 3.7C).

The specific involvement of FMRP in TTX+APV- and RA-induced synaptic scaling, but not in TTX-induced scaling, suggests that RA may not be involved in the slow, transcription-dependent form of homeostatic plasticity. Indeed, when we used the RARE reporter to measure RA synthesis after 48 hours of TTX treatment in dissociated neurons, we found no increase in

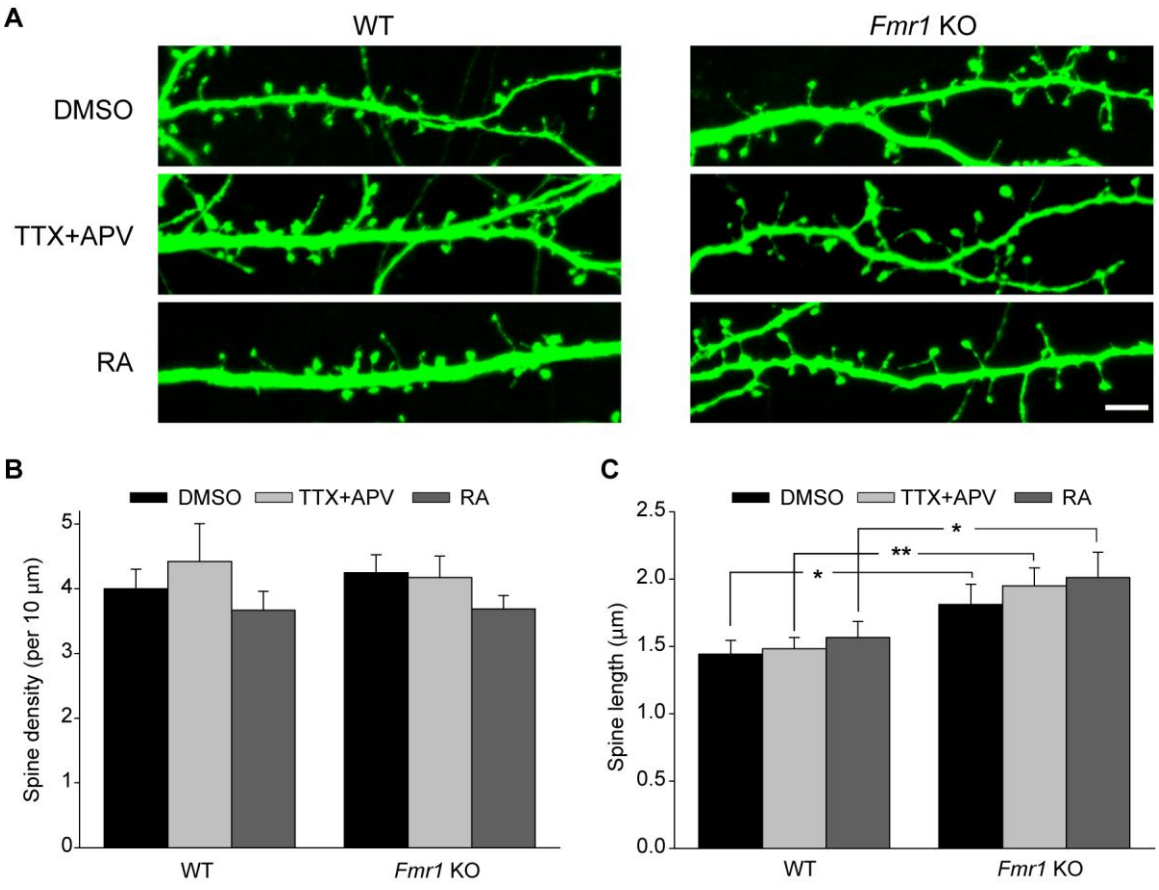

Figure 3.5. Synaptic AMPAR mRNA levels are not affected by FMRP or by RA treatment. Fold change in GluR1 **(A)** and GluR2 **(B)** SNS mRNA level after 1 or 2 hours of RA treatment in cultured slices. Expression was normalized to GAPDH. (n = 3, p>0.5).

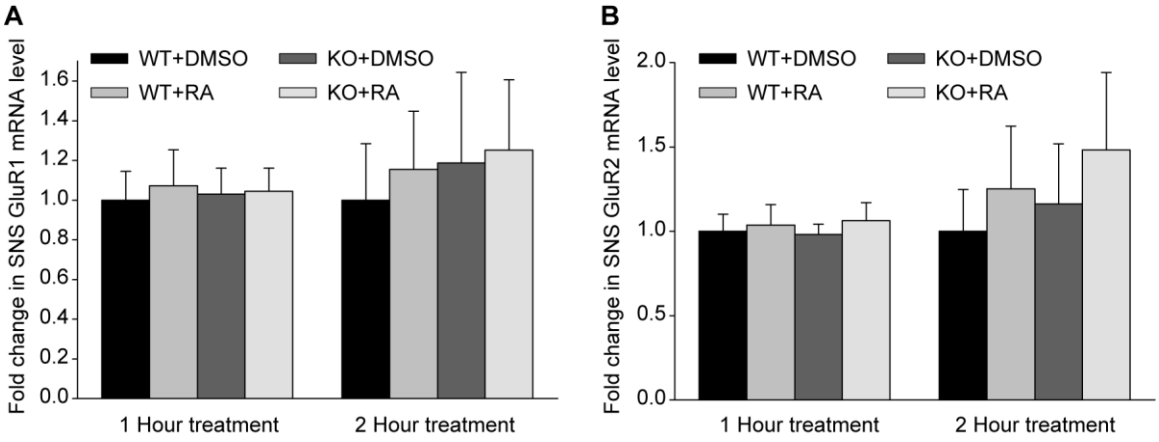

Figure 3.6. RA does not affect spine morphology in WT or KO neurons. (A) Sample images of GFP-expressing WT or KO neurons treated with TTX+APV or RA. Scale bar = 5 μm. **(B and C)** Quantification of spine density and spine length in WT and KO neurons treated with 24 hours of TTX+APV or 30 minutes (plus 1 hour washout) of RA. (n = 9-10 cells/group, 2-3 branches/cell. For spine density, $p>0.5$. For spine length *, $p<0.05$, **, $p<0.01$).

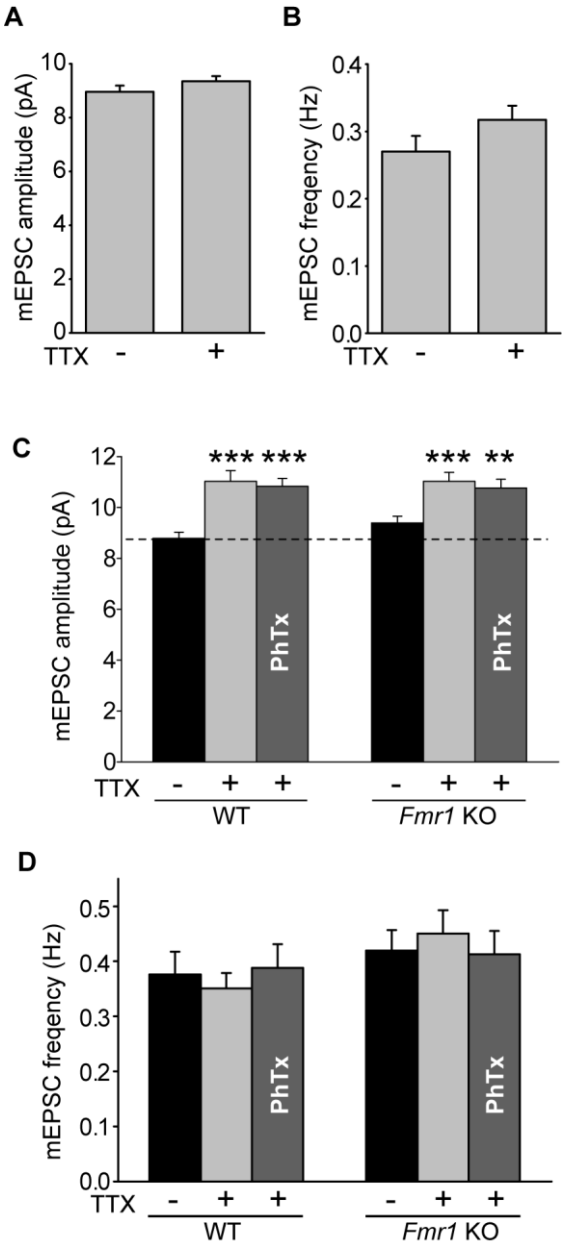

Figure 3.7. FMRP is not required for TTX alone-induced synaptic scaling. (A) Quantification of mEPSC amplitude, and **(B)** mEPSC frequency, from WT neurons treated with 36 hours of TTX only (n = 36-38, p>0.1). **(C)** 60 hours of TTX induced synaptic scaling in WT and KO neurons. Philanthotoxin-433 was used to block GluR2-lacking AMPA receptor-mediated responses. (n = 18-39; **, p<0.01; ***, p<0.001). **(D)** Quantification of mEPSC frequency from WT and KO neurons treated with 60 hours of TTX only (n = 18-39, p>0.5).

GFP fluorescence in either wild-type or knockout neurons (Figure 3.8A, B), indicating no change in RA levels. Moreover, blocking RA synthesis with 4-(diethylamino)-benzaldehyde (DEAB), an inhibitor of retinal dehydrogenase (an enzyme in the RA synthesis pathway), blocked TTX+APV-induced scaling, but did not prevent synaptic scaling induced by long-term TTX-alone treatment in wild-type or *Fmr1* knockout slices (Figure 3.8C, D). Thus, FMRP and RA synthesis are both specifically required for the form of synaptic scaling that is induced by TTX+APV and accomplished via local translation.

Translation of RARα target mRNAs requires FMRP

To probe the mechanism by which FMRP acts downstream of RA in synaptic scaling, we examined the local synthesis of synaptic proteins in dendrites in response to RA. We isolated total lysates and synaptoneurosomes from wild-type and *Fmr1* knockout hippocampal slices treated with DMSO or RA, and examined the levels of synaptic proteins by Western blotting. We found that in synaptoneurosomes from wild-type slices, RA significantly increased the levels of GluR1, GluR2, and eEF2 proteins (Figure 3.9A, C). This effect was blocked by cycloheximide, indicating that the change is dependent on new protein translation (Figure 3.9D). RA also marginally increased the levels of FMRP, but this was not statistically significant (Figure 3.9C). RA had no effect on the levels of other synaptic proteins examined, including phosphorylated eEF2, PSD-95, Stargazin, or NR1 (Figure 3.9A, C).

Strikingly, RA treatment failed to elicit changes in any synaptic protein in synaptoneurosomes from *Fmr1* knockout slices (Figure 3.9A, C), indicating that FMRP is indeed required for RA-stimulated increases in synaptic protein levels. In contrast to synaptoneurosomes, we observed no RA-dependent changes in the abundance of any protein in whole cell lysates from either wild-type or *Fmr1* knockout slices (Figure 3.9A, B), consistent with the notion that RA-induced translation in wild-type slices is a local phenomenon, occurring in dendrites near synapses.

We also examined the effect of RA on the synaptic levels of two verified FMRP target proteins, MAP1b and Arc. Although some groups have reported increased baseline MAP1b levels in FMRP knockout animals at some (but not all) developmental stages (Lu et al., 2004; Hou et al., 2006), others have seen decreased levels in knockout tissue (Chen et al., 2003; Wei et al., 2007). We saw no detectable differences in MAP1b levels between wild-type and knockout slices at this developmental stage, and no effect of RA on MAP1b levels (Figure 3.9A-C). The immediate early gene Arc, which promotes internalization of AMPARs, is not only thought to be regulated by FMRP (Zalfa et al., 2003), but is also known to play a role in synaptic scaling (Shepherd et al., 2006). We saw no change in Arc levels after RA treatment and no baseline differences in protein level between wild-type and knockout slices (Figure 3.9A-C). It should be noted, however, that Arc has been implicated only in the form of scaling induced by long-term TTX-alone treatment (Shepherd et al., 2006), and has not been examined in the context of TTX+APV-induced scaling.

RA-induced GluR1 translation is dependent on FMRP

Though our analysis of specific proteins after RA treatment showed an increase in synaptic AMAPR levels that was dependent on both FMRP and new protein translation (Figure 3.9), we wished to more directly assay the effect of RA on the translation of new AMPA receptors. ^{35}S-labeled methionine and cysteine were added to wild-type and knockout neurons along with DMSO or RA. Dissociated cultures were used for these experiments in order to

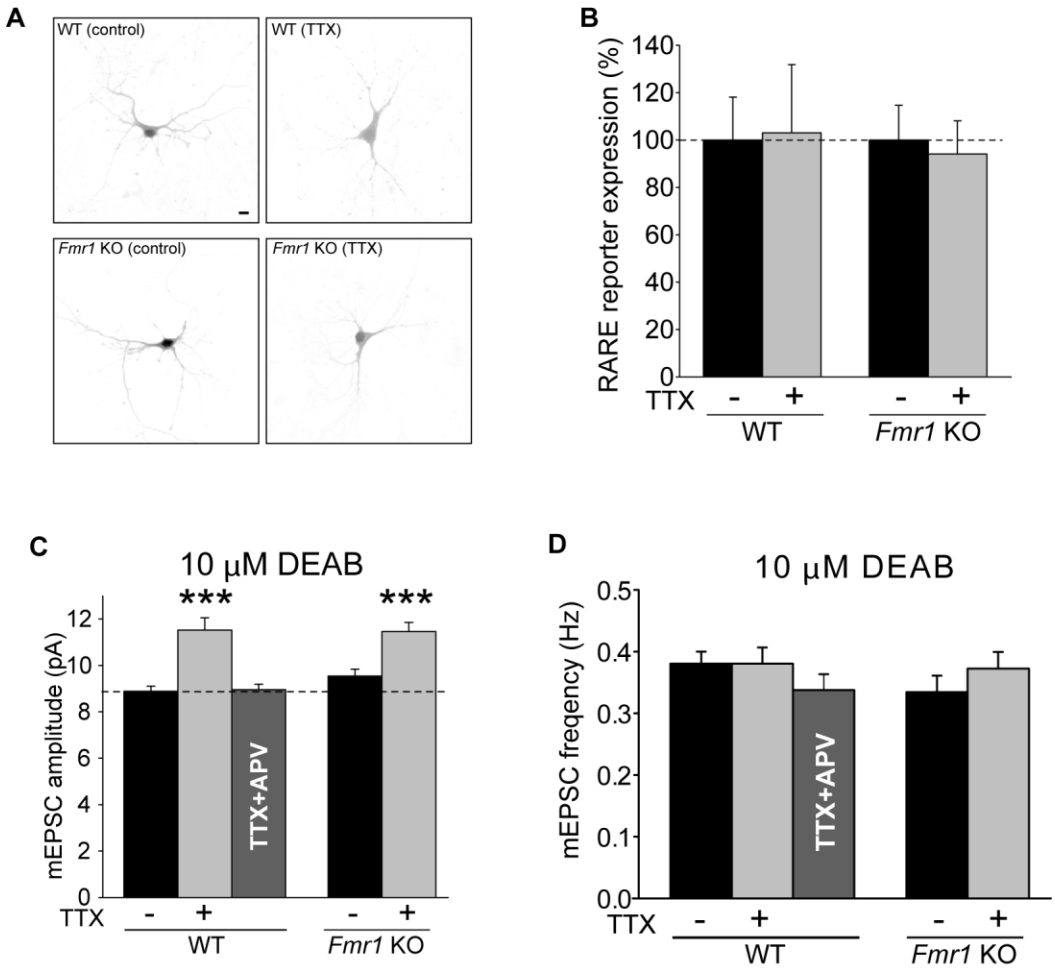

Figure 3.8. RA is not required for TTX alone-induced synaptic scaling. (A) Representative images of RARE-GFP reporter expression in WT and KO neurons with and without 48 hours TTX treatment. Scale bar, 10 μm. **(B)** Quantification of **A** (n = 22-28). **(C)** Effect of RA synthesis blocker DEAB on 60 hour TTX-induced synaptic scaling and 36 hour TTX+APV-induced scaling (n = 21-27; ***, $p < 0.001$). **(D)** Average mEPSC frequency in WT and KO neurons treated with DEAB and 60 hours of TTX or 36 hours of TTX+APV (n = 21-27, $p>0.4$).

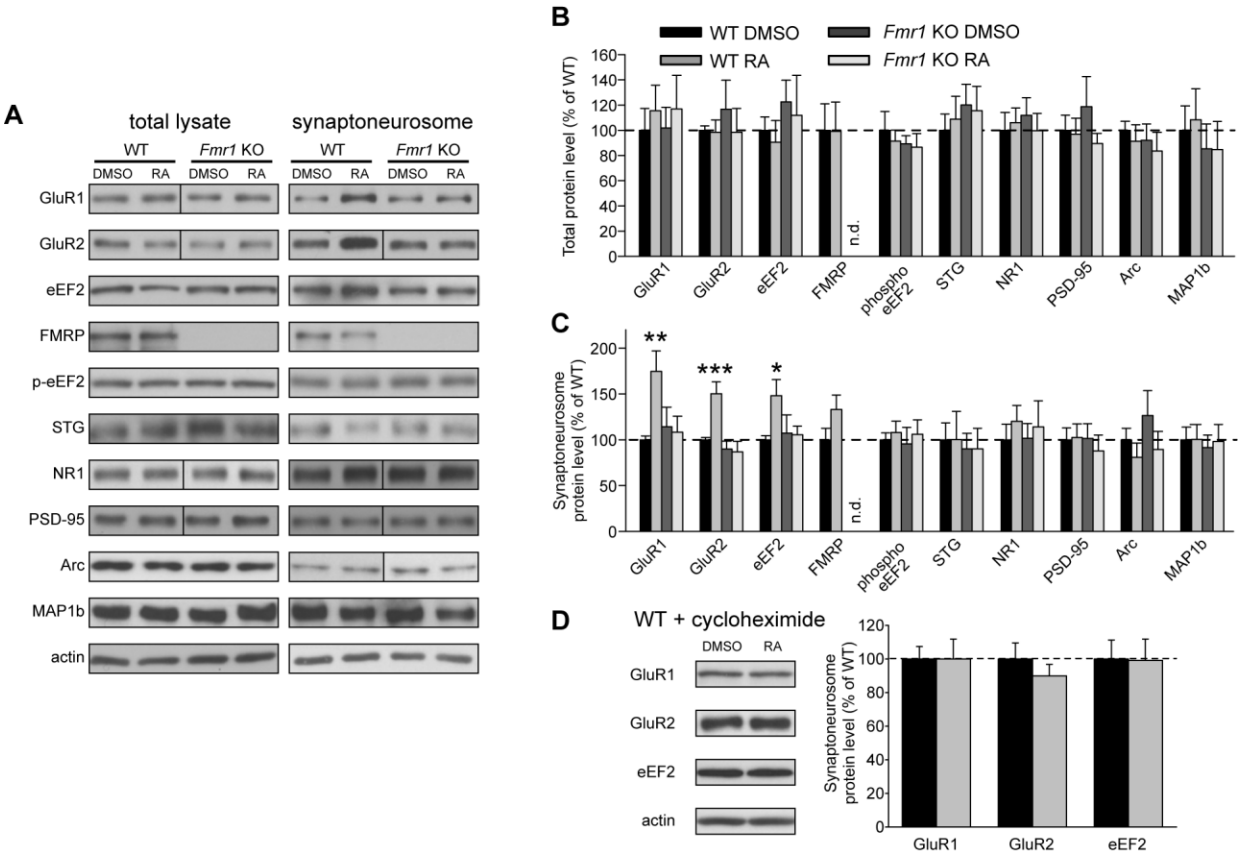

Figure 3.9. FMRP is required for RA-induced local translation of specific synaptic proteins. (A) Representative blots of synaptic proteins from whole lysates and SNS fractions of WT and KO hippocampal slices, treated with 4 hours DMSO or RA. A vertical line indicates the removal (for ease of viewing) of extra lanes between WT and KO lanes. **(B)** and **(C)** Quantification of synaptic proteins in the total lysate (B) and SNS fraction (C) from treated hippocampal slices. Band intensities were normalized to actin (n.d. = not detected; n = 5-16; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). **(D)** Representative blots and quantification of proteins from the synaptic fraction of WT slices pre-treated with translation blocker cycloheximide prior to DMSO or RA treatment (n = 5).

ensure a rapid and complete penetration of the labeling mix to all cells. After two hours of treatment, cell lysates were collected and radioimmunoprecipitation was used to analyze synthesis of GluR1 and GluR2 proteins (Muddashetty et al., 2007).

First, total cell lysate samples were subjected to gel electrophoresis and autoradiography to verify effective labeling. No obvious effect of RA on global translation was seen in either wild-type or knockout neurons, consistent with our observation that RA only affects the local translation of specific proteins (Figure 3.10A). Consistent with previous reports (Dolen et al., 2007), we did observe a slight increase in overall ^{35}S incorporation in knockout neurons compared to wild-type (Figure 3.10A), indicating globally elevated baseline translation in knockout cells.

Immunoprecipitation of GluR1 and quantification of ^{35}S incorporation showed a significant increase in radiolabeled GluR1 after RA treatment in wild-type, but not knockout neurons (Figure 3.10B). This demonstrates both that RA induces translation of GluR1 and that FMRP is required for this translation to occur. We saw no increase in radiolabeled GluR2 protein after RA treatment (Figure 3.10B), even though we saw an increase in GluR2 protein in synaptoneurosomes from RA-treated slice cultures (Figure 3.9). Although we cannot rule out the possibility that the constitutive somatic translation of GluR2 in the absence of RA masks induced translation of GluR2 in neuronal dendrites, this may also reflect a difference between dissociated and slice preparations, or may imply that the increased GluR2 seen at the synapse in slices is due to altered trafficking or degradation of existing protein, not new protein translation.

Indeed, the concurrent increase of both GluR1 and GluR2 proteins seen in RA-treated wild-type synaptoneurosomes (Figure 3.9) was somewhat surprising, because the increase in mEPSC amplitude that follows TTX+APV or RA treatment is due to synaptic insertion of GluR2-lacking AMPA receptors (Figure 3.4B; Aoto et al., 2008). Surface biotinylation confirmed that RA treatment only increased GluR1, but not GluR2 protein level on the cell surface (Figure 3.10C, D), indicating that the increased GluR2 protein seen in synaptoneurosomes after RA treatment is not reaching the surface or contributing to synaptic transmission at the time point examined.

FMRP and RARα proteins do not interact directly

Because of the previously demonstrated role for RARα in binding to GluR1 mRNA and regulating its translation (Aoto et al., 2008; Poon and Chen, 2008), we wondered whether FMRP might affect GluR1 translation by interacting directly with RARα protein. To test for a possible interaction under permissive conditions, we expressed FLAG-tagged FMRP and Myc-tagged RARα proteins in HEK293T cells and attempted co-immunoprecipitation in both directions. Though we were able to demonstrate co-IP of FMRP and FXR1 (a known binding partner), we found no evidence of direct interaction between FMRP and RARα (Figure 3.11).

Acute postsynaptic expression of FMRP in knockout neurons rescues synaptic scaling

Is FMRP required directly for TTX+APV and RA-induced synaptic scaling, or are the deficits seen in *Fmr1* knockout mice due to altered development in the absence of FMRP? To answer this question, we used lentiviral delivery to express GFP-tagged FMRP in CA1 neurons of slices obtained from *Fmr1* knockout mice, and tested whether this could restore synaptic scaling. We also tested two mutant forms of FMRP in an attempt to identify which domains of the protein might be critical for the regulation of homeostatic plasticity. FMRP has two major RNA binding domains: an RGG box, which binds RNAs containing a G-quartet structure

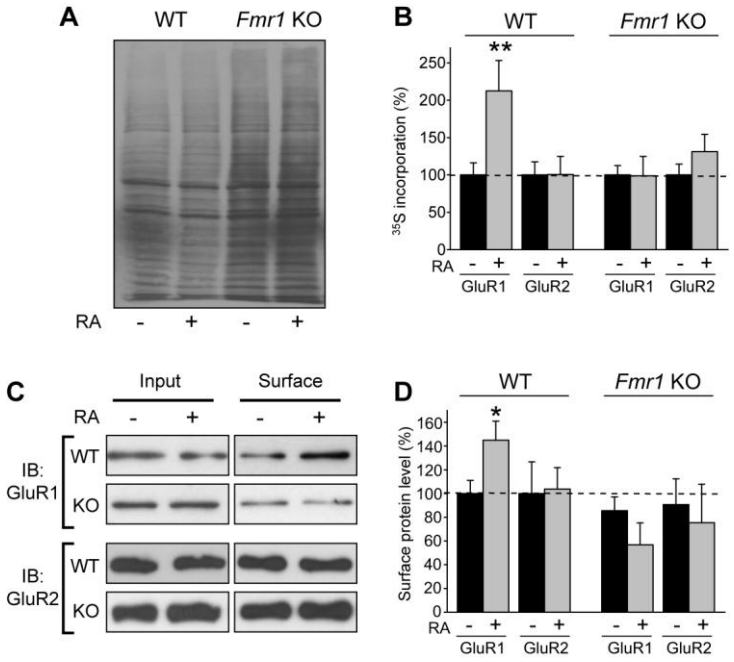

Figure 3.10. Metabolic labeling shows increased GluR1 translation with RA treatment (A) Autoradiography of total protein lysate from dissociated neurons treated with 2 hours DMSO or RA in the presence of ^{35}S-labeled amino acids. **(B)** Quantification of incorporated radioactivity using liquid scintillation counting after immunoprecipitation of AMPARs. Each genotype was normalized separately to its DMSO group (n = 5-8; **, $p<0.01$). **(C)** Representative blots for biotinylation of surface AMPARs in primary cultured neurons after RA treatment. **(D)** Quantification of surface GluR1 and GluR2 protein levels after RA treatment. Surface band intensity was normalized to input, and all groups were compared to WT DMSO (n = 3-9; *, $p<0.05$).

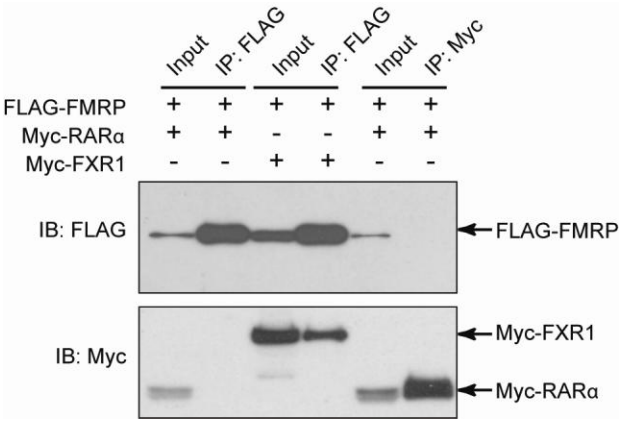

Figure 3.11. FMRP and RARα do not interact directly. Attempted co-immunoprecipitation of FMRP and RARα. Tagged constructs were expressed in HEK293T cells as indicated, and immunoprecipitation of either FMRP (anti-FLAG) or RARα (anti-Myc) was performed. Though pulldown of FMRP was able to coimmunoprecipitate known binding partner FXR1 (positive control), no interaction was seen between FMRP and RARα.

(Darnell et al., 2001), and the tandem KH domains (KH1 and KH2), which bind RNAs containing a kissing complex structure (Darnell et al., 2005b). In order to separate the functions of these two domains, we tested an FMRP construct that was missing the RGG box (FMRPΔRGG-GFP), and one containing a point mutation (I304N) in the KH2 domain (FMRP(I304N)-GFP). The pathogenic mutation I304N does not prevent FMRP from localizing to dendrites or binding G-quartet RNAs, but does inhibit binding with KH2-interacting RNAs (Darnell et al., 2005b; Zang et al., 2009). The I304N mutation also prevents the association of FMRP with actively translating polyribosomes, possibly by inhibiting homo-oligomerization of the protein (Feng et al., 1997; Laggerbauer et al., 2001; Wang et al., 2008).

We first expressed these constructs in wild-type dissociated neurons and quantified their expression levels with immunoblotting. All three constructs expressed at similar levels and none of them altered the endogenous FMRP expression level compared to the GFP-expressing cells (Figure 3.12A). The exogenous expression levels were approximately equal to total endogenous FMRP levels (Figure 3.12B), and were 2 to 2.5-fold higher than expression of the largest FMRP isoform (isoform 1) alone (Figure 3.12C). Neither the wild-type nor the mutant FMRP constructs altered AMPAR abundance (Figure 3.12D, E). Additionally, overexpression of exogenous FMRP and its mutant forms did not change basal synaptic transmission or prevent synaptic scaling in wild-type neurons in response to TTX+APV (Figure 3.12F-H).

We next introduced these wild-type and mutant FMRP constructs into knockout neurons. Consistent with the reported localization of FMRP to RNA granules (Antar et al., 2004; Aschrafi et al., 2005), we found that expression of FMRP-GFP in knockout neurons yielded a distinct punctate pattern in dendrites, resembling that of the endogenous protein (Figure 3.13A). FMRPΔRGG-GFP exhibited a similar expression pattern, as has been reported (Pfeiffer and Huber, 2007). By contrast, expression of FMRP(I304N)-GFP in knockout neurons yielded a more diffuse, less punctate expression pattern in dendrites (Figure 3.13A), which is consistent with its altered function, and similar to previous reports (Schrier et al., 2004; Pfeiffer and Huber, 2007).

Introduction of FMRP-GFP into knockout neurons caused a small reduction in the baseline amplitude of mEPSCs, compared to cells expressing GFP alone (Figure 3.13B). No change in mEPSC frequency was observed (Figure 3.14B, D). Similarly, FMRP(I304N)-GFP expression also reduced the baseline amplitude of mEPSCs, but FMRPΔRGG-GFP had no effect on mEPSC amplitude (Figure 3.13B). These results differ somewhat from a previous report (Pfeiffer and Huber, 2007) of decreased frequency, rather than amplitude, of mini events after FMRP expression in knockout neurons. These disparities are possibly due to differences in the age of slices used, or in the time course or level of FMRP expression.

Our data are consistent, however, with the finding that GluR1 and GluR2 mRNA levels are elevated in polyribosomes of *Fmr1* knockout mice (Muddashetty et al., 2007), indicating overactive baseline translation of these proteins. The acute re-introduction of FMRP into *Fmr1* knockout neurons may reduce this elevated translation back down to wild-type levels, thus temporarily decreasing the amount of these proteins and, by consequence, synaptic strength. In support of this hypothesis, we found that expression of FMRP-GFP or FMRP(I304N)-GFP in dissociated neurons from knockout animals led to a significant reduction in both GluR1 and GluR2 protein levels, though NR1 and PSD-95 levels were unchanged (Figure 3.13C, D). Surface AMPAR levels, as measured by biotinylation and pulldown, were reduced proportionally with total AMPAR protein levels, so that the ratio of surface to total fractions remained constant across all conditions (Figure 3.13E, F). Consistent with our observation that

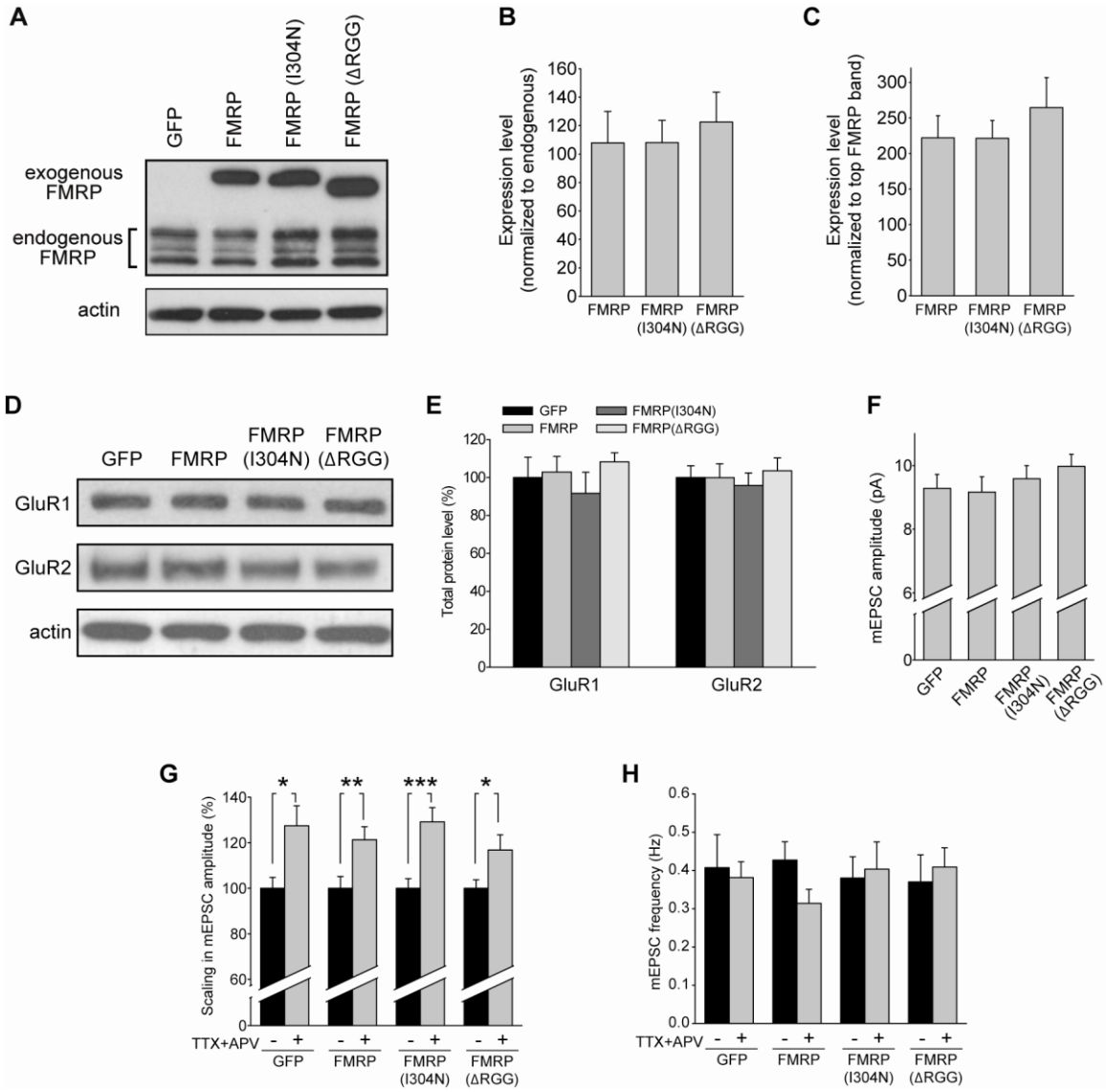

Figure 3.12. Viral overexpression of FMRP in wild-type neurons does not affect AMPAR levels or synaptic scaling. (A) Representative blot and **(B)** quantification of exogenous FMRP-GFP, FMRP(I304N)-GFP, and FMRPΔRGG-GFP expression compared to endogenous FMRP protein levels after 6 days of virus expression in wild-type dissociated neurons (n = 6). **(C)** Ratio of exogenous FMRP expression to endogenous levels of FMRP isoform 1 only (n=6). **(D)** Representative blots and **(E)** quantification of protein levels in lysates collected from WT dissociated neurons infected with virus expressing GFP or different FMRP constructs. Band intensities were normalized to actin. (n = 5-6). **(F)** Amplitude of mEPSC events in WT neurons expressing GFP, FMRP, FMRP(I304N), or FMRPΔRGG (n = 11-12). **(G)** Percent scaling after TTX+APV treatment in WT neurons expressing GFP or different FMRP constructs. DMSO groups for each construct were set to 100% (n = 11-12; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). **(H)** Quantification of mEPSC frequency from WT neurons expressing indicated constructs and treated with TTX+APV (n = 11-12)

Figure 3.13 (see legend on following page)

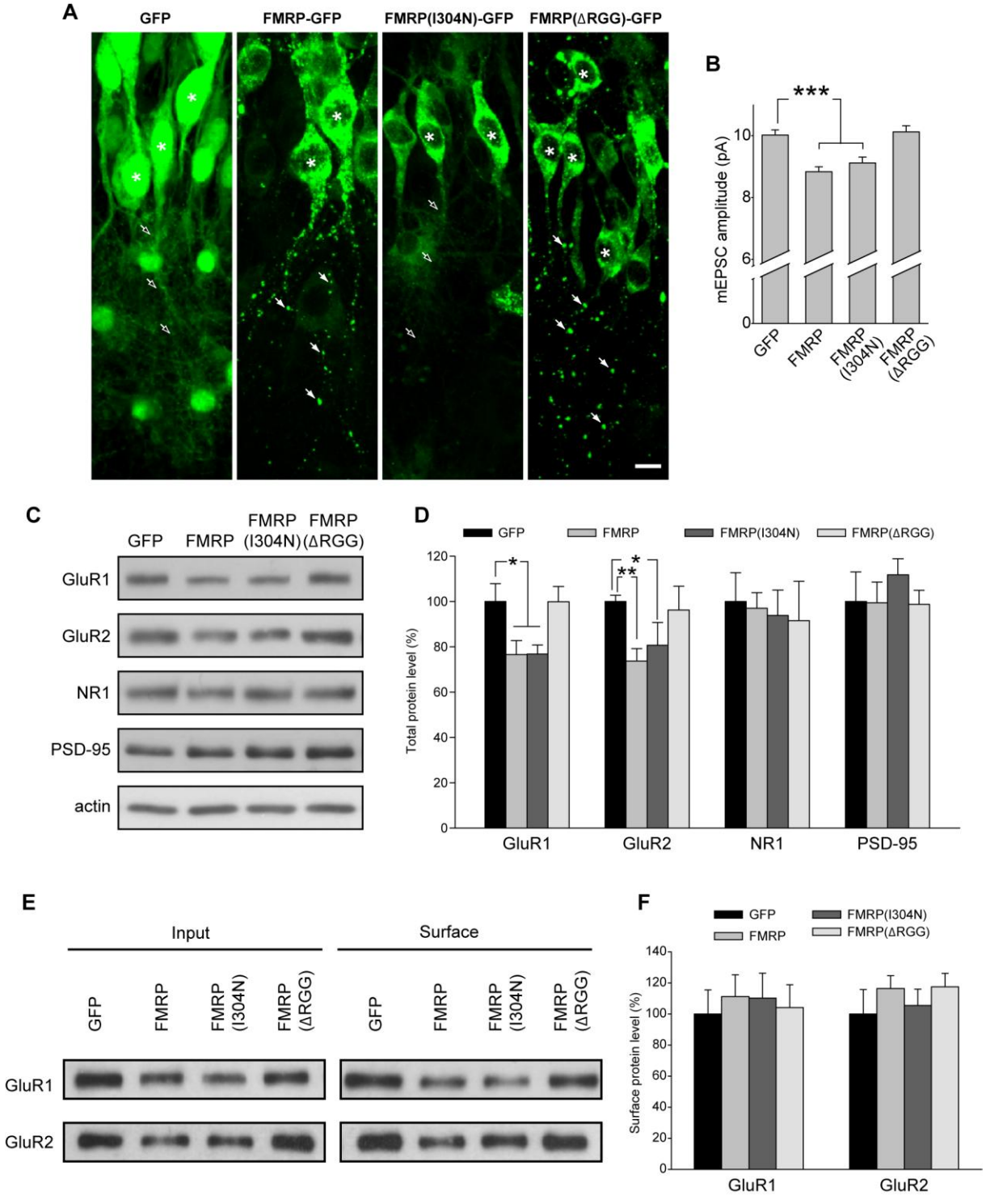

Figure 3.13. Viral expression of FMRP in knockout neurons affects AMPAR abundance. **(A)** Images from the CA1 region of hippocampal slices infected with lentiviral constructs expressing GFP, FMRP-GFP, FMRP(I304N)-GFP, or FMRPΔRGG-GFP. Asterisks indicate cell body. FMRP-GFP and FMRPΔRGG-GFP exhibit a punctate expression pattern in neuronal dendrites (solid arrows) while GFP or FMRP(I304N)-GFP are diffusely expressed in neuronal dendrites (open arrows). Scale bar: 10 µm. **(B)** Amplitude of mEPSC events in KO neurons expressing GFP, FMRP, FMRP(I304N), or FMRPΔRGG (n = 38-66; ***, $p<0.001$). **(C)** Representative blots and **(D)** quantification of protein levels in lysates collected from KO dissociated neurons infected with virus expressing GFP, FMRP-GFP, FMRP(I304N)-GFP, or FMRPΔRGG-GFP. Band intensities were normalized to actin. (n = 4-10, *, $p<0.05$, **, $p<0.01$). **(E)** Representative blots and **(F)** quantification of surface biotinylation of AMPARs in knockout dissociated neurons infected with virus expressing GFP or different FMRP constructs. Surface band intensity was normalized to input (n=5-7).

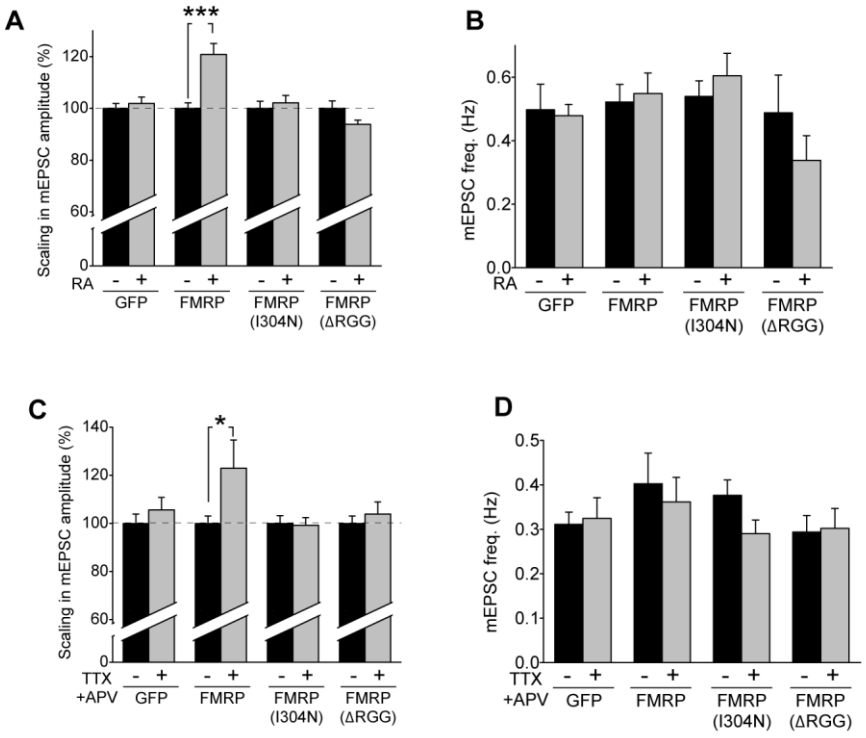

Figure 3.14. Expression of wild-type FMRP in knockout neurons restores synaptic scaling. **(A)** Percent scaling after RA treatment in neurons expressing GFP or different FMRP constructs. DMSO groups for each construct were set to 100% in order to account for altered baseline amplitudes (n = 18-49; ***, $p<0.001$). **(B)** Frequency of mEPSCs after viral expression and RA treatment (n = 18-49, $p>0.3$) or **(C)** Percent scaling after treatment with 36 hours TTX+APV. (n = 17-20; *, $p<0.05$). **(D)** Frequency of mEPSCs after viral expression and TTX+APV treatment (n = 17-20, $p>0.5$).

the RGG box is required for the reduction in mEPSC amplitude caused by FMRP reintroduction, FMRPΔRGG-GFP had no effect on levels of any of the proteins measured (Figure 3.13C-F). These data suggest that FMRP does indeed regulate the abundance of AMPARs, either through direct binding to AMPAR mRNAs or through controlling the translation of other regulatory proteins.

Importantly, FMRP-GFP restored the ability of *Fmr1* knockout neurons to undergo synaptic scaling after RA treatment (Figure 3.14A) or TTX+APV treatment (Figure 3.14C). By contrast, neither GFP alone, FMRP(I304N), nor FMRPΔRGG rescued synaptic scaling induced by RA or TTX+APV (Figure 3.14A, C). This confirms that FMRP is required acutely in the postsynaptic cell for induction of the form of synaptic scaling mediated by RA/RARα, and that the ability of FMRP to both repress and permit the translation of specific transcripts is critical for RA-induced local translation and synaptic scaling in neurons.

DISCUSSION

Homeostatic plasticity, specifically synaptic scaling, maintains network stability and the coding capacity of neural circuits (Turrigiano and Nelson, 2004; Davis, 2006). Activity blockade by TTX and APV induces a form of synaptic scaling that requires dendritic protein synthesis (Ju et al., 2004; Sutton et al., 2006), which we have shown to be mediated by RA signaling (Aoto et al., 2008). In the present study, we identify FMRP as a critical factor required for homeostatic plasticity and regulation of synaptic strength by RA. FMRP is not essential for RA production, but mediates RA-induced protein synthesis, and is specifically involved in the form of homeostatic plasticity that requires dendritic translation of discrete synaptic proteins. We also demonstrate that RA-dependent homeostatic plasticity in *Fmr1* knockout neurons is rescued by wild-type FMRP, but not by FMRP(I304N) or FMRPΔRGG. This result indicates that FMRP regulation of protein translation mediates the induction of homeostatic plasticity triggered by RA.

The involvement of FMRP in homeostatic plasticity and in RA signaling is unexpected, and raises several new questions. How do FMRP and RARα work together to regulate RA-mediated translation during homeostatic plasticity? Although we were unable to demonstrate direct binding between these two proteins, it is possible that they interact by binding to the same RNA molecules. Deciphering the functional interplay between FMRP and RARα will be a critical step toward understanding the molecular basis of FMRP-mediated translational regulation and the *Fmr1* knockout phenotype.

The ability of FMRP to function as a translational repressor has been well-described (Comery et al., 1997; Laggerbauer et al., 2001; Li et al., 2001). Numerous studies have, with some success, attempted to identify mRNAs that bind directly to FMRP (Sung et al., 2000; Brown et al., 2001; Chen et al., 2003; Miyashiro et al., 2003; Zou et al., 2008), and some specific mRNA targets have been verified, including PSD-95, MAP1b, and CaMKII (Brown et al., 2001; Hou et al., 2006; Zalfa et al., 2007). Although FMRP has not been reported to directly bind GluR1 or GluR2 mRNAs (Zalfa et al., 2007), our data and those of others (Muddashetty et al., 2007; Schuett et al., 2009) do support its involvement in regulating AMPAR translation, possibly via indirect binding or regulation of secondary factors.

Our viral expression data support a model whereby elevated baseline translation of AMPARs in the absence of FMRP could partially account for the failure of *Fmr1* knockout

neurons to respond to RA treatment. This elevated translation could impose a "ceiling effect," masking or inhibiting any additional increases in translation. Reintroducing FMRP into knockout cells lowers translation to normal levels, thus reducing AMPAR protein levels and mEPSC amplitude, allowing cells to then respond to TTX+APV or RA treatment. However, our results with I304N mutant FMRP show that simply reducing AMPAR protein levels is not sufficient to rescue plasticity. Therefore, wild-type FMRP must also participate in the activation of AMPAR translation in response to RA, which in turn leads to increased synaptic strength.

The fact that baseline AMPAR levels are unaltered in knockout neurons does not inherently contradict our model. The effect of FMRP on AMPAR translation is intricate; our results suggest that FMRP not only represses basal translation of AMPARs, but also is required for activity blockade-induced activation of AMPAR translation. Therefore, it is difficult to predict the effect that the constitutive absence of FMRP would have on total protein levels. Second, compensatory effects during development, such as altered protein degradation, could adjust the abundance of AMPARs over time in knockout animals. This can be seen with some other validated FMRP targets, such as MAP1b (Lu et al., 2004; Hou et al., 2006; Wei et al., 2007) and PSD-95 (Todd et al., 2003); Schuett et al., 2009), which do not show consistently elevated levels in knockout neurons.

We were also able to use mutant FMRP constructs to further understand the mechanism of FMRP's action in homeostatic plasticity. FMRP lacking the RGG box RNA-binding domain failed to affect baseline AMPAR levels or to restore synaptic scaling, indicating that this domain is critical for the dynamic regulation of AMPAR levels, particularly in the context of homeostatic plasticity. This is not surprising, as the RGG box binds RNAs specifically and with high affinity (Darnell et al., 2001) and mediates the interaction of FMRP with several validated target mRNAs, including those encoding MAP1b, semaphorin 3F, and FMRP itself (Schaeffer et al., 2001; Menon and Mihailescu, 2007; Menon et al., 2008). Results with the FMRP(I304N) mutant support this model. This form of FMRP, with an intact RGG box, was still able to bind RNA and downregulate AMPAR levels, but because this mutant protein cannot enter actively translating polyribosomes it did not restore the ability of neurons to increase AMPAR translation in response to activity blockade.

When overexpressed in wild-type neurons, none of the forms of FMRP tested had an effect on AMPAR abundance or the ability of the neurons to undergo synaptic scaling. This is to be expected, as neither FMRP(I304N) nor FMRPΔRGG is expected to act as a dominant negative in this context. FMRP(I304N) is able to heterodimerize with wild-type FMRP and subsequently be recruited to RNA granules (Laggerbauer et al., 2001; Levenga et al., 2009), and FMRPΔRGG is unable to bind G-quartet RNA, and thus should not interfere with endogenous FMRP regulation of these transcripts.

We saw changes in the synaptic levels of three proteins (GluR1, GluR2, and eEF2) in response to RA. Interestingly, these three proteins are each encoded by an mRNA that binds directly to RARα through a motif in its 5'UTR which confers RARα binding ability (Poon and Chen, 2008). Previous *in vitro* study of the GluR1 UTR showed that RARα binding inhibits translation, but that this inhibition is relieved upon addition of RA. Our results suggest, therefore, that FMRP may be required for this RA-induced release of inhibition by RARα.

One intriguing observation is that despite the change in GluR2 protein levels near the synapse in response to RA, we were unable to directly detect RA-induced GluR2 translation, and found no evidence for trafficking of newly synthesized GluR2 protein to the cell surface, or its insertion into the postsynaptic membrane. This result points to a differential regulation of GluR1

and GluR2 receptors during synaptic scaling, possibly due to specific trafficking or degradation of the two receptor types. In fact, this differential trafficking has already been observed, as it was previously shown that the GluR1 homomeric receptors initially inserted after activity blockade are subsequently replaced by GluR2 containing receptors (Sutton et al., 2006). The precise mechanism at work in this case requires further investigation, and may offer broader insights into the mechanism of AMPAR trafficking.

We show here that FMRP is selectively required for translation-dependent, but not transcription-dependent synaptic scaling. This observation agrees with previous findings that different protocols used to induce homeostatic plasticity operate via distinct subcellular mechanisms (Sutton et al., 2006; Yu and Goda, 2009), similarly to what has been observed for LTP and LTD induction (Malenka and Bear, 2004). What will be critical for the future of the homeostatic plasticity field is to increase our understanding of how these different protocols correspond to various *in vivo* situations, and how their mechanisms converge to regulate synaptic strength.

Traditionally, Hebbian-type synaptic plasticity is considered the cellular mechanism for learning and memory. As an animal model for Fragile-X mental retardation, *Fmr1* knockout mice have been studied extensively for defects in neuronal function and learning and memory. *Fmr1* knockout mice have impaired Hebbian-type synaptic plasticity (Huber et al., 2002; Larson et al., 2005), which may contribute to their learning deficits (Mineur et al., 2002; Yan et al., 2004; Koekkoek et al., 2005). Our study reveals an additional requirement for FMRP in homeostatic plasticity and RA-mediated translational regulation of synaptic proteins, suggesting that FMRP and its regulation of protein synthesis participate in multiple forms of activity-dependent synaptic plasticity, though seemingly through distinct mechanisms.

Our finding of impaired homeostatic synaptic plasticity provides a new perspective on the phenotype in *Fmr1* knockout mice and on the symptoms of human Fragile-X patients. It may explain, for example, the global alterations of neural activity that have been observed in *Fmr1* knockout mice and Fragile-X syndrome patients (Berry-Kravis, 2002; Yan et al., 2004). Moreover, although homeostatic synaptic adjustment may not be directly involved in encoding memory, its ability to influence network stability and neuronal coding capacity nonetheless could contribute significantly to cognitive function. It is plausible that lack of homeostatic regulation destabilizes neural networks and compromises the capacity of the network to undergo Hebbian-type plasticity, which in turn may produce behavioral and learning defects in *Fmr1* knockout mice. Understanding the interplay between these different processes will provide significant further insight into the molecular mechanisms guiding both homeostatic plasticity and Fragile-X syndrome.

Chapter 4
Regulation of GluR1 translation by microRNAs

INTRODUCTION

MicroRNAs (miRNAs) are small (approximately 22 nucleotides), non-coding RNA molecules which regulate protein synthesis by binding to miRNA recognition elements (MREs) in the 3'UTRs of mRNA transcripts and inhibiting their translation. miRNAs can be encoded either in intergenic regions of the genome or within introns of protein-coding genes, and are generally under the control of Polymerase II promoters (Lee et al., 2004). The initial miRNA transcript, called a primary miRNA (pri-miRNA), forms a hairpin loop, and its ends are cleaved in the nucleus by the protein Drosha to form a 70-100 nucleotide precursor miRNA (pre-miRNA) (Lee et al., 2003). The pre-miRNA is exported from the nucleus via the Exportin-5 transporter (Yi et al., 2003) and is cleaved again in the cytosol by Dicer, forming a double stranded RNA with no hairpin loop (Hutvagner et al., 2001).

After cleavage by Dicer, one or both strands of the miRNA are incorporated into the RNA-induced silencing complex (RISC), an RNA-protein complex which forms the functional unit for miRNA-induced translational repression. The precise composition of the RISC remains unclear, but one known member is the Argonaute (AGO) family of proteins. Most organisms have a number of AGO homologs, and different homologs have been implicated in different RISC functions (Okamura et al., 2004).

The miRNA pathway has many parallels to the short interfering RNA (siRNA) pathway. siRNAs are cleaved by Dicer from larger double-stranded RNA molecules (either endogenous or exogenous), and are typically thought of as binding perfectly to their target mRNAs and inducing cleavage. By contrast, miRNAs are thought to bind with imperfect base pairing and are usually believed to induce repression without cleavage, though there are exceptions to this rule (Pillai et al., 2007). In humans, AGO2 is the only member of the Argonaute family which is capable of inducing cleavage of the target mRNA (Meister et al., 2004).

In recent years miRNAs have emerged as critical players in the regulation of neuronal function, and have been implicated in processes ranging from neuronal patterning to cell fate determination to regulation of dendritic spine size (Aboobaker et al., 2005; Krichevsky et al., 2006; Schratt et al., 2006). Tight control of protein translation in neuronal dendrites is critical for regulation of synaptic strength and plasticity, and with this in mind a number of studies have investigated a possible role for miRNAs at the synapse. One group showed that in *Drosophila* degradation of the RISC complex was required for the increases in dendritic protein synthesis necessary for long-term memory formation (Ashraf et al., 2006). Additionally, Dicer has been shown to localize to the post-synaptic density, and can be activated by Calpain cleavage in an activity- and calcium-dependent manner (Lugli et al., 2005). Populations of dendritically localized miRNAs have been identified (Kye et al., 2007; Lugli et al., 2008), and microarray screens have also identified miRNAs which change expression levels after induction of LTP (Park and Tang, 2009; Wibrand et al., 2010), though what function these miRNAs may have remains to be seen.

A number of specific miRNAs have been implicated in regulation of dendritic spine size and morphology, including miR-134, miR-138, miR-125b and miR-132 (Edbauer et al.; Schratt et al., 2006; Siegel et al., 2009). Though these miRNAs all converge to regulate one cellular function, they act on different sets of mRNAs. Several of these miRNAs are regulated by activity changes in neurons. For example, transcription of miR-132 is enhanced after increased neuronal activity and is regulated by the transcription factor cyclic-AMP response element binding protein (CREB). miR-132 affects activity-dependent dendritic outgrowth by regulating the Rho family GTPase-activating protein, p250GAP (Vo et al., 2005; Wayman et al., 2008). By contrast, miR-134 represses LIMK1, a protein involved in synapse remodeling, and this repression is relieved after BDNF stimulation (Schratt et al., 2006).

Local translation of GluR1 and other proteins is clearly an essential component of synaptic plasticity, and the miRNA pathway provides an attractive potential system by which this translation may be regulated. miRNAs could maintain mRNA transcripts in a repressed state in the dendrite until activated by a specific trigger, such as local changes in calcium. In addition, miRNAs can provide specificity in regulating targets, which would allow for increased production of specific proteins required for plasticity without necessitating a global increase in translation.

Here, we identify miRNAs which regulate translation of the critical AMPAR subunit GluR1. We present a pair of miRNAs, miR-96 and miR-182, which are similar in sequence, are expressed on the same primary transcript, and which can bind to the 3'UTR of GluR1 and repress its translation. Overexpression of these miRNAs decreases total and extrasynaptic surface GluR1 levels, and prevents the induction of synaptic scaling by activity blockade treatment. Both miR-96 and miR-182 are expressed in the rat cortex throughout development, but we were unable to detect any activity-dependent changes in their expression levels. Additionally, attempted knockdown of these miRNAs did not reveal any subsequent changes in GluR1 abundance or impairments in homeostatic plasticity.

RESULTS

Screen of miRNAs predicted to bind GluR1

We asked whether inhibition of GluR1 translation by one or more miRNAs might contribute to the regulation of synaptic strength under basal conditions or during synaptic plasticity. To address this question we initially took a bioinformatics approach, using an online database (Targetscan) to search for miRNAs predicted to bind to the 3'UTR of the mouse GluR1 mRNA. Our initial search revealed 17 miRNAs or miRNA families which are predicted to bind to this 3'UTR (Table 4.1). A number of large-scale studies have examined miRNA expression in various tissues (Barad et al., 2004; Baskerville and Bartel, 2005; Kim and Nam, 2006; Tang et al., 2007), and we compared our list of potential GluR1-binding miRNAs with those miRNAs which had been identified by at least one group as being either uniquely expressed or enriched in the brain. Six potential miRNAs or miRNA families were identified (Table 4.1).

In order to examine the effects of each of these miRNAs in hippocampal neurons, we cloned approximately 300 base pairs of the genomic region surrounding each miRNA and inserted this primary sequence into a mammalian expression vector under the control of a CMV (Pol II) promoter. The vector also contained the coding sequence for EGFP under the control of a separate promoter, to be used as a marker for transfected cells. We transfected cultured

Table 4.1. miRNAs predicted to bind the GluR1 3'UTR

miRNA	Expressed in brain?	Reference
346	Yes	(Baskerville and Bartel, 2005, Kim and Nam, 2006, Tang et al., 2007)
691		
671		
762		
96	Yes	(Barad et al., 2004, Baskerville and Bartel, 2005, Kim and Nam, 2006, Tang et al., 2007)
182		
713		
764-3p		
694		
412		
103/107	Yes	(Barad et al., 2004, Baskerville and Bartel, 2005, Kim and Nam, 2006, Tang et al., 2007)
25/32/92/363/367	Yes	(Baskerville and Bartel, 2005)
137		
151		
136	Yes	(Baskerville and Bartel, 2005, Tang et al., 2007)
323	Yes	(Baskerville and Bartel, 2005, Kim and Nam, 2006)
743		

hippocampal neurons with each miRNA, and after 3 to 4 days of expression fixed and permeabilized the cells and stained for total GluR1 protein. Examining the intensity of GluR1 staining in the dendrites of transfected cells (compared to the intensity of staining in neighboring untransfected cells), we found that expression of only one miRNA, miR-96, had a significant effect on GluR1 levels, reducing signal to approximately 60% of untransfected levels (Figure 4.1A, C). We included miR-124, a well-studied miRNA known to be highly expressed in neurons, as a control that is not expected to affect GluR1 abundance (Krichevsky et al., 2006; Xu et al., 2007).

miR-96 regulates surface and total GluR1 levels

To test whether this effect of miR-96 was specific for GluR1, we also examined the effect of overexpression of this miRNA on levels of GluR2, including miR-103 as a control which did not affect GluR1 levels. Staining for GluR2 after 3 to 4 days of miR-96 expression revealed a no significant change in levels of GluR2 (Figure 4.1B, D). Expression of miR-103 also had no effect on levels of GluR2.

We next asked whether this decrease in total GluR1 protein levels induced by miR-96 was reflected as a decrease in synaptic AMPAR levels. We expressed miR-96 (or a control miRNA) in hippocampal neurons and recorded mEPSCs from transfected cells. We saw no change in mEPSC amplitude in cells expressing miR-96 compared with cells expressing empty vector (Figure 4.2A), indicating that baseline synaptic AMPAR levels are not affected by the global decrease in GluR1 protein caused by miR-96 expression. We also saw no change in mEPSC frequency in cells expressing miR-96, indicating no effect of this miRNA on synapse number or presynaptic release probability (Figure 4.2B).

In order to examine the extrasynaptic surface expression of AMPARs in the presence or absence of miR-96, we puffed glutamate onto outside-out membrane patches. Patches taken from neurons expressing miR-96 showed significantly reduced glutamate-evoked currents compared to patches taken from neurons expressing empty vector (Figure 4.2C, D). Taken together, these results indicate that miR-96 expression reduces the total and extrasynaptic levels of GluR1, but does not affect the abundance of AMPARs at the synapse.

Genomic location of miR-96 and target site conservation

We next examined more closely the genomic locus encoding miR-96, as well as the predicted miR-96 binding site in the GluR1 3'UTR. miR-96 is located on mouse chromosome 6, and is part of a cluster with two other miRNAs, miR-182 and miR-183 (Xu et al., 2007). All three miRNAs are expressed as one primary transcript which is subsequently processed into the three separate precursor and mature miRNAs. The three miRNAs share some sequence similarity, particularly in the seed region (base pairs 2 through 8), which is critical for binding to mRNA targets (Figure. 4.3A). Interestingly, miR-182 was identified in our original Targetscan screen as potentially targeting GluR1 at the same site as miR-96 (Table 4.1), but at that time its expression in the brain had not been reported by any group.

We also looked more closely at the predicted binding site for miR-96/182 in the GluR1 3'UTR. Compared to the predicted binding sites for the other brain-expressed potential GluR1 target miRNAs, the binding site for miR-96/182 was the most highly conserved across mammalian species, both in the seed region and across the entire binding site (Figure 4.3B, C). Only miR-25 had comparable conservation in the seed region, but was much less conserved across the rest of the binding site.

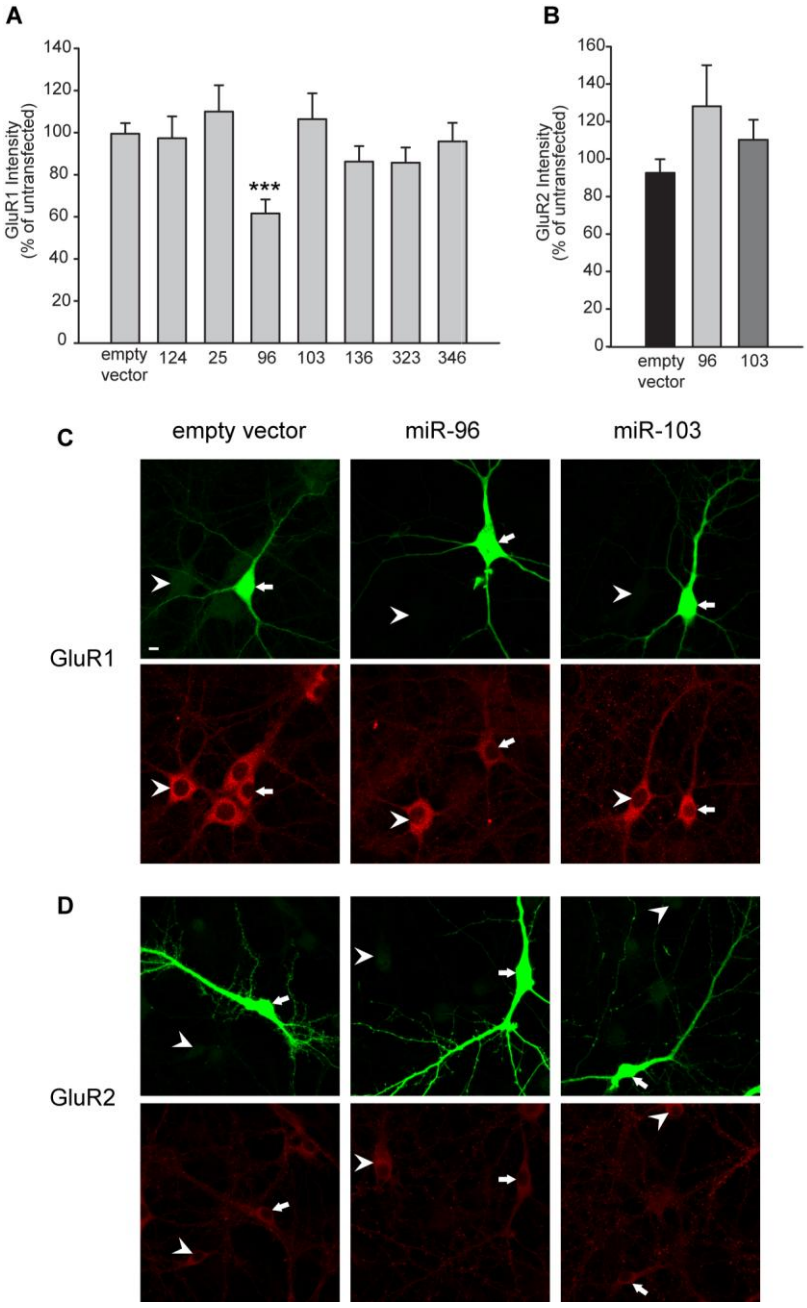

Figure 4.1. Screen of miRNAs predicted to bind to GluR1. (A) Quantification of GluR1 staining intensity in the dendrites of hippocampal neurons 3-4 days after transfection with the indicated miRNA. The signal from transfected dendrites was normalized to the signal from neighboring untransfected cells (n = 10-20 cells/group; ***, $p<0.001$). **(B)** Quantification of GluR2 staining intensity in the dendrites of hippocampal neurons 3-4 days after transfection with the indicated miRNA (n = 12 cells/group). **(C)** Representative images of GluR1 staining. Arrows point to transfected cells, arrowheads to neighboring untransfected cells used for normalization. **(D)** Representative images of GluR2 staining. Scale bar, 10 μm.

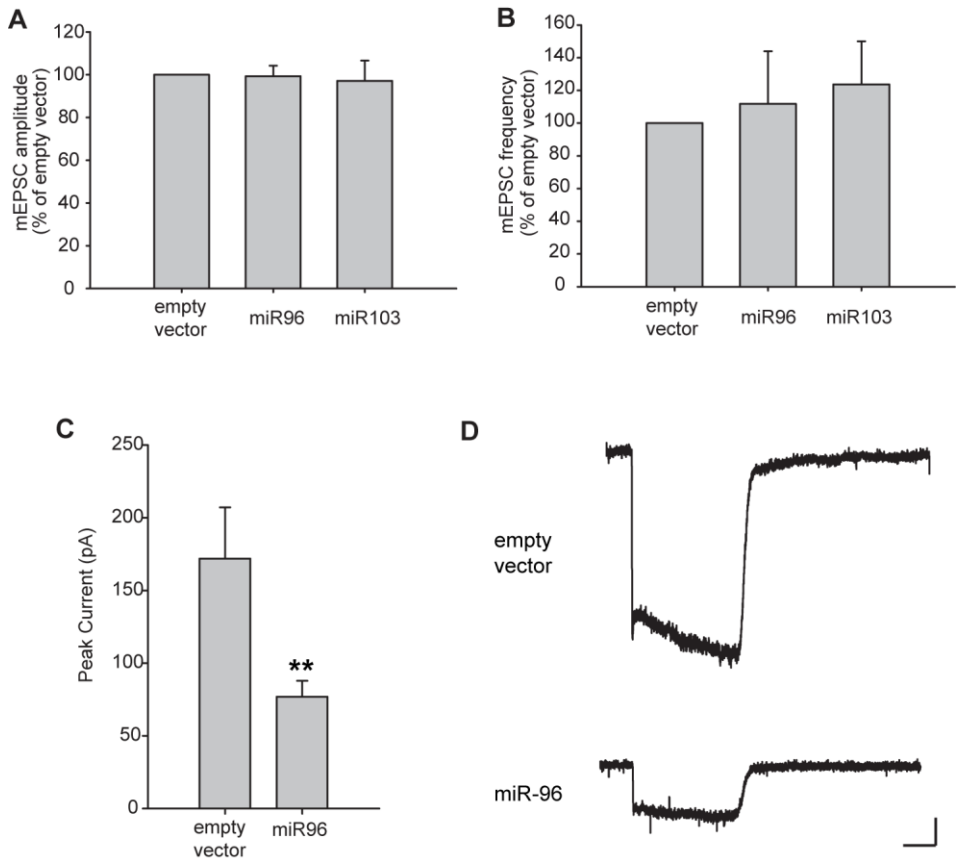

Figure 4.2. miR-96 overexpression reduces surface, but not synaptic glutamate receptors. **(A)** Quantification of average mEPSC amplitude recorded from neurons expressing the indicated miRNA. Amplitudes were normalized to those recorded from cells expressing empty vector (EGFP) alone (n = 11-16 cells/group). **(B)** Quantification of mEPSC frequency. **(C)** Quantification of peak currents evoked by glutamate puff onto outside-out patches taken from cells expressing empty vector or miR-96 (n = 14-16 cells/group, **, $p<0.01$). **(D)** Example traces. Scale bar, 20 pA, 0.5 s.

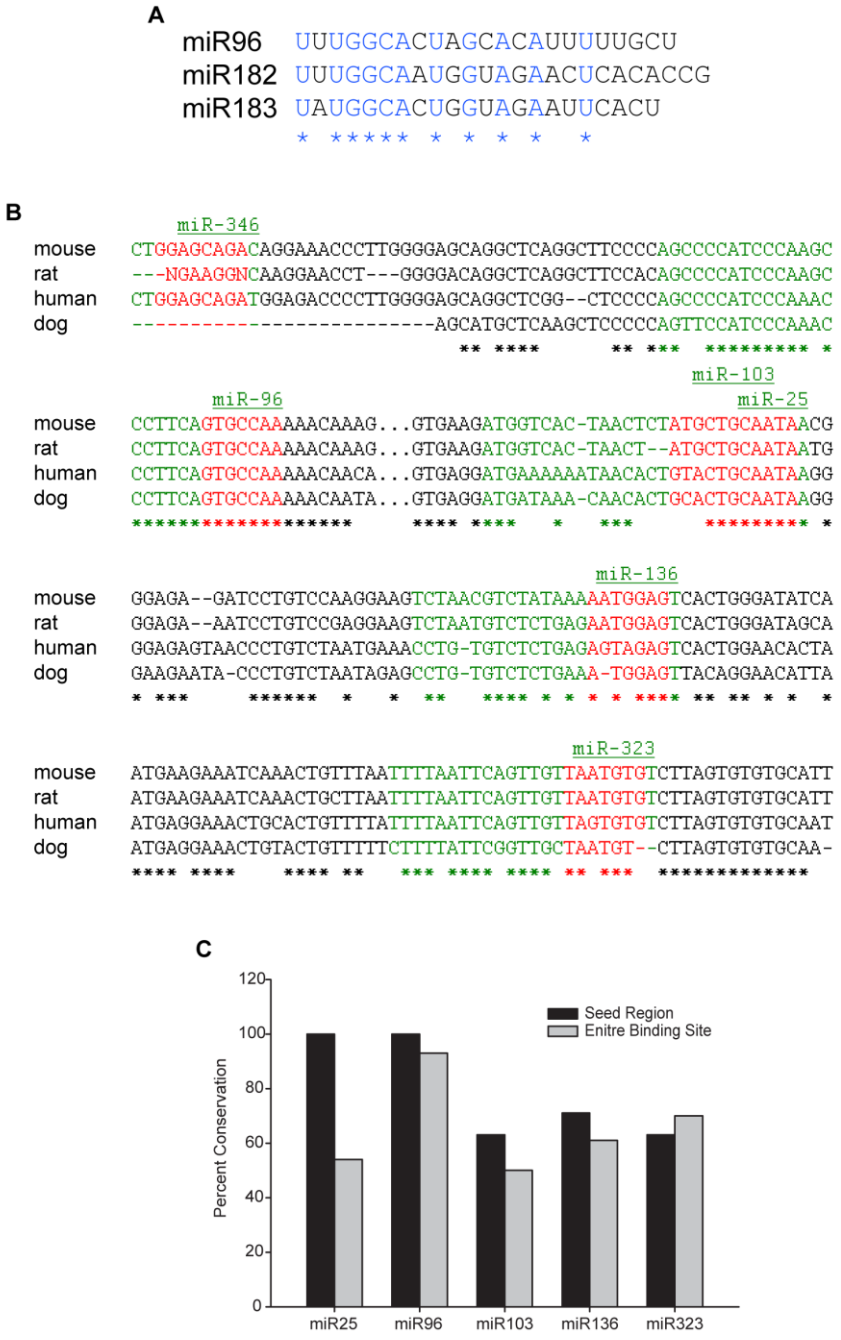

Figure 4.3. Conservation of miRNA binding sites in the GluR1 3'UTR. (A) Sequence alignment of mature miRNAs 96, 182, and 183. * indicates perfect alignment among all sequences. **(B)** Conservation of GluR1 3'UTR from mouse, rat, human, and dog, and identification of predicted miRNA binding sites. The seed region is indicated in red, and the rest of the binding site in green. The miR-346 binding site extends into the coding sequence of GluR1. **(C)** Percent conservation across the four species for the seed region and the entire binding site of each predicted miRNA.

Repression of translation by miR-96/182 depends on predicted binding site

In order to verify that miR-96 was in fact regulating GluR1 expression by binding to the predicted site in the GluR1 3'UTR, we generated a reporter system to test this regulation in a non-neuronal cell culture system. We attached the 3'UTR of GluR1 to the coding sequence for destabilized mCherry (dsmCherry) (Figure 4.4A), and expressed this construct in HEK293T cells along with miR-96, miR-182, a control miRNA (miR-124), or empty EGFP-expressing vector. We also generated a version of the reporter in which the predicted miRNA96/182 MRE was deleted (Figure 4.4B). After 24 hours of expression cells were fixed and imaged using confocal microscopy. The intensity of the mCherry signal in each cell was normalized to expression of EGFP in the same cell, as a control for transfection efficiency. Both miR-96 and miR-182 reduced the expression of the full length reporter when compared to either empty vector or miR-124 (Figure 4.4C, E). However, this reduction was absent when the miRNAs were coexpressed with the reporter lacking the MRE (Figure 4.4D, F). This indicates that both miR-96 and miR-182 can bind to the 3'UTR of GluR1 and inhibit mRNA translation.

miR-96/182 are expressed throughout development

Because both miR-96 and miR-182 can bind to and regulate translation of GluR1 mRNA, we wished to more closely examine the expression of these miRNAs in brain tissue. We collected RNA from the cortex of male rats at various postnatal time points and used end-point RT-PCR to examine miRNA expression. Stem-loop RT primers were used in order to provide specificity for the mature form of each miRNA (Varkonyi-Gasic et al., 2007). As a positive control we included RNA from thymus, where high expression of miR-96/182 has been reported (Kim and Nam, 2006), and as negative controls we included RNA from heart, as well as no RNA and no RT control reactions.

We saw expression of both miR-96 and miR-182 at all developmental time points examined (Figure 4.5A). Expression levels varied between different animals of the same age, though expression of miR-96 and miR-182 seemed to correspond. This variability was not due to differences in the quality or amount of RNA collected from each sample, as expression of a control microRNA (miR-134) was uniform across all samples (Figure 4.5A).

We hypothesized that the large variability in miR-96 and 182 expression across samples may be due to activity-dependent changes in expression of this miRNA cluster. To address this question, we examined miR-182 expression in the context of two forms of activity-dependent plasticity: homeostatic plasticity and Hebbian plasticity. We used long-term treatments of either TTX+APV (to silence neuronal activity) or picrotoxin (to enhance neuronal activity) in organotypic slice culture in order to examine miRNA expression during homeostatic plasticity. Brief (3 minute) treatments with either depolarizing KCl or glutamate in acute slices, followed by washout of treatment for various lengths of time, were used to mimic Hebbian plasticity. We were unable to detect consistent changes in miR-182 expression following either short-term or long-term plasticity treatments (Figure 4.5B, C), though the technical limitations of the end-point PCR method may have masked subtle changes in expression.

Overexpression of miR-96/182 blocks synaptic scaling

Local translation of GluR1 is a critical step during the induction of certain forms of homeostatic plasticity. In response to activity blockade with TTX+APV, GluR1 translation occurs in dendrites near synapses, and homomeric GluR1-containing receptors are inserted into the plasma membrane and the synapse (Sutton et al., 2006; Aoto et al., 2008). We therefore

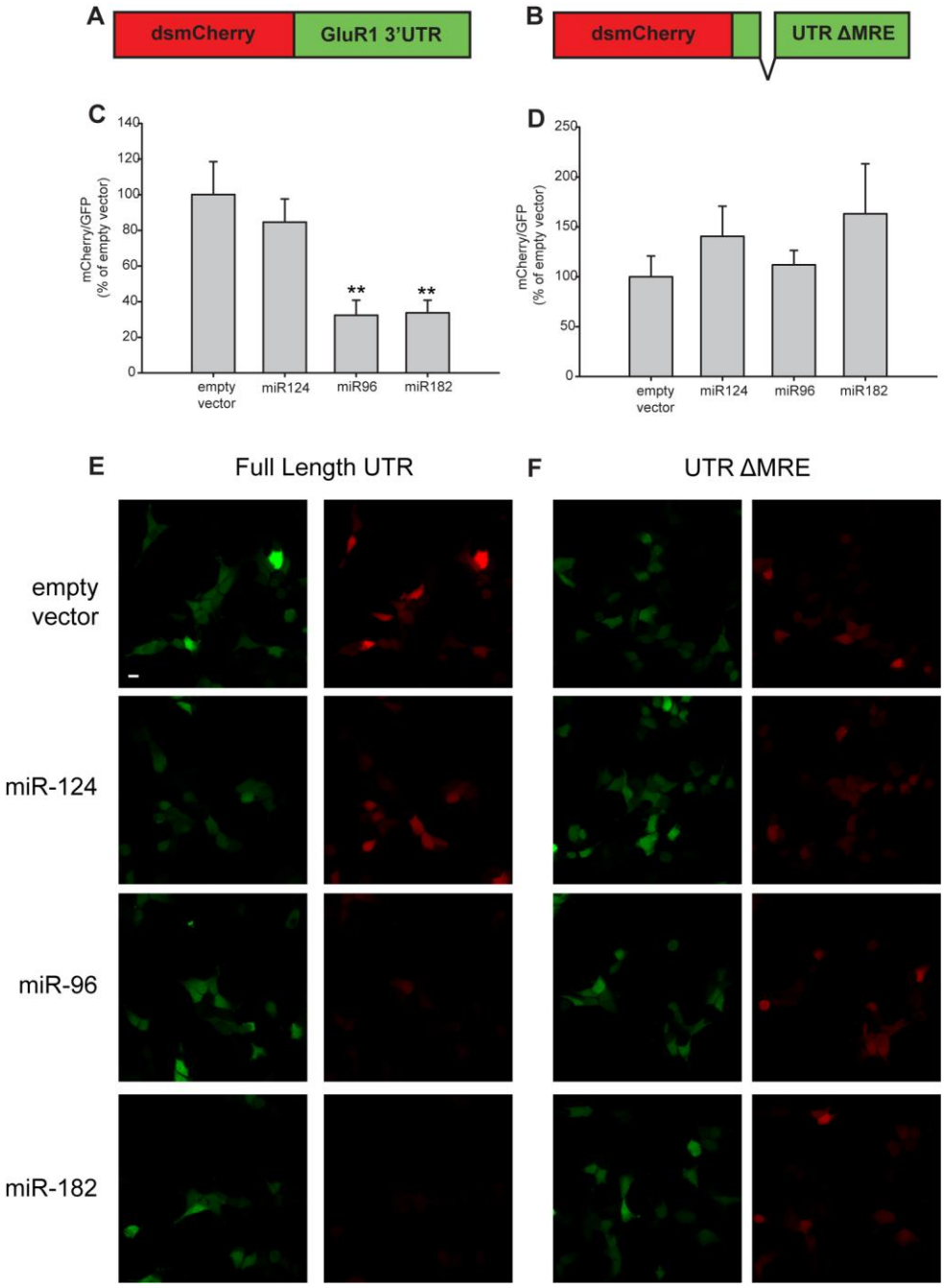

Figure 4.4. Repression of translation by miR-96/182 is dependent on presence of predicted binding site. (A and B) Schematics of the mCherry-UTR reporter constructs. **(C)** Quantification of dsmCherry signal, normalized to GFP signal from same cell, from HEK293T cells expressing the intact dsmCherry-UTR reporter along with the indicated miRNA (n = 24 cells/group; **, $p<0.01$). **(D)** Quantification of mCherry signal, normalized to GFP, from cells expressing the mCherry-ΔMRE reporter along with the indicated miRNA (n = 24 cells/group). **(E and F)** Representative images. Scale bar, 10 μm.

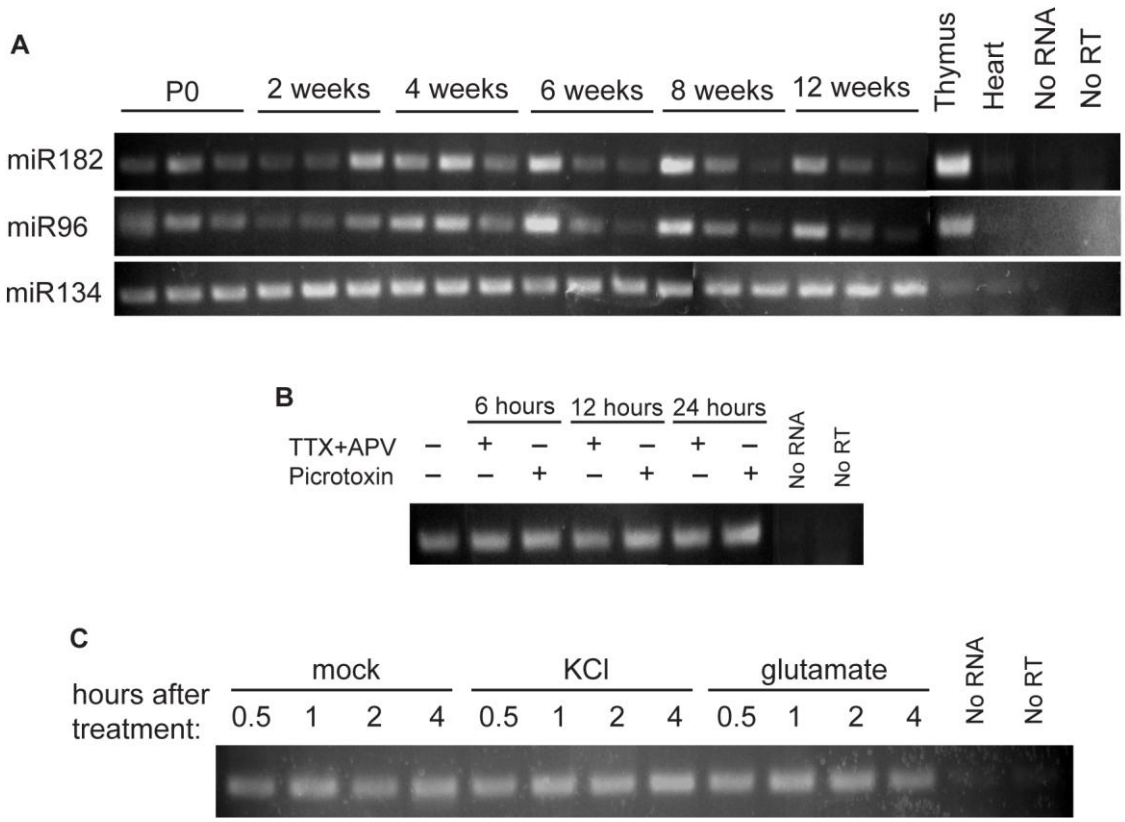

Figure 4.5. Developmental and activity-dependent expression of miR-96/182. (A) Stem-loop RT-PCR for the indicated miRNA from samples of rat cortex collected at the indicated ages. **(B)** Stem-loop RT-PCR of miR-182 from cultured hippocampal slices treated with either TTX+APV or picrotoxin for the indicated time points. **(C)** Stem-loop RT-PCR of miR-182 from acute cortical slices treated with KCl or glutamate for 3 minutes and harvested at the indicated time after treatment.

wished to test whether overexpression of miR-96 or miR-182 could prevent this new GluR1 translation and the subsequent increase in synaptic strength induced by TTX+APV treatment. While cells expressing empty vector or control miR-124 showed an increase in mEPSC amplitude after 24 hours of TTX+APV treatment, neurons expressing miR-96 or miR-182 failed to respond to activity blockade (Figure 4.6), implying that miRNA-mediated repression of GluR1 translation is sufficient to prevent synaptic scaling.

Knockdown of miR-96/182 does not affect GluR1 abundance or prevent synaptic scaling

Having demonstrated that overexpression of miRNAs 96 and 182 has a significant effect on GluR1 abundance and is capable of preventing TTX+APV-induced GluR1 translation, we wished to examine the effect of knocking down these miRNAs. One technique which has been effective in the knockdown of miRNAs is to express a miRNA "sponge" comprised of the coding sequence for a reporter, such as EGFP, followed by a 3'UTR containing multiple binding sites for a miRNA of interest (Ebert et al., 2007) (Figure 4.7A). The binding sites in the sponge are designed to bind the 5' and 3' regions of each miRNA with perfect complementarity, but an intentional mismatch is present at nucleotides 9-12 of the miRNA, in order to prevent cleavage of the sponge (Figure 4.7B). When the sponge is expressed, it will bind to and "soak up" endogenous miRNAs, preventing them from regulating their normal targets in the cell.

We designed a sponge with four binding sites each for miR-96 and miR-182 following the coding sequence for destabilized EGFP (dsEGFP; Figure 4.8A). As a proof of principle, we expressed dsEGFP or dsEGFP-sponge along with one or more miRNAs in HEK293T cells. If the sponge is recognized by the expressed miRNA, translation of the dsEGFP will be repressed and protein levels should decrease. Indeed, we saw that both miR-96 and miR-182 (expressed alone or together) were able to decrease the levels of translated dsEGFP-sponge (Figure 4.7C). miR-183, though it has some sequence similarity to both miR-96 and miR-182, did not reduce dsEGFP-sponge levels to the same extent, indicating the specificity of the sponge for miRNAs 96 and 182.

When the sponge was expressed in cultured neurons for 3 to 4 days, however, we were unable to detect a significant change in total GluR1 levels compared to neighboring untransfected cells (Figure 4.7D). Additionally, expression of the sponge did not prevent neurons from undergoing synaptic scaling in response to TTX+APV (Figure 4.7E). We conclude that either sponge expression was not sufficient to significantly affect miRNA levels in these neurons, or that the presence of additional miRNAs which regulate GluR1 masked any potential effect.

DISCUSSION

The regulation of protein translation by miRNAs is an increasingly common theme in the study of numerous cellular processes, and the list of established miRNA functions in neurons continues to grow. Here we present evidence that translation of the AMPA receptor subunit GluR1 can be inhibited by a co-regulated pair of miRNAs, miR-96 and miR-182. These two miRNAs have a high degree of sequence similarity, and they bind specifically to a designated MRE in the GluR1 3'UTR and repress translation of transcripts containing that sequence. Overexpression of either miR-96 or miR-182 is sufficient to block the homeostatic increase in synaptic strength induced by activity blockade with TTX+APV. miR-96 and miR-182 are

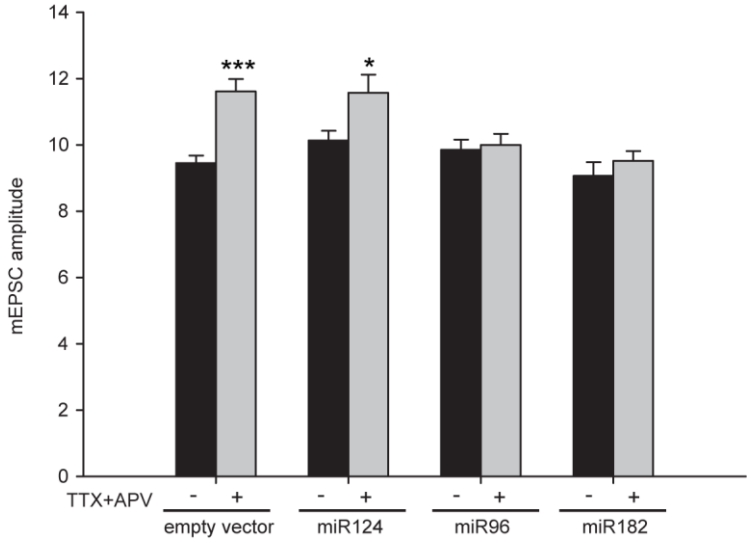

Figure 4.6. Overexpression of miR-96/182 blocks synaptic scaling. Average mEPSC amplitude from dissociated hippocampal neurons expressing the indicated miRNA and treated with 24 hours TTX+APV (n = 10-20 cells/group; *, $p<0.05$; ***, $p<0.001$).

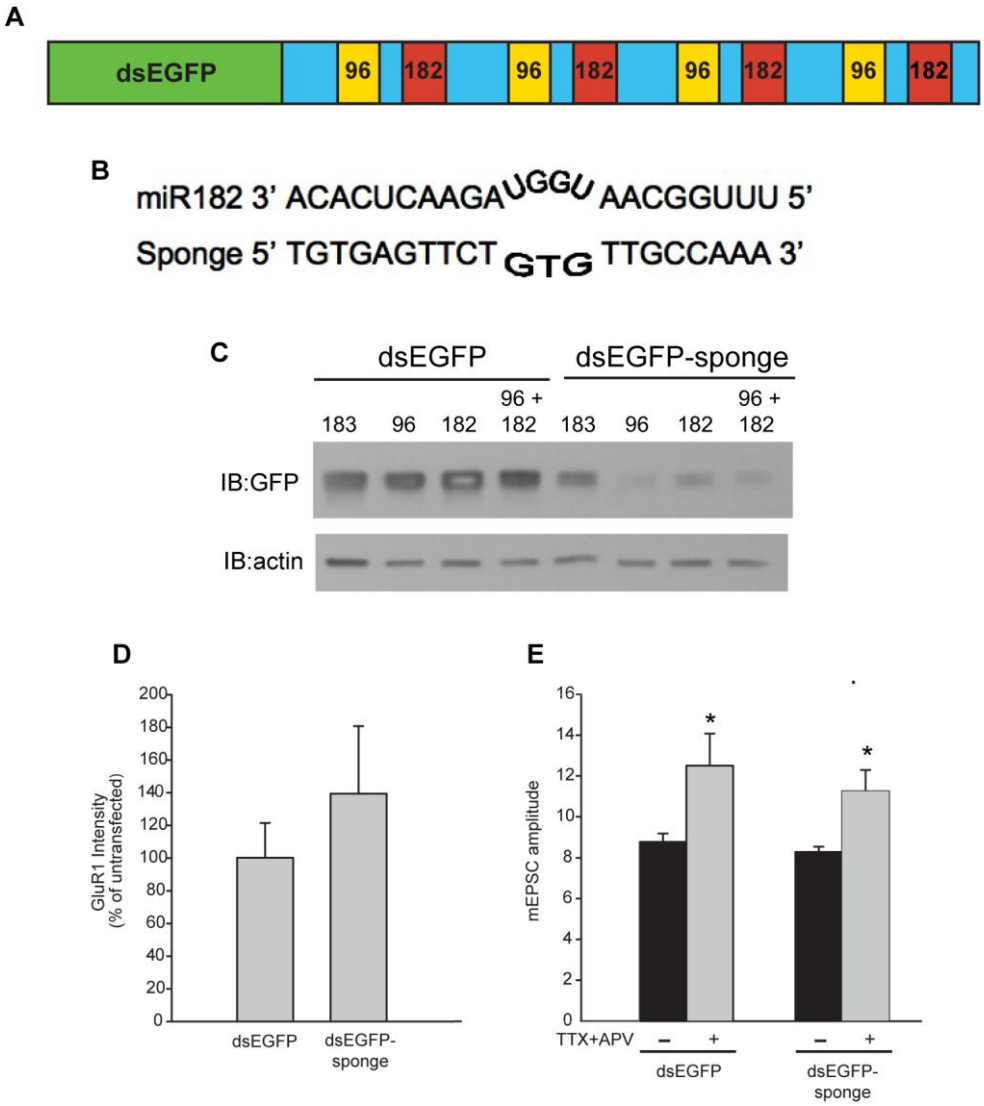

Figure 4.7. miRNA sponge does not affect GluR1 abundance or synaptic scaling. (A) Schematic of miRNA sponge, containing four binding sites each for miR-96 and miR-182. **(B)** Example of sponge binding site with bulge at bases 9-12 of the miRNA. **(C)** Expression of dsEGFP or dsEGFP-sponge in HEK293T cells co-transfected with the indicated miRNA(s). **(D)** Quantification of GluR1 staining intensity in the dendrites of hippocampal neurons 3-4 days after transfection with dsEGFP or dsEGFP-sponge (n = 8 cells/group). **(E)** Average mEPSC amplitude from neurons expressing dsEGFP or dsEGFP-sponge, treated with 24 hours of TTX+APV (n = 7-9 cells/group; *, $p<0.05$).

generated off a single primary genomic transcript, and are detectable in the cortex at all stages of postnatal development. Though expression levels varied somewhat between animals, no obvious activity-dependent changes in expression levels were detected. Additionally, attempts to knock down this pair of miRNAs did not reveal any detectable changes in GluR1 protein level or any impairments in homeostatic plasticity.

Though target prediction software was able to identify a number of predicted MREs in the 3'UTR of GluR1, only one set of miRNAs tested noticeably affected GluR1 abundance when overexpressed. However, our data do not rule out the possibility that other miRNAs may still bind to GluR1 and regulate its translation under some circumstances. A more detailed analysis using reporter constructs with different mutated MREs will be necessary in order to determine the potential for each predicted miRNA to regulate GluR1 translation.

Interestingly, while miR-96 and miR-182 were able to downregulate both total and surface levels of GluR1 protein, synaptic protein levels, as measured by mEPSC amplitude, were unaffected. This points to a tight regulation of synaptic glutamate receptor levels which is not necessarily affected by global protein levels. Indeed, unpublished observations from our own lab have found that when siRNA is used to significantly knock down global AMPA receptor levels in neurons, synaptic protein levels are only decreased slightly while extrasynaptic levels are decreased dramatically. These data support previous work demonstrating that regulation of the quantity and composition of AMPA receptors at the synapse is complex and is influenced by a number of factors, including phosphorylation of the c-terminus and the availability and state of post-synaptic scaffolding proteins (Kessels and Malinow, 2009).

We were able to detect mature miR-96 and miR-182 using RT-PCR in cortical tissue from rats at many stages of development. The presence of these miRNAs in the brain has also been confirmed by independent studies (Barad et al., 2004; Baskerville and Bartel, 2005; Kim and Nam, 2006; Tang et al., 2007). However, expression levels of both miRNAs were relatively low compared to other miRNAs tested, and varied between animals tested. Though this variability may be partially attributed to the low resolution of the end-point PCR technique used, it is interesting to note that miR-96 and miR-182 seemed to be expressed in parallel with each other, as would be predicted of miRNAs expressed from the same primary transcript.

We were curious as to whether this variability could be attributed to activity-dependent regulation of transcription of these miRNAs. Binding sites for the transcription factor Elk-1, which is known to induce activity-dependent transcription of immediate early genes such as c-fos, are present upstream of this cluster (Xu et al., 2007). However, treatments which altered neuronal activity over either short or long time periods failed to induce a noticeable change in the expression level of either miRNA. A more detailed examination of activity-dependent changes, using quantitative PCR methods and separating synaptic from somatic tissue may be more successful in illuminating potential regulatory factors controlling the expression of these miRNAs.

Regulation of this miRNA cluster also has implications beyond the control of GluR1. A number of other key synaptic proteins are predicted targets of one or more of these miRNAs (Table 4.2), and thus the potential for regulation of a much larger network of genes is substantial. Though most miRNAs are predicted to bind a large number of targets, in many cases only one specific target has been verified for each studied miRNA. It remains to be seen how broad the regulation by different miRNAs can be, and how variable the size the mRNA pool affected by each miRNA is. Whether the dominant function of miRNAs is to serve as constitutive regulators

Table 4.2. Potential targets of miR96/182/183 cluster

Italicized are targets of two miRs
Bold are targets of three miRs

Glutamate Receptors
GluR1
GluR3
mGluR1
mGluR5
GluRdelta1

Signaling Cascade Molecules
BDNF
CREB
CREB3-like2
CaMKII inhibitor 1
PKA R1alpha, *Cbeta*
PKC alpha, *epsilon*
PP1 R2, R9A, *R9B, R11, R12A*, R12C, R14B
PP2 R2A, R5C, CA, CB
PP3 (formerly PP2B) R1
MAP2K1
MAP2K3
MAP3K3
MAP3K4
MAPK9
MAP3K7 interacting protein 3
MAPK8 interacting protein 1
RICTOR
RAPGEF4

Translation Factors
FMR1
FXR1
CPEB1
CPEB2
CPEB4
*PABP interacting proteins 1 and **2***
EIF2 S1, C1, C2
EIF3 S1, S10
EIF4A2
EIF4EBP2
EIF5

Transsynaptic Molecules
Neurexin
Neuroligin 2
EPHA3
EPHA4
EPHA7
EPHB1
Ephrin B2
Ncam1
Nrcam

Ion Channels
CACN A1C (L-type), **A1E** (R-type), *A2D1*, *A2D2*, B1, *B2*, *B4*
KCNA1 (shaker K channel)
KCNJ14 (inwardly rectifying K channel)
KCNK2, K10 (voltage dependent)

Other
DLG2
DLG associated protein 2
Neurocalcin delta
Plasticity related gene 1
Syntaxin5
SNAP23
VAMP ass'd proteins B, C
CBLN4

of translation or whether they play a more significant role as activity-dependent regulators also merits further investigation

Though overexpression of miR-96 or miR-182 can block the induction of synaptic scaling, this may or may not implicate these miRNAs as members of the endogenous homeostatic plasticity machinery. miRNA repression of GluR1 translation may be a key factor in holding dendritic transcripts in an untranslated state until the appropriate signal is received from the synapse. This is an attractive model, and if true, could play a role in both Hebbian and homeostatic forms of plasticity. Conversely, overexpression of these miRNAs may simply place an artificial constraint on the translation of GluR1 mRNAs that would normally not be under miRNA regulation. An unbiased microarray screen for miRNAs which are regulated during long-term activity changes would provide valuable information for understanding what role miRNAs have in homeostatic plasticity.

The absence of a detectable phenotype after knockdown of miR-96 and miR-182 with the sponge technique was disappointing, though it does not rule out that this miR cluster has an endogenous effect on GluR1 regulation. One possible explanation is that the sponge was not expressed at high enough levels to ensure complete knockdown of both miRNAs. Knockdown of miRNAs is not a trivial matter, as the targeting of such small molecules is difficult. and it is possible that alternative methods of miRNA knockdown would prove more effective. Another likely possibility is that additional unknown miRNAs bind to and regulate GluR1, and that this regulation was maintained even during the knockdown of miR-96 and miR-182. A more precise method of investigating GluR1 translation, for example using pulse-chase labeling with radioactive amino acids, would provide more definitive evidence regarding the true endogenous effects of these miRNAs.

Clearly questions remain regarding the specifics of GluR1 regulation by miR-96/182. Do these miRNAs bind only to a subset of GluR1 mRNAs? Is binding dependent on the presence or absence of any additional factors? Is the primary function of this regulation to maintain baseline abundance of receptors, or is there a plasticity-related function? And more generally, is there sub-cellular regulation of miRNA dynamics, at the single synapse or dendrite level? Once an miRNA binds to its target, what regulates the release of that target to allow for translation? Only by addressing these questions will we gain an better understanding of the function of these specific miRNAs, and also of the rules which govern the entire neuronal cohort of miRNAs.

Chapter 5
Conclusions and Perspectives

A neuron's ability to maintain precise control over the translation of proteins near the synapse is a critical mechanism for regulating synaptic strength. Here I have investigated the translational control of the AMPA-type glutamate receptor subunit GluR1 and identified two regulatory elements that contribute to GluR1 abundance and the control of its translation.

FMRP and homeostatic plasticity

First, I demonstrated that FMRP is required for local translation of GluR1 induced either by activity blockade with TTX and APV or by direct application of RA. The requirement for FMRP is specific to the form of synaptic scaling mediated by RA and expressed via local GluR1 translation. The ability of FMRP to repress translation by binding mRNAs via its RGG box domain is critical for this regulation. Equally essential is the ability of FMRP to enter actively translating polyribosomes and permit translation of its bound mRNA transcripts.

Mechanism of FMRP's involvement in synaptic scaling

Though the requirement for FMRP in RA-mediated synaptic scaling is definitive, many questions remain about the precise mechanism of this function. Critically, we still do not understand how FMRP and RARα work together to mediate GluR1 translation during synaptic scaling. Though FMRP and RARα proteins do not interact directly, it is possible that they may bind to the same RNA molecules. This is particularly intriguing because RARα binds to the 5'UTR of GluR1, whereas FMRP typically binds to the 3'UTRs of its regulated transcripts. One possible mechanism is that even after RA binds to RARα and causes it to release its bound mRNAs, FMRP is still required in order to shuttle those transcripts into actively translating polyribosomes. Some evidence suggests that the phosphorylation state of FMRP determines whether it is found in translationally active or translationally repressed complexes. Whether activity blockade or RA affects phosphorylation of FMRP remains to be seen.

Another possibility is that FMRP does not regulate GluR1 directly, but instead regulates the translation of other proteins required for GluR1 synthesis or trafficking. Though no group has identified GluR1 mRNA as a direct substrate of FMRP, it has been noted that both GluR1 and GluR2 mRNAs are elevated in the polyribosomes of *Fmr1* knockout neurons (Muddashetty et al., 2007), indicating some effect of FMRP on the translational regulation of these transcripts. A definitive answer to the question of whether FMRP and GluR1 mRNA interact directly will be critical for our understanding of this regulatory mechanism.

The inability of RA to induce measurable GluR1 translation in the absence of FMRP may also be attributable to excessive baseline GluR1 translation. Our experiments reintroducing FMRP into knockout neurons imply that AMPA receptor translation is elevated in these cells, and one possibility is that we simply can not detect any additional translation on top of these already elevated levels. Close examination of the polysome profile of both wild-type and *Fmr1* knockout neurons in the presence or absence of RA may shed some light on the precise translational state of AMPAR mRNAs during synaptic scaling, and how FMRP may affect this state.

Translation of new proteins is only one of many cellular processes which contribute to regulating the abundance of any given protein in the cell or at the synapse. When considering translation, we must also consider its counterpart: protein degradation. Indeed, if AMPA receptor translation is in fact elevated in FMRP knockout neurons, but levels of AMPA receptor proteins are unchanged from wild-type cells, it follows that the degradation of these receptors must also be altered in order to provide compensation. If this is the case, it may be that altered degradation also contributes to the impairments in synaptic scaling seen in the absence of FMRP.

Multiple forms of homeostatic plasticity, in vitro and in vivo

Our results also raise interesting questions about the various forms of homeostatic plasticity which can be induced *in vitro*. First, how do the signaling cascades underlying each type of plasticity interact? We have observed that short-term treatment with TTX+APV leads to local translation and insertion of GluR1 homomeric receptors, but after a prolonged treatment these receptors are replaced with GluR2-containing receptors. Is this a manifestation of the same intracellular process stimulated by long-term TTX treatment alone, or is this a separate mechanism? And how does the addition of APV lead to the synthesis of RA? Ongoing studies in our laboratory are currently addressing these critical questions.

Additionally, it is essential that we achieve a greater understanding of the function of homeostatic plasticity *in vivo*. Which form or forms of synaptic scaling occur in the intact brain? Does this vary by region or across development? On what time scale and over what physical area does synaptic scaling occur *in vivo*? Is scaling controlled by activity levels of a stretch of dendrite, or an entire cell, or a network of cells? At present, no satisfactory paradigms exist which can be used to ask these questions in the intact animal, but the development of such model systems would be of tremendous benefit to the field.

Link between homeostatic plasticity and Hebbian plasticity

The study of FMRP's role in homeostatic plasticity is complicated by the large number of functions this protein has in the neuron, and by the fact that it is known to be involved in Hebbian forms of plasticity as well. Is there a potential link between impaired synaptic scaling and impaired LTP or LTD? The inability of *Fmr1* knockout neurons to adjust their global synaptic strengths and firing properties may be a contributing factor to the inability of these cells to adjust individual synaptic strengths as well. Neurons which are not scaled up or scaled down correctly may end up outside the dynamic range for both the detection of plasticity-inducing stimuli and for the proper expression of synaptic strength changes. Do Hebbian and homeostatic plasticities utilize overlapping intracellular mechanisms or signaling cascades? If so, does one form of plasticity occlude the other? Does homeostatic synaptic strengthening affect the induction or magnitude of Hebbian synaptic weakening (LTD), and vice versa? Investigations regarding the link between homeostatic and Hebbian plasticity are ongoing in our laboratory, and will provide valuable answers to these questions.

The primary form of Hebbian plasticity which is studied in the context of FMRP is mGluR-induced LTD, which is both enhanced in magnitude and uncoupled from its dependence on new protein translation in *Fmr1* knockout neurons (Huber et al., 2002). The "mGluR theory" of Fragile-X syndrome proposes that most if not all of the phenotypes found in Fragile-X patients can be attributed to hyperactive signaling of mGluR receptors through its downstream signaling cascades (Bear et al., 2004). This theory is supported by evidence showing that reducing activity of group I mGluR receptors, either with pharmacology or genetics, can rescue

many phenotypes in the *Fmr1* knockout mouse (Dolen et al., 2007). The contribution of mGluR receptors to homeostatic plasticity, however, has not been investigated. It is certainly possible that excessive signaling from these receptors alters the state of the cell or the synapse in a way that inhibits synaptic scaling. A critical next step will be to test whether inhibition of mGluR receptors can partially or completely restore homeostatic plasticity to these neurons.

Contribution of homeostatic plasticity to the Fragile-X phenotype

It is intriguing to consider the possible contribution that impaired homeostatic plasticity has to the phenotype of Fragile X patients or *Fmr1* knockout animals. The most obvious connection is the reports of altered neuronal excitability in both Fragile-X humans and model mice. Approximately 10 to 20% of Fragile-X patients experience epilepsy, which has been attributed to increased neuronal excitability (Berry-Kravis, 2002). Similarly, *Fmr1* knockout mice have an increased susceptibility to seizures, specifically audiogenic seizures (Yan et al., 2004), as well as an enhanced startle response to auditory stimuli (Nielsen et al., 2002; Yan et al., 2004). A recent report has found direct evidence of altered excitation and inhibition ratios in the somatosensory cortex of *Fmr1* knockout animals (Gibson et al., 2008). Attributing these excitability alterations and seizures to a loss of homeostatic adjustment is an attractive hypothesis, but one which remains to be proven. Critically, though we have demonstrated a definitive role for FMRP in upscaling of synaptic strength, the mechanisms governing synaptic *down*scaling are much less well understood, and a possible role for FMRP in this process has not been investigated. An improved understanding of the full contribution of FMRP to bidirectional synaptic scaling will greatly increase our knowlege of how altered homeostatic plasticity may contribute to the seizure phenotype seen in Fragile-X patients.

Up to 30% of Fragile-X patients are also diagnosed with autism (Hatton et al., 2006), and many behavioral phenotypes seen in the *Fmr1* knockout mouse correlate with autistic symptoms, including defects in social interactions, perseverance, anxiety, learning, hyperactivity, and responses to sensory stimuli (Bernardet and Crusio, 2006). Notably, it has been proposed that the symptoms of autism could be caused by an increased ratio of excitation to inhibition in critical brain regions (Rubenstein and Merzenich, 2003). A loss of control over homeostatic plasticity could be one mechanism that would lead to this imbalance in Fragile-X patients and contribute to the high incidence of autism spectrum disorders found in this group.

miRNA regulators of GluR1 translation

I also identified a pair of GluR1-regulating miRNAs. miR-96 and miR-182, generated from the same primary transcript, lead to a decrease in total and surface GluR1 levels when overexpressed in neurons. This overexpression also blocks the induction of synaptic scaling, and repression of translation was confirmed to be dependent on the presence of the predicted MRE in the GluR1 3'UTR. However, the endogenous role of these miRNAs remains somewhat elusive. If this pair of miRNAs is truly involved in regulating GluR1 translation during plasticity, one would expect that the levels of miR-96 and miR-182 would vary during changes in activity level. Though we could not detect any significant changes, this could be a reflection of the technique used, or the time points examined. Another possibility is that the localization, rather than the abundance, of these miRNAs is affected by synaptic activity. A closer examination of expression in synaptoneurosomes may be able to address this issue.

A role for miRNAs in plasticity is not novel. A recent microarray identified miRNAs in the hippocampus which change their expression levels after induction of either chemical LTP or

mGluR-LTD (Park and Tang, 2009). Interestingly, most miRNAs which showed expression changes displayed increased levels after plasticity induction, implying a possible role for these miRNAs in keeping translation in check during this critical time period. A similar analysis of changes in miRNA expression after induction of synaptic scaling would yield valuable information regarding the role that this class of molecules plays during this critical form of plasticity.

Interaction between FMRP and miRNAs

One discovery that is particularly fascinating in the context of this work is that FMRP seems to interact directly with miRNAs (Jin et al., 2004; Plante et al., 2006; Edbauer et al., 2010). FMRP associates with miRNA processing proteins and members of the RISC complex, and it has been proposed that miRNAs may mediate the binding between FMRP and its mRNA targets. A number of FMRP-associated miRNAs were recently identified, and FMRP is required for a change in spine morphology caused by overexpression of certain miRNAs (Edbauer et al., 2010). In fact, the number of miRNAs which have thus far been identified as regulators of neuronal spine morphology is intriguing, as altered spine morphology is a hallmark symptom found both in Fragile-X patients and model mice. These findings raise the question of whether altered miRNA function in the absence of FMRP is in fact the primary cause of the spine abnormalities found in *Fmr1* knockout neurons. Numerous studies have shown direct interaction between FMRP and mRNAs, but it remains to be seen how universal and bidirectional this mechanism will be: do all miRNAs targets depend on FMRP for repression, and does repression of all FMRP targets require binding through a miRNA? Regardless of whether they function independently or in concert, both miRNAs and FMRP are critical regulators of translation, and both are necessary to maintain the proper functioning of each neuron and every synapse.

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