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Supplementary Information for

mTORC1 pathway inhibition restores PSD95 induction in neurons lacking Fragile X mental retardation protein

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This PDF file includes:

Supplementary materials and methods
Figs. S1 to S2
Captions for movies S1 to S2
References for SI reference citations

Other supplementary materials for this manuscript include the following:

Movies S1 to S2

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Supplementary Materials and Methods

Compartmentalized chamber fabrication. Silicon wafer masters were made by soft photolithography on two layers of photoresist with patterning provided by two transparency masks created in CAD software and printed on a 20,000 dots-per-inch printer. Polydimethylsiloxane (PDMS) prepolymer and catalyst (Dow Corning) were mixed at a 10:1 ratio and allowed to polymerize on the masters at 55°C overnight. Allowing the PDMS molds to cure at room temperature for 7-30 days increase the possibility of successful fabrication of culture chambers in the following steps. Blocks were then cut out, sterilized, and adhered to washed coverglasses, and then the channels were coated with poly-D-lysine (Sigma) at 0.05mg/ml dissolved in Borate buffer (10 mM sodium borate, 150 mM NaCl, pH 8). After overnight coating at 37ºC and 1 week coating at 4 ºC, the compartmentalized culture chambers were washed with sterile water for 3 times before cell culture use.

Animals. For rat glial feeder layer culture, Sprague Dawley time-pregnant female rats were ordered from Charles River Laboratories, and glia were obtained from P0 pups of both sexes. For neuronal cultures, wild-type (WT) mice (C57BL/6J, #000664) and FMRP-deficient mice (B6.129P2-Fmr1^{tm1Cgr}/J, #003025) were obtained from the Jackson Laboratory, and maintained as two separate lines. Hippocampi were dissected from P0 pups of both sexes. This was necessary for obtaining sufficient numbers of neurons for transfection. For example, if littermates of mixed genotypes were desired, then a *Fmr1*-heterozygous female would need to mated with either a *Fmr1*-hemizygous male or a wild-type male. In either case, due to the location of *Fmr1* on the X chromosome, female progeny cannot provide homozygous *Fmr1*-mutant and homozygous wild-type groups: a *Fmr1*-hemizygous father cannot produce homozygous wild-type daughters while a wild-type father cannot produce homozygous *Fmr1*-mutant daughters. Thus only male pups could then be used in this experiment, and litters often lack enough male pups of one of the genotypes. To minimize the possible effects of epigenetic divergence between these two lines, both were obtained from the same commercial source at the same time, and were maintained in parallel in the same rooms. We also did not observe any changes over mouse generations in responses to BDNF stimulation within a genotype that would suggest drift. Animal procedures were approved by the Stanford Institutional Animal Care and Use Committee.

Glial culture. All cell culture reagents were obtained from Life Technologies unless otherwise specified. A rat glia feeder layer was used for the experiments in compartmentalized culture chambers. Dissociated rat hippocampal cells were plated on T75 flask in 5% DMEM with B-27; neurons were removed by hitting the flask against a hard surface and changing the medium 1 hour after plating, repeated at 1 day after plating and thereafter every 3–4 days. FUDR (Sigma) was added to the culture medium after glia reached 80% confluence. Glia were ready for use 4–10 weeks after plating. Rat glia were plated into the compartmentalized chambers 1-3 days prior to mouse neuronal plating.

Neuronal culture. Hippocampal neurons were dissected from postnatal day 0 mice and transfected with a bidirectional expression vector, expressing the PSD95-TS2:YFP reporter (with the original 5' and 3' UTR of PSD95) in the forward direction and a RFP or FMRP-IRES-RFP in the reverse direction (*SI Appendix,* Fig. S1*B*), by electroporation with Amaxa reagent (Lonza). Neurons were plated directly into 12- or 24-well tissue-culture plates, or were mixed with un-transfected neurons at a ratio of 1:2 or 1:10 (for FMRP genetic rescue experiments) for plating in compartmentalized culture chambers. They were cultured in Neurobasal A medium with B27 supplement, 2 mM GlutaMAX, and 1% FBS with 4 μg/mL FUDR. Cultures were maintained at 37ºC in 5% carbon dioxide and 100% humidity. The culture medium was replaced at 1 day in vitro (DIV) only until imaging or drug treatment at 14-18 DIV.

Time-lapse microscopy. To visualize new PSD95 synthesis in neurons, an Olympus FV1000 confocal scanning system and a 40× 0.95 NA objective were used. Images were acquired with a 515-nm argon-ion laser line and a 559-nm laser diode with the following settings: pinhole 110 μm, scan resolution 2048 x 2048 pixels, scanning speed 2 μs per pixel, photomultiplier voltage 750V, digital gain 1. On the day of imaging or drug treatment, medium was changed to a CO_2-independent medium composed of HBSS with B27, 2mM GlutaMAX and 1 mM sodium pyruvate. 1.5 μM ASV was added to both compartments and 50 ng/ml BDNF was added to the distal compartment of the transfected neurons at time 0. The entire chamber was then sealed with parafilm to minimize evaporation and maintained at 33ºC. In each imaging experiment, RFP fluorescence was used to identify transfected neurons, to estimate the percentage of neurons being transfected and to outline the morphology of a neuron. Time-lapse images

were acquired for 24 h at 1.5-h intervals (Fig. 1). Single time point experiments were performed at 21-24 h after drug addition (Fig. 2-4). A stack of optical sections at 1 μm intervals through each neuron was obtained and then flattened in a maximum intensity projection using ImageJ (NIH).

Chemicals. The following chemicals were used at the concentration specified: Asunaprevir (ASV, BMS 650032) 1.5 μM; BDNF (EMD Millipore #GF029) 50 ng/ml; okadaic acid (Tocris Bioscience #1136) 100 nM; U0126 (Tocris Biosciences #1144) 20 μM; rapamycin (Tocris Bioscience #1292) 10 μM; LY294002 (Tocris Biosciences #1130) 10 μM; Ro 31-8820 (Tocris Bioscience #2002) 2 μM; GF109203X (Tocris Bioscience #0741) 2 μM; PF-4708671 (Tocris Bioscience #4032) 10 μM.

Immunoblotting. To analyze FMRP-dependent PSD95 synthesis induced by BDNF, Fmr1-deficient mouse hippocampal neurons were transfected with PSD95-TS2:YFP before plating and treated with 1.5 μM ASV at DIV14 for 21 h with or without the presence of 50 ng/mL BDNF (EMD Millipore #GF029). Inhibitors used were: 10 μM rapamycin (Tocris Bioscience #1292); 20 μM U0126 (Tocris Biosciences #1144); 0.3 uM SCH772984 (Selleck Chemicals S7101); 3 μM Vx-11e (Selleck Chemicals S7709); 10 μM PF-4708671 (Selleck Chemicals S2163). BDNF was dissolved in water while other drugs were dissolved in DMSO. Final concentration of DMSO in each condition treated neuron culture was 0.1%. After rinsing with PBS, cells were lysed in boiling Laemmli sample buffer. Lysates were sonicated to shear DNA and then run on 4-15% Criterion TGX SDS-polyacrylamide gels (Bio-Rad #5671084). Proteins were transferred onto polyvinylidene fluoride membranes (Bio-Rad #1704275) using the Transblot Turbo system (Bio-Rad). Membranes were blocked with 5% nonfat dried milk in Tris-buffered saline (TBS) for 1 hour, incubated in primary antibodies mouse anti-PSD95 (1:1000, Neuromab cloneK28/43) and rabbit anti-NeuN (1:2000, Abcam ab177487) in 5% milk in TBS with 0.1% Tween-20 (TBST) at 4 °C, washed 3 times for 10 min each in TBST, incubated in secondary antibodies goat anti-mouse IRDye800CW (1:10,000, Li-COR Biosciences 926-32210) and goat anti-rabbit IRDye680RD (1:10,000, Li-COR Biosciences 926-68071) in 5% milk in TBST for 1 hour at room temperature, and finally rinsed 3 times for 10 min each in TBST. Fluorescent signals were acquired by an Odyssey CLx system and analyzed by Image Studio software (LiCOR Biosciences).

Experimental design and statistical analysis. Two major sets of experiments were performed in this study, time-lapse experiments and single time point experiments. Time-lapse experiments were performed to examine the accumulation of newly synthesized PSD95 over a 24-hour time course. In these pilot experiments, we cultured neurons from 6 litters of each genotype. Data was pooled from all 6 experiments then analyzed together, with mouse genotype unknown to the investigator. For single time point experiments, power analyses were performed beforehand with wild-type time-lapse results to determine the minimal sample number required to observe a 50% difference ($n > 9$). Experiments were repeated until the desired n number has been reached. Data analysis was performed with mouse genotype, construct and/or drug identities unknown to the investigator and all analyzed data included. For each condition, data were from at least 2 dissections. Because the cell-to-cell variation was larger than variation between dissections, neurons from different dissections were pooled and analyzed together. Each single data point represents one neuron, and all summary data are presented as mean ± SEM. For time-lapse imaging results, mixed effect repeated measures ANOVA was performed. For single time point imaging results, ANOVA tests were performed (Fig. 2, $p = 3.7 \times 10^{-5}$, 7 groups; Fig. 3, $p = 0.0087$, 8 groups; Fig. 4, $p = 5.9 \times 10^{-5}$, 7 groups) before subsequent *ad hoc* comparisons with Student's *t* test with Tukey's correction. *p* values from Student's t test are reported unless indicated otherwise. In all figures, an asterisk (*) indicates *p* value < 0.05.

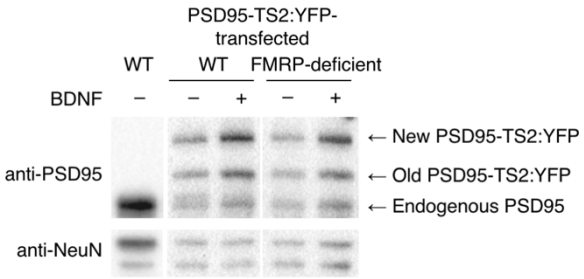

Fig. S1. Immunoblotting of neurons transfected with the PSD95:TS2-YFP reporter reveals that total cellular amounts of new PSD95 protein are increased after 21 h of global BDNF stimulation in both wild-type and FMRP-deficient neurons.

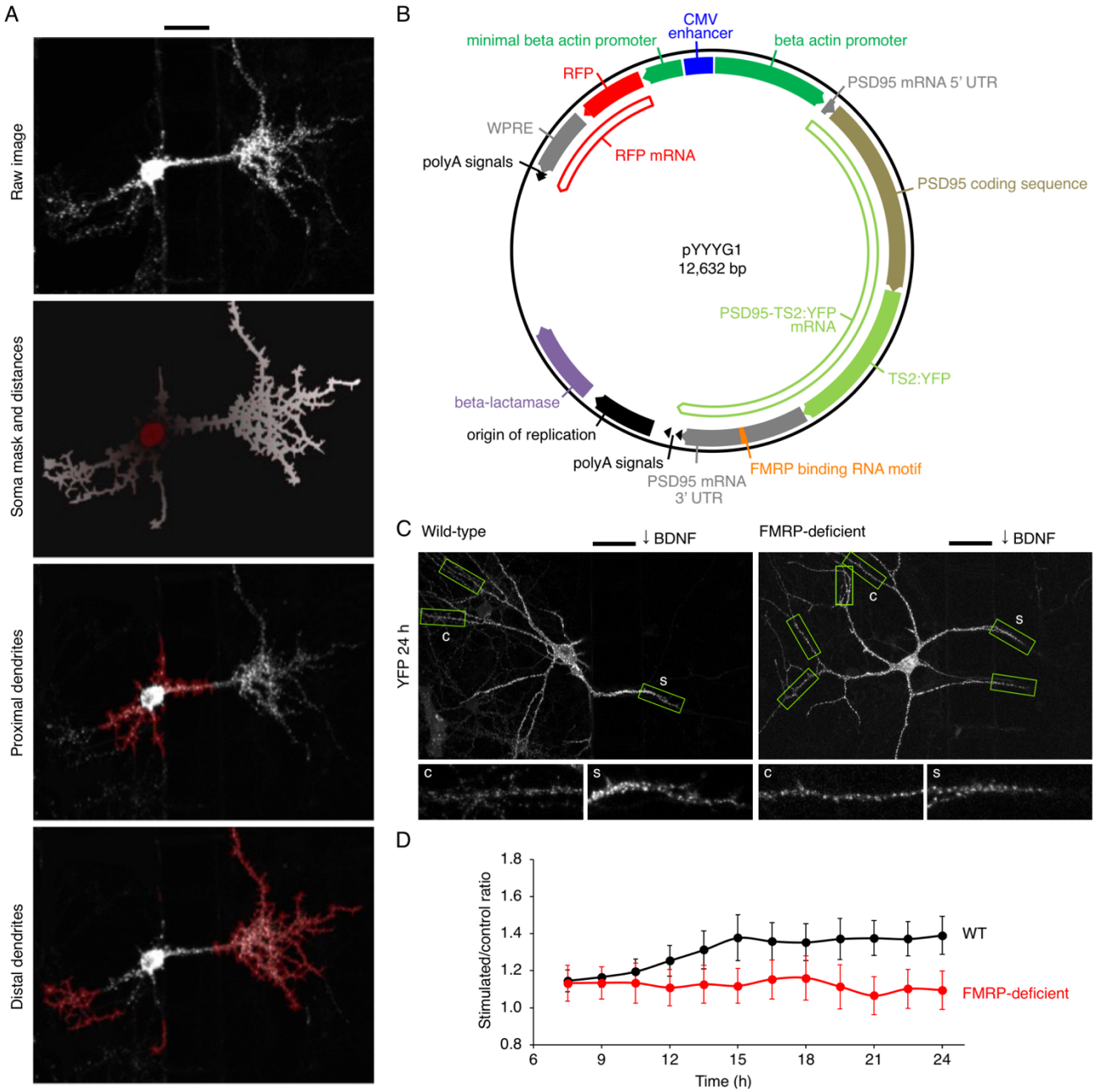

Fig. S2. Dendritic BDNF stimulation locally induces new PSD95 expression in a FMRP-dependent manner. (*A*) Automatic image analysis procedure. From top to bottom are raw image, soma mask (red) and point-wise distance to soma (gray scale), mask for stimulated proximal spines and dendrites and matching control, and mask for stimulated distal spines and dendrites and matching control. Analysis was performed by a custom-written Matlab program. Black bar above the images indicates the location of the barrier and provides a 50-μm scale bar. (*B*) Bidirectional expression plasmid for TimeSTAMP quantitation co-expressing a RFP marker and a PSD95-TS2:YFP reporter mRNA in opposite directions. (*C*) BDNF-induced expression of new PSD95 in distal dendrites requires FMRP. Stimulated distal segments were defined as the first 50 μm in the stimulation chamber, then control distal segments were defined as those segments in the unstimulated side located an equal distance away from the cell body in terms of path length along the dendrite. Insets show a stimulated (s) region and a control (c) region. (*D*) Relative PSD95-TS2:YFP intensities in the first 50-μm segment of dendrite on the stimulated side vs. equidistant control segments on the unstimulated side. Stimulated/control intensity ratios in FMRP-deficient neurons were significantly different from WT at 24 h ($p < 0.05$ by Student's t test, n = 27 WT neurons and 22 FMRP-deficient neurons). Error bars represent standard error of the mean (SEM).

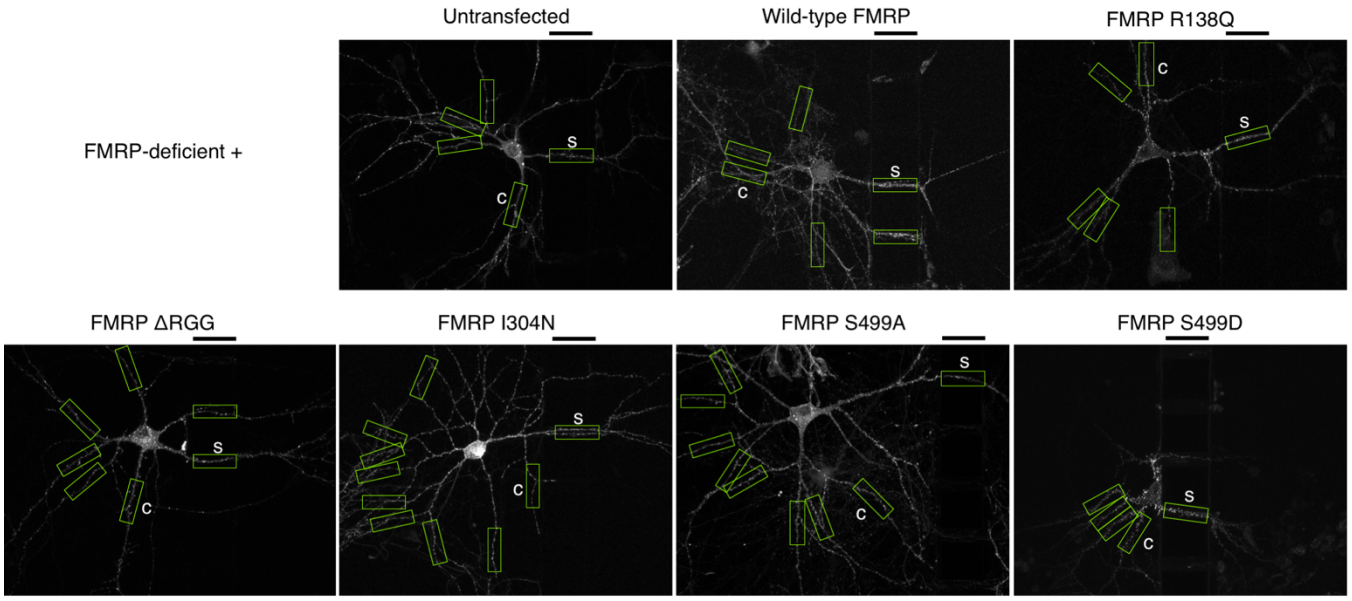

Fig. S3. Representative whole-cell images of FMRP-deficient neurons transfected with FMRP variants. Black bar above the images indicates the location of the barrier and provides a 50-μm scale bar. BDNF was applied to the right of the barrier. Boxes indicate stimulated (s) and equidistant control (c) regions identified and analyzed by automated algorithm. These regions were shown in Fig. 2*C*.

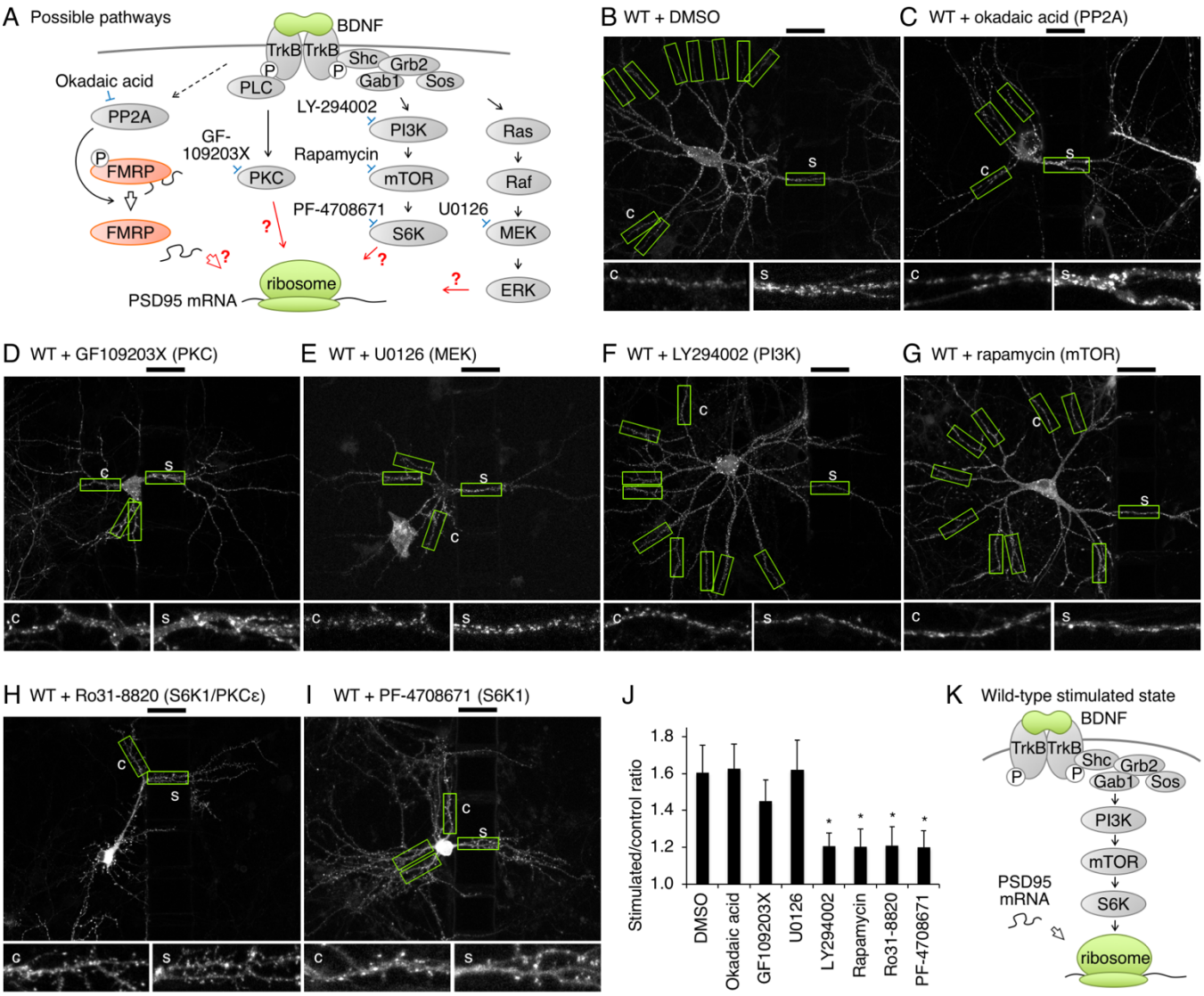

Fig. S4. Local BDNF-induced new PSD95 expression in WT neurons requires the PI3K-mTORC1-S6K1 pathway. (*A*) Models of signaling pathways regulating FMRP and PSD95 synthesis. Note simple arrows indicate positive regulatory steps, while open block arrows indicate transitions from one regulatory state to another made by the same molecule. (*B-I*) Representative images of newly synthesized PSD95 in WT neurons 21-24 h after local BDNF stimulation with (*B*) DMSO, (*C*) 100 nM PP2A inhibitor okadaic acid, (*D*) 2 μM PKC inhibitor GF109203X, (*E*) 20 μM MEK inhibitor U0126, (*F*) 10 μM PI3K inhibitor LY294002, (*G*) 10 μM mTOR inhibitor rapamycin, (*H*) 2 μM S6K1/PKC inhibitor Ro31-8820, (*I*) 10 μM S6K1 inhibitor PF-4708671 added to the stimulated dendrites together with BDNF. Black bar above the images indicates the location of the barrier and provides a 50-μm scale bar. BDNF was applied to the right of the barrier. Boxes indicate regions identified and analyzed by automated algorithm. Insets show a stimulated (s) region and an equidistant control (c) region. (*J*) Relative intensities of stimulated versus unstimulated control dendritic segments. Error bars represent SEM. DMSO, n = 12 neurons; okadaic acid, n = 12 neurons, p = 0.92; GF109203X, n = 13 neurons, p = 0.41; U0126, n = 13 neurons, p = 0.95; LY294002, n = 13 neurons, p = 0.03; rapamycin, n = 15 neurons, p = 0.04; Ro31-8820, n = 11 neurons, p =0.04; PF-4708671, n = 17 neurons, p =0.03 (each vs. DMSO by Student's t test with Tukey's correction, after ANOVA test with F (7, 99) = 2.89, p = 0.0087). (*K*) Deduced major signaling pathways in WT neurons for BDNF-induced PSD95 synthesis.

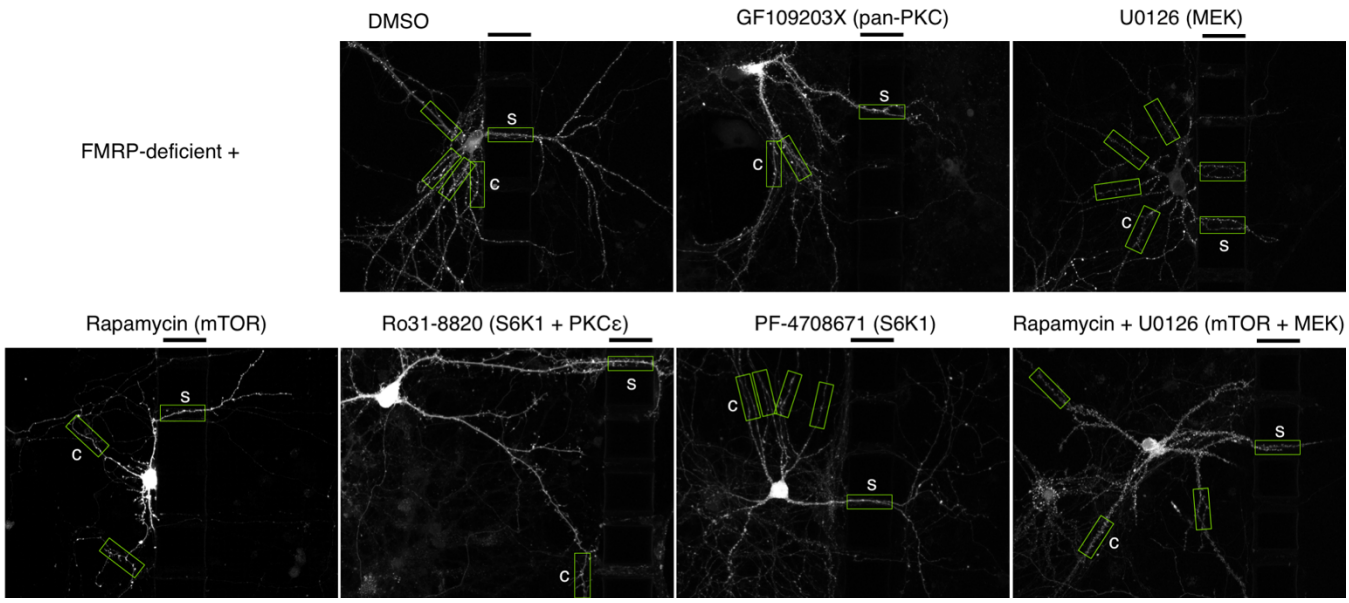

Fig. S5. Representative whole-cell images of FMRP-deficient neurons treated with various kinase inhibitors. Black bar above the images indicates the location of the barrier and provides a 50-μm scale bar. BDNF was applied to the right of the barrier. Boxes indicate stimulated (s) and equidistant control (c) regions identified and analyzed by automated algorithm. These regions were shown in Fig. 3*C*.

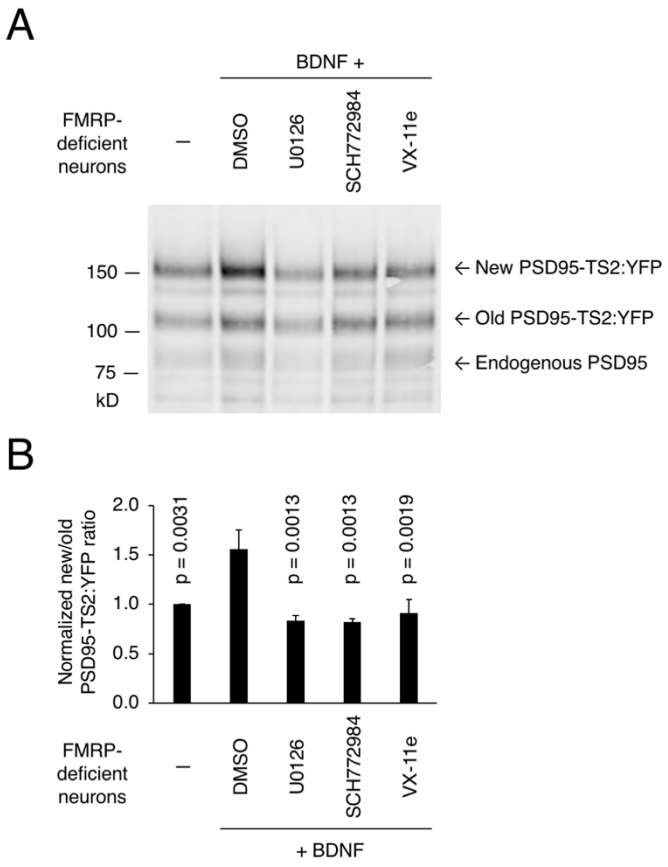

Fig. S6. The Raf-MEK-ERK pathway is required for global induction of new PSD95 by BDNF in FMRP-deficient neurons. (*A*) Representative immunoblots of FMRP-deficient neurons expressing PSD95-TS2:YFP reporter, either unstimulated or after BDNF stimulation in the presence of vehicle (DMSO) or Raf-MEK-ERK pathway inhibitors (MEK inhibitor U0126, ERK inhibitor SCH772984, or ERK inhibitor Vx-11e). BDNF and inhibitors were added for 21 h before lysis, and 1.5 μM ASV was also applied in all groups to preserve the TS2:YFP on new copies of PSD95. *(B)* New/old PSD95-TS2:YFP ratio, normalized to the unstimulated value. Error bars represent SEM, n = 4 biological replicates. Differences were significant by one-way ANOVA, with overall p = 0.0016. Individual p-values above each bar are for pairwise comparisons to the BDNF- and DMSO-treated group with Holm-Sidak correction.

Movie S1. Local dendritic BDNF stimulation induces local accumulation of new PSD95 in a wild-type neuron. Time-lapse images of newly synthesized PSD95 in a representative wild-type neuron after local BDNF stimulation. Black bar above the images indicates the location of the barrier and provides a 50-μm scale bar. BDNF was applied to the right of the barrier.

Movie S2. Local dendritic BDNF stimulation fails to induce local accumulation of new PSD95 in FMRP-deficient neurons. Time-lapse images of newly synthesized PSD95 in a representative FMRP-deficient neuron after local BDNF stimulation. Black bar above the images indicates the location of the barrier and provides a 50-μm scale bar. BDNF was applied to the right of the barrier.