

Fragile X Protein FMRP Is Required for Homeostatic Plasticity and Regulation of Synaptic Strength by Retinoic Acid

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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 of AMPA-type glutamate receptors (AMPA receptors) in dendrites and promotes synaptic insertion of newly synthesized AMPARs. Here, we show that 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* knock-out neurons, RA-dependent dendritic translation of GluR1-type AMPA receptors is impaired. Intriguingly, FMRP is only required for the form of homeostatic plasticity that is dependent on both RA signaling and local protein synthesis. Postsynaptic expression of wild-type or mutant FMRP(I304N) in knock-out neurons reduced the total, surface, and synaptic levels of AMPARs, implying a role for FMRP in regulating AMPAR abundance. Expression of FMRP lacking the RGG box RNA-binding domain had no effect on AMPAR levels. Importantly, postsynaptic expression of wild-type FMRP, but not FMRP(I304N) or FMRP Δ RGG, restored synaptic scaling when expressed in knock-out neurons. Together, these findings identify an unanticipated role for FMRP in regulating homeostatic synaptic plasticity downstream of RA. Our results raise the possibility that at least some of the symptoms of fragile X syndrome reflect impaired homeostatic plasticity and impaired RA signaling.

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 synthesis and insertion of AMPA-type glutamate receptors (AMPA receptors) (Ju et al., 2004; Thiagarajan et al., 2005; Sutton et al., 2006; Aoto et al., 2008).

We 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 tetrodotoxin (TTX), along with blockade of NMDA receptors with aminophosphonovalerate (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 RAR α , which inhibits protein translation through direct binding to specific target mRNAs (Poon and Chen, 2008). Addition of RA reverses the RAR α -dependent repression of translation of target mRNAs (Maghsoodi et al., 2008; Poon and Chen, 2008), and acute knockdown of RAR α completely blocks synaptic scaling (Aoto et al., 2008). One of the RAR α 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).

Fragile X mental retardation protein (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 knock-out (KO) mice exhibit normal baseline synaptic transmission but have altered spine morphology (Comery et al., 1997; Irwin et al., 2000), impairments in certain forms of long-term potentiation (LTP) (Li et al., 2002; Larson et al., 2005), and exaggerated metabotropic glutamate receptor (mGluR)-dependent long-term depression (LTD) (Huber et al., 2002). FMRP is associated with both translationally repressed messenger ribonucleoprotein particles and 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

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al., 2001; Bassell and Warren, 2008). Consistent with this notion, dysregulated translation and elevated basal protein synthesis are found in *Fmr1* knock-out neurons (Dolen et al., 2007; Muddashetty et al., 2007). However, whether FMRP is involved in translational regulation during homeostatic plasticity is unknown.

Here we report that FMRP is required postsynaptically for the form of synaptic scaling that is mediated by RA. Although RA synthesis is normal in *Fmr1* knock-out 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. Our data reveal an unanticipated role for FMRP in homeostatic synaptic plasticity and RA signaling.

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 enhanced green fluorescent protein (EGFP). All FMRP constructs used were the full-length isoform 1 (Ashley et al., 1993). For coimmunoprecipitation (CO-IP) experiments, FMRP was tagged with FLAG at the N terminus, RAR α with Myc at the N terminus, and FXR1 with Myc at the C terminus. The lentiviral transfer vector JHUG was derived from the original L307 vector. The internal ribosomal entry site sequence downstream of a ubiquitin promoter in L307 was deleted and replaced with a multiple cloning site (MCS) followed by the EGFP coding sequence. Mouse FMRP and FMRP(I304N) coding sequences were then inserted into the MCS. The RGG box [amino acids RRGDGRRRGGGGRRGQGGRRGGGFKGN (as described by Darnell et al., 2005a)] was removed using PCR deletion.

Antibodies. The following mouse monoclonal primary antibodies were used in this study: actin, FMRP, GluR1 N terminus, GluR2, and RAR α (Millipore Corporation), PSD-95 (Affinity Bioreagents), NR1 (BD Biosciences Pharmingen), Arc (Santa Cruz Biotechnology), FLAG (Sigma), and Myc (Roche). The following rabbit polyclonal primary antibodies were used: GluR1 (Millipore Corporation), EF2 and phospho-EF2 (Thr56) (Cell Signaling Technology), Stargazin and Myc (Abcam), and MAP1b 750 (a generous gift from Dr. Itzhak Fischer, Drexel University, Philadelphia, PA).

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

Mice. Wild-type (WT) and *Fmr1* knock-out mice in the FVB background were obtained from The Jackson Laboratory.

Cell cultures and drug treatment. Primary hippocampal cultures were prepared from mice at postnatal day 0 (P0) or P1 and maintained in serum-free Neurobasal medium supplemented with B-27 and Glutamax (Invitrogen) 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 μ M TTX and 100 μ M APV was used to induce synaptic scaling in dissociated cultures, and 36 h treatment of 10 μ M TTX and 1 mM APV was used to induce synaptic scaling in slice cultures. Four hour treatment of 2 μ M RA was used to induce synaptic scaling in slice cultures, and 30 min treatment of 1 μ M RA followed by 1 h of washout was used to induce synaptic scaling in dissociated cultures. When indicated, 100 μ M cycloheximide or 0.5 μ g/ml actinomycin D was applied for 30 min before RA treatment and remained in the media during RA treatment. To induce RA-independent synaptic scaling, 48 h treatment of 1 μ M TTX in dissociated culture or 60 h treatment of 10 μ M TTX in slice culture was used. DEAB at 10 μ M was applied when indicated.

Retinoic acid response element assay. Dissociated cultures used for retinoic acid response element (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 described previously (Aoto et al., 2008). Briefly, human embryonic kidney 293T (HEK293T) cells were transfected using calcium phosphate with the transfer vector and three helper plasmids. After 48 h, supernatants were pooled, spun at 25,000 rpm through a sucrose cushion for 1.5 h, and resuspended in PBS. Virus was injected into the CA1 region of slices on the day of cutting. For imaging, slices at 6–7 d *in vitro* (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 7 DIV and lysates were harvested at 13 DIV 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 M Ω patch pipette filled with an internal solution containing the following (in mM): 140 CsCl, 2 MgCl₂, 5 EGTA, 10 HEPES, 0.3 Na₂-GTP, and 4 Na₂-ATP, pH 7.35. Slices were continuously superfused with external solution containing the following (in mM): 120 NaCl, 26 NaHCO₃, 2.5 KCl, 11 glucose, 2.5 CaCl₂, 1.3 MgSO₄, and 1.0 NaH₂PO₄. Tetrodotoxin (1 μ M) and picrotoxin (100 μ M) were included in the external saline, along with 5 μ M philanthotoxin when indicated. Cells were held at –60 mV. Miniature responses were analyzed with Mini Analysis Program (Synaptosoft).

Surface biotinylation assay. Cultured hippocampal cells were washed with cold PBS/Mg²⁺/Ca²⁺, and surface proteins were biotinylated with 1 mg/ml Ez-link sulfo-NHS-SS-biotin (Pierce) in PBS/Mg²⁺/Ca²⁺ for 25 min on ice. Cells were washed with 0.1 M glycine in ice-cold PBS/Mg²⁺/Ca²⁺ to stop additional 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, and protease inhibitor cocktail). Lysates were centrifuged to remove cell debris and nuclei at 14,000 rpm for 20 min, and supernatants were rotated with Ultralink-immobilized streptavidin beads (Thermo Fisher Scientific) for 2 h at 4°C 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 \times g for 5 min at 4°C, and the supernatant was filtered through three layers of 100 μ m pore nylon mesh (Millipore Corporation) and a 5 μ m pore polyvinylidene difluoride (PVDF) syringe filter (Millipore Corporation). The filtrate was then centrifuged for 10 min at 10,000 \times g at 4°C, and the supernatant was removed. The synaptoneurosome-containing pellet was then resuspended in lysis buffer (see above).

Spine morphology assay. Neurons were transfected as described above at 12–13 DIV with pSUPER, a plasmid that expresses high levels of EGFP. Cells were then treated, fixed, and imaged as described above. Two to three 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.

Quantitative PCR. RNA from cultured slices or synaptoneurosome 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 the protocol of the manufacturer. Quantitative PCR (qPCR) was performed using Sybr Green supermix (Bio-Rad) on an iQ5 thermal cycler (Bio-Rad). Cycle threshold 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GluR1, GluR2, and GAPDH primer sequences were adapted for mouse from those used by Dijk et al. (2004). All primers were tested for speci-

ficity and efficiency using melting curve and dilution curve analysis. Primer sequences are available in supplemental Table 1 (available at www.jneurosci.org as supplemental material).

³⁵S 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 (Invitrogen). RA or DMSO was added along with EasyTag Express ³⁵S protein labeling mix (PerkinElmer Life and Analytical Sciences). After 2 h, media were 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, and 0.5% Na-deoxycholate). Debris was pelleted with centrifugation, and supernatants were rotated for 1 h 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 (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.1% NP-40, and 0.05% Na-deoxycholate), three times in wash buffer 3 (50 mM Tris-HCl, pH 7.4, 0.1% NP-40, and 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 appropriately sized band (identified by Western blot) was cut out from the membrane and analyzed for incorporated radioactivity using liquid scintillation counting. Background counts per minute (determined by analyzing a similar-sized band cut out from an unstained region of each lane) were subtracted from the AMPAR counts per minute 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. At 24 h after transfection, cells were washed and resuspended in PBS, pelleted, and lysed with rotating for 30 min at 4° (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.5% NP-40, and protease inhibitors). Samples were spun to pellet nuclei, and lysate was precleared with Protein-G beads (Invitrogen). Cleared lysates were rotated at 4° with antibody for 4 h and then with beads overnight. Beads were washed three times in lysis buffer, and bound protein was eluted in SDS sample buffer.

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

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* knock-out mice. Although 24 h of TTX + APV is sufficient to induce homeostatic plasticity in dissociated neurons, 36 h of treatment is required for robust scaling in slice culture (Aoto et al., 2008). TTX + APV treatment increased the amplitude of miniature EPSC (mEPSC) events in slices obtained from wild-type mice (Fig. 1A,B). In contrast, TTX + APV had no effect on mEPSCs in slices from *Fmr1* knock-out mice (Fig. 1A,B), indicating that loss of FMRP causes a defect in synaptic scaling. Consistent with previous reports (Braun and Segal, 2000), the baseline amplitude and fre-

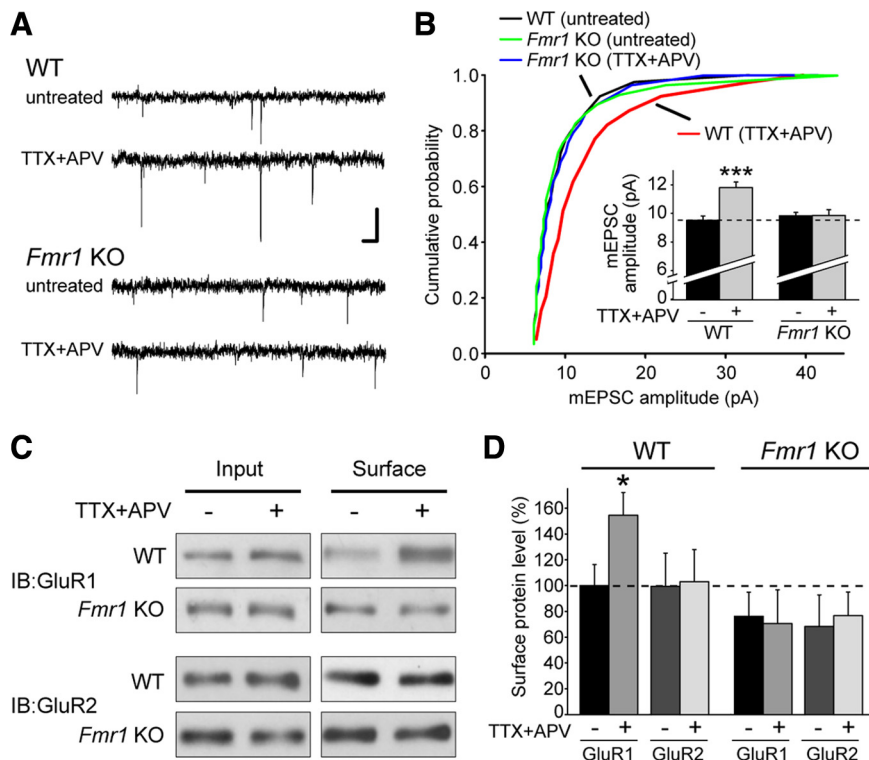


Figure 1. FMRP is required for TTX + APV-induced synaptic scaling. **A**, Representative mEPSC traces from wild-type and *Fmr1* knock-out (untreated and TTX + APV treated) neurons in hippocampal slice culture. Calibration: 10 pA, 40 ms. **B**, Cumulative distribution of mEPSC amplitudes from WT and KO neurons treated with 36 h of TTX + APV ($p < 0.001$, Kolmogorov–Smirnov test). Inset, Quantification of average mEPSC amplitude ($n = 28–34$; $***p < 0.001$). **C**, Representative blots for biotinylation of surface AMPARs in primary cultured neurons after 24 h of TTX + APV treatment. IB, Immunoblot. **D**, Quantification of **C**. Surface band intensity was normalized to input, and all groups were compared with WT untreated ($n = 4–6$; $*p < 0.05$). Error bars represent SEM.

quency of mEPSC events was not different between wild-type and knock-out slices (Fig. 1B) (supplemental Fig. 1A, available at www.jneurosci.org as supplemental material). Neither genotype showed a change in the frequency of mEPSCs after treatment (supplemental Fig. 1A, available at www.jneurosci.org as supplemental material).

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 (Fig. 1C,D). However, TTX + APV treatment failed to increase the surface levels of either GluR1 or GluR2 protein in neurons from *Fmr1* knock-out mice (Fig. 1C,D). 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* knock-out 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 knock-out mice (supplemental Fig. 1B,C, available at www.jneurosci.org as supplemental material). In addition, we saw no difference in the levels of RAR α protein (supplemental Fig. 1B,C, available at www.jneurosci.org as

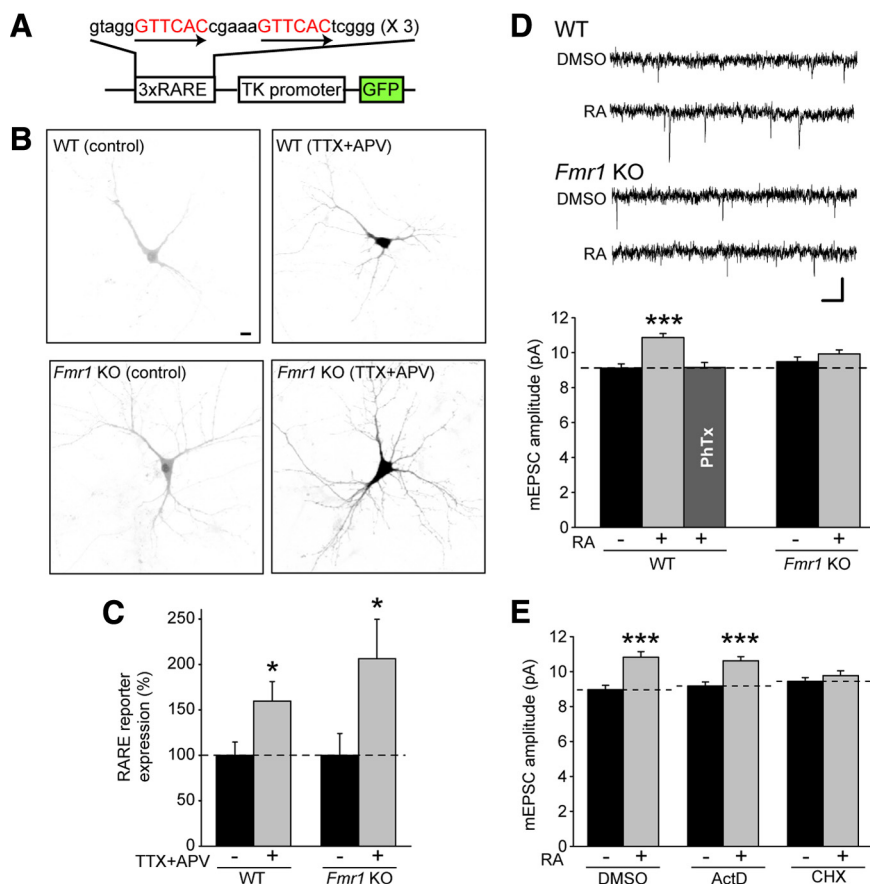


Figure 2. FMRP is not required for RA synthesis but is specifically required for RA-induced local translation-dependent synaptic scaling. **A**, Schematic of the 3xDR5–RARE–GFP reporter construct. **B**, Representative images of RARE–GFP reporter expression in WT and KO neurons with and without 24 h TTX + APV treatment. Scale bar, 10 μ m. **C**, Quantification of **B** ($n = 16–18$; $*p < 0.05$). **D**, Representative traces and quantification of mEPSC amplitude in WT and KO neurons after 4 h DMSO or RA treatment ($n = 31–33$; $***p < 0.001$). Philanthotoxin-433 was used to block GluR2-lacking AMPA receptor-mediated responses in the WT–RA group ($n = 22$). Calibration: 10 pA, 40 ms. **E**, Effect of transcription inhibitor actinomycin D and translation inhibitor cycloheximide on RA-induced synaptic scaling in WT neurons ($n = 22–27$; $***p < 0.001$). Error bars represent SEM.

supplemental material), which is also required for synaptic scaling (Aoto et al., 2008).

RA synthesis is normal in *Fmr1* knock-out neurons

Because RA synthesis is both necessary and sufficient for synaptic scaling (Aoto et al., 2008), we tested whether impaired synaptic scaling in *Fmr1* knock-out neurons is attributable to altered RA synthesis, using a genetic reporter system (Aoto et al., 2008). Dissociated hippocampal neurons from wild-type or *Fmr1* knock-out mice were transfected with a plasmid containing multiple copies of an RARE driving transcription of GFP (Fig. 2A). RAR α 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 knock-out neurons (Fig. 2B,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

Because RA synthesis is maintained in *Fmr1* knock-out neurons, we wondered whether direct application of RA is still capable of

upscaling synaptic strength in these neurons. In cultured hippocampal slices from wild-type mice, 4 h of RA treatment caused a significant increase in mEPSC amplitude without affecting event frequency (Fig. 2D) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). The increase in mEPSC amplitude in wild-type neurons was fully reversed by PhTx, a blocker of GluR2-lacking AMPA receptors (Fig. 2D), 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 upscaling in wild-type slices was unaffected by transcription blockers but required *de novo* translation of preexisting mRNAs (Fig. 2E). 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* knock-out mice (Fig. 2D). Thus, FMRP is required for synaptic scaling induced by TTX + APV or RA and acts downstream of RA.

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 (Dichtenberg et al., 2008). We wondered whether RA induces the movement of mRNAs toward synapses and, if so, whether FMRP is required for this process. Synaptoneuroosomes were collected from wild-type or knock-out 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 knock-out synaptoneuroosomes (supplemental Fig. 3A,B, available at www.jneurosci.org as supplemental material). Also, no effect of RA on mRNA levels was found in either genotype (supplemental Fig. 3A,B, available at www.jneurosci.org as supplemental material). 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 knock-out 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 knock-out animals. Analysis of GFP-expressing wild-type and knock-out neurons treated with TTX + APV or RA found no changes in spine density between genotypes or between treatments (Fig. 3A,B), confirming our physiology results showing no change in mEPSC frequency during homeostatic plasticity (supplemental Figs. 1A, 2, available at www.jneurosci.org as supplemental material).

org as supplemental material). Consistent with the literature, we found an increased average spine length in knock-out neurons compared with wild-type, but neither TTX + APV nor RA treatment had any effect on spine length in either genotype (Fig. 3*A, C*). Although this does not rule out that subtle spine shape changes may occur during synaptic scaling, we find no obvious link between the FMRP knock-out 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 h in dissociated neurons or 36 h 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 h in dissociated neurons (Turriano et al., 1998) or 60 h in slice culture (our results)]. Different from the rapid GluR1-dependent upscaling induced by TTX + APV, upscaling 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 h of TTX-alone treatment in wild-type slice cultures is insufficient to induce synaptic scaling (supplemental Fig. 4*A, B*, available at www.jneurosci.org as supplemental material). Intriguingly, however, we found that long-term (60 h) TTX treatment induced synaptic upscaling of mEPSC amplitudes even in the absence of FMRP (Fig. 4*A*) (supplemental Fig. 4*C*, available at www.jneurosci.org as supplemental material). 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 (Fig. 4*A*).

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 h of TTX treatment in dissociated neurons, we found no increase in GFP fluorescence in either wild-type or knock-out neurons (Fig. 4*B, C*), indicating no change in RA levels. Moreover, blocking RA synthesis with 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* knock-out slices (Fig. 4*D*) (supplemental Fig. 4*D*, available at www.jneurosci.org as supplemental material). 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 RAR α 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

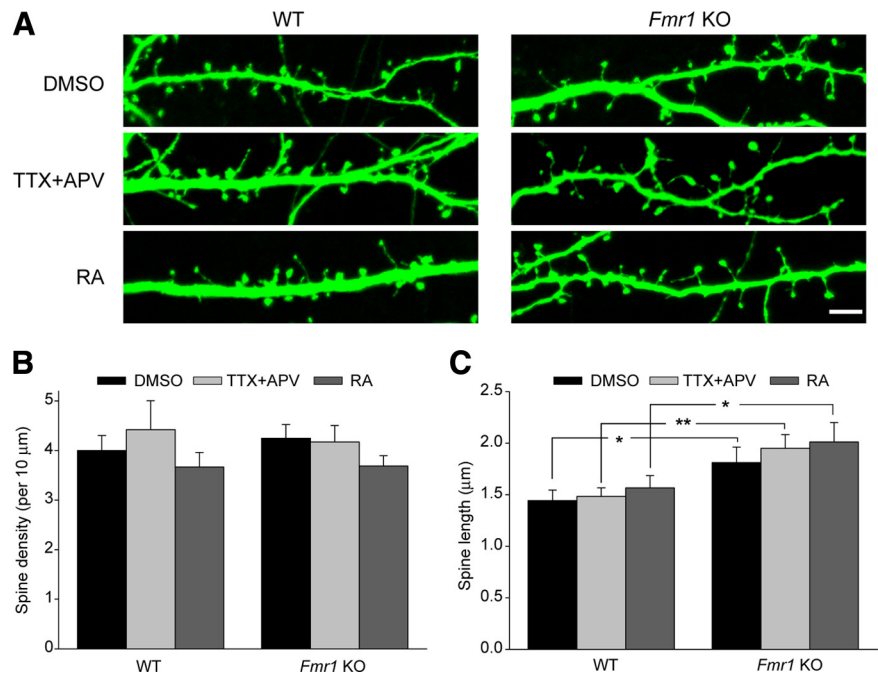


Figure 3. RA does not affect spine morphology in WT or KO neurons. *A*, Sample images of GFP-expressing WT or KO neurons treated with TTX + APV or RA. Scale bar, 5 μ m. *B, C*, Quantification of spine density and spine length in WT and KO neurons treated with 24 h of TTX + APV or 30 min (plus 1 h washout) of RA ($n = 9–10$ cells per group, 2–3 branches per cell). For spine density, $p > 0.5$. For spine length * $p < 0.05$, ** $p < 0.01$. Error bars represent SEM.

proteins in dendrites in response to RA. We isolated total lysates and synaptoneuroosomes from wild-type and *Fmr1* knock-out hippocampal slices treated with DMSO or RA and examined the levels of synaptic proteins by Western blotting. We found that, in synaptoneuroosomes from wild-type slices, RA significantly increased the levels of GluR1, GluR2, and eEF2 proteins (Fig. 5*A, C*). This effect was blocked by cycloheximide, indicating that the change is dependent on new protein translation (Fig. 5*D*). RA also marginally increased the levels of FMRP, but this was not statistically significant (Fig. 5*C*). RA had no effect on the levels of other synaptic proteins examined, including phosphorylated eEF2, PSD-95, Stargazin, or NR1 (Fig. 5*A, C*).

Strikingly, RA treatment failed to elicit changes in any synaptic protein in synaptoneuroosomes from *Fmr1* knock-out slices (Fig. 5*A, C*), indicating that FMRP is indeed required for RA-stimulated increases in synaptic protein levels. In contrast to synaptoneuroosomes, we observed no RA-dependent changes in the abundance of any protein in whole-cell lysates from either wild-type or *Fmr1* knock-out slices (Fig. 5*A, 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 knock-out animals at some (but not all) developmental stages (Lu et al., 2004; Hou et al., 2006), others have seen decreased levels in knock-out tissue (Chen et al., 2003; Wei et al., 2007). We saw no detectable differences in MAP1b levels between wild-type and knock-out slices at this developmental stage and no effect of RA on MAP1b levels (Fig. 5*A–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 differ-

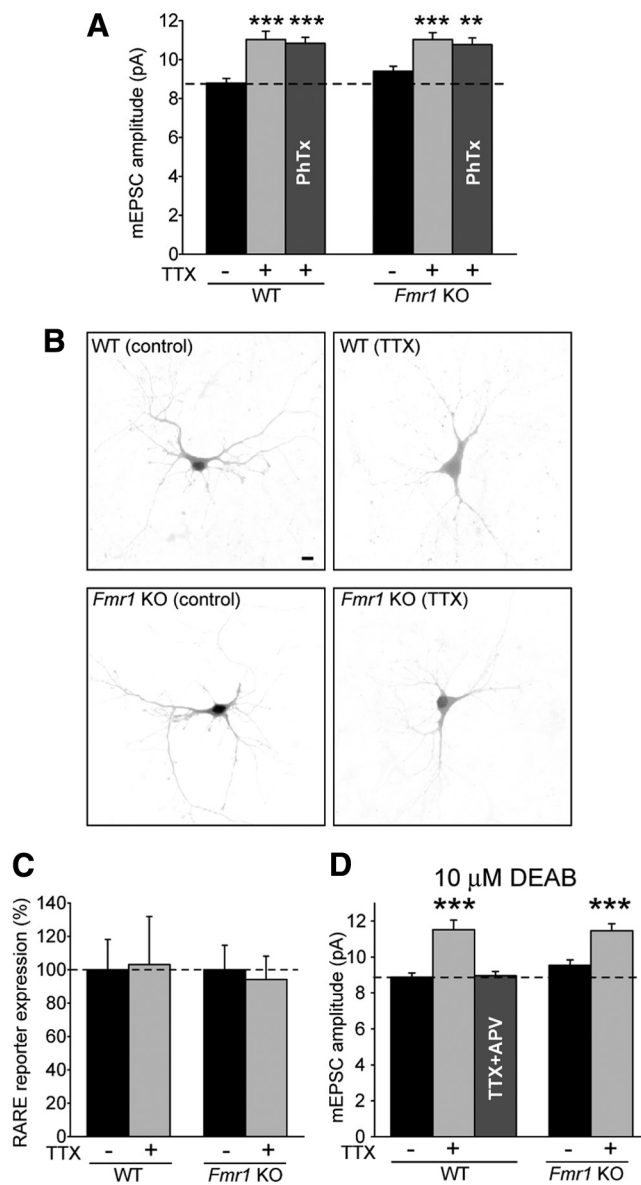


Figure 4. Neither FMRP nor RA is required for TTX-alone-induced synaptic scaling. **A**, Sixty hours of TTX induced synaptic scaling in WT and KO neurons. PhTx was used to block GluR2-lacking AMPA receptor-mediated responses ($n = 18-39$; $*p < 0.01$; $***p < 0.001$). **B**, Representative images of RARE-GFP reporter expression in WT and KO neurons with and without 48 h of TTX treatment. Scale bar, 10 μm . **C**, Quantification of **B** ($n = 22-28$). **D**, Effect of the RA synthesis blocker DEAB on 60 h of TTX-induced synaptic scaling and 36 h of TTX + APV-induced scaling ($n = 21-27$; $***p < 0.001$). Error bars represent SEM.

ences in protein level between wild-type and knock-out slices (Fig. 5A–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

Although our analysis of specific proteins after RA treatment showed an increase in synaptic AMPAR levels that was dependent on both FMRP and new protein translation (Fig. 5), we wanted 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 knock-out neurons along with DMSO or RA. Dissociated cultures were used for these experi-

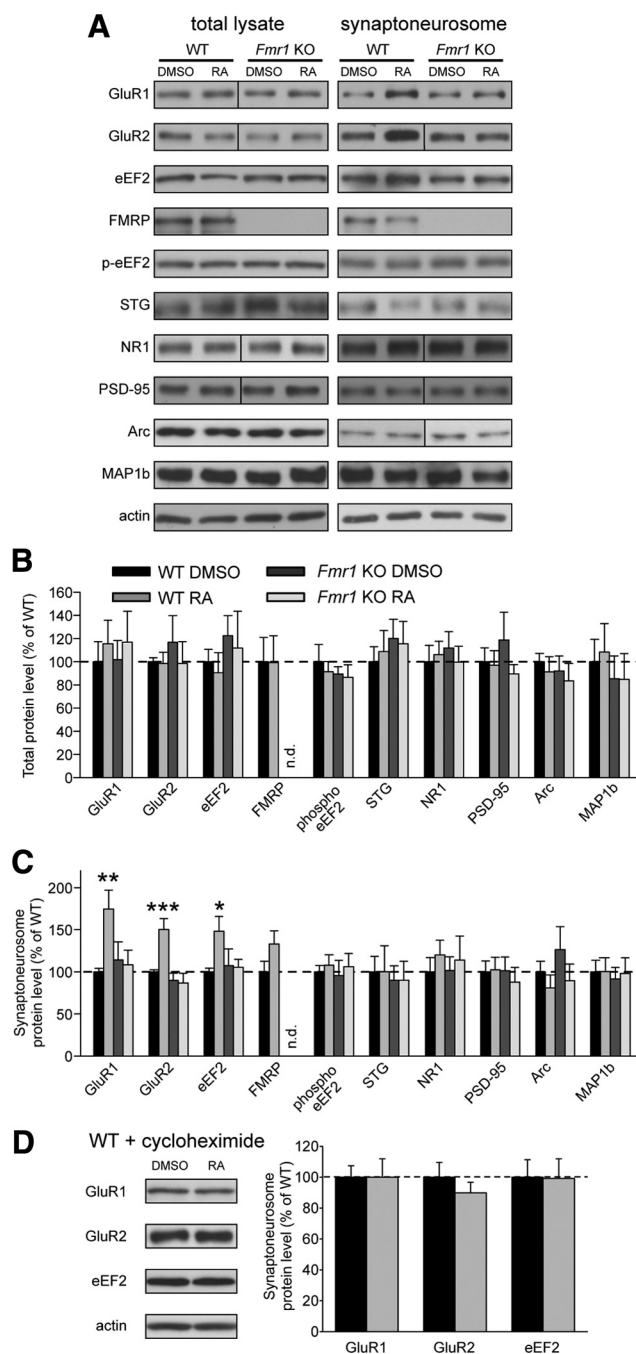


Figure 5. FMRP is required for RA-induced local translation of specific synaptic proteins. **A**, Representative blots of synaptic proteins from whole lysates and synaptoneurosomes (SNS) fractions of WT and KO hippocampal slices, treated with 4 h of DMSO or RA. A vertical line indicates the removal (for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

ments to ensure a rapid and complete penetration of the labeling mix to all cells. After 2 h 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

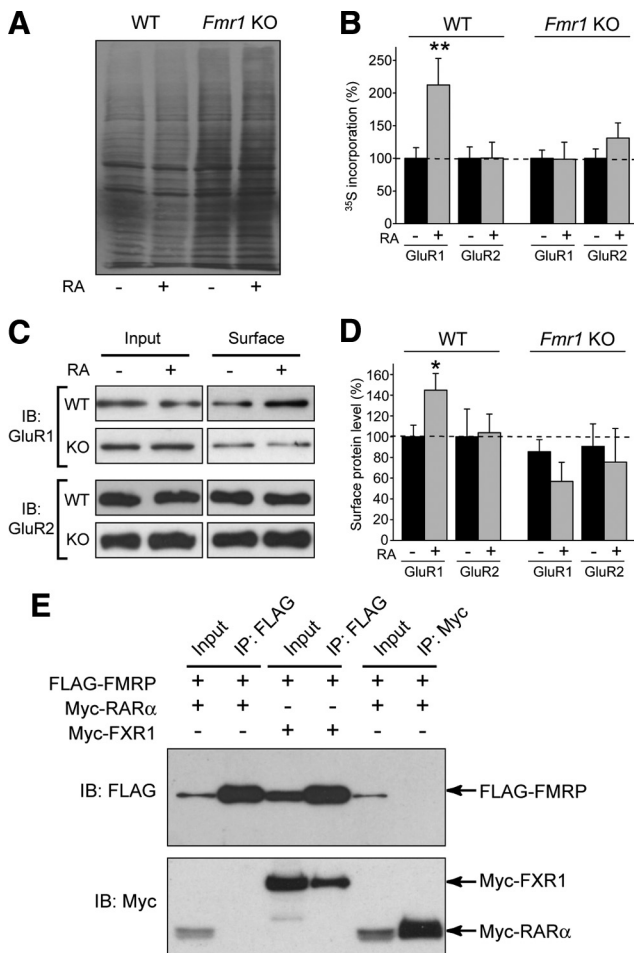


Figure 6. Metabolic labeling shows increased GluR1 translation with RA treatment, and FMRP and RAR α do not interact directly. **A**, Autoradiography of total protein lysate from dissociated neurons treated with 2 h of DMSO or RA in the presence of ³⁵S-labeled amino acids. **B**, Quantification of incorporated 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. IB, Immunoblot. **D**, Quantification of surface GluR1 and GluR2 protein levels after RA treatment. Surface band intensity was normalized to input, and all groups were compared with WT DMSO ($n = 3-9$; * $p < 0.05$). **E**, Attempted coimmunoprecipitation of FMRP and RAR α . Tagged constructs were expressed in HEK293T cells as indicated, and immunoprecipitation (IP) of either FMRP (anti-FLAG) or RAR α (anti-Myc) was performed (Input: 10% of total). Although pull down of FMRP was able to coimmunoprecipitate the known binding partner FXR1 (positive control), no interaction was seen between FMRP and RAR α . Error bars represent SEM.

obvious effect of RA on global translation was seen in either wild-type or knock-out neurons, consistent with our observation that RA only affects the local translation of specific proteins (Fig. 6A). Consistent with previous reports (Dolen et al., 2007), we did observe a slight increase in overall ³⁵S incorporation in knock-out neurons compared with wild-type (Fig. 6A), indicating globally elevated baseline translation in knock-out cells.

Immunoprecipitation of GluR1 and quantification of ³⁵S incorporation showed a significant increase in radiolabeled GluR1 after RA treatment in wild-type, but not knock-out, neurons (Fig. 6B). 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 (Fig. 6B), although we saw an increase in GluR2 protein in synaptoneurosome from RA-treated slice cultures (Fig. 5). Al-

though 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 attributable 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 synaptoneurosome (Fig. 5) was somewhat surprising, because the increase in mEPSC amplitude that follows TTX + APV or RA treatment is attributable to synaptic insertion of GluR2-lacking AMPA receptors (Fig. 2D) (Aoto et al., 2008). Surface biotinylation confirmed that RA treatment only increased GluR1, but not GluR2, protein levels on the cell surface (Fig. 6C,D), indicating that the increased GluR2 protein seen in synaptoneurosome after RA treatment is not reaching the surface or contributing to synaptic transmission at the time point examined.

FMRP and RAR α proteins do not interact directly

Because of the previously demonstrated role for RAR α 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 RAR α protein. To test for a possible interaction under permissive conditions, we expressed FLAG-tagged FMRP and Myc-tagged RAR α proteins in HEK293T cells and attempted coimmunoprecipitation in both directions. Although 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 RAR α (Fig. 6E).

Acute postsynaptic expression of FMRP in knock-out neurons rescues synaptic scaling

Is FMRP required directly for TTX + APV- and RA-induced synaptic scaling, or are the deficits seen in *Fmr1* knock-out mice attributable 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* knock-out 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 (Darnell et al., 2001), and the tandem KH domains (KH1 and KH2), which bind RNAs containing a characteristic “kissing complex” structure (Darnell et al., 2005b). To separate the functions of these two domains, we tested an FMRP construct that was missing the RGG box (FMRP Δ RGG-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; Lagerbauer 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 with GFP-expressing cells (Fig. 7A). The exogenous expression levels were approximately equal to total endogenous FMRP levels (Fig. 7A) and were 2-fold to 2.5-fold higher than expression of the

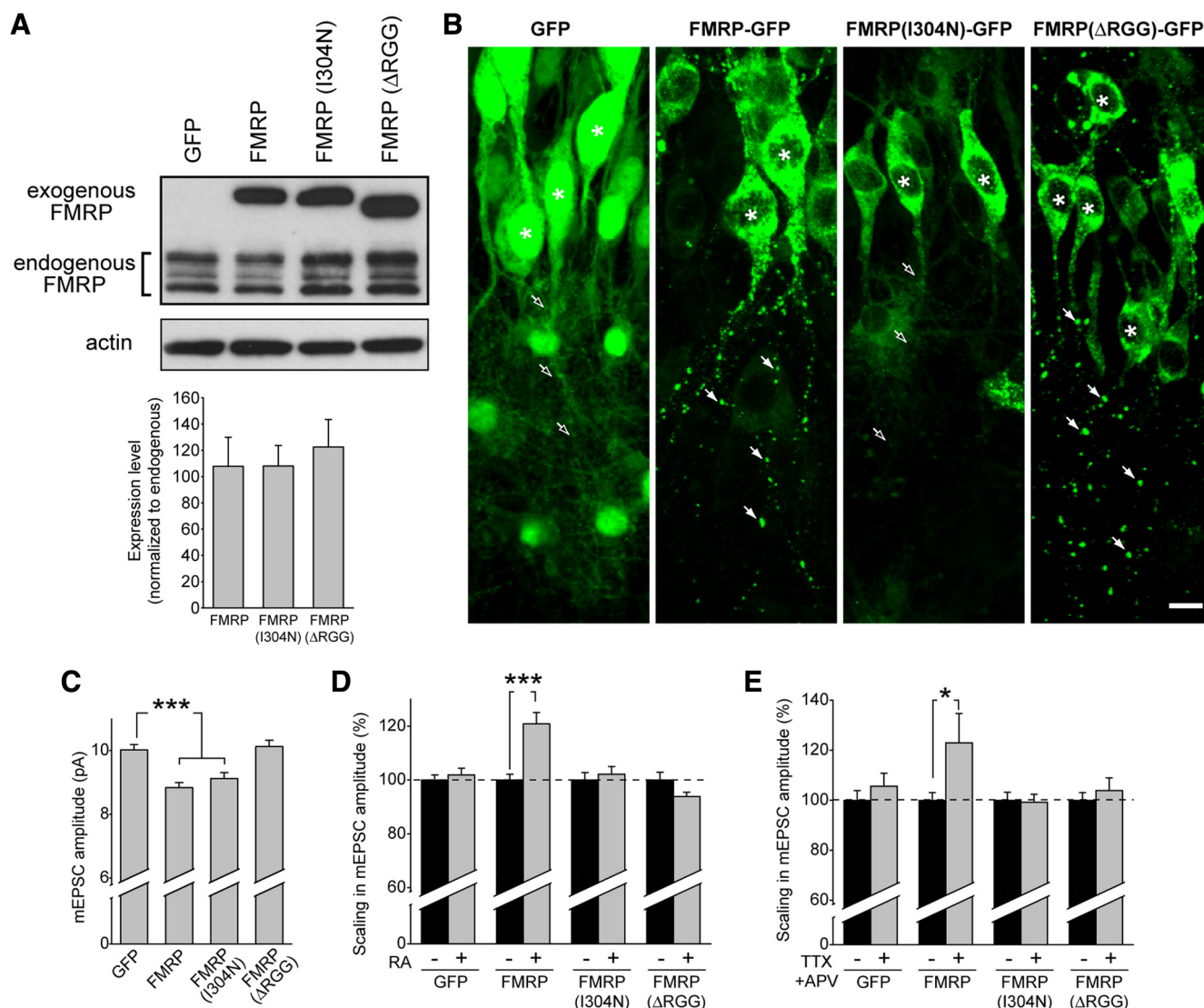


Figure 7. Viral expression of FMRP in knock-out slices restores synaptic scaling. **A**, Representative blot and quantification of exogenous FMRP–GFP, FMRP(I304N)–GFP, and FMRPΔRGG–GFP expression compared with endogenous FMRP protein levels after 6 d of virus expression in wild-type dissociated neurons ($n = 6$). **B**, 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 and FMRPΔRGG exhibit a punctate expression pattern in neuronal dendrites (filled arrows), whereas GFP or FMRP(I304N) are diffusely expressed in neuronal dendrites (open arrows). Scale bar, 10 μ m. **C**, Amplitude of mEPSC events in KO neurons expressing GFP, FMRP, FMRP(I304N), or FMRPΔRGG ($n = 38–66$; $***p < 0.001$). **D**, Percentage scaling after RA treatment in neurons expressing GFP or different FMRP constructs. DMSO groups for each construct were set to 100% to account for altered baseline amplitudes (see **C**) ($n = 18–49$; $***p < 0.001$). **E**, Percentage scaling after treatment with 36 h of TTX + APV ($n = 17–20$; $*p < 0.05$). Error bars represent SEM.

largest FMRP isoform (isoform 1) alone (supplemental Fig. 5A, available at www.jneurosci.org as supplemental material). Neither the wild-type nor the mutant FMRP constructs altered AMPAR abundance (supplemental Fig. 5E,F, available at www.jneurosci.org as supplemental material). Additionally, overexpression of exogenous FMRP and its mutant forms did not change the basal synaptic transmission or prevent synaptic scaling in wild-type neurons in response to TTX + APV (supplemental Fig. 5B–D, available at www.jneurosci.org as supplemental material).

We next introduced these wild-type and mutant FMRP constructs into knock-out 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 in knock-out neurons yielded a distinct punctate pattern in dendrites, resembling that of the endogenous protein (Fig. 7B) (supplemental Fig. 6A, available at www.jneurosci.org as supplemental material). FMRPΔRGG exhibited a similar expression pattern, as

has been reported (Pfeiffer and Huber, 2007). In contrast, expression of FMRP(I304N) in knock-out neurons yielded a more diffuse, less punctate expression pattern in dendrites (Fig. 7B), which is consistent with its altered function and similar to previous reports (Schrier et al., 2004; Pfeiffer and Huber, 2007).

Introduction of FMRP into knock-out neurons caused a small reduction in the baseline amplitude of mEPSCs compared with cells expressing GFP alone (Fig. 7C). No change in mEPSC frequency was observed (supplemental Fig. 6B,C, available at www.jneurosci.org as supplemental material). Similarly, FMRP(I304N) expression also reduced the baseline amplitude of mEPSCs, but FMRPΔRGG had no effect on mEPSC amplitude (Fig. 7C). 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 knock-out neurons. These disparities are possibly attributable to differences

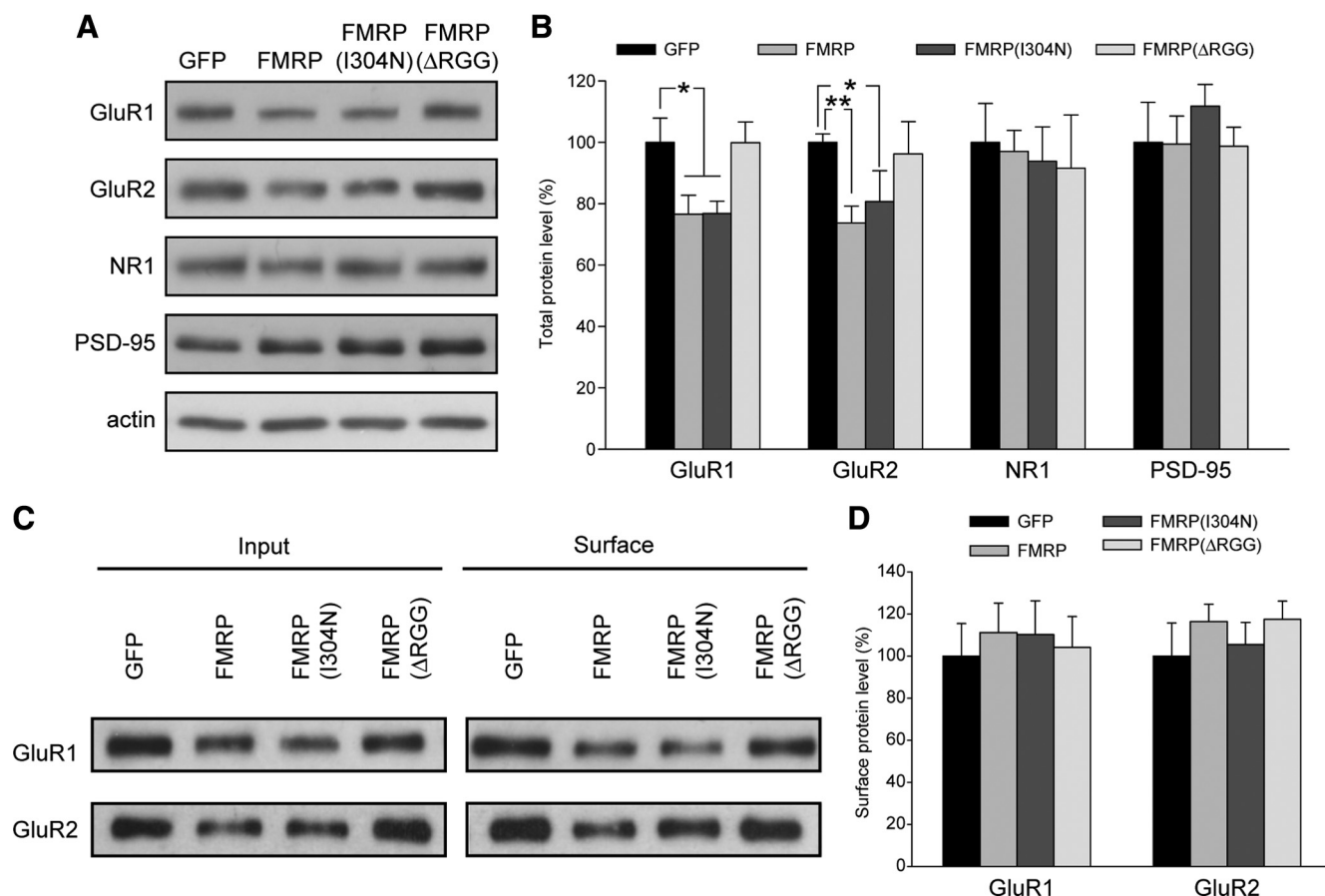


Figure 8. Viral expression of FMRP in knock-out neurons affects AMPAR abundance. Representative blots (**A**) and quantification of protein levels in lysates (**B**) collected from KO dissociated neurons infected with virus expressing GFP, FMRP, FMRP(I304N), or FMRP Δ RGG. Band intensities were normalized to actin ($n = 4-10$; * $p < 0.05$; ** $p < 0.01$). Representative blots (**C**) and quantification of surface biotinylation of AMPARs (**D**) in knock-out dissociated neurons infected with virus expressing GFP or different FMRP constructs. Surface band intensity was normalized to input ($n = 5-7$). Error bars represent SEM.

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* knock-out mice (Muddashetty et al., 2007), indicating overactive baseline translation of these proteins. The acute reintroduction of FMRP into *Fmr1* knock-out 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 or FMRP(I304N) in dissociated neurons from knock-out animals led to a significant reduction in both GluR1 and GluR2 protein levels, although NR1 and PSD-95 levels were unchanged (Fig. 8*A,B*). Surface AMPAR levels, as measured by biotinylation and pull down, were reduced proportionally with total AMPAR protein levels, so that the ratio of surface to total fractions remained constant across all conditions (Fig. 8*C,D*). Consistent with our observation that the RGG box is required for the reduction in mEPSC amplitude caused by FMRP reintroduction, FMRP Δ RGG had no effect on levels of any of the proteins measured (Fig. 8). 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 restored the ability of *Fmr1* knock-out neurons to undergo synaptic scaling after RA treatment (Fig. 7*D*) or TTX + APV treatment (Fig. 7*E*). In contrast, neither GFP alone, FMRP(I304N), nor FMRP Δ RGG rescued synaptic scaling

induced by RA or TTX + APV (Fig. 7*D,E*). This confirms that FMRP is required acutely in the postsynaptic cell for induction of the form of synaptic scaling mediated by RA/RAR α 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 (Turriano and Nelson, 2004; Davis, 2006). It has been shown that 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* knock-out neurons is rescued by wild-type FMRP but not by FMRP(I304N) or FMRP Δ RGG. 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

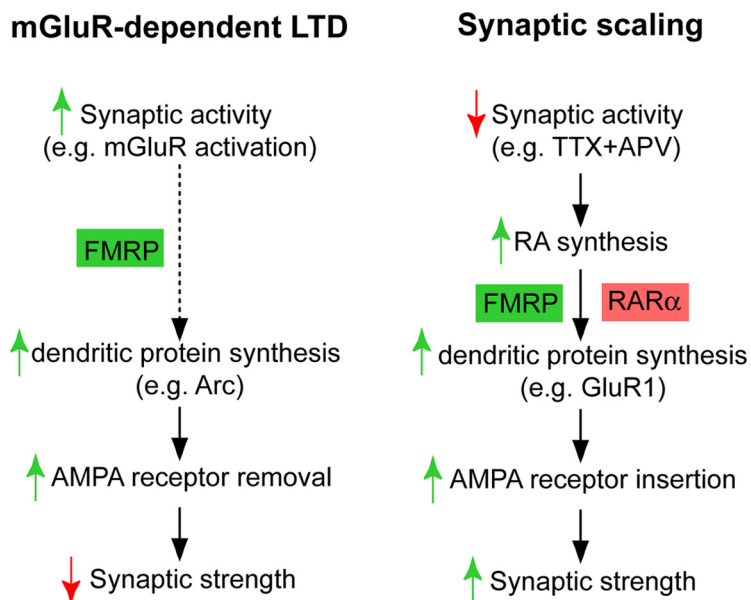


Figure 9. FMRP plays a critical role in multiple forms of synaptic plasticity. Synaptic activation leads to FMRP-dependent protein synthesis and the eventual removal of AMPARs from the synapse (mGluR-dependent LTD). By contrast, blockade of synaptic activity causes synthesis of new GluR1 receptors and a subsequent increase in synaptic strength, in a process that requires both FMRP and RAR α .

FMRP and RAR α 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 RAR α will be a critical step toward understanding the molecular basis of FMRP-mediated translational regulation and the *Fmr1* knock-out phenotype.

The ability of FMRP to function as a translational repressor has been well described (Comery et al., 1997; Lagerbauer 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; Schütt 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* knock-out 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 knock-out 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 (Fig. 7) 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 knock-out 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 knock-out animals. This can be seen with some other validated FMRP targets, such as MAP1b (Chen et al., 2003; Lu et al., 2004; Hou et al., 2006; Wei et al., 2007) and PSD-95 (Todd et al., 2003; Schütt et al., 2009), which do not show consistently elevated levels in knock-out neurons.

We were also able to use mutant FMRP constructs to further understand the mechanism of the action of FMRP 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 homeo-

static plasticity. This is not surprising, because 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 not surprising, because neither FMRP(I304N) nor FMRP Δ RGG 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 (Lagerbauer et al., 2001; Levenga et al., 2009), and FMRP Δ RGG 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 RAR α through a motif in its 5' untranslated region, which confers RAR α binding ability (Poon and Chen, 2008). Previous *in vitro* study of the GluR1 untranslated region showed that RAR α binding inhibits translation but that this inhibition is relieved during addition of RA. Our results suggest, therefore, that FMRP may be required for this RA-induced release of inhibition by RAR α .

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 as a result of specific trafficking or degradation of the two receptor types. In fact, this differential trafficking has already been observed, because it was shown previously 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 additional 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* knock-out mice have been studied extensively for defects in neuronal function and learning and memory. *Fmr1* knock-out 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, although seemingly through distinct mechanisms (Fig. 9). Our finding of impaired homeostatic synaptic plasticity provides a new perspective on the phenotype in *Fmr1* knock-out 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* knock-out 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 the cognitive function of an organism. 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* knock-out mice. Understanding the interplay between these different processes will provide significant additional insight into the molecular mechanisms guiding both homeostatic plasticity and fragile X syndrome.

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