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Research Article

RESEARCH ARTICLE

R-Baclofen Reverses a Social Behavior Deficit and Elevated Protein Synthesis in a Mouse Model of Fragile X Syndrome

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Abstract

Background: Fragile X syndrome (FXS) is the most common known inherited form of intellectual disability and the single genomic cause of autism spectrum disorders. It is caused by the absence of a fragile X mental retardation gene (*Fmr1*) product, FMRP, an RNA-binding translation suppressor. Elevated rates of protein synthesis in the brain and an imbalance between synaptic signaling via glutamate and γ-aminobutyric acid (GABA) are both considered important in the pathogenesis of FXS. In a mouse model of FXS (*Fmr1* knockout [KO]), treatment with R-baclofen reversed some behavioral and biochemical phenotypes. A remaining crucial question is whether R-baclofen is also able to reverse increased brain protein synthesis rates.
Methods: To answer this question, we measured regional rates of cerebral protein synthesis *in vivo* with the L-[1-^{14}C]leucine method in vehicle- and R-baclofen–treated wildtype and *Fmr1* KO mice. We further probed signaling pathways involved in the regulation of protein synthesis.
Results: Acute R-baclofen administration corrected elevated protein synthesis and reduced deficits on a test of social behavior in adult *Fmr1* KO mice. It also suppressed activity of the mammalian target of rapamycin pathway, particularly in synaptosome-enriched fractions, but it had no effect on extracellular-regulated kinase 1/2 activity. Ninety min after R-baclofen treatment, we observed an increase in metabotropic glutamate receptor 5 expression in the frontal cortex, a finding that may shed light on the tolerance observed in human studies with this drug.
Conclusions: Our results suggest that treatment via activation of the GABA (GABA receptor subtype B) system warrants further study in patients with FXS.

Keywords: Fragile X syndrome, mTOR, protein synthesis, R-baclofen, social behavior

Introduction

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability and known single genomic cause of autism. A complex of neuropsychiatric and physical phenotypes associated with FXS (Hagerman et al., 2009) is caused by

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silencing the fragile X mental retardation 1 gene (*FMR1*), resulting in the absence of the gene product, fragile X mental retardation protein (FMRP; Oostra and Willemsen, 2003). FMRP is an RNA-binding protein that associates with targeted mRNAs and stalls ribosome translocation along targeted mRNAs (Darnell et al., 2011). The absence of FMRP results in a loss of translational control that is thought to be at the core of the disease.

Consistent with a loss of translational control, rates of protein synthesis measured *in vivo* are increased in some regions of the brain in an *Fmr1* knockout (KO) mouse model of FXS (Qin et al., 2005a). *De novo* protein synthesis is required for many forms of long-term alterations in synaptic strength: e.g. long term depression (LTD) and long term potentiation. In dendrites where rapid alterations occur, protein synthesis is thought to be particularly important. Since intellectual disability is a key feature of FXS, research on the *Fmr1*KO mouse model has focused largely on synaptic functioning. Results have led to an increased understanding of synaptic pathophysiology in FXS and indicate several avenues for development of therapeutics.

One of the most studied changes in *Fmr1*KO mice is the elevation in group 1 (Gp1) metabotropic glutamate receptor (mGluR)-dependent LTD (Huber et al., 2002). Gp1 mGluRs are coupled to activation of the phosphoinositide-3 kinase (PI3K) mammalian target of rapamycin (mTOR) pathway. Through this pathway, translation is initiated via phosphorylation of eukaryotic initiation factor 4E (eIF4E) binding protein (4EBP). Activation of mTOR also results in phosphorylation of p70 ribosomal S6 kinase1 (S6k1), which stimulates translation of 5'terminal-oligopyrimidine tract mRNAs that encode ribosomal proteins and translation factors, thus increasing the translational capacity of the cell. Antagonists and allosteric modulators of mGluRs have been studied extensively in *Fmr1* KO mice and have been shown to reverse many behavioral, physiological, and pathological phenotypes. Despite the success in animal models, clinical trials of some of these compounds have been less promising.

Changes are also found in the inhibitory γ-aminobutyric acid (GABA) pathway (D'Hulst and Kooy, 2007). Presynaptic $GABA_B$ receptors are involved in the regulation of glutamate release (Vigot et al., 2006) and, in the forebrains of *Fmr1* KO mice, the $GABA_BR1$ subunit is down-regulated (Pacey et al., 2011). R-baclofen, a selective $GABA_B$ agonist, has been studied in patients with FXS (Berry-Kravis et al., 2012) and in the mouse model (Henderson et al., 2012). In *Fmr1* KO mice, R-baclofen decreased labeled amino acid incorporation in hippocampal slices, corrected dendritic spine density in cortices, and reduced repetitive behaviors and susceptibility to audiogenic seizures (Henderson et al., 2012). A crossover study with R-baclofen in 63 FXS patients showed improvement on a newly validated, FXS-specific Aberrant Behavior Checklist-Social Avoidance (ABC-SA) scale (Berry-Kravis et al., 2012). A Phase 3 clinical trial of FXS patients was undertaken, and by some measures R-baclofen showed efficacy in younger children (5–11 years). Primary endpoints did not show effectiveness in patients with autism, however, and the sponsoring company folded, consequently ending the trial in patients with FXS.

We report herein that acute R-baclofen administration reversed elevated rates of cerebral protein synthesis (rCPS) *in vivo* and reduced deficits in a test of social behavior in *Fmr1* KO mice. We further report that treatment with R-baclofen had measureable effects on signaling pathways involved in the regulation of protein synthesis in the frontal cortex and that even an acute dose of R-baclofen caused an increase in the cortical concentration of mGluR5.

Methods

Animals

Male wild-type (WT) and *Fmr1* KO mice on a C57Bl/6J background were bred and genotyped as previously described (Qin et al., 2005a). Mice were group-housed in a central facility, and naïve mice (80–121 days old) were used for each test. All procedures were carried out in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals and an animal study protocol approved by the National Institute of Mental Health Animal Care and Use Committee.

Drug and Dose Selection

R-baclofen and its pharmacokinetic information were provided by Seaside Therapeutics, Inc. We determined the appropriate dose by measurement of open field behavior in 12 groups (6–18/group) of mice: WT and *Fmr1* KO at doses of 0.0, 1.0, 1.5, 2.0, 3.0, and 6.0 mg/kg. To achieve maximum levels in the brain, R-baclofen dissolved in saline was administered i.p. 30 min before placing the mouse in the open field.

In all subsequent measurements animals were injected i.p. with either vehicle (C) or R-baclofen (Bf; 1.5mg/kg), and four groups were compared: (1) WT-C; (2) KO-C; (3) WT-Bf; and (4) KO-Bf.

Open Field Test

Thirty min after i.p. injection of either R-baclofen or vehicle, locomotor activity was evaluated as previously described (Liu et al., 2011). Activity was recorded for 30 min, quantified by a computer-operated tracking system (TruScan System, Coulbourn Instruments), and analyzed at 6-min intervals. Total distance moved and the % distance moved in the margins (within 6.25 cm of walls) were analyzed.

Test of Social Behavior

Thirty min after i.p. injection of either R-baclofen (1.5mg/kg) or vehicle, mice were tested in an automated three-chambered social approach apparatus as previously described (Liu et al., 2011). The test had three consecutive phases: (A) habituation, in which the test mouse was allowed to freely explore the apparatus for 5 min; (B) sociability, in which an unfamiliar mouse (Stranger-1) was placed inside a wire cup in one of the side chambers, an empty cup was placed in the other chamber, and the test mouse freely explored the apparatus for 5 min; and (C) preference for social novelty, in which a second unfamiliar mouse (Stranger-2) was placed inside the cup in the opposite chamber, and the test mouse was allowed to freely explore the apparatus for 5 min. Total time spent in each chamber and times spent sniffing a stranger mouse or an empty cup were recorded. During habituation, 12 mice that spent three or more min in any one chamber were eliminated from the study.

Regional Rates of Cerebral Protein Synthesis

We used the autoradiographic L-[1-^{14}C]leucine method to determine rCPS in WT-C (n = 8), KO-C (n = 8), WT-Bf (n = 7), and KO-Bf (n = 9) mice as described previously (Smith et al., 1988; Qin et al., 2005a). Briefly, the experimental period was initiated 30 min after an i.p. injection of either R-baclofen (1.5mg/kg) or vehicle by an intravenous pulse of 100 µCi/kg L-[1-^{14}C]leucine (60 mCi/mmol, Moravek Biochemicals, Inc.). Arterial blood samples

were collected during the following 60 min for determination of the time courses of plasma concentrations of leucine and [^{14}C] leucine. At the end of the experimental interval, brains were removed, frozen, and 20 µm sections were prepared for quantitative autoradiography. Autoradiograms were digitized (MCID Analysis, Interfocus Imaging Ltd), the concentration of ^{14}C in each region of interest was determined, and rCPS was calculated by means of the operational equation (Qin et al, 2005a). Brain regions were identified by reference to a mouse brain atlas (Paxinos and Franklin, 2001).

Western Blot Analysis

Ninety min after treatment, mice were anesthetized with sodium pentobarbital, rapidly decapitated, and the left and right frontal cortices were separated for total and synaptosome-enriched protein extraction, respectively. For total protein extraction, the tissue was weighed, homogenized in 5% (w/v) ice-cold Tissue Protein Extraction Reagent (Thermo-Scientific) with 1% Halt Protease Inhibitor Cocktail (Thermo-Scientific) and 1% Phosphatase Inhibitor Cocktail (Sigma-Aldrich), centrifuged (12 000 × g, 4°C, 15 min), and supernatant fractions were collected as protein samples. For synaptic protein extraction, tissue was weighed, homogenized (glass Dounce tissue grinder) in 20% (w/v) ice-cold Synaptic Protein Extraction Reagent (Syn-PER; Thermo Scientific) with 1% Protease and Phosphatase Inhibitor (Sigma-Aldrich), and centrifuged (1 200 x g 4°C, 10 min). Supernatant fractions were centrifuged (15 000 x g, 4°C, 20min) and synaptosome-enriched pellets were resuspended in Syn-PER (1–3 ml/g tissue).

To avoid confounding effects of genotype and drug on housekeeping proteins, we employed Stain-Free technology (Bio-Rad Laboratories, Inc.). Protein extracts (20 or 30 µg) were treated with Laemmli buffer (1:1), incubated (95°C, 5 min), and subjected to electrophoresis (4–20% Mini-PROTEIN TGX Stain-Free gels, Bio-Rad Laboratories, Inc.). Gels underwent ultraviolet activation for later assessment of total protein in each lane. Protein was transferred to nitrocellulose membranes and incubated with primary antibody (4°C, overnight), which was followed by incubation (RT, 1 h) with goat anti-rabbit horseradish peroxidase-linked secondary antibody (1:10 000; Bio-Rad Lab, Inc.). Prior to reaction with Clarity substrate (Bio-Rad Lab Inc.), the stain-free image of the blot for total protein loading control was captured. Finally, the chemiluminescence signal for each specific immunoreactive band was determined (ChemiDoc MP Imager, Bio-Rad) and normalized by the total protein loading control (Imager Lab Software, Bio-Rad). Antibodies (dilution) used were: phospho-protein kinase B (*p*-Akt; Ser 473; 1:2 000), protein kinase B (Akt; 1:2000), phospho-4E-BP1 (Ser65; 1:1 000), 4E-BP1 (1:1 000), phospho-p44/42 extracellular-regulated kinase (*p*-ERK1/2; Thr202/Tyr204; 1:2 000), ERK1/2 (1:2000), phospho-mTOR (Ser2448; 1:250), mTOR (1:500), phospho-S6k1 (Thr389; 1:500), S6k1 (1:500), and mGluR5 (1:500). All were rabbit antibodies obtained from Cell Signaling Technology Inc., except mGluR5, which was purchased from Abcam Inc.

Statistical Analysis

Data are expressed as means ± standard error of the mean (SEM). Data were analyzed by means of repeated measures analyses of variance (RM ANOVA) and, when appropriate, further probed by means of post hoc Bonferroni *t*-tests. The significance level was set at $p \le 0.05$. We used the SPSS program (IBM) for statistical computations.

Results

Determination of Optimal Dose of R-Baclofen

Following a dose of 1 mg/kg, mice of both genotypes showed increased activity in the open field compared to vehicle-injected controls (Figure 1A and B). In both WT and KO mice, activity levels were very similar to vehicle-injected mice following doses of either 1.5 or 2.0 mg/kg. Following a dose of 3 mg/kg, activity in WT mice was similar to vehicle-injected animals until the last 6 min epoch, during which activity decreased; at this dose in KO mice, activity was decreased throughout the 30 min period of measurement compared with vehicle-injected controls. Following a dose of 6.0 mg/kg, mice of both genotypes showed markedly decreased activity levels, suggesting some level of sedation. Based on these observations, we chose a dose of 1.5 mg/kg for further study because this was the lowest dose that did not increase activity levels and because it showed no evidence of sedative effects in either genotype.

Acute R-Baclofen Treatment on Locomotor Activity and General Anxiety

We measured total distance traveled as an indicator of hyperactivity and % distance moved in the margins of the field as an index of general anxiety. In all four groups, the total distance traveled per 6 min epoch gradually decreased dur-ing the 30 min session (Figure 1C) and the percent distance moved in the margins gradually increased (Figure 1D). Data were analyzed by repeated measures (RM) ANOVA with genotype, treatment, and epoch as factors with RM on epoch (Table 1). For both total distance moved and the percent distance moved in the margins, neither the three-way nor the two-way interactions were statistically significant. For both measures, the main effect of genotype was the only statistically significant result. For total distance moved, the epoch × distance curves of KO mice were higher than those of WT (Figure 1C), indicating hyperactivity of KO mice; hyperactivity was not reversed by R-baclofen treatment. Regardless of treatment, the epoch × percent distance in the margins curves of KO mice were lower than those of WT (Figure 1D), indicating lower general anxiety of KO mice; reduced general anxiety was not reversed by R-baclofen treatment. Overall our results of open field analyses indicate that KO mice are hyperactive and exhibit reduced general anxiety; neither attribute was affected by R-baclofen treatment (1.5 mg/kg).

Improvement on a Test of Social Behavior by R-Baclofen

During the sociability phase of the test, mice were exposed to a stranger mouse in Chamber-1; Chamber-2 contained an empty cup. Vehicle- and R-baclofen–treated WT and KO mice spent most of the time in Chamber-1 (Figure 2B). Moreover, for all four groups the preponderance of time spent sniffing was with the stranger mouse in Chamber-1 rather than the empty cup in Chamber-2 (Figure 2D). Data (time in chamber and sniffing time) were analyzed by RM ANOVA with genotype, treatment, and chamber as factors with RM on chamber (Table 1). Neither the three-way nor any of the two-way interactions were statistically significant. For both variables, only the main effect of chamber was statistically significant—reflecting less time spent in Chamber-2 and less time sniffing the empty cup in Chamber-2—regardless of genotype or treatment.

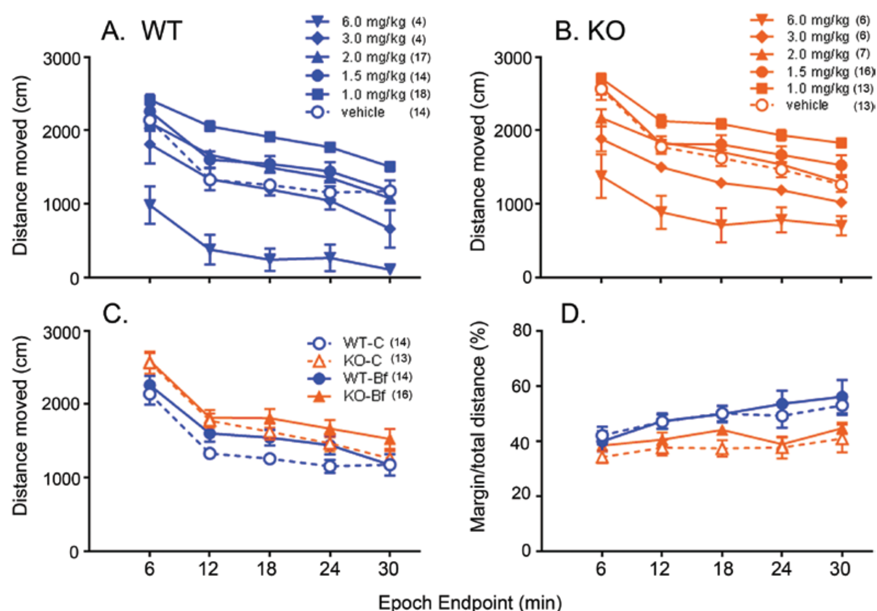

Figure 1. Behavior in the open field. Each point represents the mean ± standard error of the mean during each 6 min epoch for the number of mice indicated in parentheses. Effect of dose of R-baclofen administered on horizontal distance moved by (A) wildtype (WT) and (B) knockout (KO) mice 30 min after i.p. injection of vehicle or R-baclofen (doses from 1.0–6.0 mg/kg). (C) Horizontal distance moved by WT (circles) and KO (triangles) mice 30 min after i.p. injection of vehicle (open symbols) or R-baclofen (1.5 mg/kg; filled symbols). Data were analyzed by repeated measures analyses of variance, and exact results are given in Table 1. There were no statistically significant interactions (genotype x treatment x epoch, treatment x epoch, genotype x epoch, genotype x treatment), but main effects of genotype and epoch were both statistically significant. (D) Percent distance moved in the margins of the field by WT (circles) and KO (triangles) mice 30 min after i.p. injection of vehicle (open symbols) or R-baclofen (1.5 mg/kg; filled symbols). There were no statistically significant interactions (genotype x treatment x epoch, treatment x epoch, genotype x epoch, genotype x treatment), but main effects of genotype and epoch were both statistically significant. KO-Bf, knockout R-baclofen group; KO-C, knockout control group; WT-Bf, wildtype R-baclofen group; WT-C, wildtype control group.

During the preference for social novelty phase, mice were exposed to a novel stranger mouse in Chamber-2. The mouse in Chamber-1 from the sociability phase remained. In this phase of the test, KO mice behaved differently from WT mice. For both chamber time and sniffing time, the genotype x treatment x chamber interactions were statistically significant ($p < 0.01$; Table 1). Post hoc pairwise comparisons indicate statistically significant differences between times in Chamber-1 and -2 for all groups except the KO-C mice (Figure 2C). KO-C mice did not show the expected preference for Chamber-2 with the novel mouse, but this behavior was reversed by R-baclofen treatment. In WT mice, times in either Chamber-1 or -2 were not affected by R-baclofen treatment. Similarly, KO-C mice did not show the expected preference for sniffing Stranger-2, and this behavior was also reversed by R-baclofen treatment (Figure 2E). Post hoc pairwise comparisons indicate statistically significant differences between times sniffing Stranger-1 and -2 for all groups except the KO-C mice (Figure 2E). In WT mice, times sniffing either Stranger-1 or -2 were not affected by R-baclofen treatment, but in KO mice, time sniffing Stranger-2 was increased by R-baclofen treatment ($p < 0.01$) and time sniffing Stranger-1 was decreased ($p < 0.05$).

Acute R-Baclofen Treatment Reverses Elevated Protein Synthesis Rates *In Vivo*

Mice (total n = 32) in all four groups studied were well-matched with respect to age (97 ± 2 days, mean ± SEM) and body weight (28 ± 0.4 g, mean ± SEM), and were physiologically normal at the time of the study (hematocrit, 45 ± 0.5 %; arterial plasma glucose concentration, 6.07 ± 0.2 mM; mean arterial blood pressure, 110 ± 1 mm Hg). Arterial plasma leucine concentrations were within normal limits (116 ± 3 noml/ml). rCPS were higher in all 23 regions examined in KO-C mice (Table 2) compared with WT-C mice; differences ranged from 12% in the dorsal hippocampus as a whole to 29% in the paraventricular nucleus of the hypothalamus. Following treatment with R-baclofen, rates decreased in KO-Bf mice and tended to increase in WT-Bf mice. Data were analyzed by RM ANOVA and the three-way interaction (genotype x treatment x region) was statistically significant ($F_{(4.6,122.9)} = 9.713$, $p < 0.001$). Post hoc pairwise comparisons indicate statistically significant differences between WT-C and KO-C mice in all regions, between KO-C and KO-Bf mice in 16 of the 23 regions, and between WT-C and WT-Bf mice in two regions of the cortex. Some of the biggest effects of R-baclofen treatment were found in the dorsal hippocampus (Table 2, Figure 3); rCPS in both the cell layers (pyramidal and granular layers) and neuropil-rich areas (*stratum radiatum*) were decreased by R-baclofen treatment in KO mice by 19–25%. In areas of the cortex, R-baclofen treatment decreased rCPS by 14–17% in KO mice. In contrast, rCPS were increased in WT mice following R-baclofen, and in the frontal association and motor cortex these changes were statistically significant (Table 2, Figure 4).

Akt/mTOR and MAPK/ERK1/2 Signaling

The phosphorylation states of key translational control molecules in the cortex have been shown to be affected in *Fmr1* KO mice (Michalon et al., 2012). We examined phosphorylation states of some of these molecules in extracts of total and synaptosome-enriched fractions of the frontal cortex from vehicle- and R-baclofen—treated WT and KO mice. Our purpose was to try to understand whether R-baclofen effects changes in rCPS through these signaling pathways. Initially

Table 1. Repeated Measures Analyses of Variance Results

Behavior	Interaction	Main Effect	$F_{(df, error)}$ value	P-value
Open Field				
Total distance moved	Genotype x treatment x epoch		$F_{(2.7,144.3)} = 1.284$	0.282
	Treatment x epoch		$F_{(2.7,144.3)} = 0.760$	0.507
	Genotype x epoch		$F_{(2.7,144.3)} = 0.545$	0.635
	Genotype x treatment		$F_{(1,53)} = 0.084$	0.773
		Genotype	$F_{(1,53)} = 11.958$	0.001
		Treatment	$F_{(1,53)} = 3.695$	0.060
		Epoch	$F_{(2.7,144.3)} = 107.896$	0.000
% Margin distance	Genotype x treatment x epoch		$F_{(3.1,164.9)} = 0.627$	0.604
	Treatment x epoch		$F_{(3.1,164.9)} = 0.156$	0.931
	Genotype x epoch		$F_{(3.1,164.9)} = 1.550$	0.202
	Genotype x treatment		$F_{(1,53)} = 0.351$	0.556
		Genotype	$F_{(1,53)} = 16.114$	0.000
		Treatment	$F_{(1,53)} = 1.093$	0.300
		Epoch	$F_{(3.1,164.9)} = 7.823$	0.000
Social Behavior				
Habituation				
Chamber time	Genotype x treatment x chamber		$F_{(1,61)} = 0.470$	0.626
	Treatment x chamber		$F_{(1,61)} = 1.747$	0.179
	Genotype x chamber		$F_{(1,61)} = 0.191$	0.826
	Genotype x treatment		$F_{(1,61)} = 0.508$	0.479
		Genotype	$F_{(1,61)} = 1.688$	0.199
		Treatment	$F_{(1,61)} = 1.762$	0.189
		Chamber	$F_{(1,61)} = 1.025$	0.362
Sociability				
Chamber time	Genotype x treatment x chamber		$F_{(1,61)} = 0.531$	0.469
	Treatment x chamber		$F_{(1,61)} = 0.674$	0.415
	Genotype x chamber		$F_{(1,61)} = 1.033$	0.313
	Genotype x treatment		$F_{(1,61)} = 0.709$	0.469
		Genotype	$F_{(1,61)} = 0.531$	0.403
		Treatment	$F_{(1,61)} = 0.040$	0.841
		Chamber	$F_{(1,61)} = 6.888$	0.000
Sniffing time	Genotype x treatment x stranger		$F_{(1,61)} = 0.064$	0.801
	Treatment x stranger		$F_{(1,61)} = 0.655$	0.421
	Genotype x stranger		$F_{(1,61)} = 0.037$	0.849
	Genotype x treatment		$F_{(1,61)} = 0.080$	0.779
		Genotype	$F_{(1,61)} = 0.669$	0.417
		Treatment	$F_{(1,61)} = 0.137$	0.721
		Stranger	$F_{(1,61)} = 497.520$	0.000
Social novelty				
Chamber time	Genotype x treatment x chamber		$F_{(1,61)} = 9.396$	0.003
	Treatment x chamber		$F_{(1,61)} = 3.279$	0.075
	Genotype x chamber		$F_{(1,61)} = 4.356$	0.041
	Genotype x treatment		$F_{(1,61)} = 1.544$	0.167
		Genotype	$F_{(1,61)} = 2.780$	0.101
		Treatment	$F_{(1,61)} = 1.956$	0.167
		Chamber	$F_{(1,61)} = 24.681$	0.000
Sniffing time	Genotype x treatment x stranger		$F_{(1,61)} = 13.558$	0.000
	Treatment x stranger		$F_{(1,61)} = 6.076$	0.017
	Genotype x stranger		$F_{(1,61)} = 8.251$	0.006
	Genotype x treatment		$F_{(1,61)} = 0.285$	0.595
		Genotype	$F_{(1,61)} = 2.229$	0.141
		Treatment	$F_{(1,61)} = 0.815$	0.370
		Stranger	$F_{(1,61)} = 75.384$	0.000

we considered the possibility that R-baclofen may act through effects on the mitogen-activated protein kinase (MAPK)/ ERK1/2 pathway. We assessed the effects of R-baclofen treatment on *p*-ERK1/2/ERK1/2 in total and synaptosome-enriched extracts (Figure 5A and B). We found no genotype x treatment interaction and no main effects of either genotype or treatment (Table 3), suggesting that this pathway is not the primary mediator of the changes in protein synthesis in the cortex.

Next we examined the PI3K/Akt pathway. We measured *p*-Akt(Ser 473)/Akt and saw no effects of either genotype or treatment in total tissue extracts (Figure 5C). In synaptosome-enriched

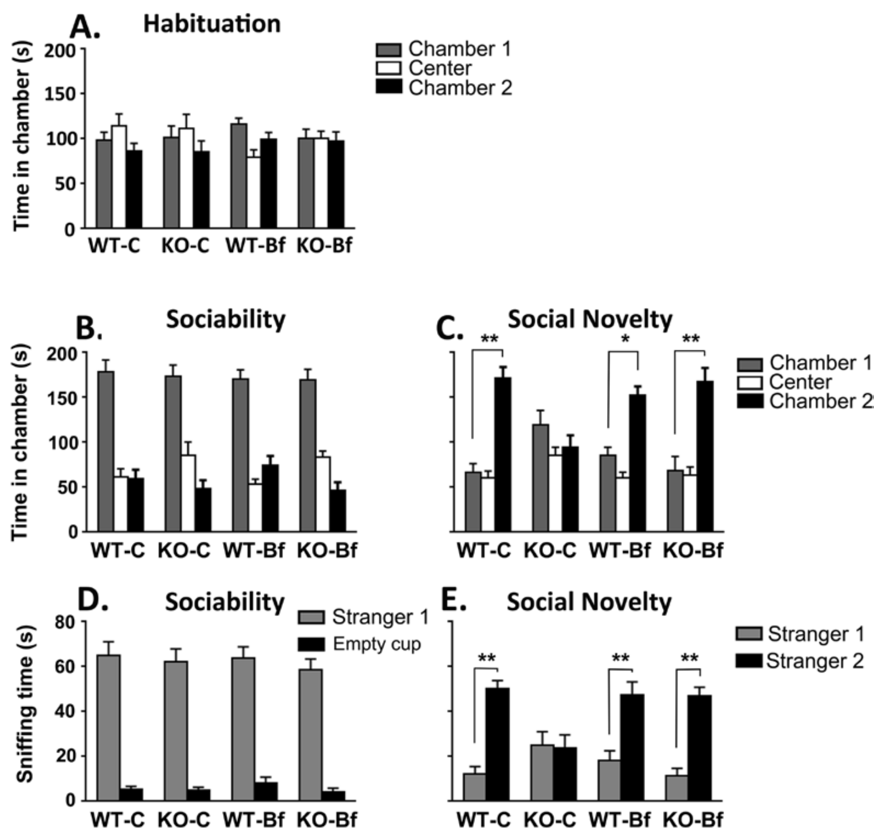

Figure 2. Effect of R-baclofen treatment on a test of social behavior in WT-C (n = 18), KO-C (n = 15), WT-Bf (n = 15), and KO-Bf (n = 17) mice. Bars represent means ± standard error of the mean of (A–C) time spent in each chamber and (D and E) time spent sniffing either the mouse in the wire cage or the empty wire cage in the chamber. The test had three phases: (1; A) habituation, in which the test mouse was allowed to freely explore all three chambers with no other mice in the apparatus; (2; B and D) sociability, in which a stranger mouse was in the cage in Chamber-1 but in Chamber-2 the cage was empty; and (3; C and E) preference for social novelty, in which a second stranger mouse was in the cage in Chamber-2. Data were analyzed by means of repeated measures analyses of variance (RM ANOVA, in which genotype and treatment were between-subjects factors and either chamber or stranger mouse was the within-subjects factor. Exact results of RM ANOVA are given in Table 1. In the habituation phase there were no statistically significant interactions or main effects for chamber time. In the sociability phase there were no statistically significant interactions for either chamber time (genotype x treatment x chamber, treatment x chamber, genotype x chamber, genotype x treatment) or sniffing time (genotype x treatment x chamber, treatment x chamber, genotype x chamber, genotype x treatment). The main effect of chamber was statistically significant for both measures. In the preference for social novelty phase, the genotype x treatment x chamber (or stranger) interaction was statistically significant for both variables. Post hoc *t*-test results are indicated on the figure. *$p < 0.01$; **$p < 0.001$. We tested for statistically significant differences between times spent in Chambers 1 & 2 and times sniffing Stranger 1 & 2 in each group. KO-Bf, knockout R-baclofen group; KO-C, knockout control group; WT-Bf, wildtype R-baclofen group; WT-C, wildtype control group.

extracts (Figure 5D) the genotype x treatment interaction approached statistical significance ($p = 0.092$; Table 3); mean *p*-Akt/Akt was 50% higher in KO-C compared to WT-C mice, but effects of R-baclofen treatment were not statistically significant.

We also examined the phosphorylation state of mTOR (Figure 5E and F). The signaling kinase mTOR downstream from PI3K/Akt is a key regulator of protein synthesis. mTOR has been shown to participate in controlling cell growth and proliferation and is thought to be involved in synaptic plasticity (Jaworski and Sheng, 2006). In total tissue extracts, we found no genotype x treatment interaction and no main effect of genotype, but the main effect of treatment was statistically significant ($p = 0.041$; Table 3). In both WT and KO mice, *p*-mTOR(ser 2448)/mTOR was decreased following R-baclofen treatment by 11 and 18%, respectively. In synaptosome-enriched extracts, the genotype x treatment interaction approached statistical significance ($p = 0.109$). Post hoc *t*-tests indicate that *p*-mTOR/mTOR was 32% higher in KO-C compared to WT-C mice, and was decreased by 30% following R-baclofen treatment in KO mice (Figure 5F).

We also measured the activity of the downstream effectors of mTOR and ERK1/2, S6k1 and 4EBP1. We found a statistically significant genotype x treatment interaction in *p*-S6k1/S6k1 in synaptosome-enriched extracts (Table 3). Post hoc *t*-tests indicate that mean *p*-S6k1/S6k1 in synaptosome-enriched extracts was 86% higher in KO-C compared to WT-C mice. R-baclofen increased *p*-S6k1/S6k1 in WT mice by 113% in synaptosome-enriched extracts but had no effect in KO mice (Figure 5H). The genotype x treatment interaction for *p*-4EBP1/4EBP1 approached statistical significance for the synaptosome-enriched extract ($p = 0.054$; Table 3). In vehicle-treated mice the mean *p*-4EBP1/4EBP1 ratio was 23% higher in KO mice compared with WT mice, and in R-baclofen-treated KO mice the mean ratio was decreased by 26% compared with KO-C mice ($p = 0.041$) (Figure 5J). Effects of R-baclofen on the levels of activation of these downstream effectors in the frontal cortex parallel effects on rCPS with R-baclofen increasing rCPS in WT and decreasing rCPS in KO mice.

mGluR5

Presynaptic $GABA_B$ receptors on glutaminergic terminals can inhibit glutamate release and thus reduce post-synaptic mGluR5 activation. We examined the effects of R-baclofen treatment on

Table 2. Effects of Acute R-Baclofen Treatment on rCPS

REGION	rCPS (nmol/g/min)			
	WT-C (8)	KO-C (7)	WT-Bf (8)	KO-Bf (9)
Cortical regions				
Medial prefrontal	4.4±0.2	5.2±0.2*	5.0±0.2	4.4±0.2†
Anterior cingulate	4.1±0.2	4.9±0.2**	4.5±0.1	4.1±0.1††
Frontal association	3.4±0.1	4.0±0.1**	3.8±0.1*	3.4±0.1††
Primary motor	3.6±0.1	4.3±0.1*	4.0±0.1*	3.6±0.1††
Primary somatosensory	3.8±0.1	4.6±0.2**	4.2±0.1	3.9±0.1††
Parietal	3.8±0.1	4.5±0.2**	4.1±0.2	3.8±0.1††
Cerebellar	4.9±0.2	5.8±0.2**	5.4±0.2	4.9±0.2††
Dorsal hippocampus				
Whole hippocampus	3.4±0.1	3.8±0.2*	3.6±0.2	3.3±0.1†
CA1, pyramidal cell layer	5.5±0.2	6.7±0.3**	6.0±0.2	5.3±0.1††
CA2/CA3 pyramidal cell layer	7.4±0.3	9.1±0.4**	8.2±0.2	7.2±0.2††
Stratum radiatum	2.0±0.1	2.5±0.1**	2.0±0.1	1.9±0.1††
Dentate gyrus, granular cell layer	5.8±0.3	7.0±0.3**	6.4±0.2	5.7±0.1††
Ventral hippocampus				
Whole hippocampus	3.2±0.1	3.7±0.2*	3.4±0.2	3.1±0.1††
CA1, pyramidal cell layer	5.0±0.1	6.1±0.3**	5.3±0.2	5.0±0.1††
CA2/CA3 pyramidal cell layer	7.5±0.2	9.0±0.5**	7.6±0.2	7.3±0.2††
Stratum radiatum	1.6±0.1	2.0±0.1**	1.7±0.1	1.6±0.1††
Dentate gyrus, granular cell layer	5.7±0.3	6.9±0.3**	6.0±0.2	5.6±0.1††
Other areas				
Caudate-putamen	2.4±0.1	2.8±0.1*	2.6±0.1	2.5±0.1†
Thalamus	3.4±0.1	4.1±0.2**	3.8±0.2	3.4±0.1††
Amygdala, anterior nucleus	5.2±0.2	6.4±0.3**	5.5±0.2	5.2±0.1††
Amygdala, basolateral nucleus	4.0±0.1	4.8±0.2**	4.4±0.2	3.9±0.1††
Bed nucleus of stria terminalis	2.5±0.1	2.9±0.1**	2.8±0.2	2.5±0.1††
Hypothalamus, paraventricular nucleus	7.5±0.2	9.8±0.4**	7.9±0.2	7.4±0.1††

Values are the means ± standard error of the mean for the number of mice indicated in parentheses, except for the anterior nucleus of the amygdala and the paraventricular nucleus of the hypothalamus, in which there are seven animals in the WT-Bf group. Results were analyzed by repeated measures analyses of variance, and both the genotype x treatment x region ($F_{(4.6, 122.9)} = 9.713$, $p \leq .001$) and the genotype x treatment ($F_{(1, 27)} = 18.056$, $p \leq .001$) interactions were statistically significant. We further probed these data for statistically significant differences between groups within a region: we compared WT-C v KO-C, WT-C v WT-Bf, and KO-C v KO-Bf.
KO-Bf, knockout R-baclofen group; KO-C, knockout control group; rCPS, rates of cerebral protein synthesis; WT-Bf, wildtype R-baclofen group; WT-C, wildtype control group; CA1, Cornu Ammonis Area 1; CA2/CA3, Cornu Ammonis Areas 2 & 3.
*Statistically significantly different from WT-C, Bonferroni-corrected *t*-tests, $p \leq 0.05$.
**Statistically significantly different from WT-C, Bonferroni-corrected *t*-tests, $p \leq 0.01$.
†Statistically significantly different from KO-C, Bonferroni-corrected *t*-tests, $p \leq 0.05$.
††Statistically significantly different from KO-C, Bonferroni-corrected *t*-tests, $p \leq 0.01$.

mGluR5 levels in protein extracts of frontal cortex homogenates and of synaptosome-enriched fractions from the frontal cortices in WT and KO mice (Table 3, Figure 6). Genotype x treatment interactions were not statistically significant in either extract, but main effects of treatment in total tissue extracts and main effects of genotype in synaptosome-enriched fractions were both statistically significant. In total tissue extracts, mGluR5 levels were elevated in both genotypes following R-baclofen treatment, whereas in synaptosome-enriched fractions the levels of mGluR5 were lower in KO mice regardless of treatment. These results suggest that mGluR5 levels are lower in synapses in the frontal cortex in *Fmr1* KO mice and that as little as 90 min after treatment with R-baclofen an up-regulation in mGluR5 occurs in cells.

Discussion

Dysregulation of protein synthesis may be at the heart of FXS, and many of the phenotypes seen in FXS and in animal models of FXS are considered consequences of this change. A treatment that reverses the protein synthesis phenotype may be able to normalize many of the symptoms of FXS. The central finding of this present study is that a single administration of a low dose of the $GABA_B$ agonist, R-baclofen, can normalize the increased rCPS seen in *Fmr1* KO mice *in vivo*. Moreover, R-baclofen treatment also reversed the impairment on a test of social behavior. At the low dose used in our study, R-baclofen had no effect on activity in the open field. At higher doses R-baclofen did decrease activity levels, but it is difficult to distinguish this effect from the sedating effects of the drug. Our examination of the effects of R-baclofen treatment in the frontal cortex on some of the signaling pathways that regulate protein synthesis indicates that the ERK1/2 pathway does not appear to be involved, but R-baclofen suppression of the mTOR pathway was noteworthy in synaptosome-enriched fractions. Finally, the increase in mGluR5 following a single dose of R-baclofen that we observed may shed light on the tolerance seen in human studies with this drug. Overall, the results of our study indicate that use of medication acting at the $GABA_B$ receptor in the treatment of patients with fragile X syndrome warrants further study.

The dose of R-baclofen (1.5 mg/kg) used in our studies was carefully chosen to avoid any sedating effects of the drug. In WT

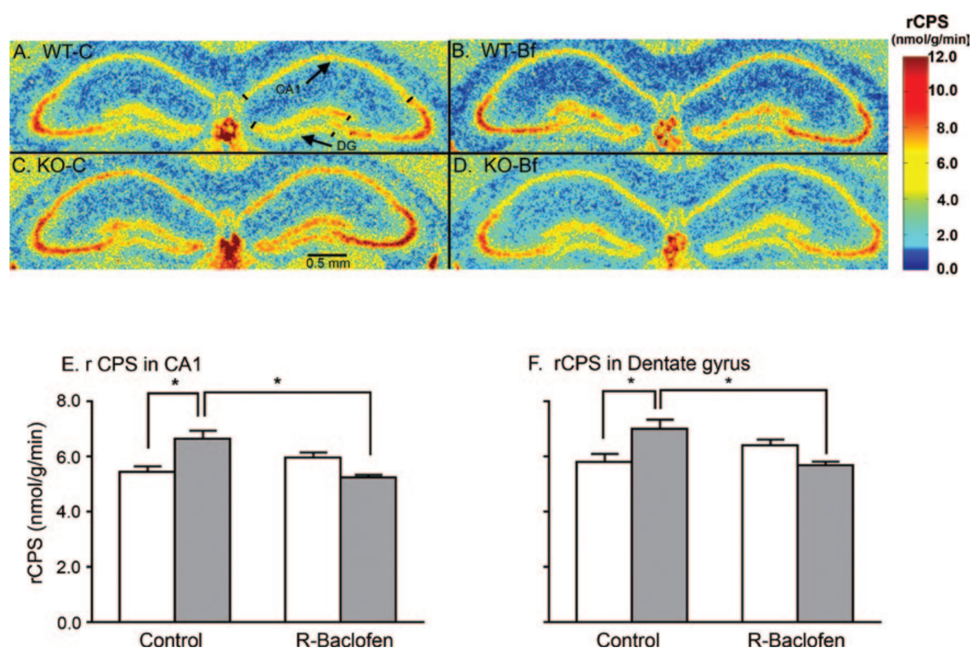

Figure 3. Effect of R-baclofen treatment on rates of cerebral protein synthesis (rCPS) in the dorsal hippocampus. Representative digitized autoradiographic images color-coded for rCPS at the level of the dorsal hippocampus in (A) WT-C, (B) WT-Bf, (C) KO-C, and (D) KO-Bf mice. The color scale in the color bar at the right and the scale bar (0.5 mm) in C apply to all four images. In A, the arrows identify the Cornu Ammonis Area 1 (CA1) pyramidal cell layer and the granular layer of the dentate gyrus. Mean ± standard error of the mean rCPS for each group is given for (E) the CA1 pyramidal cell layer and (F) the granular layer of the dentate gyrus. The genotype x treatment x region interaction was statistically significant (Table 2), so we tested for differences between vehicle and R-baclofen treatment in each genotype and for a genotype difference in the vehicle-treated animals by means of post hoc *t*-tests; $^{*}p < 0.01$. KO-Bf, knockout R-baclofen group; KO-C, knockout control group; WT-Bf, wildtype R-baclofen group; WT-C, wildtype control group.

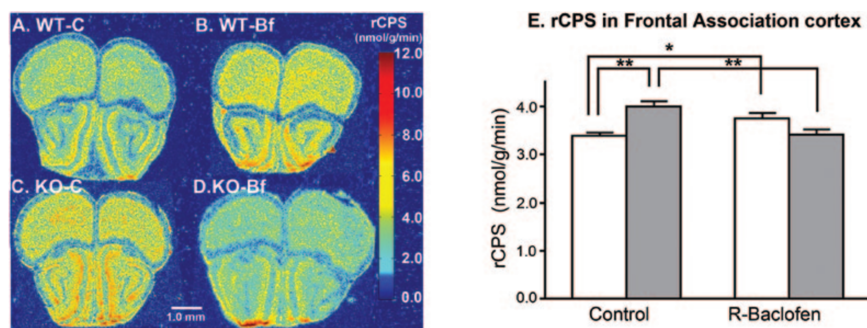

Figure 4. Effect of R-baclofen treatment on rates of cerebral protein synthesis (rCPS) in frontal association cortex. Representative digitized autoradiographic images color-coded for rCPS in (A) WT-C, (B) WT-Bf, (C) KO-C, and (D) KO-Bf mice. The color scale in the color bar at the right and the scale bar (1 mm) in C apply to all four images. (E) rCPS (mean ± standard error of the mean) for each group is shown in the bargraph. The genotype x treatment x region interaction was statistically significant (Table 2), so we tested for differences between vehicle and R-baclofen treatment in each genotype and for a genotype difference in the vehicle-treated animals by means of *post hoc t*-tests; $^{*}p < 0.05$; $^{**}p < 0.001$. KO-Bf, knockout R-baclofen group; KO-C, knockout control group; WT-Bf, wildtype R-baclofen group; WT-C, wildtype control group.

mice, we saw a clear sedating effect of R-baclofen at the highest dose tested (6 mg/kg); in *Fmr1* KO mice, we also found a decrease in activity levels at 3 mg/kg. We do not know whether this effect in the *Fmr1* KO mice is due to sedation or to a positive effect of the treatment on hyperactivity. Interestingly, our data indicate that the lowest dose of R-baclofen tested (1 mg/kg) appeared to increase the level of activity in both genotypes. To avoid any possible influence of sedation or activation we chose a dose of 1.5 mg/kg for our studies. This dose had no effect on behavior in the open field. As has been reported previously (Peier et al., 2000; Qin et al., 2002, 2005b; Liu et al., 2011), *Fmr1* KO mice showed hyperactivity in the open field and a decrease in anxiety-like behavior. Neither activity level nor anxiety appeared to be affected by treatment at this low dose.

Our results support and extend a previous study in *Fmr1* KO mice (Henderson et al., 2012), in which acute administration of 1.5 mg/kg R-baclofen reduced the incidence of audiogenic seizures. In accord with our findings that higher doses of R-baclofen (3 and 6 mg/kg) produce some sedation, Henderson et al. (2012) reported decreased repetitive behavior, activity in the open field, and rotorod performance in both WT and *Fmr1* KO mice at these doses. High doses of R-baclofen also affected indices of translation, suggesting a reduction in protein synthesis and a corrected increased spine density in the visual cortex (Henderson et al., 2012). These results are in agreement with our *in vivo* studies.

Our results on a test of social behavior replicate our previous findings, in which *Fmr1* KO mice behave like WT mice during the

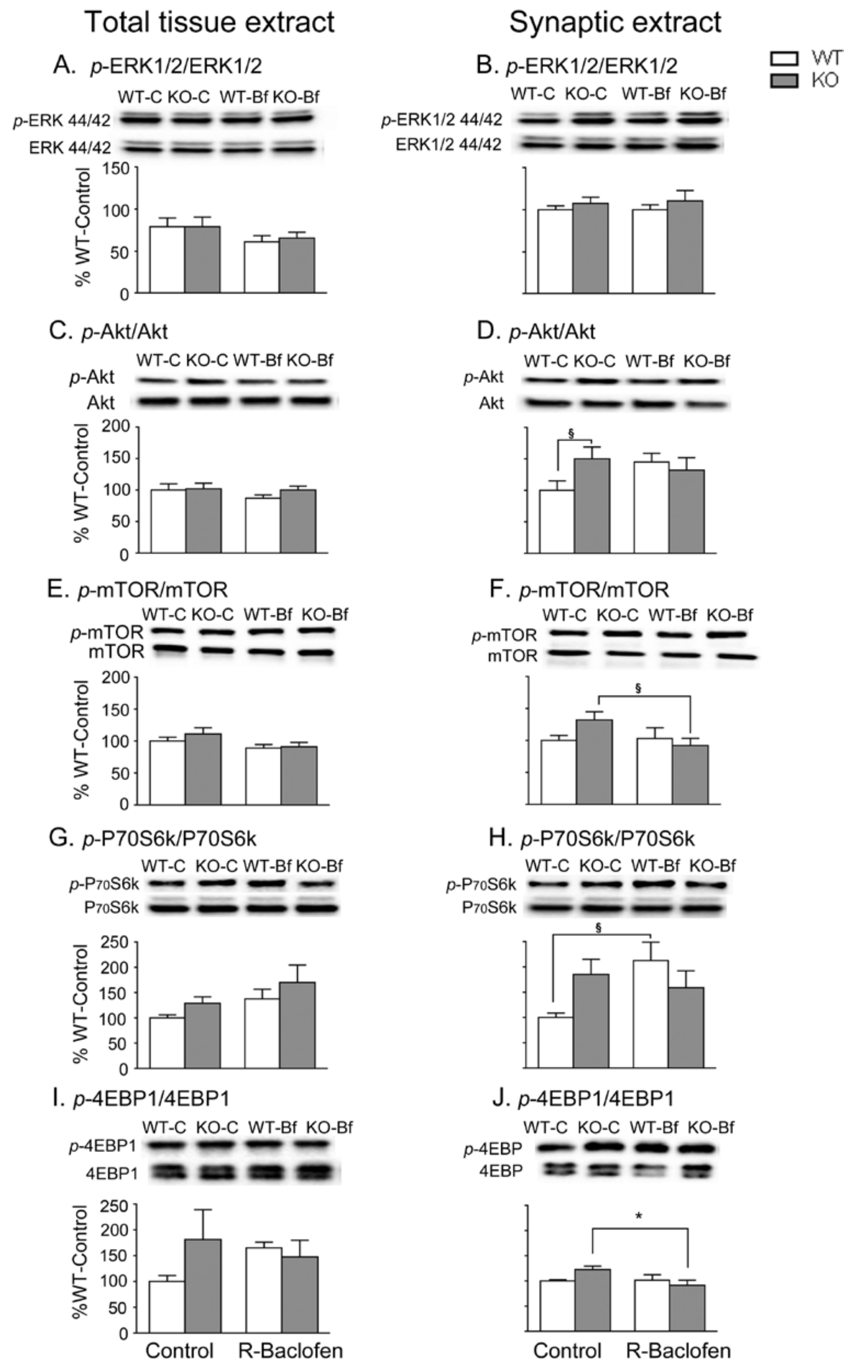

Figure 5. (A and B) Western blot analysis and representative blots of phospho-ERK1/2 (*p*-ERK1/2) and extracellular-regulated kinase (ERK1/2), (C and D) phospho-Akt (*p*-Akt) and protein kinase B (Akt), (E and F) phospho-mTOR (*p*-mTOR) and mammalian target of rapamycin (mTOR), (G and H) phospho-P70 S6k1 (*p*-P70 S6k1) and p70 ribosomal S6 kinase1 (S6k1), and (I and J) phospho-4EBP (*p*-4EBP) and eukaryotic initiation factor 4E (eIF4E) binding protein (4EBP) in total tissue and synaptosome-enriched extracts, respectively. Each bar represents the mean ± standard error of the mean in five-seven mice per group for ERK1/2 and Akt, three-six per group for S6k1 and 4EBP, and five-ten for mTOR. Values are expressed as a percent of WT-C. Genotype x treatment interactions were statistically significant for p-S6k1/S6k1 in synaptosome-enriched extracts (Table 3). In synaptosome-enriched extracts, genotype x treatment interactions for *p*-Akt/Akt, p-mTOR/mTOR, and p-4EBP/4EBP approached statistical significance (Table 3). We tested for differences between vehicle and R-baclofen treatment in each genotype and for a genotype difference in the vehicle-treated animals by means of post hoc *t*-tests; §$p < 0.1$; *$p < 0.05$. KO-Bf, knockout R-baclofen group; KO-C, knockout control group; WT-Bf, wildtype R-baclofen group; WT-C, wildtype control group.

sociability phase of the test, but diverge from WT mice during the preference for social novelty phase (Liu et al., 2009, 2011; Mines et al., 2010; Heitzer et al., 2013). In contrast to WT mice, *Fmr1* KO mice appeared to have no chamber or stranger preference during the social novelty phase of the test. This could be interpreted as a memory deficit, in that the *Fmr1* KO mice did not remember which mouse they had interacted with previously. Or it could demonstrate a social anxiety phenotype. In the presence of the two stranger mice, the KO mice have decreased (22%) total sniffing interaction, altogether suggestive of social anxiety. Following acute R-baclofen treatment this social behavior deficit was reversed, and behavior of *Fmr1* KO mice was

Table 3. Two-Way ANOVA Results on Immunoblot Analyses

Protein Target	Interaction	Main Effect	$F_{(df, error)}$ value	P-value
p-ERK/ERK				
Total extract	Genotype x treatment		$F_{(1,24)} = 0.070$	0.793
		Genotype	$F_{(1,24)} = 0.072$	0.791
		Treatment	$F_{(1,24)} = 2.757$	0.110
Synaptic extract	Genotype x treatment		$F_{(1,19)} = 0.031$	0.863
		Genotype	$F_{(1,19)} = 1.259$	0.276
		Treatment	$F_{(1,19)} = 0.029$	0.867
p-Akt/Akt				
Total extract	Genotype x treatment		$F_{(1,24)} = 0.386$	0.540
		Genotype	$F_{(1,24)} = 0.861$	0.363
		Treatment	$F_{(1,24)} = 0.847$	0.367
Synaptic extract	Genotype x treatment		$F_{(1,17)} = 3.196$	0.092
		Genotype	$F_{(1,17)} = 1.170$	0.294
		Treatment	$F_{(1,17)} = 0.637$	0.436
p-mTOR/mTOR				
Total extract	Genotype x treatment		$F_{(1,35)} = 0.413$	0.525
		Genotype	$F_{(1,35)} = 1.015$	0.321
		Treatment	$F_{(1,35)} = 4.520$	0.041
Synaptic extract	Genotype x treatment		$F_{(1,20)} = 2.821$	0.109
		Genotype	$F_{(1,20)} = 0.687$	0.417
		Treatment	$F_{(1,20)} = 2.064$	0.166
p-P_{70}S6 kinase/P_{70}S6 kinase				
Total extract	Genotype x treatment		$F_{(1,8)} = 0.005$	0.946
		Genotype	$F_{(1,8)} = 2.076$	0.188
		Treatment	$F_{(1,8)} = 3.550$	0.096
Synaptic extract	Genotype x treatment		$F_{(1,17)} = 5.335$	0.034
		Genotype	$F_{(1,17)} = 0.277$	0.606
		Treatment	$F_{(1,17)} = 1.996$	0.176
p-4EBP1/4EBP1				
Total extract	Genotype x treatment		$F_{(1,9)} = 2.493$	0.149
		Genotype	$F_{(1,9)} = 1.023$	0.338
		Treatment	$F_{(1,9)} = 0.235$	0.639
Synaptic extract	Genotype x treatment		$F_{(1,20)} = 4.177$	0.054
		Genotype	$F_{(1,20)} = 0.569$	0.459
		Treatment	$F_{(1,20)} = 3.093$	0.094
mGluR5				
Total extract	Genotype x treatment		$F_{(1,24)} = 0.006$	0.939
		Genotype	$F_{(1,24)} = 0.016$	0.899
		Treatment	$F_{(1,24)} = 6.920$	0.015
Synaptic extract	Genotype x treatment		$F_{(1,32)} = 0.795$	0.379
		Genotype	$F_{(1,32)} = 3.878$	0.058
		Treatment	$F_{(1,32)} = 0.595$	0.446

4EBP, eukaryotic initiation factor 4E (eIF4E) binding protein; Akt, protein kinase B; ERK, extracellular-regulated kinase; mGluR metabotropic glutamate receptor; mTOR, mammalian target of rapamycin; *p*-4EBP, phospho-4EBP; *p*-Akt, phospho-Akt; *p*-ERK, phospho-p44/42 ERK1/2; *p*-mTOR, phospho-mTOR

indistinguishable from that of WT, suggesting that activation of $GABA_B$ receptors can normalize this social anxiety/memory deficit response. Improvements in social function have also been reported in a placebo-controlled clinical trial of R-baclofen, in which significant changes were reported in the ABC-SA (Berry-Kravis et al., 2012).

Our primary goal in this study was to determine whether a single dose of R-baclofen could reverse the increased *in vivo* rCPS found in *Fmr1* KO mice (Qin et al., 2005a). Measurement of rCPS in the intact and functioning nervous system is a reflection of ongoing steady state processes. Protein synthesis, particularly in cell bodies, may be performing many housekeeping functions, such as maintenance and replacement of structural and functional components of the cell. Cell body protein synthesis may also be reacting to events taking place at its synapses. At the synapse, protein synthesis is likely an integral part of the plasticity response and affected by synaptic signaling. To be effective, plasticity changes must be highly localized, and with the autoradiographic method we are able to determine rCPS in neuronal cell body-rich areas (e.g. hippocampal pyramidal cell layer) and in synapse-rich areas (e.g. hippocampal *stratum radiatum*). We focused our analysis on the hippocampus because it is in the hippocampus that we previously observed some of the largest and most reproducible increases in rCPS in *Fmr1* KO mice (Qin et al., 2005a; Liu et al., 2012). We also analyzed 13 additional areas of the cortex and midbrain. Physiological, biochemical, and/or morphological abnormalities have been observed in *Fmr1* KO mice in all of these regions.

For our study of signaling molecules that might be involved in response to the drug, we chose the frontal cortex because we

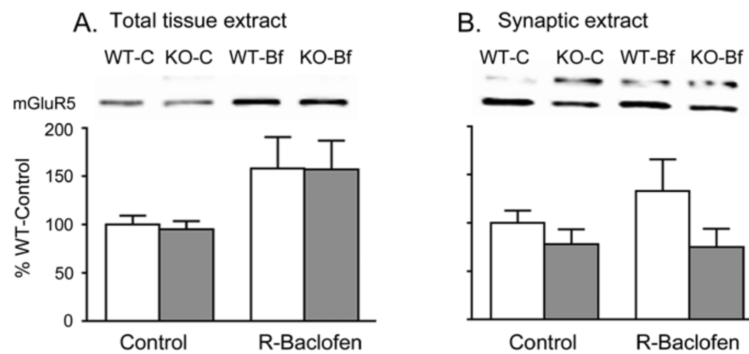

Figure 6. Western blot analysis and representative blots of mGluR5 in (A) total tissue and (B) synaptosome-enriched extracts. Each bar represents the mean ± standard error of the mean. For the total tissue extracts we analyzed the protein in seven mice per group. For the synaptosome-enriched fraction we analyzed the protein in nine WT-C, ten KO-C, eight WT-Bf, and nine KO-Bf mice. KO-Bf, knockout R-baclofen group; KO-C, knockout control group; WT-Bf, wildtype R-baclofen group; WT-C, wildtype control group.

found robust changes in rCPS with treatment in this part of the cortex in both WT and *Fmr1* KO mice. We extracted protein from homogenates of the entire frontal cortex: this included the prefrontal, frontal association, motor, and somatosensory cortices. We also prepared a synaptosome-enriched fraction of this cortical area because it is the synapse that is thought to be primarily affected in FXS. We used total protein (Stain Free Technology) as the normalization factor because it has been demonstrated to be more reliable and sensitive compared to the traditional use of "housekeeping proteins" (Gürtler et al., 2013). Further, by use of this normalization method we avoided the possibly confounding consequences of genotype and/or drug effects on the housekeeping protein itself.

Whether R-baclofen is acting pre- or post-synaptically and how it might effect a reduction in rCPS in *Fmr1* KO mice is not known. Activation of post-synaptic $GABA_B$ receptors can reduce excitability through an effect on voltage-gated potassium channels (Lüscher et al., 1997), and activation of presynaptic $GABA_B$ receptors can inhibit the release of glutamate (Isaacson and Hille, 1997). Decreasing the activation of mGluR5 via either pre- or post-synaptic mechanisms may decrease up-regulated protein synthesis, thought to occur via both the mTOR and ERK1/2 pathways. We considered both of these synaptic signaling pathways in trying to understand the mechanisms underlying our findings. Our results indicate that, in *Fmr1* KO mice, R-baclofen treatment has no effect on ERK1/2, but the mTOR pathway is dampened, particularly in synaptosome-enriched extracts. Consistent with this effect, we also found decreased activity downstream of mTOR in *p*-4EBP1 in synaptic extracts from KO-Bf mice. In synaptic frontal cortical extracts, the most robust effects of R-baclofen treatment were found in WT mice, in which *p*-S6k1/S6k1 was elevated by R-baclofen treatment. Similarly, *p*-4EBP1/4EBP1 was also elevated in total cortical extracts, but effects did not reach statistical significance. These effects in WT mice are in accord with the increased rCPS in the cortex following R-baclofen treatment.

Our results in vehicle-treated mice on the lack of an effect on ERK1/2 phosphorylation in *Fmr1* KO mice contrast with previously reported results in which the vehicle-treated *Fmr1* KO mice had a higher (23%) level of *p*-ERK1/2/ERK1/2 compared with vehicle-treated WT mice (Michalon et al., 2012). Differences between the two studies include techniques used, ages of mice studied, and area of cortex analyzed. The major difference between the two studies is the vehicle treatment. In our study mice received a single i.p. injection of normal saline 90 min before tissue removal, whereas in the previous study vehicle treatment was chronic administration of a mixture of normal saline and Tween-80 by oral gavage every other day for 4 weeks. It is possible that the vehicle treatments may have had effects on ERK1/2 activity. Our finding of unaffected *p*-ERK1/2 in the frontal cortices of *Fmr1* KO mice is consistent with our own previous findings in hippocampal lysates (Liu et al., 2012).

Most previous work on signaling pathways that may be involved in the regulation of protein synthesis has focused on the hippocampus (Osterweil et al., 2010; Sharma et al., 2010; Bhattacharya et al., 2012; Liu et al., 2012), and several of these studies are primarily analyses of hippocampal slices studied *in vitro* (Osterweil et al., 2010; Sharma et al., 2010). Our findings of increased phosphorylated forms of mTOR, and S6k1 in *Fmr1* KO mice cortices are in accord with reported changes in hippocampal lysates (Bhattacharya et al., 2012; Liu et al., 2012).

Our finding of significantly increased levels of mGluR5 in cortical extracts following R-baclofen treatment points to a possible explanation for the observed tolerance to this medication. This increase may be a compensatory change in response to the enhanced inhibitory signaling. Excessive glutamatergic signaling at mGluR5 synapses is a well-characterized FXS phenotype (Bear et al., 2004). Moreover, it is known that mGluR5 can be rapidly down-regulated in response to overstimulation (Javitt et al., 2011). Our finding of reduced levels of mGluR5 in the synaptosome-enriched fraction in *Fmr1* KO mice is in accord with this idea and is in agreement with a previous study (Giuffrida et al., 2005). It is likely that the mGluR5 effects in the synaptosome-enriched fractions are more a reflection of the state of the animals prior to treatment, whereas the effects in the total tissue extracts may reflect regulation occurring primarily in cell bodies, where synthesis of receptors is likely taking place. We would expect that with chronic treatment and chronic activation of $GABA_B$ receptors, synthesis of mGluR5 would be chronically elevated. With time, mGluR5 synthesized in the cell bodies, transported to terminals, and inserted into synaptic membranes may be responsible for tolerance to chronic treatment with R-baclofen.

Overall, results of this preclinical study indicate that the $GABA_B$ system is a promising target for therapeutic development in FXS, but that issues of compensatory responses to receptor activation over the long term must be taken into consideration. The efficacy of R-baclofen at a low dose is encouraging

in that reversal of both the increased rCPS and deficit in social interaction phenotypes occur without any sedative effects of the medication. Our studies of signaling pathways confirm the importance of the mTOR pathway for regulation of protein synthesis, particularly at the synapse.

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Statement of Interest

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