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

Sally M. Till,1,5 Hsiu-Ling Li,1,2 Maria Concetta Miniaci,1,6 Eric R. Kandel,1,2,3,7 and Yun-Beom Choi1,4

1Department of Neuroscience, College of Physicians and Surgeons of Columbia University, New York, New York 10032, USA; 2Howard Hughes Medical Institute, College of Physicians and Surgeons of Columbia University, New York, New York 10032, USA; 3Kavli Institute for Brain Science, College of Physicians and Surgeons of Columbia University, New York, New York 10032, USA; 4Department of Neurology, College of Physicians and Surgeons of Columbia University, New York, New York 10032, USA

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 (mGluR1)-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; Christie et al. 2009), and that altered levels of FMRP affect growth cone dynamics (Antar et al. 2006; Greenough 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 FMR 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 (Ashley et al. 1993). Importantly, ApFMRP contains all of the key structural elements found in human FMRP (hFMRP) including amino acids corresponding to regions that are absent in the Fragile X-related family members FXR1P and FXR2P. Scores are expressed as percent identity to hFMRP. (NLS) Nuclear localization signal; (KH) K homology domain; (NES) nuclear export signal; (RGG) arginine glycine box; (NoS) nucleolar signal sequence.

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 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; Wan 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.
ApGluR2 were used as internal controls (Li et al. 2009b). Representative triplicates of the experiments performed (Aplysia demonstrates that they are expressed in various tissues including neurons (Fig. 3B; Antar et al. 2005, 2006). These structures extend tens of FMRP localization in primary cultures of rodent neurons uniformly (Fig. 3A), ApFMRP-iso6–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, −17.8% ± 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). Oligonucleotide injection did not affect EPSPs recorded 24 h post-injection in the absence of FMRF-amide treatment (V %EPSP, +5.29% ± 5.74%, n = 7; S %EPSP, +3.38% ± 6.16%, n = 8; antisense oligo [AS] %EPSP, +2.30% ± 3.62%, n = 10; P > 0.05), indicating that postsynaptic ApFMRP does not modulate basal synaptic transmission. The enhancement of long-term depression in Aplysia due to the depletion of ApFMR1 is similar to findings in Fmr1 knockout mice that identify enhanced forms of late-phase LTD in the hippocampus and cerebellum (Huber et al. 2002; Koekkoek et al. 2005) that are thought to have a predominantly postsynaptic mechanistic basis of expression (Huber et al. 2001; Koekkoek et al. 2005).

To determine whether FMRP also has a presynaptic contribution to long-lasting forms of synaptic depression, we looked at the effect of injecting presynaptic sensory neurons in sensory–motor neuron co-cultures with an ApFMR1 antisense oligonucleotide on

Figure 2. ApFMR1 is alternatively spliced. (A) Alternatively spliced isoforms of ApFMR1 result from alternate 5′ donor and 3′ acceptor splice sites of adjacent exons. (B) Schematic depiction of the resulting six possible isoforms and their relative abundance in neurons. (C) RT-PCR for specific ApFMR1 isoforms demonstrates that they are expressed in various tissues including neurons. (D) ApFMR1 isoforms 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).

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. ApFMRP 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
the expression of long-term synaptic depression induced by stimulation with five pulses of 1 μM FMRF-amine (Fig. 4B). Whereas the mean amplitude of the EPSP at the sensory–motor neuron synapse decreased 24 h following repeated exposure to FMRF-amine in cultures with sensory neurons injected with vehicle alone or with a sense control oligonucleotide (S %EPSP, −24.32% ± 7.35%, n = 18; V %EPSP, −21.67% ± 6.99%, n = 18), injection of sensory neurons with ApFMR1 antisense oligonucleotides significantly enhanced the decrease in mean EPSP amplitude in response to FMRF-amine (%EPSP, −40.59% ± 3.62%, n = 27; P = 0.024 vs. V; P = 0.048 vs. S; Fig. 4B). Again, oligonucleotide injection did not affect basal synaptic transmission recorded 24 h post-injection in the absence of FMRF-amine treatment (V %EPSP, +0.77% ± 7.40%, n = 13; S %EPSP, +2.06% ± 3.93%, n = 18; AS %EPSP, +0.056% ± 7.19%, n = 18; P > 0.05).

Taken together, these results suggest a general role for FMRP in processes specific to regulating the maintenance of synaptic depression. Furthermore, while experiments in Fmr1 knockout mice have not been able to distinguish pre- from postsynaptic roles of FMRP in hippocampal LTD, our data in Aplysia offer direct evidence for a presynaptic as well as a postsynaptic role for FMRP in long-term synaptic depression.

Reduction of ApFMRP does not affect FMRF-amine-induced short-term depression

We next asked whether ApFMRP has a general role in regulating processes that underlie synaptic depression or whether its role is specific to long-lasting forms of depression. To test whether FMRP plays a postsynaptic 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-amine. The mean amplitude of the EPSP in the sensory–motor neuron synapse decreased 5 min after exposure to one pulse of FMRF-amine (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-amine 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-amine (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-amine 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 targeted against 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% ± 18.61%, 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% ± 4.9%, 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
A presynaptic role of FMRP in long-term plasticity

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...
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 cDNAs 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 isolectine 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 inserts 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 (Dennan 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-amide-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; Pardee 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-amide 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 LTf. 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 presynaptic 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. 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 mRNAs (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 (Napoli 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 2006).

**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 KH2 domain corresponding to KVIGKNG (AARGTNAHGNNAAAYGGG) and KEVEQLR (GNKARYGTYCNACYCCTYT). 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 dFXRP accession number NP_7311443. Scores are measures of amino acid identity and are calculated as percent identity of a length of sequence.

**Subcloning**

For expression of ApFMRP506–ECFP in *Aplysia* neurons, *ApFmr1* full-length cDNA was amplified from *Aplysia* cDNA using primers HindIII–FMRstart (CCCCAAGCTCTGATGGAGGATCTTCGTT GAAATC) and FMRstop–BamHI (CGGGATCCCTATTCCCATT CACTATGCTG) and cloned into the HindIII/BamHI sites of pcDNA3 (Clontech). An NheI/BamHI fragment from pECFP–C1–ApFMR1 was subcloned into XbaI/BamHI sites in *ApSia* expression vector pNE3.

**RT-PCR**

RNA was extracted from multiple tissues using RNAqueous–4–PCR (Ambion), and RT-PCR was performed using HF-PROSTAR RT-PCR kit (Strategene) with 50 ng of total RNA per reaction and an ApFMRP universal reverse primer (RT-QAKLE: TCTGCTGTGG CTTGCC) and isoform unique forward primers (iso1: AGGAGA GGGTGCAAGTTA; iso2: AGACACATGCTTCTCTTCT; iso5: AGACACATTGCCTCCTCTTCT; iso6: AGGAGAAGGCT TCTTTC). Single-cell RT-PCR was performed as previously described (Li et al. 2009b). The nested primer pairs are as follows: F1: CAGCG GACGCAAGGAGGAAAAAC; F2: AGGAGAAACACCGCTTCC CGAGGC; R1: GGCCCTGAAAGTGGCGACGCAGT; R2: GGGCAAGGCGACGTCGAGGT.

**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: GTCCTGAGATGTCCGCAGTCC; sense: ATGGGGCAAGGAGGACAC. 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/μl (oligo) and 1 μg/μl (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 pico-spritzer (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. 1988).
Confocal imaging
Fluorescent images of sensory neurons expressing injected DNA constructs were acquired with Zeiss Axiosvert 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.

Acknowledgments
We thank C.H. Bailey for his critical comments on the manuscript, L. Moroz, A.B. Kohn, and T.J. Ha for assistance in cloning full-length ApFMR1; and Huixiang “Vivian” Zhu and Edward Konstantinov for Aplysia culture preparation. This work is supported by grants from the FRAXA Research Foundation (to S.M.T.), the Howard Hughes Medical Institute (to E.R.K.), the Simons Foundation (to E.R.K.), and the National Institutes of Health grant NS053415 (to Y.-B.C.).

References


A presynaptic role of FMRP in long-term plasticity


Received July 24, 2010; accepted in revised form November 19, 2010.
A presynaptic role for FMRP during protein synthesis–dependent long-term plasticity in *Aplysia*

Sally M. Till, Hsiu-Ling Li, Maria Concetta Miniaci, et al.

Access the most recent version at doi:10.1101/lm.1958811

**Supplemental Material**
http://learnmem.cshlp.org/content/suppl/2010/12/21/18.1.39.DC1.html

**References**
This article cites 94 articles, 38 of which can be accessed free at:
http://learnmem.cshlp.org/content/18/1/39.full.html#ref-list-1

**Email Alerting Service**
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or **click here.**

To subscribe to *Learning & Memory* go to:
http://learnmem.cshlp.org/subscriptions

© 2011 Cold Spring Harbor Laboratory Press