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SYNAPTIC NMDA RECEPTOR-MEDIATED CURRENTS IN ANTERIOR PIRIFORM CORTEX ARE REDUCED IN THE ADULT FRAGILE X MOUSE

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Abstract—Fragile X syndrome is a neurodevelopmental condition caused by the transcriptional silencing of the fragile X mental retardation 1 (*FMR1*) gene. The *Fmr1* knockout (KO) mouse exhibits age-dependent deficits in long term potentiation (LTP) at association (ASSN) synapses in anterior piriform cortex (APC). To investigate the mechanisms for this, whole-cell voltage-clamp recordings of ASSN stimulation-evoked synaptic currents were made in APC of slices from adult *Fmr1*-KO and wild-type (WT) mice, using the competitive N-methyl-D-aspartate (NMDA) receptor antagonist, CPP, to distinguish currents mediated by NMDA and AMPA receptors. NMDA/AMPA current ratios were lower in *Fmr1*-KO mice than in WT mice, at ages ranging from 3–18 months. Since amplitude and frequency of miniature excitatory post-synaptic currents (mEPSCs) mediated by AMPA receptors were no different in *Fmr1*-KO and WT mice at these ages, the results suggest that NMDA receptor-mediated currents are selectively reduced in *Fmr1*-KO mice. Analyses of voltage-dependence and decay kinetics of NMDA receptor-mediated currents did not reveal differences between *Fmr1*-KO and WT mice, suggesting that reduced NMDA currents in *Fmr1*-KO mice are due to fewer synaptic receptors rather than differences in receptor subunit composition. Reduced NMDA receptor signaling may help to explain the LTP deficit seen at APC ASSN synapses in *Fmr1*-KO mice at 6–18 months of age, but does not explain normal LTP at these synapses in mice 3–6 months old. Evoked currents and mEPSCs were also examined in senescent *Fmr1*-KO and WT mice at 24–28 months of age. NMDA/AMPA ratios were similar in senescent WT and *Fmr1*-KO mice, due to a decrease in the ratio in the WT mice, without significant change in AMPA receptor-mediated mEPSCs.

Key words: Fmr1, FMRP, olfactory cortex, glutamate, long-term potentiation.

INTRODUCTION

Fragile X syndrome (FXS) is the leading cause of inherited intellectual disability and accounts for nearly 5% of autism spectrum disorders (Kelleher and Bear, 2008). In addition to cognitive impairment, FXS is often accompanied by other neuropsychiatric symptoms, including hyperactivity, attention deficits, and seizure disorders. FXS typically results from a trinucleotide expansion in the 5′ UTR of the fragile X mental retardation 1 (*FMR1*) gene, leading to hyper-methylation and transcriptional silencing (Jin and Warren, 2000). *FMR1* encodes the fragile X mental retardation protein (FMRP), an RNA-binding protein that is highly expressed in brain neurons and associates with a variety of mRNA species to regulate their translation.

Neurobiological studies using mouse models of FXS (*Fmr1*-KO mice) have focused on synaptic function, development, and plasticity, since synaptic communication is critical for the cognitive functions affected in the human disorder. Postmortem studies show immature dendritic spines in FXS cerebral cortex, as seen in mental retardation syndromes generally; these spine abnormalities are also observed in cortex of *Fmr1*-KO mice (Rudelli et al., 1985; Wisniewski et al., 1991; Comery et al., 1997; Irwin et al., 2000). These findings suggest that normal spine development or plasticity depends on FMRP. Many studies have now identified synaptic plasticity deficits involving both long term potentiation (LTP) (Li et al., 2002; Larson et al., 2005; Zhao et al., 2005; Lauterborn et al., 2007; Wilson and Cox, 2007) and long-term depression (LTD) (Huber et al., 2002; Hou et al., 2006) in hippocampus and other cortical regions in *Fmr1*-KO mice. However, the molecular mechanisms responsible for these deficits are not well understood.

Olfactory cortex is an attractive model system for investigating the synaptic basis for behavioral and cognitive dysfunctions. Olfactory discrimination learning is impaired by lesions of primary olfactory (piriform) cortex (Staubli et al., 1987) and olfactory training induces synaptic plasticity in piriform cortex (Saar et al., 2002). In *Fmr1*-KO mice, olfactory discrimination learning is impaired, although detection thresholds for odors are normal (Larson et al., 2008). Synapses in anterior piriform cortex (APC) in *Fmr1*-KO mice show an age-dependent impairment of LTP: potentiation at associational (ASSN) system synapses is normal in mice aged 3–6 months,

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Abbreviations: aCSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; APC, anterior piriform cortex; ASSN, association; BMI, bicuculline methiodide; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPP, 3-[(R)-2-carboxypiperazin-4-yl]-prop-2-enyl-1-phosphonic acid; EPSC, excitatory post-synaptic current; Fmr1, fragile X mental retardation 1; FMRP, fragile X mental retardation protein; FXS, fragile X syndrome; IPI, inter-pulse interval; LOT, lateral olfactory tract; LTD, long-term depression; LTP, long-term potentiation; mEPSC, miniature excitatory post-synaptic current; NMDA, N-methyl-D-aspartate; PSD, post-synaptic density; TTX, tetrodotoxin.

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but progressively declines in *Fmr1*-KO mice 6–18 months old (Larson et al., 2005).

LTP in APC depends on activation of N-methyl-D-aspartate (NMDA) receptors for induction and modification/insertion of AMPA receptors for long-term expression. The present study was undertaken to investigate the possibility that LTP deficits at ASSN synapses in APC of *Fmr1*-KO mice are due to functional changes in synaptic AMPA or NMDA receptors. FMRP binds to ribonucleoprotein complexes in a phosphorylation-dependent manner and influences constitutive and activity-dependent protein translation (Ceman et al., 2003; Narayanan et al., 2008; Darnell et al., 2011). Although FMRP is not known to regulate the translation of ionotropic glutamate receptor mRNAs, it may participate in receptor localization by regulating translation of receptor-binding proteins such as post-synaptic density (PSD)-95 (Muddashetty et al., 2007). We find that NMDA receptor currents are smaller in APC associational synapses of *Fmr1*-KO mice than in those of wild-type (WT) mice, without detectable alterations in their kinetic properties. These results suggest a mechanism that may contribute to both LTP impairment in piriform cortex and defective olfactory learning in mice lacking FMRP.

EXPERIMENTAL PROCEDURES

Animals

Fmr1-KO mice were developed by the Dutch-Belgian Fragile X Consortium (Bakker et al., 1994). Experiments were conducted on littermate *Fmr1*-KO and WT mice bred in the laboratory from congenic C57BL/6J stock obtained from Jackson Laboratories (Bar Harbor, ME, USA). The mutation had been back-crossed at least 10 generations into the C57BL/6 background. Experimental mice (both mutant and control) were obtained from heterozygous *Fmr1*-KO dams mated with either WT or hemizygous *Fmr1*-KO sires. Mice were genotyped by PCR of DNA from ear snips as described previously (Bakker et al., 1994). Experiments and analyses were both conducted blind with respect to genotype. All procedures were in accordance with NIH guidelines and protocols were approved by the Animal Care Committee of the University of Illinois at Chicago.

In vitro slice preparation and electrophysiology

Parasagittal slices (300 μm) of APC were prepared from adult (3–28 month old) WT and *Fmr1*-KO mice. Mice were decapitated and brains removed in oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (120), KCl (3.1), K_2HPO_4 (1.25), $NaHCO_3$ (26), dextrose (5.0), L-ascorbate (2), $MgCl_2$ (1.0), $CaCl_2$ (2.0) at ~4 °C. The brain was then sectioned into blocks, mounted on a cutting stage and sliced on a vibrating cutter (Vibratome, St. Louis, MO, USA). The slices were incubated at 32 °C for 1 h and then allowed to cool to room temperature (25–27 °C). Slices were then transferred to a submerged recording chamber and perfused at a rate of 1 ml/min. with aCSF. All drugs and chemicals were applied via the perfusate by a solenoid-controlled gravity-feed system (ValveLink 8, AutoMate Scientific, Inc., Berkeley, CA, USA). Recordings were obtained from principal cells in layer II at room temperature. Patch electrodes (1.8–3 MΩ) contained (in mM): cesium methanesulfonate (145), $MgCl_2$ (1), HEPES (10), BAPTA (1.1), MgATP (5), and phosphocreatine (20) adjusted to pH 7.2 with CsOH, 290 mOsm. The following drugs were added via the perfusate: 3-[(R)-2-carboxypiperazin-4-yl]-prop-2-enyl-1-phosphonic acid (CPP), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 1(S),9(R)-(−)-bicuculline methiodide (BMI), and tetrodotoxin (TTX) (all obtained from Sigma, St. Louis, MO, USA). Cells were visualized with differential interference contrast (DIC) optics on a Nikon Eclipse E600FN "PhysioStation" (Nikon, Melville, NY, USA). Twisted bipolar electrodes (custom made) with a tip diameter ~50 μm were positioned in layer Ib 150–200 μm away from the recorded neuron. Stimulation in this layer activates recurrent association (ASSN) fibers projecting from principal cells in layer II. Responses at these synapses display paired-pulse depression whereas stimulation of afferent fibers in layer Ia exhibit facilitation (Bower and Haberly, 1986). Therefore, stimulation of ASSN fibers in layer Ib was confirmed after obtaining whole-cell recording by non-facilitating responses to paired-pulse stimulation at a 200 ms inter-pulse interval (IPI). ASSN fibers were stimulated at 20 s intervals during recordings. Constant current stimulation (0.1 ms) was adjusted to between 70 and 1000 μA in order to elicit a 300 pA response. Evoked excitatory post-synaptic currents (EPSCs) and miniature EPSCs (mEPSCs) were recorded with an Axopatch-1D and pClamp software (Molecular Devices, Sunnyvale, CA, USA), filtered at 1 kHz, digitized at 10 kHz, and stored on the computer hard drive. Input and series resistance were checked every 20 s throughout experiments. Recordings were rejected if series resistance exceeded 15 MΩ or if input resistance changed more than 20%. Series and whole-cell capacitance compensation were not used. No corrections were made for liquid junction potentials.

Histology

After obtaining electrophysiological recordings, some slices were fixed in 4% paraformaldehyde and cryoprotected in 30% sucrose in PBS. These slices were then resectioned at 30 μm, processed, and stained with 0.1% Cresyl Violet.

Data analysis

NMDA currents were calculated by digitally subtracting composite responses from pharmacologically isolated AMPA currents at +40 mV. Decay time constants were obtained from single and double exponential fits of spontaneous and evoked responses using ClampFit software (Molecular Devices). Evoked responses (10 consecutive traces averaged) and mEPSCs (>100 traces averaged) were fit with the standard exponential equation $I(t) = I * \exp(-t/\tau)$, where I is the peak amplitude and τ is the decay time constant. A weighted time constant (τ_w) was calculated for each trace, except for the mEPSCs, with the equation $\tau_w = [I_F/(I_F + I_S)] * \tau_F + [I_S/(I_F + I_S)] * \tau_S$. The percentage of the decay kinetics mediated by the fast component (τ_F) relative to the slow component (τ_S) was calculated as $\%\tau_F = I_F/(I_F + I_S)$. Current–voltage plots (four traces averaged) were obtained by ramping membrane potentials sequentially from −80 to +40 mV (or from +40 to −80 mV) in 10 mV intervals. All data are presented as means ± SEM. Statistical differences were calculated using Student's unpaired *t*-test or the Kolmogorov–Smirnov test if the data were not normally distributed. Analysis of variance was used for comparisons involving more than two groups.

RESULTS

Associational system synaptic currents in APC slices

Voltage clamp recordings in the whole-cell configuration were obtained from layer II cells in slices of APC (Fig. 1A). At a holding potential of −80 mV using pipettes filled with a cesium-based internal solution, stimulation of associational (ASSN) system fibers in layer Ib elicited inward currents consisting of an early component mediated by AMPA receptors and a slower component