Published in final edited form as:

Eur J Neurosci. 2014 May ; 39(10): 1602–1612. doi:10.1111/ejn.12546.

Activity-dependent regulation of release probability at excitatory hippocampal synapses: a crucial role of FMRP in neurotransmission

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Abstract

Transcriptional silencing of the *Fmr1* gene encoding fragile X mental retardation protein (FMRP) causes Fragile X Syndrome (FXS), the most common form of inherited intellectual disability and the leading genetic cause of autism. FMRP has been suggested to play important roles in regulating neurotransmission and short-term synaptic plasticity at excitatory hippocampal and cortical synapses. However, the origins and the mechanisms of these FMRP actions remain incompletely understood, and the role of FMRP in regulating synaptic release probability and presynaptic function remains debated. Here we used variance-mean analysis and peak scaled nonstationary variance analysis to examine changes in both pre- and postsynaptic parameters during repetitive activity at excitatory CA3-CA1 hippocampal synapses in a mouse model of FXS. Our analyses revealed that loss of FMRP did not affect the basal release probability or basal synaptic transmission, but caused an abnormally elevated release probability specifically during repetitive activity. These abnormalities were not accompanied by changes in EPSC kinetics, quantal size or postsynaptic AMPA receptor conductance. Our results thus indicate that FMRP regulates neurotransmission at excitatory hippocampal synapses specifically during repetitive activity via modulation of release probability in a presynaptic manner. Our study suggests that

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FMRP function in regulating neurotransmitter release is an activity-dependent phenomenon that may contribute to the pathophysiology of FXS.

Keywords

EPSC; FMRP; Fragile X syndrome; hippocampus; release probability; short-term plasticity; mouse

Introduction

Fragile X Syndrome (FXS), the most common form of inherited intellectual disability and the leading genetic cause of autism (Bear *et al.*, 2004; Bassell & Warren, 2008), is caused by transcriptional silencing of *Fmr1* gene encoding fragile X mental retardation protein (FMRP) (Bassell & Warren, 2008; Pfeiffer & Huber, 2009). FMRP is expressed in the dendrites as well as axons (Antar *et al.*, 2006; Bassell & Warren, 2008; Till *et al.*, 2012). It modulates expression of nearly a third of pre- and postsynaptic proteome (Liao *et al.*, 2008; Darnell *et al.*, 2011; Klemmer *et al.*, 2011) and functions at both pre- and post-synaptic compartments (Till *et al.*, 2010; Deng *et al.*, 2013; Patel *et al.*, 2013).

The postsynaptic role of FMRP has been extensively investigated and is summarized in “mGluR theory” of FXS (Bear *et al.*, 2004). Increasing evidence suggests important roles of FMRP in regulating presynaptic function. For example, studies using mosaic *Fmr1* KO mice demonstrated that loss of FMRP has a target-cell specific presynaptic effect on the basal release probability in excitatory cortical synapses onto fast spiking interneurons (Patel *et al.*, 2013) and also causes neuronal connectivity defects with a presynaptic origin in the hippocampal circuit (Hanson & Madison, 2007). Our recent studies revealed a number of ultrastructural and functional changes in presynaptic terminals of hippocampal excitatory neurons. Specifically, *Fmr1* knockout mice exhibits elevated responses to high-frequency stimulation, enhanced synaptic vesicle recycling, and enlarged readily-releasable and reserved vesicle pools (Deng *et al.*, 2011). Loss of FMRP causes reduced activity of Ca^{2+} -activated K^+ (BK) channels and excessive action potential (AP) prolongation during repetitive activity, leading in turn to exaggeratedly elevated presynaptic Ca^{2+} influx, and enhanced synaptic transmission and short-term plasticity (STP) (Deng *et al.*, 2011). In most central synapses, the enhanced neurotransmission during repetitive activity is thought to arise from elevation in presynaptic calcium levels, and subsequent increase in the probability of vesicle release (Zucker & Regehr, 2002). However, a number of studies that examined presynaptic parameters in excitatory neurons in the hippocampus and several other brain regions in *Fmr1* knockout mice did not observe changes in the basal state (Pfeiffer & Huber, 2007; Gibson *et al.*, 2008; Deng *et al.*, 2011; Patel *et al.*, 2013). The role of FMRP in regulating release probability (*Pr*) remains controversial, with reports of changes in *Pr* ranging from decreased, unaltered to increased in *Fmr1* KO mice (Pfeiffer & Huber, 2007; Gibson *et al.*, 2008; Suvrathan *et al.*, 2010; Deng *et al.*, 2011; Klemmer *et al.*, 2011; Patel *et al.*, 2013). On the other hand, the elevated mGluR-LTD in the hippocampus of *Fmr1* knockout mice is believed to be related to enhanced internalization of AMPA receptors (Bear *et al.*, 2004). It has also been reported that the postsynaptic AMPA receptors are

developmentally altered in a mouse model of FXS (Pilpel *et al.*, 2009). Thus, it is incompletely known that whether the abnormal neurotransmission and enhanced STP in FXS are caused by changes in presynaptic transmitter release or postsynaptic receptors. To elucidate these questions, we used variance-mean analysis and peak scaled nonstationary variance analysis to examine changes in both pre- and postsynaptic parameters during repetitive activity at excitatory CA3-CA1 hippocampal synapses in a mouse model of FXS.

Our results indicate that FMRP modulates release probability and synaptic transmission in an activity-dependent manner during repetitive activity at hippocampal excitatory CA3-CA1 synapses, but it does not regulate basal release probability at these synapses. These actions of FMRP occur without any detectable changes in EPSC kinetics, vesicle quantal size and postsynaptic AMPA receptor (AMPA) conductance. These results provide evidence that in hippocampal excitatory neurons, FMRP regulates neurotransmitter release via a presynaptic suppression of release probability in an activity-dependent manner. These actions of FMRP may have important implications to understanding the neuronal dysfunction in FXS.

Materials and Methods

Animals and slice preparation

Fmr1 knockout (KO, FVB.129P2-*Fmr1*^{tm1Cgr/J}) and wildtype control (WT, FVB.129P2-Pde6b⁺Tyr^{c-ch}/AntJ) mice were generated by breeding *Fmr1* heterozygous females with either WT or *Fmr1* KO male mice obtained from The Jackson Laboratory. Both male and female 16- to 21-day-old mice (littermate- and age-matched controls) were used. Genotyping was performed according to The Jackson Laboratory protocols and analyzed in a blind manner. After being deeply anesthetized with CO₂ or isoflurane, mice were decapitated and their brains were dissected out in ice-cold saline 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 (400 μm) including the hippocampi were cut using a vibrating microtome (Leica VT1100S). 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 were in compliance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals, and conformed to the guidelines approved by Animal Care and Use Committee of Central South University or Washington University Animal Studies Committee.

Electrophysiology

Whole-cell patch-clamp recordings using an Axopatch 200B or a Multiclamp 700B amplifier (Molecular Devices) in voltage-clamp mode were made from CA1 pyramidal neurons visually identified with infrared video microscopy (Olympus BX51WI) 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) (Deng *et al.*, 2011; Deng *et al.*, 2013): 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). AMPA excitatory

postsynaptic currents (EPSCs) were recorded from CA1 pyramidal neurons (held at -65 mV) by stimulating Schaffer collaterals (SC) in CA1 stratum radiatum every 5 s, in the presence of NMDA receptors blocker APV ($50\text{ }\mu\text{M}$) to prevent long-term effects, and GABA_A receptor blocker bicuculine ($10\text{ }\mu\text{M}$). For train stimulation, each train was presented 4–6 times in each cell, and each presentation was separated by ~ 2 min of low-frequency ($0.1\text{--}0.2$ Hz) control stimuli to allow complete EPSC recovery to the baseline. Data were filtered at 2 kHz, digitized at 20 kHz, acquired using pClamp9 software (Axon Instruments) or custom software written in LabView, and analyzed using Clampex9 or programs written in MATLAB. EPSC measurement was performed as previously (Deng *et al.*, 2011).

Amino-5-phosphonopentanoic acid (APV, an NMDA antagonist) and gabazine (a GABA_A receptor antagonist) were from Tocris Bioscience. All other chemicals were purchased from Sigma-Aldrich.

Variance–mean analysis

Variance–mean (V–M) analysis was conducted following the methods described previously (Clements & Silver, 2000; Clements, 2003; Silver, 2003; Wang *et al.*, 2012). Mean EPSC amplitude (M) was averaged from 60–100 successive EPSCs recorded at different extracellular Ca^{2+} concentrations to alter release probability after stabilization of the EPSCs at each Ca^{2+} concentration. Variance (V) of EPSC was calculated as the square of the standard deviation of the corresponding EPSC amplitudes in each condition after correction for baseline variance. Note that, in our experimental conditions ($\sim 33^\circ\text{C}$), receptor desensitization and saturation are insignificant (Wesseling & Lo, 2002; Klyachko & Stevens, 2006). For bursts during train stimulation, EPSCs were averaged from the last 20 stimuli of the bursts (a total of 80–120 EPSCs for a given cell were used). The relationship between V and M can be described by a parabola equation: $V = qM - M^2/N$, where q is the quantal size and N is the number of independent release sites, as opposed to the number of synaptic contacts.

Peak-scaled nonstationary variance analysis

EPSC amplitudes during decay part differ not only as a result of random channel gating, but also from variations in release of transmitter and differences in the numbers of receptors activated in the postsynaptic membrane. To isolate the fluctuations in the EPSC due to stochastic channel gating properties from those due to changes in the total number of postsynaptic AMPARs (i.e., channels) activated by transmitter, we used peak-scaled nonstationary variance (PSNSV) analysis to estimate the properties of postsynaptic AMPARs, as we and others did previously (Traynelis *et al.*, 1993; Benke *et al.*, 1998; Traynelis & Jaramillo, 1998; Lei & McBain, 2002; Lei *et al.*, 2003; Deng & Lei, 2007). The EPSCs were initially inspected visually to exclude those responses contaminated by spontaneous synaptic activity. In order to minimize the contribution to our measurements of variance that arises from asynchronous release of transmitter, we only selected EPSCs showing fast rise time ($10\text{--}90\%$ rise time < 2.5 ms) and smooth decay for analysis (without contamination by spontaneous events and successful fitted by double exponentials) (Lei *et al.*, 2003; Deng & Lei, 2007). For bursts during train stimulation, EPSCs were selected from the last 20 stimuli of the bursts (a total of 80–100 EPSCs were used for any given cell). The

selected EPSCs were aligned along the steepest rise points of their rise parts and then averaged. The average response (EPSC waveform) was scaled to the peaks of and subtracted from individual responses to compute the variance. The decay part of EPSC waveform was divided into 100 equally sized bins along the amplitude and the corresponding variances of individual EPSCs were pooled. The binned variance was plotted against the binned mean current amplitude, and the single-channel current and the number of AMPARs activated were calculated by fitting the data according to the equation: $\sigma^2 = iI - I^2/N + \sigma_{\text{base}}$, where σ^2 is the variance, I is the mean current, N is the average number of AMPARs activated at the peak of EPSC (as opposed to the number of total AMPARs in the postsynaptic membrane), i is the single-channel current, and σ_{base} is the background variance. The single-channel conductance was measured by $\gamma = i/(E - E_{\text{rev}})$, where γ is the single-channel conductance, E the holding potential, and E_{rev} the reversal potential that was measured to be close to 0 mV under our recording conditions.

Statistics

Statistical analysis was carried out using Origin 7.5 (OriginLab, Northampton, MA, USA). Student's unpaired *t*-test or one-way ANOVA (and Tukey test for post hoc multiple comparison) was used as appropriate. Statistical methods and results were reported throughout the text, and significance was set as $P < 0.05$. The n number in the text represents the cells examined. Data were presented as mean $\pm$ SEM.

Results

Abnormal synaptic strength is related to increased presynaptic transmitter release, but not accompanied by changes in EPSC kinetics at CA3-CA1 synapses of *Fmr1* KO mice

We have previously reported that FMRP loss causes abnormally elevated STP at the excitatory CA3-CA1 hippocampal synapses (Deng *et al.*, 2011). Whether these defects are accompanied by changes in postsynaptic function has not been determined. To examine this question we recorded AMPA EPSCs from hippocampal CA1 pyramidal cells (PCs) in WT or *Fmr1* KO mice by stimulating Schaffer collaterals (SC) at 20 Hz for 100 stimuli in CA1 stratum radiatum (Figs. 1A, B), at near-physiological temperatures (33–34°C) (Klyachko & Stevens, 2006; Deng *et al.*, 2011) in the presence of APV (50 μ M) and gabazine (5 μ M) to block NMDA and GABA_A receptors, respectively. 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. As we reported previously, synaptic strength exhibited excessive enhancement in the *Fmr1* KO relative to WT mice (peak gain: WT 2.41 ± 0.16 -fold of baseline; KO 2.95 ± 0.12 -fold of baseline, $t_{15} = 2.4504$, $P = 0.01351$; Average gain during the trains: WT 2.11 ± 0.12 -fold of baseline; KO 2.75 ± 0.10 -fold of baseline, $t_{15} = 2.6198$, $P = 0.00966$; $n = 8$ for WT, $n = 9$ for KO, unpaired *t*-test; Figs. 1A–C). To assess changes in postsynaptic function during these high-frequency trains, we first examined the EPSC kinetics during the trains. We found that the EPSC latency, rise (10%–90%) and decay times were not significantly altered in *Fmr1* KO mice compared to WT controls (Latency at baseline: WT 1.65 ± 0.04 ms, KO 1.67 ± 0.05 ms, $t_{15} = 0.5490$, $P = 0.59106$; Latency at the end of train: WT 1.86 ± 0.04 ms, KO 1.84 ± 0.03 ms, $t_{15} = 0.3906$, $P = 0.70155$; Rise time at baseline: WT 2.06 ± 0.13 ms; KO 2.19 ± 0.11 ms, $t_{15} = 0.5323$, P

= 0.60234; Rise time at the end of train: WT 2.44 ± 0.08 ms; KO 2.27 ± 0.07 ms, $t_{15} = 1.2671$, $P = 0.22445$; Decay time at baseline: WT 9.28 ± 0.53 ms; KO 8.48 ± 0.29 ms, $t_{15} = 0.5901$, $P = 0.56389$; Decay time at the end of train: WT 10.13 ± 0.40 ms; KO 9.60 ± 0.31 ms, $t_{15} = 0.8268$, $P = 0.42133$; $n = 8$ for WT, $n = 9$ for KO, unpaired t -test; Fig. 1E), indicating that the time-course of transmitter release, the activation of postsynaptic AMPARs, and the rate of transmitter clearance from the synaptic cleft are not affected significantly by the loss of FMRP.

The excessively enhanced STP in *Fmr1* KO mice could be attributable to an increase in presynaptic glutamate release or/and to a rapid upregulation of postsynaptic AMPAR function during the train stimulation. Changes in presynaptic transmitter release are usually concomitant with an alteration in coefficient of variation (CV) of EPSCs (Fitzjohn *et al.*, 2001; Deng & Lei, 2007; Deng *et al.*, 2010). We found that CV of EPSC amplitude during the trains was significantly decreased within WT or KO mice (4 time-points as indicated in Fig. 1C: WT, $F_{3,28} = 4.6657$, $P = 0.00912$; KO, $F_{3,32} = 4.6050$, $P = 0.00866$; $n = 8$ for WT, $n = 9$ for KO, one-way ANOVA; Fig. 1D), supporting changes in presynaptic transmitter release (Zucker & Regehr, 2002). To further elucidate the mechanisms of abnormal STP and neurotransmitter release in *Fmr1* KO mice, we next examined changes in the release probability (Pr), quantal size (q), and postsynaptic AMPAR properties both at basal transmission and during repetitive activity.

Baseline synaptic transmission is unaffected at CA3-CA1 synapses in *Fmr1* KO mice

To estimate the effects of FMRP deficiency on release probability and quantal parameters, we performed variance-mean (V-M) analysis as described previously (Clements & Silver, 2000; Clements, 2003; Silver, 2003; Wang *et al.*, 2012). Various release probabilities were achieved by using 3 different ratios of $\text{Ca}^{2+}/\text{Mg}^{2+}$ (1:1, 2:1 and 4:1 mM) in the extracellular solution (Wang *et al.*, 2012). EPSCs were recorded from CA1 PCs by stimulating SC at 0.2 Hz. Keeping the stimulus intensity unchanged for the same recordings, the EPSC amplitude were significantly altered under different $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentrations within WT or KO mice (Fig. 2A,B). However, the EPSC latency, rise and decay time were not significantly affected among different $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratios, and between WT and KO mice (Fig. 2C). In contrast, the CV of EPSC was significantly decreased when the extracellular Ca^{2+} concentration increased (among 3 Ca^{2+} concentrations: WT, $F_{2,24} = 5.9349$, $P = 0.00805$; KO, $F_{2,33} = 5.7718$, $P = 0.00708$; $n = 9$ for WT, $n = 12$ for KO, one-way ANOVA; Fig. 2D), in line with increasing presynaptic release probability by elevating extracellular Ca^{2+} . We then plotted the variance of EPSC against corresponding mean amplitude for each Ca^{2+} concentration. The V-M plot was then fitted by a parabolic function. The V-M analysis showed that the quantal size (WT 10.86 ± 1.02 pA, KO 10.77 ± 1.05 pA; $t_{19} = 0.0587$, $P = 0.95381$; $n = 9$ for WT, $n = 12$ for KO, unpaired t -test; Fig. 2F) and the basal Pr were unaffected in the *Fmr1* KO mice (1 mM Ca^{2+} : $Pr_{\text{WT}} = 0.0627 \pm 0.00959$, $Pr_{\text{KO}} = 0.07034 \pm 0.01128$, $t_{19} = 1.2259$, $P = 0.2352$; 2 mM Ca^{2+} : $Pr_{\text{WT}} = 0.13453 \pm 0.03062$, $Pr_{\text{KO}} = 0.15092 \pm 0.0319$, $t_{19} = 0.8359$, $P = 0.4136$; 4 mM Ca^{2+} : $Pr_{\text{WT}} = 0.45203 \pm 0.034$, $Pr_{\text{KO}} = 0.50713 \pm 0.040$, $t_{19} = 0.6673$, $P = 0.5126$; $n = 9$ for WT, $n = 12$ for KO, unpaired t -test; Fig. 2G). Since facilitation is strongly related to baseline release probability, this result is consistent with the previous observations that facilitation is not altered at hippocampal synapses of *Fmr1* KO mice

(Zhang *et al.*, 2009; Deng *et al.*, 2011). For better comparisons across neurons, V–M relationships were normalized by dividing V and M by the corresponding maximum EPSC estimated from the parabolic fit within individual neurons. Normalized scatter plots and curves were similar between WT and KO mice (Fig. 2H), further supporting the above observation that quantal size and basal release probability are not significantly altered in *Fmr1* KO mice.

We then conducted peak-scaled non-stationary variance (PSNSV) analysis to calculate the single channel conductance and number of postsynaptic AMPARs activated under different Ca^{2+} concentrations (Figs. 3A–D). This analysis has demonstrated that loss of FMRP did not alter the single channel conductance of the AMPARs (pooled data at different Ca^{2+} levels because of no significant difference among them: WT 16.88 ± 1.01 pS, KO 17.38 ± 0.91 pS, $F_{5,57} = 0.7141$, $P = 0.61542$; $n = 9$ for WT, $n = 12$ for KO, one-way ANOVA among 3 different Ca^{2+} concentrations and 2 genotypes; Figs. 3B and C) and the number of postsynaptic AMPARs activated at the same Ca^{2+} concentration (number of open channels: 1 mM Ca^{2+} , $N_{\text{WT}} = 64 \pm 4$, $N_{\text{KO}} = 59 \pm 5$, $t_{19} = 0.1719$, $P = 0.86533$; 2 mM Ca^{2+} , $N_{\text{WT}} = 112 \pm 12$, $N_{\text{KO}} = 123 \pm 6$, $t_{19} = 0.3970$, $P = 0.69581$; 4 mM Ca^{2+} , $N_{\text{WT}} = 251 \pm 28$, $N_{\text{KO}} = 246 \pm 11$, $t_{19} = 0.1254$, $P = 0.90152$; $n = 9$ for WT, $n = 12$ for KO, unpaired *t*-test; Fig. 3D). Together, V–M analysis and PSNSV analysis suggest that the basal synaptic transmission and AMPAR properties at CA3–CA1 synapses are not affected significantly by the loss of FMRP.

Loss of FMRP causes abnormally increased release probability during repetitive activity, without changes in quantal size and postsynaptic AMPAR conductance

We next analyzed the potential changes in the quantal parameters and in postsynaptic AMPAR properties during train stimulation. Because calculation of quantal parameters by V–M analysis requires several different *Pr* measurements, we took advantages of the fact that high frequency train stimulation may transiently increase *Pr* (at synapses with low basal *Pr*) via a buildup of residual Ca^{2+} in the presynaptic terminals during the train, without requiring changes in extracellular Ca^{2+} concentrations (Zucker & Regehr, 2002; Silver, 2003; Huang *et al.*, 2010; Bagnall *et al.*, 2011).

When using V–M analysis, one may presume that the single channel conductance of AMPARs is unchanged under different *Pr* settings, and thus the calculated quantal size is reliable. While this was tested when EPSCs were evoked by single stimuli at low frequency (Larkman *et al.*, 1997), it has not been well established whether the single-channel AMPAR conductance is stable during high-frequency stimulation. If the AMPAR conductance varies during the train stimulation, then the calculated quantal size is no longer reliable. Thus, we first calculated the AMPAR conductance to ensure we can perform V–M analysis for EPSCs from train stimulation. EPSCs were recorded from CA1 PCs by stimulating SC with a complex train consisting of 4 tandem segments: 10 stimuli at 0.2 Hz, 50 stimuli at 20 Hz, 50 stimuli at 40 Hz, and 10 stimuli at 0.2 Hz, representing the basal state, 1st increased *Pr* setting, 2nd increased *Pr* setting and recovery from the train stimulation, respectively (Figs. 4A and B). In agreement with our previous results, loss of FMRP significantly increased the EPSC responses during the train stimulation at both 20Hz and 40Hz (20 Hz: WT $2.26 \pm$

0.15-fold of baseline, KO 3.01 ± 0.10 -fold of baseline, $t_{16} = 2.9927$, $P = 0.00861$; 40 Hz WT 2.88 ± 0.12 -fold of baseline, KO 4.05 ± 0.14 -fold of baseline, $t_{16} = 3.2198$, $P = 0.00535$; $n = 8$ for WT, $n = 10$ for KO, unpaired t -test; Figs. 4A and B). To calculate the AMPAR parameters at baseline (before high-frequency burst) and during the burst, we performed the PSNSV analysis as described above. As shown in Figs. 4C and D, the AMPAR conductance was not significantly altered during high-frequency stimulation in either WT or *Fmr1* KO neurons. More importantly, loss of FMRP did not affect the AMPAR conductance (pooled data for 0.2, 20 and 40 Hz stimulation because of no significant difference, WT 18.25 ± 1.37 pS; KO 17.36 ± 1.27 pS, $F_{5,48} = 1.8236$, $P = 0.12602$; $n = 8$ for WT, $n = 10$ for KO, one-way ANOVA among 3 frequencies and 2 genotypes; Fig. 4D).

We then performed V-M analysis to calculate quantal size and Pr . Our analysis revealed that the quantal sizes calculated from the train stimulation were very close to those estimated from EPSCs evoked by single-stimulus (Fig. 2F) and no significant difference was observed between WT and *Fmr1* KO mice (quantal size calculated from train stimulation: WT 11.55 ± 0.91 pA, KO 12.57 ± 0.57 pA, $t_{16} = 0.4499$, $P = 0.6588$; $n = 8$ for WT, $n = 10$ for KO, unpaired t -test; also shown in Fig. 5D), consistent with the previous report that during high-frequency activity quantal size remained unchanged in these synapses (Zhou *et al.*, 2000).

Loss of FMRP did not affect the baseline Pr when calculated from the train stimulation (at 0.2 Hz: WT 0.14978 ± 0.0146 ; KO 0.15529 ± 0.00934 , $t_{16} = 0.3091$, $P = 0.7612$; $n = 8$ for WT, $n = 10$ for KO, unpaired t -test; Fig. 4F), which is consistent with the estimates from low frequency single-stimulus recordings at the same Ca^{2+} levels (Fig. 2G). However, in both WT and KO mice the Pr was not only significantly increased during the 20Hz and 40 Hz stimulus epochs, but, most importantly, the Pr was excessively elevated in the *Fmr1* KO mice during both of these high-frequency epochs (Pr at 20 Hz: WT 0.33152 ± 0.02142 ; KO 0.42898 ± 0.01949 , $t_{16} = 2.4400$, $P = 0.0267$; Pr at 40 Hz: WT 0.41967 ± 0.02161 ; KO 0.55106 ± 0.02169 , $t_{16} = 3.0697$, $P = 0.00733$; $n = 8$ for WT, $n = 10$ for KO, unpaired t -test; Fig. 4F). These data suggest that FMRP regulates release probability specifically during high-frequency stimulation without affecting the quantal size or AMPAR conductance.

Excessively elevated release probability during repetitive activity results in abnormal short-term plasticity in *Fmr1* KO mice

The above analysis indicates that loss of FMRP leads to excessive increase in Pr , without changes in quantal size and postsynaptic AMPAR conductance. Abnormal STP in the absence of FMRP may then arise either from rapid changes in presynaptic Pr and/or in the number of functional postsynaptic AMPARs. The latter possibility, however, requires AMPAR saturation in response to quantal release. In other words, only if AMPARs are saturated by quantal release, increase in the number of functional AMPARs can lead to an increase in EPSC amplitude. Considering extensive evidence that AMPARs at hippocampal CA3-CA1 synapses are not saturated by quantal release (Liu *et al.*, 1999; McAllister & Stevens, 2000), it appears unlikely that increase in the number of functional postsynaptic AMPARs can account for the excessively increased EPSCs observed in *Fmr1* KO mice (see discussion). Therefore, we hypothesized that abnormally elevated STP in the absence of FMRP results predominately from the excessively enhanced transmitter release that causes

activation of more postsynaptic AMPARs. If this is the case, the increase in the number of AMPARs activated should linearly scale with the increase in Pr in *Fmr1* KO mice. We tested this idea by combining results of V-M analysis with those of PSNSV analysis cell by cell (i.e., pairing parameters for the same cells). We note that V-M analysis uses the fluctuations of EPSC peak values (variations from the total number of opened AMPARs at the time-point of EPSC wave peak) to calculate quantal parameters, while PSNSV analysis uses the fluctuations of the decay part of EPSC about the scaled mean waveform (variations from the stochastic closing of AMPARs during the EPSC decay part) to calculate AMPAR parameters. In other words, V-M and PSNSV analyses use different elements of the data to evaluate pre- and postsynaptic parameters, respectively. Thus, if the presynaptic changes in Pr can fully account for the postsynaptic changes in the number of AMPARs activated in the absence of FMRP, then we can conclude that the abnormal STP in *Fmr1* KO mice is a result of changes in transmitter release.

In this analysis, the EPSCs that we originally used for PSNSV analysis were used to perform V-M analysis. Here we focused on the Pr , the number of total independent release sites (N_{sites} , as opposed to the synaptic contacts), the number of AMPARs activated (N_{AMPA} , as opposed to the number of total postsynaptic AMPARs, summarized in Fig. 5A, from PSNSV analysis). Due to a variable amount of presynaptic inputs being stimulated in different recordings, the actual sites that release quanta may be different even if the Pr is the same (Pr is a ratio, not an absolute value). Thus, it is not feasible to directly assess the correlation between Pr (a ratio) and the actual number of AMPARs activated (an absolute value) across all recordings. To overcome this impracticality, we introduced the actual number of independent sites that release quanta (N_{released}) corresponding to Pr . For any specific recording, the total independent release site (N_{site}) does not vary because the stimulation intensity was kept unchanged. Thus, for a given Pr value, the N_{released} is estimated by $N_{\text{released}} = Pr \times N_{\text{site}}$ (summarized in Fig. 5B; samples shown in Fig. 4E, numbers in parentheses). We then determined the correlation of the actual number of presynaptic sites that release quanta (N_{released}) and the number of AMPARs activated (N_{AMPA}) by plotting N_{AMPA} against N_{released} cell by cell (Fig. 5C). Plots were closely fitted by linear regression lines both in WT and *Fmr1* KO mice (correlation coefficient: WT, $R = 0.855$; KO, $R = 0.905$; Fig. 5C). This analysis reveals that the number of AMPARs activated is highly correlated with the number of presynaptic quanta released. Since the actual number of sites that release quanta is derived from the corresponding Pr measurements (Fig. 5B), this close correlation between N_{released} and N_{AMPA} indicates that the increase in Pr during the train stimulation leads to the increase in the number of postsynaptic AMPAR activated. This analysis supports the notion that abnormally elevated STP in the absence of FMRP results predominately from increase in presynaptic release probability during repetitive activity.

We noted that the number of AMPARs activated by a single quantum can be estimated from the above fits (Fig. 5C) by the slope of the fitted lines. This measurement gives ~10–11 AMPARs being activated in response to release of a presynaptic quantum (regression line slope: WT 10.33; KO 10.91; Fig. 5C), which is consistent with previous estimates in glutamatergic synapses (Traynelis *et al.*, 1993). Therefore, an alternative way to estimate the

quantal size is available. In this case, the quantal size can be determined by $q = N_{\text{AMPA}/q} \times \gamma \times V$, where $N_{\text{AMPA}/q}$ is the number of AMPARs activated in response to a single quantum release (i.e., the slope of the fitted lines in Fig. 5C), γ is the single channel conductance of AMPARs, V is the driving force (holding potential minus reversal potential, holding potential is -65 mV and reversal potential is assumed 0 in our settings). Using the AMPAR conductance values calculated from train stimulation above, we estimated the quantal size to be 12.25 ± 0.92 pA for WT and 12.31 ± 0.90 pA for *Fmr1* KO mice (Fig. 5D). This is in a close agreement with the estimates calculated by V-M analysis from train stimulation (WT 11.55 ± 0.91 pA; KO 12.57 ± 0.57 pA; Fig. 5D), as well as from single-stimulus (WT 10.86 ± 1.02 pA; KO 10.77 ± 1.05 pA; Fig. 2F).

The amplitude of EPSC is given by $\text{EPSC} = N_{\text{site}} \times Pr \times q$, and the quantal size $q = N_{\text{AMPA}/q} \times \gamma \times V$, thus EPSC is also estimated by $\text{EPSC} = N_{\text{site}} \times Pr \times N_{\text{AMPA}/q} \times \gamma \times V$ (Fig. 5E). Because the quantal size q (both estimated by V-M analysis and correlation analysis, i.e., $N_{\text{AMPA}/q} \times \gamma \times V$), postsynaptic AMPAR conductance (γ), and total independent release sites for a given recording (N_{site}) are unchanged during train stimulation, our analyses thus suggest that the abnormal STP in the absence of FMRP is attributed predominately to an abnormally increased Pr during repetitive activity (Fig. 5E), indicating that FMRP regulates Pr at excitatory CA3-CA1 synapses in an activity-dependent manner.

Discussion

Here we demonstrate an activity-dependent role of FMRP in regulating release probability at excitatory hippocampal CA3-CA1 synapses specifically during repetitive activity under nearly physiological temperature, without affecting basal release probability. These actions of FMRP are not accompanied by changes in EPSC kinetics, quantal size or postsynaptic AMPAR conductance. We base these assertions on the following observations: (i) During repetitive activity, neurotransmission and STP are abnormally elevated in CA3-CA1 synapses of *Fmr1* KO mice, which are caused by excessively elevated release probability. (ii) EPSC kinetics, quantal size and postsynaptic AMPAR conductance are not significantly altered in *Fmr1* KO mice in either the basal state or during repetitive activity. (iii) Correlation analyses of quantal and AMPAR parameters indicate that the increased number of AMPARs activated during repetitive activity in *Fmr1* KO mice results from increased quantal release (i.e., increase in Pr); and (iv) This increase in quantal release/ Pr accounts well for the altered STP. Taken together, these results support the role of FMRP in activity-dependent regulation of release probability specifically during repetitive activity. Loss of this FMRP function may play important roles in neuropathology of FXS.

Presynaptic role of FMRP in regulating release probability

Increasing evidence supports the multifaceted roles of FMRP in synaptic function, which, in addition to its well established roles in controlling mRNA trafficking and translation in dendrites, include regulation of many presynaptic properties, such as synaptic strength, intrinsic excitability, action potential waveform, vesicle pool sizes as well as connectivity (Zhang *et al.*, 2001; Hanson & Madison, 2007; Gatto & Broadie, 2008; Gibson *et al.*, 2008;

Pfeiffer & Huber, 2009; Deng *et al.*, 2011; Deng *et al.*, 2013; Wijetunge *et al.*, 2013). The role of FMRP in regulation of synaptic release probability remains, however, a matter of substantial debate. This fundamental synaptic parameter determines the basal synaptic strength and is commonly assessed using measurements of a paired-pulse ratio, which has been shown to inversely correlate with the basal release probability (Zucker & Regehr, 2002). Studies in *Fmr1* KO mice over the last decade led to conflicting views on changes in release probability in the absence of FMRP. No changes in facilitation (or paired-pulse ratio) were observed in a majority of studies in hippocampal and cortical excitatory synapses (Pfeiffer & Huber, 2007; Gibson *et al.*, 2008; Deng *et al.*, 2011; Patel *et al.*, 2013), suggesting lack of significant involvement of FMRP in controlling basal release probability. However, Klemmer *et al.*, (2011) reported that paired-pulse ratio is reduced in the absence of FMRP in CA3-CA1 hippocampal excitatory synapses, suggesting that FMRP normally positively regulates the basal release probability. In contrast, Suvrathan *et al.*, (2010) showed enhanced paired-pulse facilitation and decreased presynaptic release in the amygdale in *Fmr1* KO mice. More direct measurements of *Pr* in cortical excitatory synapses also suggested that FMRP positively regulate the basal release probability at excitatory synapses onto fast spiking interneurons, but not onto excitatory neurons, and without detectable changes in the paired-pulse ratio (Patel *et al.*, 2013). On the other hand, recordings of synaptic transmission in the hippocampal excitatory neurons (Deng *et al.*, 2011; Deng *et al.*, 2013) indicated an increase in glutamate release during train stimulation in the absence of FMRP, suggesting that FMRP negatively regulate neurotransmitter release during repetitive activity. However, the *Pr* was not directly assessed in these studies.

Here we performed multiple analyses to determine changes in both pre- and post synaptic parameters in hippocampal excitatory synapses caused by loss of FMRP, both at the basal state and during repetitive activity. This approach allowed us to estimate changes in *Pr* as well as to determine whether abnormalities in neurotransmitter release in the absence of FMRP are accompanied by changes in postsynaptic function. Indeed, our V-M analysis indicates that *Pr* is abnormally elevated in the absence of FMRP in CA3-CA1 synapses, but only during high-frequency stimulation, while the basal *Pr* remains unaffected. These findings thus provide an explanation for the lack of changes in basal synaptic function in the mouse model or zebra fish model of FXS (Gibson *et al.*, 2008; Deng *et al.*, 2011; Deng *et al.*, 2013; Ng *et al.*, 2013; Patel *et al.*, 2013). Furthermore, our results demonstrate that the EPSC kinetics is unaffected in *Fmr1* KO mice compared with WT controls both in the basal state and during repetitive activity, indicating that loss of FMRP does not significantly affect the time-course of neurotransmitter release, AMPAR activation or neurotransmitter clearance at this synapse.

The abnormally enhanced synaptic transmission and STP during repetitive activity in *Fmr1* KO mice could in principle also arise from an increase in quantal size, the number of AMPARs or/and AMPAR conductance. Our results, however, strongly argue against these possibilities. First, our nonstationary variance analysis found no significant changes in the AMPAR conductance. Second, increase in the number of AMPARs is unlikely to contribute to the observed rapid changes in EPSC amplitudes for the following reasons: (i) It is unlikely that the number of AMPARs can increase several folds and then go back, on a

millisecond-timescale, on which the changes in EPSCs are observed during high-frequency trains. (ii) Only if the AMPARs are saturated by quantal release, increase in the number of AMPARs can lead to increase the EPSC amplitude. Previous studies have elegantly demonstrated that the AMPARs are not saturated by quantal release (Liu *et al.*, 1999; McAllister & Stevens, 2000) and our observations that the EPSC amplitude increased immediately with increase in stimulation frequency from 0.2 to 20 Hz (or from 20 to 40 Hz) within the same stimulus train also strongly argue against this possibility, because it is unlikely that the number of AMPARs will increase without any signal being given in advance to indicate the ensuing changes in stimulus frequency; and (iii) CV of EPSCs during the train is significantly altered, suggesting that changes in EPSCs have a presynaptic origin. Third, our two independent estimates of the quantal size indicated no changes in postsynaptic parameters attributable to the loss of FMRP. Therefore, given that the quantal size, EPSC kinetics and postsynaptic AMPAR conductance are unaffected by the loss of FMRP, our results suggest that excessive increase in *Pr* represents the main mechanism underlying abnormally elevated STP in the hippocampal synapses of *Fmr1* KO mice.

We note that our conclusions are supported by two independent estimates of quantal and AMPAR parameters: the V-M analysis and PSNSV analysis. Indeed, these two approaches use different data to evaluate synaptic parameters. Variations in EPSC waveform arise both from quantal variability as well as the stochastic gating of AMPARs. While V-M analysis bases the estimation of quantal parameters on the fluctuations of EPSC wave peaks, PSNSV analysis uses fluctuation around the scaled mean waveform of the decay part of EPSC to bypass variation arising from quantal properties, and provide estimates for AMPAR single-channel conductance and the number of open channels (i.e., activated AMPARs). Our estimates of quantal size, based on PSNSV analysis and correlation fits, are in a close agreement to that calculated from V-M analysis, which provide further confirmation that the changes in *Pr*, but not in quantal size or AMPAR properties, account for the abnormal STP in the absence of FMRP. If the CA3-CA1 synapse is typical of central glutamate synapses, then our results supports the emerging view that the presynaptic function of FMRP plays an important role in STP via regulation of *Pr*.

Mechanisms responsible for FMRP regulation of release probability and STP

Our previous studies revealed that among several forms of STP, augmentation is the predominant STP component affected by the loss of FMRP in CA3-CA1 synapses (Deng *et al.*, 2011). Augmentation is thought to arise from elevation in presynaptic calcium levels during repetitive activity, and subsequent increase in *Pr* (Zucker & Regehr, 2002), suggesting that FMRP might be involve in modulation of presynaptic Ca^{2+} dynamics, including action potential (AP)-driven Ca^{2+} influx, cytoplasmic Ca^{2+} buffering, uptake/release by internal stores, and calcium extrusion (Scott, 2007). Our previous analysis that examined these possibilities points to abnormal increase in AP-driven Ca^{2+} influx as the major process responsible for excessive elevation of presynaptic Ca^{2+} levels during repetitive activity in *Fmr1* KO mice (Deng *et al.*, 2011). Moreover, we recently found that FMRP regulates presynaptic calcium influx via modulation of AP duration in CA3 pyramidal neurons. This regulation is translation-independent and is mediated by BK channels. In the absence of FMRP, the activity of BK channels is reduced, leading to

excessive AP broadening during repetitive activity, thus prolonging the open time of voltage-gated Ca^{2+} channels and enhancing presynaptic Ca^{2+} influx (Contractor, 2013; Deng *et al.*, 2013). Our observations that these actions of FMRP are prominent mainly during high-frequency activity (due to calcium-dependent nature of BK channels), provide a plausible reason for activity-dependent changes in the *Pr* observed in the current study and the lack of significant changes in the basal *Pr* in the absence of FMRP observed in these synapses by us and others (Pfeiffer & Huber, 2007; Gibson *et al.*, 2008; Deng *et al.*, 2011; Patel *et al.*, 2013).

CA3-CA1 synapses can maintain a sustained synaptic transmission during high frequency stimulation. This property relies on the availability of release-ready synaptic vesicles. Due to the limited size of synaptic vesicle pools, the vesicle replenishment is thus critical for maintenance of synaptic transmission during repetitive activity. Indeed, presynaptic terminals are able to efficiently recycle synaptic vesicles, which can be reused for hundreds or even thousands of exo-endocytic cycles (Saheki & De Camilli, 2012). The readily releasable pool is selectively replenished by vesicles generated by clathrin-mediated endocytosis, whereas activity-dependent bulk endocytosis generates additional vesicles to increase reserved pool capacity (Cheung *et al.*, 2010). Presynaptic Ca^{2+} level is thought to be critical for vesicle replenishment/recycling and mobilization (Sudhof, 1995; Balaji *et al.*, 2008; Saheki & De Camilli, 2012). Thus, in *Fmr1* KO mice the excessively elevated presynaptic Ca^{2+} during repetitive activity might contribute to accelerate vesicle recycling and vesicle pool refilling. Indeed, we observed an increased vesicle recycling rate in *Fmr1* KO mice (Deng *et al.*, 2011). Also, it has been shown that the readily releasable vesicle pool and reserved pool are larger in *Fmr1* KO mice (Deng *et al.*, 2011). Collectively, these morphological findings (larger size of vesicle pools) and functional analyses (enhanced Ca^{2+} influx and faster vesicle recycling) support the notion that *Fmr1* KO mice have an essential basis for maintaining a higher release probability during high frequency stimulation.

It remains to be determined whether other translation-dependent and -independent functions of FMRP contribute to the regulation of presynaptic calcium and release probability. Given the critical importance of release probability in determining the amount and specificity of synaptic information transmission (Rotman *et al.*, 2011), our results suggest that FMRP activity-dependent regulation of release probability might play an important role in understanding neuropathology of FXS.

Acknowledgments

This work was partly supported by Shenghua Scholar Program of Central South University, grants from National Natural Science Foundation of China (81370248 and 81070941), the NINDS R01 NS081972, FRAXA Foundation, and McDonnell Center for Systems Neuroscience, USA.

Abbreviations

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMPAR	AMPA receptor
AP	action potential

APV	Amino-5-phosphonopentanoic acid
BK	large conductance Ca^{2+} -activated K^{+} channel
CV	coefficient of variation
EPSC	excitatory postsynaptic current
FMRP	fragile X mental retardation protein
FXS	fragile X syndrome
γ	conductance
GABA	γ -aminobutyric acid
M-V analysis	variance-mean analysis
mGluR-LTD	metabotropic glutamate receptors-dependent long-term depression
NMDA	N-methyl-D-aspartate
N_{site}	number of total independent release sites
N_{released}	actual number of independent release sites that released quanta
N_{AMPA}	number of AMPARs activated
$N_{\text{AMPA}/q}$	number of AMPARs activated in response to a single quantum release
PC	pyramidal cell
Pr	neurotransmitter release probability
PSNSV analysis	peak-scaled nonstationary variance analysis
SC	Schaffer collateral
STP	short-term plasticity

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FIG. 1. Abnormal synaptic strength is related to increased presynaptic transmitter release, but not accompanied by changes in EPSC kinetics at CA3-CA1 synapses of *Fmr1* KO mice (A) and (B), Sample traces of EPSCs recorded from CA1 pyramidal cells evoked by stimulation of Schaffer collaterals in CA1 stratum radiatum with 100 stimuli at 20 Hz, preceded by 5 and followed by 10 stimuli at 0.2 Hz. Stimulus artifacts were removed for clarification. Upper traces show the whole burst of EPSCs, the first preceding and last followed EPSCs; lower traces show the first 10 EPSCs of the burst. Gray dashed lines show the folds of baseline EPSC amplitude. Data of WT (A) were plotted in black and those of

KO (B) in red, from here on, unless stated otherwise. Note the enhanced increase in EPSC amplitude of KO mice.

(C) Normalized EPSC amplitude was exaggeratedly increased in *Fmr1* KO mice during the train.

(D) Coefficient of variation (CV) of EPSCs was decreased during the train stimulation, indicating that the increase in EPSCs amplitudes during repetitive activity are, at least in part, attributed to changes in presynaptic transmitter release. The letters a, b, c and d indicate the time points as shown in (C). ** $P < 0.01$.

(E) EPSC latency (*upper*), rise time (10–90%, *middle*), and decay time (*lower*) during the train. Though the EPSC kinetics varied along the train (especially the latency), no significant differences were observed between WT and KO mice, indicating that the kinetics of transmitter release, diffusion and clearance, as well as the kinetics of gating of postsynaptic AMPARs, are not affected significantly in the *Fmr1* KO mice.

FIG. 2. Baseline synaptic transmission is unaffected at CA3-CA1 synapses in *Fmr1* KO mice

(A) Sample traces of EPSCs recorded from CA1 pyramidal cells evoked by stimulation of SC at 0.2 Hz in different bath Ca^{2+} concentrations, 100 EPSCs were superimposed. Gray traces in left panel and red traces in right panel are average of corresponding EPSCs.

(B) Summarized data of EPSC amplitudes. ns, not significant.

(C) Kinetics of EPSCs at different bath Ca^{2+} concentrations. $[\text{Ca}^{2+}]_o$ 1, 2 and 4 denote the bath Ca^{2+} concentration 1, 2 and 4 mM, respectively. ns, not significant.

(D) Coefficient of variation (CV) of EPSCs at difference bath Ca^{2+} concentrations. Note the significantly decreased CV along with the bath Ca^{2+} concentration increase, in agreement with that rising bath calcium concentration increases presynaptic release probability. ** $P < 0.01$.

(E) Sample plot (from WT) of variance-mean (V-M) of EPSC were used to calculate quantal size and Pr . Digital around the scatters are Pr at different Ca^{2+} concentrations for this specific sample.

(F) and (G), Summarized quantal size (F), circles are individual values; bars represent average) and summarized Pr (G). Note there are no significant differences of quantal size and Pr between WT and KO mice.

(H) Variance and mean values from each neuron were normalized by dividing each value by the corresponding predicted maximum EPSC. Normalized scatter plots and curves are similar between WT and KO mice, indicating that quantal size and Pr are not significantly altered in KO mice.

FIG. 3. Postsynaptic AMPAR conductance is not affected at CA3-CA1 synapses in *Fmr1* KO mice at basal state

(A) Schematic illustration of peak-scaled non-stationary variance (PSNSV) analysis. All EPSCs were aligned according to the steepest rise points, and then averaged them as the EPSC waveform. The EPSC waveform was scaled to the same amplitude of individual EPSCs, according to the peak position of EPSC waveform. The decay part of scaled EPSC waveform was divided into 100 equal sections along the amplitude. The corresponding 100 time-intervals (which are not equal in time duration) were used to calculate the difference between scaled EPSC waveform and individual EPSCs. Variance is the squared difference. The variance and their decay parts of EPSCi were binned in 100 sections according to the 100 time-intervals; the binned variance and EPSCi of all EPSCs were pooled in corresponding bins and averaged. Finally, the averages of binned variance was plotted against averages of binned EPSC, which were then fitted by a parabolic function (see B) and the AMPAR single channel conductance and the number of AMPARs activated were calculated. (B) Sample plots of variance against current of PSNSV analysis were used to calculate AMPAR conductance and number at different Ca²⁺ concentration. Digits around parabolas are numbers of AMPARs activated at the peak of EPSCs (in parentheses) and conductance (with the unit pS) for these sample cells.

(C) Summarized data of AMPAR single channel conductance. Note no significant difference among different Ca^{2+} concentration, as well as between WT and KO mice.

(D) Summarized data of number of AMPARs (channels) activated. Note the numbers of AMPARs activated significantly increase along with the increasing Ca^{2+} concentration, but no significant differences were found between WT and KO mice at the same Ca^{2+} concentrations.

FIG. 4. Loss of FMRP causes abnormally increased release probability during repetitive activity, without changes in quantal size and postsynaptic AMPAR conductance

(A) Sample traces of EPSCs recorded from CA1 pyramidal cells evoked by stimulation of SC with multiple frequency train (50 stimuli at 20 Hz followed by 50 stimuli at 40 Hz, leaded and followed by 10 stimuli at 0.2 Hz, only the first leading stimulus and last following stimulus were shown). Stimulus artifacts were removed for clarification. Gray dashed lines show the folds of baseline EPSC amplitude.

(B) Summarized data of EPSCs for the train stimulation. Note the exaggerated increase in EPSC amplitude in *Fmr1* KO mice for both 20 Hz and 40 Hz stimulations. Inserts are

individual EPSCs of 0.2 Hz (*thin traces*), at the end of 20- (*medium size traces*) and 40-Hz (*thick traces*), corresponding to the time points indicated by a, b and c, respectively. EPSCs of 20- and 40-Hz were normalized to corresponding EPSCs of 0.2 Hz. Stimulation artifacts were removed for clarification. WT in black and KO in red.

(C) Sample plots of PSNSV analysis were used to calculate AMPAR conductance and number during complex train stimulation. Digitals around parabolas are numbers of AMPARs activated (in parentheses) and conductance (with the unit pS) for these sample cells (see Fig. 5A for summarized data).

(D) Summarized data of AMPAR conductance during complex train stimulation. Note there are no significant differences between WT and KO mice, as well as under different stimulation frequency.

(E) Sample plots of V-M analysis were used to calculate quantal size and actual sites that released quanta during complex train stimulation. Digitals around the scatters are Pr at different stimulation frequency for these sample cells. Digitals in parentheses are mean actual number of sites that released quanta (the production of $N_{\text{sites}} \times Pr$) for these sample cells (see Fig. 5B for summarized data).

(F) Summarized Pr during complex train stimulation. Note exaggerated increase in Pr in *Fmr1* KO mice at 20 Hz and 40 Hz. * $P < 0.05$; ** $P < 0.01$.

FIG. 5. Excessively elevated release probability during repetitive activity results in abnormal short-term plasticity in *Fmr1* KO mice

(A) Summarized data of number of AMPARs activated (N_{AMPA}) during complex train stimulation. Note that rising stimulus frequency increases N_{AMPA} .

(B) Summarized data of actual number of sites that released quanta ($N_{\text{released}} = N_{\text{sites}} \times Pr$) during complex train stimulation. Note that rising stimulus frequency increases N_{released} .

(C) N_{AMPA} was plotted against N_{released} cell by cell. Scatters were well fitted linearly, yielding a slope factor around 10~11. Note that the slope factors between genotypes are very close, supporting that quantal size is not changed significantly in *Fmr1* KO mice.

(D) Quantal sizes estimated from two independent approaches are very close both within or between genotypes.

(E) The amplitude of EPSC is given by $\text{EPSC} = N_{\text{site}} \times Pr \times q$, or $\text{EPSC} = N_{\text{site}} \times Pr \times N_{\text{AMPA}/q} \times \gamma \times V$. Because the quantal size (q estimated by V-M analysis, or by correlation fits, i.e., $N_{\text{AMPA}/q} \times \gamma \times V$), postsynaptic AMPAR conductance (γ), and total independent release sites for a given recording (N_{site}) are unchanged during train stimulation, our analysis thus suggests that the abnormal STP in the absence of FMRP is attributed predominately to an abnormally increased Pr during repetitive activity. Single yellow arrows indicate increase, double yellow arrows excessive increase. nc, not changed.