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Abnormal Presynaptic Short-Term Plasticity and Information Processing in a Mouse Model of Fragile X Syndrome

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Fragile X syndrome (FXS) is the most common inherited form of intellectual disability and the leading genetic cause of autism. It is associated with the lack of fragile X mental retardation protein (FMRP), a regulator of protein synthesis in axons and dendrites. Studies on FXS have extensively focused on the postsynaptic changes underlying dysfunctions in long-term plasticity. In contrast, the presynaptic mechanisms of FXS have garnered relatively little attention and are poorly understood. Activity-dependent presynaptic processes give rise to several forms of short-term plasticity (STP), which is believed to control some of essential neural functions, including information processing, working memory, and decision making. The extent of STP defects and their contributions to the pathophysiology of FXS remain essentially unknown, however. Here we report marked presynaptic abnormalities at excitatory hippocampal synapses in *Fmr1* knock-out (KO) mice leading to defects in STP and information processing. Loss of FMRP led to enhanced responses to high-frequency stimulation. *Fmr1* KO mice also exhibited abnormal synaptic processing of natural stimulus trains, specifically excessive enhancement during the high-frequency spike discharges associated with hippocampal place fields. Analysis of individual STP components revealed strongly increased augmentation and reduced short-term depression attributable to loss of FMRP. These changes were associated with exaggerated calcium influx in presynaptic neurons during high-frequency stimulation, enhanced synaptic vesicle recycling, and enlarged readily-releasable and reserved vesicle pools. These data suggest that loss of FMRP causes abnormal STP and information processing, which may represent a novel mechanism contributing to cognitive impairments in FXS.

Introduction

Fragile X syndrome (FXS) is recognized as the most common inherited form of intellectual disability often associated with autism. FXS is caused by the lack of fragile X mental retardation protein (FMRP) attributable to loss-of-function mutations in the *Fmr1* gene (Bassell and Warren, 2008; Pfeiffer and Huber, 2009). Despite compelling evidence for both presynaptic and postsynaptic effects of FMRP loss (Brown et al., 2001; Miyashiro et al., 2003; Liao et al., 2008; Christie et al., 2009), research on FXS has traditionally focused on dendritic abnormalities leading to defects in long-term forms of synaptic plasticity (Huber et al., 2002), as summarized in the “mGluR theory” of FXS (Bear et al., 2004). This postsynaptic theory of FXS does not, however, seem

to fully account for the marked structural and functional abnormalities observed at the presynaptic neuromuscular terminals in a *Drosophila* model of FXS (Zhang et al., 2001; Gatto and Broadie, 2008) or for the changes in synaptic strength and intrinsic excitability in excitatory cortical neurons of *Fmr1* knock-out (KO) mice (Gibson et al., 2008). Moreover, a recent study of mosaic *Fmr1* KO mice (Hanson and Madison, 2007) revealed that the reduced connectivity observed in the hippocampal circuit have a cell-autonomous presynaptic origin rather than a secondary homeostatic or trans-synaptic one. Indeed, immunoelectron microscopy localized FMRP in presynaptic terminals and axons in many brain circuits (Christie et al., 2009), and FMRP has been shown to directly interact with and modulate activity of a mostly presynaptic sodium-activated potassium channel, Slack (Brown et al., 2010). These findings suggest that regulatory functions of FMRP are not limited to controlling protein translation and indicate that at least some FXS abnormalities arise from the requirement for FMRP in presynaptic function. However, how absence of FMRP affects basic presynaptic processes, including neurotransmitter release and short-term plasticity (STP), is essentially unknown.

The potential link between FMRP loss and dysfunctions in STP is of particular interest because STP is believed to play important roles in information processing (Abbott and Regehr, 2004), working memory (Mongillo et al., 2008), and decision making (Deco et al., 2010). STP dysregulation may thus contrib-

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ute to the cognitive impairments of FXS. Previous studies that considered some of the STP components in *Fmr1* KO mice came to conflicting conclusions (Centonze et al., 2008; Gibson et al., 2008; Zhang et al., 2009; Olmos-Serrano et al., 2010), and no coherent picture of STP dysfunction in FXS has thus far emerged.

Here we demonstrate that loss of FMRP causes marked morphological and functional presynaptic abnormalities leading to altered STP in excitatory hippocampal synapses. *Fmr1* KO mice exhibited greatly increased augmentation, presumably as a result of excessive calcium influx observed in the presynaptic neurons during spike trains, whereas faster vesicle recycling and enlarged vesicle pools led to reduced short-term depression. These presynaptic changes resulted in abnormal processing of natural stimulus trains, specifically excessive enhancement during high-frequency spike bursts associated with hippocampal place fields. These data suggest that FXS has a significant presynaptic component associated with abnormal STP and information processing that may contribute to the pathophysiology of FXS.

Materials and Methods

Animals and slice preparation. *Fmr1* KO (FVB.129P2-*Fmr1*^{tm1Cgr/J}); stock #4624) and control [FVB.129P2-*Pde6b*⁺*Tyr*^{cre-*cl*/Ant}]; stock #4828, hereafter referred to as wild-type (WT)] strain mice were obtained from The Jackson Laboratory. Both male and female 15- to 25-d-old mice were used. Genotyping was performed according to The Jackson Laboratory protocols. Both littermate- and age-matched controls were used and found to be indistinguishable; data for controls were thus pooled. After being deeply anesthetized with CO₂, mice were decapitated and their brains were dissected out in ice-cold saline solution that contained the following (in mM): 130 NaCl, 24 NaHCO₃, 3.5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂, 5.0 MgCl₂, and 10 glucose, pH 7.4 (saturated with 95% O₂ and 5% CO₂). Horizontal brain slices (350 μm) including the hippocampus were cut using a vibrating microtome (Leica VT1200S). Slices were initially incubated in the above solution at 35°C for 1 h for recovery and then kept at room temperature (~23°C) until use. All animal procedures conformed to the guidelines approved by the Washington University Animal Studies Committee.

For single-synapse imaging experiments, primary cultures of hippocampal neurons were created as described previously (Kaeck and Banker, 2006). Briefly, hippocampi were surgically isolated from the P0–P1 pups, dentate gyrus was removed, and tissue was dissociated after a brief papain treatment. Neurons were plated on astrocyte feeder layers in the Neurobasal media supplemented with B27 and held in culture for 3 weeks.

Electrophysiology. Whole-cell patch-clamp recordings using an Axopatch 200B amplifier (Molecular Devices) in current- or voltage-clamp mode were made from CA1 or CA3 pyramidal neurons visually identified with infrared video microscopy (Olympus BX50WI; Dage-MTI) and differential interference contrast optics. All the recordings were conducted at near-physiological temperature (33–34°C). The recording electrodes were filled with the following (in mM): 130 K-gluconate, 0.5 EGTA, 2 MgCl₂, 5 NaCl, 2 ATP₂Na, 0.4 GTPNa, and 10 HEPES, pH 7.3. The extracellular solution contained the following (in mM): 130 NaCl, 24 NaHCO₃, 3.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, and 10 glucose, pH 7.4 (saturated with 95% O₂ and 5% CO₂). In all experiments, NMDA receptors were blocked with AP-5 (50 μM) to prevent long-term effects. For some experiments, Ca²⁺ currents were recorded with intracellular solution containing the following: 100 mM Cs-gluconate, 5 mM NaCl, 2 mM MgCl₂, 20 mM tetraethylammonium (TEA)-Cl, 0.5 mM EGTA, 10 mM HEPES, 20 mM phosphocreatine, 50 U/ml phosphocreatine kinase, 2 mM MgATP, 0.1 mM NaGTP, and 0.1 mM leupeptin, pH 7.3. In these experiments, the extracellular solution was supplemented with 20 mM TEA-Cl and 3 mM 4-AP (replacing equivalent amounts of NaCl) and 1 μM TTX. EPSCs were recorded from CA1 pyramidal neurons (held at –65 mV) by stimulating Schaffer collaterals with a bipolar electrode. For action potential (AP)-clamp recordings in CA3 pyramidal neurons, APs were first evoked by current injections in a desired temporal pattern

(with the resting potential set at –60 mV to avoid spontaneous AP firing); these AP waveforms were then used as voltage command patterns in the same neurons. For AP-clamp experiments, cell capacitance was compensated. Series resistance compensation was enabled with ~90% correction and 10–20 μs lag.

Data were filtered at 2 kHz, digitized at 20 kHz, acquired using custom software written in LabView, and analyzed using programs written in MATLAB or MiniAnalysis. EPSCs during the stimulus trains were normalized to an average of five low-frequency (0.2 Hz) control stimuli preceding each train, to give relative changes in synaptic strength. Each stimulus train was presented four to six times in each cell, and each presentation was separated by ~2 min of low-frequency (0.2–0.1 Hz) control stimuli to allow complete EPSC recovery to the baseline. To correct for the overlap of EPSCs at short interspike intervals (ISIs), a normalized template of EPSC waveform was created for each stimulus presentation by averaging all EPSCs within a given train that were separated by at least 100 ms from their neighbors and normalized to their peak values. Every EPSC in the train then was approximated by a template waveform scaled to the peak of the current EPSC, and its contribution to synaptic response was digitally subtracted. The natural stimulus trains used in this study represent the firing patterns recorded *in vivo* from the hippocampal place cells of awake, freely moving rats [generously provided by Fenton and Muller (1998)]. Spikes with the ISIs <10 ms were treated as a single stimulus, because the delay between the action potential firing and the peak of postsynaptic currents/potentials prevented resolution of individual synaptic responses at shorter ISIs. Such treatment does not significantly affect synaptic responses to natural stimulus trains as we have shown previously (Klyachko and Stevens, 2006b). Note that, in our experimental conditions, receptor desensitization and saturation are insignificant and voltage-clamp errors are also small and do not provide a significant source of nonlinearity (Wesseling and Lo, 2002; Klyachko and Stevens, 2006a). Therefore, postsynaptic responses can be used as a linear readout of transmitter release in the relevant frequency range.

Transmission electron microscopy. Four- to five-week-old mice were used for transmission electron microscopy analysis. Mice were anesthetized with sodium pentobarbital and perfused with PBS, pH 7.4, followed by 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed, and horizontal sections were cut at 500 μm with a vibratome in 0.1 M PBS. The hippocampal CA1 region was dissected from appropriate sections, postfixed for 1 h with 1% osmium tetroxide in 100 mM cacodylate buffer, pH 7.4, dehydrated using a series of ethanol dilutions, rinsed in propylene oxide, and embedded in Epon 812. Ultrathin sections (60–80 nm) were cut and stained with uranyl acetate and lead citrate. Complete profiles of nonperforated asymmetric synapses on dendritic spines in the stratum radiatum of CA1 were selected and photographed using a digital camera in a Hitachi 7500 electron microscope operated at 80 kV at a final magnification of 20,000 or 80,000. A total area of 5350 μm² from WT and 5671 μm² from KO mice were counted for quantification of synapse density. A total of 132 asymmetric synapses from three WT and 148 synapses from four KO mice were analyzed. The counts of small synaptic vesicles were separated into two groups defined according to criteria developed by Dickinson-Nelson and Reese (1983) as “docked” vesicles (located within 50 nm of the presynaptic active zone) and “reserved” vesicles (located 50–550 nm from the presynaptic active zone). Vesicle counts were normalized to the length of the active zone as determined by the length of postsynaptic density (Pozzo-Miller et al., 1999; Bamji et al., 2003).

Fluorescence imaging. Day 14–18 cultured hippocampal neurons were mounted on an inverted Olympus microscope (Olympus IX70) equipped with 60×, 1.4 NA oil-immersion objective. Fluorescence was excited with a xenon lamp via a filter cube containing 480/40 nm excitation filter, 515 nm dichroic mirror, and 575/60 nm emission filter. Fluorescent signal was captured using a cooled EM CCD Hamamatsu camera and Simple PCI software (Hamamatsu). Neurons were stimulated using a pair of electrodes positioned ~1 cm apart in the bath and controlled by the computer software via Master-8 stimulus generator (A.M.P.I.). Only one cycle of dye staining and destaining was performed for each coverslip. Each episode of activity-evoked dye destaining was

preceded by a set of 10 control measurements at 0.5 Hz in the absence of stimulation. All recordings were performed at 37°C using a whole-microscope incubator equipped with a rapid feedback temperature controller (In Vivo Scientific). Image analysis was performed in NIH ImageJ (Wayne S. Rasband, NIH, Bethesda, MD; <http://rsb.info.nih.gov/ij>) and MATLAB. The background in recorded images was subtracted using the rolling-ball algorithm (Sternberg, 1983). The rigid-body transformation was used to align the images (Thévenaz et al., 1998), and Kalman filter was used to improve ROI selection. Dye bleaching was determined and compensated for based on the monoexponential fits to the fluorescence decay in the dendritic regions not containing ROIs. Circular ROIs of the same diameter covering the entire synapse were drawn manually in the first frame of each movie and then propagated through the entire image stack using Time Series Analyzer ImageJ plug-in (J. Balaji, Cornell University, Ithaca, NY). The activity-evoked dye destaining was determined as the difference between the ROI fluorescence intensity in a given frame and the intensity of the ROI in the last frame, normalized to the control fluorescence intensity, which was calculated for each synapse as an average ROI intensity in the first 10 frames of each movie before the stimulation onset. The stimulus protocol of 1000 AP at 10 Hz was chosen because it has been shown previously to release all recycling vesicles. Based on previous estimates (Ryan and Smith, 1995), ROIs in which the overall decrease in fluorescence intensity was below 25% were unlikely to represent active synapses, and these ROIs were excluded from analysis. The kinetics of vesicle turnover was assessed as described previously (Ryan and Smith, 1995) based on the dye destaining time constant determined from the monoexponential fits in MATLAB.

Statistics. Data are presented as mean \pm SEM. Student's paired or unpaired *t* test or ANOVA were used for statistical analysis as appropriate; *p* values are reported throughout the text, and significance was set as *p* < 0.05.

Results

Altered STP in excitatory hippocampal synapses of *Fmr1* KO mice

The major changes in the levels of many presynaptic proteins involved in excitability, neurotransmitter release, vesicle recycling, and calcium homeostasis that are observed in *Fmr1* KO mice (Liao et al., 2008; Akins et al., 2009) suggest that various forms of STP may be affected by FMRP loss. To examine the effects of FMRP loss on STP and synaptic information processing, we used CA3–CA1 hippocampal synapses as a model system. The hippocampal circuit is a well-characterized model system to study synaptic and circuit mechanisms and for these reasons is widely used in FXS research (Braun and Segal, 2000; Huber et al., 2002; Hanson and Madison, 2007; Pfeiffer and Huber, 2007; Zhang et al., 2009; Zhang and Alger, 2010; Gross et al., 2011). From the circuit perspective, because CA3 pyramidal cells (PCs) are presynaptic to CA1 pyramidal cells, to simplify the description of changes at the CA3–CA1 synapse, below we will refer to the processes that occur in CA3 neurons as “presynaptic.”

We first assessed overall changes in STP attributable to loss of FMRP by comparing synaptic dynamics during high-frequency stimulus trains in *Fmr1* KO mice and WT controls. EPSCs were recorded from hippocampal CA1 PCs in response to the constant-frequency stimulation of Schaffer collaterals at 2–100 Hz (Fig. 1). Whole-cell patch-clamp recordings were performed at near-physiological temperatures (33–34°C) (Klyachko and Stevens, 2006a) and with intact inhibitory circuits. EPSCs during the stimulus train were normalized to an average of five low-frequency controls preceding each train, thus representing relative changes in synaptic strength (also referred to as synaptic gain). We found that synaptic dynamics was markedly altered in the *Fmr1* KO mice that exhibited excessive synaptic enhancement particularly at frequencies of and above 20 Hz (Fig. 1*B–D*). The peak gain reached 4.98 ± 0.37 -fold increase at 100 Hz in *Fmr1*

KO mice versus 3.21 ± 0.40 -fold increase in controls (*p* < 0.001, *t* test). The average gain during the trains was also significantly larger in KO than WT mice for stimulus trains at and above 20 Hz (Fig. 1*D*). These results demonstrate that FMRP loss enhances synaptic response to high-frequency stimulation.

Abnormal processing of natural stimulus patterns in *Fmr1* KO mice

The marked changes in STP we observed during constant-frequency stimulation in *Fmr1* KO mice may have a significant impact on the synaptic information processing and may thus contribute to the cognitive impairment in FXS. To examine this possibility, we recorded EPSCs from CA1 PCs evoked by the natural spike pattern recorded *in vivo* from hippocampal place cells in exploring rodents (provided by Drs. A. Fenton and R. Muller, State University of New York, Brooklyn, NY) (Fenton and Muller, 1998). Place cells are known to encode information about the animal's position via high-frequency spike discharges at certain places in the environment, known as place fields (O'Keefe and Dostrovsky, 1971). Here, we used a long segment of such natural spike pattern, consisting of 256 spikes with a mean burst frequency of 26.0 ± 1.8 Hz and duration of 12.9 ± 2.2 spikes (Fig. 2*A*, top trace).

Our previous studies have shown that STP in excitatory CA3–CA1 synapses acts as a high-pass filter that selectively amplifies synaptic strength during high-frequency spike bursts associated with place fields (Klyachko and Stevens, 2006a,b). We observed the same response pattern both in WT and *Fmr1* KO mice (Fig. 2*A*), in which excitatory synaptic strength alternates between a basal near-constant value during low-frequency stimulation and another elevated near-constant value during the discharges. As during constant-frequency stimulation, however, the responses to the natural stimulus trains were markedly enhanced in the *Fmr1* KO neurons selectively during the spike discharges (Fig. 2*A,B*) (the average gain during the discharge in WT was 2.13 ± 0.06 , *n* = 11; in KO 2.60 ± 0.10 , *n* = 13; *p* < 0.001). This excessive enhancement in the *Fmr1* KO mice is more apparent when synaptic gain is plotted as a function of instantaneous stimulus frequency, i.e., the reciprocal of the interspike interval (Fig. 2*C*). These data were fitted with the sigmoidal Boltzmann equation, giving a significantly larger elevated gain value during the discharges in the KO mice (WT, 2.55 ± 0.02 ; KO, 3.29 ± 0.04 ; *p* < 0.01). The transition frequencies between the gain levels were not significantly different (WT, 7.30 ± 0.30 Hz; KO, 7.65 ± 0.37 Hz; *p* > 0.5) and are similar to previously reported ones (Klyachko and Stevens, 2006b). The frequency dependence of excessive synaptic gain in *Fmr1* KO mice during natural stimulus trains (Fig. 2*E*) was similar to that during constant-frequency stimulation (Fig. 1*D*). Together, these results indicate that excitatory hippocampal synapses in *Fmr1* KO mice exhibit hypersensitive responses to natural stimulus trains and specifically to high-frequency spike bursts associated with hippocampal place fields.

Because we recorded EPSCs in the presence of intact inhibitory circuits in the above experiments, the enhanced responses could result from possible alterations of GABAergic transmission in *Fmr1* KO mice (Centonze et al., 2008; Olmos-Serrano et al., 2010). To investigate this possibility, we performed the same recordings as those described above using a constant 60 Hz stimulus train (Fig. 3*A*) or a natural spike pattern (Fig. 3*B–F*) in the presence of the GABA_A receptor (GABA_AR) blocker gabazine (5 μ M) and the GABA_BR blocker CGP55845 [(2*S*)-3-[(1*S*)-1-(3,4-dichlorophenyl)ethyl]-amino-2-hydroxypropyl](phenylmethyl)phosphinic acid] (2 μ M). The excessive increase in synaptic strength observed in *Fmr1* KO

mice remained unaltered when inhibitory transmission was blocked (average gain during the discharge: WT, 1.96 ± 0.03 , $n = 7$; KO, 2.44 ± 0.05 , $n = 11$; $p < 0.001$, t test), indicating that the observed enhancement of excitatory responses is not attributable to alterations of GABAergic transmission in the KO mice. These data confirm that loss of FMRP causes abnormal STP in excitatory hippocampal synapses during both constant frequency and natural stimulus trains.

Enhanced augmentation, but not facilitation, in excitatory synapses of *Fmr1* KO mice

The rapid synaptic dynamics during stimulus trains has been shown to result from the interplay of several presynaptic forms of STP (Zucker and Regehr, 2002). The excessive increase in synaptic strength during constant-frequency and natural stimulus trains we observed in *Fmr1* KO mice can arise from the increase in short-term facilitation/augmentation, decrease in short-term depression, or a combination of both. To distinguish these possibilities, we used our previously developed approach to separate contributions of different STP components to synaptic dynamics (Klyachko and Stevens, 2006a) and to assess changes in individual components attributable to FMRP loss. Paired-pulse protocol was used first to examine changes in facilitation. Although we found a tendency toward increased paired-pulse ratio (PPR) at short interspike intervals (7–40 ms) in *Fmr1* KO mice, the overall differences in the paired-pulse facilitation were not significant between WT and KO mice (Fig. 4A, B), consistent with the previous report in the hippocampus (Zhang et al., 2009).

Augmentation represents a longer-lasting component of short-term enhancement than facilitation (Zucker and Regehr, 2002). It operates on a timescale of several seconds and has been shown to serve as a major component of STP that sustains synaptic strength during high-frequency spike discharges (Kalkstein and Magleby, 2004; Kandaswamy et al., 2010). We examined contribution of augmentation using our previously developed protocol (Klyachko and Stevens, 2006a) and found a marked increase in augmentation amplitude in *Fmr1* KO mice, particularly at frequencies of 20 Hz and above (Fig. 5A–D) (extrapolated augmentation for 80 Hz stimulus train: WT, 2.70 ± 0.2 ; KO, 4.63 ± 0.46 ; $n = 8$ for each; $p < 0.01$) (Fig. 5B, C), without significant changes in the decay time constant (WT, 6.08 ± 0.83 s; KO, 4.76 ± 0.47 s; $n = 8$ for each; $p = 0.32$) (Fig. 5C). The frequency-dependent increase in augmentation in *Fmr1* KO mice (Fig. 5D) closely paralleled that of overall synaptic enhancement during high-frequency trains (Figs. 1D, 2C), suggesting that changes in augmentation may be one of the main contributors to abnormal synaptic dynamics in *Fmr1* KO mice.

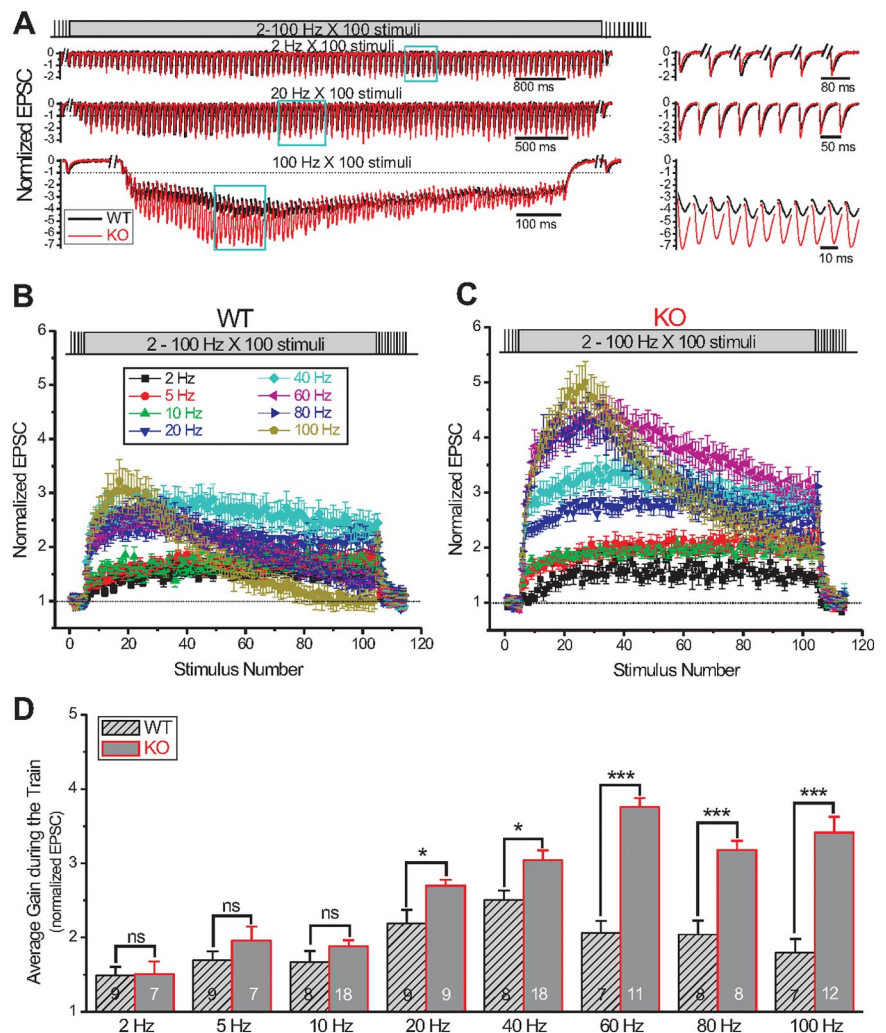


Figure 1. Altered STP in excitatory hippocampal synapses of *Fmr1* KO mice. **A**, EPSCs were recorded from CA1 pyramidal neurons in response to 100-stimulus trains at different frequencies. Examples of synaptic responses during the 100 stimuli at 2, 20, and 100 Hz are shown on the left, and short epochs of box-highlighted traces are shown at increased timescales on the right for WT (shown in black here and in subsequent figures) and *Fmr1* KO mice (shown in red here and in subsequent figures). Traces shown are an average of four to six train presentations in the same neuron (here and in subsequent figures). Stimulation artifacts were digitally removed for presentation. **B**, **C**, Average synaptic responses to high-frequency stimulation at 2–100 Hz in WT (**B**) and *Fmr1* KO (**C**) mice, recorded as described in **A**. $n = 7$ –9 for WT and $n = 7$ –18 for KO. **D**, Average synaptic gain during stimulus trains for the data in **B** and **C**. The numbers of neurons tested are shown for each frequency. * $p < 0.05$, *** $p < 0.001$; ns, not significantly different (here and in subsequent figures).

Increased calcium influx during high-frequency stimulation in *Fmr1* KO mice

What are the mechanisms of these changes? Augmentation is commonly believed to arise from elevation in residual presynaptic calcium levels during repetitive activity (Zucker and Regehr, 2002). Presynaptic calcium levels are controlled by several mechanisms that include calcium influx, release and uptake from internal stores, and calcium buffering/clearance processes. The absence of significant changes in facilitation/augmentation decay kinetics suggests that calcium clearance is not likely to be significantly affected by the loss of FMRP (Zucker and Regehr, 2002), whereas release/uptake by internal stores has been shown not to play a significant role in STP in the CA3–CA1 synapse on the timescales relevant to the current study (Carter et al., 2002). Thus, augmentation may be enhanced in the absence of FMRP if presynaptic calcium influx is increased to a larger extent during high-frequency stimulation in *Fmr1* KO mice than in WT mice.

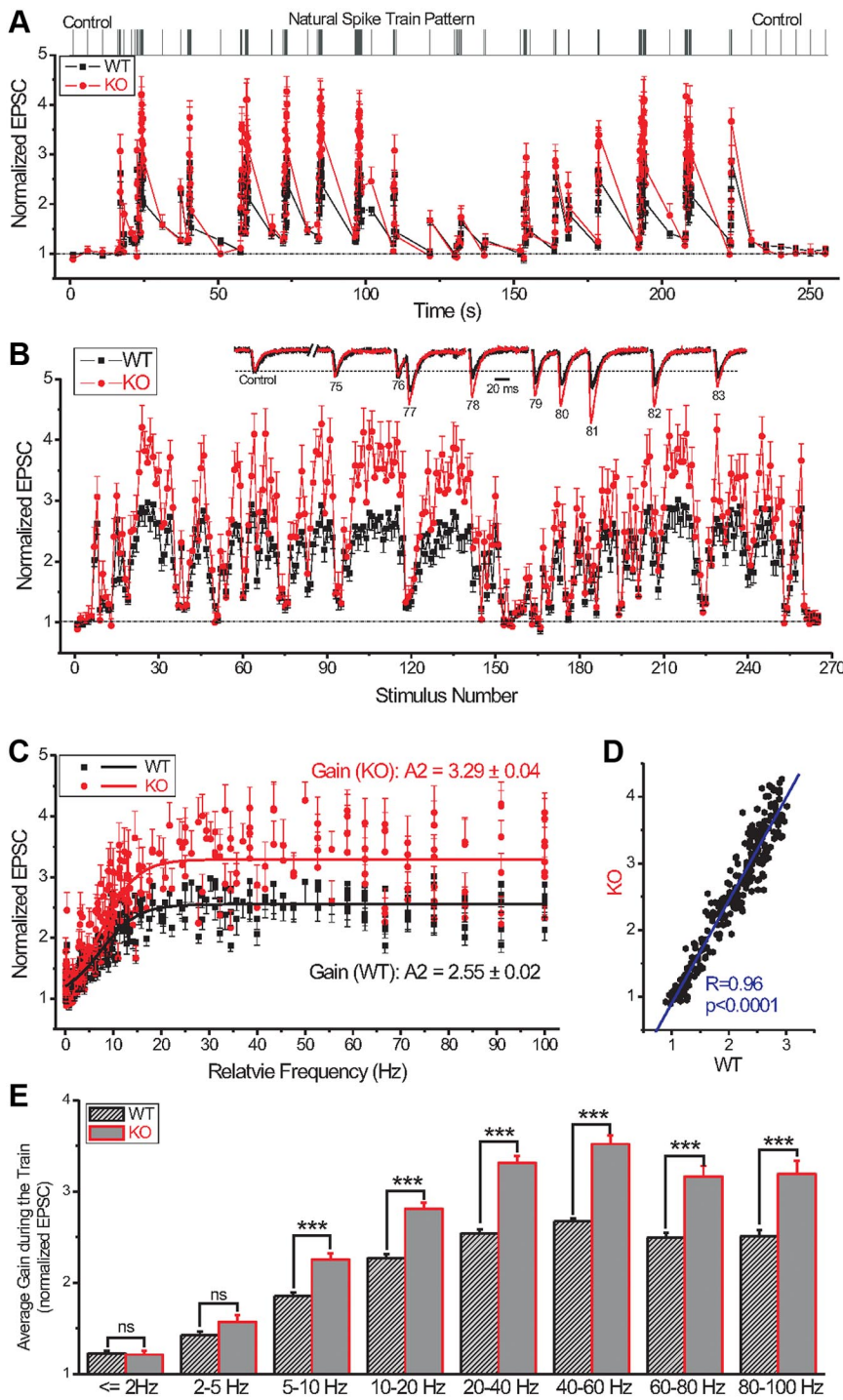


Figure 2. Abnormal processing of natural stimulus patterns in *Fmr1* KO mice. **A**, Changes in synaptic strength during natural stimulus trains plotted as a function of time for WT and *Fmr1* KO mice. Note the excessive increases in synaptic strength during bursts in the *Fmr1* KO mice. The top shows the natural stimulus pattern used (Fenton and Muller, 1998), preceded by four and followed by six control stimuli at 0.2 Hz. $n = 11$ and 13 for WT and *Fmr1* KO, respectively. **B**, Same as in **A** but plotted as a function of stimulus number. Inset shows EPSCs 75–83 during the natural stimulus trains, scaled to their own controls for comparison. **C**, Synaptic strength during natural stimulus trains (data from **A**) plotted as a function of instantaneous stimulus frequency, i.e., reciprocal of interspike intervals. Solid lines represent fits to a Boltzmann equation: $y = (A1 - A2)/(1 + \exp((x - x_0)/dx)) + A2$, where $A1$ is the basal gain level, $A2$ is the elevated gain level, and x_0 is the transition frequency. **D**, Normalized EPSC amplitudes during the natural spike trains in WT and KO animals are plotted point by point against each other; the correlation coefficient is determined by linear regression (blue line). Correlation analysis shows that FMR1 loss alters the amplitude but not the pattern of changes in synaptic strength during natural stimulus trains. **E**, Average synaptic gain during natural stimulus trains plotted as a function of frequency bands. The frequency dependence of abnormal synaptic enhancement in *Fmr1* KO is similar to that seen in Figure 1D during constant-frequency trains. *** $p < 0.001$.

To test this hypothesis, we used an AP-clamp approach to measure calcium influx during high-frequency spike trains in presynaptic CA3 neurons. In AP clamp, the voltage-gated calcium channels (VGCCs) experience the same trajectory of voltage changes as during the AP trains, and thus calcium influx occurs with the time course and amplitude that closely approximate what occurs during high-frequency neuronal firing (Bean, 2007). N-type and P/Q-type calcium channels, the two predominant VGCCs in presynaptic terminals of CA3 neurons (Wheeler et al., 1994; Wu and Saggau, 1994; Elliott et al., 1995), jointly carry the bulk of presynaptic calcium transients that trigger neurotransmitter release (Dunlap et al., 1995). Previous studies revealed highly differential spatial distribution of different types of VGCCs in CA3 pyramidal cells and other central neurons: N-type and P/Q-type channels were found to be localized predominantly at the nerve terminals with substantially lower density at the soma, whereas the L-type channels are expressed almost exclusively in the soma and proximal dendrites but not at the synaptic terminals (Westenbroek et al., 1992, 1995; Elliott et al., 1995). Because of this highly specialized spatial distribution of VGCCs and inaccessibility of presynaptic terminals to patch-clamp recording in most central neurons, somatic recordings in combination with pharmacology have been widely used to infer about the properties of presynaptic calcium currents (Luebke et al., 1993; Wheeler et al., 1994; Dunlap et al., 1995; Evans and Zamponi, 2006).

We thus recorded APs evoked in CA3 PCs by a 60 Hz train through current injections in the whole-cell recording configuration and then used these AP trains as voltage commands for the same (corresponding genotype) neurons. The total calcium current was isolated first using the calcium channel blocker Cd^{2+} in the presence of TEA (20 mM), 4-AP (3 mM), and TTX (1 μM) (Fig. 6A). The peak of total calcium current (I_{Ca}) had a transient increase at the very beginning of the train that was similar in both groups (WT, 1.53 ± 0.24 nA; KO, 1.59 ± 0.08 nA; $p > 0.05$; $n = 6$ for each) (data not shown), followed by a decline in amplitude during the train. This decline, however, was significantly smaller in the KO mice (average I_{Ca} for the last 10 stimuli in the train: WT, $74.2 \pm 0.5\%$ of control; KO, $82.7 \pm 0.6\%$ of control; $p < 0.001$; $n = 6$ for each group) (data not shown). Most importantly, the calcium charge transfer (i.e., calcium influx), calculated as a time inte-

gral of the calcium transient, was markedly larger in the KO mice relative to WT mice during the train (Fig. 6B) (WT, $74.5 \pm 0.9\%$ of control; KO, $107 \pm 1.2\%$ of control, measured as the average for the last 10 spikes of the train; $p < 0.001$; $n = 6$ for each group), without significant difference for the first two APs. As a result, the cumulative calcium influx was significantly larger in the *Fmr1* KO mice during the trains, except for the first few stimuli (Fig. 6C).

To more specifically examine changes in calcium influx through N- and P/Q-type VGCCs, we performed the above experiments with the L-type calcium channel blocker nimodipine ($10 \mu\text{M}$) added to the external solution. In addition, the above AP-clamp experiments were performed using K^+ -based pipette solution to first record AP trains from CA3 pyramidal neurons and then use those AP trains as voltage commands applied to the same neurons. The presence of high K^+ concentration in the pipette might influence the accuracy of measuring calcium currents. We thus measured calcium currents from CA3 pyramidal cells using the previously recorded AP trains as voltage commands and a Cs^+ -based pipette solution containing TEA (20 mM), as well as the external solution containing nimodipine ($10 \mu\text{M}$), TEA (20 mM), 4-AP (3 mM), and TTX ($1 \mu\text{M}$). Under these conditions, N- and P/Q-type currents represent $\sim 50\%$ of the total calcium current, similar to previous reports (Elliott et al., 1995). With the L-type currents blocked, we also observed significantly larger Ca^{2+} charge transfer during high-frequency trains in *Fmr1* KO mice relative to WT controls (Fig. 6D,E) (WT, $61.0 \pm 1.3\%$ of control, $n = 8$; KO, $85.3 \pm 2.1\%$ of control, $n = 11$; measured as the average for the last 10 spikes of the train; $p < 0.001$). These results show that hippocampal CA3 neurons in *Fmr1* KO mice exhibit enhanced calcium influx through N-type and P/Q-type VGCCs during high-frequency stimulus trains longer than a few stimuli. Because N-type and P/Q-type VGCCs mediate the bulk of synaptic transmission in CA3 neurons, if the above phenomenon is also present in presynaptic terminals, it could explain the observed increase in augmentation, but not in facilitation, measured with the paired-pulse protocol (Figs. 4, 5).

Reduced short-term depression in *Fmr1* KO mice

In addition to increased augmentation, the excessive enhancement observed in *Fmr1* KO mice during high-frequency stimulation can arise in part from reduced short-term depression. We

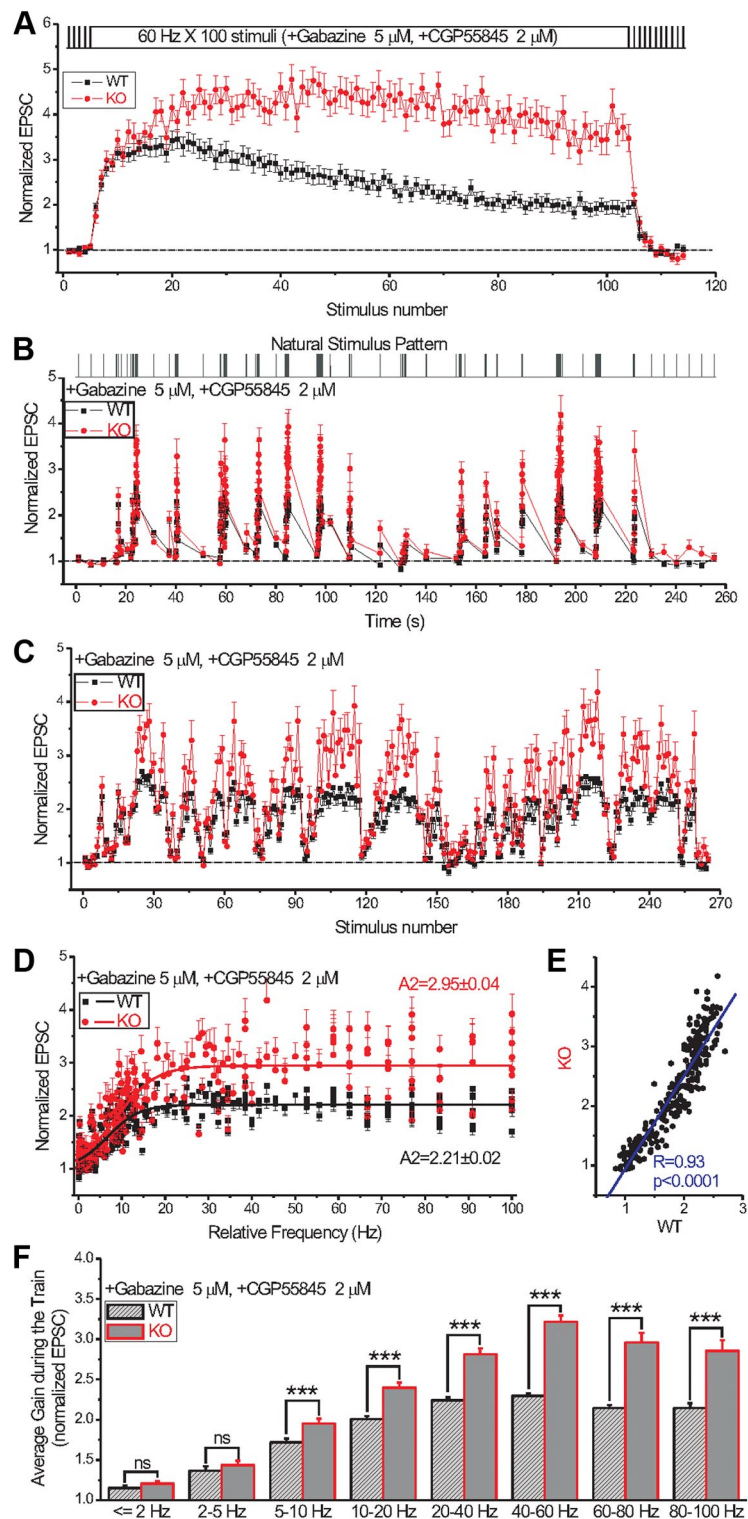


Figure 3. Enhanced excitatory responses to high-frequency stimulation in *Fmr1* KO mice are not attributed to changes in inhibitory GABAergic transmission. **A**, In the presence of GABA_A blocker gabazine and GABA_B blocker CGP55845, CA3–CA1 synapses in *Fmr1* KO mice still exhibited enhanced responses to 60 Hz train stimulation similar to that observed with inhibition intact. $n = 7$ for WT and $n = 9$ for KO. **B–F**, In the presence of GABA_A and GABA_B blockers, CA3–CA1 excitatory synapses in *Fmr1* KO mice exhibited the same abnormally enhanced responses to natural stimulus trains as when GABA blockers were absent (see Fig. 2). $n = 7$ for WT and $n = 11$ for KO. Panels are aligned as in Figure 2. *** $p < 0.001$.

thus examined the recovery from depression using single test pulses applied at different intervals after a high-frequency train (60 Hz, 150 stimuli) (Fig. 7A). Because recovery from depression overlaps with the decay of augmentation, the actual measured

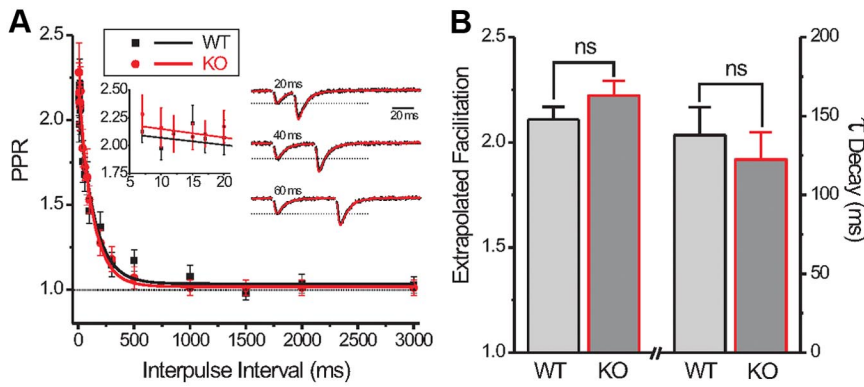


Figure 4. Facilitation is not altered in hippocampal synapses of *Fmr1* KO mice. **A, B**, PPR was measured at intervals from 7 to 3000 ms in *Fmr1* KO mice and WT controls. Monoexponential fits to the data were used to determine the amplitude of facilitation and the decay time constant; both were not significantly different between WT and KO animals. $n = 9$ for WT; $n = 15$ for KO. **A**, Left inset shows PPR values measured at very short ISIs (7–20 ms) on an expanded timescale. Inset on the right shows examples of paired-pulse traces at 20, 40, and 60 ms intervals, scaled to the first EPSC in the pair for each interval.

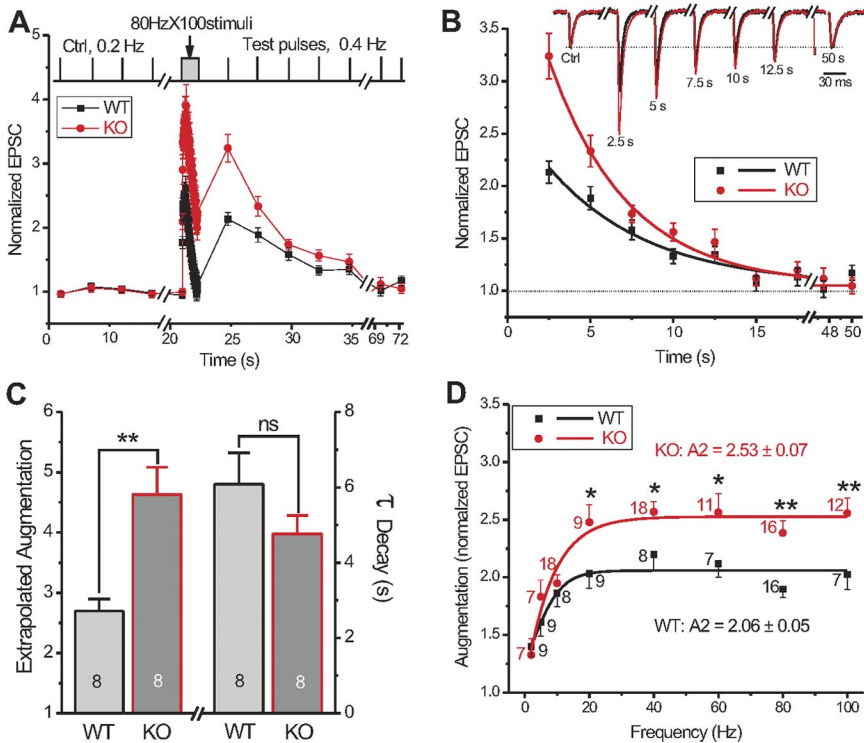


Figure 5. Augmentation is enhanced in excitatory synapses of *Fmr1* KO mice. **A–C**, Augmentation was examined using single test pulses at 2.5 s intervals after 100 stimuli at 80 Hz (**A**). Parameters of augmentation were extracted using monoexponential fits to normalized EPSC decay as described previously (Klyachko and Stevens, 2006a) (**B, C**). Inset in **B** shows EPSCs recorded in response to test stimuli at indicated time points, revealing significantly enhanced augmentation in KO mice relative to WT mice. The time course of augmentation decay was not significantly altered in KO mice ($p = 0.32$) (**C**), $n = 8$ for each. **D**, Augmentation amplitude was measured 5 s after the end of 100 stimuli at 2–100 Hz. Data were fitted by Boltzmann equation, as shown by the solid lines. Augmentation was significantly enhanced in *Fmr1* KO mice at stimulus frequencies ≥ 20 Hz. Number of neurons tested is shown for each frequency. Data at 80 Hz combines two sets of recordings using either 2.5 or 5 s test pulse intervals pooled together. * $p < 0.05$, ** $p < 0.01$.

EPSC amplitude reflects the net effect of these two components rather than depression alone. To isolate depression, we corrected synaptic responses for contributions from augmentation as we described previously (see figure legend and Klyachko and Stevens, 2006a). This analysis revealed a significant decrease in the amplitude of synaptic depression in *Fmr1* KO mice (Fig. 7*B, C*) (extrapolated depression amplitude was 0.83 ± 0.04 in WT vs

0.68 ± 0.02 in KO; $p < 0.01$), without significant changes in the recovery time constant (Fig. 7*C*). These data suggest that loss of FMRP causes abnormally potentiated synaptic responses during stimulus trains in part by reducing short-term depression.

Facilitated kinetics of vesicle turnover in *Fmr1* KO mice

What is the mechanism of reduced short-term depression in *Fmr1* KO mice? In hippocampal synapses, vesicle depletion is believed to be the dominant contributor to short-term depression (Zucker and Regehr, 2002). A number of postsynaptic processes, such as receptor desensitization and saturation, have also been shown to contribute to short-term depression in some experimental systems (Chen et al., 2002; Wong et al., 2003), but these mechanisms have been found not to play a significant role in hippocampal STP (Wesseling and Lo, 2002; Klyachko and Stevens, 2006a). Reduced vesicle depletion and synaptic depression in *Fmr1* KO may thus arise in part from changes in vesicle recycling or in the number of vesicles available for release. We examined the first possibility using an imaging approach to study the kinetics of vesicle turnover in individual synaptic terminals of cultured hippocampal neurons (Fig. 8*A*), following protocols developed previously (Ryan and Smith, 1995). Synaptic vesicles were labeled with the fluorescent lipophilic marker FM1-43 [*N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide] (10 μ M) via compensatory endocytosis using a 10 Hz, 100-stimuli train; vesicle release was subsequently evoked by 1000 APs at 10 Hz, shown previously to release all recycling vesicles (Ryan and Smith, 1995). These imaging experiments were performed at 37°C. Note that our loading protocol is expected to label the majority of recycling vesicles, based on previous estimates at room temperature ($\sim 40\%$ of total recycling pool) (Ryan and Smith, 1995) and taking into account that facilitation and particularly augmentation (and thus release probability during the stimulus train) are increased several-fold at body temperature, at which our recordings were performed (Klyachko and Stevens, 2006a). Using this approach, we found that the kinetics of

FM1-43 destaining is accelerated significantly in *Fmr1* KO relative to WT mice (Fig. 8*B*) (WT, 19.3 ± 1.4 s; KO, 12.3 ± 0.8 s; $n = 320$ WT and 455 KO synapses from 10 and 15 animals, respectively; $p < 0.01$). Because the dye unloading under these conditions has been shown previously to reflect the kinetics of vesicle turnover (Ryan and Smith, 1995), these data suggest that loss of FMRP accelerates vesicle recycling. Faster vesicle recycling in *Fmr1* KO is indeed likely to lead

to reduced vesicle depletion and short-term depression (Zucker and Regehr, 2002).

Enlarged vesicle pools in *Fmr1* KO mice

In addition to faster vesicle recycling, reduced short-term depression in *Fmr1* KO mice can also arise, in part, from changes in the size of vesicle pools (Zucker and Regehr, 2002). To measure the morphological vesicle pool size directly, we performed ultrastructural analysis of CA1 excitatory synapses of 4- to 5-week-old *Fmr1* KO and WT mice using electron microscopy (Fig. 9A). We found a significant increase in the number of both docked and undocked vesicles in EM micrographs of *Fmr1* KO mice (Fig. 9B) (docked vesicles: WT, 13.8 ± 0.4 ; KO, 15.6 ± 0.5 , $p < 0.01$; undocked vesicles: WT, 67.7 ± 3.4 ; KO, 87.0 ± 4.3 , $p < 0.001$; $n = 132$ synapses from 3 WT mice and $n = 148$ synapses from 4 KO mice). No significant differences in synaptic density and active zone length were found between the two groups (Fig. 9C). The number of docked vesicles has been shown to strongly correlate with the size of the functionally defined readily-releasable vesicle pool (RRP) (Rosenmund and Stevens, 1996; Schikorski and Stevens, 2001), whereas the number of undocked vesicles represents mostly the reserved vesicle pool. Together, these data suggest that loss of FMRP leads to an increase in the size of both the RRP and the reserved vesicle pool, which may contribute to reduced depletion in *Fmr1* KO mice.

Our morphological findings of an increased RRP size are supported by the functional estimates of the RRP size that we obtained using analysis of cumulative release during steady-state depression (Schneggenburger et al., 1999). This approach is based on the assumption that, under conditions of steady-state depression during prolonged high-frequency trains, the transmission is limited mostly by a resupply of vesicles. In this case, the linear back-extrapolation of the steady-state release levels during prolonged high-frequency activity has been shown to be proportional to the product of the RRP size (n) and the quantal size (q) (Schneggenburger et al., 1999). Our analysis of cumulative release during high-frequency trains using this approach indicates a significantly increased $n * q$ value in *Fmr1* KO mice during steady-state depression (WT, 76.8 ± 5.2 , $n = 50$; KO, 132.2 ± 6.7 , $n = 82$; $p < 0.0001$) (Fig. 9D,F). To determine whether this increase arises mostly from the changes in the RRP size or in the quantal size, we estimated the quantal size q by recording the spontaneous miniature EPSCs (mEPSCs) from CA1 pyramidal cells (Fig. 9E). Both amplitude and frequency of mEPSCs were not significantly altered in the *Fmr1* KO mice [amplitude: WT, 26.4 ± 2.0 pA; KO,

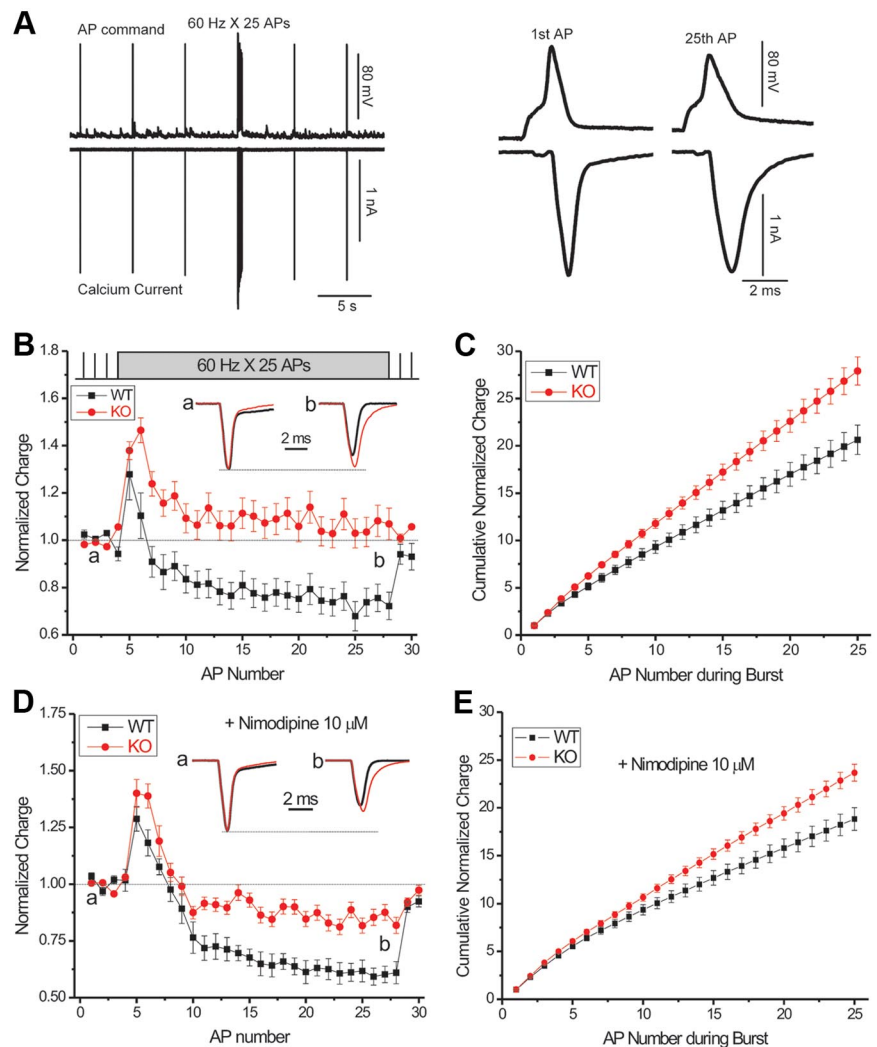


Figure 6. Calcium influx is enhanced during high-frequency stimulation in *Fmr1* KO mice. **A**, Example of an AP-clamp experiment in *Fmr1* KO CA3 pyramidal cell. A 60 Hz, 25-AP train was evoked by injecting brief positive current pulses into CA3 pyramidal cell with a resting potential of -60 mV. The recorded AP train was then used as the voltage command for the same cell to evoke calcium currents. Calcium currents were isolated using digital subtraction of traces recorded before and after Cd²⁺ application in the presence of TTX, TEA, and 4-AP. The entire train of APs (top trace) and the corresponding evoked calcium currents (bottom trace) are shown on the left; the first and 25th AP during the train and corresponding Ca²⁺ currents are shown on an expanded timescale on the right. Note the broadening of APs and Ca²⁺ current during the train. **B**, Ca²⁺ charge transfer (Ca²⁺ influx) during the AP train is plotted versus AP number in the train. Charge transfer was calculated as an integral of AP-evoked calcium transients. Insets show Ca²⁺ current traces for the denoted points, which were normalized and scaled to their own control (**a**). Calcium current broadened to a larger extent in *Fmr1* KO mice at the **b** point, leading to the greater Ca²⁺ influx in the KO neurons than that of WT. $n = 6$ each for WT and KO mice. **C**, Cumulative Ca²⁺ charge transfer plotted versus AP number during the 25-AP burst, indicating larger intracellular Ca²⁺ buildup in *Fmr1* KO mice during the AP trains after the first few stimuli. **D**, **E**, Same as (**B**, **C**) for the Ca²⁺ influx and cumulative calcium influx through non-L-type voltage-gated Ca²⁺ channels. Recordings were performed using Cs⁺-based pipette solution. Nimodipine (10 μM) was added to the bath to block L-type Ca²⁺ channels. As in **B** and **C**, greater Ca²⁺ influx was found through non-L-type voltage-gated Ca²⁺ channels during high-frequency stimulation. $n = 9$ for WT and $n = 11$ for KO animals.

24.0 ± 1.6 pA; $n = 6$ for each, $p = 0.36$ (Fig. 9F); frequency: WT, 0.65 ± 0.13 Hz; KO, 0.48 ± 0.18 Hz; $n = 6$ for each, $p = 0.42$ (data not shown)]. Therefore, the quantal size q is not significantly altered by FMRP loss at CA3–CA1 synapses, in agreement with previous studies (Braun and Segal, 2000; Pfeiffer and Huber, 2007), thus confirming our morphological findings of increased RRP size attributable to FMRP loss.

Discussion

Our results demonstrate that excitatory CA3–CA1 hippocampal synapses in *Fmr1* KO mice exhibit abnormal presynaptic STP and

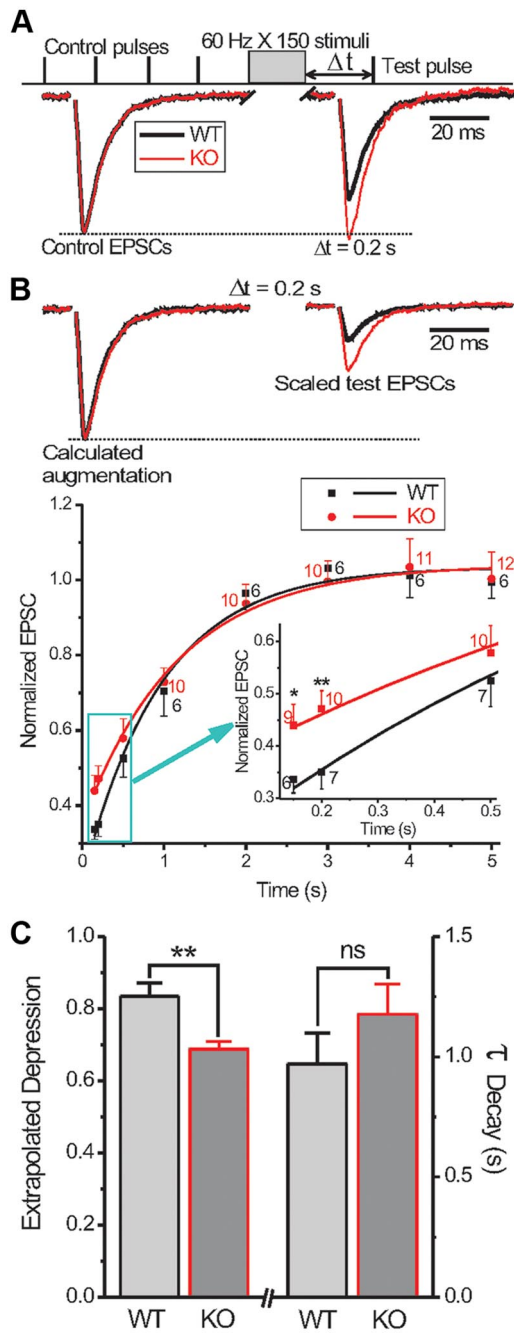


Figure 7. Short-term depression is reduced in *Fmr1* KO mice. **A**, Recovery from depression was examined using single test pulses at variable intervals (0.15–5 s) after 150 stimulus trains at 60 Hz as described previously (Klyachko and Stevens, 2006a). Traces show an example of EPSCs recorded 0.2 s after the end of the stimulus trains in WT and KO mice. EPSCs were scaled to their own controls and showed larger extent of recovery in the KO animals than in WT animals. **B**, To correct recovery from depression for overlapping decay of augmentation, the latter was also examined in the same recordings using test pulses at 5 s intervals after the depression-inducing train (i.e., 150 stimuli at 60 Hz). Augmentation parameters were determined from monoexponential fits to the data as described previously (Fig. 5) and extrapolated to the earlier time points (0.15–5 s) used to estimate depression. At each time point, the depression then was corrected by scaling the measured EPSCs by the calculated augmentation at the same time points (Klyachko and Stevens, 2006b). The top shows an example of calculated augmentation EPSCs (reconstructed from EPSC template) at 0.2 s after the depression-inducing train and the scaled test EPSCs at the same time point. For presentation only the calculated augmentation EPSCs of WT and KO mice were normalized to the same level for comparison. This analysis revealed the true extent of depression as shown in the bottom for each time point. Depression amplitude and recovery time constant were determined from monoexponential fits shown by the solid lines. The number of cells tested is shown for each time point. Inset shows

altered information processing. We observed strongly enhanced augmentation and reduced short-term depression attributable to loss of FMRP. These changes were associated with heightened Ca^{2+} influx in presynaptic CA3 neurons during high-frequency spike trains, faster synaptic vesicle recycling, and increased vesicle pools. As a result, hippocampal synapses in *Fmr1* KO mice exhibited abnormal processing of natural stimulus patterns, specifically hypersensitivity to high-frequency bursts associated with hippocampal place fields. Our results thus demonstrate major functional and morphological presynaptic defects leading to abnormal STP in the absence of FMRP. Given the important roles of STP in information processing, working memory, and decision making (Fioravante and Regehr, 2011), the observed STP dysfunction may contribute to cognitive deficits in FXS.

Abnormal STP in *Fmr1* KO mice

STP represents a rapid, use-dependent modulation of synaptic strength and comprises several dynamic processes—namely facilitation, augmentation, and depression—that have a predominantly presynaptic origin in most small central synapses (Zucker and Regehr, 2002). We found that augmentation, but not facilitation, is significantly increased in excitatory hippocampal synapses in *Fmr1* KO mice, whereas short-term depression is decreased. These STP defects in *Fmr1* KO mice have been mostly overlooked previously because the majority of studies of presynaptic function rely on so-called PPR measurements as a sole indicator of presynaptic changes. In agreement with the previous studies (Pfeiffer and Huber, 2007; Zhang et al., 2009), we did not observe significant changes in PPR in hippocampal synapses (Fig. 4). Augmentation, however, represents a longer-lasting form of short-term enhancement than facilitation and requires more extensive stimulation to be evoked in detectable quantities. The excessive frequency-dependent increase in augmentation in *Fmr1* KO mice (Fig. 5D) closely parallels that of the overall increase in synaptic strength during high-frequency trains (Figs. 1D, 2C). This result suggests that increased augmentation may serve as the dominant mechanism of abnormal STP in *Fmr1* KO animals. Interestingly, the only previous study that directly examined effects of FMRP loss on augmentation using *Drosophila* neuromuscular junction (NMJ) did not find significant changes (Repicky and Brodie, 2009). These apparent discrepancies may result from the stimulus frequencies used, mostly outside of the high-frequency range within which we detected significant changes in augmentation. In addition, augmentation appears to have, at least in part, different mechanisms in the NMJ and the hippocampal synapses (Magleby and Zengel, 1982; Kandaswamy et al., 2010).

In addition to enhanced augmentation, we observed reduced short-term depression in *Fmr1* KO mice (Fig. 7). In hippocampal synapses, short-term depression is thought to be driven mostly by the depletion of the readily-releasable vesicles (Zucker and Regehr, 2002). Our findings of faster vesicle recycling concomitant with increased vesicle pools at CA3–CA1 synapses (Figs. 8, 9) thus provide likely mechanisms for reduced short-term depression in *Fmr1* KO mice. An elevated rate of vesicle cycling attributable to loss of FMRP has also been described previously in the *Drosophila*

← the earlier time points on expanded timescale. * $p < 0.05$, ** $p < 0.01$. **C**, Depression amplitude extrapolated to $t = 0$ (revealing extent of depression at the end of the high-frequency train) was significantly reduced in KO mice, without change in the recovery time constant. ** $p < 0.01$.

NMJ (Gatto and Brodie, 2008), but whether changes in vesicle pool sizes occurred has not been reported. It remains to be determined whether increased vesicle pools is a secondary effect resulting from faster vesicle recycling or whether these presynaptic changes arise from independent mechanisms.

Mechanisms of STP dysfunction in FXS

What are the mechanisms of these presynaptic changes associated with the loss of FMRP? Elevation in residual presynaptic Ca^{2+} levels that drive augmentation during repetitive activity is determined by a combination of several processes, namely AP-driven Ca^{2+} influx, cytoplasmic Ca^{2+} buffering, uptake/release by internal stores, and calcium extrusion (Scott, 2007). Because we did not observe significant changes in the kinetics of augmentation decay, which is widely believed to reflect calcium clearance/sequestration processes (Zucker and Regehr, 2002), these processes may not be strongly affected by FMRP loss. Moreover, at the CA3–CA1 synapse, the Ca^{2+} release/uptake by internal stores have been shown not to play significant roles in STP on the timescales relevant to the current study (Carter et al., 2002). Calcium influx through VGCCs thus represents the major process driving buildup of residual calcium on these timescales in CA3–CA1 synapses (Dunlap et al., 1995). Our data show that Ca^{2+} influx during high-frequency stimulation is significantly increased in CA3 neurons as a result of loss of FMRP after the first few stimuli in the train (Fig. 6). This result is consistent with the observed increase in augmentation but not paired-pulse facilitation in *Fmr1* KO animals (Figs. 4, 5).

One possible mechanism of the enhanced Ca^{2+} influx is the increase in calcium channel open time caused by prolongation of APs in *Fmr1* KO animals. This possibility is supported by the evidence that expression of the voltage-gated potassium channels Kv3.1b (Strumbos et al., 2010) and Kv4.2 (Gross et al., 2011) is positively regulated by FMRP in the brainstem and hippocampal neurons, respectively. Decreased number of voltage-gated K^+ channels in the absence of FMRP could cause excessive AP broadening, in turn leading to increased presynaptic Ca^{2+} influx. Another potential mechanism of elevated calcium influx is direct modulation of VGCC properties by FMRP. A recent study reported that FMRP can directly interact with and modulate gating of at least one type of ion channel, the sodium-activated K^+ channel Slack (Brown et al., 2010). It remains to be determined whether FMRP also modulates the activity of other

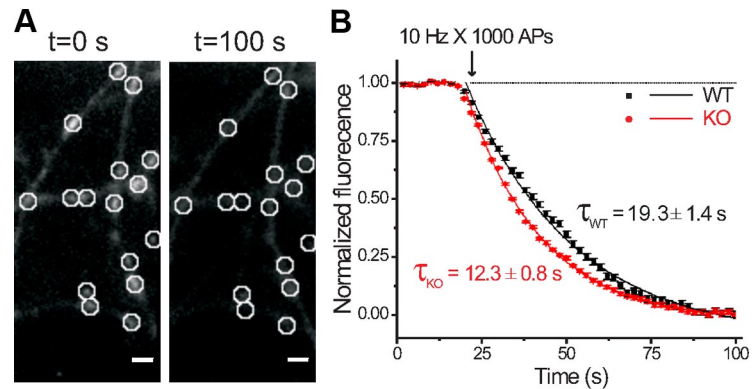


Figure 8. Vesicle pool turnover is faster in *Fmr1* KO mice. **A**, Hippocampal synapses in culture (days 14–18) were labeled with FM1-43 ($10 \mu\text{M}$) presented during and for 90 s after a 100 stimulus train at 10 Hz ($t = 0$). After a washing period, the dye was released with 1000 stimuli at 10 Hz ($t = 100$ s). An example of dye loading and unloading in WT neurons is shown. Scale bar, $2 \mu\text{m}$. **B**, Averaged dye release kinetics in WT and *Fmr1* KO mice at 37°C . Note the faster destaining kinetics in KO synapses ($n = 320$ WT and 455 KO synapses from 10 and 15 animals, respectively; $p < 0.01$).

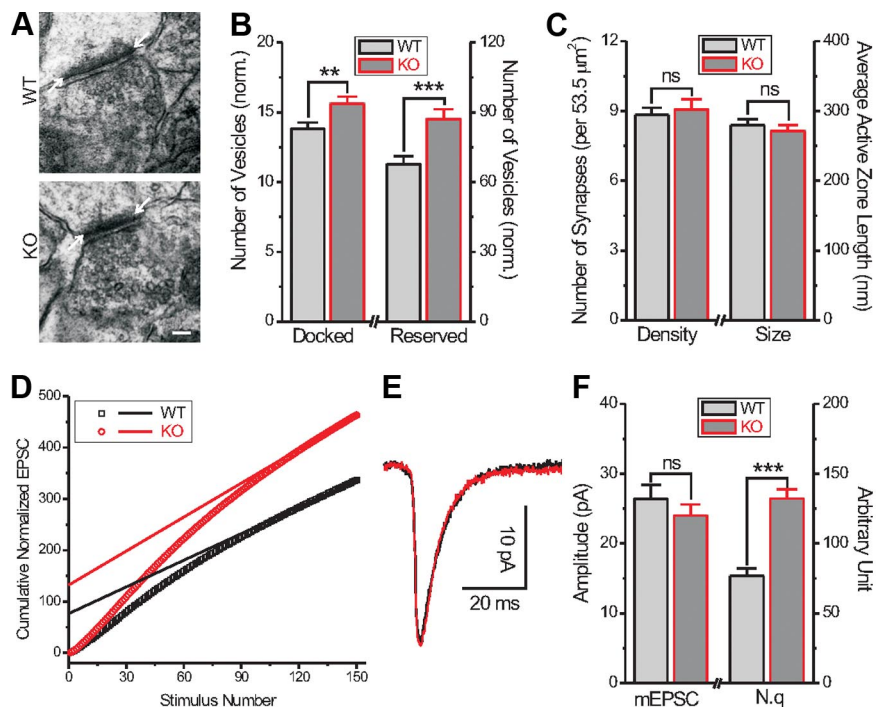


Figure 9. Vesicle pools are enlarged in excitatory hippocampal synapses of *Fmr1* KO mice. **A**, Transmission electron microscopy images of asymmetric excitatory synapses in the CA1 stratum radiatum. Arrows show the points used to measure the length of postsynaptic density. Scale bar, 100 nm . **B**, Analysis of vesicle counts in excitatory CA3–CA1 synapses shows significant increases in the numbers of both docked and reserved vesicles in *Fmr1* KO mice. Vesicle counts were normalized to the active zone size (vesicles per micrometer) as evaluated by the postsynaptic density length. $**p < 0.01$ or $***p < 0.001$; $n = 132$ synapses from 3 WT mice and $n = 148$ synapses from 4 KO mice. **C**, No significant differences in the density of asymmetric synapses and length of postsynaptic density (as an estimate for the length of the active zone) were found. **D**, Analysis of cumulative normalized EPSCs during 150 stimuli at 60 Hz was used to estimate changes in the functional RRP size. For each cell, the last 10 cumulative EPSC points were fitted with a linear regression line and extrapolated to the time $t = 0$ to estimate the N^*q value, as shown in **F**. $n = 50$ for WT, $n = 82$ for KO. **E**, The average mEPSC traces recorded in the presence of TTX in the WT and KO animals. **F**, The amplitude of mEPSC ($n = 6$ for each WT and KO; $p = 0.36$) used to evaluate changes in quantal size q and estimated N^*q values ($n = 50$ for WT, $n = 82$ for KO; $***p < 0.001$). No significant changes in q were found, confirming that N , the functional RRP size, is increased in the *Fmr1* KO animals.

types of ion channels, including presynaptic VGCCs. FMRP loss has also been shown to downregulate the expression of a major calcium buffer, calbindin D28K, in the hippocampal neurons (Real et al., 2011). Decreased levels of the presynaptic Ca^{2+} buffers have been shown to result in increased residual calcium levels in *Drosophila*

mushroom body neurons (Tessier and Broadie, 2011) and may thus also contribute to heightened augmentation in hippocampal synapses.

Increased calcium levels are also known to facilitate vesicle recycling (Dittman and Regehr, 1998) and may thus represent a mechanism leading to reduced short-term depression in the absence of FMRP. Other potentially overlapping mechanisms may lead to reduced depression via increase in the size of vesicle pools in the *Fmr1* KO mice (Fig. 9). Proteomics and translational profile analyses revealed that the levels of many presynaptic proteins involved in vesicle release and endocytosis/recycling are affected by loss of FMRP (Brown et al., 2001; Miyashiro et al., 2003; Liao et al., 2008). Changes in expression levels and/or activity of these proteins may lead to facilitated vesicle recycling and increased vesicle accumulation, thereby reducing synaptic depression in the absence of FMRP.

Alternative or concurrent with these potential presynaptic mechanisms of FMRP actions is trans-synaptic retrograde signaling arising from the postsynaptic site. The presence of a trans-synaptic mechanism is suggested by evidence in the *Drosophila* model of FXS that constitutive presynaptic dFMRP expression could rescue presynaptic structural deficits in *dFmr1* null mutants but not the functional ones (Gatto and Broadie, 2008). Retrograde signaling mechanisms have indeed been shown to regulate quantal size and vesicle release in *Drosophila* NMJ synapses (Davis et al., 1998). Whether trans-synaptic mechanisms can alter calcium dynamics, vesicle recycling, or size of the vesicle pools remains to be determined. Given the variety of known FMRP functions at synapses, it is also conceivable that both cell-autonomous and trans-synaptic mechanisms combine to determine presynaptic STP abnormalities arising from FMRP loss.

Implications of STP dysfunction for information processing in FXS

Our observation of abnormal STP in hippocampal synapses during physiologically relevant activity patterns may have important implications for cognitive dysfunctions in FXS individuals. Indeed, firing patterns of excitatory hippocampal neurons used here as a “natural” input to CA3–CA1 synapses were recorded from place cells of awake, freely moving rodents (Fenton and Muller, 1998), and their characteristics are typical of the input that hippocampal synapses may encounter *in vivo*. The function of place cells extends well beyond their well-described role in space representation. They have been shown to respond to various nonspatial sensory inputs and are generally believed to combine sensory information and spatial context to provide encoding of new context-specific or episodic information (Leutgeb et al., 2005). Abnormal synaptic information processing in the hippocampus may lead to dysfunctional learning abilities and thus contribute to cognitive deficits in FXS individuals. STP dysfunction in hippocampal synapses thus represents a first example of major presynaptic abnormalities leading to altered information processing in FXS.

Although they vary in relative abundance, the core mechanisms of STP are preserved in most central synapses (Zucker and Regehr, 2002). Our findings thus suggest the possibility that STP dysfunction is present in other brain regions, in which in addition to information processing, STP is thought to play important roles in working memory (Mongillo et al., 2008) and decision making (Deco et al., 2010). Moreover, spike bursts are a prominent feature of sensory coding in primary sensory and cortical neurons (Metzner et al., 1998; Leutgeb et al., 2005), and abnormal STP in these neurons, in combination with other factors, may explain

hypersensitivity to sensory stimuli and hyperactivity observed in FXS individuals (Bassell and Warren, 2008). Future studies in other brain areas will establish the overall impact of STP abnormalities in cognitive impairments in FXS.

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