Fragile X mental retardation protein regulates heterosynaptic plasticity in the hippocampus

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Silencing of a single gene, FMR1, is linked to a highly prevalent form of mental retardation, characterized by social and cognitive impairments, known as fragile X syndrome (FXS). The FMR1 gene encodes fragile X mental retardation protein (FMRP), which negatively regulates translation. Knockout of Fmr1 in mice results in enhanced long-term depression (LTD) induced by metabotropic glutamate receptor (mGluR) activation. Despite the evidence implicating FMRP in LTD, the role of FMRP in long-term potentiation (LTP) is less clear. Synaptic strength can be augmented heterosynaptically through the generation and sequestration of plasticity-related proteins, in a cell-wide manner. If heterosynaptic plasticity is altered in Fmr1 knockout (KO) mice, this may explain the cognitive deficits associated with FXS. We induced homosynaptic plasticity using the β-adrenergic receptor (β-AR) agonist, isoproterenol (ISO), which facilitated heterosynaptic LTD that was enhanced in Fmr1 KO mice relative to wild-type (WT) controls. To determine if enhanced heterosynaptic LTP in Fmr1 KO mouse hippocampus requires protein synthesis, we applied a translation inhibitor, emetine (EME). EME blocked homo- and heterosynaptic LTD in both genotypes. We also probed the roles of mTOR and ERK in boosting heterosynaptic LTD in Fmr1 KO mice. Although heterosynaptic LTD was blocked in both WT and KOs by inhibitors of mTOR and ERK, homosynaptic LTD was still enhanced following mTOR inhibition in slices from Fmr1 KO mice. Because mTOR will normally stimulate translation initiation, our results suggest that β-AR stimulation paired with derepression of translation results in enhanced heterosynaptic plasticity.

Fragile X syndrome (FXS), a leading cause of mental retardation (Hagerman et al. 2009), is linked to silencing of the FMR1 gene (Verkerk et al. 1991; Eichler et al. 1994; Feng et al. 1995). FXS is characterized by mild to severe cognitive deficits, including impaired learning and memory (Macleod et al. 2010) resulting from altered synaptic function (Huber et al. 2002; Bear et al. 2004; Hou et al. 2006; Zhang et al. 2009). The Fmr1 knockout (KO) mouse recapitulates the primary molecular pathology associated with FXS: a reduction in expression of the FMR1 protein product, fragile X mental retardation protein (FMRP) (Bakker et al. 1994). FMRP is an RNA binding protein that is implicated in processes critical for synaptic plasticity, including regulation of mRNA trafficking and translation (Corbin et al. 1997; Feng et al. 1997; Huber et al. 2002; Bear et al. 2004; Stefani et al. 2004; Weiler et al. 2004). Studies of the hippocampus, a brain structure involved in memory formation (Scoville and Milner 1957; Zola-Morgan et al. 1986), revealed enhanced metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD) in the absence of FMRP (Hou et al. 2006). mGluR-LTD is rendered insensitive to translation inhibitors in Fmr1 KO mouse hippocampus, suggestive of altered regulation of protein synthesis (Hou et al. 2006; Nosyreva and Huber 2006).

Despite the evidence implicating FMRP in mGluR-LTD, it is less clear whether FMRP significantly impacts the expression of long-term potentiation (LTP), an activity-induced increase in synaptic strength believed to underlie specific types of learning and memory (Bliss and Collingridge 1993; Godfraind et al. 1996; Paradae et al. 1999; Larson et al. 2005; Lauterborn et al. 2007).

β-Adrenergic receptors (β-ARs) are G protein-coupled receptors that enhance hippocampal homosynaptic LTP and long-term memory formation through translation regulation (Gelinas and Nguyen 2005; Gelinas et al. 2007). As β-ARs and mGluRs both couple to signaling cascades that regulate translation (Banko et al. 2006; Gelinas et al. 2007), we asked whether β-AR-dependent heterosynaptic LTD was altered in Fmr1 KO mouse hippocampus. Frey and Morris (1997) have shown that the induction of protein synthesis-dependent LTP at one synaptic pathway (S1), can facilitate the generation of heterosynaptic LTD at a second set of synapses (S2). However, to date, heterosynaptic processes have not been assessed in any mouse model of FXS. To test the hypothesis that β-AR-dependent heterosynaptic LTD is altered in Fmr1 KO mice, we induced heterosynaptic plasticity using a novel protocol consisting of β-AR activation paired with brief stimulation in one synaptic pathway followed later with milder stimulation at a neighboring pathway.

We report that β-AR-dependent heterosynaptic LTD is altered in the hippocampus of Fmr1 KO mice. Although homosynaptic LTD was similar to wild-type (WT) controls, heterosynaptic LTD was enhanced in Fmr1 KO mouse hippocampus. This promotion of heterosynaptic plasticity may contribute to cognitive abnormalities in FXS, such as impaired learning during periods of heightened attention or arousal that recruit the noradrenergic neuromodulatory system and engage β-ARs.

Results

Synaptic tagging induced by HFS alone is normal in Fmr1 knockout mouse hippocampus

Induction of translation-dependent LTP at one synaptic pathway can facilitate the induction of heterosynaptic LTD following the
activity-dependent setting of “tags,” which capture plasticity-related proteins (PRPs) necessary for expression of LTP (Frey and Morris 1997; for review, see Barco et al. 2008; Frey and Frey 2008). Synaptic tagging and capture provides a mechanistic explanation for how translational and transcriptional products are appropriately targeted to previously active dendritic compartments, thereby maintaining synaptic specificity believed to be critical for cellular processes underlying memory formation (Morris 2006; Barco et al. 2008; Frey and Frey 2008). Regulated protein synthesis produces PRPs necessary for heterosynaptic transfer of LTP (Frey and Morris 1997). Knockout of Fmr1 reduces the expression of the translational repressor FMRP, which binds to several mRNAs that encode PRPs involved in synaptic plasticity (Hou et al. 2006; Muddashetty et al. 2007; Park et al. 2008; Schütt et al. 2009). Thus, we asked whether reduced expression of FMRP would alter synaptic tagging and capture of LTP, by using a stimulus protocol similar to one that is commonly implemented to probe tagging (Frey and Morris 1997).

Two stimulating electrodes were placed in CA1 straddling a recording electrode positioned in stratum radiatum. Independence of synaptic pathways was confirmed using paired-pulse facilitation both before and at the conclusion of the experiments. To determine if synaptic tagging was altered in Fmr1 knockout mouse hippocampus we first established that synaptic tagging and capture could be induced in WT controls. A high-frequency stimulation protocol consisting of four trains of 100-Hz stimulation at 3-sec intertrain intervals was applied to one synaptic pathway (S1; homosynaptic), in WT hippocampal slices, which induced long-lasting LTP (Fig. 1A). The magnitude of LTP in S1 as assessed 120 min after high-frequency stimulation was 154 ± 10% of the baseline (n = 9). When the same stimulation protocol was applied to Fmr1 KO mouse hippocampal slices, long-lasting LTP was induced (fEPSPs were potentiated to 158 ± 8% of the baseline, 120 min after HFS), which was not significantly different from WT controls (P > 0.05) (Fig. 1C). Following a 30-min delay, one train of 100-Hz stimulation (1-sec duration) was applied to a second synaptic pathway (S2), which converged on the same group of post-synaptic cells. One train of 100-Hz stimulation normally induces decremental LTP in mouse hippocampal slices, which returns to baseline in <2 h (Duffy et al. 2001; Gelinas and Nguyen 2005). When preceded by multiple HFS trains of LTP homosynaptically, a single train of HFS applied heterosynaptically generated long-lasting LTP in both WT (mean fEPSP slope was 142 ± 8%) and Fmr1 KO (mean fEPSP slope was 144 ± 10%) (Fig. 1C) slices as assessed 2 h post-stimulation. Although, no significant differences were detected between KO and WT slices at the second synaptic pathway (P > 0.05) (Fig. 1C), these results suggest that in both WT and Fmr1 KO mouse hippocampi, 4 × 100-Hz stimulation generates long-lasting LTP, which can be captured at a second synaptic pathway in an activity-dependent manner.

To confirm that heterosynaptic facilitation of LTP had occurred, two control experiments were conducted in Fmr1 KO mouse hippocampal slices in which each pathway was stimulated alone. Either 4 × 100 or 1 × 100 Hz alone was applied to S1, while monitoring heterosynaptic baseline responses at S2. Consistent with previous results (Frey and Morris 1997), application of multiple high-frequency trains (4 × 100 Hz) induced LTP (fEPSPs were 156 ± 14% 120 min after HFS at S1, n = 6) (data not shown) that did not transfer to a second (S2) synaptic pathway receiving baseline stimulation (1 pulse per minute; fEPSPs were 101 ± 4% at 120 min). Next, we applied brief, HFS (1 × 100 Hz, 1 sec) alone, which failed to induce long-lasting synaptic changes at either synaptic pathway (fEPSPs were 107 ± 9% of baseline at S1 and 98 ± 8% of baseline at S2 120 min post-stimulation; n = 4). Our results show that HFS-dependent synaptic tagging and capture of LTP in

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Figure 1. HFS-induced synaptic tagging and capture are not altered in Fmr1 knockout mice. (A) Application of 4 × 100-Hz stimulation to S1 (*) facilitates the induction of LTP by brief, HFS (1 × 100 Hz; □) at S2 (n = 9). (B) In Fmr1 KO slices, HFS-induced synaptic tagging and capture are similar to WT. Stimulation with repeated HFS at one synaptic pathway (4 × 100 Hz; *) induces long-lasting LTP, which can be captured heterosynaptically following a single train of HFS (1 × 100 Hz; □; n = 8) at a second set of synapses converging on the same post-synaptic cells. (C) Summary histogram comparing fEPSP slopes obtained in WT and KO slices 120 min after HFS at S1 and LFS at S2. No statistically significant differences were detected between WT and KO fEPSPs when homo- and heterosynaptic LTP were compared. Sample traces were taken 10 min after the commencement of the baseline recordings and 120 min after stimulation at S1 and S2. Results in C represent mean ± SEM.
Fmr1 KO mouse hippocampus is intact and similar to slices from wild-type mouse.

Activation of β-ARs facilitates heterosynaptic transfer of LTP

β-AR activation in area CA1 of mouse hippocampal slices facilitates induction of homosynaptic LTP by stimulation protocols normally subthreshold for inducing persistent, translation-dependent LTP (Thomas et al. 1996; Gelinas and Nguyen 2005). As β-AR-dependent LTP requires de novo protein synthesis for its expression (Straube et al. 2003; Gelinas and Nguyen 2005; Gelinas et al. 2007), we wanted to determine if inducing β-AR-dependent LTP at one synaptic pathway would enhance LTP at a second, independent set of synapses in WT slices. To do this, we repeated the two-pathway regimen previously described. Briefly, two stimulating electrodes were placed in stratum radiatum on either side of a recording electrode and staggered relative to two stimulating electrodes were placed in stratum radiatum on its expression (Straube et al. 2003; Gelinas and Nguyen 2005; Gelinas et al. 2007). We applied the β-AR-dependent LTP protocol to Fmr1 KO mouse hippocampal slices for 15 min. Application of ISO induced a modest, transient increase in fEPSPs at both synaptically pathways, which returned to baseline, consistent with previously reported data (Gelinas and Nguyen 2005). Sixty minutes after ISO application, the mean fEPSP slopes at S1 were 103 ± 5% of baseline (n = 7) (Fig. 2C). After a 25-min delay, low-frequency stimulation (5 Hz for 10 sec) was applied to S2, which induced short-lasting LTP that decayed to baseline in <1 h: mean fEPSP slopes were 105 ± 5% of baseline 90 min after 5 Hz stimulation (Fig. 2C,F). Next, we sought to determine if a single tetanus of 100 Hz (1-sec duration) to Fmr1 KO mouse hippocampal slices could facilitate the expression of heterosynaptic LTP induced by ISO. Consistent with previous results, a 1 × 100-Hz stimulation induced transient LTP that returned to baseline in <2 h (mean fEPSP slopes were 104 ± 8% at S1 120 min after ISO, n = 8) (Fig. 2D). Following a 30-min delay, low-frequency stimulation (5 Hz, 10 sec) was applied to a second group of synapses converging on the same post-synaptic cells. Heterosynaptic low-frequency stimulation failed to induce long-lasting LTP (mean fEPSPs were 102 ± 7% at S2 when assessed 2 h after stimulation; n = 8) (Fig. 2D,F). Taken together, these data suggest that application of either ISO alone or 1 × 100 Hz stimulation without ISO, does not facilitate the future induction of heterosynaptic LTP in Fmr1 KO mouse hippocampal slices.

We applied the β-AR-dependent heterosynaptic plasticity protocol to Fmr1 KO mouse hippocampal slices. Briefly, following a 20-min baseline, ISO was applied for 10 min, then paired with 1 × 100 Hz stimulation at S1 (homosynaptic) and maintained in ISO for an additional 5 min. When this protocol was applied to Fmr1 hippocampal slices, homosynaptic, long-lasting LTP was induced which was not significantly different from WT slices (mean fEPSP slopes were 152 ± 6% in KO; n = 8, and 147 ± 7% in WT, 120 min after stimulation; n = 8) (Fig. 2B,E). Thirty minutes after HFS in S1, low-frequency stimulation (5 Hz, 10 sec) was applied to a second, independent set of synapses converging on the same post-synaptic cells (S2). LFS applied heterosynaptically generated long-lasting LTP that was significantly enhanced in Fmr1 KO slices relative to WT controls (mean fEPSP slopes in KO = 139 ± 8%, WT = 119 ± 6%, as assessed 120 min post-LFS; P < 0.05) (Fig. 2F). These results suggest that heterosynaptic plasticity is enhanced in response to β-AR stimulation in Fmr1 KO mouse hippocampus.

To determine if β-AR activation alone is sufficient for enhancing heterosynaptic plasticity, ISO (1 μM) was applied to Fmr1 knockout hippocampal slices for 15 min. Application of ISO induced a modest, transient increase in fEPSPs at both synaptic pathways, which returned to baseline, consistent with previously reported data (Gelinas and Nguyen 2005). Sixty minutes after ISO application, the mean fEPSP slopes at S1 were 103 ± 5% of baseline (n = 7) (Fig. 2C). After a 25-min delay, low-frequency stimulation (5 Hz for 10 sec) was applied to S2, which induced short-lasting LTP that decayed to baseline in <1 h: mean fEPSP slopes were 105 ± 5% of baseline 90 min after 5 Hz stimulation (Fig. 2C,F). Next, we sought to determine if a single tetanus of 100 Hz (1-sec duration) to Fmr1 KO mouse hippocampal slices could facilitate the expression of heterosynaptic LTP induced by ISO. Consistent with previous results, a 1 × 100-Hz stimulation induced transient LTP that returned to baseline in <2 h (mean fEPSP slopes were 104 ± 8% at S1 120 min after ISO, n = 8) (Fig. 2D). Following a 30-min delay, low-frequency stimulation (5 Hz, 10 sec) was applied to a second group of synapses converging on the same post-synaptic cells. Heterosynaptic low-frequency stimulation failed to induce long-lasting LTP (mean fEPSPs were 102 ± 7% at S2 when assessed 2 h after stimulation; n = 8) (Fig. 2D,F). Taken together, these data suggest that application of either ISO alone or 1 × 100 Hz stimulation without ISO, does not facilitate the future induction of heterosynaptic LTP in Fmr1 KO mouse hippocampal slices.

Altered inhibition is not the primary cellular mechanism mediating β-AR-dependent heterosynaptic facilitation in Fmr1 KO hippocampus

Alterations to GABAergic inhibition have previously been associated with neuropathologies observed in FXS rodent models (Burgard and Servey 1991; El Idrissi et al. 2005; Curia et al. 2009). Hippocampal tissue from adult male Fmr1 KO mice exhibits reduced expression of the GABA-A receptor β subunit, which is required for normal receptor function (El Idrissi et al. 2005). In addition, recent evidence suggests that ISO can reduce inhibitory drive within the hippocampus (Ziros and Maccarferri 2008). Therefore, we investigated whether altered inhibitory circuit function could account for the enhanced heterosynaptic LTP observed in Fmr1 KO hippocampus. To examine the effects of altered inhibitory function, we preapplied the GABA-A receptor antagonist, bicuculline (BICU; 10 μM), for 20 min prior to and overlapping with, ISO. If altered GABA-A receptor function is the mechanism responsible for enhanced heterosynaptic plasticity, then prior application of BICU should occlude the LTP enhancement generated by β-AR stimulation. First, Fmr1 KO mouse hippocampal
slices were exposed to a 20-min preincubation in BICU. Subsequently, a cocktail consisting of ISO and BICU was added to the bath for 15 min. Ten minutes into ISO + BICU application, 1 × 100 Hz, 1-sec stimulation was applied to S1. This protocol generated long-lasting LTP that was potentiated to 164 ± 12% (n = 6) 2 h after stimulation in Fmr1 slices (Fig. 3B). To test if heterosynaptic plasticity was altered by BICU, 25 min after homosynaptic 100-Hz stimulation, 5-Hz, 10-sec stimulation was given to a second synaptic pathway. Heterosynaptic plasticity was significantly potentiated (denoted as +) relative to both controls (C,D) and WT slices treated with ISO + 100 Hz (A) 120 min after LFS at S2 (E; n = 8). (C) Application of ISO alone to Fmr1 KO slices prior to LFS induces a modest, short-lasting increase in basal synaptic response, which has no long-lasting effects on synaptic transmission (n = 7). (D) 100-Hz stimulation alone induces transient LTP, which returns to baseline in < 2 h and does not facilitate heterosynaptic LTP following LFS at a second synaptic pathway in Fmr1 KO mouse slices (n = 8). Importantly, LFS (5 Hz, 10 sec) does not last more than 45 min. (E) Summary histogram comparing fEPSP slopes obtained 120 min after HFS at S1. (F) Summary histogram comparing fEPSP slopes 120 min after LFS at S2. Sample traces were taken 10 min after commencement of baseline recordings and 120 min after S1 stimulation. Results in panels E and F represent mean ± SEM, P < 0.05.

**Figure 2.** β-AR-dependent heterosynaptic plasticity is enhanced in Fmr1 KO mice. (A) In WT slices, pairing one train of 100-Hz stimulation with ISO application induces LTP (†), which facilitates LTP at a second synaptic pathway (□) following LFS (5 Hz, 10 sec; n = 8). (B) Heterosynaptic LTP (□) was similarly enhanced (†) relative to controls treated with ISO alone (C) or 100 Hz alone (D). Heterosynaptic LTP (□) was significantly enhanced (†, as shown in F) relative to controls when preceded by ISO paired with 100 Hz homosynaptically. (B) Similar to WT, homosynaptic long-lasting LTP was generated in Fmr1 KO hippocampal slices when 100 Hz was applied with ISO (†). However, heterosynaptic LTP was significantly potentiated (denoted as +) relative to both controls (C,D) and WT slices treated with ISO + 100 Hz (A) 120 min after LFS at S2 (E; n = 8). (C) Application of ISO alone to Fmr1 KO slices prior to LFS induces a modest, short-lasting increase in basal synaptic response, which has no long-lasting effects on synaptic transmission (n = 7). (D) 100-Hz stimulation alone induces transient LTP, which returns to baseline in < 2 h and does not facilitate heterosynaptic LTP following LFS at a second synaptic pathway in Fmr1 KO mouse slices (n = 8). Importantly, LFS (5 Hz, 10 sec) does not last more than 45 min. (E) Summary histogram comparing fEPSP slopes obtained 120 min after HFS at S1. (F) Summary histogram comparing fEPSP slopes 120 min after LFS at S2. Sample traces were taken 10 min after commencement of baseline recordings and 120 min after S1 stimulation. Results in panels E and F represent mean ± SEM, P < 0.05.
Next, wild-type slices were treated with the same regimen. Preincubation with BICU followed by ISO paired with HFS induced long lasting LTP at S1 (fEPSPs were potentiated to 144 $\pm$ 10% 2 h after stimulation; $n = 6$) (Fig. 3A). Subsequent low-frequency stimulation (5 Hz) applied heterosynaptically generated long-lasting LTP, which was potentiated to 154 $\pm$ 11% at 2 h post-tetanus (Fig. 3A). When homosynaptic (S1) $\beta$-AR-dependent LTP in slices from WT (Fig. 2A) was compared to WT slices exposed to BICU (Fig. 3A) 2 h after stimulation, no significant differences were detected ($P > 0.05$) (Fig. 3C). Comparisons between homosynaptic LTP in Fmr1 KO slices in the presence or absence of BICU failed to reveal any significant differences ($P > 0.05$) (Fig. 3C). When homosynaptic comparisons were conducted between fEPSPs 2 h after LFS in WT slices either exposed (Fig. 3A) or not exposed (Fig. 2A) to BICU, a significant enhancement of LTP in the presence of BICU was observed ($P < 0.01$) (Fig. 3D). Likewise, comparisons between heterosynaptic LTP in Fmr1 KO slices treated or not treated with BICU showed that BICU contributed to a significant increase in potentiation ($P < 0.05$) (Fig. 3D) 2 h after low-frequency stimulation. These results suggest that decreasing inhibition results in a further enhancement of heterosynaptic LTP in both genotypes and that stimulation of $\beta$-ARs engages cellular plasticity mechanisms that are not primarily dependent on altered GABAergic receptor function.

$\beta$-AR activation is required for isoproterenol-induced heterosynaptic plasticity

Genetic and pharmacological inhibition of $\beta$-ARs in area CA1 can interfere with expression of LTP and long-term memory formation (Winder et al. 1999; Ji et al. 2003). To test if $\beta$-ARs are required for heterosynaptic plasticity induced with isoproterenol, hippocampal slices from wild-type and Fmr1 knockout mice were pre-treated with a $\beta$-AR antagonist, propranolol (PROP; 50 $\mu$M). In wild-type mice, following a 10-min baseline, PROP was bath-applied 20 min prior to, overlapping with, and 10 min after ISO application. When high-frequency stimulation was applied homosynaptically, LTP was induced, which decayed to baseline in <2 h (Fig. 4A) (mean fEPSP slopes were 101 $\pm$ 8%, 120 min after
mGluR activation is not required for enhanced heterosynaptic LTP in Fmr1 KO mouse hippocampus

mGluR-dependent LTD is enhanced in Fmr1 KO mouse hippocampus. Normally, mGluR LTD requires protein synthesis for maintenance of GluR internalization (Nosyreva and Huber 2006; Nakamoto et al. 2007). In Fmr1 KO mouse hippocampus, mGluR LTD does not require translation, suggestive of an increase in basal protein synthesis (Hou et al. 2006; Nosyreva and Huber 2006). It is unclear whether heterosynaptic LTD in Fmr1 KO mouse hippocampus requires mGluR activation. To determine whether mGluRs are required for heterosynaptic facilitation of LTP by β-ARs in Fmr1 mouse hippocampal slices, we used the mGlu5 antagonist, MPEP (10 μM). Application of MPEP 20 min prior to and overlapping with β-AR stimulation paired with 100 Hz tetanus in S1, did not affect LTP expression in either synaptic pathway: mean fEPSP slopes in S1 were 147 ± 8%/2 h after tetani- zation in Fmr1 KO slices (n = 6) (Fig. 6A). When compared to WT controls not exposed to MPEP (144 ± 6%/90 min after HFS in S1 and 101 ± 5%/90 min after LFS at S2, n = 8) (Fig. 4C). Thus, heterosynaptic plasticity produced by ISO application requires β-AR activation.
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KO mice were 144 ± 8% of baseline, which is significantly elevated relative to wild-type slices (mean fEPSPs were 118 ± 6% of baseline), when compared 120 min after stimulation ($P < 0.05$) (Fig. 6C). Thus, the enhancement of heterosynaptic LTP in slices from Fmr1 KO mice does not appear to require mGluR5 activation.

**β-AR-dependent heterosynaptic LTP requires translation in Fmr1 KO mice**

Protein synthesis critically involved in the generation of LTP and long-term memory formation. Results from Auerbach and Bear (2010) suggest that priming of LTP by prior activation of mGluRs, involves a reduced requirement for translation in Fmr1 KO mice, consistent with enhanced synthesis of PRPs. Previously, cap-dependent translation was shown to be a critical target for regulation during β-AR-induced synaptic plasticity (Gelinas et al. 2007). As β-ARs and mGluRs both engage cap-dependent translation, we asked whether β-AR activation in Fmr1 knock-out mice renders LTP immune to protein synthesis inhibition. To test this hypothesis, we preincubated slices in a translational inhibitor, emetine (EME). Homosynaptic LTP elicited by pairing β-AR activation with 1 × 100 Hz high-frequency stimulation in S1 decayed in the presence of EME. Mean fEPSP slopes were 115 ± 10% in KO ($n = 8$) and 107 ± 13% in WT ($n = 7$), $P > 0.05$, 120 min after HFS (Fig. 7A,B). When compared to homosynaptic LTP induced with β-ARs in WT controls (mean fEPSP slopes were 144 ± 9% 120 min after HFS, $n = 6$) (data not shown), Tukey-Kramer post hoc tests revealed that emetine significantly inhibited LTP in both WTs and KOs ($P < 0.05$). Bath application of emetine overlapping with ISO likewise inhibited LTP heterosynaptically when 5-Hz, 10-sec LFS was applied 30 min later in S2 relative to EME-free control slices (Fig. 7A,B) (mean fEPSP slopes in the presence of EME were 106 ± 6% in KO ($n = 8$) and 104 ± 10% in WT ($n = 7$), $P > 0.05$, 90 min after LFS) (Fig. 7C). When compared to heterosynaptic LTP induced with β-AR stimulation in WT controls (127 ± 7%, 90 min after LFS) (data not shown), Tukey-Kramer post hoc tests revealed that heterosynaptic LTP in both wild-type and Fmr1 knockout slices ($P < 0.05$) (Fig. 7D) decayed in the presence of EME. Thus, when compared 2 h post-stimulation, Fmr1 KO mouse slices exhibited significant decreases in potentiation at both S1 and S2, which was similar to WT slices exposed to EME.

**Knockout of Fmr1 confers immunity to inhibition of mTOR but not ERK**

β-ARs recruit the mammalian target of rapamycin (mTOR) signaling pathway to facilitate LTP maintenance through translation initiation (Gelinas et al. 2007). We assessed whether heterosynaptic LTP was affected by an mTOR inhibitor, rapamycin, in Fmr1 knockout mouse hippocampus. Wild-type slices treated with rapamycin (RAP) demonstrated reduced homo- and heterosynaptic plasticity. Application of RAP 30 min prior to, overlapping with, and 10 min after ISO paired with high-frequency stimulation inhibited the expression of homosynaptic LTP as assessed 2 h after HFS (mean fEPSP slopes in S1 were 108 ± 12%). Subsequent (30 min post-HFS) stimulation with low-frequency stimulation at S2 generated decremental LTP which was 108 ± 9% 90 min post-LFS (Fig. 8A) ($n = 9$). Interestingly, Fmr1 KO mouse slices treated with RAP demonstrated intact homosynaptic LTP. Homosynaptic LTP was significantly enhanced (mean fEPSP slope was 140 ± 9% in S1, 120 min post-stimulation) in rapamycin-treated slices (Fig. 8B,E) ($n = 6$, $P < 0.02$) relative to wild-type slices exposed to RAP. However, inhibition of mTOR still attenuated LTP heterosynaptically in Fmr1 mouse slices. Mean fEPSP slope at S2 90 min after low-frequency stimulation was 115 ± 9% ($P > 0.05$).
MPEP does not block the β-AR-dependent enhancement of heterosynaptic plasticity in Fmr1 KO mice. (A) Induction of β-AR-dependent LTP at one synaptic pathway (S1; *) facilitates the subsequent expression of heterosynaptic LTP (S2; □) in WT slices (n = 8). (B) The same protocol applied to Fmr1 KO mouse hippocampal slices in the presence of the mGlur5 antagonist MPEP, induces heterosynaptic plasticity (S2; □) that is still enhanced relative to WT controls (n = 6). (C) A summary histogram comparing fEPSP slopes obtained 120 min after HFS at S1 (black bars) revealed no significant differences in homosynaptic LTP. Heterosynaptic LTP (S2; white bars) was significantly elevated (*) 120 min after LFS in Fmr1 KO slices treated with MPEP when compared to WT slices not treated with MPEP. Sample traces were taken 10 min after commencement of baseline recordings and 120 min after HFS. Results in C represent mean ± SEM, P < 0.05.

Discussion

Fragile X syndrome is characterized by behavioral (Hagerman and Hagerman 2002; Spencer et al. 2005; Moon et al. 2006) and cognitive (Tsouiris and Brown 2004; Macleod et al. 2010) abnormalities that have been linked to altered metabotropic receptor signaling and translation regulation (Huber et al. 2002; Bear et al. 2004; Kelleher and Bear 2008; for review, see O'Donnell and Warren 2002; Pfeiffer and Huber 2009). The noradrenergic system, acting through metabotropic adrenergic receptors, regulates multiple processes including sleep, attention, learning, memory, and arousal (Gelinas and Nguyen 2007; Berridge and Waterhouse 2003). β-ARs also mediate synaptic plasticity involved in encoding the association of disparate events over time (Shapiro et al. 2006). Heterosynaptic facilitation provides a mechanism for associating stimuli over time by integrating synaptic events following induction of persistent synaptic plasticity (Barco et al. 2008; Frey and Frey 2008). A previously defined heterosynaptic protocol known as “synaptic tagging and capture” is commonly initiated by applying HFS (4 × 100 Hz) at one pathway (S1), which generates plasticity-related proteins (PRPs) that can be captured by activity-dependent tags set at a second synaptic pathway (S2) (Frey and Morris 1997). Neuromodulators gate both protein synthesis and activation of second messengers that can support this type of heterosynaptic plasticity (Gelinas and Nguyen 2005; Navakkode et al. 2007). Application of a cAMP phosphodiesterase inhibitor, rolipram, enabled the heterosynaptic transformation of translation-independent LTP induced by one 100-Hz train into translation-dependent LTP, suggestive of a cAMP-mediated increase in plasticity products (Navakkode et al. 2004). Noradrenergic receptors can facilitate the generation and regulation of plasticity proteins capable of prolonging LTP (Winder et al. 1999; Straube et al. 2003; Gelinas and Nguyen 2005) and boosting long-term memory (Cahill et al. 1994; Hu et al. 2007; Kemp and Manahan-Vaughan 2008). Interestingly, prior exposure of rats to novel experiences engages the noradrenergic system (Kitchigina...
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et al. 1997) to promote the consolidation of short-term memory into protein synthesis-dependent long-term memory, that is likely to involve “behavioral tagging” (Moncada and Viola 2007; Ballarini et al. 2009). Taken together, these results suggest that β-AR activation engages intracellular pathways critical for heterosynaptic facilitation of LTP. Herein, we describe a novel heterosynaptic plasticity protocol that relies upon activation of β-ARs for its induction. We found that pairing β-AR activation with high-frequency stimulation induces LTP that could be transferred to a second synaptic pathway provided that stimulation sufficient for generating a molecular activity trace was applied.

How does β-AR activation facilitate heterosynaptic plasticity in Fmr1 KO mouse hippocampus? One of the core phenotypes of FXS models is enhanced epileptogenesis as a result of dysregulated inhibitory circuit function (Burgard and Sarvey 1991; El Idrissi et al. 2005; Curia et al. 2009). This fact, coupled with the ability of isoproterenol to depress inhibitory interneurons, suggests that altered inhibitory circuit function may be responsible for enhanced heterosynaptic LTP in Fmr1 KO mice. However, we failed to find that disinhibition through application of the GABA-A receptor antagonist bicuculline failed to occlude the effects of β-AR activation. Indeed, consistent with previous data (Burgard and Sarvey 1991), co-application of ISO and BICU resulted in further enhancement of heterosynaptic LTP (additionally, there was a trend for enhanced homosynaptic LTD in Fmr1 KO slices, but this failed to reach statistical significance). Our results do not support the idea that altered GABAergic inhibition could account for enhanced heterosynaptic LTP in Fmr1 KO mice.

The mGluR theory of FXS states that stimulation of mGluR1/5 leads to enhanced synthesis of LTD-related plasticity proteins, which are normally repressed through FMRP-dependent mRNA regulation (Bear et al. 2004; Pfieffer and Huber 2007). The enhancement of heterosynaptic LTP in Fmr1 KO mice observed following our β-AR-dependent heterosynaptic protocol did not require mGluR activation, as application of the mGluR5 antagonist MPEP had no effect on the magnitude of LTD at either synaptic pathway in Fmr1 KO slices. The mGluR theory has been extended to include signaling molecules that affect cap-dependent translation, including ERK and mTOR (Hou et al. 2006; Volk et al. 2007; Sharma et al. 2010). Importantly, β-AR activation has been linked to regulation of cap-dependent translation through ERK and mTOR pathways, both of which are dysregulated in Fmr1 KO mouse hippocampus (Hou et al. 2006; Kim et al. 2008; Sharma et al. 2010). These pathways are critical for coupling mGluRs and β-ARs to protein synthesis (Banko et al. 2006; Gellinas et al. 2007) through interactions with eIF4E, a translation initiation factor required for eIF4F initiation complex formation and translation initiation (Costa-Mattioli et al. 2009; Richter and Klann 2009). Our data suggest that activation of the ERK pathway is required for homosynaptic LTD and for heterosynaptic facilitation of LTD in both WT and Fmr1 KO mouse hippocampus. In contrast, activation of mTOR is required for heterosynaptic LTD, but not for homosynaptic LTD, in Fmr1 KO mouse hippocampus. Although the mTOR inhibitor rapamycin prevented the expression of heterosynaptic LTD, homosynaptic LTD was still intact. Up-regulated mTOR signaling may lower the threshold for initiation of protein synthesis by elevating the levels of “free” eIF4E (Sharma et al. 2010), thereby boosting heterosynaptic plasticity.

As inhibition of protein synthesis, but not inhibition of mTOR, blocked heterosynaptic facilitation of LTD, decreased levels of FMRP appear to affect processes upstream of mTOR-mediated translation regulation. Previous results suggest that in Fmr1 KO mice, mTOR signaling is elevated, which may facilitate mGluR-dependent LTD (Hoefner and Klann 2010; Sharma et al. 2010). Hyperphosphorylation of eIF4E-binding proteins (4E-BPs)
by mTOR regulates translation initiation by increasing levels of unbound eIF4E. Once dissociated from the 4E-BPs, eIF4E is able to complex with other initiation factors to engage translation (Klann et al. 2004; Richter and Klann 2009). Dysregulation of the PI3K-mTOR pathway in the absence of FMRP has recently been reported (Sharma et al. 2010) and could provide a mechanism for enhanced β-AR-dependent synaptic facilitation (Fig. 9A). As β-AR-dependent synaptic plasticity was partially immune to mTOR inhibition, our results suggest that, in a broader perspective, dysregulation of mTOR signaling may be an important cause...
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Figure 9. (A) Increased mTOR signaling in the absence of FMRP enhances heterosynaptic plasticity. In wild-type mouse hippocampus, FMRP represses mTOR activity (left side). The mTOR signaling cascade can regulate protein synthesis mechanisms necessary for heterosynaptic transfer of LTP. Without the repressive effects of FMRP (right side), mTOR activity is elevated resulting in dysregulated translation and exaggerated heterosynaptic plasticity. (B) Activation or β-ARs confers sensitivity to protein synthesis inhibition. (a) This schematic diagram represents the sensitivity of mouse hippocampal synaptic plasticity to protein synthesis inhibition. In wild-type mice, the fulcrum is centered, indicative of situations in which the ability of synapses to undergo translation-dependent changes is modifiable. (b) β-AR activation shifts the fulcrum to the left through up-regulation of protein synthesis, thereby increasing the sensitivity of synaptic changes (LTP) to translation inhibition. (c) In Fmr1 knockout mouse hippocampus, the fulcrum is shifted to the right under basal conditions, rendering certain forms of synaptic plasticity (mGluR-dependent LTD) insensitive to inhibition of protein synthesis. (d) Activation of β-ARs enhances activation of signaling cascades that regulate translation, resetting the sensitivity to protein synthesis inhibition (shifting the fulcrum to the left) in Fmr1 knockout mouse hippocampus, thereby restoring the translation-dependence of synaptic plasticity.

of altered synaptic plasticity, which both mGluRs and β-ARs critically modulate.

Interestingly, β-AR-dependent heterosynaptic facilitation in Fmr1 mouse hippocampus is blocked by a protein synthesis inhibitor, in contrast to mGluR-LTD, which is immune to translational inhibition (Nosyreva and Huber 2006). Evidence suggests that FMRP may act as a brake on cap-dependent translation, evident from the increased basal elf4F initiation complex formation observed in Fmr1 KO mice (Sharma et al. 2010). FMRP suppresses translation through interaction with cytoplasmic FMRP-interacting protein (CYFIP1), which binds to elf4F, thereby preventing formation of the elf4F initiation complex (Napoli et al. 2008). Our data suggest that translation is still required for heterosynaptic plasticity mediated by β-ARs in both WT and Fmr1 KO mice. These results contrast with mGluR-LTD (Nosyreva and Huber 2006) and mGluR-primed LTD (Auerbach and Bear 2010) in Fmr1 knockout mice, which persist in the presence of translation inhibitors. The disparity in these data could be the result of a higher demand for plasticity proteins in heterosynaptic plasticity protocols relative to single pathway experiments. Enhanced basal protein synthesis may be sufficient for overcoming translational inhibition using protocols that do not exhaust the availability of PRPs. Indeed, previous research has described a form of intersynaptic competition for plasticity proteins under conditions of reduced protein synthesis (Fonseca et al. 2004). When plasticity protein synthesis is limited, the maintenance of LTP at one synaptic pathway occurs at the expense of a second previously potentiated synaptic pathway, in a process known as “competitive maintenance” (Fonseca et al. 2004). Thus, even in circumstances of enhanced basal protein synthesis, the sharing of plasticity proteins required for heterosynaptic facilitation of synaptic changes may require additional translation to maintain LTP in two pathways.

Temporally and spatially restricted translation gates persistent synaptic plasticity by generating proteins necessary for long-term modifications of synaptic structure and function. Fmr1 knockout mice exhibit alterations in translation regulation (Nimchinsky et al. 2001). Enhanced protein synthesis has been demonstrated in the hippocampus of Fmr1 KO mice (Qin et al. 2005; Dölen and Bear 2008) with several of the dysregulated mRNAs encoding proteins involved in LTP, LTD, and long-term memory (Glur1, Arc, PSD-95, MAP1B) (Hou et al. 2006; Muddashetty et al. 2007; Park et al. 2008; Schütt et al. 2009). As β-ARs couple to both mTOR and ERK signaling cascades, which can co-regulate translation, we propose that activation of β-ARs resets the sensitivity of long-term synaptic changes to translational inhibition (Fig. 9B). Previous data have demonstrated an abnormally rapid ERK dephosphorylation in response to mGluR1/5 stimulation in synaptoneurosomes isolated from Fmr1 knockout mouse cortical tissue (Kim et al. 2008). Thus, β-AR activation may compensate for down-regulated ERK activity observed in Fmr1 knockouts, thereby re-establishing the homeostasis between ERK and mTOR signaling necessary for regulated translation. As a result, the sensitivity of long-term synaptic modifications to translational control would be reset to levels similar to that observed in wild-type mice. The impact of β-AR-mediated tagging and enhanced heterosynaptic plasticity on cognition in Fmr1 knockout mice remains to be determined.

Overall, our data show an enhancement of heterosynaptic plasticity in Fmr1 knockout mouse hippocampus following the induction of homosynaptic β-AR-dependent LTD. This form of synaptic plasticity requires translation, suggestive of a β-AR-mediated re-establishment of sensitivity to protein synthesis inhibition. Given that β-AR antagonists are already being used clinically with positive results (Rosenberg 2007), β-ARs and their...
downstream effectors are logical targets for the pharmacologic treatment of fragile X syndrome (Restivo et al. 2005; Kelley et al. 2008). Increased heterosynaptic plasticity in response to noradrenergic system activation may promote hyperconsolidation of synaptic changes across multiple neural networks that would normally be repressed when FMRP is present. Synaptic hyperconsolidation could stem from a lowered threshold for heterosynaptic plasticity, thereby compromising the network- and synapse-specific changes in synaptic weights that are believed to be necessary for normal learning to occur. This may lead to the cognitive impairments observed in fragile X syndrome.

Materials and Methods

Animals

C57BL/6 congenic Fmr1 KO mice and their WT littermates (3−4 mo) were used for all experiments. Animals were socially housed with food and water available ad libitum and were maintained on a 12 h on/off light cycle. Animals were housed at the University of Alberta using guidelines approved by the Canadian Council on Animal Care. Fmr1 KO mice and their WT littermates were generated in harem crosses using hemizygous Fmr1 mutant or WT males crossed to heterozygote Fmr1 (+/−) females. Mice were weaned between 21 and 28 d and genotyped using standard methods using primer sequences and PCR instructions supplied by Jax Labs (http://jaxmice.jax.org/protocolsdb).

Electrophysiology

After cervical dislocation and decapitation, transverse hippocampal slices (400 μm in thickness) were prepared as described by Nguyen and Kandel (1997). Slices were maintained in an interface chamber at 28 °C, which is a consistently used temperature for recording field responses on extended (hours) time scales (Gelinas and Nguyen 2005; Young et al. 2006; Gelinas et al. 2007). Slices were perfused at 1−2 mL/min with artificial cerebrospinal fluid (ACSF) composed of (mM): 124 NaCl, 4.4 KCl, 1.3 MgSO4, 1.0 NaH2PO4, 26.2 NaHCO3, 2.5 CaCl2, and 10 glucose, aerated with 95% O2 and 5% CO2. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass micro-electrode filled with ACSF (resistances, 2−3 MΩ) and positioned in the stratum radiatum of area CA1. fEPSPs were elicited by using two bipolar nickel−chromium electrodes placed on either side of the recording electrode in stratum radiatum to stimulate two separate sets of inputs converging onto the same postsynaptic population of neurons. Stimulating electrodes were staggered within stratum radiatum relative to each other to maximize independence of synaptic pathways. For simplicity, “S1” refers to the first or homosynaptic pathway and “S2” refers to the second or heterosynaptic pathway. The independence of the two pathways was confirmed by the absence of interpathway paired-pulse facilitation, elicited by successive stimulation through the two electrodes at 75-, 100-, 150- and 200-ms intervals. Interepathway paired-pulse facilitation was assessed during baseline acquisition and at the conclusion of experiments. Stimulation intensity (0.08-msec pulse duration) was adjusted to evoke fEPSP amplitudes that were 40% of maximal size (Woo and Nguyen 2003; Gelinas and Nguyen 2007). Subsequent fEPSPs were elicited at the rate of once per minute at this “test” stimulation intensity, with S1 stimulation preceding S2 stimulation by 200 msec. Our first series of experiments were conducted using a standard “synaptic tagging and capture” protocol (Frey and Morris 1997) in which repeated high-frequency stimulation (HFS) was induced by applying four trains (4 × 100-Hz, 1-sec duration at test strength, 3-sec interval) to S1. After 30 min, one train of HFS (1 × 100-Hz, 1-sec duration) was applied to the second set of synaptic inputs (S2) (Woo and Nguyen 2003; Young et al. 2006). β-AR-dependent LTP was induced by applying one train of HFS (1000-Hz, 3-sec duration at test strength) following a 10-min application of the β-AR agonist, isoproterenol (ISO; 1 μM) (Gelinas and Nguyen 2005). Post-HFS, ISO was applied for an additional 5 min. Thirty minutes after HFS at S1, low-frequency stimulation (LFS: 5-Hz, 10-sec duration) was applied to S2. All experiments were performed blind to genotype.

Drugs

The β-AR agonist, R (−)-isoproterenol (±)-bitartrate (ISO; Sigma) was prepared daily as concentrated stock solutions at 1 mM in distilled water. The β-AR antagonist (±)-propranolol hydrochloride (PROP; 50 μM; Sigma) was also prepared daily in distilled water as a 50-mM stock solution. The GABA-A receptor antagonist, bicuculline (BICU; 10 μM; Sigma) was prepared as a 1-mM stock solution and was used to assay for altered GABAergic inhibition. The mGluR5 antagonist, 6-methyl-2-((phenylethynyl) pyridine (MPEP, 10 μM; Sigma) was prepared as a 10-mM stock solution and perfused for 20 min prior to application of ISO. A translation inhibitor, emetine (EME; Sigma), was dissolved to a stock concentration of 20 mM in distilled water and perfused at 20 μM, 20 min prior to ISO application. At lower concentrations than 20 μM, EME blocked protein synthesis by >80% in hippocampal slices (Stanton and Savry 1984). A MEK inhibitor, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD 98059) (50 μM; Sigma), was prepared in DMSO at a concentration of 10 mM. An mTOR inhibitor, rapamycin (Rap; 1 μM; Sigma), was dissolved in DMSO to make stock solutions at 1 mM. PD98059 and Rap were perfused for 30 min prior to commencing experiments, overlapping with ISO application, and 10 min following ISO. All drug experiments were performed under dimmed light conditions due to photosensitivity of the drugs. Drug experiments were interleaved with drug-free controls.

Data analysis

The initial slope of the fEPSP was measured as an index of synaptic strength (Johnston and Wu 1995). The average “baseline” slope values were acquired for 20 min before experimental protocols were applied. fEPSP slopes were measured at either 120 or 90 min after LFS or HFS for comparisons of LTP. Student’s t test was used for statistical comparisons of mean fEPSP slopes between two groups, with a significance level of P < 0.05 (denoted with an asterisk on the graphs). One-way ANOVA and Tukey−Kramer post hoc tests were done for comparison of more than two groups to determine which groups were significantly different from the others. The Welch correction was applied in cases in which the SDs of compared groups was significantly different. All values shown are mean ± SEM, with n = number of slices.

Acknowledgments

Special thanks to Sabyasachi Maity for his technical assistance. This research was supported by grants from the Canadian Institutes of Health Research (to P.V.N.), the National Institutes of Health, and the FRAXA Research Foundation (to E.K.). Salary support was received from a graduate scholarship (to S.A.C.) from the Natural Sciences and Engineering Research Council of Canada and a Scientist Award (to P.V.N.) from the Alberta Heritage Foundation for Medical Research.

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Cold Spring Harbor Laboratory Press on July 20, 2018 - Published by learnmem.cshlp.org Downloaded from learnmem.cshlp.org on July 20, 2018 - Published by Cold Spring Harbor Laboratory Press
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Received October 18, 2010; accepted in revised form December 28, 2010.
Fragile X mental retardation protein regulates heterosynaptic plasticity in the hippocampus

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Learn. Mem. 2011, 18:
Access the most recent version at doi:10.1101/lm.2043811

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