Supporting Information

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SI Case Report

The patient was born full-term without complication but was hospitalized for 5 d immediately after birth for feeding and respiratory problems, as well as to receive phototherapy for jaundice. No further complications were experienced until 1 mo of age, when the patient suffered nonaccidental trauma, developed seizures immediately after the event, and was diagnosed with shaken baby syndrome. The MRI and computed tomography scans were negative for any gross abnormalities at the time. At 18 mo, the patient experienced another seizure episode that was associated with fever. At 24 mo, the patient experienced a third seizure episode, without any precipitating event, and was started on Trileptal (Novartis Pharmaceuticals) antiseizure medication. From 2–8 y of age, the patient continued to have episodes of complex partial seizures every 3–4 mo, with about 12 seizures per day for 2–3 d at a time. The seizures did not localize or lateralize to any particular brain region and remained uncontrolled despite treatment with multiple antiepileptic medications. The patient was consequently diagnosed with intractable unclassified epilepsy; however, he is currently taking Trileptal and has not had any seizure episodes for the past 2.5 y.

In addition to epilepsy, the patient has a history of developmental delay and ID. Some early motor developmental milestones were met, such as rolling over and sitting without support, although walking independently was not achieved until 18 mo (mildly delayed). By 8 y of age, the patient had poor handwriting skills, could not write his name or numbers, and could not use utensils to feed himself. His language developmental milestones were also delayed, because he did not combine words until 3 y of age and did not form complete sentences until 7 y of age. Psychoeducational assessments were conducted at ∼8 y of age with two different tests, which revealed a full-scale IQ of 42 (moderately impaired function) by the Stanford–Binet Intelligence Scales, fifth edition, and an IQ of 71 (mild ID) by the Kaufman Brief Intelligence Test, second edition. The Vineland Adaptive Behavior Scales, second edition, revealed a score of 70 on communication, 104 on socialization, and 81 on adaptive behavior. The Child Behavior Checklist was also conducted and found that the patient did not display any maladaptive behaviors commonly associated with FXS or autism, such as stereotypic behaviors, hyperactivity, impulsivity, physical aggressiveness, difficulty with changes or transitions, or problems with sleeping or eating. Based on the clinical evaluation and Diagnostic and Statistical Manual of Mental Disorders (DSM) IV criteria (DSM V was not available at the time), the patient did not meet criteria for autism spectrum disorder, and therefore was not further evaluated by the Autism Diagnostic Observation Schedule. Currently, the patient is in a seventh-grade special education class and reading at the kindergarten level.

On physical examination, the patient is a nondysmorphic male with height and weight in 25th percentile, head circumference in 95th percentile (mildly macrocephalic), and ears in the 75th to 95th percentile range. The patient did not display an elongated face, prominent forehead or jaw (Fig. 1*A*), macroorchidism, or any other dysmorphic feature commonly associated with FXS, except for one café-au-lait spot on his lower left chest.

There is a family history of learning problems on both the maternal and paternal sides (Fig. 1*B*). The patient's father attended special education classes throughout childhood, with delays in reading and writing. He did not complete the 10th grade. The patient's mother completed 12th grade; however, she had significant difficulty throughout school and also has a history of anxiety. The patient's maternal grandfather, now deceased, also had learning problems as a child and was only able to complete middle school. The mother was the only family member available for genetic testing, and she was found to be a carrier of the R138Q mutation, indicating maternal transmission.

SI Experimental Procedures

Constructs. For lentivirus production, full-length human *FMR1* [iso-13 variant that uses a second splice acceptor site in exon 17 (1)] with an aminoterminal Flag tag was cloned into the BamHI/EcoRI sites of the FUGW lentiviral vector, and the R138Q mutation was introduced using a Quikchange Lightning Site-Directed Mutagenesis Kit (Agilent). WT and R138Q mutant lentiviral constructs were verified by restriction enzyme digest and sequencing of the *FMR1* insert before being sent to the Emory University Viral Vector Core for lentivirus production.

For *Drosophila* transgene injection, we first excised the *dfmr1* coding sequence from the pUAST-*dfmr1* vector by double-restriction digestion with EcoRI-high fidelity and XbaI, and then ligated this fragment into the TOPO v2.1 vector backbone with the same digested ends using a Takara DNA Ligation Kit. The *Drosophila* equivalent of the human R138Q mutation requires two nucleotide substitutions, c.419_420delinsAG (R140Q), and they were introduced using the Quikchange Lightning Site-Directed Mutagenesis Kit. Mutated *dfmr1*-R140Q was then excised from the TOPO vector by EcoRI-high fidelity and XbaI, ligated back into the pUAST vector, and confirmed by sequencing of the *dfmr1* insert.

For protein expression, constructs were sent to the Emory Custom Cloning Core Facility for cloning. Truncated WT and R138Q human *FMR1* (residues 1–298) were cloned into the NdeI/BamHI sites of a modified pET28b expression vector (Novagen) harboring an upstream 6xHis-Smt3 (yeast SUMO) between the NcoI and NdeI sites. The 6xHis-SUMO-*FMR1* fusion constructs were verified by sequencing the *FMR1* insert. For binding assay experiments, an additional 6xHis tag was inserted between the SUMO and *FMR1* sequences to generate 6xHis-SUMO-6xHis-*FMR1*.

Animals. For cell culture experiments, control mice (C57BL/6J, stock no. 664; The Jackson Laboratory) and *Fmr1* KO mice (generated on the same background) were housed and maintained according to the Emory University Institutional Animal Care and Use Committee guidelines. For electrophysiology, *Fmr1* KO (FVB.129P2-*Fmr1*^{tm1Cgr}/J; stock no. 4624; The Jackson Laboratory) and control (FVB.129P2-*Pde6b*$^{+}$*Tyr*$^{c\text{-}ch}$/AntJ; stock no. 4828; The Jackson Laboratory) mice were housed and maintained according to the Washington University Animal Studies Committee guidelines.

Cell Culture. Immortalized MEFs were generated from *Fmr1* KO mice and maintained in culture with DMEM (Corning) supplemented with 10% (vol/vol) FBS (HyClone).

Primary cortical neuronal cultures were generated from embryonic day 16.5 *Fmr1* KO mice. Cortices were dissected in Hibernate E without Ca^{2+} (BrainBits) and digested in 2 mg/mL papain (Worthington Biochemical), and the dissociated neurons were plated at 8.4e4 cells per square centimeter directly onto poly-L-lysine–coated (0.2 mg/mL), 100-mm dishes in Adhesion medium [1× MEM (Gibco) supplemented with 10% (vol/vol) FBS, 30 mM glucose, and 10 mM Hepes]. After 4 to 6 h, Adhesion medium was replaced with Neurobasal medium (Gibco) supplemented with B27 (Invitrogen) and Glutamax (Invitrogen).

Polyribosome Profiling and Western Blotting. *Fmr1* KO MEFs were plated in T225 flasks (Corning), transduced with lentivirus at ∼40–50% confluency for 16 h, and collected for polyribosome assay 24 h after virus removal. Immediately before harvest, cells were treated with cycloheximide (100 μg/mL; Acros Organics) for 15 min at 37 °C and then lysed in 20 mM Tris·HCl (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, and 0.3% IGEPAL (Sigma), supplemented with EDTA-free complete mini-protease inhibitor (Roche), 100 U/mL SUPERaseIn (Ambion), and 100 μg/mL cycloheximide. Lysates were clarified by centrifugation at 13,000 × *g* for 10 min at 4 °C, and the supernatants loaded on top of 15–45% wt/wt linear sucrose gradients containing 20 mM Tris·HCl (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, 20 U/mL SUPERaseIn, and 100 μg/mL cycloheximide, which were made using a Gradient Master 108 (BioComp), and centrifuged at 247,600 × *g* for 2 h at 4 °C using a SW41Ti rotor (Beckman Coulter). After centrifugation, the samples were fractionated into 10 × 1.1-mL fractions by bottom displacement using a Teledyne ISCO fractionator, with continuous monitoring at OD_{254} using an ISCO UA-6 UV detector, and then stored at −80 °C. Before Western blotting, the samples were thawed on ice and 500 μL of each fraction was concentrated to ∼40 μL with Amicon Ultra 30K centrifugal filters (Millipore). Concentrated samples were denatured in SDS sample buffer, separated by SDS/PAGE on 4–15% gradient polyacrylamide gels (BioRad), transferred onto nitrocellulose membranes, and probed with primary antibodies against FMRP (1:2,000, MAB2160; Millipore) and S6 ribosomal protein (1:1,000, no. 2317; Cell Signaling Technologies) using standard techniques. Primary antibodies were labeled by HRP-conjugated secondary antibody (1:50,000; Millipore), and the enhanced chemiluminescence signal was detected using SuperSignal Femto Max Sensitivity Substrate (Thermo Scientific).

RNA Co-IP and Quantitative RT-PCR. *Fmr1* KO primary cortical neuron cultures were transduced with lentivirus at 10 days in vitro (DIV) for 18 h and collected for RNA co-IP 72 h after virus removal. Neurons were lysed in 50 mM Tris·HCl (pH 7.5), 300 mM NaCl, 30 mM EDTA, and 0.5% Triton X-100 (Sigma), supplemented with complete mini-protease inhibitor and 100 U/mL SUPERaseIn. Lysates were clarified by centrifugation at 20,000 × *g* for 15 m at 4 °C, and the supernatant protein concentrations were determined by Bradford assay (BioRad). Equal quantities of protein were used for all input (∼150 μg) and IP (∼1,100 μg) samples within an experiment. For IP, the samples were incubated with EZview Red Anti-Flag M2 Affinity Gel (Sigma) for 2 h at 4 °C and washed with the above lysis buffer four times, and RNA was extracted using TriReagent (Ambion). For input samples, RNA was extracted using TRIzol LS (Invitrogen). RNA was then stored at −80 °C until processed for quantitative RT-PCR.

Before RNA extraction, a portion of both the input and IP samples was saved for Western blotting to verify that lentiviral infection produced equal FMRP expression and pull-down across samples. Western blotting was performed as described above with the following exceptions: Proteins were transferred onto PDVF membranes, probed with primary antibodies against FMRP (1:500, F4055; Sigma) and β-actin (1:1,000, no. 4970; Cell Signaling Technologies), and labeled by HRP-conjugated secondary antibody (1:10,000; Millipore), and the enhanced chemiluminescence signal was detected using HyGlo Quick Spray (Denville Scientific). Densitometry was quantified using ImageJ (NIH).

For quantitative RT-PCR, 700 ng of total RNA was treated with DNase I (Invitrogen), and reverse-transcribed using random hexamers and the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. PCR was performed with iQ SYBR Green Supermix (BioRad) and 100 nM forward and reverse primers (primer sequences are provided in Table S1). mRNA quantification was determined using the standard curve method for relative quantification. A standard curve was established for each primer set, and the relative mRNA level for each input and IP sample was determined after normalizing each sample to its β-actin mRNA level and adjusting for FMRP protein expression level as determined by Western blot densitometry. The ratio between IP and input mRNA quantification was normalized to the WT value for each experiment to allow comparison across different experiments. Data were analyzed by two-way ANOVA with Dunnett's post hoc analysis ($n = 5$, $n = 4$, $n = 3$, and $n = 2$ for GFP-, WT-, R138Q-, and G266E-infected groups, respectively).

***Drosophila* NMJ Immunostaining and Analysis.** All flies were maintained under standard culture conditions on cornmeal-molasses-yeast medium at 25 °C. Transgenic *dfmr1* WT and R140Q mutant flies were generated by standard P-element transgene injection (Bestgene, Inc.) into a w^{1118} strain and then crossed with either a Da-GAL4 or Elav-GAL4 line (nos. 8641 and 458, respectively; Bloomington Stock Center).

To perform immunostaining of the NMJ, third-stage wandering larvae were harvested, washed, and dissected in PBS (Ca^{2+}-free) as described (2). Dissected larvae were fixed for 1 h on ice with 4% (wt/vol) paraformaldehyde, followed by permeabilization at room temperature with three to four 10-min washes using 0.3% PTX (PBS + 0.3% Triton X-100). Tissues were then blocked for 30 min to 1 h with 0.1% PTX + 5% (vol/vol) normal goat serum, incubated overnight at 4 °C with antidiscs large primary antibody (no. 4F3; Developmental Studies Hybridoma Bank), washed 3 × 5 min with 0.1% PTX, and labeled for 1–2 h with Alexa Fluor 488 anti-mouse secondary antibody (A-11001; Invitrogen). Tissues were then mounted onto slides using Vectashield (Vector Laboratories), and NMJ length and number of branches were measured from micrographs taken of muscles 6/7 of abdominal segment 2 or 3 using Axiovision software (Zeiss). Approximately 30 larvae were analyzed per genotype, and data were analyzed by one-way ANOVA with a Tukey post hoc analysis.

Brain Slice Preparation. Both male and female 15- to 20-d-old mice were used. After being deeply anesthetized with CO_2, mice were decapitated and their brains were dissected out in ice-cold saline solution that contained the following: 130 mM NaCl, 24 mM $NaHCO_3$, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 5.0 mM $MgCl_2$, and 10 mM glucose (pH 7.4) (saturated with 95% O_2 and 5% CO_2). Horizontal brain slices (350 μm), including the hippocampus, were cut using a vibrating microtome (Leica). Slices were initially incubated in the above solution at 35 °C for 1 h for recovery and then kept at room temperature until use.

AP Recordings and Analysis. APs were recorded using an Axopatch 700B amplifier (Molecular Devices) in whole-cell configuration from hippocampal CA3 pyramidal neurons or layer 5 pyramidal neurons in the entorhinal cortex visually identified with IR video-microscopy (Olympus BX50WI; Dage-MTI) and differential interference contrast optics. All of the recordings were performed at near-physiological temperature (33–34 °C). The recording electrodes contained the following solution: 130 mM K-gluconate, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM NaCl, 2 mM ATP_2Na, 0.4 mM GTPNa, and 10 mM Hepes (pH 7.3). The extracellular solution contained the following: 130 mM NaCl, 24 mM $NaHCO_3$, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM glucose (pH 7.4) (saturated with 95% O_2 and 5% CO_2). DL-2-amino-5-phosphonopentanoic acid (AP-5) (50 μM), 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX) (10 μM), and gabazine (5 μM) were also included in the perfusate. APs were evoked by repetitive injection of a 1-ms current to evoke a 25-AP train at 60 Hz or 62.5 Hz. Sixty-hertz trains have fractional 16.67-ms ISIs, whereas 62.5-Hz trains have 16-ms ISIs. Because of software rounding of submillisecond timing to a whole millisecond, every

third ISI is 1 ms shorter in 60-Hz stimulus trains, causing apparent periodicity in the data (3). This periodicity was no longer present in 58.8-Hz trains (constant 17-ms ISIs) (3) or 62.5-Hz trains (constant 16-ms ISIs; Fig. 4 *A*–*C*). This effect is very small (<2%) and is present in all recordings at 60 Hz. Membrane potential was set at −65 mV by automatic slow current injection to ensure stability of the resting potential and to prevent spontaneous AP firing. Each AP train was evoked four to six times in each neuron, and each train was separated by ∼2-min rest periods. AP duration during bursts was normalized to an averaged duration of four low-frequency baseline APs (0.2 Hz) that were evoked before each burst. FMRP fragments were introduced into the neurons via patch pipette using a microperfusion system (Bioscience Tools). Only recordings in which no significant AP amplitude rundown was observed after FMRP perfusion were used in this analysis. Recordings were filtered at 2 kHz, digitized at 20 kHz, acquired using custom software written in LabView, and analyzed using programs written in MATLAB.

Protein Expression and Purification. All proteins were expressed as 6xHis-SUMO fusion proteins to facilitate protein expression and purification. WT and R138Q FMRP fragments (residues 1–298), with or without an additional 6xHis tag immediately amino-terminal to FMRP, were expressed in *E. coli* BL21 (DE3)-Gold cells with the RIL-Codon plus plasmid (Stratagene). Cultures were first grown at 37 °C in LB supplemented with 50 μg/mL kanamycin until the OD_{600} reached between 0.4 and 0.8. The temperature was then shifted to 16 °C, and protein expression was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside for 16 h. Cells were harvested and resuspended in 4 vol of 300 mM NaCl, 20 mM sodium phosphate (pH 7.4), 20 mM imidazole, 1 mM DTT, and 0.3 mM phenylmethyl-sulphonyl fluoride; they were then lysed using sonication for 5 min (1 s on, 2 s off), followed by centrifugation at 38,000 × *g* for 1 h. The fusion proteins were isolated on a nickel-charged HiTrap chelating column (GE Healthcare) and eluted with 500 mM imidazole, and the 6xHis-SUMO tag was removed by overnight incubation with yeast ubiquitin-like–specific protease 1 (Ulp1; purified in-house) at 4 °C. The cleaved proteins were then diluted with 6 vol of 20 mM Hepes (pH 7.0) and 1 mM DTT, loaded over a HiTrap-Q column (GE Healthcare), and eluted at ∼170–280 mM NaCl with a linear gradient of 100 to 800 mM NaCl. Fractions containing the FMRP fragments were pooled together and concentrated using Spin-X UF 30K MWCO concentrators (Corning). The proteins were then loaded onto a Superdex 75 (16/60) sizing column (GE Healthcare) that was preequilibrated with 200 mM NaCl, 20 mM Hepes (pH 7.0), and 1 mM DTT. The fractions containing purified FMRP fragment were pooled together, concentrated to 100 μM, and stored in aliquots at −80 °C.

Pull-Down Assay. Whole mouse brain from WT C57BL/6J mice was dissected and transferred to a Dounce homogenizer containing 5 mL of lysis buffer on ice [50 mM Hepes (pH 7.4), 300 mM NaCl, and 1% Triton X-100, supplemented with protease and phosphatase inhibitors (Roche)]. After homogenization, brain lysates were clarified by centrifugation at 20,000 × *g* for 1 h at 4 °C. Supernatants were then precleared to reduce nonspecific binding by incubation with HisPur Cobalt Resin (Pierce) for 1 h at 4 °C. Beads were removed, and total protein concentration was determined by the bicinchoninic acid (BCA) method. One hundred micrograms of His-tagged WT-$FMRP_{298}$ or R138Q-$FMRP_{298}$ fragments was incubated with 1 mg of lysate with rotation for 4 h to overnight at 4 °C. Twenty microliters of 50% slurry HisPur Cobalt Resin was then added and incubated for 1 h at 4 °C, followed by centrifugation at 350 × *g* for 1 min. Beads were washed five times with washing buffer [50 mM Hepes (pH 7.4), 500 mM NaCl, 1% Triton X-100, 10 mM imidazole] and denatured with 1× SDS/PAGE loading buffer, and the supernatants were separated by SDS/PAGE on 4–12% Bis-Tris gels (Invitrogen). Proteins were then transferred onto nitrocellulose membranes, probed with primary antibodies against BK channel α-subunit (1:500, NBP1-48250; Novus Biological) and BK channel β4-subunit (1:500, ab105587; Abcam) labeled by HRP-conjugated goat anti-mouse secondary antibody (1:5,000; Invitrogen) and detected using SuperSIgnal West DURA (Thermo Scientific).

1. Sittler A, Devys D, Weber C, Mandel JL (1996) Alternative splicing of exon 14 determines nuclear or cytoplasmic localisation of fmr1 protein isoforms. *Hum Mol Genet* 5(1):95–102.
2. Brent JR, Werner KM, McCabe BD (2009) Drosophila larval NMJ dissection. *J Vis Exp* 24:pii: 1107.
3. Deng P-Y, et al. (2013) FMRP regulates neurotransmitter release and synaptic information transmission by modulating action potential duration via BK channels. *Neuron* 78(1):205.

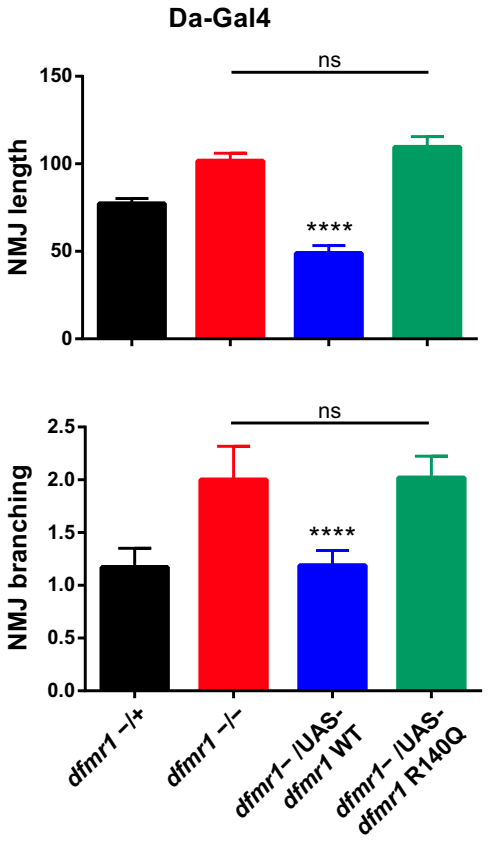

Fig. S1. Ubiquitously driven R140Q-FMRP expression in *Drosophila* does not rescue NMJ synaptic overgrowth. Quantification of NMJ length and branching in *dfmr1*-deficient flies with ubiquitously driven (Da-Gal4) expression of WT or R140Q-FMRP. WT-FMRP expression effectively reduces both NMJ length and branching, whereas R140Q fails to do so and has synaptic overgrowth similar to *dfmr1*-deficient flies. Data are represented as mean ± 95% confidence interval. ****$P < 0.0001$ for comparison with $dfmr1^{-/-}$; ns, not significant.

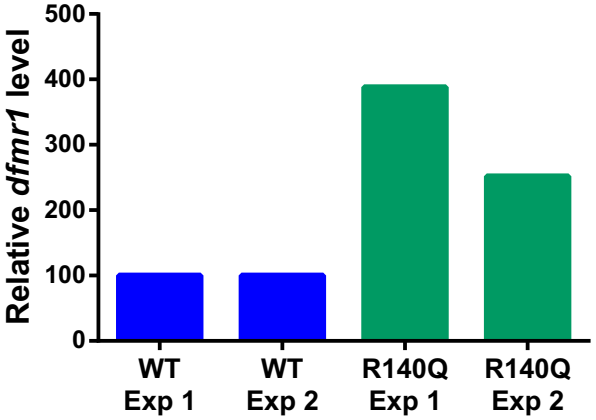

Fig. S2. Elav-Gal4 drives sufficient expression of UAS-*dfmr1*-R140Q. Relative *dfmr1* levels measured from whole larvae in Elav-Gal4–driven UAS-*dfmr1*-WT or UAS-*dfmr1*-R140Q flies. In two independent experiments, R140Q mutants expressed *dfmr1* at higher levels than WT, indicating the lack of rescue in NMJ length or branching was not due to inadequate R140Q-FMRP expression.

Table S1. List of primers

Map1b	Forward: 5′-CGATCGTGGGACACAAACCT-3′
	Reverse: 5′-GTGATCATCAAACGCACCTCA-3′
Dlg4 (Psd95)	Forward: 5′-CTATGAGACGGTGACGCAGA-3′
	Reverse: 5′-CGGGAGGAGACAAAGTGGTA-3′
CamKIIα	Forward: 5′-AATGGCAGATCGTCCACTTC-3′
	Reverse: 5′-ATGAGAGGTGCCCTCAACAC-3′
Actb (β-actin)	Forward: 5′-TGTTACCAACTGGGACGACA-3′
	Reverse: 5′-GGGGTGTTGAAGGTCTCAAA-3′

Map1b, microtubule-associated protein 1b; Dlg4, discs large homolog 4; CamKIIα, calcium/calmodulin-dependent protein kinase II alpha.