ABSTRACT: Fragile X syndrome (FXS) is the most common form of inherited intellectual disability in humans. This X-linked disorder is caused by the transcriptional repression of a single gene, Fmr1. The loss of Fmr1 transcription prevents the production of fragile X mental retardation protein (FMRP) which in turn disrupts the expression of a variety of key synaptic proteins that appear to be important for intellectual ability. A clear link between synaptic dysfunction and behavioral impairment has been elusive, despite the fact that several animal models of FXS have been generated. Here we report that Fmr1 knockout mice exhibit impaired bidirectional synaptic plasticity in the dentate gyrus (DG) of the hippocampus. These deficits are associated with a novel decrease in functional NMDARs (N-methyl-D-aspartate receptors). In addition, mice lacking the Fmr1 gene show impaired performance in a context discrimination task that normally requires functional NMDARs in the DG. These data indicate that Fmr1 deletion results in significant NMDAR-dependent electrophysiological and behavioral impairments specific to the DG. © 2010 Wiley-Liss, Inc.

KEY WORDS: dentate gyrus; fragile X syndrome; synapse; long-term potentiation; long-term depression

INTRODUCTION

Fragile-X syndrome (FXS) is a form of mental retardation that is initiated by cytosine-guanine-guanine (CGG) trinucleotide repeat expansion (>200) and is associated with autism in males. The trinucleotide expansion causes hypermethylation of the human Fmr1 gene (Turner et al., 1996), resulting in complete transcriptional repression, and loss of production of the fragile X mental retardation protein (FMRP; Pieretti et al., 1991; Verkerk et al., 1991; Ashley et al., 1993). This protein is expressed throughout the body, but is highly expressed in dentate gyrus (DG) granule neurons (Hinds et al., 1993). FMRP itself can shuttle several mRNAs from the nucleus to the cytoplasm and then to distal postsynaptic sites (Zalfa et al., 2007; Bassell and Warren, 2008). Among the mRNAs that FMRP normally binds to are several that are important for synaptic plasticity, including those that code for NMDAR (N-methyl-D-aspartate receptor) subunits NR1 and NR2B, and components of the postsynaptic density, such as PSD-95 and CaMKIIα (Brown et al., 2001; Darnell et al., 2004; Zalfa et al., 2007; Schutt et al., 2009). NMDAR subunits, in particular, are thought to play a vital role in learning and memory processes (Bliss and Collingridge, 1993). Thus it seems logical that a loss of FMRP should alter the expression of synaptic proteins integral to synaptic plasticity and lead to cognitive deficits.

Surprisingly, it has proven difficult to elucidate a robust impairment in learning and memory in mice that lack FMRP (1994; D’Hooge et al., 1997; Yan et al., 2004; Eadie et al., 2009). Impairments in synaptic plasticity have been equally elusive (Godfraind et al., 1996; Huber et al., 2002), with the exception of an increase in mGluR (metabotropic glutamate receptor)-mediated LTD in the CA1 subfield of the hippocampus (Huber et al., 2002; Bear et al., 2004). While this does indicate that some learning and memory mechanisms could be altered, a link between mGluR-mediated LTD and any intellectual impairment in these mice remains unclear (Dolen et al., 2007). Indeed, adult Fmr1 KO mice show normal learning in the Morris water maze, a task dependent on the CA1 subfield of the hippocampus (D’Hooge et al., 1997; Eadie et al., 2009).

Recently, a number of studies have indicated that developing neurons in the brain may be disproportionately affected by a loss of FMRP expression. Significant structural deficits are selectively apparent in the dendrites of young neurons in the neocortex of Fmr1 KO mice, and the transient nature of these spine abnormalities suggests that this could be a deficit that might impact learning capacity during early critical periods (Nimchinsky et al., 2001). Similarly, young cultured hippocampal neurons exhibit marked decreases in morphological complexity (Braun and Segal, 2000; Castren et al., 2005), and recently it has been reported that very young Fmr1 KO mice show robust impairments in CA1 synaptic plasticity (Hu et al., 2008; Pilpel et al., 2009). While deficits in the CA1 subfield of the adult hippocampus have been difficult to document, we have recently shown that the process of neurogenesis in the adult DG is abnormal in mice that lack FMRP (Eadie et al., 2009). These newly generated neurons contribute disproportionately
to NMDAR-dependent synaptic plasticity in the DG (van Praag et al., 1999; Farmer et al., 2004; Ge et al., 2008). Despite this, investigations into synaptic plasticity in the DG of Fmr1 KO mice have been lacking, as have an assessment of the putative behavioral ramifications of impaired structural and functional plasticity in the DG of Fmr1 KO mice. The present work demonstrates that a loss of FMRP in the mammalian brain alters NMDAR-dependent synaptic plasticity in the DG. In addition, we show that context discrimination, a behavior reliant on intact NMDAR function in the DG (McHugh et al., 2007; pp 1640), is altered in Fmr1 KO mice. Therefore, this study introduces NMDARs in the DG as a novel target in the pathology of FXS.

**MATERIALS AND METHODS**

**Animals**

Eighty-six C57BL/6, male Fmr1 knockout (KO) (n = 42) and Fmr1 wild-type (WT) (n = 44) littermate mice were produced by breeding WT male C57BL/6 (Jackson Laboratories) mice with female C57BL/6 mice heterozygous for the Fmr1 gene. All animals were sexed, weaned, ear-punched, and tail-snipped at postnatal day 24 and group-housed with minimal enrichment (tubes and/or nesting materials). Adult male mice (2–4 months) were randomly assigned to electrophysiology or behavioral experiments (Fig. 1). Experiments were conducted on young, adult male mice between 2 and 4 months of age.

**Genotyping**

**DNA extraction and purification**

For DNA extraction and purification, tissue from each animal was placed in 180 μl digestion buffer and 20 μl Proteinase K in a DNase/RNase-free 1.5 ml Eppendorf tube and incubated overnight in a thermomixer at 55°C while being agitated at 300 RPM. The sample was centrifuged at 21,000 RCF for 3 min and the supernatant was transferred into a new tube. About 20 μl RNase A was then added, vortexed, and incubated at room temperature for 2 min. First 200 μl lysis buffer and then 200 μl 100% EtOH was added to each tube and vortexed. The lysate was transferred to a clean spin column and centrifuged at 10,000 RCF for 1 min at room temperature. The spin column was then placed in a fresh tube and washed by adding 500 μl Wash Buffer I and centrifuged at 10,000 RCF for 1 min at room temperature. This process was repeated with 500 μl Wash Buffer II and centrifuged at 21,000 RCF for 3 min at room temperature. About 100 μl Elution Buffer was added to the spin column and incubated for 1 min followed by centrifugation at 21,000 RCF for 1 min at room temperature.

**PCR analysis**

The reaction was performed by mixing 11 μl nuclease-free H2O, 2.5 μl 10× PCR reaction buffer, 2.5 μl (50 mM) MgCl2, 2.0 μl (2.5 mM) dNTP, 1.25 μl of each forward and reverse primer, 2 μl DNA, and 0.5 μl Taq DNA polymerase (Invitrogen Canada; Burlington, Ontario, Canada). The cycling parameters employed were: first cycle of 5 min at 94°C, then 35 cycles of 60 s at 94°C, 90 s at 65°C, and 150 s at 72°C. Primers M2 = 5’ ATCTAGTCATGCTATGGATATCAGC 3’ and N2 = 5’ GTGGGCTCTATGGCTTCTGAGG 3’ were used to test for KO allele (amplified fragments of 800 base pairs). Primers S1 = 5’ GTGGTTAGCTAAAGTGAGGATGAT 3’ and S2 = 5’ CAGGTTTTGTTGGGATTAAACAGATC 3’ were used to test for the WT mouse allele amplifying a fragment of 465 base pairs. PCR products were run on a 1.5% agarose gel with 10,000× SYBR-safe (1:13,333 in 1× TAE) and visualized under a BioRad trans-illuminator.

**FIGURE 1.** Outline of experimental procedures. Schematic showing the progression of the experiments and group assignments. Male Fmr1 knockout (KO) and wild-type (WT) littermate mice were weaned and ear-punched at postnatal Day 24. Mice were pseudorandomly assigned to either electrophysiology or behavior experiments commencing at postnatal Day 60. All experiments were conducted on young, adult male mice between 2 and 4 months of age.
Electrophysiology

**Slice preparation**

*Fmr1* KO (*n* = 19) and WT littermate (*n* = 21) mice were used for the electrophysiology experiments. Mice were anesthetized with isoflurane, rapidly decapitated, and their brains removed in oxygenated (95% O₂/5% CO₂), ice-cold normal ACSF (nACSF) [(in mM) 125 NaCl, 2.5 KCl, 1.25 NaHPO₄, 25 NaHCO₃, 2 CaCl₂, 1.3 MgCl₂, and 10 dextrose, pH 7.3]. Transverse hippocampal slices (350 μm) were sectioned using a Vibratome 1500 (Ted Pella, Redding, CA). Sections were kept in order using a modified 24-well plate and incubated in continuously oxygenated nACSF at 30°C. Sections were allowed to rest for a minimum of 1 h before recordings commenced.

**Field electrophysiology recordings**

Field recordings were collected in nACSF using an Axon MultiClamp 700B amplifier connected to a PC running Clampex 10.2 software (Molecular Devices, CA). Electrodes were placed under visual guidance using an Olympus BX51 microscope and motorized micromanipulators (Siskyou Design, OR). Field excitatory postsynaptic potentials (fEPSPs) were elicited by delivering a 120 μs (10–40 μA) current pulse to the medial perforant path (MPP) of the DG using a digital stimulus amplifier (Getting Instruments, CA) and a single, concentric bipolar stimulating electrode (FHC, Bowdoin, ME). fEPSPs were recorded using a single glass recording electrode (0.5–1.5 MΩ) filled with nACSF, and placed in the MPP about 200 μm from the stimulating electrode. Stimulation magnitude was set to elicit a response of ~50% and an input–output experiment was conducted with increasing stimulation magnitude (30- to 300-μs pulse width; 30-μs intervals). Paired-pulse recordings were subsequently performed using an interpulse interval of 50 μs (5×; 20 s between pairings). The GABA<sub>A</sub> receptor antagonist bicuculline methiodide (5 μM; Sigma-Aldrich, Oakville, ON, Canada) was included to isolate the excitatory component of synaptic transmission for all recordings. A stable baseline (minimum 20 min) of the slope of fEPSPs elicited every 15 s was then achieved before a conditioning stimulus (CS) was delivered to induce synaptic plasticity. Baseline stimulation parameters were returned to following the CS (minimum 60 min). All analyses were conducted with Axon ClampFit 10.2 software (Molecular Devices, CA).

**Conditioning stimulation protocols**

Long-term potentiation (LTP) of fEPSPs was induced using a CS consisting of four trains of 50 pulses at 100 Hz, 30 s apart (high-frequency stimulation; HFS); whereas, long-term depression (LTD) of fEPSPs was induced using a CS of 900 pulses delivered at 1 Hz over 15 min (low-frequency stimulation; LFS).

**Whole cell electrophysiology**

Whole-cell recordings were obtained using an Axon Axopatch 200B amplifier connected to a PC running Clampex 10.2 software (Molecular Devices, CA). The intracellular recording solution consisted of (in mM) 20 KCl, 120 KGluc-o-nate, 4 NaCl, 0.10 EGTA, 4 ATP, 0.3 TrisGTP, 14 Phospho-creatine. Biocytin (0.2%) was also included for post hoc staining of the recorded neuron. The resistance of recording electrodes was 5–7 MΩ. Only recordings with a series resistance of less than 30 MΩ were used in analyses.

The resting membrane potential (RMP) was obtained immediately following break-in in voltage clamp mode. Series resistance (*R*) was calculated from back-fitting recordings from a +5 mV (−90 to −85 mV) 300 μs step (average of 100 recordings) using Axon ClampFit 10.2 software. AMPAR (α-amino-3-hydroxyl-5-methyl-isoxazole-propionate receptor)-mediated currents were evoked using extracellular stimulation, while granule cell was held at −70 mV. Following an estimation of the maximum EPSC amplitude, an input–output function was obtained. NMDAR-mediated EPSCs were obtained by washing over a low Mg<sup>2+</sup> (0.1 mM) ACSF containing the AMPAR antagonist NBQX (5 μM; Sigma-Aldrich, MO). NMDAR-mediated EPSCs were blocked using APV (50 μM).

**Behavior**

**Modified SHIRPA**

A separate cohort of mice (WT: *n* = 10, KO: *n* = 10) was assessed using a modified version of the SHIRPA test battery (Rogers et al., 1997). The measures were: weight, body length, body position, spontaneous activity, respiratory rate, tremor, urination, defecation, transfer arousal, piloerection, startle, gait, provoked biting, righting reflex, contact righting reflex, negative geotaxis, fearfulness, irritability, aggression, and vocalization.

**Contextual fear acquisition and generalization**

As part of an assay to assess contextual fear discrimination, the ability of *Fmr1* KO (*n* = 14) and WT (*n* = 14) mice to acquire contextual fear was first evaluated (as described previously by McHugh et al., 2007). On Days 1 through 4 of testing, a single animal was exposed to a single type of context for a 4-min, 2-s session. Three minutes into the session, a 2 s, 650-μA shock was delivered through stainless steel rods composing the floor of the apparatus to induce conditioned fear. The mice were left in the context for another minute. The dependent measure was freezing behavior within the first 3 min (i.e., preshock) of each session. Freezing was measured using the Video Freeze software with the freezing threshold set at 19 and the minimum freeze duration set at 1 s. Two types of contexts were used. Context A (used as the S+ context) consisted of four identical conditioning chambers (30 × 25 × 25 cm<sup>3</sup>; Med-Associates) placed in a sound-attenuating cubicle with a 60-dB back ground noise provided by a fan and scented with Windex original glass cleaner. An overlay of two white plastic panels created a continuous curve to the side and back walls.

**Hippocampus**
The floor of each chamber was made up of 16 stainless steel rods of alternating diameter (0.4 and 1.0 cm) spaced 1.5-cm apart (center to center) wired to a shock generator and scrambler (Med-Associates) to deliver foot shock. Each chamber was wiped down with 70% ethanol before conditioning and between animals. Context B (used as the S+ context) consisted of four identical conditioning chambers (30 × 25 × 25 cm³; Med-Associates) placed in a sound-attenuating cubicle in a different room from Context A with a fan providing a background noise of 60 dB and scented with Simple Green. The grid floors were the same as those used in A to increase the similarity of the contexts. The side walls consisted of two black plastic panels joined at the top and sloping down to the side of the conditioning chamber to form an A-frame.

Assessment of generalization of contextual fear occurred on Days 5 and 6 of testing and involved two 3-min sessions separated by 2 h. Mice were exposed to both the context that they were previously shocked (S+) and a novel context (S−); however, the mice did not receive any shocks during these sessions. The sessions on Day 6 were conducted in reverse order. Freezing behavior was assessed as described for assessment of acquisition of contextual fear.

**Contextual fear discrimination**

Days 7 through 14 of testing consisted of discrimination training where animals were placed in both the S+ and S− contexts each day. Again, in the S+ context, mice received a 2 s, 650-μA shock after 3 min and were left in the chamber for 1 min following shock. In the S− context, mice were simply placed in the chamber for an equivalent 4 min and 2 s. Freezing was measured during the 3-min preceding shock on all days in which shock was administered and the equivalent period of time in the S− context. The order of training followed a double alternation schedule: Day 7 S−→S+, Day 8 S+→S−, Day 9 S+→S−, Day 10 S−→S+, etc. For statistical analysis and graphical presentation the data was collapsed into consecutive 2-day blocks so that each block consisted of 1 day of S+ → S− and 1 day of S− → S+.

**Extinction of contextual fear**

During the first 3 min of Days 5, 6, and 7 of testing, all mice were exposed to the S+ context (i.e., the context that was paired with a shock during acquisition training), but did not receive a shock. Note that on Day 7, the mice do receive a shock in the S+ that affects freezing behavior in subsequent sessions, but not the current session. Extinction can thus be measured as decreased freezing in the S+ context over Days 5, 6, and 7 of testing.

**Statistical Analyses**

Group data are presented as mean ± standard error of the mean (SEM). Differences between mean values of experimental groups were compared using Student’s t test, with Bonferroni correction, or analysis of variance (ANOVA) followed by Tukey’s post hoc tests as appropriate using Statistical 7.0 software (StatSoft, Tulsa, OK). Statistical significance: P < 0.05.

**RESULTS**

**Normal Basal Synaptic Transmission and Short-Term Synaptic Plasticity in the Dentate Gyrus of Fmr1 KO Mice**

Stimulating and recording electrodes were placed in the molecular layer of the DG to elicit excitatory postsynaptic potentials (fEPSPs) in transverse hippocampal sections obtained from young adult Fmr1 knockout (KO) and wild-type (WT) littermate mice. Input/Output (IO) functions and paired-pulse measures of presynaptic transmitter release were recorded for WT and Fmr1 KO mice (Fig. 2). The slope of the fEPSP significantly increased with increasing stimulation (repeated measures ANOVA; WT n = 10, Fmr1 KO n = 12; F(8,160) = 362.68, P = 0.000). A significant interaction between stimulation strength and genotype (F(8, 160) = 1.560, P = 0.141) or a significant main effect of genotype (F(1,20) = 1.795, P = 0.195) were not apparent. These data suggest that basal synaptic transmission is not significantly altered in the dentate gyrus (DG) by loss of FMRP. Short-term synaptic plasticity was then assayed using a paired-pulse protocol with a 50-μs interpulse interval. Paired-pulse depression in the medial perforant path (MPP) of the DG was clear in both genotypes (WT: −9.23 ± 3.63, n = 6; Fmr1 KO: −7.49 ± 4.66, n = 5) but not significantly different between genotypes (t test; t(9) = 0.102, P = 0.921). These data suggest that there is no obvious difference in basal synaptic transmission or short-term synaptic plasticity in the DG of adult Fmr1 KO and WT mice.

**Decreased NMDAR-Dependent LTP in the Dentate Gyrus of Fmr1 KO Mice**

The application of a conditioning stimulus, consisting of four trains of high-frequency stimuli (HFS; 50 pulses at 100 Hz), produced a robust LTP in WT animals (Fig. 3; 50–60 min Post-HFS: 73.01% ± 8.64% (n = 15); t test, P < 0.001). The magnitude of LTP was significantly decreased in Fmr1 KO mice (44.27% ± 7.51%, n = 15; t test, P = 0.034). The inclusion of the NMDAR antagonist APV (50 μM) during HFS severely attenuates LTP in both WT and Fmr1 KO slices (WT: 8.17% ± 3.75%, n = 14; Fmr1 KO: 3.79% ± 6.98%, n = 7), indicating that the LTP observed in both groups was primarily NMDAR-dependent.

**Decreased NMDAR-Dependent LTD in the Dentate Gyrus of Fmr1 KO Mice**

The application of low frequency stimulation (LFS) induced a significant LTD in the DG of sections obtained from WT mice (Fig. 4; 50- to 60-min Post-LFS: −21.63% ± 4.03% (n = 14); t test, P < 0.000). In contrast, LFS failed to induce
magnitudes (Fig. 5; WT: 328.6 ± 6.6 pA; Fmr1 KO: 399.8 ± 16.8 pA (n = 5); t test, P = 0.04577). This is consistent with our observation of comparable IO functions between genotypes. Following collection of AMPAR-mediated IOs, NMDAR-mediated currents were obtained by application of the AMPAR antagonist NBQX (5 μM) in low-Mg²⁺ ACSF. In all cells, the EPSC was challenged with APV (50 μM) at the end of the experiment to confirm that these were indeed NMDAR-mediated responses. Following acquisition of NMDAR-mediated EPSCs, an IO function was then obtained in a manner similar to that described for AMPAR-mediated EPSCs. The absolute maximum NMDAR-mediated EPSC was significantly decreased in Fmr1 KO mice relative to WT littermates [WT: 281.3 ± 18.2 pA (n = 5), Fmr1 KO: 168.5 ± 48.0 pA (n = 5); t test, P = 0.0197]. A comparison of AMPAR/NMDAR current ratios between the genotypes revealed a significant increase in the ratio in DG neurons from Fmr1 KO mice [WT = 1.190 ± 0.198 (n = 5), Fmr1 KO = 2.714 ± 0.617 (n = 5); t test, P = 0.0302]. Loss of FMRP appears to decrease NMDAR-mediated currents in DG granule neurons, and it is likely that this is responsible for the impaired NMDAR-dependent bidirectional synaptic plasticity.

Decreased NMDAR-Mediated Synaptic Transmission in the DG of Fmr1 KO Mice

The data thus far indicate that a loss of FMRP substantially limits bidirectional synaptic plasticity in the DG. The NMDAR makes a significant contribution to both forms of synaptic plasticity in the DG (Vasuta et al., 2007), thus it may be that NMDAR function is downregulated in these animals. To determine if a loss of FMRP alters NMDAR function, we performed whole-cell voltage clamp experiments to examine both AMPAR- and NMDAR-mediated components of the excitatory post synaptic currents (EPSCs). All experiments were performed with inhibition blocked, and each neuron was filled with biocytin (0.2%) to allow for morphological confirmation of cell identity at the end of the experiment. AMPAR-mediated EPSCs were obtained by holding the neurons at −70 mV in artificial cerebrospinal fluid (nACSF). AMPAR-mediated EPSCs were evoked with increasing afferent stimulation to obtain reliable maximum responses. Both genotypes showed absolute maximal AMPAR-mediated currents of comparable magnitudes [Fig. 5; WT: 328.6 ± 39.6 pA (n = 5), Fmr1 KO: 399.8 ± 94.1 pA (n = 5); t test, P = 0.4577]. This is consistent with our observation of comparable IO functions for fEPSPs between genotypes. Following collection of AMPAR-mediated IOs, NMDAR-mediated currents were obtained by application of the AMPAR antagonist NBQX (5 μM) in low-Mg²⁺ ACSF. In all cells, the EPSC was challenged with APV (50 μM) at the end of the experiment to confirm that these were indeed NMDAR-mediated responses. Following acquisition of NMDAR-mediated EPSCs, an IO function was then obtained in a manner similar to that described for AMPAR-mediated EPSCs. The absolute maximum NMDAR-mediated EPSC was significantly decreased in Fmr1 KO mice relative to WT littermates [WT: 281.3 ± 18.2 pA (n = 5), Fmr1 KO: 168.5 ± 48.0 pA (n = 5); t test, P = 0.0197]. A comparison of AMPAR/NMDAR current ratios between the genotypes revealed a significant increase in the ratio in DG neurons from Fmr1 KO mice [WT = 1.190 ± 0.198 (n = 5), Fmr1 KO = 2.714 ± 0.617 (n = 5); t test, P = 0.0302]. Loss of FMRP appears to decrease NMDAR-mediated currents in DG granule neurons, and it is likely that this is responsible for the impaired NMDAR-dependent bidirectional synaptic plasticity.

Intact Passive and Active Membrane Properties of Granule Neurons in the Dentate Gyrus of Fmr1 KO Mice

The resting membrane potential (RMP), input resistance and whole cell capacitance were not significantly different between genotypes (Table 1). In current clamp mode, the rheobase and action potential frequency were determined and were also not significantly different between genotypes. In voltage clamp mode, a depolarizing voltage step protocol (−70 to +70 mV; 10-mV intervals) was employed to assess both a clear fast inward current (i.e., Na⁺) and a slower outward current (i.e., K⁺) and a hyperpolarizing voltage step protocol (−70 to −120 mV; 5 mV intervals) was employed to assess an inward...
rectifying current (i.e., K+ IR; Fig. 6). The peak inward current to the depolarizing step protocol changed significantly across holding potentials [WT (n = 7) and KO (n = 5); repeated measures factorial ANOVA, F(14,140) = 68.383, P = 0.000]; however, there was no significant interaction (F14,126) = 0.850, P = 0.614) or main effect of genotype (F1,9) = 0.1685, P = 0.227). These data suggest that there is no obvious difference in outward K+ conductance in dentate granule neurons lacking FMRP. The peak inward current resulting from the hyperpolarizing step protocol changed significantly across holding potentials [WT (n = 7) and KO (n = 5); repeated measures factorial ANOVA,
$F_{(1,160)} = 2.157, P = 0.008$); however, there was no significant interaction ($F_{(1,160)} = 1.117, P = 0.343$) or main effect of genotype ($F_{(1,10)} = 1.191, P = 0.301$). These data suggest that there is no obvious difference in inward-rectifying K$^+$ conductance in dentate granule neurons lacking FMRP.

**Modified SHIRPA Indicates That Fmr1 KO Mice are Behaviorally Similar to Wild-Type Animals**

No significant differences were observed between WT and Fmr1 KO mice in any of the measures in the SHIRPA test battery ($t$ tests, $P > 0.05$ for all measures; Table 2). This indicates that there are no significant behavioral abnormalities that could interfere with the performance of these animals in the context fear discrimination procedure used here.

**Normal Acquisition and Generalization of Contextual Fear in Fmr1 KO Mice**

NMDAR function in the hippocampus is critical to contextual fear learning and NMDARs in the DG contribute to the ability to learn to discriminate between contexts (Young et al., 1994). Prior to the assessment of context discrimination, both groups were assessed on acquisition and generalization of contextual fear (Fig. 7). The acquisition of the context-shock association in Fmr1 KO and WT mice was measured over four training days. Repeated measures ANOVA across training days showed no overall effect of genotype ($F_{(1,26)} = 2.87, P = 0.102$) and the effect of training day ($F_{(3,78)} = 16.38, P = 0.000$) and the genotype by training day interaction were not significant ($F_{(3,78)} = 2.09, P = 0.108$). Overall, Fmr1 KO
mice appear to show normal acquisition of contextual fear. Conditional fear responses will occur not only in response to the original conditional stimuli but also to stimuli which show similar or overlapping features, a process referred to as generalization. The extent to which Fmr1 KO and WT mice generalize to a context that was similar, but distinct from, the original training context was tested over a 2-day period. Freezing data from S+ and S− over the two generalization testing days were averaged. Two-way ANOVA indicated a significant effect of context ($F_{(1,26)} = 182.4, P < 0.001$) but the effect of genotype ($F_{(1,26)} = 2.866, P = 0.102$) and the genotype by context interaction ($F_{(1,26)} = 1.298, P = 0.265$) did not reach statistical significance. This indicates that Fmr1 KO mice show normal levels of freezing in the S+ context and equivalent generalization of contextual fear to the S− context.

**Impaired Context Discrimination in Fmr1 KO Mice**

The ability of Fmr1 KO and WT mice to discriminate between the S+ context (paired with shock) and the S− context (never paired with shock) was assessed over 8 days of discrimination training (Fig. 8). Learning this discrimination is thought to require a process of “pattern separation” where the pattern of multi-modal stimuli that defines the S+ context is separated from the pattern of stimuli that define the S− context. This information is utilized to determine the “safe” and the “dangerous” context. Repeated measures ANOVA for freezing in the S+ context showed a significant effect of Training Block ($F_{(3,78)} = 16.38, P = 0.000$) but did not show a Training Block by Genotype interaction ($F_{(3,78)} = 2.09, P = 0.108$) or an overall effect of Genotype ($F_{(1,26)} = 2.87, P = 0.102$).

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### TABLE 1.

**Passive Membrane Properties and Intrinsic Excitability of Dentate Granule Neurons From Wild-Type and Fmr1 Knockout Mice**

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Fmr1 knockout</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (mV)$^a$</td>
<td>−88.64 (±3.73)</td>
<td>−89.87 (±3.19)</td>
<td>0.710</td>
</tr>
<tr>
<td>Input resistance (MΩ)$^b$</td>
<td>173.03 (±34.11)</td>
<td>195.60 (±30.88)</td>
<td>0.478</td>
</tr>
<tr>
<td>Capacitance (pF)$^c$</td>
<td>20.04 (±2.11)</td>
<td>18.41 (±2.58)</td>
<td>0.872</td>
</tr>
<tr>
<td>Rheobase (pA)$^d$</td>
<td>102.86 (±8.33)</td>
<td>103.50 (±6.21)</td>
<td>0.955</td>
</tr>
<tr>
<td>AP frequency (Hz)$^e$</td>
<td>6.70 (±0.44)</td>
<td>6.88 (±0.48)</td>
<td>0.708</td>
</tr>
</tbody>
</table>

Passive membrane properties and intrinsic excitability of dentate granule neurons from adult, male wild-type ($n = 9$ cells; three mice) and Fmr1 knockout ($n = 8$ cells; three mice) mice.

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This indicates that Fmr1 KO and WT mice freeze to a similar extent in the S+ context. Repeated measures ANOVA for freezing in the S− context showed a significant effect of Training Block ($F_{(3,78)} = 28.42, P = 0.000$) and an overall effect of genotype ($F_{(1,26)} = 10.52, P = 0.003$), but did not show a training block by genotype interaction ($F_{(3,78)} = 0.444, P = 0.722$). This analysis indicates that Fmr1 KO mice show elevated freezing in the S− context.

A discrimination ratio was calculated for each individual animal for each training block using the following formula: (Freezing in S+)/((Freezing in S+) + (Freezing in S−)). Discrimination ratios above 0.5 are indicative of significant discrimination. Repeated measures ANOVA on the discrimination ratios indicated a significant effect of training block ($F_{(3,78)} = 20.96, P = 0.000$).
The present work indicates that a loss of FMRP can profoundly impact the capacity for DG to exhibit synaptic plasticity and facilitate discrimination learning. We show for the first time that deletion of the Fmr1 gene impairs long-term potentiation (LTP) and depression (LTD) in the DG subfield of the hippocampus. Both forms of DG synaptic plasticity are NMDAR-dependent, and appear to be associated with NMDAR hypofunction. A significant decrease in NMDAR-mediated currents was apparent in DG granule cells, while AMPAR-mediated currents did not differ significantly between Fmr1 KO and WT mice. Assessment of the ability of Fmr1 KO mice to discriminate between two similar contexts, a task requiring contextual fear, however, they have difficulty in learning to suppress responding to a similar, but safe, context (impaired extinction and discrimination).

**DISCUSSION**

The basic behavioral phenotype of WT and FMR1 KO mice was assessed using the SHIRPA test battery (Reproduced with permission from Rogers et al., *Mammalian Genome*, 1997, 8, 711–713, 1997). No significant differences were observed after Bonferroni correction (*P* < 0.001 required for significance). Abbreviations are Body Position, Spontaneous Activity, Respiratory Rate, Transfer Arousal, Piloerection, Pelvic Elevation, Tail Elevation, Touch Escape, Positional Passivity, Abnormal Behavior, Visual Placing, Pinna Reflex, Corneal Reflex, Wire Maneuver, Abdominal Tone, Salivation, Provoked Biting, Righting Reflex, Contact Righting Reflex, Negative Geotaxis.

28.42, *P* = 0.000) and a significant effect of genotype (*F*<sub>1,26</sub> = 5.02, *P* = 0.034) but no training block by genotype interaction (*F*<sub>3,78</sub> = 0.71, *P* = 0.549). This analysis indicates that Fmr1 KO mice show a significantly reduced discrimination ratio relative to WT mice.

### TABLE 2.

**Modified SHIRPA Assessment of Fmr1 KO and WT Mice**

<table>
<thead>
<tr>
<th></th>
<th>Weight</th>
<th>Squares</th>
<th>Body length</th>
<th>Body posit</th>
<th>Spont act</th>
<th>Resp rate</th>
<th>Tremor</th>
<th>Urination</th>
<th>Defecation</th>
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<th>Piloerec</th>
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<td>0</td>
<td>5</td>
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<tr>
<td></td>
<td>SE</td>
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<td>0.89</td>
<td>0.12</td>
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<tr>
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<td>18</td>
<td>9.03</td>
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</tr>
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</table>

The basic behavioral phenotype of WT and FMR1 KO mice was assessed using the SHIRPA test battery (Reproduced with permission from Rogers et al., *Mammalian Genome*, 1997, 8, 711–713, 1997). No significant differences were observed after Bonferroni correction (*P* < 0.001 required for significance). Abbreviations are Body Position, Spontaneous Activity, Respiratory Rate, Transfer Arousal, Piloerection, Pelvic Elevation, Tail Elevation, Touch Escape, Positional Passivity, Abnormal Behavior, Visual Placing, Pinna Reflex, Corneal Reflex, Wire Maneuver, Abdominal Tone, Salivation, Provoked Biting, Righting Reflex, Contact Righting Reflex, Negative Geotaxis.

Impaired Extinction of Contextual Fear in Fmr1 KO Mice

WT mice showed clear evidence of extinction across Days 5, 6, and 7 in the above behavioral paradigm (Fig. 9). In contrast, Fmr1 KO mice did not exhibit extinction. Repeated measures ANOVA across Days 6 and 7 showed significant main effects of genotype (*F*<sub>1,26</sub> = 5.79, *P* = 0.0235) and day (*F*<sub>1,26</sub> = 9.60, *P* = 0.0046) as well as a genotype by day interaction (*F*<sub>1,26</sub> = 5.90, *P* = 0.0224). Post hoc comparisons indicated that Fmr1 KO mice show greater freezing on Day 7 compared to WT littermates (*t*<sub>28</sub> = 3.205, *P* < 0.01), suggesting that Fmr1 KO mice show impaired fear extinction. Overall, the behavioral analyses indicate that Fmr1 KO mice are able to acquire contextual fear, however, they have difficulty in learning to suppress responding to a similar, but safe, context (impaired extinction and discrimination).
dependent on functional NMDARs and NMDAR-dependent synaptic plasticity specifically in the DG, revealed a robust learning impairment (McHugh et al., 2007). In addition, an impairment in extinction of contextual fear was apparent in Fmr1 KO mice. These data illuminate robust impairments in major forms of synaptic plasticity associated with learning impairment in the mouse model of FXS.

The most influential theory of the pathophysiology of FXS has been the mGluR theory (Bear et al., 2004). The theory is based on the finding that application of the group 1 mGluR agonist DHPG induces LTD of ~23% in Fmr1 KO mice compared to 12% in controls (Huber et al., 2002). Although many interesting parallels exist between mGluR activation and symptoms of FXS, the theory does not account for the key symptom of FXS: intellectual disability. For example, the enhanced mGluR-LTD in Fmr1 KO mice was found in the CA1 subfield of the hippocampus but Fmr1 KO mice perform as well as controls on learning tasks dependent on this brain region such as acquisition of the classic Morris water maze and one-trial inhibitory avoidance (D’Hooge et al., 1997; Eadie et al., 2009).

It has recently been suggested that enhanced mGluR-LTD is associated with impaired inhibitory-avoidance extinction (IAE) in Fmr1 KO mice (Dolen et al., 2007); however, this behavioral link is not direct. The association is based on the postulate that enhanced mGluR-LTD is protein synthesis dependent and IAE is also protein synthesis dependent (Power et al., 2006). This relationship is confounded by the fact that the enhanced mGluR-LTD in Fmr1 KO mice is not protein synthesis dependent (Nosyreva and Huber, 2006), despite the fact that mGluR-LTD is protein synthesis in WT mice (Nosyreva and Huber, 2006). The mGluR theory of FXS has also been called into question because the enhanced LTD observed in Fmr1 KO mice can also occur in response to metabotropic receptor activation in general (Volk et al., 2007). As attractive as the theory is, it does not seem to fully explain the deficits observed in humans and animals, and thus it may be that.
FMRP plays different roles in the cell during development and adulthood (Bear et al., 2004).

NMDA receptors have largely been ignored in the study of FXS, perhaps due to an early study reporting normal NMDAR-dependent LTP in the CA1 subfield of Fmr1 KO mice (Godfraind et al., 1996). Consistent with this observation was the report that NMDAR-dependent LTD is normal in the CA1 subfield of the hippocampus (Huber et al., 2002). In contrast to these results conducted in adult mice, recent studies investigating NMDAR-dependent LTP and LTD in young mice has revealed deficits. Hu et al. (2008) show impaired NMDAR-dependent LTP in the CA1 subfield of 2-week-old Fmr1 KO mice. In a separate recent study, Pilpel et al. (2009) noted abnormally enhanced NMDAR-dependent LTP in the CA1 subfield of 2-week-old, but not 6- to 7-week-old, Fmr1 KO mice. In addition, the latter study reported a decrease in the AMPAR/NMDAR ratio only in 2-week postnatal Fmr1 KO mice (Pilpel et al., 2009). This appeared to be primarily a function of a decrease in AMPAR-mediated synaptic transmission; however, the relationship between enhanced NMDAR-dependent LTP in Fmr1 KO mice and learning was not investigated in these studies. In the present study, input-output functions for AMPAR and NMDAR-mediated EPSCs from dentate granule neurons revealed decreased NMDAR-mediated synaptic transmission and normal AMPAR-mediated synaptic transmission. These results are consistent with the hypothesis that the AMPAR/NMDAR ratio is altered by a loss of the FMRP in developing regions of the brain; even if our findings in the adult DG are the opposite of the findings in the CA1 subfield of 2-week postnatal Fmr1 KO mice (Pilpel et al., 2009). That said, another group has also reported results consistent with ours (i.e., decreased NMDAR-mediated synaptic transmission and normal AMPAR-mediated synaptic transmission in the DG of Fmr1 KO mice) (Yun et al., 2009 SFN abstract) and it may be that some of these changes are developmentally regulated (Harlow et al., 2010). In the present study, decreased NMDAR-mediated synaptic transmission appears to underlie impaired bidirectional, NMDAR-mediated synaptic plasticity in the DG of adult Fmr1 KO mice.

**FIGURE 8.** Impaired context discrimination in Fmr1 KO mice. (a) Experimental design for discrimination training. Data was collapsed over each consecutive 2-day period into trial blocks 1–4 for the S+ and S− contexts. (b) Percent freezing in the 3 min prior to shock in the S+ context and in the equivalent 3-min period in the S− context across discrimination training. Fmr1 KO mice show a persistent elevation of freezing in the S− context relative to WT mice. (c) Discrimination ratios, calculated as (Percent freezing in S+)/(Percent freezing in S+) + (Percent freezing in S−)) across discrimination training. Fmr1 KO mice showed reduced discrimination ratios relative to WT mice. (* denotes P < 0.05 between genotypes).
more time in the exposed, well-lit regions of behavioral apparatus such as the open field (Eadie et al., 2009). In addition, a consistent lack of difference across trials in an IA task may reflect an inability of mice to maintain the ability to discriminate between two similar contexts. Thus our data provide some insight into the potential cause of greater extinction of IA reported (Dolen et al., 2007). Further studies into the effects of loss of FMRP on extinction are warranted.

The physiological basis of fear extinction may be related to abolished NMDAR-dependent LTD in the DG of Fmr1 KO mice. Recently, Dalton et al. (2008) reported that administration of a peptide that interferes with NMDAR-dependent LTD impairs the extinction, but not acquisition, of contextual fear (Dalton et al., 2008). Interestingly, these authors reported that this effect was dependent on NR2B-containing NMDARs. A separate recent study has shown that FMRP normally binds mRNA for the NR2B subunit suggesting that loss of FMRP may specifically lead to a decrease in NR2B-containing NMDARs (Schutt et al., 2009). Future studies should elucidate the subtype of NMDAR that is decreased in the DG of Fmr1 KO mice and the relationship to LTD and impaired fear extinction.

Learning impairments in Fmr1 KO mice parallel learning impairments in mice lacking functional NMDARs specifically in the DG (DG-NR1 KO mice; McHugh et al., 2007). We have shown that Fmr1 KO mice, similar to DG-NR1 KO mice, are not impaired on acquisition of the Morris water maze (Eadie et al., 2009). DG-NR1 KO mice do show impairments when assessed on a contextual fear discrimination task. This is consistent with modern theories that the function of the DG is pattern separation (Lisman, 2003; Rolls and Kesner, 2006). Assessment of Fmr1 KO mice using a similar paradigm revealed a novel learning impairment in the ability to discriminate between two similar or overlapping contexts. In fact, the impairment in context discrimination was strikingly similar between DG-NR1 KO and Fmr1 KO mice: both transgenic mice appear to manifest impaired context discrimination because of an increase in freezing behavior in the context that they were not previously shocked. These results extend upon our previous findings suggesting that a reduction in functional NMDARs in the DG is sufficient to impair context discrimination and illuminate a novel learning impairment in the mouse model of FXS.

Decreased NMDAR-mediated synaptic transmission in the DG may extend beyond impaired learning ability. Niewoehner et al. (2007) have recently shown that DG-NR1 KO mice exhibit decreased anxiety-like behavior (Niewoehner et al., 2007). This is consistent with the observation of anxiolysis resulting from intrahippocampal infusion of NMDAR antagonists. These data have led to the suggestion that NMDARs in the DG are at the interface between cognition and emotion (Barkus et al., 2010). Our group and several others have shown that Fmr1 KO mice exhibit decreased anxiety-like behavior similar to that observed in DG-NR1 KO mice and rodents receiving intrahippocampal infusions of NMDAR antagonists (Hackl and Carobrez, 2006; Niewoehner et al., 2007; Eadie et al., 2009; Barkus et al., 2010). It is possible that decreased NMDA-mediated synaptic transmission and plasticity reported here underlies...
other key behavioral abnormalities observed in the mouse model of FXS.

Intellectual disability is generally assumed to result from deleterious alterations in several genes combined with, often unidentified, environmental factors. The resulting abnormalities in the brain that underlie the intellectual disability are often assumed to be equally complex. Here we demonstrated that robust learning impairments can result from mutation of a single gene, and that the effects on the brain can be clear and robust. The illumination of this brain-behavior relationship was dependent upon investigation of a brain region previously uninvestigated in Fmr1 KO mice: the dentate gyrus. We show that loss of FMRP decreases NMDAR-mediated synaptic transmission and NMDAR-dependent bidirectional synaptic plasticity specifically in the DG. We show that learning dependent on NMDA receptors specifically in the DG is also impaired. These experiments improve our understanding of the pathophysiology of intellectual disability in general and identify a novel target for therapeutics aimed at ameliorating intellectual disability in FXS and related neurodevelopmental disorders.

Acknowledgments

The authors thank E. Wiebe, N. Jacobs and Drs. J. Johnston, C. Brown, P. Nahiriey, A. Titterness, and Y.T. Wang for assistance with this work. BDE holds a Vancouver Coastal Health-CIHR-UBC MD/PhD Studentship Award. TSK holds a NSERC-CGS award. BRC is a MSFHR Senior Scholar. This work was initiated with funding from Fragile-X Canada and the Scottish Rites Charitable Foundation.

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Hippocampus


