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Impaired bidirectional NMDA receptor dependent synaptic plasticity in the dentate gyrus of adult female *Fmr1* heterozygous knockout mice

Abbreviated title: Hippocampal NMDA receptor hypofunction in female Fragile X syndrome mice

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Abstract: (262 words)

Fragile-X syndrome (FXS) is caused by the transcriptional repression of the *Fmr1* gene resulting in loss of the Fragile-X mental retardation protein (FMRP). This leads to cognitive impairment in both male and female patients, however few studies have focused on the impact of FXS in females. Significant cognitive impairment has been reported in approximately 35% of women who exhibit a heterozygous Fmr1 gene mutation, however to date there is a paucity of information regarding the mechanistic underpinnings of these deficits. We, and others, have recently reported that there is significant impairment in N-methyl-D-aspartate receptor (NMDAR)-dependent long-term potentiation (LTP) and long-term depression (LTD) in the hippocampal dentate gyrus (DG) of male *Fmr1* knock out mice. Here we examined if female mice displaying a heterozygous loss of the *Fmr1* gene (*Fmr1*$^{+/-}$) would exhibit similar impairments in DG-dependent spatial memory processing and NMDAR hypofunction. We found that Female *Fmr1*$^{+/-}$ mice did not show impaired metabotropic glutamate receptor (mGluR)-LTD in the CA1 region, and could perform well on a temporal ordering task that is thought to involve this brain region. In contrast, female *Fmr1*$^{+/-}$ mice showed impairments in a pattern separation task thought to involve the DG, and also displayed a significant impairment in both NMDAR-dependent LTD and LTP in this region. The LTP impairment could be rescued by administering the NMDAR co-agonist, glycine. Our data suggests that NMDAR hypofunction in the DG may partly contribute to learning and memory impairment in female *Fmr1*$^{+/-}$ mice. Targeting NMDAR-dependent mechanisms may offer hope as a new therapeutic approach for treating female FXS patients with learning and memory impairments.

Introduction

Fragile-X Syndrome (FXS) is the most common X-linked form of inherited intellectual impairment (Bagni and Greenough, 2005), affecting approximately 1 in 4000 males and 1 in 8000 females (Hagerman et al., 1999; Turner et al., 1996). It is caused by an expanded cytosine-guanine-guanine (CGG) (>200 repeats, knowns as full mutation) repeat tract in the Fragile X mental retardation syndrome 1 (*Fmr1)* gene, which, in turn, causes DNA hypermethylation and subsequent loss of the Fragile-X mental retardation protein (FMRP) (Hansen et al., 1992; Pieretti et al., 1991).

FXS patients display varied cognitive performance ranging from normal to severe impairment (Alanay et al., 2007; Kabakus et al., 2006; Rousseau et al., 1991). These cognitive impairments appear to be sexually dimorphic, as the majority of FXS males show moderate to severe cognitive impairments (Hagerman et al., 1999) while affected females often have a less severe phenotype than males (Abrams et al., 1994; Sobesky, 2002) and exhibit varied degrees of behavioral deficits. Approximately 50% of homozygous females (Mazzocco et al., 1993; Rousseau et al., 1991) and 35% of heterozygous females (Kemper et al., 1986; Miezejeski et al., 1986) show significant mental retardation. Furthermore, emotional dysfunction has most been reported in FXS females (Hagerman et al., 1999; Reiss et al., 1988), suggesting that FXS females appear most vulnerable to specific psychopathology and behavioral deficits, such as social anxiety and depression (Freund et al., 1993).

The causes for wide variation in mental impairment among female patients remain undetermined; however, the number of CGG repeats in the Fmr1 gene may be a factor influencing cognitive affectedness of female carriers (Taylor et al., 1994). A correlation between CGG expansion size and spatial performance was found in FXS males (Cornish et al., 1999). Similarly, an association between size of CGG repeats and severity of social anxiety was found in FXS females (Freund et al., 1993). Levels of FMRP in the brain may correlate with the severity of behavioral impairment in FXS individuals. Understanding the female condition in FXS can be challenging since FXS females differ greatly in their degree of mental impairment and CGG repeat number. Information regarding behavioural abnormalities in female FXS patients is still very limited. Female patients

with FXS display reduced hippocampal activity during visual memory encoding tasks as well as deficits in episodic memory (Greicius et al., 2004) and often exhibit emotional dysfunction (Freund et al., 1993). Together, these findings suggest that hippocampal dysfunction underlies the symptoms of FXS in female patients. With the development of *Fmr1* knockout (KO) mice (The Dutch-Belgian Fragile X Consortium, 1994), the majority of animal studies using *Fmr1* KO mice have focused on males, leaving the effects of a loss of FMRP on females largely unexplored.

The hippocampus, which is important for emotion as well as learning and memory (Squire, 1992), has high expression for *Fmr1* mRNA in human fetal brains (Abitbol et al., 1993). The granule neurons of the dentate gyrus (DG) express the highest levels of *Fmr1* mRNA in the mouse brain (Hinds et al., 1993). Disruptions in FMRP production may introduce synaptic impairments in the hippocampus that could result in deficits in learning and memory in *Fmr1* KO mice (Eadie et al., 2012; Franklin et al., 2014; King and Jope, 2013). In the *cornu ammonis* region 1 (CA1) of the hippocampus, exaggerated metabotropic glutamate receptor-dependent (mGluR), but intact N-methyl-D-aspartate receptor (NMDAR)-dependent long-term depression (LTD) and long-term potentiation (LTP), have been reported in male *Fmr1* KO mice (Godfraind et al., 1996; Huber et al., 2002; Lauterborn et al., 2007). In the DG, we have reported that male *Fmr1* KO mice show impairments in pattern separation performance, reduced NMDAR-dependent LTD and LTP, and decreased NMDAR subunit protein expression (Bostrom et al., 2013; Eadie et al., 2012). While little work has been done in females, *Fmr1* heterozygous KO (*Fmr1*$^{+/-}$) mice have been shown to exhibit a significant increase in susceptibility to audiogenic seizures (Musumeci et al., 2000). Whether the hippocampi of female FXS mice are affected in the same way as males remains unknown. Because female patients with FXS are usually heterozygous carriers, we examined whether female *Fmr1*$^{+/-}$ mice show parallel impairments in hippocampal synaptic plasticity and behavioral deficits to male KO mice.

Materials and Methods

Transgenic Mice

Female *Fmr1*$^{+/-}$ and wild-type (WT) littermate mice with C57Bl/6J genetic background were generated by breeding a female *Fmr1*$^{+/-}$ mouse with a WT male mouse from established breeding colonies. All experiments were carried out in adult mice (2 - 4 months of age) in accordance with international standards on animal welfare and guidelines set by the Canadian Council on Animal Care and the Animal Care Committees at the University of Victoria.

Genotyping

Genotyping was performed as previously described (Bostrom et al., 2013; Eadie et al., 2012). Ear snips were used to isolate DNA using the DNA isolation kit according to the manufacture's instructions (Invitrogen, Ontario, Canada), followed by PCR analysis with cycling parameters as follows: denaturation at 94^{0}C for 5 min, followed by 35 cycles of 60s at 94 ^{0}C, 90 s at 65 ^{0}C and 150 s at 72 ^{0}C. Primers used for probing the Fmr1 KO allele (800 base pairs) were: M2 = 5'-ATCTAGTCAYGCTATGGATATCAGC-3' and N2 = 5'-GTGGGCTCTATGGCTTCTGAGG-3'. Primers for probing the WT allele (450 base pairs) were: S1 = 5'-GTGGTTAGCTAAAGTGAGGATGAT-3' and S2 = 5'-CAGGTTTGTTGGGATTAACAGATC-3' (**Suppl. Fig. 1**). PCR products were run in a 1.5 % agarose gel with SYBR-safe and visualized using a BioRad trans-illuminator.

Analyses of Estrus Cycle

Mice that were in the proestrus stage of their estrus cycle at the time of decapitation for electrophysiology were excluded from data analysis, since the proestrus stage has been associated with alterations in functional plasticity (Warren et al., 1995). All female mice subjected to electrophysiology were subjected to vaginal lavage immediately before decapitation while anesthetized with isoflurane. Papanicolaou (PAP) histochemistry was performed to confirm the stage of the estrus cycle as illustrated in **Suppl. Fig 2**. In brief, slides were immersed into Gills hematoxylin solution 1 for 3 min, followed by washes in tap water and then immersed in Scott's tap water substitute (0.3 % sodium bicarbonate and 2% anhydrous magnesium sulfate in distilled water). Slides were then immersed in PAP stain (1: 1 Orange G6 and Eosin-azure 50; Sigma-Aldrich, Ontario, Canada) for 3 min and immersed in 100% ethanol, then a xylene substitute (Citrisolv; Fisher Scientific Company, Ontario, Canada), coverslipped with permount

and analyzed by conventional light microscopy (Model BX51TF, Olympus Corporation, Pennsylvania, US).

Behavioral tests

A separate cohort of animals was used for behavioral tests. All mice were subjected to 7 days of pre-handling, followed by two days where they were habituated to the empty testing apparatus for 15 minutes. Pre-handling and habituation can significantly reduce stress that is associated with handling and exposure to novel environments. This is critical as the Fmr1 KO mice are hypersensitive to stress (Ghilan et al., 2015). Mice were transferred to the behavioral room 2 hr before conducting behavioral tests each day. Each mouse was subjected to a battery of behavioral tests, however only one behavioral test was performed on any single day.

Categorical spatial processing task

A Plexiglas circular apparatus (diameter 100 cm X height 30 cm) with four visual cues attached to the wall was used. This task assesses categorical spatial memory which depends on the DG (Goodrich-Hunsaker et al., 2005). It consists of a 15-min habituation session with two different, but highly similar objects spaced 40 cm apart. Following a 5-min inter-trial interval in an empty, opaque transfer cage, mice were allowed to explore the objects with their locations interchanged without altering the distance between objects for 5 min. Increased exploration of the objects during the test session compared to the last 5 min of the habituation indicates that the mice remembered the object positions (**Fig. 1A$_1$**). The exploration ratio was calculated as (exploration time of object in 5 min test or last 5 min of habituation) / (exploration time of object in 5-min test + last 5 min of habituation). Exploration index was calculated as (exploration time of object in 5-min test - last 5 min of habituation) / (exploration time of object in 5-min test + last 5 min of habituation).

Temporal order task

This test assesses temporal order memory and requires the CA1 region to be intact (Hoge and Kesner, 2007). Mice received three sessions to explore two copies of a new set of objects (sets 1, 2, 3, respectively) and were allowed to explore these objects (40 cm apart) for 5 min, followed by 5 min intertrial interval in an opaque transfer cage (as above). During the 5-min test session,

mice were allowed to explore a copy of object set 1 and a copy of object set 3 (**Fig. 1B$_1$**). More time spent on exploring the first object (Object 1) presented relative to the most recent explored object (Object 3) indicates normal temporal order memory. Exploring ratio of the objects was calculated as (exploration time of Object 1 or 3) / (exploration time of Object 1 + 3). Exploration index was calculated as (exploration time of Object 1 – 3) / (exploration time of Object 1 + 3).

Tail suspension test

The tail suspension test was conducted as previously described (Yau et al., 2014). Briefly, the mouse was suspended with a climb-stopper placed around its tail prior to applying the tape. A 6-min session was video-taped and analyzed for immobility time by observers in a blind manner.

Forced swim test

Mice were placed in a 5 L beaker filled with 4 L room temperature tap water for 6 min. The session was video-taped for scoring by an observer blind to the treatment conditions. The immobility time in the last 4-min testing session was analyzed and presented as an indicator for depression-like behaviour as previously described (Yau et al., 2014). Immobility was defined as any movement beyond what was needed to keep the head above the water.

Field electrophysiological recordings

Mice were deeply anesthetized with isoflurane, and were immediately decapitated. Their brains were removed directly into oxygenated (95% O_2/5% CO_2) ice-cold normal artificial cerebrospinal fluid (nACSF) consisting of (in mM) 125 NaCl, 2.5 KCl, 1.25 $NaHPO_4$, 25 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgCl_2$, and 10 dextrose, pH 7.3. Transverse hippocampal slices (350 μm) were obtained using a Vibratome 1500 (Ted Pella Inc., Redding, California, U.S.A.). Slices were incubated in continuously oxygenated nACSF at 30 ^oC and were allowed to recover for a minimum of 1 hr before recordings commenced.

Slices were perfused at a rate of approximately 2 ml / min. All drugs used were dissolved in nACSF before bath application. Field EPSPs were collected using an Axon MultiClamp 700B amplifier connected to a Windows computer running Clampex 10.2 software (Molecular Devices, Sunnyvale, CA). Electrodes were placed under the visual guidance of an upright microscope. The slope from a single fEPSP trace was calculated from the initial slope of the

fEPSP relative to the slope of the 10 ms interval immediately preceding afferent stimulation. The current magnitude (10-50 μA) was delivered through a digital stimulus isolation amplifier (Getting Instruments Inc., CA) and set to elicit a fEPSP approximately 40-50% of maximum for synaptic potentiation experiments and 50-60% for synaptic depression experiments. A stable baseline (for a minimum of 20 minutes) was obtained by delivering single pulse stimulation at 15s interstimulus intervals.

NMDAR-dependent LTP of field excitatory post-synaptic potentials (fEPSPs) in the DG was induced using a conditioning stimulus (CS) consisting of 4 trains of 50 pulses at 100 Hz, 30 s apart (high-frequency stimulation; HFS). Medial perforant path evoked fEPSPs were obtained using a concentric bipolar stimulating electrode (FHC Inc., Bowdoin, Maine, USA) and a glass recording electrode (1-2 MΩ) filled with nACSF and placed in the medial molecular layer. HFS-LTP was conducted in the presence of 5 μM bicuculline methiodide (BIC; Sigma-Aldrich, Ontario, Canada) to block the inhibitory effects of the gamma-aminobutyric acid receptor type A (GABAa) on synaptic plasticity in this region of the hippocampus. BIC was bath applied before recording the baseline. Experiments assessing the role of NMDAR in HFS-LTP were also conducted in the presence of 50 μM (2R)-amino-5-phosphonovaleric acid (APV; Sigma-Aldrich, Ontario, Canada) in addition to BIC. APV was bath applied 5 min prior to and during CS. For experiments assessing the use of glycine to rescue deficits in NMDAR dependent LTP, glycine (10 μM) was bath applied during baseline and HFS conditioning protocols. LTD in the DG was induced by administering 900 pairs of pulses (50 ms interstimulus interval) delivered at 1 Hz (PP-LFS). PP-LTD was conducted in the presence of 5 μM BIC. We have shown that difference in LTD between male WT and *Fmr1*$^{-/-}$ mice can be abolished by application of a selective NMDAR antagonist APV (data not shown)(Bostrom et al., 2013).

Evoked fEPSPs in the CA1 were obtained using a glass-recording electrode (1-2 MΩ) filled with nACSF and placed in the stratum radiatum. mGluR-dependent LTD of fEPSPs in the CA1 region was induced with a CS consisting of 900 pairs of stimuli (50 ms interstimulus interval) delivered at 1 Hz over 15 min (PP-LFS). PP-LTD was conducted in the presence of 5 μM BIC. Additionally, 50 μM APV was bath applied to the slice for a minimum of 5 min before and

during the application of PP-LFS CS to block any possible NMDAR mediated effects on PP-LTD.

Input-output experiments were conducted to measure basal dendritic excitation in response to increasing applied current in nACSF (using an increasing pulse width from 30 to 300 μs with 30 μs interstimulus intervals). Paired-pulse recordings were also conducted to measure presynaptic release probability. Paired-pulse recordings consisted of 5 sets of two pulses each with an interpulse interval of 50 ms (20 s between paired stimuli) delivered in nACSF.

Evoked fEPSP responses were digitized and the initial slope of the fEPSP was analyzed using pClamp 10 software (Molecular Devices, Sunnyvale, CA). All data are represented as percentage change from the initial average baseline fEPSP slope, which was defined as the average slope obtained for the 20 min prior to CS application. Percentage potentiation or depression was calculated as follows: (average fEPSP 50 to 60 min post-CS) / (average fEPSP between -20 to 0 min pre-CS) x 100.

Whole cell patch clamping recording

Transverse hippocampal slices (350 μm) were obtained using high Mg^{2+}, low Ca^{2+} slicing ACSF which consisted of (in mM) 123 NaCl, 25 $NaHCO_3$, 3 KCl, 1.25 $NaH_2PO_4.H_2O$, 1 $CaCl_2$, 6 $MgCl_2$. Following a minimum recovery in nACSF for at least 1 hour at 33 ^{0}C, whole cell recording were obtained using an Axon Axopatch 200B amplifier and digitized with DigiData 1440A connected to a PC running Clampex 10.3 software (Molecular Devices, Sunnyvale, CA). The intracellular solution consisted of (in mM) 135 $CsMeSO_4$, 8 NaCl, 10 HEPES, 7 phosphocreatine, 0.3 Na_3GTP., 2 Mg_2ATP, 10 QX-314, pH 7.3. Liquid conjunction potential was adjusted to 9.5 mV. The resistance of the recording electrode was 5 – 7 MΩ. Synaptic responses were evoked by electrical stimulation in the middle molecular layer (medial perforant pathway) of the suprapyramidal blade. Recordings were performed in the suprapyramidal limb of the granule cell layer located midway between the crest and the distal end of the limb. Dentate granule cells with series resistance larger than 30 MΩ and change of series resistance larger than 30% were discarded. Once whole-cell recording was achieved in nACSF, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated excitatory postsynaptic currents

(EPSCs) were obtained by holding cells at -70 mV, and NMDAR-mediated EPSCs were obtained by holding cells at +40 mV with a 0.05 ms pulse in voltage clamp mode. The peak current observed at -70 mv represents the maximum AMPAR-EPSC, whereas NMDAR-EPSC was measured at +40 mV after 50 ms of the simulating pulse when the AMPAR-EPSC is gone. Pharmacological isolation of NMDAR-mediated EPSCs was obtained using low Mg^{2+} ACSF (0.1 mM) containing AMPAR antagonist NBQX (5 µM; Tocris Bioscience) and 100 µM Picrotoxin (Sigma-Aldrich, Ontario, Canada) after break-in in the cells. An approximately 100 pA evoked NMDAR-EPSC was obtained without glycine. Glycine potentiated NMDA currents were obtained following bath application of glycine for 15 min, and were confirmed by bath application of the glycine binding site antagonist 5,7-Dichorokynurenic acid (Tocris Bioscience, Ontario, Canada).

Statistical Analyses

Differences between the mean values of experimental groups were compared with two-tailed Student's *t* test for two group comparison. Repeated measures ANOVA or two-way ANOVA with Tukey's post-hoc test was applied for electrophysiological data when appropriate using SPSS 16.0 software. Data are presented as means ± standard error of the mean (SEM). Statistical significance was set at $P < 0.05$.

Results:

Female *Fmr1*$^{+/-}$ mice displayed impairment in spatial memory processing task

Adult male Fmr1 knockout mice displayed impaired temporal order memory and spatial pattern separation processing (Franklin et al., 2014; King and Jope, 2013). Here we tested if similar learning and memory impairment could be found in female *Fmr1*$^{+/-}$ mice. The categorical spatial learning task is a DG dependent task that tests topological spatial pattern separation (Goodrich-Hunsaker et al., 2005). It assesses the time spent exploring two objects after the positions of these two objects are transposed in the test phase (**Fig. 1A$_1$**). *Fmr1*$^{+/-}$ mice spent significantly less time exploring the objects in the test phase when compared to WT mice (**Fig. 1A$_2$ & A$_3$**. $t_{(12)} = 4.230$, $P = 0.001$), indicating impairment in categorical spatial memory in *Fmr1*$^{+/-}$ mice.

In the temporal order test, control mice spent less time exploring a most recently presented object in habituation phase (**Fig. 1B$_1$**). Both WT and *Fmr1*$^{+/-}$ mice spent more time exploring the first object presented than the most recent object presented (**Fig. 1B$_2$**). Exploration index indicated that there was no significant difference in temporal ordering for objects between WT and *Fmr1*$^{+/-}$ mice (**Fig. 1B$_3$**. $t_{(14)} = -0.153$, P = 0.880).

There was no significant difference in depressive despair in either the forced swim test (**Fig. 1C.** $t_{(14)} = -0.104$, P = 0.919) or tail suspension test (**Fig. 1D.** $t_{(14)} = 0.242$, P = 0.812), as indicated by comparable time spent in immobility between WT and *Fmr1*$^{+/-}$ mice.

Intact basal synaptic transmission and short-term synaptic plasticity in the DG and CA1 regions of *Fmr1*$^{+/-}$ Mice

Basal synaptic transmission was measured by Input/Output functions while short-term synaptic plasticity was measured by pair-pulse measures of presynaptic neurotransmitter release. The slope of the fEPSP significantly increased when the applied current was increased from a pulse width of 30 to 300 μs (**Fig. 2A**. repeated measures ANOVA: $F_{(8,88)} = 109.317$, $P < 0.001$;). However, no statistical differences were found between genotypes (**Fig. 2A.** repeated measures ANOVA: $F_{(1,11)} = 0.064$, $P = 0.805$), suggesting no difference in basal synaptic transmission in the DG between WT and *Fmr1*$^{+/-}$ mice. Paired-pulse conditioning stimulation was used as a measure of short-term plasticity. There was no significant difference between WT and *Fmr1*$^{+/-}$ responses to paired-pulse assay of presynaptic release probability in the DG (**Fig. 2B.** t(13) = -0.120, P = 0.906), suggesting that WT and female *Fmr1*$^{+/-}$ mice have an equal probability of neurotransmitter release. Intact basal synaptic transmission (**Fig. 2C.** repeated measures ANOVA: $F_{(1,13)} = 0.019$, P = 0.894) and short-term plasticity (**Fig. 2D.** $t_{(19)} = -2.38$, P = 0.815 . WT: Pulse 2 = 143.6 ± 6.1 % of pulse 1; *Fmr1*$^{+/-}$: Pulse 2 = 162.3 ± 7.3 % of pulse 1) were also observed in the CA1 region of female *Fmr1*$^{+/-}$ mice when compared to WT mice.

Impaired NMDAR-dependent LTP and LTD in the DG, but intact mGluR-LTD in the CA1 of *Fmr1*$^{+/-}$ mice

LTP was induced by HFS and LTD was induced by LFS in the DG of WT and *Fmr1*$^{+/-}$ mice (**Fig. 3**). *Fmr1*$^{+/-}$ mice displayed significantly less LTP (**Fig. 3A$_1$ & A$_2$** WT: 84.10 ± 2.51 %; *Fmr1*$^{+/-}$: 40.7 ± 6.5 %; $t_{(15)} = 2.987$, P = 0.01) and LTD (**Fig. 3B$_1$ & B$_2$**, WT: -29.73 ± 4.05 %; *Fmr1*$^{+/-}$: -12.19 ± 4.10%; $t_{(18)} = -3.162$, P < 0.005) in the DG when compared to WT mice. There was no significant difference in mGluR-LTD in the CA1 region between WT and *Fmr1*$^{+/-}$ mice (**Fig. 3C$_1$ & C$_2$**, WT: -24.37 ± 8.70%; *Fmr1*$^{+/-}$: -19.92 ± 6.35%; $t_{(12)} = -0.526$, P = 0.608).

Decreased AMPAR and NMDAR-mediated EPSCs in the dentate gyrus of *Fmr1*$^{+/-}$ mice

Since female *Fmr1*$^{+/-}$ mice showed impairment in spatial pattern separation, a task dependent on the dentate gyrus of the hippocampus (Goodrich-Hunsaker et al., 2005), we investigated if this behavioral deficit was associated with a decrease in AMPAR- or NMDAR-mediated EPSCs in dentate granule cells. Whole cell patch clamping revealed that *Fmr1*$^{+/-}$ mice showed significant decreases in both AMPAR- (**Fig. 4A & B.** repeated measure ANOVA: $F_{(1,37)} = 4.847$, P = 0.034) and NMDAR-mediated EPSCs (**Fig. 4A & C.** repeated measure ANOVA: $F_{(1, 37)} = 9.695$, P = 0.004) when compared to WT mice. Furthermore, both maximum AMPAR- (WT = 590.67 ± 33.29 pA; *Fmr1*$^{+/-}$ = 451.04 ± 39.36 pA) and NMDAR- (WT = 358.97 ±38.68; *Fmr1*$^{+/-}$ = 205.94 ± 24.74 pA) mediated EPSCs in *Fmr1*$^{+/-}$ mice were significantly lower than that in WT mice (**Fig. 4D**. AMPAR: $t_{(36)} = 3.467$, P = 0.001; NMDAR: $t_{(36)} = 3.0714$, P = 0.004).

Glycine potentiated NMDA currents in both WT and *Fmr1*$^{+/-}$ mice

Glycine, a co-agonist of the NMDAR, may potentiate the NMDAR and enhance NMDA currents (Johnson and Ascher, 1987). Bath application of glycine increased NMDAR-mediated EPSCs in both WT and *Fmr1*$^{+/-}$ mice, though *Fmr1*$^{+/-}$ mice showed significantly lower NMDAR-mediated EPSCs than WT mice (**Fig. 5.** WT = 200.84 ± 28.75%; *Fmr1*$^{+/-}$ = 181.85 ± 16.91%; $t_{(16)} = 2.783$, P = 0.013).

Impairments in NMDAR-dependent LTP can be reversed by glycine in *Fmr1*$^{+/-}$ mice

We have previously reported that glycine rescues impaired synaptic plasticity in male Fmr1 KO mice (Bostrom et al., 2013). We therefore tested if glycine exerts similar effects on female *Fmr1*$^{+/-}$ mice. Two-way ANOVA revealed no effect of genotype ($F_{(1,29)} = 2.452$, P = 0.13), but significant effect of glycine ($F_{(1,29)} = 5.382$, P = 0.029); and interaction ($F_{(1,29)} = 10.599$, P =

0.003). Posthoc analysis revealed that the same conditioning stimulation protocol induced significantly less LTP in female *Fmr1*$^{+/-}$ mice when compared to WT mice (WT: 84.10 ± 2.51 %; *Fmr1*$^{+/-}$: 40.7 ± 6.5 %; Tukey post-hoc test $P < 0.05$). Glycine significantly normalized LTP in *Fmr1*$^{+/-}$ mice (**Fig. 6A$_1$ & A$_2$** *Fmr1*$^{+/-}$+Glycine: 91.81 ± 41.51 %; $P < 0.005$), and showed no effect on WT mice (WT+Glycine: 71.84 ± 25.58 %; $P > 0.05$).

Bath application of the NMDAR antagonist APV (50 μM) attenuated LTP to the same level in both WT and *Fmr1*$^{+/-}$ mice ((**Fig 6B$_1$ & B$_2$** WT: -4.3 ± 11.4 %; *Fmr1*$^{+/-}$: -4.3 ± 4.6 %; $t_{(11)} = -1.778$, $P = 0.103$), indicating that the LTP induced through HFS in both WT and *Fmr1*$^{+/-}$ slices was predominantly NMDAR dependent.

Discussion

While females with FXS often have a less severe behavioral phenotype than males (Abrams et al., 1994; Sobesky, 2002), approximately 35% of heterozygous females show significant cognitive impairment (Kemper et al., 1986; Miezejeski et al., 1986). To date, the underlying mechanism(s) of how the loss of FMRP affects synaptic function in female brains remains unclear. To the best of our knowledge, this is the first study examining hippocampal synaptic plasticity in an adult female mouse model of FXS with partial loss of FMRP. The results show that female *Fmr1*$^{+/-}$mice exhibit impairments in a pattern separation task that requires normal functioning of the DG, but no significant changes in depression-like behaviors. The memory processing deficit in *Fmr1*$^{+/-}$ mice is associated with a DG-specific impairment in bidirectional NMDAR-dependent synaptic plasticity; a result that is congruent with those previously reported in male Fmr1 KO mice (Bostrom et al., 2013; Eadie et al., 2012; Franklin et al., 2014; Yun and Trommer, 2011). However, females differ in that the exaggerated mGluR-LTD that has been reported in the CA1 region of male Fmr1 KO mice (Bear et al., 2004; Huber et al., 2002) was absent. Female *Fmr1*$^{+/-}$ mice also displayed normal temporal order processing. Application of the NMDAR co-agonist glycine restored NMDAR-dependent LTP in the DG, suggesting that a pharmacological approach to increase endogenous glycine concentration in the brain may serve as an effective treatment for improving learning and memory deficits in both male and female FXS patients.

Impaired spatial memory processing in female Fmr1$^{+/-}$ mice

Here we demonstrated that female *Fmr1*$^{+/-}$ mice on a C57BL/6 background showed significant impairment in a DG-dependent categorical spatial task, similar to previous observations in male *Fmr1* KO mice (Franklin et al., 2014; King and Jope, 2013). Male *Fmr1* KO mice with complete loss of FMRP show severe impairments in the temporal order test (Franklin et al., 2014; King and Jope, 2013), a visual task reliant on an intact CA1 region (Hoge and Kesner, 2007). In contrast, female *Fmr1*$^{+/-}$ mice displayed intact temporal order memory in this study. This may be due to the fact that partial loss of FMRP is not sufficient to impair CA1 synaptic function, as no impairment of mGluR-dependent synaptic plasticity was found in this region in female *Fmr1*$^{+/-}$ mice. Although female FXS patients show emotional dysfunction more often than their male counterparts (Freund et al., 1993), depression-like behavior as assessed by forced swim test and tail suspension test was not altered in our female *Fmr1*$^{+/-}$ mice, similar to what is seen in male *Fmr1* KO mice (Yuskaitis et al., 2010). A recent study using male and female *Fmr1 KO* mice on a C57BL/6J-*Tyr*$^{c\text{-}Brd}$ (C57 albino) background, show similar impairments between sexes in spatial learning and memory in the Morris Water Maze (MWM), contextual memory deficits, and decreased anxiety in the open field test (Baker et al., 2010). This suggests that there may be negligible sex differences in regards to behavioral impairments in mice with a full mutation of the *Fmr1* gene. However, our results suggest that female mice with a partial loss of FMRP show less severe behavioral deficits than male mice with a full *Fmr1* mutation. This is in line with the clinical findings which report female patients to be less severely affected by the loss of FMRP when compared to male patients.

Impaired NMDAR-dependent bidirectional synaptic plasticity in the DG of Fmr1$^{+/-}$ mice

Understanding the mechanism(s) underlying impairments in hippocampal plasticity will be critical for the success of future therapeutic treatment of FXS patients with learning and memory impairments. Intact basal transmission and short-term plasticity were observed in female *Fmr1*$^{+/-}$ mice, indicating that behavioral and synaptic impairments may be linked to dysfunction at postsynaptic sites rather than at pre-synaptic sites. Our previous findings in male mice indicate that an absence of FMRP can produce impairments in bidirectional synaptic plasticity in the DG that may be due to decreased NMDAR subunits (Bostrom et al., 2013). NMDAR hypofunction

and impaired NMDAR-dependent LTP and LTD in the MPP-DG synapses (Bostrom et al., 2013; Eadie et al., 2012; Franklin et al., 2014; Yun and Trommer, 2011) may contribute to these deficits in a DG-dependent pattern separation task. This is supported by the fact that female *Fmr1*$^{+/-}$ mice also displayed DG-dependent behavioral deficits that are correlated with impaired NMDAR-LTP and LTD. Future research warrants a closer examination of NMDAR subunits expression, which may also underlie NMDAR-LTP and LTD findings in the female *Fmr1*$^{+/-}$ mouse. Female *Fmr1*$^{+/-}$ mice displayed intact mGluR-mediated LTD in the CA1 region, which correlated with normal behavioral performance in the temporal order task. Exaggerated mGluR-mediated LTD in the CA1 region has been reported in male *Fmr1* KO mice (Godfraind et al., 1996; Huber et al., 2002), in association with impaired temporal order memory (Franklin et al., 2014; King and Jope, 2013). Interestingly, partial loss of FMRP in female *Fmr1*$^{+/-}$ mice may not be sufficient to cause impairments in synaptic plasticity in the CA1 region. Since the DG has the highest FMRP mRNA expression among hippocampal regions (Hinds et al., 1993), partial loss of FMRP may disproportionately affect this brain region, causing severe impairments in synaptic plasticity in the DG, but not the CA1 region.

Glycine potentiates NMDAR and restores LTP in the DG of Fmr1*$^{+/-}$ *mice

Pharmacological rescue of NMDAR hypofunction in the hippocampal DG should take its place alongside current research into inhibition of mGluR5 at the forefront of the search to enhance cognitive function in FXS (Bostrom et al., 2016). Inhibition of mGluR5 by its antagonist MPEP have repeatedly shown to rescue LTP deficits in the CA1 (Dölen et al., 2007; Michalon et al., 2014), but have failed to rescue impaired LTP in the DG and hippocampal-dependent learning in male FXS mice (Franklin et al., 2014). Similarly, inhibition of mGluR5 by MPEP has failed to rescue LTP deficits in the amygdala (Suvrathan et al., 2010). These studies suggest that only targeting mGluR5 may not be sufficient to rescue all hippocampal synaptic deficits in FXS.

However, pharmacological blockade of GSK3β, a negative regulator of NMDAR-dependent LTP (Hooper et al., 2007; Peineau et al., 2007), has been shown to reverse NMDAR-dependent synaptic deficits in MPP-DGC synapses and improve cognitive deficits in male *Fmr1* KO mice (Franklin et al., 2014). We observed a similar benefit of glycine in rescuing synaptic dysfunction

in the DG of female *Fmr1*$^{+/-}$ and male *Fmr1* KO mice. Whole cell recordings revealed that glycine was able to potentiate NMDAR-mediated EPSCs in *Fmr1*$^{+/-}$ mice and NMDAR-LTP was restored by the addition of glycine in female *Fmr1*$^{+/-}$ mice (without affecting control levels of LTP). This data suggests that enhancing NMDAR function may be able to effectively reverse synaptic deficits in FXS mice regardless of sex differences. Possible behavioral benefits of potentiating NMDAR *in vivo* in the hippocampus of female *Fmr1*$^{+/-}$ mice warrant further investigation. Since impairment in synaptic plasticity and behavioral deficit observed in the present study is observational, it would be of great interest to examine whether increasing NMDAR activation specifically in the DG rescues behavioral deficiency in both female and male *Fmr1* KO mice, or DG region-specific reduction of NMDAR activity in the WT mice would result in behavioral deficit that is similar to the *Fmr1* KO mice.

The present findings reinforce the integral role of the NMDAR in both male and female FXS mice, and speak to the potential benefit of pharmacological therapy aimed at reversing NMDAR dependent deficits for hippocampal dependent learning and memory in FXS.

Conclusions

This study has corroborated and extended the theory of NMDAR hypofunction in FXS by revealing impaired synaptic plasticity in female *Fmr1* transgenic mice with partial loss of FMRP. Taking our previous findings with male *Fmr1* KO mice and our current findings together, impairments in NMDAR-dependent LTD and LTP which are possibly linked to NMDAR hypofunction in the DG may contribute to hippocampal-dependent learning and memory impairment observed in male and female FXS patients. Our present study indicates that increasing NMDAR function by the use of the co-agonist glycine can rescue impaired synaptic plasticity specifically in female *Fmr1*$^{+/-}$ mice. This suggests that modulating the NMDAR may serve as a potent treatment for learning and memory impairment in FXS patients. Though further investigation will be required to fully elucidate the mechanisms underlying altered synaptic plasticity in the DG, our results solidify the important contribution of NMDARs to hippocampal pathophysiology in both male and female FXS.

Conflicts of Interest

The authors have no conflicts of interest to declare regarding this manuscript.

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Figure legends:

Fig. 1. Behavioral performance of female *Fmr1*$^{+/-}$ mice and their WT littermates. (A$_1$) An illustration of the task procedure with two similar objects presented to mice for 15 mins, followed by a 5-min test phase with swapped object positions**. (A$_2$)** WT mice spent more time exploring the transposed objects in test phase of the categorical spatial test, while *Fmr1*$^{+/-}$ mice spent equal amount time exploring the objects in the test phase. **(A$_3$)** *Fmr1*$^{+/-}$ mice displayed significant impairment in categorical spatial processing. (**B$_1$**) An illustration of temporal order task with three different object sets presented to mice for 5 mins, followed by a 5-min test phase with a presence of a copy of the first and last object**. (B$_2$)** Both WT and *Fmr1*$^{+/-}$ mice showed a higher exploration ratio to the first object presented (object 1) than to the most recently explored object (object 3) in the temporal order test. **(B$_3$)** No deficit was observed in temporal order memory in *Fmr1*$^{+/-}$ mice when compared to WT mice. **(C & D)** *Fmr1*$^{+/-}$ mice did not show an increase in depression-like behavior in the forced swim test or tail suspension test, respectively. * $P < 0.005$ n = 7-9 per group.

Fig. 2. Intact basal synaptic transmission and short-term synaptic plasticity in *Fmr1*$^{+/-}$ mice in the dentate gyrus. (**A**) *Fmr1*$^{+/-}$ mice showed no difference in synaptic responses to single pulse stimuli in the medial perforant path when compared to WT mice. WT: n = 4 (7), *Fmr1*$^{+/-}$: n = 5 (6) (**B**) *Fmr1*$^{+/-}$ mice showed no significant difference in paired-pulse plasticity when compared to WT mice. WT: n = 5 (7); *Fmr1*$^{+/-}$: n = 5 (8). **(C & D)** *Fmr1*$^{+/-}$ mice showed no significant difference in synaptic responses to single pulse stimuli and paired-pulse plasticity when compared to WT mice. WT: n =6(9), *Fmr1*$^{+/-}$: n = 4 (10).

Fig. 3. Impaired NMDAR-dependent LTD in the DG of *Fmr1*$^{+/-}$ mice. (A$_1$) WT mice showed significant NMDAR-LTP in the dentate gyrus, whereas *Fmr1*$^{+/-}$ mice showed an attenuated NMDAR-LTP when compared to WT mice. $P < 0.05$; **(A$_2$)** Bar chart showing the averaged NMDAR-LTP at 50-60 min post HFS. **(B$_1$)** WT mice showed significant NMDAR-LTD in the dentate gyrus, whereas *Fmr1*$^{+/-}$ mice showed an attenuated NMDAR-LTD when compared to WT mice. $P < 0.005$; WT n = 10(10); *Fmr1*$^{+/-}$ n = 7(9) **(B$_2$)** Bar chart showing the averaged NMDAR-LTD at 50-60 min post LFS **(C$_1$)** There was no significant difference in mGluR-LTD

in the CA1 region between WT and *Fmr1*$^{+/-}$ mice. WT: n = 4(7); *Fmr1*$^{+/-}$: n = 5 (7). **(C_2)** Bar chart showing the averaged mGluR-LTD at 50-60 min post LFS. Scale bar: Y: 0.2 mV, X: 3 ms.

Fig. 4. Decreased NMDAR-mediated EPSCs in the DG of *Fmr1*$^{+/-}$ mice. (A) Representative traces of maximum AMPA and NMDA currents. **(B)** *Fmr1*$^{+/-}$ mice showed lower amplitudes of AMPAR and **(C)** NMDAR-mediated EPSCs with increasing stimulation intensity. **(D)** *Fmr1*$^{+/-}$ mice showed significant decreases in both AMPAR- and NMDAR-mediated EPSCs. **(E)** There was no different in AMPAR / NMDAR ratio between *Fmr1*$^{+/-}$ mice and WT mice. * $P < 0.005$. WT: n = 6(19); *Fmr1*$^{+/-}$: n = 8(20)

Fig. 5. Glycine potentiated NMDAR-mediated EPSCs in both WT and *Fmr1*$^{+/-}$ mice. (A) WT mice showed larger glycine potentiated NMDA currents in WT than *Fmr1*$^{+/-}$ mice. **(B)** Representative traces of glycine-potentiated NMDA currents. WT: n = 5(7); *Fmr1*$^{+/-}$: n = 9(11). * $P < 0.05$.

Fig. 6. Impaired NMDAR-dependent long-term potentiation in the DG of *Fmr1*$^{+/-}$ mice was reversed by glycine. (A_1) Induction of LTP was significantly less in *Fmr1*$^{+/-}$mice compared to WT mice (Data duplicated from Fig 2A). Application of glycine normalized LTP induction in *Fmr1*$^{+/-}$ mice, without affecting LTP induction in WT mice. **(A_2)** Bar chart showing the changes in averaged LTP at 50-60 min post HFS. WT: n =6 (8); *Fmr1*$^{+/-}$: n = 8(8). **(B_1)** Application of the NMDAR antagonist APV blocked the induction of LTP in both WT and *Fmr1*$^{+/-}$ mice. WT: n =5 (7); *Fmr1*$^{+/-}$: n = 5(7) **(B_2)** Bar chart showing changes of LTP with APV application.