Title
Spaced training rescues memory and ERK1/2 signaling in fragile X syndrome model mice.

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Recent studies have shown that short, spaced trains of afferent stimulation produce much greater long-term potentiation (LTP) than that obtained with a single, prolonged stimulation episode. The present studies demonstrate that spaced training regimens, based on these LTP timing rules, facilitate learning in wild-type (WT) mice and can offset learning and synaptic signaling impairments in the fragile X mental retardation 1 (Fmr1) knockout (KO) model of fragile X syndrome. We determined that 5 min of continuous training supports object location memory (OLM) in WT but not Fmr1 KO mice. However, the same amount of training distributed across three short trials, spaced by one hour, produced robust long-term memory in the KOs. At least three training trials were needed to realize the benefit of spacing, and intertrial intervals shorter or longer than 60 min were ineffective. Multiple short training trials also rescued novel object recognition in Fmr1 KOs. The spacing effect was surprisingly potent: just 1 min of OLM training, distributed across three trials, supported robust memory in both genotypes. Spacing also rescued training-induced activation of synaptic ERK1/2 in dorsal hippocampus of Fmr1 KO mice. These results show that a spaced training regimen designed to maximize synaptic potentiation facilitates recognition memory in WT mice and can offset synaptic signaling and memory impairments in a model of congenital intellectual disability.

Fmr1 KO | hippocampus | object location memory | massed training | novel object recognition

Fragile X syndrome (FXS) is the most common cause of inherited intellectual disability (ID) (1). Currently no treatments exist for cognitive deficits associated with FXS or other neurodevelopmental disorders with ID. Research on the fragile X mental retardation 1 (Fmr1) KO mouse model of FXS has identified impairments in synaptic signaling required to produce lasting synaptic modifications (2–6) with corresponding disturbances in the activation threshold and stabilization of hippocampal long-term potentiation (LTP) (7, 8). These findings suggest specific synaptic disturbances underlie learning problems in FXS as well as targets for therapeutic interventions to improve cognitive function.

The present experiments tested predictions from LTP studies as to how modified training paradigms might rescue synaptic signaling and learning in Fmr1 KO mice. There is a deep literature demonstrating that individuals learn better when trained in short trials spaced in time than in a single, extended training episode (9). We recently found that LTP exhibits a synaptic analog for this “spaced trials effect” (10). Specifically, in hippocampal field CA1 short trains of theta burst afferent stimulation spaced by 60 min elicit far greater synaptic potentiation than can be achieved with long theta trains or by repeated trains applied at shorter intervals. As LTP is considered a mechanism of memory encoding, we propose that spaced training regimens that use the same 60-min periodicity should facilitate hippocampus-dependent learning and, thereby, may offset deficits associated with congenital ID. This hypothesis was tested for Fmr1 KO mice using the object location memory (OLM) task that both is highly sensitive to the duration of training and depends on dorsal hippocampal field CA1 (11) which exhibits LTP impairments in the KOs (7).

Our results show that given short training trials spaced by 1 h, wild-type (WT) mice learn object location in a fraction of the time needed with continuous training, and Fmr1 KOs perform at WT levels. In KOs, this robust behavioral rescue is accompanied by restoration of training-induced synaptic activation of ERK1/2, a kinase required for hippocampal LTP and learning (12, 13).

Results

Fmr1 KOs Have an Elevated Threshold for Enduring OLM. Mice were exposed to two identical objects in an open arena during training, and later returned to the arena in which one object had been moved to a novel location (Fig. 1A); preferential exploration of the novel location object indicates learning of the original location. In WT mice, a single 5- or 10-min training session elicits both short-term (90-min latency) and long-term (24-h latency) OLM whereas 3 min of training is not sufficient (14). Accordingly we used near-threshold, 5-min training to test the prediction from LTP studies that Fmr1 KO mice have an elevated threshold for this form of memory. WTs exhibited robust long-term OLM, whereas KOs did not (Fig. 1B). The deficit in KO mice was not due to initial encoding impairments; retention tested after 90 min was comparable between genotypes (Fig. 1C). As expected from the threshold argument, training for 10 min produced similar retention scores in KOs and WTs (Fig. 1D), suggesting that the KOs defect involves the amount of training needed to transfer information into long-term storage.

We tested whether the above results, obtained using FVB129-background mice, also hold for KOs on the C57BL/6 background: Short-term OLM was again comparable for KO and WT mice but long-term OLM was absent in KOs given 5-min massed training (Fig. 1E).

Potential Contributors to the OLM Defect in KO Mice. Like FXS patients, Fmr1 KOs are excessively anxious (15), which could reduce object exploration during training. However, object

Significance

There are no treatments for congenital intellectual disabilities. Here we show that newly discovered timing rules for maximizing hippocampal long-term potentiation predict training regimens that offset defects in synaptic chemistry and memory in the fragile X mental retardation 1 (Fmr1) KO model of fragile X syndrome. Wild-type mice required far less training to form stable memories when given three training trials separated by 1 hour as opposed to one extended session; shorter or longer intervals were ineffective. The same spaced training protocol rescued memory in Fmr1 KO mice and restored activation of synaptic ERK1/2, a kinase critical for both LTP and learning. These results suggest a readily implementable, neurobiologically based therapeutic strategy for a prevalent form of intellectual disability.

Author contributions: R.R.S., G.L., and C.M.G. designed research; R.R.S., K.W., and Y.Q.Y. performed research; R.R.S., G.L., and C.M.G. contributed new reagents/analytic tools; R.R.S., K.W., G.L., and C.M.G. analyzed data; and R.R.S., G.L., and C.M.G. wrote the paper.

The authors declare no conflict of interest.

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interaction times during training and retention trials were comparable in WTs and KOs (Fig. S1). 

Fmr1 KOs are also hyperactive in open-field tests (16). In accord with this, distance traveled for KOs decayed more slowly than for WTs across habituation sessions, resulting in greater locomotion on pretraining days 2–5. However, with objects present, the genotypes traveled the same distance (Fig. 1F).

To assess the robustness of the OLM impairment, we tested mice during the dark phase of their day/night cycle. Long-term OLM was still absent in KOs (Fig. 1G) although total object exploration times were not different between genotypes during training or retention testing.

Next we tested whether 5 min of continuous (i.e., massed) training produces a memory trace that is too weak to elicit quantifiable retention. If so, then additional training should increase trace strength, producing measurable discrimination indices. Mice were trained for 5 min and 24 h later for 5 min more. Tested 1 d later, WT mice showed strong long-term OLM but KOs did not (Fig. 1H). We gave additional mice 5-min training daily for 6 d: OLM was still absent in the KOs indicating that they do not form a partial memory trace after 5 min of training.

Fmr1 KOs Have Impaired, Long-Term Novel Object Recognition (NOR).

We focused on OLM because its retrieval depends on hippocampal field CA1 (11, 14), which exhibits an elevated LTP threshold in Fmr1 KOs (7, 8) and enhanced potentiation with spaced stimulation in WTs (10, 17). However, Fmr1 KOs have more robust LTP impairments in other regions including cortex (6, 18–21). Therefore, we tested if the KOs have deficits in enduring NOR memory which depends, at least in part, on perirhinal and insular cortices for retrieval (14, 22, 23). Short-term NOR impairments have been described for Fmr1 KOs (6, 24). Mice were trained as for OLM for 5 min. For retention testing 24 h later, one of the familiar objects was replaced with a novel object but there was no change in arena-context or object location. WT mice spent more time sampling the novel object but KOs did not (Fig. 1I). The total time spent exploring objects was comparable for WTs and KOs during training but somewhat longer for KOs during retention testing (Fig. S2). NOR deficits in the KOs persisted after 10 min of training (Fig. S3). We conclude that in Fmr1 KO mice impairments in OLM and NOR reflect defects in long-term object recognition memory and, as with LTP impairments, the effects of genotype are more robust for measures with greater dependence upon cortical function.

Spaced Training Facilitates WT Learning and Rescues Memory in Fmr1 KOs.

As WT mice do not form OLM with 3 min of continuous training (14), we first tested whether dividing training into three 60 s long trials produces enduring memory (Fig. 2A). A 60-min intertrial interval was selected because in LTP studies successive stimulation trains separated by this interval enhance potentiation in normal rodents (10). As shown (Fig. 2B), in WT mice, three 60-s trials supported robust OLM, whereas 3 min of continuous training did not.

To test whether spaced training also decreases the learning threshold in Fmr1 KOs, mice were given 5 min of training divided into three 100-s trials spaced by 60 min. This regimen produced robust OLM in KOs that was comparable to measures in WTs (Fig. 2C). The same amount of training in a single 300-s trial did not support OLM in KO mice (Fig. 2B). A potent spacing effect was also observed for NOR: KOs that received three 100 s trials training spaced by 60 min performed as well as WTs when tested 24 h later (Fig. 2D). These results constitute, to our knowledge, the first evidence that spaced training modeled on LTP timing rules can rescue long-term memory in a mouse model of ID and further show the approach is effective in tasks that depend on dorsal hippocampus (OLM) or on both hippocampal and cortical fields (NOR) for retrieval.

One hypothesis for the advantages of spacing posits that the animal’s interest is greatest at the beginning of training, so that
We next sought to define minimum conditions for lowering the training threshold for OLM with spaced training. For both genotypes, two 100-s trials, or two 150-s trials (5 min total), spaced by 1 h, did not support OLM, whereas at least three trials spaced by 1 h did (Fig. 2F). Thus, at least three trials are needed to facilitate encoding. To assess the minimum duration of training required, three trials separated by 60 min were used but each trial was decreased from 100 to just 20 s. Remarkably, when appropriately spaced, a total of 1-min training was sufficient for both WT and KO mice to encode robust, long-term memory (Fig. 2G). These results point to an unprecedented potency for spaced training, and indicate that spacing can normalize this form of learning in Fmr1 KOs.

**Spaced Training Rescues Synaptic ERK1/2 Activation in Fmr1 KOs.** The above results suggest that in KOs spaced training overcomes defects in synaptic mechanisms that promote encoding. Several points implicate disturbances in ERK1/2 signaling. First, the kinase helps stabilize LTP and memory (13) and is critical for recognition memory (12). Second, although effects of the FXS mutation on ERK1/2 activation are variably described (2, 4, 26, 27), we found that synaptic, but not overall, levels of the activated kinase are elevated, and activity-driven increases in synaptic ERK1/2 phosphorylation are stunted in KO relative to WT mice (6). Here, we tested whether (i) object location information activates synaptic ERK1/2 in WTs, (ii) the effect is impaired in Fmr1 KOs, and (iii) spaced training offsets the signaling defect in the KOs.

Synaptic levels of activated (Thr202/Tyr204 phosphorylated, p-) ERK1/2 were measured in CA1 stratum radiatum (SR) using quantitative fluorescence deconvolution tomography (5, 28). Sections were dual-immunolabeled for the excitatory synapse scaffold protein PSD95 (29) and p-ERK1/2 (Fig. 3 A–C). The KOs had elevated numbers of densely p-ERK1/2 immunopositive (+) synapses in CA1 SR (Fig. 3D), although total numbers of ERK1/2+ synapses were not different between genotypes. As synaptic ERK1/2 is activated 2 min after LTP induction in WT mice (5), we used this latency to test whether spaced training, which supports OLM, produces similar activation in WT mice (Fig. 3E). Synaptic p-ERK1/2 levels were evaluated in every 10th section through hippocampus to determine where OLM-driven effects map onto the septotemporal arc of field CA1 (Fig. 3F). There was a significant elevation in p-ERK1/2+ PSDs ~2.16 mm posterior to Bregma. Therefore, as a conservative measure of effects map onto the septotemporal arc of field CA1 (Fig. 3F).

As expected, densely p-ERK1/2+ PSDs were twice as numerous in control KOs than in control WT mice (Fig. 3G). For the KOs, a single 5-min training session with objects present, which does not elicit long-term OLM, failed to further increase numbers of densely p-ERK1/2+ synapses. Indeed, a significant decrease, relative to KO controls, was observed (Fig. 3G). However, 10 min of continuous training, which support OLM in KOs, increased numbers of p-ERK1/2+ synapses in CA1 SR (Fig. 3H). Thus, in both WT and KO mice, effective training activated synaptic ERK1/2 in CA1 of midrostral hippocampus.

We next tested if three 100-s training trials spaced by 1 h increase synaptic p-ERK1/2 in Fmr1 KOs (Fig. 4A). In contrast to effects of 5-min massed training, spaced trials significantly increased numbers of densely p-ERK1/2+ PSDs. The increase was located in the caudal two-thirds of the segment in which increases were found after massed training in WT (Fig. 4B) and involved a rightward shift in the p-ERK1/2 immunolabeling intensity frequency distribution (Fig. 4C) suggesting an increase in levels of activated ERK1/2 as opposed to an increase in numbers of contacts that are p-ERK1/2+. Finally, we tested if ERK1/2 activation was required for the rescue of OLM with spaced training in KOs using the brain-permeable ERK1/2 activation
inhibitor SL327 (13). Mice received three 100-s-long training trials spaced by 1 h, and were injected with vehicle or SL327 (at a dose verified to depress synaptic p-ERK1/2 levels, Fig. S4) 30 min before the last trial (Fig. 4D). When tested 24 h later, vehicle-treated KOs exhibited robust OLM whereas those receiving SL327 did not (Fig. 4E); exploration times and distance traveled were comparable between groups (Fig. 4F and Fig. S5).

**Discussion**

The present results demonstrate that a spaced training regimen modeled on timing rules that optimize hippocampal LTP dramatically facilitates two forms of memory in WT and Fmr1 KO mice. Indeed, short training sessions spaced by one hour enabled learning with a fraction of the total training time normally required for WTs and seemingly normalized learning threshold in the KOs. Moreover, in Fmr1 KO mice, spaced training rescued otherwise deficient synaptic signaling (i.e., ERK1/2 activation) thought to be critical for memory encoding.

Fmr1 KO mice model the most common cause of inherited ID (1), but their cognitive impairments are subtle and inconsistently observed (19, 21, 30). As increased anxiety likely disrupts performance in many tasks (31), we sought a low-stress paradigm in which the KOs have a robust memory impairment. OLM satisfied these criteria. Extensive handling, several days of habituation, and an absence of strong stimuli or salient rewards during training likely minimized anxiety-inducing features. Despite the expected hyperactivity over pretraining days, measures of object interaction and exploration were comparable in KOs and WTs during training and retention trials, as was short-term memory. Nevertheless long-term memory in both OLM and NOR tasks was completely and consistently absent with conventional massed training in Fmr1 KO mice.

In the KOs, enduring LTP is impaired in the same hippocampal field required for OLM retrieval (11, 14) although this potentiation defect can be overcome by increasing the duration of inducing stimulation above that needed in WTs (7, 8). A similar phenomenon was found for the memory deficit in KO mice: Increasing massed OLM training from 5 to 10 min yielded WT retention levels. These findings suggest that the FXS mutation impairs plasticity by raising the threshold of a normally used signaling cascade or relies upon a “redundant” pathway with a high threshold. Our results support the former hypothesis: (i) KOs have more densely p-ERK1/2+ synapses than WTs, (ii) their memory impairment following a single massed trial was associated with an absence of training-induced synaptic ERK1/2 activation, (iii) spaced training, which fully restores OLM in the KOs, induced robust synaptic ERK1/2 activation, and (iv) re-alization of the spacing effect on learning in KO mice required ERK1/2 activation. These findings build on a rapidly expanding literature implicating ERK1/2 signaling in neurodevelopmental disabilities, including FXS (5, 32). How might abnormal regulation of synaptic ERK1/2 signaling affect long-term encoding events? One possibility is suggested by studies showing that in association with LTP the stabilization of changes to the actin cytoskeleton is impaired in Fmr1 KOs (3); ERK1/2 normally contributes to this process via signaling to actin cross linking proteins including cortactin (5, 33). Thus, disturbances in synaptic ERK1/2 regulation and filamentous (F-) actin stabilization with massed training may underlie, or importantly contribute to, the Fmr1 KO’s elevated threshold for memory encoding.

Although, in WT rodents, hippocampus-dependent forms of memory rely on activity-induced ERK1/2 phosphorylation (13),
provide, to our knowledge, the first demonstration of synaptic ERK1/2 activation with learning in WT mice and its impairment in a model of intellectual disability. Moreover, evidence that training-induced ERK1/2 activation is localized to a particular septotemporal segment of hippocampus is unprecedented but accords with evidence that spatial learning activates LTP-related signaling in a limited span in the septal third of hippocampal field CA1 in rat (34). Although our results do not shed light on the basis of this topography, they raise intriguing possibilities regarding the distribution of synapses encoding different forms of memory.

Whatever its origins, the memory impairment in Fmr1 KO mice was corrected by spaced training, a well-described process in which brief, temporally separated training episodes are used to enhance memory (9, 25, 35, 36). The studies also demonstrate that the facilitating effects of spacing, for both genotypes, align with the periodicity of afferent stimulation required to enhance hippocampal LTP (10, 17). This results in a narrow time window for augmenting learning: Spaced trials were effective with a minimum of three trials spaced by 60 min while 20- or 120-min intervals did not improve retention scores. The theory that spaced training focuses learning on constant, memory-relevant intervals did not improve retention scores. The theory that the delay in attention that accompanies prolonged learning (14, 22). Beyond this, the extremely simple conditions used for the present OLM and NOR tasks may not be predictive of spaced training. For OLM, three 20-s trials produced robust memory whereas three times (WTs) or five times (KOs) this amount of training was ineffective when given in a single session. The FXS mutation appears to have more severe consequences in humans than in mice and many of these symptoms are extrahippocampal in nature (1). It is thus noteworthy that in the KOs spaced training also rescued enduring NOR, a task that relies upon perirhinal and insular cortices for retrieval (14, 22). Beyond this, the extremely simple conditions used for the present OLM and NOR tasks may not be predictive of results for everyday circumstances in which distracting stimuli and alternative choices are present. Studies incorporating these elements constitute a next step in evaluating the translational potential of spaced training.

Materials and Methods
For greater detail, refer to SI Materials and Methods.

Animals. Adult (3–5 mo old) male Fmr1 KO and WT mice on FVB129 and C57BL/6 backgrounds were used. Unless otherwise specified, experiments were performed on the light cycle in the FVB129 line. Studies were conducted in accordance with NIH guidelines for the care and use of laboratory animals.
animals and protocols approved by the Institutional Animal Care and Use Committee.

Behavioral Analysis. Animal handling, training, and testing for LOM and NOR tasks were performed as described (14) with 5 min retention testing 90 min or 24 h after the last training. Arena sessions were video recorded with an overhead camera; movements were tracked, and total distance traveled was assessed, using ANY-Maze software (Stoelting). Mice were scored as interacting with an object when sniffing with nose touching or within 0.5 cm from the object. To assess preferential visitation to an object, a discrimination index was calculated as 100 × (IFM1 − IFM2)/(IFM1 + IFM2); A positive discrimination index represents a preference for the novel object (NOR) or novel object location (OLM). In studies involving inhibition of ERK1/2 activation, the MEK inhibitor SL327 (50 mg/kg by 2.5 mg/mL; Tocris Bioscience) (13), or vehicle (DMSO) was given 30 min before the third training trial in spacing studies or 30 min before sacrifice for immunofluorescence.

Immunofluorescence/Fluorescence Deconvolution Tomography. Mice were euthanized 2 min after the last training session and brains were cryostat sectioned (20 μm, coronal) through hippocampus. Every 10th section was processed for dual immunofluorescence localization of PSD95 and either p-ERK1/2 and TH2r/2/Tyr240 or total ERK1/2 as described (5, 28, 39). To quantify synaptic immunolabeling, 2–3 nonoverlapping image z-stacks per section were taken at 1.4 μm (NA 1.4) from CA1 SR (Fig. 5G). Stacks were individually processed for FDT (28) which entailed restorative deconvolution, normalization of background fluorescence and construction of a 3D montage of each z-stack (which constituted each 42,840 μm³ sample field). Automated systems then counted and measured in 3D the size and fluorescence intensity of all synapse-sized, single- and double-labeled elements per sample field. Contacts were designated double-labeled if there was any overlap in the boundaries of labeling with the different fluorophores as assessed in 3D. Double-labeled PSD counts were normalized to the total number of PSD95+ elements in a given sample field; normalized values for each field were averaged to obtain a mean value for each brain. Intensity frequency distributions were constructed for p-ERK1/2 immunolabeling. Elements considered to be “densely” p-ERK1/2 immunoreactive had a labeling intensity of 100 or above.

Statistical Analysis. Two-way repeated-measures ANOVAs and two-tailed Student t tests were used to assess statistical significance (considered as P ≤ 0.05). A single N was one animal for behavioral and immunofluorescence analyses. Data points that were more than four SDs removed from the group mean were not included in analyses. Values in text and figures show group mean ± SEM.

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