Delayed Development of Dendritic Spines in *Fxr2* Knockout Mouse

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Abstract: Fragile X syndrome, the most common form of inherited mental retardation is caused by silencing of the Fmr1 (fragile x mental retardation-1) gene. Two mammalian homologues of Fmr1 have been identified: fragile X-related Protein 1 (*Fxr1*) and Protein 2*Fxr2*. Aberrations in dendritic spines of Fragile X syndrome patients and *Fmr1* null mice implicate FMRP in synapse fo rmation and function. However, no structural analysis has been performed on *Fxr2* null mice. Here we examined dendritic spines in brains of *Fxr2* KO mouse. We report that at the age of 2 weeks, unlike in the *Fmr1* null mice, spines in the somatosensory cortex and the hippocampus of *Fxr2* null mice are less dense compared to wild type mice. On the other hand, there is an increase in spine length similar to that reported in the *Fmr1* null mice. These differences in spine density and morphology are no longer detected by the age of 4 weeks. Our results indicate for the first time that *Fxr2* plays a role in spine development and further suggest that *Fxr2* has only partially overlapping function with *Fmr1*.

Keywords: Pyramidal neuron, somatosensory cortex, Hippocampus, Fragile X, *Fmr1*, FMRP.

INTRODUCTION

Fragile X syndrome is the most common form of inherited mental retardation. In most of the affected individuals, an expansion of a CGG trinucleotide repeat in the 5' untranslated region of the *Fmr1* gene causes transcriptional silencing and subsequent lack of expression of the *Fmr1* gene product, the Fragile X Mental retardation Protein (FMRP) [1-4]. *Fxr1* and *Fxr2* are two autosomal homologues of *Fmr1* [5-7] that together form the Fragile X-related (FXR) family of proteins. These proteins share similar gene structure and amino acid sequence and show partially overlapping patterns of expression [7, 8]. In neurons, FXR proteins are found in the cytoplasm [8] and FMRP is found in dendrites and synapses [9]. FMRP is known to associate with translating polyribosomes and is believed to function as a regulator of protein synthesis at the synapse [9, 10]. The similarities in protein structure and expression of the FXR proteins as well as similarities in some neurobehavioral and synaptic plasticity deficits in mice lacking the *Fmr1* or the *Fxr2* genes suggest partially complementary functions [11-14].

Analysis of neuronal morphology in fragile-X patients [15-17] and of transgenic mice lacking the *Fmr1* gene [18-22], detected abnormalities in dendritic spines, sites of most excitatory inputs in the central nervous system [23, 24]. Spines were described to be longer compared to control tissue, reminiscent of dendritic filopodia-like protrusions prominent during development [25, 26]. Some studies also observed an increase in the density of dendritic spines, suggesting decreased pruning of synaptic structures. Abnormal synaptic structures and neuronal connectivity could mediate the cognitive and other deficits exhibited by FXR patients and the KO mice [27, 28]. Although the behavioral pheno type of *Fxr2* KO mice has been characterized, the neuronal cytoarchitecture has not yet been studied.

To better understand the function of FXR2P we analyzed dendritic spines in intact brains of *Fxr2* KO mouse. We report that dendritic spines on pyramidal neurons from the somatosensory barrel cortex and the hippocampus are longer and less dense in the *Fxr2* KO brains at postnatal day (P) 14 compared to WT mice. Interestingly, by P30 the difference in spine density and spine length is no longer observed between the two genotypes. These observations suggest that FXR2P plays an important role during the development of synapses and has partially overlapping functions with FMRP.

MATERIAL AND METHODOLOGY

Animals

Fxr2 KO (*Fxr2*$^{-/-}$) mice [12] in a C57BL/6 genetic background were a gift of Dr. David Nelson from Baylor College of Medicine. All experiments were carried out in accordance with institutional guidelines for animal welfare. Male and female mice were placed in breeding cages in a standard laboratory animal housing environment with a light cycle of 12 hr on, 12 hr off.

Tissue Preparation

At postnatal day (P) 14 and 30, animals were anesthetized with a Ketamine/Xylazine cocktail and transcardiac perfusion was performed with 4% paraformaldehyde (PFA) in 0.1 M Phosphate Buffer Saline (PBS). The brains were exposed and immersion-fixed in the skulls for additional 1-2 days at 4°C. Brain hemispheres were sectioned coronally on a vibratome at 100 μm. Sections containing the hippocampus and somatosensory cortex were collected and stored in PBS.

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Diolistic Labeling of Brain Sections

Preparation of Gene Gun Bullets

The protocol was adapted from [29]. Briefly, 8 mg of gold particles (1.6 μm in diameter) were mixed with 2.5 mg DiI (Sigma) and dissolved in 250 μl methylene chloride. After drying, the coated particles were collected into 1.5 ml eppendorf tube with water and placed into a sonicator for 5 minutes. The solution was vortexed for 15 seconds, and immediately transferred to 1 mm diameter gene gun tubing (BioRad). The tube was sealed with parafilm on both ends and the gold particles were allowed to adhere to the tubing at 4°C for 1-2 days. After withdrawing the solution, the tubing was dried with constant nitrogen flow for 1 hour. The tube was cut into small sections and stored in a dessicated container at 4°C for up to one month.

Delivery of Particles

Brain sections were transferred to a Petri dish and most of the PBS was removed. DiI- coated particles were delivered into the tissue using the helios gene gun system (Bio-Rad) at a pressure of 150 psi. After shooting, the slices were incubated in PBS at 4°C overnight to allow diffusion of the dye along the neuronal processes.

Nissl Counterstaining

For identification of neuronal cell bodies in cortical layers, DiI labeled sections were counterstained with Nissl. Sections were incubated in 0.5% fluorescent Nissl stain solution (NeuroTrace 500/525 green fluorescent Nissl stain solution, Molecular Probes, Oregon) in 0.1 M PB for 20 min at room temperature. After rinsing in PBS, the counterstained sections were mounted onto glass slides using 65% glycerin in PBS [30].

Confocal Imaging

All images were acquired blind to the genotype using the Leica TCS SP2 AOBS microscope with a 63x 1.4 N.A. objective. DiI labeled pyramidal neurons in layer 5 of the somatosensory barrel cortex and in the CA1 area of the hippocampus were identified with Nissl staining (and distance from the pial surface). Images were first collected at low magnification to capture the full extent of the labeling of the imaged cell. Spine images were collected with a voxel resolution of 116 x 116 x 285 nm^3 on secondary and tertiary basal dendrites (1-4 dendrites/ cell).

Measurements and Statistical Test

All image analysis was performed using MetaMorph (Molecular Devices, Sunnyvale, CA). Dendritic length was measured using a bent-line tool on a maximum intensity projections of the Z-stacks. Spines were counted by scrolling through the Z-stack and marking each spine. Spine density was computed as number of spines per dendrite length in microns. Maximum intensity projections made from z-stacks were also used to measure spine length. Length was measured from shaft to tip using the bent-line tool. Only spines projecting mainly into the XY plane were measured. Spine morphological measurements were made on WT and *Fxr2* KO animals at P14 and P30 (n=3-5 animals for each group). Data was analyzed using Graph Pad Prism 4. Normality was tested using the Shapiro–Wilkes test. Depending on the distributions, we used either an unpaired t-test or the Mann-Whitney test to look for significant differences between spines from the KO and wild type mice.

RESULTS

Pyramidal neurons from CA1 region of the hippocampus and layer 5 somatosensory cortex were identified by combining diolistic labeling of neurons with Nissl staining (Fig. **1**). Although many dendrites are labeled with the diolistic method, the origin of some dendrites was not always possible to determine due to partial labeling of cells. Only basal dendrites of clearly identifiable pyramidal neurons in the cortex (Fig. **1C**) and the hippocampus (Fig. **1D**) with typical morphology of a large triangular cell body with multiple basal dendrites and a single apical shaft that branched distally were used for this analysis. The partial labeling of neurons achieved with diolistics precluded us from performing an analysis of dendritic branch length, yet the measurement of the width of secondary and tertiary basal dendrites in the cortex and the hippocampus indicated no significant changes between mutant and wild type mice at P14 or P30 (Fig. **1E** and **1F**, $P>0.05$, n= 18-30 dendrites).

We next determined if the density of dendritic spines was altered in the *Fxr2* KO mice at P14 and P30. Spines on secondary and tertiary basal dendrites were counted. In the barrel cortex at P14 (Fig. **2 A-E**), spine density was 30.8% lower in the *Fxr2* KO mice compared to wild type mice ($P<0.0001$, 10.5 ± 1.1 spines/10 microns, n=23/550 dendrites/spines and 15.2 ± 0.7 spines/10 microns, n=30/1302 dendrites/spines in *Fxr2* KO and wild type mice respectively). At P30 the difference in spine density was smaller, and no longer significant, ($P>0.05$, 18.3±1 spines/10 microns, n=18/948 dendrites/spines and 21.4 ± 1.1 spines/10 microns, n=18/1227 dendrites/spines in *Fxr2* KO and wild type mice respectively).

Similar observations were made in the hippocampus. At P14, spine density was 41% lower in the *Fxr2* KO mice (Fig. **3**), $P<0.001$, 8.8 ± 0.4 spines/10 microns, n=27/767 and 15.1 ± 0.9 spines/10 microns, n=15/820 dendrites/spines in *Fxr2* KO and wild type mice respectively). Like in the cortex, in the hippocampus spine density was not different in the *Fxr2* KO and wild type mice at P30 ($P>0.05$, 23.4± 0.9 spines/10 microns n=17/998 dendrites/spines and 23.5 ± 1 spines/10 microns, n=26/1569 dendrites/spines in *Fxr2* KO and wild type mice respectively). These results indicate that in *Fxr2* KO mice there is a transient effect on spine numbers.

Since spine morphology is altered in *Fmr1* KO mice [18-20, 31], we next examined the effects of the absence of *Fxr2* on spine length. At P14, dendritic spines in the somatosensory cortex were 13% longer in the *Fxr2* KO mice than in wild type mice (Fig. **2**, $P<0.001$, 2 ± 0.05 μm, n= 24/258 dendrites/ spines and 1.8 ± 0.03, n=30/521 dendrites/spines in *Fxr2* KO and wild type mice respectively). At this age, the distribution of spine lengths in the cortex differed significantly between the two genotypes (Kolmogorov-Smirnof comparison of two samples, $P<0.01$), with *Fxr2* KO mice having fewer short spines (0.5-1.5 μm) and more medium to long spines (2-4 μm) (Fig. **2G**). By P30, the difference in spine length between wild type and mutant mice was no longer significant ($P>0.05$, 1.6 ± 0.03 μm, n=18/409 den-

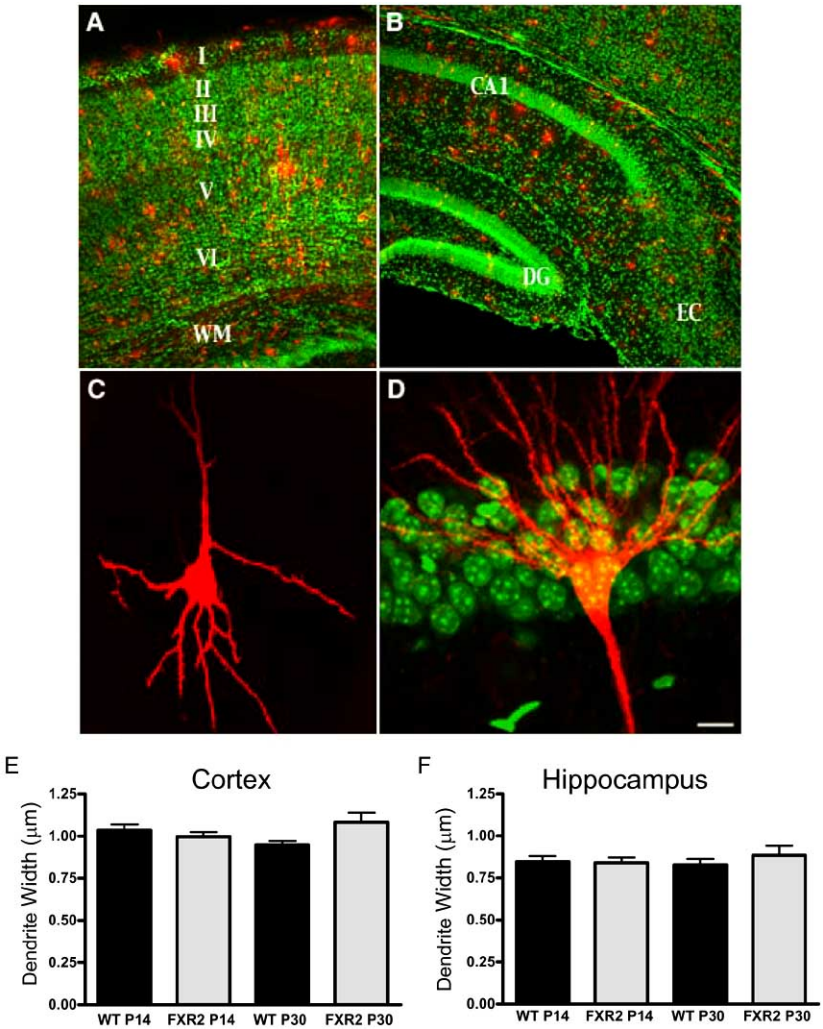

Fig. (1). Pyramidal neurons in barrel cortex and hippocampus identified with diolistic and Nissl counterstaining. **A**. The laminar organization (I-VI) of neurons in the barrel cortex (P7) (red-DiI, green-Nissl). **B**. Hippocampal section (P7) showing the dentate gyrus (DG), CA1 area, and entorhinal cortex (EC). **C**. DiI labeled pyramidal neuron (P14) in layer V of somatosensory cortex. **D**. DiI labeled pyramidal neuron (P14) in CA1 area of hippocampus. Measurement of width of basal dendrites at P14 and P30 in the somatosensory cortex **E** and the CA1 hippocampus **F**. Bar: 100 μm in A and B, 1 5μm in C and 17 μm in D. WM-white matter.

drites/spines and 1.6 ± 0.03, n= 17/390 dendrites/spines in *Fxr2* KO and wild type mice respectively) (Fig. **2F**, **H**).

Spine length was also altered in the hippocampus of *Fxr2* KO mice. At P14, spines were 11.2% longer in the *Fxr2* KO mouse than in the wild type mice (Fig. **3A**, **C**, **F**, **G**, $P<0.001$, 1.6 ± 0.04 μm, n=24/301 dendrites/spines and 1.4 ± 0.03 μm, n=15/275 in *Fxr2* KO and wild type mice respectively). Accordingly, at P14, the distribution of spine lengths differed significantly between the two genotypes, (Kolmogorov-Smirnof comparison of two samples, $P<0.05$). At P30, a time synaptogenesis is almost complete, spine length was no longer significantly different between the cells from mutant and the control hippocampal cells ($P>0.05$, 1.2 ± 0.02 μm, n=17/280 dendrites/spines and 1.3 ± 0.02, n=25/348 dendrites and spines in *Fxr2* KO and wild type mice respectively).

DISCUSSION

The present study demonstrates that dendritic spines of *Fxr2* deficient neurons in the somatosensory cortex and the hippocampus develop abnormally. In normal development, during the first few postnatal weeks the density of dendritic spines increases while spine length is reduced [25, 26, 32, 33]. Although this developmental trend was evident in both control and Fxr2 mutant mice, spine density in the *Fxr2* mouse was 30-40% lower in the second postnatal week in both hippocampus and somatosensory cortex. At P30, the difference in spine density between mutant and wild type mice was no longer observed. In addition, we report that dendritic spines are longer in the *Fxr2* mutant mice. As with spine density, we find that alterations in spine length are transient. Spines are longer at P14, a peak time of synaptogenesis but not at P30, a time when synaptogenesis wanes. This developmental phenotype was observed in both the somatosensory cortex and the hippocampus of *Fxr2* null mice suggesting a similar role of FXR2 in those distinct brain regions. Since longer spines and dendritic filopodia are generally associated with younger cells the increased spine length in the second postnatal week suggests a lag in the maturation of synaptic structures.

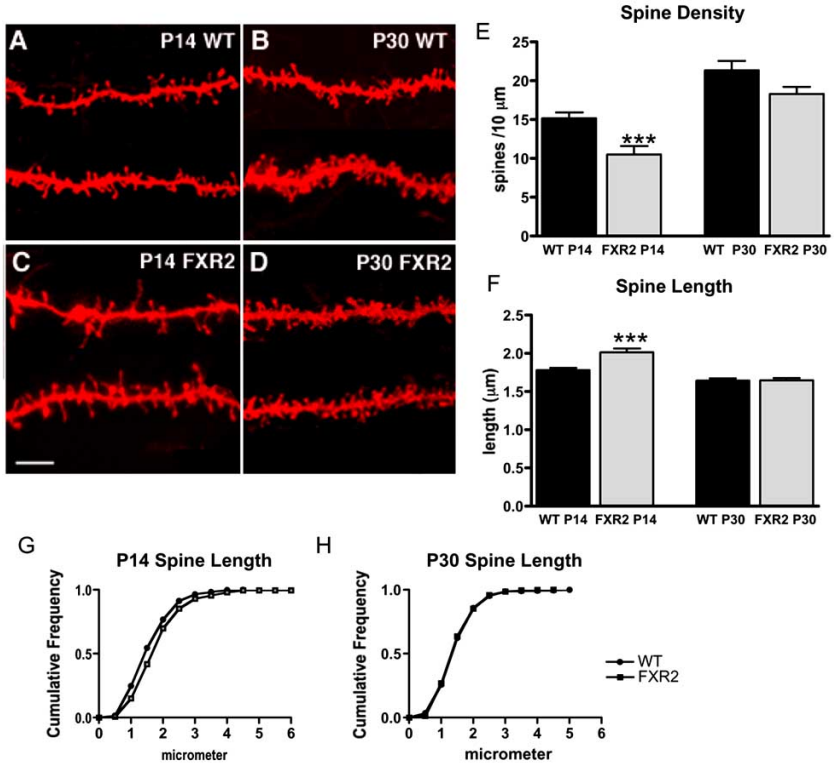

Fig. (2). Developmental delay of dendritic spines in the somatosensory cortex of *Fxr2* KO mice. Confocal micrographs of DiI labeled secondary and tertiary basal dendrites of layer V pyramidal neurons from wild type (**A**, **B**) and *Fxr2* KO (**C**, **D**) mice at P14 (**A**, **C**) and P30 (**B**, **D**). **E**. Mean spine density is reduced in *Fxr2* KO mice at P14 but not at P30. **F**. Spine length is increased in *Fxr2* KO mice at P14 but not at P30. Cumulative frequency distribution of spine lengths. **G**: at P14, and **H**: at P30. Bar: 25 μm in A-D. Error bars indicate SEM. ***P<0.001.

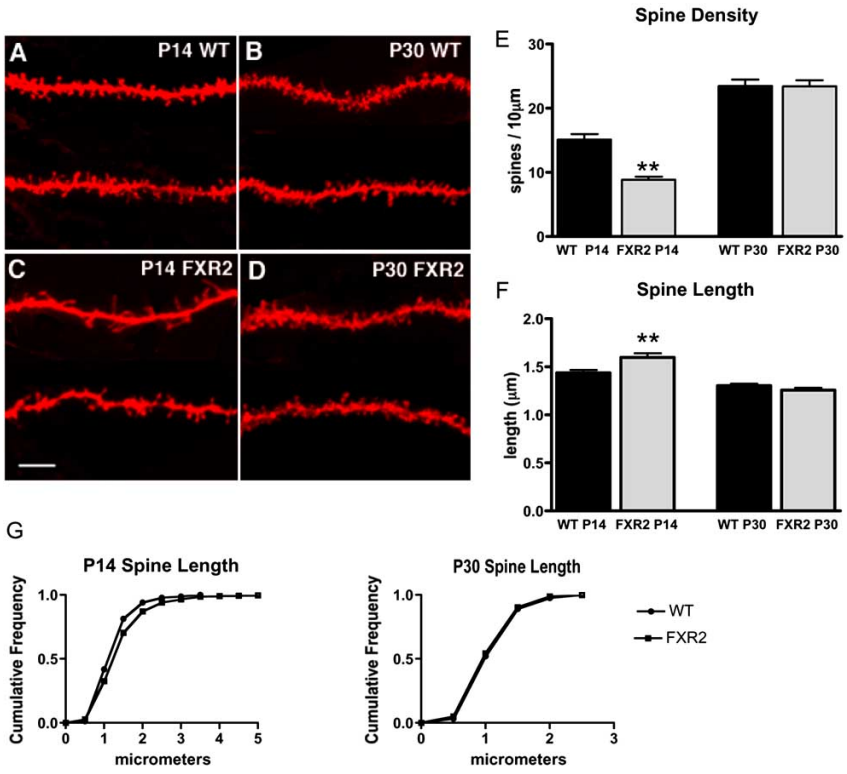

Fig. (3). Developmental delay of dendritic spines in the hippocampus of *Fxr2* KO mice. Confocal micrographs of DiI-labeled secondary and tertiary basal dendrites of CA1 pyramidal neurons from wild type (**A**, **B**) and *Fxr2* KO (**C**, **D**) mice at P14 (**A**, **C**) and P30 (**B**, **D**). **E**. Mean spine density is reduced in *Fxr2* mice at P14 but not at P30. **F**. Spines are longer in *Fxr2* KO mice at P14 but not at P30. Cumulative frequency distribution of spine lengths. **G**: at P14, and **H**: at P30. Bar: 25 μm in A-D. Error bars indicate SEM. **p<0.01

Fxr1 and *Fxr2*, are homologues of *Fmr1* and have a similar functional domains (including RNA binding domains), as well as similar tissue and subcellular expression pattern [7, 8]. The three proteins interact with each other *in vitro* [34], suggesting that they might have analogous functions. Behavioral analysis of the *Fxr2* KO mouse shows a partially overlapping phenotype with the *Fmr1* KO mouse (although some behaviors were affected in opposite manners in the two KO mice) [12]. *Fmr1* and *Fxr2* double-knockout mice have exaggerated impairments in a subset of neurobehavioral phenotypes relative to the single gene KO, further suggesting overlapping functions between the FXR proteins [13]. However, the developmental reduction in spine density in *Fxr2* null mice reported here is the opposite phenotype of what was reported for *Fmr1* null mice. Most studies of the *Fmr1* null mouse, as well as studies of the fragile X patients, report an increase in spine density [but see 20]. Our finding of decreased spine density in the *Fxr2* null mouse suggest that *Fmr1* and *Fxr2* have opposite effects on spine numbers.

During the first few postnatal weeks, dendritic spines are highly dynamic with dendritic protrusions extending and retracting [25, 35-38]. The static imaging method used in the current study precludes us from determining if reduced spine density in the *Fxr2* null mice results from fewer spines emerging and/or more spines retracting during the first two postnatal weeks. It is interesting to note that long-term depression (LTD), a form of synaptic plasticity that is associated with spine retraction [39] is increased in the Fmr1 null mouse [40, 41]. It would be interesting to determine if altered synaptic function, such as increased LTD, also occurs in *Fxr2* and could mediate the reduced spine density reported here.

The phenotype of increased spine length in the *Fxr2* null mouse is consistent with that observed in the *Fmr1* null mice: a 11-13% increase in spine length was observed at P14 in our study as well as in the somatosensory cortex of *Fmr1* null mice [19]. This finding points to a similar function of FMRP and FXR2P in maintaining spine length. We observe here transient aberrations in spine numbers and morphology in the *Fxr2* null mice. Transient changes in spines and other synaptic properties have been previously reported in the *Fmr1* null mice [19, 42]. One question is how such delay in development of spines results in permanent deficits observed in adult mice [12]? One possibility is that although the number of synaptic connections eventually formed in these mice is not different, the delay in spine maturation might cause altered connectivity between neurons that might be responsible for the neurological defects in these mice.

CONCLUSION

The present study demonstrates that dendritic spines of *Fxr2* deficient neurons in the somatosensory cortex and the hippocampus develop abnormally. Overall this study is consistent with previous conclusions that FXR2P and FMRP have partially but not completely overlapping functions in central nervous system. Although levels of FXR2P are not altered in FXS patients [7] or in the *Fmr1* KO mice this study highlights the importance of this protein for normal development of synaptic structures.

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