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# Astrocytic Contributions to Synaptic and Learning Abnormalities in a Mouse Model of Fragile X Syndrome

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## ABSTRACT

**BACKGROUND:** Fragile X syndrome (FXS) is the most common type of mental retardation attributable to a single-gene mutation. It is caused by *FMR1* gene silencing and the consequent loss of its protein product, fragile X mental retardation protein. *Fmr1* global knockout (KO) mice recapitulate many behavioral and synaptic phenotypes associated with FXS. Abundant evidence suggests that astrocytes are important contributors to neurological diseases. This study investigates astrocytic contributions to the progression of synaptic abnormalities and learning impairments associated with FXS.

**METHODS:** Taking advantage of the Cre-lox system, we generated and characterized mice in which fragile X mental retardation protein is selectively deleted or exclusively expressed in astrocytes. We performed in vivo two-photon imaging to track spine dynamics/morphology along dendrites of neurons in the motor cortex and examined associated behavioral defects.

**RESULTS:** We found that adult astrocyte-specific *Fmr1* KO mice displayed increased spine density in the motor cortex and impaired motor-skill learning. The learning defect coincided with a lack of enhanced spine dynamics in the motor cortex that normally occurs in response to motor skill acquisition. Although spine density was normal at 1 month of age in astrocyte-specific *Fmr1* KO mice, new spines formed at an elevated rate. Furthermore, fragile X mental retardation protein expression in only astrocytes was insufficient to rescue most spine or behavioral defects.

**CONCLUSIONS:** Our work suggests a joint astrocytic-neuronal contribution to FXS pathogenesis and reveals that heightened spine formation during adolescence precedes the overabundance of spines and behavioral defects found in adult *Fmr1* KO mice.

***Keywords:*** Astrocytes, Dendritic spines, Fragile X syndrome, *Fmr1*, Motor cortex, Motor learning

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Fragile X syndrome (FXS) is the most frequent form of inherited mental retardation, affecting approximately 1 in 4000 males and 1 in 6000 females of all races and ethnic groups (1). Patients with FXS display a wide spectrum of phenotypes, including moderate to severe mental retardation, autistic behavior, macroorchidism, predisposition to epileptic seizures, and facial abnormalities (2–4). They also experience huge social challenges, placing a great economic and emotional burden on their families. Nearly all FXS cases are caused by a trinucleotide repeat expansion in the X-linked *FMR1* gene, which silences its transcription and abolishes the expression of its protein product, fragile X mental retardation protein (FMRP) (5). FMRP regulates the transportation and translation of messenger RNAs (mRNAs) that are important for dendritic growth, synapse development, and plasticity (6).

Spine morphology and density are paramount to synaptic function and connectivity (7,8). In vivo imaging studies on global *Fmr1* knockout (KO) mice reveal elevated spine dynamics along apical dendrites of layer II/III and layer V pyramidal neurons in motor and barrel cortices at various ages, suggesting that the absence of FMRP reduces the stability of synapses (9–11). Furthermore, in both patients with FXS (12) and adult global *Fmr1* KO mice (13,14), the density of long and thin spines on apical dendrites of cortical pyramidal neurons is abnormally high, reminiscent of the abundance of immature spines found during early development (15). Thus, it has been hypothesized that the absence of FMRP causes abnormalities in spine development, which in turn alter synaptic connectivity and ultimately result in behavioral impairments, including learning defects (14).

The aforementioned spine phenotypes have elicited copious research on the neuronal mechanisms of FXS (16). However, little is known regarding the contribution of non-neuronal cells in the brain, for example, glia, to FXS pathogenesis. As the most abundant glial cells in the mammalian brain, astrocytes modulate synaptic structure and function and are implicated in many neurodevelopmental diseases (17). Although FMRP expression among individual cell types in

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the human brain has not been examined, FMRP has been found in astrocytes of the mouse brain (18–20), which suggests a possible astrocytic role in FXS pathogenesis. In support of this notion, culturing wild-type neurons with *Fmr1*-deficient astrocytes leads to the development of abnormal dendritic morphologies, reduces synaptic protein clusters, and increases levels of extracellular glutamate (19,21). These studies provide evidence that astrocytic FMRP is vital to the development of neurons and synapses in vitro. However, the contribution of astrocytes to the progression of dendritic spine and behavioral defects in FXS in vivo remains elusive.

## METHODS AND MATERIALS

### Experimental Animals

The Institutional Animal Care and Use Committee of University of California Santa Cruz approved all animal care and experimental procedures. The *Fmr1*$^{fl}$ and *Fmr1*$^{neo}$ mice were obtained from Dr. David L. Nelson, Baylor College of Medicine; the global *Fmr1* KO mice, from Dr. Stephen T. Warren, Emory University; the *mGFAP-Cre*$^{+}$ mice (line 73.12), from Dr. Michael V. Sofroniew, University of California, Los Angeles; and the *S100β-GFP* mice, from Dr. Wesley J. Thompson, Texas A&M University. *Thy1*-YFP-H and *Rosa26*$^{tdTomato}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were backcrossed with C57BL/6 mice more than 10 generations to produce congenic strains. Male mice were used in all experiments.

### Cortical Astrocyte Culture and Immunocytochemistry

The protocol to prepare primary astrocyte cultures has been previously described (22). Detailed procedures of culture preparation and immunocytochemistry are described in the Supplemental Methods.

### Immunohistochemistry for Cortical Sections

Mice were transcardially perfused with 4% paraformaldehyde fixative in 0.1 mol/L phosphate-buffered saline (PBS). Brains were postfixed in 4% paraformaldehyde fixative/PBS overnight at 4°C and cryoprotected in 30% sucrose. For most experiments, 40-μm brain sections were used. Sections were permeabilized and blocked with 0.5% Triton X-100 and 10% normal goat serum/PBS, then incubated with the following primary antibodies in 0.5% Triton X-100/PBS at 4°C overnight: rabbit anti-S100β (1:1000; Cat. no. Z0311; DakoCytomation, Carpinteria, CA), mouse anti-NeuN (1:2000; Cat. no. MAB377; Millipore, Darmstadt, Germany), rabbit anti-Olig2 (1:500; Cat. no. AB9610; Millipore), goat anti-Iba1 (1:100; Cat. no. ab5076; Abcam, Cambridge, United Kingdom), or rabbit anti–glial fibrillary acidic protein (GFAP) (1:500; Cat. no. Z0334; DakoCytomation). For FMRP colabeling, 25-μm sections were incubated in 10 mmol/L sodium citrate (pH 6.0) with 0.05% Tween-20 at 85°C for 20 minutes, followed by 30 minutes in blocking solution (0.01% Triton X-100, 5% goat serum, 1% bovine serum albumin) at room temperature. Sections were labeled with mouse anti-FMRP 2F5-1 (1:1; Developmental Studies Hybridoma Bank, Iowa City, IA) and rabbit anti-NeuN (1:500; Cell Signaling Technology, Danvers, MA) at 4°C overnight. Sections were then incubated with Alexa Fluor 488- and 594-conjugated secondary antibodies (1:1000; Life Technologies, Carlsbad, CA) in 10% normal goat serum/PBS for 2 hours at room temperature for fluorescence imaging or with biotinylated secondary antibody (1:400; Vector Laboratories, Burlingame, CA), avidin-biotin complex (Vector Laboratories), and diaminobenzidine (Vector Laboratories) for bright-field imaging. Sections were mounted with Fluoromount-G mounting medium (Southern Biotechnology Associates, Inc., Birmingham, AL) or Vectashield hardening mounting medium (Vector Laboratories).

### Western Blot

Cortical tissues were dissected from adult mice and homogenized in ice-cold radioimmunoprecipitation assay lysis buffer containing protease inhibitors (Roche, Basel, Switzerland). Nuclei were pelleted by centrifugation at 15,000 rpm, 4°C, for 15 minutes, and the supernatant was denatured in 2X Laemmli buffer. Cultured astrocytes were directly lysed and denatured in hot 2X Laemmli buffer. Denatured lysates were electrophoretically separated by a sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). The following primary antibodies were used at 4°C overnight: mouse anti-FMRP 2F5-1 (1:1; Developmental Studies Hybridoma Bank), mouse anti-tubulin (1:5000; Cat. no. 8328; Sigma-Aldrich, St. Louis, MO), and rabbit anti-actin (1:1000; Cat. no. A2066; Sigma-Aldrich). Horse radish peroxidase–conjugated secondary antibody (anti-mouse immunoglobulin G or anti-rabbit immunoglobulin G; 1:5000; Cell Signaling Technology) was used for detection. All images shown are representative of at least three replications.

### Fluorescence-Activated Cell Sorting Purification of Astrocytes and Quantitative Reverse-Transcriptase Polymerase Chain Reaction

Acute isolation of astrocytes from postnatal day 30 to 50 (P30–50) mice by immunopanning and fluorescence-activated cell sorting was adapted from previously established protocols (23,24). RNA was extracted from sorted astrocytes and prepped for reverse-transcriptase polymerase chain reaction. Details on the procedures are described in the Supplemental Methods.

### Optical Imaging and Image Analysis for Brain Sections and Cultured Cells

Bright-field images were collected on a Zeiss Axio Imager.M2 microscope with either a 20X/NA 0.8 objective or a 40X/NA 1.4 oil-immersion objective, using the AxioVision software (Zeiss, Oberkochen, Germany). Confocal images were acquired on a Leica SP5 confocal system with either a 20X/NA 0.75 objective or a 63X/NA 1.4 oil-immersion objective (Leica, Wetzlar, Germany). Imaging settings were identical between samples in which fluorescence intensity was analyzed. Astrocyte number and morphology were analyzed from bright-field images using Stereo Investigator (MBF Bioscience, Williston, VT). Confocal images were used to analyze tdTomato-positive cells colabeled with various cell-specific markers by manual counting in