



Published in final edited form as:

Nat Med. 2013 November ; 19(11): 1473–1477. doi:10.1038/nm.3353.

## Genetic and Acute CPEB Depletion Ameliorate Fragile X Pathophysiology

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### Abstract

Fragile X Syndrome (FXS), the most common cause of inherited mental retardation and autism, is caused by transcriptional silencing of *Fmr1*, which encodes the translational repressor protein FMRP. FMRP and CPEB, an activator of translation, are present in neuronal dendrites, are

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### Author Contributions:

T.U. and J.D.R. conceived of the initial project with much input from L.J.L. T.U., N.G.F., and M.J. designed and performed the majority of the experiments. H.K. and E.K. performed the electrophysiology experiments in Figure 1c,d and Supplementary Fig. 4a–b. J.M.A. performed the electrophysiology experiments in Supplementary Fig. 4c–e. S. Anilkumar and S.C. performed spine density analysis in Fig. 1e,f and Supplementary Fig. 5a–b. M.I. created and tested the CPEB antibody used in Supplementary Fig 2f. J.A.H. performed the bioinformatics analysis in Supplementary Table 1. V.C.N. and G.J.B. performed the immunocytochemistry analysis in Supplementary Fig. 2a–b. N.G.F. and J.D.R. wrote the manuscript. All authors contributed to interpretation and discussion of results and to editing of the manuscript.

The authors declare no competing financial interests.

predicted to bind many of the same mRNAs, and may mediate a translational homeostasis that, when imbalanced, results in FXS. Consistent with this possibility, *Fmr1*<sup>-/-</sup> *Cpeb*<sup>-/-</sup> double knockout mice displayed significant amelioration of biochemical, morphological, electrophysiological, and behavioral phenotypes associated with FXS. Acute depletion of CPEB in the hippocampus of *Fmr1*<sup>-/-</sup> mice rescued working memory deficits, demonstrating reversal of this FXS phenotype in adults. Finally, we find that FMRP and CPEB balance translation at the level of polypeptide elongation. Our results suggest that disruption of translational homeostasis is causal for FXS, and that the maintenance of this homeostasis by FMRP and CPEB is necessary for normal neurologic function.

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Fragile X Syndrome (FXS), the most common inherited form of mental retardation and syndromic autism<sup>1,2</sup>, is caused by inactivation of the *Fmr1* gene<sup>3</sup>. FXS phenotypes include cognitive dysfunction, repetitive behaviors, anxiety, seizures and morphological abnormalities<sup>1</sup>. *Fmr1* encodes the Fragile X Mental Retardation Protein (FMRP), a widely expressed RNA binding protein (RBP) that is found in neuronal soma and synaptodendrites<sup>4</sup>. FMRP binds target mRNAs predominantly in coding regions and likely represses translation by tempering transit of polypeptide-elongating ribosomes<sup>5</sup>. FMRP responds to signaling from group I metabotropic glutamate receptors (mGluRs)<sup>6,7</sup>, which mediate its translation-repressing activity<sup>8</sup>. In *Fmr1*<sup>-/-</sup> (*Fmr1* KO) mice, protein synthesis is elevated by ~20%<sup>7,9-11</sup>, which most probably is responsible for the synapse dysmorphogenesis, aberrant synaptic plasticity<sup>7,12</sup>, and behavioral and cognitive dysfunctions displayed by FXS children and animal models. FMRP binds >1000 mRNAs in the brain<sup>5</sup> and determining which contribute to FXS pathophysiology is a daunting task.

The cytoplasmic polyadenylation element binding protein (CPEB) also affects synapse function<sup>13-16</sup>; it binds the 3' UTR cytoplasmic polyadenylation element (CPE) of target mRNAs and stimulates polyadenylation-induced translation<sup>13,17</sup>. In neurons, CPEB is localized to synaptodendrites<sup>13,18</sup> and activates translation in response to synaptic stimulation<sup>17,18</sup>. *Cpeb*<sup>-/-</sup> knockout (KO) mice display neurological phenotypes including perseverative hippocampal-dependent memory<sup>19</sup> and defects in long-term potentiation<sup>15</sup>, underscoring the importance of CPEB in regulating translation and neural function.

We hypothesized that the translational imbalance and associated pathophysiologies in FXS animals might be rescued if the loss of FMRP was accompanied by ablation of a factor that stimulates rather than represses translation. In the normal condition, translation could be balanced by FMRP and factor(s) X; unbalanced translation due to the loss of FMRP yields FXS but perhaps ablation of the counter-balancing factor(s) could rebalance translation and restore normal neurologic function (Supplementary Fig. 1a). We surmised that factor X could be CPEB because about one-third of FMRP-bound mRNAs<sup>5</sup> contain 3'UTR CPEs and thus are potential targets for CPEB (Supplementary Fig. 2b, Supplementary Table 1); indeed several mRNAs co-immunoprecipitate with both FMRP and CPEB (Fig. 1a). Moreover, CPEB and FMRP co-localize in dendrites of cultured hippocampal neurons (Supplementary Fig. 2a,b) and co-purify in neuroblastoma cells and *in vitro* (Supplementary Fig. 2c-f). Consequently, we generated *Fmr1*<sup>-/-</sup> *Cpeb*<sup>-/-</sup> double KO (DKO) mice (Supplementary

Fig. 3) and examined protein synthesis in them. Although *Fmr1* KO animals displayed a ~15% increase in protein synthesis in acute hippocampal slices, the DKO mice were similar to WT (Fig. 1b), suggesting that translational homeostasis was restored in these animals. There was no significant decrease in protein synthesis in the *Cpeb* KO animals.

We examined the levels of specific proteins in hippocampal lysates from mice of all four genotypes (Supplementary Table 2). The amounts of several proteins encoded by FMRP target mRNAs was increased in *Fmr1* KO animals. Consistent with previous reports of CPEB function, translation of many mRNAs was decreased in *Cpeb* KO animals. In DKO animals, translation of FMRP targets were either similar to WT or decreased as in the *Cpeb* KO hippocampus. These results suggest that a “brake” is placed on the runaway translation of the *Fmr1* KO brain in DKO animals. We also examined specific protein levels by Western blot in cortical samples, but found few significant differences between WT and *Fmr1* KO animals (Supplementary Table 3).

Because several studies noted an increase in mTOR and ERK1/2 signaling in *Fmr1* KO animals<sup>9,11</sup>, we examined the phosphorylation states and relative levels of several components of these signaling pathways. We did not observe a consistent pattern of enhanced signaling through either pathway. P70 ribosomal S6 kinase 1 (S6K1) lies downstream of both the ERK and mTOR pathways and while its phosphorylation was not significantly altered, phosphorylation of its immediate downstream target, ribosomal protein S6, was significantly increased in the *Fmr1* KO hippocampus<sup>9</sup>. The levels of phospho-S6 were similar in *Cpeb* KO and DKO animals, indicating that signaling through S6K1 is restored in DKO animals. By analyzing the levels of specific proteins (Supplementary Table 2) as well as general protein synthesis (Fig. 1b), we demonstrate that CPEB and FMRP do indeed balance translation in the brain.

Metabotropic glutamate receptors (mGluRs) are hyper-activated in *Fmr1* KO mice as evidenced by enhanced mGluR-dependent LTD at Schaffer collateral-CA1 synapses in the hippocampus<sup>7,12,20</sup>. *Fmr1* KO and *Cpeb* KO mice both displayed exaggerated LTD in hippocampal slices treated with DHPG, a group I mGluR agonist<sup>12</sup>. LTD was restored to WT levels in slices derived from DKO animals (Fig. 1c). Basal synaptic transmission and presynaptic function were not affected in the DKO animals (Supplementary Fig. 4a, b). Because DHPG-induced LTD is a translation-dependent process<sup>12</sup>, we determined whether application of the protein synthesis inhibitor anisomycin attenuated LTD in hippocampal slices. Anisomycin inhibited LTD in WT slices but had no effect on *Fmr1* KO, *Cpeb* KO, or DKO slices (Fig. 1d). These results suggest that the protein synthesis-dependency in the DKO slices occurs before mGluR stimulation, perhaps during synapse formation.

Long-term potentiation (LTP) is also affected in the *Fmr1* KO hippocampus under certain stimulation protocols<sup>21</sup>. We reported a deficit in LTP induced by theta burst stimulation (TBS-LTP) in *Cpeb* KO mice<sup>15,16</sup>. We therefore examined TBS-LTP in *Fmr1* KO and DKO animals (Supplementary Fig. 4c–e). Although *Fmr1* KO animals displayed normal TBS-LTP, DKO animals also displayed normal TBS-LTP, suggesting the deficit in TBS-LTP previously seen in *Cpeb* KO animals is rescued by the absence of FMRP. However, when LTP is induced by 1mM glycine (Gly-LTP) which requires mGluR receptor function<sup>21</sup>,

*Fmr1* KO animals showed a significant deficit compared to WT animals, which was again rescued in the DKO. It therefore seems that both LTP and LTD defects in synaptic plasticity in the *Fmr1* KO hippocampus are largely dependent upon mGluR signaling and are both rescued in DKO animals.

Dendritic spine density, which is commonly increased in FXS<sup>7,9</sup> was enhanced *in vivo* in both *Fmr1* KO and *Cpeb* KO mice on the primary apical dendritic branches of hippocampal CA1 pyramidal neurons (Fig. 1e,f; Supplementary Fig. 5a,b). Spine density was significantly reduced in the DKO neurons, demonstrating that FMRP and CPEB balance one another to control synapse number. We further compared synapse formation in hippocampal and cortical neuron cultures<sup>22</sup>, and found in both cases that a significant increase in synaptic puncta in *Fmr1* KO neurons was partially restored in DKO neurons (Supplementary Fig. 6a–d). Total body weight, which is increased in adolescent *Fmr1* KO animals, was reversed to WT level in DKO animals (Supplementary Fig. 5c), suggesting that the interplay between FMRP and CPEB that leads to phenotypic rescue is not limited to the brain.

To examine whether higher neural function is rescued in the DKO mice, we tested animals for their sensitivity to acoustic stimulation, which in *Fmr1* KO mice results in an initial period of wild running followed by clonic/tonic seizures, status epilepticus, and in some cases, death<sup>7,11</sup>. WT and *Cpeb* KO animals were little affected by the acoustic stimulus, yet the *Fmr1* KO mice displayed the expected running and seizure responses (Fig. 2a). The seizure phenotype was reduced by ~50% in the DKO animals as well as in *Fmr1*  $-/-$ , *Cpeb*  $+/-$  heterozygous (HKO) animals.

*Fmr1* KO mice displayed decreased anxiety as suggested by their tendency to remain in the center of an open experimental field 45% longer than WT animals, which preferentially moved about the periphery<sup>23</sup> (Fig. 2b). *Cpeb* KO animals were comparable to WT with respect to time in the center field, although they moved slightly less than WT (Supplementary Fig. 7a). The DKO animals were indistinguishable from WT for time in the center and distance moved. Reducing the CPEB dose by one-half (HKO) did not ameliorate the anxiety phenotype.

We examined learning and memory by assessing passive avoidance. Mice placed into a light chamber of a two chamber test apparatus readily moved to the dark chamber where a foot shock was administered. After 24 hours, the test was repeated and in all genotypes there was increased latency in entering the dark chamber, indicating memory of the foot shock. Although we did not observe a statistical difference in the learning ability between WT and *Fmr1* KO animals, the *Cpeb* KO animals displayed increased latency to move to the dark field, indicative of enhanced learning (Fig. 2c). This exaggerated learning was restored to WT levels in the DKO mice, demonstrating that the loss of FMRP corrected for the loss of CPEB. These results support the hypothesis that FMRP and CPEB maintain a functional homeostasis in the brain that is critical for normal cognition. Normal circadian activity was observed for all genotypes (Supplementary Fig. 7b).

Nest building is a social behavior in mice that is affected in another model of syndromic autism<sup>24</sup>. Squares of pressed cotton nesting material (Nestlet) presented to the animals were

scored 24 and 72 hours later for shape; the amount of unused Nestlet was also measured. At 24 hours, nest quality of *Fmr1* KO animals was poorer than those of WT (Fig. 2d). The *Fmr1* KO mice also used less material in nest construction (Fig. 2d, e). The nest quality and Nestlet usage of both *Cpeb* KO and DKO animals were not distinguishable from WT. Nest quality of the four genotypes was not due to time required for construction as similar results were obtained after 72 hours (Supplementary Fig. 7c,d). Therefore, this autism-related paradigm is rescued by simultaneous depletion of FMRP and CPEB.

We examined spatial working memory using a T-maze assay. Animals were placed in the start arm of a T-shaped maze and allowed to explore. Upon reaching the junction of the T arms, the animal must move right or left. Once the left/right choice was made, access to the non-chosen arm was denied by a door until the animal returned on its own to the start arm. The door was then re-opened and the paradigm repeated until the animal made 15 choices. WT mice chose right or left arms randomly on day 1 but chose alternating arms on day 2 suggesting they were aware of which arm they had most recently visited, a characteristic of working memory known as spontaneous alternation (SA) (Fig. 3f). Both *Fmr1* KO and *Cpeb* KO animals had deficient SA on day 2; the DKO and HKO animals, however, displayed SA indistinguishable from WT, indicating a rescue of working memory. Working memory is thought to involve the hippocampus, particularly in adolescent mice<sup>25,26</sup>. To determine whether acute depletion of CPEB could rescue working memory, we injected lentivirus encoding GFP and shRNA targeting *Cpeb* (sh-*Cpeb*), or a non-targeting control (sh-*Ctrl*), bilaterally into the hippocampi of 30 day old *Fmr1* KO animals (Fig. 3g,h). After allowing for efficient depletion (Supplementary Fig. 7e,f), injected animals were tested in the T-maze. Sh-*Ctrl* injected *Fmr1* KO animals displayed SA on day 2 that was not statistically distinct from random chance (50%) (Fig. 3i). Amazingly, sh-*Cpeb* injected *Fmr1* KO animals demonstrated significant improvement in SA on day 2 compared to the random chance level, demonstrating improvement in working memory upon acute depletion of CPEB.

FMRP predominantly binds the coding regions of mRNAs and may slow the translocation of ribosomes during polypeptide elongation<sup>5,27</sup>. In the absence of FMRP, ribosomes can translocate unimpeded and will produce more polypeptide from target mRNAs (Supplementary Fig 8a). Because general and specific protein synthesis is restored in DKO animals (Fig. 1b, Supplementary Table 2), we determined whether this rescue could be attributed to a restoration of ribosome translocation rate. We employed an assay to monitor the ribosome transit rate (RTR) in brain (less cerebellum and midbrain) lysates that is based on the run-off of ribosomes that are actively engaged in translation<sup>5</sup>. <sup>35</sup>S-methionine/cysteine was added to lysates from the four genotypes and the ribosomes allowed to elongate polypeptides without new initiation (Supplementary Fig. 8b, ref 7). When all active ribosomes have completed their elongation phase, the radioactive signal reaches saturation, which corresponds to the RTR<sup>28</sup>. Our results show that brain extracts derived from *Fmr1* KO mice have an enhanced RTR compared to WT (Fig. 2j, Supplementary Table 4). The RTRs in *Cpeb* KO and DKO samples were indistinguishable from WT, demonstrating complete restoration of polypeptide elongation rate. These results identify the polypeptide elongation machinery as being essential for the translational homeostasis maintained by FMRP and CPEB in the brain.

Brain-associated FXS pathophysiologies are likely caused by excessive translation in neurons<sup>2,7,9,11</sup>, which may also occur in syndromic and idiopathic forms of autism<sup>29,30</sup>. We have restored the translational landscape that is altered in FXS by genetically ablating CPEB, which we surmised acts as an mRNA-specific translational counterweight to FMRP in the brain. When coupled to the loss of FMRP, the additional loss of CPEB restores several biochemical, morphological, electrophysiological, and behavioral phenotypes associated with FXS (Supplementary Table 5). Furthermore, our data, which confirm and extend the observations of several laboratories<sup>5,27</sup> indicating that FMRP inhibits polypeptide elongation, demonstrate that ribosome transit times are excessive in FMRP KO brains but are mitigated with the concomitant loss of CPEB. This targeting of the ribosome transit machinery by FMRP and CPEB suggests that our original hypothesis, the balance of mRNA-specific translation to maintain normal neural function requires modification. Although these two proteins could act on common mRNAs that mediate polypeptide elongation such as eEF2 (Supplementary Table 2), they might also regulate distinct components to maintain elongation equilibrium. Indeed, one issue arising from our study is how the loss of CPEB, which alone has no detectable effect on ribosome transit, palliates this process upon loss of FMRP. Cytoplasmic polyadenylation-induced translation is a main CPEB activity that could regulate an elongation “sensor” that is involved in restoring protein synthesis (Supplementary Fig. 8c).

Because the *Fmr1* KO and *Cpeb* KO mice display enhanced DHPG-induced LTD, it is not intuitively obvious how this measure of synaptic efficacy is rescued in the DKO. FMRP and CPEB are both downstream of Group I mGluRs because the KO of each has a deficient response to DHPG treatment. These proteins are unlikely to be completely epistatic to one another and could reside at least partially in different regulatory pathways such that biologic responses to null mutations in each gene could be cancelled by null mutations in both genes. If such pathways include positive and negative influences on LTD, then one could envision how two wrongs (e.g., immature dendritic spines) make a right (i.e., near WT spines in the DKO).

We rescued working memory, an FXS-associated pathology in adult *Fmr1* KO mice by injecting lentivirus expressing shRNA for CPEB bilaterally into the hippocampus. Working memory requires the hippocampus as well as the prefrontal cortex but is generally thought to be independent of protein synthesis<sup>26</sup>. Although protein synthesis is not required for the short working memory time (several minutes), the CPEB shRNA was injected 10 days prior to the memory test, sufficient time for it to alter translation homeostasis. These experiments demonstrate that at least this one characteristic of FXS, which is generally thought to be a developmental disease, can be reversed in the adult. Therefore, therapies that target CPEB and/or the translational apparatus, particularly the polypeptide elongation machinery, might be efficacious in ameliorating other features of FXS.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

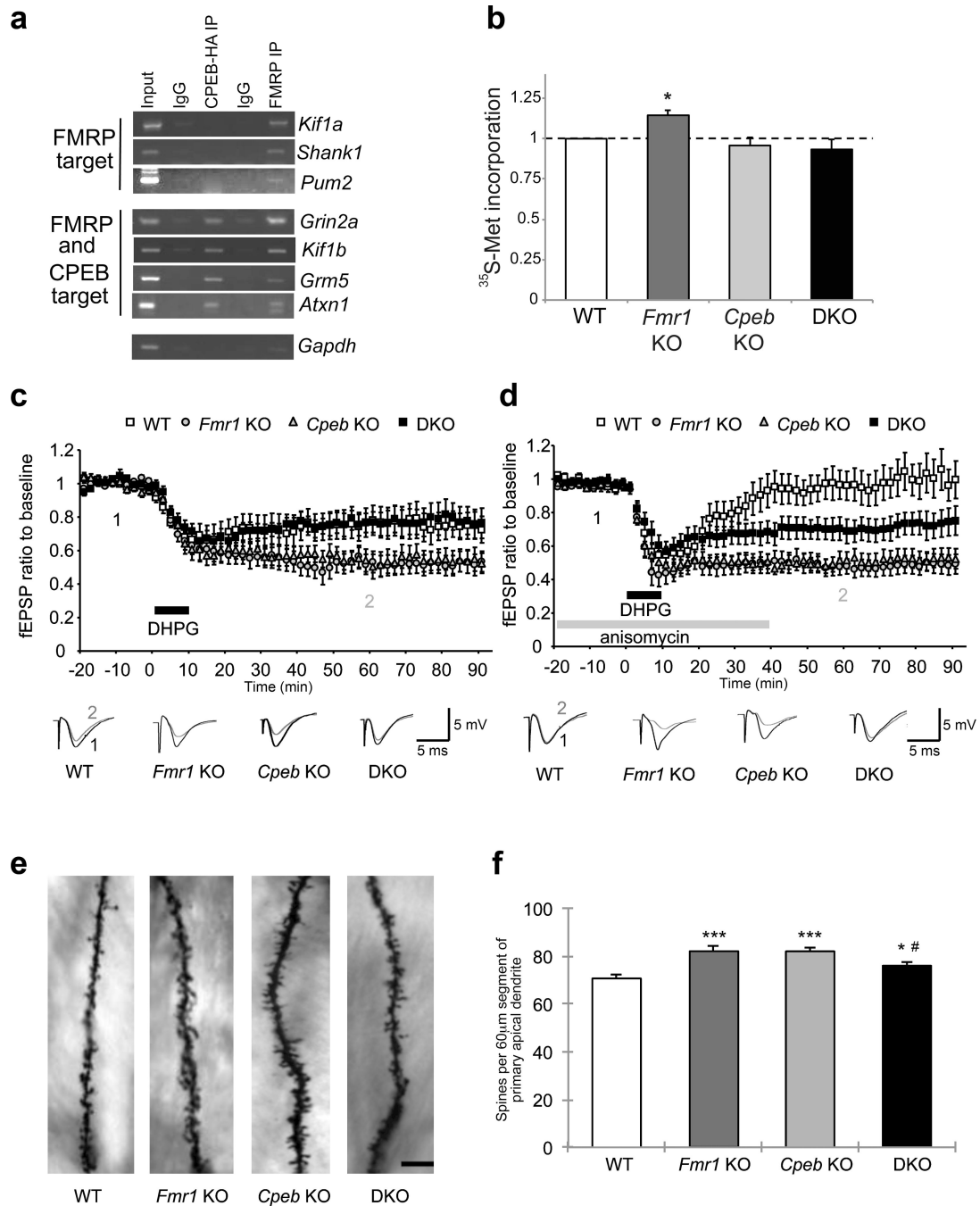
We thank N. Dawra for technical assistance, P. Lombroso and C. Proud for kind gifts of antibodies, J. Pelletier for the kind gift of hippuristanol, and members of the Richter lab for helpful discussions. T.U. and N.G.F. gratefully acknowledge fellowships from the FRAXA Research Foundation. J.A.H. was supported by NIH-NRSA Postdoctoral Fellowship F32GM095060. This work was supported by NIH grants GM46779 and NS079415 to J.D.R. and MH086509 to S. Akbarian.

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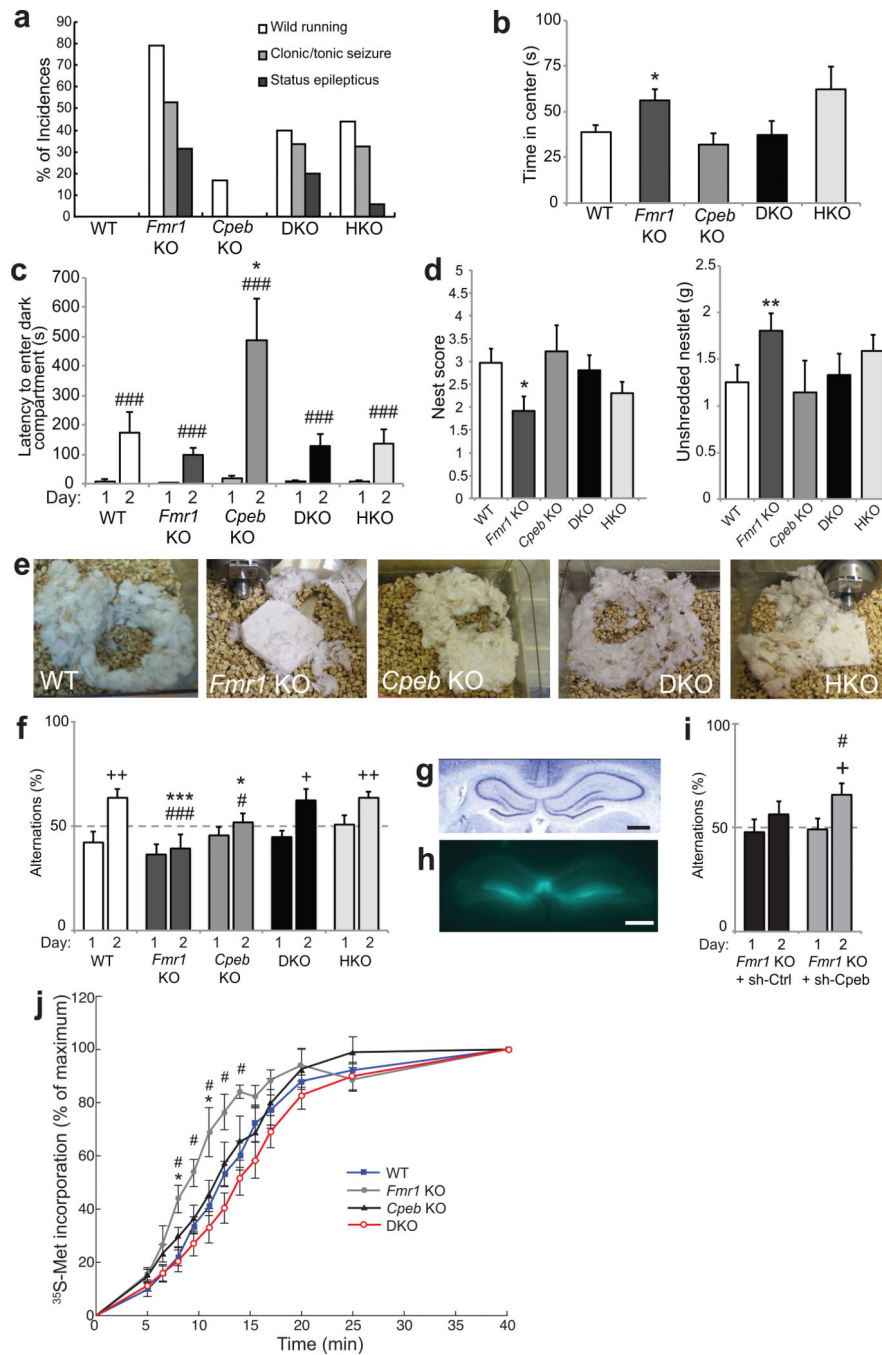




**Figure 1. Interplay between FMRP and CPEB in the brain**

**a**, Co-immunoprecipitation of RNAs with FMRP or epitope-tagged CPEB from neurons. IgG served as a negative control. **b**, Acute hippocampal slices were labeled with <sup>35</sup>S-methionine and incorporation into protein was determined by scintillation counting (n=4; ANOVA,  $F_{(3,12)}=5.458$ ,  $p<0.05$ ). \*  $p<0.05$  as compared to wild-type (student's t-test). Error bars are S.E.M. **c**, DHPG-induced LTD at Schaffer collateral-CA1 synapses was enhanced in the *Fmr1* KO (n=15, ANOVA,  $p<0.001$ ) and *Cpeb* KO (n=19, ANOVA,  $p<0.001$ ) relative to WT (n=12), however, it was nearly identical to WT in the DKO (n=14, ANOVA,

p > 0.05). **b**, Anisomycin inhibited the DHPG-induced LTD in WT (n=13), but not in the *Fmr1* KO (n=8), *Cpeb* KO (n=13) (ANOVA, p < 0.001), or DKO (n=16) (ANOVA, p < 0.001). DHPG-induced LTD with anisomycin in the DKO slices was significantly different from the LTD observed in *Fmr1*, *Cpeb* and WT groups. (ANOVA, p < 0.001). **c**, Representative images of Golgi-Cox stained dendritic segments of CA1 pyramidal neurons of the hippocampus from WT, *Fmr1* KO, *Cpeb* KO, and DKO mice. Scale bar, 20  $\mu$ m. **d**, Total number of spines were quantified along a 60- $\mu$ m segment from the origin of primary apical dendritic branches of Golgi-Cox stained CA1 pyramidal neurons. n=26 dendritic segments, \*\*\*p < 0.001, \*p < 0.05, as compared to WT (ANOVA). #p < 0.05 by two-way ANOVA (*Fmr1* KO vs. DKO). Error bars are S.E.M.



**Figure 2. *Cpeb* deletion ameliorates FXS-related behavioral abnormalities**

**a**, Audiogenic seizure in WT (n=6), *Fmr1* KO (n=19), *Cpeb* KO (n=6), DKO (n=15), and *Fmr1* KO/*Cpeb* heterozygous (HKO) (n=34) P19–21 mice. Seizure reduction in the DKO (40%) and HKO (44%) compared to *Fmr1* KO was significant (79%,  $p < 0.05$ , Fisher exact test). **b**, Time spent in the center of an open field for 15 min (n=9–11, ANOVA,  $F_{(4,46)}=3.28$ ,  $p < 0.05$ ). **c**, Passive avoidance memory measured by latency to enter a dark compartment 24 hours after foot shock in the dark (n=8–11, ANOVA on effect of day  $F_{(1,44)}=43.5$ ,  $p < 0.001$ ; on genotype  $F_{(4,44)}=5.04$ ,  $p < 0.01$ ). **d**, Nest building scored on a scale

of  $1-5^{42}$  (left) and weight of unused nesting 24 hours after 2.5g nestlet presentation (n=9–18, ANOVA,  $F_{(4,70)}=2.93$ ,  $p=0.058$  and  $F_{(4,70)}=1.63$ ,  $p=0.17$ , respectively) (right). **e**, nests after 72 hours. **f**, T-maze assay of spontaneous alternations (n=8–11, ANOVA on genotype  $F_{(4,44)}=4.48$ ,  $p<0.01$ ). **g**, Hematoxylin/eosin stained hippocampus. Bar = 1mm. **h**, hippocampus after bilateral injection with GFP/sh-RNA-expressing lentivirus. Bar = 1mm. **i**, T-maze assay after bilateral hippocampal injection of *Fmr1* KO mice with lentivirus expressing sh-Ctrl or sh-*Cpeb* (n=8–11 per treatment/genotype). **j**, Ribosome transit rate (RTR) in brain lysates. Quantification of radiolabel incorporation was plotted as a percentage of the maximum (40 min.). WT vs. *Fmr1* KO, \* $p<0.05$ ; *Fmr1* KO vs. DKO, # $p<0.05$  (ANOVA, followed by Bonferroni's post-hoc test, n= 3–4 per genotype; Supplementary Table 5). For all panels, \* and \*\*,  $p<0.05$  and  $0.01$ , respectively compared to wild-type (Mann-Whitney U test); # and ###,  $p<0.05$  and  $0.001$ , respectively compared to performance on day 1 (paired t-test); + and ++  $p<0.05$  and  $0.01$ , respectively (Wilcoxon signed-rank test to test the difference from chance level of 50%). Error bars are S.E.M.