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Correction of fragile X syndrome in mice

Gül Dölen[1,2], **Emily Osterweil**[1], **B. S. Shankaranarayana Rao**[3], **Gordon B. Smith**[1], **Benjamin D. Auerbach**[1], **Sumantra Chattarji**[4], and **Mark F. Bear**[1]

1 *Howard Hughes Medical Institute, The Picower Institute for Learning and Memory, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA, USA*

2 *Department of Neuroscience, Brown Medical School and the Division of Biology and Medicine, Providence, RI, USA*

3 *Department of Neurophysiology, National Institute of Mental Health and Neuroscience, Bangalore, India*

4 *National Center for Biological Sciences, Tata Institute of Fundamental Research, Bangalore , India*

Summary

Fragile X syndrome (FXS) is the most common form of heritable mental retardation and the leading identified cause of autism. FXS is caused by transcriptional silencing of the *FMR1* gene that encodes the fragile X mental retardation protein (FMRP), but the pathogenesis of the disease is unknown. According to one proposal, many psychiatric and neurological symptoms of FXS result from unchecked activation of mGluR5, a metabotropic glutamate receptor. To test this idea we generated *Fmr1* mutant mice with a 50% reduction in mGluR5 expression and studied a range of phenotypes with relevance to the human disorder. Our results demonstrate that mGluR5 contributes significantly to the pathogenesis of the disease, a finding that has significant therapeutic implications for fragile X and related developmental disorders.

Introduction

Despite progress understanding the etiology of fragile X, it is still unknown how disruption of brain function by the *FMR1* mutation leads to a devastating syndrome that includes altered neural development, cognitive impairment, childhood epilepsy, and autism (Bernardet and Crusio, 2006). There is no treatment for FXS and the prospects for therapy by gene replacement are not promising (Peier et al., 2000). Future therapeutic approaches must therefore be based on a more complete understanding of the basic pathogenesis of the disease.

FMRP is enriched postsynaptically in the brain, particularly at synapses that use the major excitatory neurotransmitter glutamate, so much attention has been focused on synaptic dysfunction in FXS. Recently a “metabotropic glutamate receptor (mGluR) theory” of fragile X pathogenesis was proposed (Bear et al., 2004), based on the following four observations: (i) FMRP can function as a repressor of mRNA translation at synapses (Brown et al., 2001; Qin et al., 2005); (ii) synaptic protein synthesis is stimulated potently by activation of group 1 (Gp1) mGluRs, comprising mGluR1 and mGluR5 (Weiler and Greenough, 1993); (iii) many of the lasting consequences of activating Gp1 mGluRs depend on synaptic mRNA translation (Huber et al., 2000; Karachot et al., 2001; Merlin et al., 1998; Raymond et al., 2000; Vanderklish and

To whom correspondence should be addressed: mbear@mit.edu.

Edelman, 2002; Zho et al., 2002); and (iv) in the absence of FMRP, several protein synthesis-dependent consequences of activating mGluRs are exaggerated (Chuang et al., 2005; Hou et al., 2006; Huber et al., 2002; Koekkoek et al., 2005). Together, these findings have led to the idea that FMRP and Gp1 mGluRs normally work in functional opposition, and that in the absence of FMRP, unchecked mGluR-dependent protein synthesis leads to the pathogenesis of FXS (Figure S1).

The appeal of the mGluR theory stems from its simplicity and the potentially profound therapeutic implication—that down-regulating Gp1 mGluR signaling could correct multiple symptoms of FXS. However, the theory remains controversial. To date, the strongest evidence in favor of the mGluR theory (McBride et al., 2005; Tucker et al., 2006) has been indirect, relying on drug treatments in non-mammalian species with mGluR orthologues coupled to different signaling cascades than mammalian Gp1 mGluRs (Bjarnadottir et al., 2005). It has been shown in fragile X knockout mice that acute administration of MPEP (2-methyl-6-(phenylethynyl)-pyridine), an mGluR5 antagonist, can reversibly suppress seizure phenotypes (Chuang et al., 2005; Yan et al., 2005). However, in addition to off-target activity of MPEP (Heidbreder et al., 2003; Lea and Faden, 2006), interpretation of this finding is complicated by the fact that the drug is anticonvulsant in wild type mice as well. Thus, it remains to be established if chronic down-regulation of Gp1 mGluR signaling can correct altered development in fragile X, as predicted by the mGluR theory. In the current study, we used a genetic strategy to definitively address this critical question.

Results

Rescue strategy and rationale

Because both the human *FMR1* and *GRM5* genes have functional homologues in the mouse (*Fmr1* and *Grm5*), we were able to generate *Fmr1* knockout mice with reduced expression of mGluR5, the major Gp1 mGluR in the forebrain. By crossing two mutant lines, the functional relationship between two protein products can be examined; genetic “rescue” occurs when single mutant phenotypes are attenuated in the double mutant. The power of this approach in the murine model is two-fold: (1) it is a precise and selective method to reduce mGluR5 function, (2) it permits analysis of diverse phenotypes across many developmental time points, using a variety of experimental methods both *in vitro* and *in vivo*. In addition, unlike simpler genetically modifiable organisms, endophenotypes identified in this mammalian model can serve not only to establish genetic interaction, but may also bear direct relation to the phenotype in humans with the disease.

Fmr1 mutant mice (Consortium, 1994) were crossed with *Grm5* mutant mice (Lu et al., 1997) to produce *Fmr1* knockout animals with a selective reduction in mGluR5 expression (Figure S2). To increase the therapeutic relevance, we concentrated on animals with a 50% reduction in mGluR5 rather than a complete knockout (which impairs brain function (Jia et al., 1998; Lu et al., 1997)). Littermates with 4 different genotypes were created in our cross: Wild type [*Fmr1* (+/Y) *Grm5* (+/+)], *Fmr1* knockout [*Fmr1* (−/Y) *Grm5* (+/+)], *Grm5* heterozygote [*Fmr1* (+/Y) *Grm5* (+/−)], and the knockout/heterozygote cross [*Fmr1* (−/Y) *Grm5* (+/−)]; these animals are termed WT, KO, HT, and CR, respectively. In all crossings, animals were on the C57Bl/6J clonal background.

The key question that we address in this study if a reduction of mGluR5 expression will correct diverse fragile X mutant phenotypes, as predicted by the mGluR theory (Figure S1). Our genetic rescue strategy rests on the assumption that the FMRP-regulated “readout” of mGluR5 activation is modulated by *Grm5* gene dosage. One FMRP-regulated consequence of mGluR5 activation is hippocampal long-term synaptic depression (LTD), which is approximately doubled in the KO (Huber et al., 2002). It had already been established that there is a significant

effect of mGluR5 expression level on LTD in the C57Bl/6J WT background (Huber et al., 2001), and we confirmed in the present study that a 50% reduction in mGluR5 protein expression also significantly reduces LTD in the *Fmr1* KO background (Figure S2). We therefore went on to examine diverse phenotypes with relevance to the human disorder, including experience-dependent cortical development, hippocampus-dependent memory, altered body growth, seizure, and postpubertal macroorchidism. All analyses of these mice were performed "blind", without experimenter knowledge of the genotype. Note that in each experiment, three outcomes were possible: the reduced *Grm5* gene dosage could ameliorate, exacerbate, or have no effect on *Fmr1* mutant phenotypes.

Altered ocular dominance plasticity in *Fmr1* KO mice is rescued by reducing mGluR5 expression

Ocular dominance (OD) plasticity in visual cortex, elicited by temporary monocular deprivation (MD), is the classic example of how experience modifies the brain during critical periods of development. Here, we use this paradigm to study the interaction of genes and environment in a disease model.

Visually evoked potentials (VEPs) were recorded in visual cortex of awake mice (Figure 1A), as described previously (Frenkel and Bear, 2004). We initially assessed absolute levels of visual responsiveness across genotypes on postnatal day (P) 28 and found no difference (Figure 1B). Additional mice were studied before and after MD begun on P28. Previous studies using the chronic VEP method have shown how visual responses evolve during the course of MD (Figure S3). Closure of the contralateral eyelid initially causes depression of responses to the deprived-(contralateral-) eye (apparent at 3 d. MD), followed by potentiation of nondeprived-(ipsilateral-) eye responses (apparent by 7 d. MD) (Frenkel and Bear, 2004). Because they are recorded chronically, changes in VEPs for each animal can be conveniently described by two values: the fractional change from baseline in contralateral-eye response, and the fractional change from baseline in the ipsilateral-eye response. For reference, average effects (± SEM) of 3 and 7 days of MD in WT mice from a previous study (Frenkel and Bear, 2004) appear in Figure S3.

In the current study we also found that the response to 3 d MD in WT mice was dominated by deprived-eye depression, as expected. In KO littermates, however, the response to brief MD was characterized by substantial open-eye potentiation, reminiscent of what happens in WT mice after longer periods of MD. On the other hand, the HT mice showed a "hypoplastic" response to MD, as they lacked significant deprived-eye depression. However, crossing the two mutant mice resulted in a phenotype very similar to WT that was again dominated by deprived-eye depression (Figure 1C).

Plots of the average (± SEM) fractional changes after 3 d MD in the 4 genotypes are shown in Figure 1D. The KO mice displayed increased plasticity compared to the WT (MANOVA WT:KO, $P = 0.011$); HT mice displayed diminished plasticity compared to WT (MANOVA WT:HT, $P = 0.013$); CR mice showed a rescue of the KO phenotype and were not significantly different from WT (MANOVA WT:CR, $P = 0.8268$, KO:CR $P = 0.037$, HT:CRS $P = 0.161$).

Since the KO and HT mutations affected OD plasticity in opposite directions, one could question whether the CR phenotype reflects rescue or the simple addition of two independent effects. However, a compound phenotype would be the absence of deprived-eye depression (the effect of reducing mGluR5) and an exaggeration of open-eye potentiation (the effect of reducing FMRP). Instead, we observe a phenotype in the CR mice that is significantly different from KO mice, and not significantly different from WT. Thus, reducing mGluR5 by 50% corrects the defect in plasticity caused by the absence of FMRP.

Density of dendritic spines on cortical pyramidal neurons is increased in *Fmr1* KO and rescued by reducing mGluR5 expression

Abnormalities in dendritic spines, the major targets of excitatory synapses in the brain, have long been associated with various forms of human mental retardation, including FXS. The increased spine density phenotype observed in humans has been recapitulated in the *Fmr1* KO mouse (reviewed by (Grossman et al., 2006). Because one protein synthesis-dependent consequence of activating Gp1 mGluRs on cortical neurons *in vitro* is an increase in the density of long, thin spines (Vanderklish and Edelman, 2002), we hypothesized that FMRP and mGluR5 antagonistically regulate dendritic spine density *in vivo*.

We chose to examine this question in layer 3 pyramidal neurons of binocular visual cortex at P30, since we had established that OD plasticity at this age was altered in the *Fmr1* KO mice. Dendritic spine density was analyzed separately in apical and basal branches across the four genotypes, using the Golgi-Cox silver staining method (Figure 2A). We observed a highly significant increase in total dendritic spine density in the KO, readily apparent as a rightward shift in the cumulative probability histogram (Figure 2B). Reducing mGluR5 expression had no effect on spine density in the HT mice, but the fragile X phenotype was completely rescued in the CR mice (Apical, Kruskal-Wallis test $P < 0.0001$; Kolmogorov-Smirnov test WT:KO $P < 0.0001$; WT:HT $P = 0.3920$; CR:WT $P = 0.4407$; CR:KO $P < 0.0001$; Basal, Kruskal-Wallis test $P < 0.0001$; Kolmogorov-Smirnov test WT:KO $P < 0.0001$; WT:HT $P > 0.9999$; CR:WT $P > 0.9999$; CR:KO $P < 0.0001$).

We also performed a segmental analysis of spine density across the four genotypes. Consistent with previous observations, we observed an inverted U shaped distribution of synapses in both apical and basal branches across all genotypes. However, as shown in Figure 2C, the density of spines was uniformly increased in the *Fmr1* KO and rescued in the CR (Repeated measures ANOVA: apical distance $P < 0.0001$, apical distance* genotype $P < 0.0001$, apical genotype $P < 0.0001$, basal distance $P < 0.0001$, basal distance*genotype $P = 0.0181$, basal genotype $P < 0.0001$; ANOVA genotype: apical, basal, 10–100, in 10 μm segments $P < 0.0001$; unpaired t-tests apical, basal, 10–100, in 10 μm segments WT:KO $P < 0.05$, WT:HT $P > 0.05$, WT:CR $P > 0.05$, KO:CR $P < 0.05$). These results suggest that neither the *Fmr1* KO phenotype, nor the rescue by selective reduction in gene dosage in the CR, reflects a redistribution of synapses within the segment.

Increased basal protein synthesis in hippocampus of *Fmr1* KO mice is rescued by reducing mGluR5 expression

A previous study reported an elevated basal rate of *in vivo* protein synthesis in the hippocampus of *Fmr1* KO mice (Qin et al., 2005). We asked if this difference could also be observed in hippocampal slices *in vitro* by examining the incorporation of ^{35}S-methionine/cysteine into new protein. We observed a significant effect of genotype on protein synthesis (Figure 3A). The increased protein synthesis seen in KO hippocampus was prevented by selective reduction in mGluR5 gene dosage.

Electrophoretic separation of radiolabeled translation products (Figure 3B) suggests that increased protein synthesis in the KO is not limited to one or few predominant protein species, but rather extends across a broad range of resolved molecular weights. Because the rate of protein synthesis was unaffected in the HT mice relative to WT, the rescue in the CR mice is unambiguous, and does not simply reflect an offsetting decrease in synthesis of a separate pool of proteins.

Inhibitory avoidance extinction is exaggerated in *Fmr1* KO mice and rescued by reducing mGluR5 expression

Although humans with FXS show mental retardation in the moderate to severe range, prior studies of cognitive performance in *Fmr1* KO mice on the C57Bl/6J clonal background have revealed only subtle deficits (Bernardet and Crusio, 2006). Consistent with these observations, we found that acquisition of one-trial inhibitory avoidance (IA), a hippocampus-dependent memory, did not differ from normal in the *Fmr1* KO mice. However, we were inspired to additionally investigate IA *extinction* (IAE) by a recent report that this process requires protein synthesis in the hippocampus (Power et al., 2006). We discovered that IAE is exaggerated in the *Fmr1* KO mouse, and that this phenotype is corrected by reducing expression of mGluR5.

Adult mice of all four genotypes were given IA training, followed at 6 and 24 hours by IAE training (Figure 4A). For each animal, we measured the latency to enter the dark side of the box on the first trial (baseline), the latency 6 hours later (post-acquisition) to assess IA memory, and again at 24 and 48 hours (post-extinction 1 and 2, respectively) to assess IAE. As shown in Figure 4B, animals of all four genotypes showed both significant IA acquisition at 6 h and extinction by 48 h. A global statistical test suggested that the pattern of learning across time varied across genotypes (repeated measures ANOVA genotype *time $P = 0.0239$). As shown in Figure 4C-E, these differences are likely due to extinction rather than acquisition of inhibitory avoidance. At 6 h, there was no difference across genotypes in latency to enter (6 h ANOVA $P = 0.1525$); however, at 24 h KO mice showed significantly shorter latencies, suggesting exaggerated extinction in the absence of FMRP. This phenotype was rescued by selective reduction in mGluR5 gene dosage in the CR mice (24 h ANOVA $P = 0.0013$; t-tests: WT:KO $P < 0.0001$,WT:HT $P = 0.8251$, WT:CR $P = 0.1156$, KO:CR $P = 0.0132$).

Because the primary aim of this study is to examine genetic interaction between *Fmr1* and *Grm5*, we also performed a multivariate analysis which takes into consideration both acquisition and extinction as they vary across genotypes. As shown in Figure 4F, KO animals showed significant difference in 24 h latency (memory retention) as it varied with 6 h latency (memory acquisition), and this difference was rescued by the selective reduction in mGluR5 gene dosage in the CR mice (MANOVA for genotype 6:24 $P = 0.0054$, MANOVA WT:KO $P = 0.0005$, WT:HT 0.0785, KO:CR $P = 0.0490$, WT:CR $P = 0.1863$). The difference in retention across all genotypes was not significant by 48 h. Regardless of whether this KO phenotype reflects exaggerated extinction or diminished stability of the formed memory, there clearly is a significant genetic interaction between *Fmr1* and *Grm5*: The *Fmr1* KO phenotype is rescued by the selective reduction in mGluR5 expression.

Audiogenic seizures and accelerated body growth in *Fmr1* KO mice are rescued by reducing mGluR5 expression

Consistent with neurological findings in fragile X patients, previous studies have shown increased seizure susceptibility in the *Fmr1* KO mouse, using both *in vitro* and *in vivo* epilepsy models (Bernardet and Crusio, 2006). We employed the audiogenic seizure (AGS) paradigm, which shows a robust phenotype in *Fmr1* KO mice and exhibits developmental changes consistent with epilepsy in human FXS (Yan et al., 2005). Because C57Bl/6 WT mice are normally seizure resistant (Robertson, 1980), seizures in the KO mice are a specific consequence of the absence of FMRP. As shown in Table S1, significant differences in AGS susceptibility were observed across the four genotypes examined. WT and HT mice showed zero incidences of AGS, as expected, whereas 72% of the KO mice had a seizure in response to the tone (Mann-Whitney U test WT:KO $P < 0.0001$). This mutant phenotype was significantly attenuated in the CR mice (Mann-Whitney U test CR:KO $P = 0.028$). Thus chronic reduction of mGluR5 gene dosage in KO mice produced a substantial rescue of the seizure phenotype that is caused specifically by the lack of FMRP.

Children with FXS show accelerated prepubescent growth (Loesch et al., 1995). We discovered that this phenotype is recapitulated in the KO mouse, and is rescued by reducing mGluR5 gene dosage (Figure S4). At weaning (P20-21), animals from all four genotypes had similar body weights, but by P26 KO mice showed a slight (10%) but significant increase in body weight as compared to WT animals at the same age. This difference was not observed in either the HT or CR mice (ANOVA $P = 0.048$, post-hoc t-tests WT:KO $P = 0.017$, KO:CR $P = 0.004$, CR:WT $P = 0.818$). The WT:KO body weight difference was maximal at P30 (~15%) when it was again rescued by a reduction in mGluR5 gene dosage in the CR mice (ANOVA $P = 0.005$, post hoc t-tests WT:KO $P = 0.020$, KO:CR $P = 0.001$, CR:WT $P = 0.555$). As in humans, the KO growth increase in mice was no longer apparent after adolescence (P45).

Macroorchidism in *Fmr1* KO mice is not rescued by reducing mGluR5 expression

Children with FXS (and KO mice) have dysmorphic features, including postadolescent macroorchidism. Testes express Gp1 mGluRs (Storto et al., 2001), so we wondered if this phenotype might also be rescued in our CR mice. Postadolescent testicular weight was increased by 24% in KO mice compared to WT ($P < 0.0004$; t-test); however there was no rescue of this phenotype in the CR mice (Figure S5). To investigate if the absence of rescue was a matter of gene dosage, we generated KO mice that had a complete absence of mGluR5 (*Fmr1*KO/*Grm5*KO, dKO). Again, however, there was no rescue of the testicular phenotype.

Discussion

The goal of this study was to test a key prediction of the mGluR theory—that aspects of fragile X syndrome can be corrected by down-regulating signaling through group 1 mGluRs (Bear et al., 2004). Each analysis was designed to examine a different dimension of the disorder in mice with relevance to the human syndrome, ranging from the cognitive to the somatic. The experiments assayed dysfunction in very different neural circuits; and for each, three outcomes were possible: amelioration, exacerbation, or persistence of *Fmr1* mutant phenotypes in mice with reduced expression of mGluR5. Thus, it is remarkable that by reducing mGluR5 gene dosage by 50%, we were able to bring multiple, widely varied fragile X phenotypes significantly closer to normal.

A novel aspect of the current study was the use of OD plasticity as an *in vivo* assay of how experience-dependent synaptic modification is altered by the loss of FMRP. MD sets in motion a sequence of synaptic changes in visual cortex, characterized by a rapid and persistent loss of responsiveness to the deprived eye and a slower compensatory increase in responsiveness to the non-deprived eye (Frenkel and Bear, 2004). Because MD triggers mechanisms of synaptic depression and potentiation, as well as homeostatic adaptations to an altered environment, OD plasticity is a particularly rich paradigm for understanding the interactions of genes and experience. The intersecting trends of using mice to study OD plasticity mechanisms and to model human diseases provided the opportunity to use this paradigm to get a more precise understanding of how development goes awry in a genetic disorder.

Previous work suggested that Gp1 mGluR signaling is highest in visual cortex during the period of maximal synaptic plasticity (Dudek and Bear, 1989), and the current findings strongly suggest an important role for mGluR5 in OD plasticity. Although more experiments will be required to pinpoint this role, an obvious clue comes from the genetic interaction with *Fmr1*. FMRP can act as a translational suppressor (Brown et al., 2001; Qin et al., 2005), and OD plasticity, like many forms of persistent synaptic modification, requires protein synthesis (Taha and Stryker, 2002). Thus, our findings suggest the intriguing hypothesis that the rate of plasticity in visual cortex is determined by the level of activity-dependent protein synthesis, which is stimulated by mGluR5 and inhibited by FMRP. Consistent with this model, the phenotype in *Fmr1* KO mice appears to reflect “hyperplasticity”, since 3 days of MD yielded

effects on VEPs that normally require 7 days. This exaggerated plasticity was corrected by reducing mGluR5 expression by 50%.

Although we observed an increased spine density in visual cortex of KO mice, there was no apparent difference in the amplitude of VEPs at P30. This discrepancy may be becasue VEPs were recorded in layer 4, whereas the spine measurements were made on layer 3 neurons. In any case, the clear mutant spine phenotype in layer 3 gave us the opportunity to examine if this structural defect could also be corrected by decreasing mGluR5. We observed a remarkable rescue of the fragile X spine phenotype in the CR mice, despite the fact that reducing mGluR5 expression in the HT mice had no effect on spine density. Thus, although the reduction in *Grm5* gene dosage is not sufficient to alter spine density by itself, it completely corrects the defect in fragile X mice.

Strain specific variation has confounded previous attempts to identify a behavioral learning and memory phenotype in the *Fmr1* KO (Bernardet and Crusio, 2006). Consistent with earlier findings, we were unable to detect a significant IA deficit in the *Fmr1* KO mice on the C57BL/6 background. On the other hand, we were able to detect a difference in the rate of IA extinction that could be corrected by reducing mGluR5 expression. Because IA induces LTP of Schaffer collateral synapses in area CA1 of the hippocampus (Whitlock et al., 2006), it is tempting to speculate that IA extinction is exaggerated in Fmr1 KO mice due, at least in part, to excessive mGluR-dependent synaptic weakening (Huber et al., 2002; Zho et al., 2002). Unbalanced LTD could account for the cognitive impairment that is the hallmark of fragile X.

Fragile X is a syndromic disorder. In addition to mental retardation, associated features of the disease in humans include childhood epilepsy, altered body growth, and postpubertal macroorchidism . In the case of epilepsy and macroorchidism, these phenotypes have been recapitulated in the mouse model (Bernardet and Crusio, 2006); however it was previously unknown that *Fmr1* KO mice show a similar disruption in body growth. Both the body growth and AGS phenotypes were ameliorated in the CR mice; however, there was no evidence of an interaction between FMRP and mGluR5 in the control of testicle size. These results argue against a role for mGluR5 in the pathogenesis of the macroorchidism phenotype in FXS, but we cannot rule out a contribution of the other Gp1 mGluR (mGluR1).

Conclusion

Although we studied a range of phenotypes, a simple way to conceptualize the constellation of findings is that fragile X is a disorder of excess—excessive sensitivity to environmental change, synaptic connectivity, protein synthesis, memory extinction, body growth, and excitability—and these excesses can be corrected by reducing mGluR5. Although the precise molecular basis of the interaction remains to be determined, the data show unambiguously that mGluR5 and FMRP act as an opponent pair in several functional contexts, and support the theory that many CNS symptoms in fragile X are accounted for by unbalanced activation of Gp1 mGluRs. These findings have major therapeutic implications for fragile X syndrome and autism (see Bear et al., 2008).

Experimental Procedures

Animals

Fmr1 mutant mice (Jackson Labs) were crossed with *Grm5* mutants (Jackson Labs) to produce mice of four genotypes. In all crossings, animals were on the C57Bl/6J clonal background. In an effort to reduce variability due to rearing conditions, all experimental animals were bred from *Fmr1* heterozygote mothers, group housed (animals weaned to solitary housing were

excluded), and maintained in a 12:12 h light:dark cycle. Paternal genotype varied between crossings and included WT, *Grm5* HT or *Grm5* KO.

Genotyping

See Figure S2 and supplemental online material.

Electrophysiology and spine measurements

Transverse hippocampal slices were prepared from P25-30 mice and mGluR-LTD was studied as described by Huber *et al.* 2001. VEP recordings and monocular deprivation were performed as previously described (Frenkel and Bear, 2004). Spines were analyzed using the Golgi-Cox method as described by (Hayashi et al., 2004). Animal n = 8 WT, 8 KO, 6 HT, 8 CR; dendritic segment n = 80 WT, 80 KO, 60 HT, 80 CR apical and basal branches, respectively. All protrusions, irrespective of their morphological characteristics, were counted as spines if they were in direct continuity with the dendritic shaft. In total, 68,032 spines were counted across all four genotypes.

Inhibitory avoidance extinction, metabolic labeling, and audiogenic seizure

IAE experiments were performed as previously described (Power et al., 2006). Metabolic labeling experiments were similar to those described in (Raymond et al., 2000). AGS experiments were performed as described by (Yan et al., 2005). See supplemental online material.

Statistical analysis

In all cases, post-hoc comparisons between genotypes were made only if global analysis indicated a statistically significant ($P < 0.05$) effect of genotype. Outliers (= 2 s.d. from the mean) were excluded. For AGS experiments, non-parametric statistics (Kruskall-Wallis, Mann-Whitney U) were used since incidence scores were bimodal (yes/no). For all other analysis, parametric tests (ANOVA, MANOVA, two-tailed paired and unpaired t-tests, assuming equal variance) were used. For the metabolic labeling experiments, the post-hoc paired t-test was used to eliminate the variability due to strength of radioactive label on different experimental days, and was justified by the experimental design (samples were collected with yoked, rather than randomized, controls).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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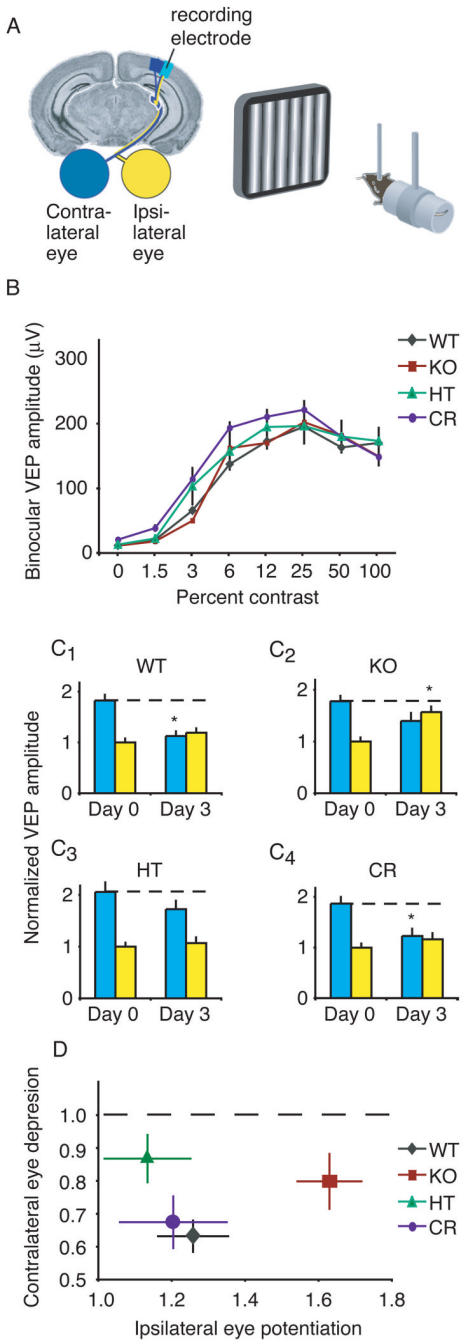

Figure 1. Genetic rescue of OD plasticity phenotype in FXS

(**A**) Schematic of the mouse visual pathway and position of the recording electrode in primary visual cortex. (**B**) Absolute VEP amplitudes recorded during binocular viewing across contrasts (0 –100%, square reversing at 1 Hz, 0.05 cycles/degree). No significant differences across genotypes. (n = 46 WT, n = 33 KO, n = 8 HT, n = 20 CR hemispheres, MANOVA P = 0.0868). (**C**) Effect of 3 day MD on VEP amplitude (data expressed as mean ± SEM, normalized to day 0 ipsilateral eye value. (**C_1**) WT mice (n = 19). Note significant deprived eye depression. (**C_2**) KO mice (n = 18). Note significant open eye potentiation. (**C_3**) HT mice (n = 16). Note absence of deprived eye depression. (**E_4**) CR mice (n = 13). Note rescue of KO phenotype. Post-hoc t-tests: * indicates significantly different from baseline (day 0). (**D**) Plots (mean ±

SEM) of the fractional change in open and deprived eye responses after 3 day MD show rescue of the KO phenotype in CR mice.

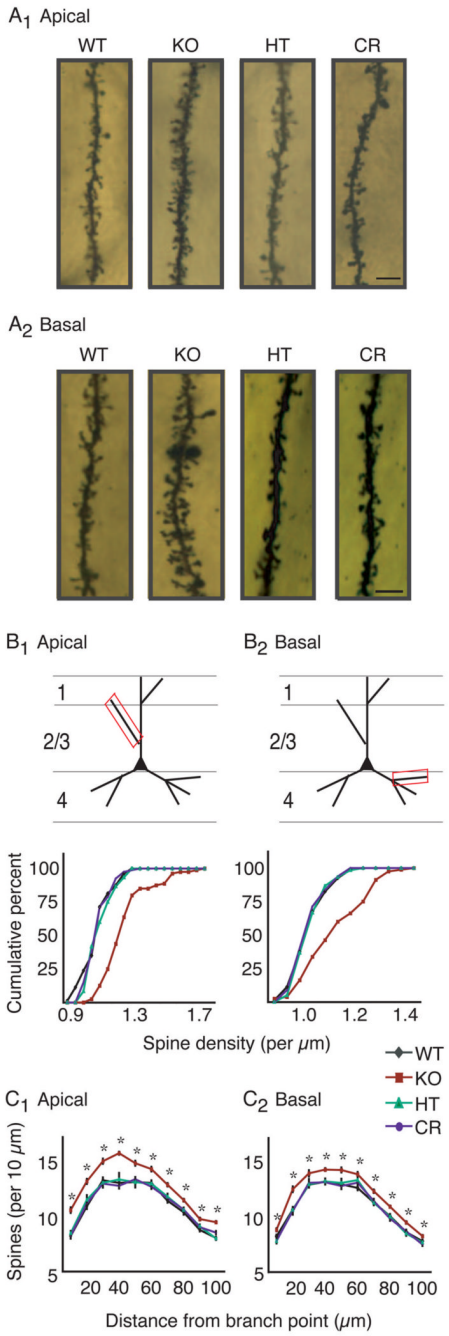

Figure 2. Genetic rescue of dendritic spine phenotype in FXS

(**A**) Representative images from apical (A_1) and basal (A_2) dendritic segments of layer 3 pyramidal neurons in the binocular region of primary visual cortex of all four genotypes collected at P30. (**B**) Cumulative percent spines per μm in each dendritic segment; apical branches, $\mathbf{B_1}$; basal branches, $\mathbf{B_2}$ (n = 80 WT, 80 KO, 60 HT, 80 CR apical and basal branches, respectively). (**C**) Segmental analysis of spine density; number of spines per 10 μm bin, given as distance from the origin of the branch, for apical ($\mathbf{C_1}$) and basal ($\mathbf{C_2}$) segments across four genotypes.

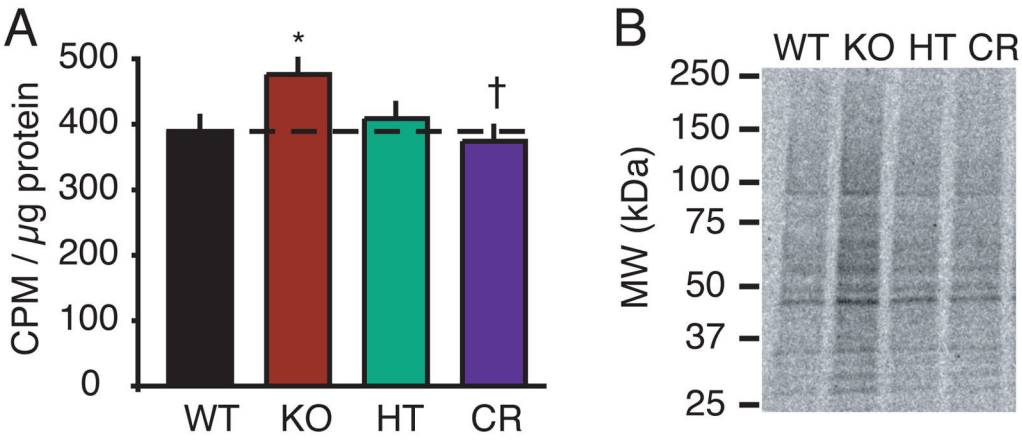

Figure 3. Genetic rescue of protein synthesis phenotype in FXS

(**A**) Significant differences in the levels of protein synthesis exist across genotypes in the ventral hippocampus (n = 10 samples, 5 animals per genotype). KO mice showed increased protein synthesis (mean ± SEM: WT 389 ± 33.77 cpm/μg; KO 476 ± 29.98 cpm/μg; post-hoc paired t-test WT:KO P = 0.004). Protein synthesis levels in the HT mice were no different than WT (HT 409 ± 42.99 cpm/μg). Increased protein synthesis seen in the KO were rescued in the CR mice (CR 374 ± 50.81 cpm/μg). Post-hoc paired t-tests: * indicates significantly different from WT, † indicates significantly different from KO. (**B**) Representative autoradiogram shows that synthesis of many protein species is elevated in the KO compared to all other genotypes.

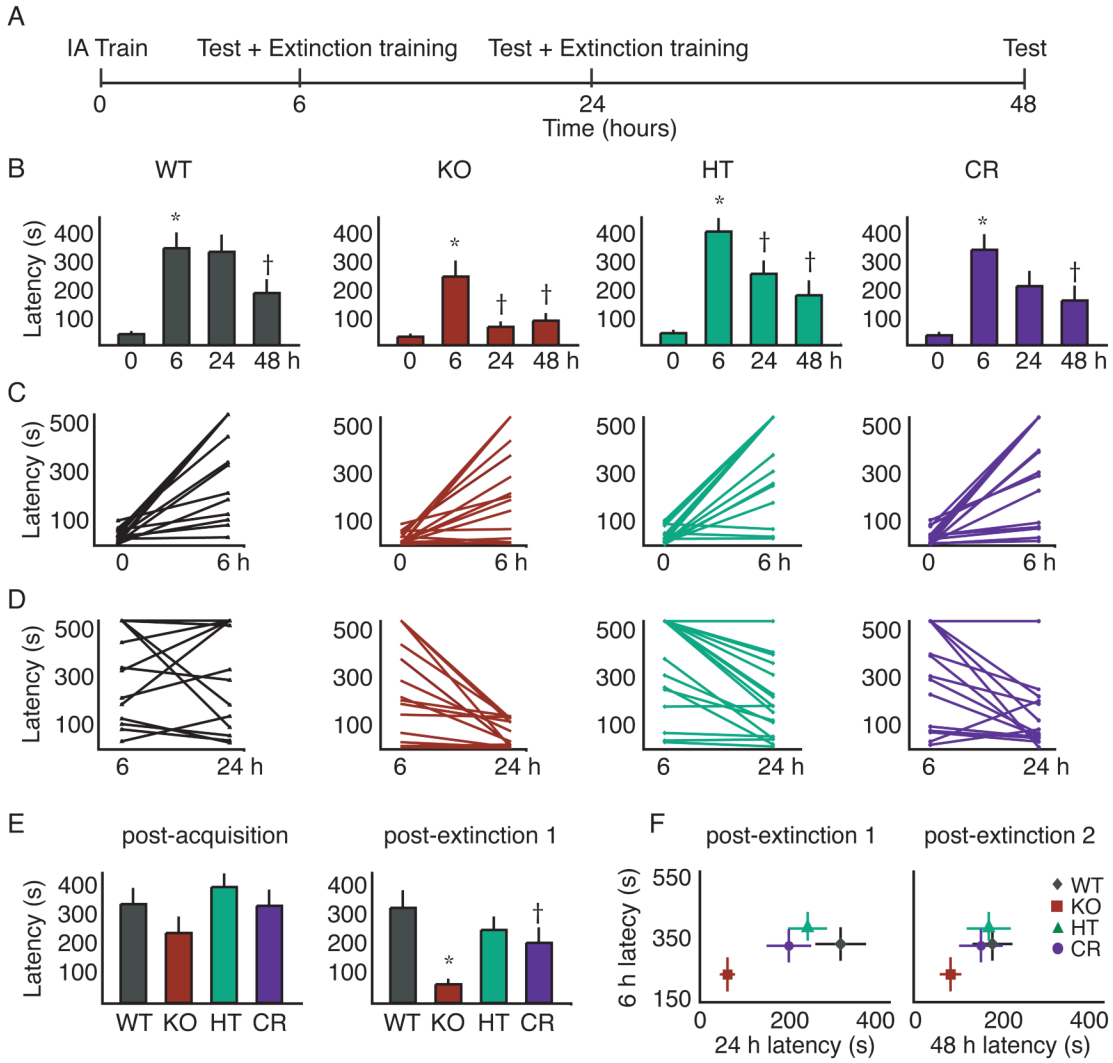

Figure 4. Genetic rescue of behavioral learning and memory phenotype in FXS

(**A**) Experimental design. Animals of all 4 genotypes (n = 15 WT, n = 15 KO, n = 20 HT, n = 17 CR) were given IA training at time 0 and the latency to enter the dark side was measured at 6 h. They were then given IAE training, and latency was again measured at 24 h. Testing was followed by another round of IAE training, and latency was measured again at 48 h. (**B**) Animals in all four genotypes showed significant acquisition and extinction. Post-hoc t-tests: * indicates significantly different from time 0h, † indicates significantly different from time 6h. (**C**) Raw data for acquisition of IA in the four genotypes. Each line represents the change in latency to enter the dark side for one mouse (data from some mice superimpose). (**D**) Raw data for extinction-1 in the four genotypes. (**E**) Comparison of latency (mean ± SEM) across genotypes for 6 hour time point (post acquisition) and 24 hour time point (post extinction 1). Post-hoc t-tests: * indicates significantly different from WT, † indicates significantly different from KO. (**F**) Multivariate analysis of extinction as a function of acquisition at 24 h time point (post extinction-1) and 48 h time point. Plotted are mean latencies ± SEM.