Supporting Information

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Materials and Methods

Animals. Adult male *Fmr1* KO mice in the FVB129 and C57BL/6 backgrounds, and sex, age, and background matched wild-type (WT) controls were used. Mice were standard group housed with litter mates (two to five per cage) in rooms at 68 °F and 55% humidity; 12 h on/12 h off light cycle; and food and water ad libitum.

Behavioral Analyses. For object location memory (OLM) and novel object recognition (NOR) studies, mice were handled for 2 min daily for 5 d and then habituated to a white chamber (24 × 30 × 30 cm) containing sawdust bedding for 5 min daily for 5 d (1, 2). On the following day, mice were subject to OLM or NOR training. For both paradigms, mice were placed in the chamber containing identical 100 mL glass beakers in two corners of the apparatus, ∼1 inch from the perimeter, for periods described in Results. OLM retention testing of experimental mice involved returning the mouse to the chamber with one beaker in its original ("familiar") location and the second in closer proximity to the center of the apparatus ("novel") with the novel location alternated across each experimental group. For OLM retention testing of "control" mice involved placing the mouse in the arena in which the two objects were in their original/training locations. For NOR retention testing, experimental mice returned to the arena containing two objects in the original training locations: one of these was identical to the training-object whereas the second was replaced with a novel object.

Experiments were performed in an otherwise dark room with direct overhead lighting (235 lx) during the animals light cycle (unless otherwise specified). Some experiments were conducted on the animals dark cycle: In these instances, all procedures (handling, training, testing) were conducted under red light and 12 h later than the light-cycle testing time. In between arena sessions, animal feces were removed from the apparatus, sawdust was thoroughly mixed, and beakers were cleaned with 70% ethanol and dried. All training and testing videos are scored offline by raters, blind to genotype and experimental group.

For both tasks, mice were scored as interacting with (i.e., exploring) an object when sniffing with their nose touching or within 0.5 cm from the object. Interaction was not scored when the animal (*i*) was within this radius and was grooming or digging or (*ii*) touched the object or was within this radius but did not show intent to interact (e.g., they fell within this zone when turning). Total exploration time was quantified as the time interacting with both objects. To assess preferential attention to an object, a discrimination index was calculated as $100 \times (t_{\text{novel}} - t_{\text{familiar}})/(t_{\text{novel}} + t_{\text{familiar}})$. As plotted, a positive discrimination index represents a preference for the novel object or novel object location for NOR and OLM, respectively. Total distance traveled was calculated via ANY-Maze.

ERK1/2 Inhibition. In some studies, starting on the final day of handling, mice were primed with i.p. injections of saline (2 d) and then DMSO vehicle (4 d). On the day of training, activation of ERK1/2 was blocked using SL327 [50 mg/kg at 2.5 mg/m (3); Tocris Bioscience], an inhibitor of the ERK1/2 activator MEK; control mice received vehicle (DMSO, Fisher Scientific). On pretreatment days, saline/vehicle injections were given 30 min before handling or habituation. On the day of training, animals underwent OLM training with three 100-s trials, each separated by 1 h. The injection of SL327 or vehicle was administered 30 min before the third training trial. Mice were then tested for retention 24 h posttraining. As in other behavioral experiments, total exploration times and total distances traveled were calculated for all training and retention trials. For immunolabeling analyses, SL327 (50 mg/kg) was administered to a separate cohort of KOs and brains were harvested 30 min later (e.g., the time at which the third spaced trial would occur in the separate behavioral experiments) for tissue processing as described below.

Tissue Collection and Immunofluorescence. To assess effects of behavior on synaptic ERK1/2 activation, tissue sections through hippocampus were processed for dual immunofluorescence and then FDT to quantify labeling within the postsynaptic compartment. Immediately at the end of the behavioral session, mice were euthanized with isoflurane 2 min following training. As described in detail elsewhere (4, 5), brains were rapidly harvested, fast frozen in 2-methylbutane (−50 °C), and cryostat sectioned (20 μm, coronal) through the rostrocaudal extent of hippocampus; the sections were thaw mounted onto microscope slides and fixed in methanol (−20 °C, 15 min). Series containing every 10th tissue section were washed through several changes of 0.1 M phosphate buffer and incubated (48 h, room temperature) in a mixture of mouse anti-PSD95 (1:1,000; Thermo Scientific MA1-045) and rabbit antisera to either phosphorylated (p-) ERK1/2 Thr202/Tyr204 (1:500; Cell Signaling 4370) or total ERK1/2 (1:500; Cell Signaling 9102). The tissue was again washed in phosphate buffer and then incubated with donkey anti-mouse AlexaFluor 488 and donkey anti-rabbit AlexaFluor 594 (1:1,000; Invitrogen). After final washes slides were coverslipped with VectaShield with DAPI (Vector Labs).

Fluorescence Deconvolution Tomography (FDT). Using a Leica DM6000 epifluorescence microscope, a 63× oil-immersion objective (NA 1.4), and an ORCA-ER (Hamamatsu) camera, digital image z-stacks were collected through a depth of 3 μm (at 0.2-μm steps which is below the Nyquist rate for the imaging conditions used) for 136 × 105 μm sample fields that were centered on hippocampal field CA1 stratum radiatum (SR; offset from the pyramidal cell layer by about 100 μm). For each section, two or three z-stacks were collected from fields within the middle depths of SR as shown in Fig. S6; these z-stacks were individually processed for restorative deconvolution (Volocity 5.0, Perkin-Elmer) and through in-house software to normalize background fluorescence intensity and construct 3D montages of each 42,840-μm^3 sample field (i.e., each z-stack generated one 3D sample field). Automated systems then counted and measured in 3D the size and fluorescence intensities of single- and double-labeled elements within size constraints of synapses. Approximately 40,000 PSDs were quantified for each sample field. Objects were counted as double-labeled if there was any overlap in the boundaries of immunolabeling with the two fluorophores as evaluated in 3D. Quantification was limited to objects that satisfied size and eccentricity constraints of synapses, were detected across multiple intensity thresholds, and were fully contained within the image z-stack (4, 5). Objects detected by only one threshold, and thus likely being fluorescent artifacts, were excluded from quantification. Counts of double-labeled PSDs were normalized to the total number of PSD95-immunopositive elements within a given sample field; normalized values from each z-stack were averaged to obtain a mean value for each brain.

1. Stefanko DP, Barrett RM, Ly AR, Reolon GK, Wood MA (2009) Modulation of long-term memory for object recognition via HDAC inhibition. *Proc Natl Acad Sci USA* 106(23): 9447–9452.
2. Haettig J, et al. (2011) HDAC inhibition modulates hippocampus-dependent long-term memory for object location in a CBP-dependent manner. *Learn Mem* 18(2):71–79.
3. Thomas GM, Huganir RL (2004) MAPK cascade signalling and synaptic plasticity. *Nat Rev Neurosci* 5(3):173–183.
4. Seese RR, et al. (2012) LTP induction translocates cortactin at distant synapses in wild-type but not Fmr1 knock-out mice. *J Neurosci* 32(21):7403–7413.
5. Seese RR, et al. (2013) Synaptic abnormalities in the infralimbic cortex of a model of congenital depression. *J Neurosci* 33(33):13441–13448.

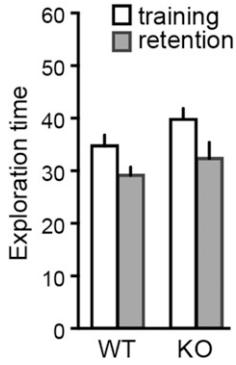

Fig. S1. Total times (in seconds) exploring objects during 5-min massed object location memory training. Retention trials were not different between WT and Fmr1 KO mice ($P > 0.40$; $n \geq 8$ per group).

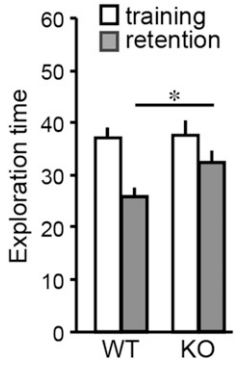

Fig. S2. Plot shows that total times (in seconds) exploring objects during 5-min continuous novel object recognition (NOR) training was comparable between genotypes whereas, during NOR retention testing, KOs spent more time than WTs in object exploration (*$P = 0.03$). Thus, in comparing training to retention sessions, the WTs show a greater reduction in object exploration time relative to the KOs. $n \geq 12$ per group.

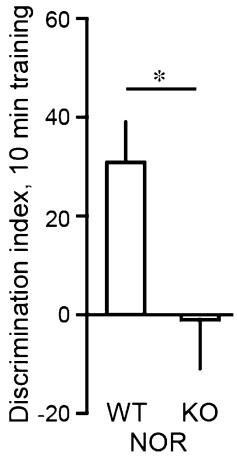

Fig. S3. After 10 min of massed training, wild-type (WT) mice exhibited novel object recognition (NOR, i.e., had a positive discrimination index) whereas *Fmr1* KOs did not. Mice were tested 24 h after training. Performance of the KOs was significantly different from that of WTs (*$P < 0.05$; $n \geq 9$ per group).

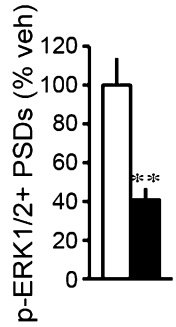

Fig. S4. *Fmr1* KO mice were injected with the ERK1/2 activation inhibitor SL327 (50 mg/kg) or DMSO/vehicle and then euthanized 30 min later to assess effects of treatment on numbers of p-ERK1/2+ synapses (double labeled with anti-PSD95) using fluorescence deconvolution tomography (as applied in Fig. 3). Quantification of densely p-ERK1/2+ PSDs showed that SL327 significantly reduced synaptic p-ERK1/2 levels (**$P < 0.01$ vs. KO vehicle; $n = 10$ per group).

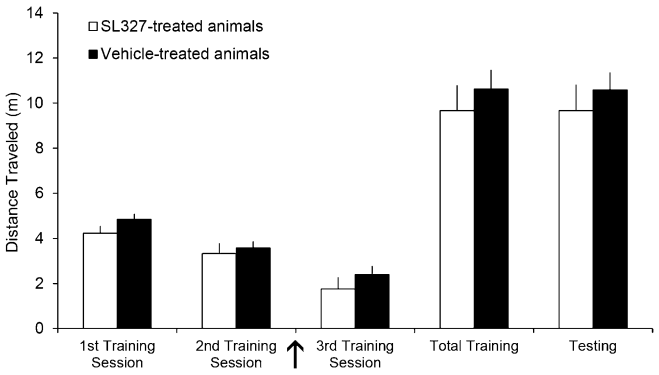

Fig. S5. Bar graph shows total distance traveled by *Fmr1* KO mice that were treated with vehicle (DMSO) or SL327 (50 mg/kg) in DMSO (see Fig. 4*D* for treatment paradigm). SL327 was administered in between the second and third training session (i.e., 30 min before the final training trial; see arrow). For all trials as well as for retention testing, total distance traveled for WT and *Fmr1* KO animals were comparable ($P > 0.05$; $n = 10$ per group).

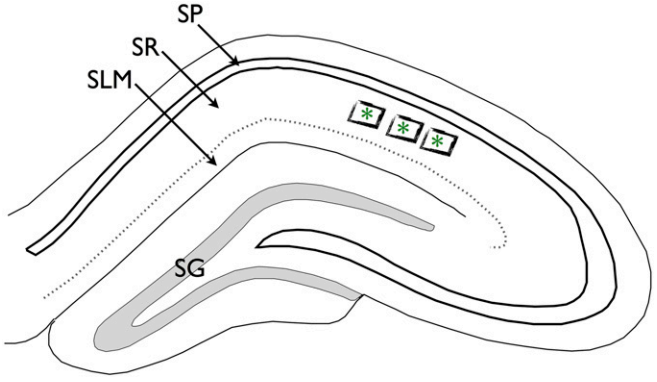

Fig. S6. Schematic showing the placement of image z-stacks (boxes with asterisks) in CA1 str. radiatum (SR). SG, str. granulosum; SLM, str. lacunosum-moleculare; SP, str. pyramidale.