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A presynaptic role for FMRP during protein synthesis–dependent long-term plasticity in *Aplysia*

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Loss of the Fragile X mental retardation protein (FMRP) is associated with presumed postsynaptic deficits in mouse models of Fragile X syndrome. However, the possible presynaptic roles of FMRP in learning-related plasticity have received little attention. As a result, the mechanisms whereby FMRP influences synaptic function remain poorly understood. To investigate the cellular locus of the effects of FMRP on synaptic plasticity, we cloned the *Aplysia* homolog of FMRP and find it to be highly expressed in neurons. By selectively down-regulating FMRP in individual *Aplysia* neurons at the sensory-to-motor neuron synapse reconstituted in co-cultures, we demonstrate that FMRP functions both pre- and postsynaptically to constrain the expression of long-term synaptic depression induced by repeated pulses of FMRF-amide. In contrast, FMRP has little to no effect on long-term synaptic facilitation induced by repeated pulses of serotonin. Since other components of signaling pathways involved in plasticity appear to be conserved between *Aplysia* and mammalian neurons, our findings suggest that FMRP can participate in both pre- and postsynaptic regulation of enduring synaptic plasticity that underlies the storage of certain types of long-term memory.

[Supplemental material is available for this article.]

Fragile X syndrome is the most common genetically inherited form of mental impairment and results from the loss of a single protein, FMRP, encoded by the *FMR1* gene (Penagarikano et al. 2007). FMRP is an RNA binding protein that is localized throughout the cell body and dendrites of neurons and is thought to regulate the translation of proteins required for synaptic plasticity, perhaps in an activity-dependent and local fashion (Bagni and Greenough 2005). In support of this idea, mice lacking FMRP have enhanced type 1 metabotropic glutamate receptor (mGluR)-dependent hippocampal long-term depression (LTD) (Huber et al. 2002), a form of plasticity that requires protein synthesis in the postsynaptic cell (Huber et al. 2001).

Despite increasing evidence regarding the postsynaptic dendritic function of FMRP, little is known about its role in the presynaptic neuron. Several findings suggest that the loss of FMRP also has presynaptic effects. For example, some Fragile X individuals exhibit structural changes in their brain that are indicative of abnormalities in both axon segregation and aberrant white matter connectivity (Barnea-Goraly et al. 2003; Haas et al. 2009). Similarly, in the hippocampus, axonal projections from granule cells in the dentate gyrus to CA3 pyramidal neurons are abnormal in *Fmr1* knockout mice (Ivanco and Greenough 2002; Mineur et al. 2002). In addition, several studies in flies and rodents have demonstrated that FMRP can localize to axons and presynaptic specializations (Feng et al. 1997a; Antar et al. 2006; Christie et al. 2009), and that altered levels of FMRP affect growth cone dynamics (Antar et al. 2006; Li et al. 2009a) and axonal morphology (Morales et al. 2002; Bureau et al. 2008), as well as synapse and circuit formation (Zhang et al. 2001; Hanson and Madison 2007; Bureau et al. 2008; Gibson et al. 2008). Furthermore, FMRP is predicted to bind several mRNAs coding for proteins that are localized to axons and are involved in path-finding and synaptic plasticity (Brown et al. 2001; Miyashiro et al. 2003; Zalfa et al. 2003; Darnell et al. 2004).

These examples suggest an additional presynaptic role for FMRP. Although presynaptic FMRP has been implicated in synaptic plasticity (Bureau et al. 2008; Gibson et al. 2008; Zhang et al. 2009), no direct test of the role of FMRP in presynaptic function in forms of long-term synaptic plasticity has been undertaken. To address this question, we have cloned the homolog of FMRP in *Aplysia californica* (ApFMRP) and have studied its regulatory role in long-term synaptic plasticity using *Aplysia* sensory-to-motor neuron co-cultures. This reduced preparation is capable of expressing multiple forms of long-term synaptic plasticity that underlie sensitization and habituation, two simple forms of learning in *Aplysia* (Montarolo et al. 1986, 1988; Rayport and Schacher 1986) and allows the selective manipulation of pre- and postsynaptic neurons. We find that in *Aplysia*, FMRP plays a modulatory role during the expression of long-term synaptic depression induced by repeated pulses of FMRF-amide. In addition, we identify a novel presynaptic locus for FMRP function and confirm a postsynaptic role of FMRP in *Aplysia* previously described in rodents.
Results

Identification of an Aplysia FMR1 homolog (ApFMR1)

ApFMR1 was cloned using a combination of degenerate primed PCR, RACE PCR-based cloning, and isolation of two overlapping clones from a cDNA library constructed from Aplysia central nervous system mRNA. The resulting full-length clone codes for a protein approximately 710 amino acids in length (Fig. 1A). Comparison of Aplysia, mammalian, and Drosophila Fragile X-related proteins reveals both marked conservation and potentially important differences (Fig. 1B). Overall, ApFMRP is 40% identical to human FMRP, with significantly higher conservation in regions containing identified functional domains. Importantly, ApFMRP contains all of the key domains found in the family of Fragile X-related proteins including RNA binding motifs and elements involved in subcellular localization. The amino-terminal region of FMRP mediates protein–protein interactions and contains two relatively well-conserved Tudor domains that are common to RNA binding proteins (Maurer-Stroh et al. 2003; Ramos et al. 2006). Human and Aplysia FMRP share ~39% identity in this region. The two KH domains in ApFMRP are ~60% identical to human FMRP and retain a conserved isoleucine residue that is important for FMRP function (De Boule et al. 1993; Feng et al. 1997b; Darnell et al. 2005b; Zang et al. 2009). ApFMRP also contains an RGG box, a poorly defined domain that is involved in RNA binding (Kiledjian and Dreyfuss 1992). Homology scores between human and Aplysia FMRP in this region are relatively low because this domain is defined only as containing a high concentration of arginines and glycines. Furthermore, it is unclear whether there are multiple distinct RGG boxes in the carboxy-terminal region of ApFMRP or if this domain is merely expanded.

**ApFMR1 is alternatively spliced**

Mammalian FMR1 undergoes significant alternative splicing giving rise to at least 12 possible protein isoforms (Ashley et al. 1993; Verkerk et al. 1993). Interestingly, many of the ApFMR1 clones identified during screening contained small insertions of variable lengths in the second KH domain. Analysis of genomic clones identified during screening contained small insertions of 1993; Verkerk et al. 1993). Interestingly, many of the isoforms give rise to at least 12 possible protein isoforms (Fig. 2B). These insertions give rise to amino acids that exhibit some identity to those coded for by exons 11 and 12 of mammalian FMR1, regions that are not present in other Fragile X-related genes, FXR1 and FXR2, or in the Drosophila FMR1 homolog (Siomi et al. 1995; Zhang et al. 1995; Wang et al. 2000; Kirkpatrick et al. 2001). Analysis of Drosophila genomic sequence in analogous regions revealed no conventional splice sites that could produce similar alternative splicing, nor are there genomic sequences that can potentially code for homologous insertions. This further underscores the similarity of Aplysia and mammalian FMRP.

**ApFMR1 is widely expressed**

To determine the expression profile of ApFMRP isoforms among multiple tissues, ApFMR1 was amplified from RNA harvested from different organs from adult Aplysia using isoform-specific primers. ApFMR1 has a widespread expression pattern among various tissues, with abundant expression in neurons (Fig. 2C). This is consistent with FMR1 expression in other species including flies, zebrafish, frogs, and mammals (Hinds et al. 1993; Wang et al. 2000; Tucker et al. 2004; Lim et al. 2005). In neurons, mRNA corresponding to ApFMRP isoform six is more abundant than other alternatively spliced forms as detected by semiquantitative RT-PCR (Fig. 2C). This finding is corroborated by quantification of the frequency of different isoforms identified among clones during ApFMR1 screening; of 109 clones identified from CNS-derived cDNA, ApFMRP isoforms were represented as...
were used as internal controls (Li et al. 2009b).

ApGluR2

representative triplicates of the experiments performed (Aplysia equally well in demonstrates that they are expressed in various tissues including neurons. (D) (Fig. 3B; Antar et al. 2005, 2006). These structures extend centered FMRP localization in primary cultures of rodent neurons uniformly (Fig. 3A), ApFMRPiso6–ECFP was found in the cell imaged by confocal microscopy. Whereas ECFP labeled neurons either ECFP or an ApFMRPiso6–ECFP fusion protein and were sensory neurons were injected with a DNA construct coding for

isoform in ApFMR1

Western FMRP localization in primary cultures of rodent neurons (Kirkpatrick et al. 2001).

four possible isoforms and their relative abundance in neurons. (D) ApFMR1 is expressed equally well in Aplysia sensory and L7 motor neurons using single-cell RT-PCR. These results show the representative triplicates of the experiments performed (n = 6). The synaptic markers ApSynapsin and ApGluR2 were used as internal controls (Li et al. 2009b).

follows: isoform 1: 36%; isoform 2: 5%; isoform 3: 4%; isoform 4: 4%; isoform 5: 1%; and isoform 6: 50%. Isoform 6 contains an insertion in the region of alternative splicing, which based on homology comparisons suggests that it is more like FMRP than the FXRs (Kirkpatrick et al. 2001).

Since Aplysia sensory-to-motor neuron co-cultures have been widely used for studies of long-term synaptic plasticity, we verified the expression of ApFMR1 in these neurons using single-cell RT-PCR (Fig. 2D). Because each isoform varies only in a small region, it is not feasible to design nested primer pairs that are specific for each isoform. Thus, we adopted nested primer pairs that are common to all ApFMR1 isoforms. We found that ApFMR1 is expressed equally well in both Aplysia sensory and L7 motor neurons (Fig. 2D).

ApFMRP is distributed throughout neuronal cell bodies and neurites

In all species studied to date, the subcellular localization of FMRP has been predominantly cytoplasmic. Because antibodies to ApFMRP do not exist, we expressed the most abundant ApFMR1 isoform in Aplysia CNS, isoform 6, as an ApFMRP-ECFP fusion protein to determine the subcellular localization of ApFMRP. Aplysia sensory neurons were injected with a DNA construct coding for either ECFP or an ApFMRPiso6–ECFP fusion protein and were imaged by confocal microscopy. Whereas ECFP labeled neurons uniformly (Fig. 3A), ApFMRPiso6–ECFP was found in the cell body and extended into neurites in a granular pattern reminiscent of FMRP localization in primary cultures of rodent neurons (Fig. 3B; Antar et al. 2005, 2006). These structures extend throughout sensory cell neurites regardless of whether cells are cultured alone (Fig. 3B) or in the presence of postsynaptic target neurons (Fig. 3C).

Reduction of ApFMRP enhances long-term depression induced by FMRFamide

Although FMRP has been shown to play a role in several long-lasting forms of synaptic plasticity in mammals (Huber et al. 2002; Li et al. 2002; Koekkoek et al. 2005; Larson et al. 2005; Zhao et al. 2005; Desai et al. 2006; Nosyreva and Huber 2006; Lauterborn et al. 2007; Meredith et al. 2007; Wilson and Cox 2007; Hu et al. 2008; Shang et al. 2009; Auerbach and Bear 2010), the relative contribution of FMRP located in either the pre- or postsynaptic compartment has not been determined. Since ApFMR1 is expressed throughout the Aplysia nervous system and pre- and postsynaptic cells can be selectively manipulated in sensory–motor neuron co-cultures, we have directly examined the role of both pre- and postsynaptic ApFMRP in regulating long-term synaptic plasticity. To deplete ApFMRP, we injected individual cultured neurons with an antisense oligonucleotide designed to selectively target and degrade all isoforms of ApFMR1 mRNA (Supplemental Fig. S1).

To evaluate possible postsynaptic roles of ApFMRP, we injected motor neurons in sensory–motor neuron co-cultures with either an antisense oligonucleotide targeted against ApFMR1, a sense (control) oligonucleotide, or vehicle alone. We then induced protein synthesis-dependent long-term synaptic depression by stimulation with five pulses of 1 μM FMRF-amide. In cultures with motor neurons injected with vehicle alone or with a sense oligonucleotide, the mean amplitude of the EPSP in the sensory–motor neuron synapse decreased 24 h following repeated exposure to FMRF-amide (sense oligo [S] %EPSP, −18.45% ± 2.2%, n = 11; vehicle alone [V] %EPSP, −17.88% ± 4.11%, n = 15; Fig. 4A). In contrast, in cultures with motor neurons injected with ApFMR1 antisense oligonucleotides, the decrease in mean EPSP amplitude 24 h after FMRF-amide treatment was significantly enhanced (%EPSP, −31.38% ± 4.14%, n = 16; P = 0.015 vs. V; P = 0.027 vs. S; Fig. 4A).

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processes that underlie synaptic depression or whether its role is specific to long-lasting forms of depression. To test whether FMRP plays a post-synaptic role in short-term depression, we injected motor neurons in sensory–motor neuron co-cultures with either antisense oligonucleotide targeted against ApFMR1, a sense control oligonucleotide, or vehicle alone and induced short-term synaptic depression by stimulating co-cultures with a single 5-min pulse of 1 μM FMRF-amide. The mean amplitude of the EPSP in the sensory–motor neuron synapse decreased 5 min after exposure to one pulse of FMRF-amide (V %EPSP, −52.20% ± 4.69%, n = 10) and was unaffected by injection of antisense or sense oligonucleotides (S %EPSP, −58.00% ± 4.23%, n = 8; AS %EPSP, −58.11% ± 3.74%, n = 9; P > 0.05; Fig. 4C). Injection of motor neurons with oligonucleotide did not affect basal synaptic transmission in the absence of FMRF-amide treatment (V %EPSP, −11.75% ± 4.73%, n = 4; AS %EPSP, −13.2% ± 6.45%, n = 5; S %EPSP, −6.17% ± 7.64%, n = 6; P > 0.05). Similarly, we found that injection of either antisense oligonucleotides targeted against ApFMR1 or control oligonucleotides into sensory neurons in sensory–motor neuron co-cultures had no effect on the mean amplitude of the EPSP in response to a single pulse of 1 μM FMRF-amide (V %EPSP, −52.13% ± 6.00%, n = 8; S, −56.55% ± 5.30%, n = 11; AS, −52.33% ± 8.40%, n = 9; P > 0.05; Fig. 4D). We again found that oligonucleotide injection into sensory neurons did not affect basal synaptic transmission in the absence of FMRF-amide treatment (V %EPSP, −2.71% ± 6.00%, n = 7; S, −12.57% ± 3.84%, n = 7; AS, −9.75% ± 8.35%, n = 8; P > 0.05). These results indicate that neither pre- nor post-synaptic ApFMRP contributes to short-term synaptic depression or basal synaptic transmission.

**Synaptic facilitation induced by 5-HT is not changed by reduction of ApFMRP**

Finally, we asked whether ApFMRP regulates processes underlying long-term synaptic plasticity in general or if it is specific to synaptic depression. We first asked whether ApFMRP plays a role in the induction of short-term facilitation induced by serotonin (5-HT). Postsynaptic motor neurons in sensory–motor neuron co-cultures were injected with an antisense oligonucleotide targeting ApFMR1, a sense control oligonucleotide, or vehicle alone, and short-term facilitation was induced by stimulation with a single 5-min pulse of 10 μM 5-HT. All groups demonstrated comparable increases in EPSP amplitude 5 min following exposure to 5-HT (V %EPSP, +88.11% ± 16.61%, n = 9; S %EPSP, +76.08% ± 16.40%, n = 12; AS %EPSP, +92.27% ± 14.13%, n = 11; P > 0.05; Fig. 5A). Oligonucleotide injection also did not affect basal synaptic transmission in the absence of 5-HT treatment (V %EPSP, −8.11% ± 4.99%, n = 9; S %EPSP, −2.80% ± 4.62%, n = 10; AS %EPSP, −9.80% ± 4.82%, n = 10; P > 0.05). Similarly, oligonucleotide injection into presynaptic sensory neurons in sensory–motor neuron co-cultures also did not affect short-term
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**Figure 4.** Down-regulation of ApFMRP enhances long-term depression induced by FMRF-amide. Repeated exposure to FMRF-amide (1 μM) causes a long-term depression of synaptic transmission measured 24 h after induction. Injection of an ApFMR1 antisense oligonucleotide (AS) into either postsynaptic motor neurons (A) or presynaptic sensory neurons (B) enhances FMRF-amide-induced long-term depression of synaptic transmission 24 h after induction, whereas injection of sense oligonucleotide (S) or vehicle alone (V) does not. In contrast, neither post- nor presynaptic injection of sense oligonucleotides affected short-term depression induced with a single 5-min pulse of 1 μM FMRF-amide (C and D, respectively). Oligonucleotide injections did not affect basal transmission recorded post-injection in the absence of FMRF-amide.

potentiation induced by one pulse of 5-HT (V %EPSP, +71.80% ± 19.69%, n = 10; S %EPSP, +64.22% ± 13.30%, n = 9; AS %EPSP, +68.30% ± 14.56%, n = 10; P > 0.05) or basal synaptic transmission (V %EPSP, −10.67% ± 5.19%, n = 9; S %EPSP, −8.25% ± 5.62%, n = 8; AS %EPSP, −0.27% ± 4.46%, n = 15; P > 0.05; Fig. 5A). These results indicate that neither pre- nor postsynaptic ApFMRP modulates the induction of short-term facilitation by 5-HT.

We next addressed whether ApFMRP has a role in the long-term facilitation induced by repeated exposure to 5-HT. Motor neurons in sensory–motor neuron co-cultures were injected with an antisense oligonucleotide targeted to ApFMR1, a sense oligonucleotide, or vehicle alone, and long-term potentiation was induced by stimulation with five pulses of 10 μM 5-HT. All groups demonstrated comparable increases in EPSP amplitude 24 h following exposure to 5-HT (V %EPSP, +63.14% ± 12.09%, n = 7; S %EPSP, +67.90% ± 24.45%, n = 10; AS %EPSP, +58.54% ± 7.95%, n = 13; P > 0.05), and oligonucleotide injection did not affect basal synaptic transmission recorded at 24 h in the absence of 5-HT treatment (V %EPSP, −3.0% ± 5.15%, n = 8; S %EPSP, −1.43% ± 7.64%, n = 7; AS %EPSP, −3.33% ± 7.50%, n = 6; P > 0.05; Fig. 5C). Similarly, injection into the presynaptic sensory neuron did not affect basal synaptic transmission recorded at 24 h in the absence of 5-HT treatment (V %EPSP, −6.78% ± 8.61%, n = 9; S %EPSP, −0.83% ± 12.45%, n = 6; AS %EPSP, −1.67% ± 6.36%, n = 6; P > 0.05), or long-term facilitation of EPSP amplitude 24 h following repeated exposure to 5-HT (V %EPSP, +94.1% ± 12.58%, n = 20; S %EPSP, +69.5% ± 13.94%, n = 18; AS %EPSP, +65% ± 11.36%, n = 17; P > 0.05; Fig. 5D). The inability of the knockdown of ApFMRP to affect processes required for the induction or expression of LTP is similar to several findings in *Aplysia* knockout mice that have largely failed to identify a role of FMRP in multiple forms of hippocampal long-term potentiation (LTP) (Godfraind et al. 1996; Paradee et al. 1999; Li et al. 2002; Larson et al. 2005; but see Lauterborn et al. 2007; Hu et al. 2008; Shang et al. 2009; Auerbach and Bear 2010).

**Discussion**

*Aplysia* express a homolog of *FMR1*

Comparison with mammalian *FMR1* demonstrates that *ApFMR1* represents the *Aplysia* homolog of the Fragile X gene family. Consistent with expression profiles of *FMR1* orthologs in other species, *ApFMR1* appears to be widely expressed, with the highest levels of expression in central neurons, and relatively low, but significant levels in all other tissues tested. This pattern of expression is compatible with

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findings in vertebrates and flies (Hinds et al. 1993; Schenck et al. 2001; Tucker et al. 2004; Lim et al. 2005).

The characterization of several CDNs identified during screening for ApFMRP did not identify additional Fragile X-related transcripts in Aplysia, nor did analysis of more than 190,000 ESTs from Aplysia CNS libraries (Moroz et al. 2006). Given the degree of sequence homology in the FMRP KH domains among multiple species, it is unlikely that these cloning methods would have missed additional family members. Thus, it appears that, as with the Drosophila FMRP homolog (Wan et al. 2000), a single gene in Aplysia represents the family of vertebrate Fragile X-related proteins. The theory of gene duplication as a major genetic force permitting the evolution of vertebrates from invertebrates (Olino 1970) is now generally accepted, and it is not uncommon for invertebrate genomes to contain single genes that are orthologous to entire vertebrate gene families (e.g., Araki et al. 1996; Stock et al. 1997). This single gene in invertebrates has most likely evolved via duplication to give rise to the Fragile X-related family of genes in mammals.

ApFMRP contains three distinct classes of highly conserved RNA binding domains that are characteristic of Fragile X-related proteins in other species: Tudor, KH, and RGG box domains. These regions share significant similarity with human FMRP in regions that confer RNA binding capacity, including the conservation of isoleucine residues important for FMRP KH domain function. ApFMRP is also homologous to the Fragile X-related proteins in regions involved in ribosome association and protein–protein interactions. In addition, the ApFMRP–ECFP fusion protein forms granular structures that are distributed throughout the neurites of cultured Aplysia neurons in a pattern similar to the distribution of mammalian FMRP in dendrites (Antar et al. 2003, 2006; Ferrari et al. 2007). The subcellular distribution and these RNA binding domains are central to the function of FMRP in processes including its regulation of RNA stability (Zalfa et al. 2007), transport and trafficking (De Diego Otero et al. 2002; Antar et al. 2005; Ferrari et al. 2007), and association with polyribosomes (Khandjian et al. 1996; Tamanini et al. 1996; Corbin et al. 1997; Feng et al. 1997b; Stefani et al. 2004; Darnell et al. 2005a). The dendritic and synaptic localization of FMRP (Feng et al. 1997b; Ferrari et al. 2007) and its association with mRNAs coding for proteins involved in synaptic plasticity (Zalfa et al. 2003; Iacoangeli et al. 2008) and synthesized in response to synaptic signals (Park et al. 2008; Waung et al. 2008) support a role for FMRP in local protein synthesis at the synapse, a cellular process thought to underlie changes in the morphology and stability of synapses associated with the expression of long-term plasticity (Martin and Zukin 2006). Indeed, studies in rodents have identified the importance of the KH2 RNA binding domain in the formation of dendritically localized FMRP granules (Darnell et al. 2005a) and elimination of dendritic spines (Pfeiffer and Huber 2007), as well as alterations in synaptic plasticity and behaviors associated with the loss of FMRP function in mice (Zang et al. 2009).

Further characterization of ApFMRP may provide valuable insight into the function of human FMRP. Human FMRP contains a stretch of amino acids encoded by exons 11 and 12 that are unique among the Fragile X-related family of proteins. While vertebrate FXRP proteins and the Drosophila Fragile X homolog do not contain these sequences, several alternatively spliced neuronal isoforms of ApFMRP contain insertions exhibiting some homology to amino acids in this unique region. Structure studies of mammalian FMRP and other proteins containing KH domains suggest that these extra amino acids are part of a variable loop region in the second KH domain of FMRP that contains charged residues in an exposed position (Valverde et al. 2007). Whereas little is known about the function of this loop, its inclusion appears to alter the affinity of FMRP for certain RNA targets (Dennman and Sung 2002; Xie et al. 2009), and alternative splicing in this region may serve to increase the functional diversity of FMRP by affecting its RNA binding and target recognition properties. In addition, since the FXRPs lack these sequences and do not appear to function as repressors of protein synthesis in vitro (Laggerbauer et al. 2001), this loop may also affect FMRP-mediated regulation of translation.

**FMRP function in synaptic plasticity**

To begin to study the pre- and postsynaptic roles of FMRP in long-term plasticity, we used a reduced neuronal culture system that reconstitutes critical components of the circuitry underlying a simple form of behavioral learning in Aplysia. Similar to findings that long-term depression is enhanced in mice lacking FMRP (Huber et al. 2002; Koekkoek et al. 2005), we find that injection of individual cultured neurons with an antisense oligonucleotide designed to selectively target and degrade ApFMR1 mRNA leads to an enhancement of FMRP-amine-induced long-term depression. In contrast, antisense oligonucleotide injection does not affect long-term facilitation induced by repeated exposure to 5-HT at these synapses. This result is consistent with findings from several studies that suggest some forms of hippocampal long-term potentiation are unaffected in Fmr1 knockout mice (Godfraind et al. 1996; Paradee et al. 1999; Li et al. 2002; Larson et al. 2005). Together, these studies highlight the similarities between forms of long-term plasticity in Aplysia and the mammalian hippocampus and suggest that FMRP normally functions to regulate molecules critical for the expression of long-term synaptic depression.

What may underlie the context specificity of the effect of FMRP knockdown on the expression of these forms of long-term plasticity? One possibility is that FMRP-amine induction of protein synthesis-dependent LTD specifically recruits signaling pathways that engage FMRP, for example, mGluR signaling. If 5-HT induction of LTD does not require the same signaling pathways, FMRP may not be recruited. Alternatively, FMRP may regulate the synthesis of a subset of mRNAs that are specifically required for the expression of LTD, but not LTD. Indeed, a recent study suggests that local protein synthesis can be both transcript- and stimulus-specific (Wang et al. 2009).

Whereas FMRP knockdown did not affect protein synthesis-dependent LTD induced by 5-HT, it remains a possibility that FMRP is involved in other forms of long-lasting synaptic facilitation. For example, some forms of LTD are altered in the cortex (Li et al. 2002; Larson et al. 2005; Zhao et al. 2005; Meredith et al. 2007; Wilson and Cox 2007; Harlow et al. 2010) and in the hippocampus (Lauterborn et al. 2007; Hu et al. 2008; Shang et al. 2009; Auerbach and Bear 2010) of Fmr1 knockout mice. It is known that distinct forms of long-term plasticity can have different mechanisms underlying their expression. Furthermore, it is possible that the function of FMRP varies in a neuron- or synapse-specific manner, depending on the signaling pathways required for the induction or expression of distinct forms of plasticity, as well as the developmental stage of the animal. Alternatively, distinct molecular profiles of populations of cells or individual synapses may influence the regulation of FMRP or the availability of its RNA targets.

What is the locus for the influence of FMRP on synaptic plasticity? Previous investigations of synaptic depression in Fmr1 knockout mice have suggested a postsynaptic role for FMRP by regulating mGluR-stimulated protein synthesis (Huber et al. 2001; Koekkoek et al. 2005). While our findings confirm a postsynaptic role of FMRP in the expression of protein synthesis-dependent long-term depression in Aplysia, they also demonstrate an additional presynaptic role for FMRP in this persistent form of plasticity. Since other mechanisms underlying plasticity appear to
be conserved between *Aplysia* and mammalian neurons (Pittenger and Kandel 2003), it is tempting to suggest that FMRP may also have a coordinated pre- and postsynaptic role in regulating some forms of long-term plasticity in the mammalian brain. For example, although the form of type 1 mGluR-dependent hippocampal LTD that is enhanced in *Fmr1* knockout mice has been shown to require de novo protein synthesis in dendrites (Huber et al. 2001), a presynaptic locus has not been directly investigated, but does contribute to other forms of mGluR-dependent LTD in the hippocampus (Zakharenko et al. 2002; Feinmark et al. 2003). Moreover, while the major postsynaptic mechanism for this form of mGluR-LTD is thought to be the internalization of postsynaptic AMPA receptors (Snyder et al. 2001), the levels of AMPA receptor surface expression in acute hippocampal slices from *Fmr1* knockouts appear to be comparable to controls in both basal conditions and following the induction and expression of mGluR-LTD (Nosyreva and Huber 2006). The discrepancy between these results could be accounted for by a presynaptic mechanism, such as the one we describe here for *Aplysia*, that also contributes to the enhanced expression of this form of plasticity in *Fmr1* knockout mice.

What, then, are the mechanisms by which FMRP regulates FMRP-amide-induced LTD presynaptically? Putative mRNA targets of FMRP have been identified that code for proteins important for postsynaptic function, including munc13 and PP2A, as well as mRNAs coding for proteins important for normal axonal morphology, including MAP1b (Darnell et al. 2001; Castets et al. 2005). As FMRP is able to interact with or regulate the production of factors involved in structural rearrangements (Brown et al. 2005). As FMRP is able to interact with or regulate the production of factors involved in structural rearrangements (Brown et al. 2001; Darnell et al. 2001; Schenck et al. 2001; Zhang et al. 2001; Miyashiro et al. 2003), it is possible that one or more of those factors is needed for the structural rearrangements leading to synapse destabilization or elimination that is associated with long-term depression (Bailey and Chen 1983, 1988; Bailey et al. 1992). In addition, since FMRP appears to inhibit the translation of its target RNAs (Laggerbauer et al. 2001; Li et al. 2001; Mazroui et al. 2002), it is possible that FMRP normally represses the synthesis of one of the proteins needed for expression of LTD, perhaps via mechanisms involving recruitment of RNAi machinery (Sionni et al. 2004) or interactions with the 4E-BP-like repressor of translation CYFIP1 (Napol et al. 2008). In this model, down-regulation of FMRP removes an inhibitory constraint on this form of plasticity by increasing the translation of specific mRNAs necessary for long-term plasticity leading to enhanced LTD.

Finally, what might be the relationship between the cognitive impairment and abnormal immature-looking dendritic spines observed in Fragile X individuals and the enhancement of LTD observed following knockdown of FMRP in animal models? It is possible that enhanced LTD leading to enhanced synapse elimination (Bailey and Chen 1983, 1988; Bailey et al. 1992) can slow down the synaptic maturation, resulting in immature-looking dendritic spines and cognitive impairment (Pfeiffer and Huber 2007).

**Materials and Methods**

**Cloning of ApFMRP**

Total RNA was extracted from *Aplysia* pleural and pedal ganglia with Trizol (Invitrogen), and cDNA was synthesized with the Stratascript First Strand cDNA Synthesis Kit (Stratagene). Degenerate primers for PCR were designed against two regions of homology in the Fragile X-related proteins among multiple species: IUPAC code sequences from the K12 domain corresponding to KVIKNGN (AARGTNATHGGNAARAAYGG) and KEVEQLR (GNKARYGTYCNACYTCTTT). Bands of predicted size were subcloned into vector PCR2.1 using TOPO-TA cloning (Invitrogen). Similarity to Fragile X-related proteins was determined using the BLAST search program (NCBI).

The 5′ end of *ApFMR1* cDNA was identified using the SMART RACE cDNA amplification system (Clontech). To identify the entire ApFMRP coding region, the 5′ half of the *ApFMR1* cDNA was then used to screen a phage cDNA library made from *Aplysia* central nervous system (a gift from Dusan Bartsch). Homology was determined using ClustalW alignments with Fragile X-related proteins from other species: hFMRP accession number NP_002015, hFXR1P accession number EAW78371, hFXR2P accession number NP_004851, and dFXR accession number NP_731443. Scores are measures of amino acid identity and are calculated as percent identity of a length of sequence.

**Subcloning**

For expression of ApFMRPls06–ECFP in *Aplysia* neurons, *ApFmr1* full-length cDNA was amplified from *Aplysia* cDNA using primers HindIII–FMStart (CCCCAAGCTCTAGAGAGCTCTGCAGTT GAAACT) and FMRstop–BamHI (Cgggaacctcatattcccctatt CACTATATGCT) and cloned into the HindIII/BamHI sites of pECFP-C1 (Clontech). An NheI/BamHI fragment from pECFP-C1–ApFMR1 was subcloned into XbaI/BamHI sites in *Aplysia* expression vector pNEX3.

**RT-PCR**

RNA was extracted from multiple tissues using RNAqueous-4–PCR (Ambion), and RT-PCR was performed using HF-PROSTAR RT-PCR kit (Stratagene) with 50 ng of total RNA per reaction and an ApFMRP universal reverse primer (RT-QAkrele: TTCAGCTTGG CTTGCC) and isoform specific forward primers (iso1: AGGGA GAAGTGGAG; iso2: AGACACATGGCCTTTTCT; iso3: TGTTGAAGTGGAG; iso4: GTTGACAGTGCCCTTCT; iso5: AGACACATGGCCTTTTCT; iso6: AGGGAAGACGCC TCTTTC). Single-cell RT-PCR was performed as previously described (Li et al. 2009b). The nested primer pairs are as follows: F1: CAGCG GACGCGAAAGGAGGAAAAAC; F2: AGGAGGAAAAACCGTCCC CGAGGC; R1: GCCCCCCCGAAGTGGCCGACGCAG; R2: GCCCGACGAGCCGACAGGCTT.

**Oligonucleotide and plasmid DNA injection**

Antisense oligonucleotides were chosen from nucleotide sequence contained in all isoforms of *ApFmr1* that exhibited the lowest degree of homology to entries in the nonredundant BLAST database; antisense: GTCTTGGAGATGTTCCCGG; sense: ATGGGCGGAAACCATCGAGAC. DNA constructs for injection were purified by equilibrium centrifugation in CsCl–ethidium bromide gradients (Sambrook et al. 1989). DNA and oligonucleotides were dissolved in water and diluted to a concentration of 25 ng/ul (oligo) and 1 μg/ul (DNA) in injection buffer (10 mM Tris-HCl at pH 7.6, 100 mM KCl, 0.1% fast green) and were pressure-injected into motor and sensory neurons with a picospritzer (General Valve) as previously described (Kaang 1996). Oligonucleotides were injected 3 h prior to FMRP-amide or 5-HT treatment.

**Sensory and motor neuron co-cultures and electrophysiology**

Co-cultures of sensory neurons and the motor neuron L7 of *Aplysia californica* were prepared as previously described (Montarolo et al. 1988). Electrical recordings were made from motor neurons using intracellular microelectrodes filled with 2.5 M KCl using an Axoclamp-2A amplifier. EPSPs were evoked by stimulating the sensory neurons extracellularly with depolarizing pulses as previously described (Montarolo et al. 1986).
Confocal imaging
Fluorescent images of sensory neurons expressing injected DNA constructs were acquired with Zeiss Axiovert microscopes mounted on an LSM Pascal (Zeiss) laser confocal scanning microscope. Images were taken with a 40 × , NA 0.75 (MRC1000) objective, and the gains and neutral density filters were adjusted to prevent saturation of the detection threshold.

Statistical analysis
All data are presented as mean percentage change ± SEM in the EPSP amplitude as compared to the initial amplitude. A two-factor (treatment/oligo) ANOVA and post hoc Fisher's LSD test were used for multiple comparisons.

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References


A presynaptic role of FMRP in long-term plasticity


