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Alterations in CA1 hippocampal synapses in a mouse model of Fragile X Syndrome

Safdar Jawaid, Grahame J. Kidd, Jing Wang, Carrie Swetlik, Ranjan Dutta, and Bruce D. Trapp

Department of Neurosciences, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195

Abstract

Fragile X Syndrome (FXS) is the major cause of inherited mental retardation and the leading genetic cause of Autism Spectrum Disorders (ASDs). FXS is caused by mutations in the Fragile X Mental Retardation 1 (Fmr1) gene, which results in transcriptional silencing of Fragile X Mental Retardation Protein (FMRP). To elucidate cellular mechanisms involved in the pathogenesis of FXS, we compared dendritic spines in the hippocampal CA1 region of adult wild-type and *Fmr1* knockout (Fmr1-KO) mice. Using diolistic labeling, confocal microscopy, and 3-dimensional electron microscopy, we show a significant increase in the diameter of secondary dendrites, an increase in dendritic spine density, and a decrease in mature dendritic spines in adult Fmr1-KO mice. While WT and Fmr1-KO mice had the same mean density of spines, the variance in spine density was three times greater in Fmr1-KO mice. Reduced astrocyte participation in the tripartite synapse and less mature post-synaptic densities were also found in Fmr1-KO mice. We investigated whether the increase in synaptic spine density was associated with altered synaptic pruning during development. Our data are consistent with reduced microglia-mediated synaptic pruning in the CA1 region of Fmr1-KO hippocampi when compared to wild-type littermates at postnatal day 21, which is the peak period of synaptic pruning in the mouse hippocampus. Collectively, these results support abnormal synaptogenesis and synaptic remodeling in mice deficient in FMRP. Deficits in the maturation and distribution of synaptic spines on dendrites of CA1 hippocampal neurons may play a role in the intellectual disabilities associated with FXS.

Graphical abstract

Keywords

tripartite synapse; astrocyte; microglia; post-synaptic density

1. Introduction

Autism Spectrum Disorders (ASDs) are a group of neurodevelopmental disorders that share common features, including social deficits, communicative difficulties, repetitive behaviors, and cognitive delay (Betancur et al., 2009; Mefford et al., 2012). One in 68 newborns in the USA (>3.5 million individuals) and approximately 1% of the world population are affected by an ASD (Buescher et al., 2014). Both genetic and environmental factors contribute to the etiology of ASDs, which include abnormalities in brain structure, neuronal function, and connectivity (Hallmayer et al., 2011; Ecker et al., 2015). In addition to those defined specifically as ASDs, many diseases with other neurological classifications are also included in the autism spectrum (Hagerman et al., 2010; Mefford et al., 2012). Fragile X syndrome (FXS) is the most common cause of inherited intellectual disability and results from mutations in the Fragile X mental retardation 1 (*Fmr1*) gene. The *Fmr1* gene encodes Fragile X mental retardation protein (FMRP), which is an RNA binding protein that plays a critical role in 1) transport of mRNAs from nucleus to cytoplasm, 2) dendritic localization of mRNAs encoding proteins that are important for dendritic growth/synaptogenesis, and 3) suppression of dendritic protein translation (Brown et al., 2001; Miyashiro et al., 2003; Liao et al., 2008). The gene contains a CGG trinucleotide repeat in the 5'UTR that is normally 6 to 45 CGGs long. In FXS patients, the 5'UTR region contains 200 or more CGG repeats, which results in hypermethylation and transcriptional silencing of the *Fmr1* gene and subsequent loss of FMRP (Fu et al., 1991; Willemsen et al., 2011). FXS patients exhibit ASD phenotypes as well as comorbid intellectual disabilities (Veltman et al., 2005; Hagerman et al., 2010; Mefford et al., 2012). FMRP is highly expressed in human hippocampus (Abitbol et al., 1993). Hippocampal volume changes have been reported in individuals with FXS (Bostrom et al., 2016) and FXS patients show reduced hippocampal activation during visual memory tasks (Greicius et al., 2004). While hippocampal neuronal changes are not well-documented in FXS brains, dendritic spine abnormalities have been

described in neocortex (Rudelli et al., 1985; Hinton et al., 1991; Wisniewski et al., 1991; Irwin et al., 2000; Irwin et al., 2001).

Fmr1-null mice (*Fmr1*-KO) are commonly used as a rodent model of FXS (Bakker, 1994). Hippocampal and behavioral changes in *Fmr1*-KO mice have been investigated in detail (for review see (Bostrom et al., 2016)). Behavioral changes mimic many described in FXS, including increased anxiety, reduced spatial and content-dependent learning, and enhanced fear conditioning (Huber et al., 2002). Exaggerated mGluR long-term depression in the CA1 region (Huber et al., 2002; Bear et al., 2004) of the hippocampus and impaired NMDA receptor long-term potentiation in the dentate gyrus (Yun et al., 2011; Franklin et al., 2014; Bostrom et al., 2015) are thought to contribute to these behavioral changes. Studies investigating hippocampal dendritic spine densities in *Fmr1*-KO mice have shown mixed results, including increases (Levenga et al., 2011; Bhattacharya et al., 2012), decreases (Braun et al., 2000; Segal et al., 2003) and no significant changes (Grossman et al., 2006). Reports regarding the status of spine morphology are also contradictory, with descriptions of more mature (Grossman et al., 2006) and less mature (Bilousova et al., 2009; Bhattacharya et al., 2012) dendritic spines in the CA1 region of *Fmr1*-KO hippocampus.

Synaptogenesis is a dynamic process during normal brain development. An overabundance of synapses is initially formed; synaptic connectivity is then refined through elimination of synapses. Microglia play an essential role in sculpting synaptic fields during postnatal brain development by a process called synaptic pruning (Paolicelli et al., 2011; Li et al., 2012; Schaefer et al. 2013). Motile microglial processes (Nimmerjahn et al., 2005) make brief contacts with synaptic structures (Wake et al., 2009; Tremblay et al., 2010), sense defective synaptic activity, and physically remove the defective synapse (Paolicelli et al., 2011). Microglia-mediated phagocytosis of defective excitatory synapses involves the expression of the complement cascade component C1q by neurons and the expression of complement receptor 3 (CD11b/CD18) by microglia (Stevens et al., 2007; Schafer et al., 2012). Microglia-mediated synaptic pruning appears to be unchanged during early development of the visual system in an animal model of Rett Syndrome (Schafer et al., 2016). Deficits in synaptic pruning have not been investigated in *Fmr1*-KO mice.

Astrocytes, which are an active component of the tripartite synapse, mediate neurotransmitter homeostasis and recycling, provide substrates for neuronal metabolism, sequester Ca^{2+} ions, and promote synaptogenesis and synaptic remodeling (Sofroniew et al., 2010; Halassa et al., 2010). As part of the tripartite synapse, astrocytic processes cover synaptic clefts. The extent of astrocytic-synaptic cleft contact varies in different brain regions: perisynaptic astrocytes are present in 90% of cerebellar synapses (Grosche et al., 1999), while approximately 50-60% of hippocampal synapses have perisynaptic astrocytes (Spacek et al., 1998; Ventura et al., 1999). Astrocytes have been implicated in the pathogenesis of FXS. When astrocytes null for *Fmr1* were cultured with wild-type (WT) neurons, synaptogenesis was impaired (Jacobs et al., 2010). FMRP is expressed in cells of non-neuronal lineage during development, indicating a potential role of glial cells lacking FMRP in the neural development of FXS (Pacey and Doering 2007). Deletion of FMRP from astrocytes causes cortical synaptic defects that include altered astroglial expression of glutamate transporter 1 (GLT1), impaired glutamate uptake, and enhanced neuronal

excitability (Higashimori et al., 2016). It is unknown whether astrocyte-synapse cleft alterations are present in individuals with FXS or in Fmr1-KO mice.

This study investigates alterations in the development and maintenance of dendritic spines in hippocampal CA1 neurons from Fmr1-KO mice. We describe data consistent with a deficit in synaptic pruning during early development as well as an increase in immature synaptic spine density and reduced astrocyte-synaptic cleft associations in adult mice.

2. Materials and Methods

2.1 Animals

Fmr1-KO (B6.129P2-*Fmr1*^{tm1Cgr/J}) and WT C57BL/6J mice, purchased from Jackson Laboratory, have been described in detail previously (Bakker, 1994). All mice were maintained under pathogen-free conditions in a temperature- and humidity-controlled environment in the biological resources facility at the Lerner Research Institute. Access to food and water was provided *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic and were consistent with NIH guidelines on the use of laboratory animals.

2.2 Diolistic labeling of neurons

Diolistic labeling fills individual neuronal perikarya, dendrites, and dendritic spines with fluorescent dye. Three-dimensional visualization of dendrites and spines is then obtained by confocal scanning microscopy. Sixty-day-old (P60) Fmr1-KO and WT mice were perfused with 10 mL of phosphate-buffered saline (PBS) and 15 mL of 1.5% paraformaldehyde. The brain was removed and the hippocampus was sectioned at a thickness of 150 µm on a vibrating microtome. Sections were “shot” with DiI/DiO-coated tungsten bullets (Molecular Probes, Eugene, OR) using a Helios Gene Gun (BioRad Laboratories, Inc., Hercules, CA). Sections were washed and placed in PBS for 24 hours at 4°C. Sections were then post-fixed in 4% paraformaldehyde, mounted on glass slides, and imaged with a Leica confocal microscope at 63x magnification. The dendritic field of CA1 neurons (Bregma -1.25mm), approximately 100 µm from the pyramidal cell layer, was imaged. Secondary dendrites of more than 10 µm in length were segmented from the confocal image stacks. Quantification and classification of dendritic spines were performed using ImageJ software. Six dendrites from each of 8 WT and 8 Fmr1-KO mice were analyzed.

2.3 Three-dimensional electron microscopy (3D-EM)

P60 WT and Fmr1-KO mice were perfused with 2.5% glutaraldehyde and 4% paraformaldehyde. Brains were removed and sliced coronally using a 0.5 mm mouse brain matrix (Electron Microscopy Sciences). Slices containing the CA1 region described above were sequentially treated with osmium ferricyanide, thiocarbohydrazide, aqueous osmium (2%), aqueous uranyl acetate, and Walton’s lead aspartate stain, dehydrated and embedded in Epon (all reagents from Electron Microscopy Sciences, Hatfield, PA). The Epon-embedded CA1 hippocampus was placed in the Carl Zeiss Sigma VP scanning electron microscope containing a 3View in-chamber ultramicrotome system and a Gatan high sensitivity, low-kV backscattered electron detector (Gatan, Warrendale, PA). Sets of 500

images were serially collected at 5-7 nm per pixel resolution, $48\ \mu\text{m} \times 48\ \mu\text{m}$ size and at 75 nm thickness using serial block-face scanning EM. Care was taken to ensure that the same population of dendrites was studied in each animal. As described above for diolistic labeling, the secondary dendritic branches of CA1 pyramidal neurons (Bregma -1.25mm) were identified where they branched from primary dendrites in the distal stratum radiatum $\sim 100\ \mu\text{m}$ from the pyramidal cell layer. Secondary dendrites were followed for $15\ \mu\text{m}$ or more, with the proximal $5\ \mu\text{m}$ branch zone excluded. Images were further processed and registered using ImageJ software with FIJI plug-in sets. Image stacks were imported into Reconstruct software (Fiala, 2005) and tracing of objects was used to obtain 3D reconstructions and meshes for analysis. Secondary dendrites were reconstructed and their volumes were measured. The diameters of secondary dendrites and the number of spines per μm length of dendrites and dendrite surface area were compared in WT and Fmr1-KO mice. The density, shape, and volume of dendritic spines, postsynaptic density (PSD), and the percent area of synaptic clefts opposed by astrocyte processes were quantified for at least $10\ \mu\text{m}$ of each secondary dendrite. Dendritic spines were classified based on shape (mushroom, stubby, thin) as described by Harris and colleagues (Harris et al., 1992). Two blinded assessors (inter-assessor variation = $\pm 5\%$) rated the spine numbers and spine shapes of the secondary dendrites from the CA1 region of hippocampus. Our classification parameters are based on a standard set of metrics using neck length and spine head size. Spines with thin necks (typically $0.2\ \mu\text{m}$ diameter) and heads that were $<2\times$ neck diameter were classified as “thin”. Spines with heads $>2\times$ neck diameter were classified as mushroom spines. Short spines ($<1\ \mu\text{m}$) with similar head and neck diameters were classified as “stubby”. All analyses were performed blinded to the genotype of the mice. Five dendrites from each of 3 WT and 3 Fmr1-KO mice were analyzed by 3D-EM.

2.4 Immunostaining and confocal microscopy

To determine the peak period of synaptic pruning in the CA1 hippocampal region, we investigated synaptic pruning between P1 and P42 in WT mice. Mice were anaesthetized and perfused with 4% paraformaldehyde in 0.08M phosphate buffer (pH=7.4). Brains were removed and stored overnight in the same fixative and then sectioned on a freezing sliding microtome at a thickness of $30\ \mu\text{m}$. Sections were stored in 12-well plates, cryoprotected in 20% glycerol, and stored at -20°C . For immunohistochemistry, free-floating sections were pre-treated with 3% H_2O_2 , 1% Triton X-100 in PBS (PBST), and blocked with 3% normal goat serum (NGS). Sections were incubated with a combination of rabbit polyclonal PSD95 (Abcam, Cambridge MA; diluted 1:500) and mouse monoclonal Iba1 (CCF Hybridoma Core; diluted 1:500) antibodies in 0.1% PBST and 3% NGS overnight at 4°C . After washing with 0.1% PBST, sections were incubated with secondary antibodies - Alexa Fluor® 594 (Abcam; diluted 1:1000) and Alexa Fluor® 488 (Abcam; diluted 1:1000) for 1 hour at room temperature. Sections were then washed with PBS and mounted on glass slides with DAPI medium. PSD95- and Iba1-stained sections were imaged by confocal microscopy. Thirty-micron, free-floating sections were imaged using an x63 (NA 1.4) lens at 0.2 micron steps (optical section thickness 0.2 microns). In ImageJ, the Iba1 image from each slice was thresholded, dilated by 0.2 microns, and the binary mask was used to select PSD95-positive puncta within Iba1-positive microglia. Our developmental study identified P21 as the peak

period of microglia-mediated synaptic pruning in the CA1 region of WT hippocampi (Fig. 5c). We compared synaptic pruning in P21 WT and Fmr1-KO hippocampi.

2.5 Statistical Analysis

Statistical analyses were conducted with the R-statistical package and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). All results are presented as mean $\pm$ standard deviation except where noted. Comparisons were made by Student's two-tailed unpaired t-tests incorporating Bonferroni's corrections for multiple comparisons where appropriate, and using F-tests for variance analysis. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Fmr1-KO mice contain more dendritic spines

Diolistic labeling is a reliable method for visualizing neuronal morphology. We used this technology to compare secondary dendritic morphology of the apical shaft of hippocampal CA1 neurons from P60 WT and Fmr1-KO mice. Figure 1a shows a labeled neuron and the dendritic shaft area analyzed (Fig. 1a, box). At higher magnification, individual dendritic spines were visualized in WT (Fig. 1b) and Fmr1-KO dendrites (Fig. 1c). The number of spines per dendrite length was significantly increased in Fmr1-KO dendrites (Fig. 1d; $p < 0.01$). We next used criteria established by Harris and colleagues (Harris et al., 1992) to characterize spines based on shape ("Mushroom", "Stubby", or "Thin"). Thin- and stubby-shaped spines are considered immature, while mushroom-shaped spines are considered mature (Bourne et al., 2007). Compared to WT mice, mushroom-shaped spines were reduced by 24% in Fmr1-KO mice (Fig. 1e; $p < 0.001$), while thin spines were increased by 27% ($p < 0.001$).

3.2 Dendritic spines in Fmr1-KO mice are immature

We utilized 3D-EM to compare dendritic morphology at higher magnification. Figure 2a shows a secondary dendritic shaft and synaptic spine reconstructed from 200 serial sections. Dendritic spines from WT (Fig. 2b) and Fmr1-KO (Fig. 2c) mice were reconstructed in 3D. The morphological shape of individual spines can be resolved upon rotation of these 3D reconstructions (see Supplemental Video 1). A 14% increase in the mean dendritic shaft diameter was found in Fmr1-KO mice compared to WT (Fig. 2d; $p < 0.05$). Similar to diolistic labeling studies, 3D-EM detected a significant increase (15.40%; $p < 0.05$) in the density of dendritic spines per micron of dendrite length in Fmr1-KO mice (Fig. 2e). We further investigated the relationship between spine number and dendritic surface area. While WT and Fmr1-KO mice had the same mean density of spines, the variance in spine density was three times greater in Fmr1-KO mice (Fig. 2f). In WT mice, spine number closely correlated with dendritic surface area (Fig. 2g; $r^2 = 0.85$, $p < 0.0001$). In contrast, spine number in Fmr1-KO mice showed little correlation with dendrite surface area (Fig. 2h; $r^2 = 0.08$). Figures 2i and 2j illustrate the variations in spine density in Fmr1-KO dendrites of similar diameter.

Compared to WT mice, the percentage of mushroom-shaped spines was significantly decreased (52% vs 36%) in Fmr1-KO mice and the percentage of thin spines was significantly increased (38% vs. 53%; Fig. 3a). Stubby spines represented ~10% in each group. It is important to note that while diolistic labeling *and* 3D-EM produced similar results, the total number of dendritic spines was greater with 3D-EM analysis. The absolute number of spines was approximately 1.8 times greater than that observed by light microscopy following diolistic labeling (Supplemental Fig. 1a). This difference is a reflection of the limited light microscopic resolution of spines projecting in the z-plane following diolistic labeling. When spine density along the dendrite was assessed by 3D-EM, the number of mushroom spines per unit length of dendrite in Fmr1-KO was reduced by 22% compared to WT, whereas the number of thin spines was increased by 57% in Fmr1-KO (Fig. 3b). These differences were detected by both diolistic labeling and 3D-EM (Supplemental Fig. 1b).

Densities attached to the cytoplasmic surface of excitatory post-synaptic membranes reflect active zones of neurotransmitter-receptor interactions (Arellano et al., 2007; Sheng et al., 2011; Gundelfinger et al., 2012; Meyer et al., 2014). The PSD is a complex structure that performs multiple functions, including positioning of neurotransmitter receptors opposite the presynaptic terminal, linkage of these receptors to the cytoskeleton, and postsynaptic signaling events (Kennedy, 2000; Okabe, 2007; Sheng et al., 2011). PSDs can be divided into two groups: macular and perforated. Macular PSDs have a continuous sheet of PSDs that have irregular shapes (Fig. 3c). Perforated PSDs are discontinuous (Fig. 3c) and synapses with perforated PSDs are considered to be more mature and active (Geinisman, 1993; Desmond et al., 1998; Toni et al., 1999; Marrone et al., 2002). We compared PSDs in WT and Fmr1-KO 3D-EM stacks. The PSD area per spine was significantly decreased in Fmr1-KO mice (Fig. 3d; $p < 0.05$). In addition, the proportion of perforated PSDs was significantly decreased and the proportion of macular PSDs was significantly increased in Fmr1-KO mice (Fig. 3e; $p < 0.05$).

3.3 Astrocytic involvement in the tripartite synapse is reduced in Fmr1-KO mice

Astrocytes are a critical component of the tripartite synapse and are essential for synaptic function and regulation of extracellular glutamate (Sofroniew et al., 2010). To investigate whether astrocytic involvement in the tripartite synapse is affected in Fmr1-KO mice, we reconstructed the distribution of astrocyte processes in our 3D-EM stacks. Astrocyte processes in individual slices were identified by the presence of intermediate filaments and glycogen granules. When the ultrastructural characteristics of individual processes were unclear, they were followed in 3D stacks and traced to regions where astrocytic features were evident. Figure 4a shows a single slice where an astrocytic process opposes a synaptic cleft (asterisks; yellow = astrocyte process; blue = presynaptic terminal; green = dendritic spine; red = PSD). Figure 4b shows 2 synaptic terminals without an astrocytic process (Fig. 4b; 1-2) and one with an astrocytic process (Fig. 4b; 3). Fifty-three percent of synaptic clefts in WT mice were opposed by astrocytic processes (Fig. 4c). In Fmr1-KO mice, 43% of the synapses had astrocyte processes opposing the synaptic cleft (Fig. 4c; $p < 0.001$). 3D reconstructions show the relationship between astrocyte processes and synapses in WT (Fig. 4d,e) and Fmr1-KO (Fig. 4f,g) mice. Figures 4d and 4f show all astrocyte processes

(yellow), while figures 4e and 4g show dendritic spines (green), PSDs (red), and astrocytic processes associated with synaptic clefts. Compared to WT mice (Figs. 4d,e), astrocytic processes at the synaptic cleft were decreased in Fmr1-KO mice (Figs. 4f,g) and were mostly found on mushroom-shaped dendrites. Involvement of astrocytes in the tripartite synapse is therefore significantly reduced in Fmr1-KO CA1 dendrites.

3.4 Aberrant synaptic pruning in P21 Fmr1-KO mice

We next investigated whether increased synaptic density in adult Fmr1-KO mice was associated with alterations in microglia-mediated synaptic pruning during early postnatal development. Synaptic pruning has been quantified by co-localizing the post-synaptic protein, PSD95, and the microglial protein Iba1 (Paolicelli et al., 2011). We first determined the peak period of synaptic pruning in WT CA1 synaptic fields. Hippocampal sections from WT mice (between P1 and P42) were co-labeled with PSD95 and Iba1 antibodies. An example of PSD95-Iba1 co-localization from WT at P15 is shown in Figures 5a-b at low and high magnification, respectively. The number of PSD95 puncta that co-localized with Iba1 in 0.2 μm -thick planes was quantified at postnatal days 1-42 (Fig. 5c). Based upon this developmental curve, we choose P21 as the time point to compare synaptic pruning in WT (Figs. 5d-f) and Fmr1-KO mice (Figs. 5g-i). The number of PSD95 puncta within microglia was significantly reduced in Fmr1-KO sections (Fig. 5j). These results support reduced synaptic pruning in P21 Fmr1-KO hippocampus.

4. Discussion

The present study investigated synaptic changes in a rodent model of FXS, the most common cause of intellectual disability and ASD in humans. Our studies focused on the CA1 region of the hippocampus, which is a brain area responsible for cognition and mood. Despite an increase in the overall density of dendritic spines in adult Fmr1-KO mice, spine number did not linearly correlate with dendritic surface area and the proportion of mature, mushroom-shaped spines was significantly decreased. Dendritic spines in adult Fmr1-KO mice also had reduced PSD area, less perforated PSDs, and less involvement of astrocytes in the tripartite synapse. These results establish that dendritic spine distribution and maturation are significantly altered in the hippocampus of adult Fmr1-KO mice and support the possibility that these synaptic changes play a role in the pathophysiology of FXS and ASDs. Our data also support the concept that microglia-mediated pruning of CA1 synapses is significantly decreased during development and may contribute to increased dendritic spine density in adult Fmr1-KO hippocampus.

While the literature on dendritic spines in Fmr1-KO mice has been contradictory, there is a growing consensus that dendritic spine density is increased in Fmr1-KO cortex and in FXS in general (for review, see (Bostrom et al., 2016)). Past discrepancies in CA1 dendritic spine densities in Fmr1-KO hippocampus are likely due to several variables, including the age of the mice and the method of spine detection. Diolistic labeling is the most-utilized method for dendritic spine analysis. While improvements in confocal microscopy have increased the visualization of individual dendritic spines, appropriate detection and classification of dendritic spines in the Z plane is still challenging. Our diolistic studies detected an overall

increase in dendritic spine densities and a reduction in more mature mushroom-shaped spines. We extended these light microscopic data by 3D-EM. This technology enables resolution of all dendritic spines in three dimensions and at nanometer resolution. 3D-EM studies provided valuable insights to our light microscopic methods and added subcellular data not provided by light microscopy. 3D-EM analysis confirmed our light microscopic data of increased total dendritic spines and reduced mushroom-shaped dendritic spines in Fmr1-KO mice. 3D-EM, however, detected an increase in the diameter of dendrites in Fmr1-KO mice. The mean density of spines per micron² of dendrite surface area was similar in WT and Fmr1-KO mice, but the variance of spine density was three times larger in Fmr1-KO mice. A linear correlation between spine number and dendrite surface area was observed in WT mice but was not found in Fmr1-KO mice. This indicates an altered distribution of spines in Fmr1-KO mice. Percent differences in spine density and spine shape between Fmr1-KO and WT mice were similar in light microscopy and 3D-EM analyses (Supplemental Fig. 1), indicating that diolistic labeling can provide valid results when only detecting a proportion of the total spines. Finally, as discussed below, 3D-EM provides information that is difficult, if not impossible, to obtain at the light microscopic level.

3D-EM enabled an in-depth comparison of the tripartite synapse in Fmr1-KO and WT mice and we detected novel changes in PSDs and astrocyte associations with the synaptic cleft. FMRP has three functions that could impact dendritic spine development: regulation of mRNA transport to dendrites, stabilization of mRNA, and inhibition of translation. Changes in PSD proteins have been described in Fmr1-KO mice (for review, see (Kindler et al., 2012)). Loss of FMRP does not appear to play an obligatory role in trafficking mRNAs to dendritic spines (Steward et al., 1998; Muddashetty et al., 2007). Since FMRP inhibits translation, its loss increases levels of PSD proteins (Schutt et al., 2009). These include the Shank family of scaffolding proteins that are considered to be master scaffolds of the PSD and dynamic regulators of synaptic spine development (Gundelfinger et al., 2006). Mutations in Shank family genes are associated with mental retardation (Bonaglia et al., 2001) and ASDs (Durand et al., 2007; Berkel et al., 2010). It has been proposed that excessive Shank expression in Fmr1-KO dendrites prevents dendritic spine maturation and results in increased immature spine density (Kindler et al., 2012). Our data extend these observations by establishing decreased PSD area in Fmr1-KO dendritic spines. In addition, the PSDs present had a more immature appearance with less perforated PSDs. In general, CA1 PSD area has been positively associated with better synaptic function and increased long-term memory (Hongpaisan et al., 2007). PSD area correlates with the abundance of glutamate receptors (Nusser et al., 1998; Racca et al., 2000; Kasai et al., 2003) and perforated synapses have a greater density of glutamate receptors than PSDs without perforations (Baude et al., 1995; Desmond et al., 1998; Nusser et al., 1998). Reduced PSD area and less perforated PSDs in CA1 synapses contribute to synaptic dysfunction in Fmr1-KO mice.

Astrocytes play important roles in synapse function. As the third component of the tripartite synapse, astrocytes regulate extracellular glutamate via GLT1 (Rothstein et al., 1994). Glutamate release at CA1 synapses induces GLT1-mediated currents in perisynaptic astrocytes (Bergles et al., 1997). By clearing glutamate from presynaptic clefts, astrocytes help protect against glutamate excitotoxicity (Rothstein et al., 1994; Sofroniew et al., 2010).

As reported previously in rat brain (Witcher et al., 2007), 53% of mouse CA1 hippocampal synapses in the present study had astrocytes (Fig. 4c), and most of these were mature mushroom-shaped synapses. This percentage was significantly reduced in Fmr1-KO mice (Fig. 4c), indicating that glutamate diffusion from mature synapses may be increased in Fmr1-KO CA1 hippocampus. Increased glutamate spillover will modulate synchronous firing of neighboring synapses and contribute to hippocampal dysfunction by reducing synaptic input specificity. Reduced GLT1 in Fmr1-KO astrocytes (Higashimori et al., 2016) may further augment glutamate spillover and hippocampal dysfunction. This hypothesis is relevant to studies indicating that FMRP may regulate cell-to-cell synaptic connections, rather than individual synapses (Patel et al., 2014).

Investigation of FXS animal models at early ages may identify pathological changes that are amenable to therapeutic manipulation. In this regard, our data support the concept that P21 Fmr1-KO mice have reduced microglia-mediated synaptic pruning of CA1 dendrites. This developmental alteration may contribute to the increased synaptic density in adult mice. Since FMRP is not highly expressed by microglia (Zhang et al., 2014), it is most likely that microglia are responding to altered CA1 synaptic activity. Abnormal short-term synaptic plasticity and immature presynaptic terminals in Fmr1-KO CA1 neurons at P14 (Klemmer et al., 2011) and exaggerated mGluR5-dependent long-term depression in juvenile Fmr1-KO hippocampi (Huber et al., 2002) support this possibility. Increased microglia-mediated synaptic pruning has been reported in adult mice that lack Mecp2 (Schafer et al., 2016), which is a rodent model of Rett Syndrome. Microglia-mediated synaptic pruning can be mitigated by minocycline, an agent that reduces microglial activation (Schafer et al., 2012). It is possible that a therapeutic increase in microglial activation will enhance microglia-mediated synaptic pruning, decrease synaptic spine density, and modulate synaptic changes in Fmr1-KO mice.

We describe several novel observations regarding synaptic changes in the CA1 region of hippocampi that lack FMRP. We establish with 3D-EM that CA1 dendritic spines are increased and more immature in Fmr1-KO mice. FMRP has several functions that may contribute to abnormal synapse formation and function, including altered gene expression, reduced mRNA transport to dendrites, and increased mRNA translation. Loss of neuronal FMRP reduced the active zone of mature postsynaptic densities. These ultrastructural changes are consistent with previous reports that loss of FMRP causes changes in translation of PSD proteins (Kindler et al., 2012). We have also established that downstream effects of FMRP loss correlate with changes in astrocytes that reduce their ability to participate in the tripartite synapse. Reduced GLT1 expression by Fmr1-KO astrocytes (Higashimori et al., 2016) are likely to exacerbate these morphological changes. Reductions in microglia-mediated synaptic pruning during early development suggest that microglia contribute to synaptic changes in FXS. It remains to be determined whether microglia are mediators of these synaptic changes or are responders to reduced synaptic activity. In either event, the pathogenesis of FXS includes alterations in glial cells and provides additional support that FXS is not a “neurocentric” disease and that therapeutics that target glial cells should be considered as part of the arsenal that may be required to treat FXS and ASDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Main Points

- Adult Fmr1-KO mice have increased density of dendritic spines in CA1 hippocampal neurons. These spines are immature with reduced PSD area.
- Reduced microglial-mediated synaptic pruning may contribute to reduced dendritic spines in Fmr1-KO mice.

Figure 1.

Decreased dendritic spines in Fmr1-KO mice. (a) Diolistic labeling of P60 WT and Fmr1-KO CA1 hippocampal neurons. DiO Dye labeled individual CA1 neurons (a, red). Secondary dendrites (a, box) were chosen for spine analysis. (b-c) At higher magnification, individual dendritic spines were visible (b, WT; c, Fmr1-KO). (d) Fmr1-KO mice had 14% more spines per μm than WT mice. (e) Based-upon shape, spines were classified as mushroom, stubby, or thin. Fmr1-KO mice had 24% fewer mushroom-shaped spines and 27% more thin spines than WT mice (N = 8 mice per group; 6 dendritic segments per mouse. ** $p < 0.01$, *** $p < 0.001$. Scale bars a=20 μm ; b, c=2.5 μm .

Figure 2.

Three-dimensional EM analysis of secondary dendrites from P60 WT and Fmr1-KO CA1 hippocampal neurons. (a) Secondary dendrites were reconstructed from stacks of up to five hundred 75 nm-thick sections collected at 7 nm/pixel resolution. (b-c) Dendritic shafts and dendritic spines from WT (b, 10 μm) and Fmr1-KO (c, 10 μm) mice were analyzed. (d) The diameter of secondary dendrites was significantly increased in Fmr1-KO mice. (e) Dendritic spine density was increased in Fmr1-KO mice. (f) The mean number of spines per dendritic surface area was similar in WT and Fmr1-KO mice, but the variance was significantly higher in the Fmr1-KO group (F-test, $p < 0.04$). (g,h) Spine numbers linearly correlate with dendrite surface area in WT mice (g; $r^2 = 0.85$). Spine number does not correlate with dendritic surface area in Fmr1-KO mice (h; $r^2 = 0.08$). Comparison of two Fmr1-KO dendrites illustrates the high variability in spine density in dendrites of similar diameter (i,j).

Figure 3.

Fmr1-KO synaptic terminals are immature and have decreased active zones. (a) The proportion of mushroom spines was reduced by 30% and that of thin spines was increased by 39% in Fmr1-KO mice compared to WT. (b) When spine density was measured per μ m of dendrite, the proportion of mushroom spines was reduced by 22% and that of thin spines was increased by 57% in Fmr1-KO mice compared to WT. The distribution, volume, and shape of post-synaptic densities was determined from 3D-EM stacks of WT and Fmr1-KO dendrites (c, Both macular PSDs and perforated PSDs were identified). (d) The total PSD area per spine was significantly decreased in Fmr1-KO mice (WT: N = 3 animals, 7 dendrites, 291 spines; Fmr1-KO: N = 3 animals, 10 dendrites, 470 spines; $*p < 0.05$). (e) The percentage of spines with macular-shaped PSDs was increased, while perforated spines were decreased in Fmr1-KO mice. (a,b; WT: N = 3 animals, 15 dendrites, 614 spines; Fmr1-KO: N = 4 animals, 18 dendrites, 738 spines. $***p < 0.001$); (c,d; WT: N = 3 animals, 3 dendrites, 123 spines; Fmr1-KO: N = 3 animals, 3 dendrites, 100 spines; $*p < 0.05$).

Figure 4.

Astrocyte participation in the tripartite synapse is decreased in Fmr1-KO mice. (a,b) Single slice electron micrographs showing a synapse with (a, b; 1-2) and without (b; 3) an astrocyte process opposing the synaptic cleft (asterisks = apposition; yellow = astrocyte process; blue = presynaptic terminal; green = dendritic spine; red = PSD). (c) Synaptic clefts with astrocyte processes were significantly greater in WT than in Fmr1-KO mice. (d-g) Three-dimensional reconstructions of astrocyte processes (yellow), dendritic spines (green), and PSDs (red) in WT mice (d, e) and Fmr1-KO (f, g) mice. d-g Reconstruction of the tripartite synapse in WT (d, e) and Fmr1-KO (f, g) mice; e and g, 3D reconstructions of astrocyte-synapse associations; d and f, all astrocyte processes, e.g. astrocyte contact zones (WT: N =

3 animals, 3 dendrites, 121 spines; Fmr1-KO: N = 3 animals, 3 dendrites, 101 spines;
*** $p < 0.001$), Scale bars (a, b) = 0.5 μm

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Figure 5.

Microglia-mediated synaptic pruning in Fmr1-KO hippocampus at postnatal day 21. (a-b) Synaptic pruning was measured by detection of PSD 95 punctae in Iba1-positive microglia. Panel b is a higher magnification of the blue box in a. (c) The peak of microglia-mediated synaptic pruning was at P21 in WT mice. (d-i) Microglia-mediated synaptic pruning was compared in P21 WT (d-f) and Fmr1-KO (g-i) mice. e and h are higher magnifications of the boxed areas in d and g, respectively. f and i are 0.2 μm -thick single confocal slices from e and h, respectively. (j) The number of PSD95-positive punctae was significantly decreased in Fmr1-KO mice (N=3 animals per group; ***p<0.001). Scale bar = 20 μm