

SCIENTIFIC REPORTS



OPEN

GABA_B receptor upregulates fragile X mental retardation protein expression in neurons

Received: 28 November 2014

Accepted: 15 April 2015

Published: 28 May 2015

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Fragile X mental retardation protein (FMRP) is an RNA-binding protein important for the control of translation and synaptic function. The mutation or silencing of FMRP causes Fragile X syndrome (FXS), which leads to intellectual disability and social impairment. γ -aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the mammalian central nervous system, and its metabotropic GABA_B receptor has been implicated in various mental disorders. The GABA_B receptor agonist baclofen has been shown to improve FXS symptoms in a mouse model and in human patients, but the signaling events linking the GABA_B receptor and FMRP are unknown. In this study, we found that GABA_B receptor activation upregulated cAMP response element binding protein-dependent *Fmrp* expression in cultured mouse cerebellar granule neurons via two distinct mechanisms: the transactivation of insulin-like growth factor-1 receptor and activation of protein kinase C. In addition, a positive allosteric modulator of the GABA_B receptor, CGP7930, stimulated *Fmrp* expression in neurons. These results suggest a role for GABA_B receptor in *Fmrp* regulation and a potential interest of GABA_B receptor signaling in FXS improvement.

Fragile X mental retardation protein (FMRP) is an RNA-binding protein that controls translation and synaptic function^{1,2}. FMRP mutation or silencing causes Fragile X syndrome (FXS), a common inherited disease associated with autism, intellectual disability, and social impairment³. Chemical compounds targeting metabotropic glutamate receptor 5 (mGluR5) and other neurotransmitter receptors such as γ -aminobutyric acid and serotonin receptors^{4,5} or downstream signaling pathways such as mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK)1/2 and phosphatidylinositol 3 kinase (PI3K)/glycogen synthase kinase 3 β /Akt⁶ have been tested for their ability to improve FXS symptoms such as anxiety, seizure, and hyperactivity. Recent studies have demonstrated that the GABA_B receptor agonist R-baclofen (STX209) can improve locomotor activity and motor coordination in patients with FXS and modify the pathophysiology induced by FMRP deficiency including the effects on protein synthesis, AMPA receptor turnover, and dendritic spine density^{7,8}, suggesting a connection between GABA_B receptor and FMRP regulation. However, the signaling events linking GABA_B receptor activation to FMRP are not well understood.

The GABA_B receptor is the metabotropic receptor of GABA, the main inhibitory neurotransmitter in the mammalian central nervous system⁹. The receptor is a seven transmembrane domain-containing protein belonging to class C G protein-coupled receptors (GPCRs)¹⁰ and is assembled as a heterodimer containing GABA_{B1} and GABA_{B2} subunits⁹. Only GABA_{B1} subunit can bind agonists, whereas GABA_{B2} subunit is responsible for G protein coupling¹¹. Positive allosteric modulators bind within the GABA_{B2}

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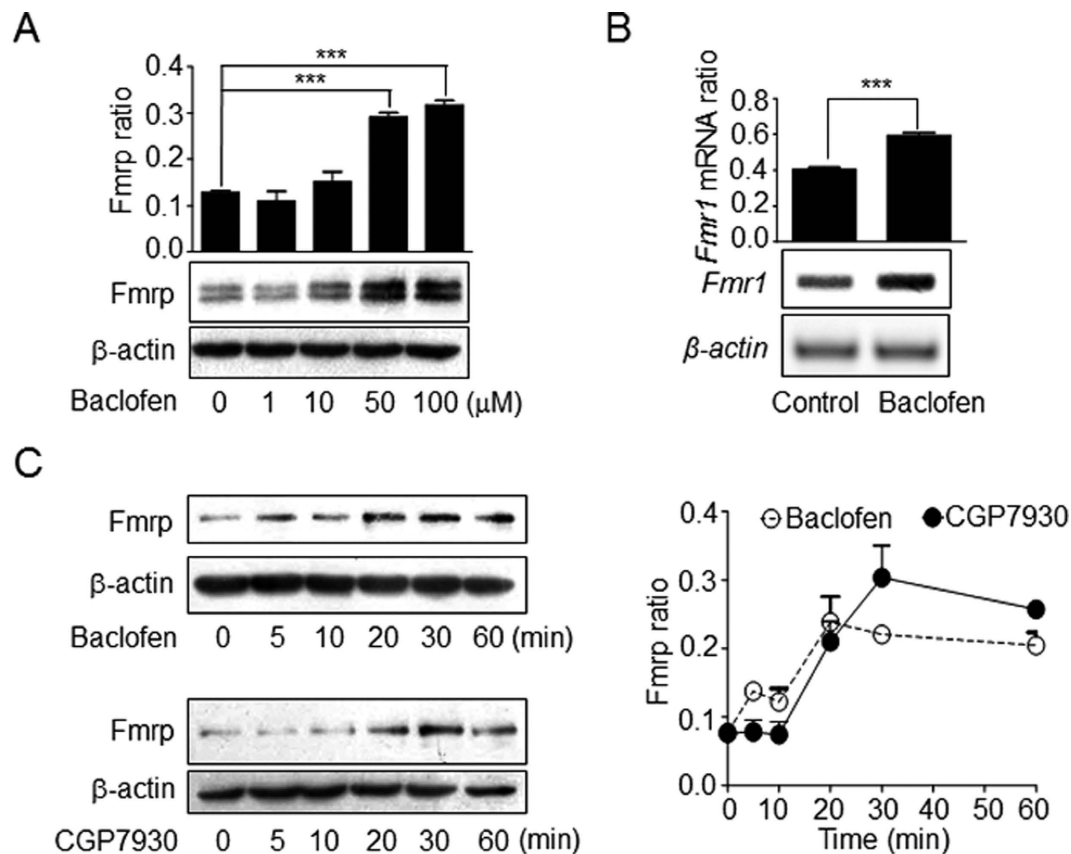


Figure 1. Activation of GABA_B receptor upregulates Fmrp expression in CGNs. (A) Fmrp expression in CGNs treated with indicated concentrations of baclofen. ***P < 0.001 vs. basal levels. (B) *Fmr1* mRNA expression upon treatment with baclofen. (C) Time course of Fmrp expression induced by baclofen and CGP7930. Fmrp expression level was quantified based on three independent experiments (mean ± SEM). ***P < 0.001 vs. basal levels. Fmrp ratio and *Fmr1* mRNA ratio were defined as the ratio between the density of each band and the sum of the densities of all the bands in a given blot. Full-size blots are shown in **Supplementary Figure S1** and the band of interest is indicated by a red box.

transmembrane domain to potentiate the effect of agonists¹². Presynaptic GABA_B receptor activation inhibits neurotransmitter release through the depression of voltage-gated Ca²⁺ channels, whereas activated postsynaptic GABA_B receptors open K⁺ channels to induce neuronal hyperpolarisation¹³. GABA_B receptor activation induces the ERK_{1/2}/cAMP response element-binding protein (CREB) signaling pathway, which is dependent on G_{i/o} protein¹⁴. GABA_B receptor also activates PI3K/Akt signaling to decrease apoptosis in cerebellar granule neurons (CGNs)^{15,16} through transactivation of the insulin-like growth factor-1 receptor (IGF-1R).

This study investigated the link between GABA_B receptor and Fmrp. The results show that activation of the GABA_B receptor upregulated *Fmr1* mRNA and protein expression via activation of CREB. IGF-1R- and protein kinase C (PKC)-dependent signaling pathways were found to be involved in CREB activation and Fmrp synthesis. In addition, we show that CGP7930, a positive allosteric modulators (PAMs) of GABA_B receptor, also upregulated Fmrp expression.

Results

GABA_B receptor activation upregulates Fmrp expression. The link between GABA_B receptor activation and Fmrp expression was investigated using the GABA_B receptor agonist baclofen. Drug treatment increased Fmrp level in a dose-dependent manner in CGNs (Fig. 1A, **Figure S1A**), and increased *Fmr1* mRNA expression as well as Fmrp protein synthesis in a time-dependent manner (Fig. 1B, C **upper panel**, **Figure S1B**, **Figure S2**) starting 20 min after drug application, with effects persisting for more than 60 min.

PAMs potentiate the GABA_B receptor activation by orthosteric agonists such as baclofen¹⁷; in the case of CGP7930, this is accomplished via binding to the transmembrane domain of GABA_{B2} subunit^{18–20}. We recently showed that CGP7930 can directly activate the GABA_B receptor in cultured cell lines and neurons in the absence of an agonist^{14,15}. We found here that the kinetics of Fmrp expression after CGP7930

treatment were similar to those induced by baclofen (Fig. 1C, **Figure S1C**). These data demonstrate that GABA_B receptor activation via the GABA_{B2} subunit increases *Fmrp* expression.

CREB is required for *Fmrp* upregulation induced by GABA_B receptor. The *Fmr1* gene promoter contains a CREB-binding site, and mGluR1 and 5 can regulate *Fmrp* expression through CREB^{21,22}. Moreover, CREB itself is regulated by various receptors via downstream effectors such as PKA, PKC, ERK_{1/2}, and Akt^{23–25}. The role of CREB in GABA_B receptor-mediated *Fmrp* upregulation was investigated by short interfering (si)RNA-knockdown of CREB in mouse embryonic fibroblasts (MEFs) co-transfected with GABA_{B1} and GABA_{B2}. CREB depletion abolished the GABA_B receptor-induced increase in *Fmrp* expression relative to the control (Fig. 2A, **Figure S3A**). These data indicate that GABA_B receptor-induced CREB activity is required for *Fmrp* synthesis.

In CGNs, baclofen and CGP7930 treatment induced a concentration-dependent increase in the level of phosphorylated CREB without altering total CREB expression level (Fig. 2B, **Figure S3B**). The rapid and transient increase in CREB phosphorylation peaked at 10 min and decreased to the basal level at 60 min after drug application (Fig. 2C, **Figure S3C**). Interestingly, pre-treatment of CGNs with the competitive GABA_B receptor antagonist CGP54626 blocked baclofen but not CGP7930-induced CREB phosphorylation (**Figure S4**). These results indicate that GABA_B receptor activation can induce a transient increase in phosphorylation of CREB, a component of signaling pathway that important for *Fmrp* expression.

GABA_B receptor-mediated transactivation of IGF-1R leads to CREB activation. IGF-1R was reported to be transactivated by GABA_B receptor through G_{i/o} protein, PLC β and focal adhesion kinase (FAK), and then further induced MEK/ERK_{1/2} and PI3K/Akt activation^{15,16}. Therefore, we investigated the role of the IGF-1R transactivation signaling pathway in the activation of CREB. ERK_{1/2}, Akt, and CREB phosphorylation were blocked by treatment with pertussis toxin (PTX), which uncouples G_{i/o} proteins from GPCRs via ADP-ribosylation of G $\alpha_{i/o}$ subunits (Fig. 3A, **Figure S5A**), suggesting that GABA_B receptor-mediated CREB activation in CGNs is G_{i/o} protein-dependent. Pre-treatment of CGNs with U73122 (PLC β inhibitor) or PF573228 (FAK inhibitor) blocked baclofen-induced ERK_{1/2}, Akt, and CREB phosphorylation (Fig. 3B, C, **Figure S5B, C**). Furthermore, the IGF-1R inhibitor AG1024 also blocked the baclofen-induced phosphorylation of CREB in CGNs, as well as that of ERK_{1/2} and Akt (Fig. 4A, **Figure S6A**). In MEFs expressing the recombinant GABA_B receptor, siRNA knockdown of endogenous IGF-1R reduced baclofen-induced phosphorylation of CREB, ERK_{1/2}, and Akt (Fig. 4B, **Figure S6B**). Similar results were obtained by short hairpin-mediated knockdown in MEFs using a shRNA targeting IGF-1R (**Figure S7**). Taken together, these results show that IGF-1R transactivation via G_{i/o} protein, PLC β , and FAK is important for baclofen-induced CREB activation.

PKC is required for GABA_B receptor-induced CREB activation independent of IGF-1R signaling. PKC was previously shown to be activated by baclofen treatment¹⁵. Phosphorylation of the PKC substrate MARCKS was increased in a time-dependent manner by baclofen treatment (Fig. 5A, **Figure S8A**). Phospho-MARCKS level was reduced by application of the FAK inhibitor PF573228 or by siRNA-mediated knockdown of FAK (Fig. 5B, C, **Figure S8B, C**), but not by IGF-1R knockdown (Fig. 5D, **Figure S8D**), suggesting that PKC acts downstream of FAK but independently of IGF-1R. Three PKC inhibitors (GF109203x, Gö-6983, and Gö-6976) were used to analyse the effect of PKC on CREB activation; GF109203x and Gö-6983 inhibit all PKC isozymes^{25,26}, whereas Gö-6976 is selective for Ca²⁺-sensitive PKC isotypes²⁶. All three inhibitors blocked baclofen-induced CREB phosphorylation but had no effect on the phosphorylation of ERK_{1/2} and Akt (Fig. 6A, B, **Figure S9A, B** and **Figure S10A**). In addition, siRNA-mediated knockdown of PKC α or PKC β in MEFs co-expressing GABA_{B1} and GABA_{B2} subunits of the GABA_B receptor decreased baclofen-mediated CREB phosphorylation, whereas no changes in IGF-1R transactivation or ERK_{1/2} and Akt phosphorylation were observed (Fig. 6C, **Figure S9C** and **Figure S10B**). These results indicate that Ca²⁺-sensitive PKCs are required for GABA_B-induced CREB activation, but that this effect is independent of IGF-1R transactivation.

IGF-1R and PKC are required for GABA_B receptor-induced upregulation of *Fmrp* expression. The role of IGF-1R and PKC in the GABA_B receptor-induced expression of *Fmrp* was assessed. GABA_B receptor-induced *Fmrp* synthesis was markedly reduced in CGNs by treatment with IGF-1R inhibitor (Fig. 7A, **Figure S11A**); siRNA-mediated IGF-1R knockdown abolished the baclofen-induced increase in *Fmrp* level in MEFs expressing the recombinant GABA_B receptor (Fig. 7B, **Figure S11B**). PKC inhibitor also reduced baclofen-induced *Fmrp* expression (Fig. 7C, **Figure S11C**). FAK acts upstream of IGF-1R¹⁶ and PKC (Fig. 5B, C) in the GABA_B receptor-mediated signaling pathway. Accordingly, pre-treatment with PF573228 decreased baclofen-induced *Fmrp* synthesis (Fig. 7D, **Figure S11D**). Taken together, these results indicate that both IGF-1R and PKC are critical for the upregulation of *Fmrp* expression induced by GABA_B receptor activation.

GABA_B receptor PAM increases *Fmrp* expression. PAMs bind to the transmembrane domain of the GABA_B receptor at a location independent of the agonist-binding site, thereby potentiating the effect of the receptor agonist. Of the three commercially available GABA_B receptor PAMs (CGP7930, GS39783,

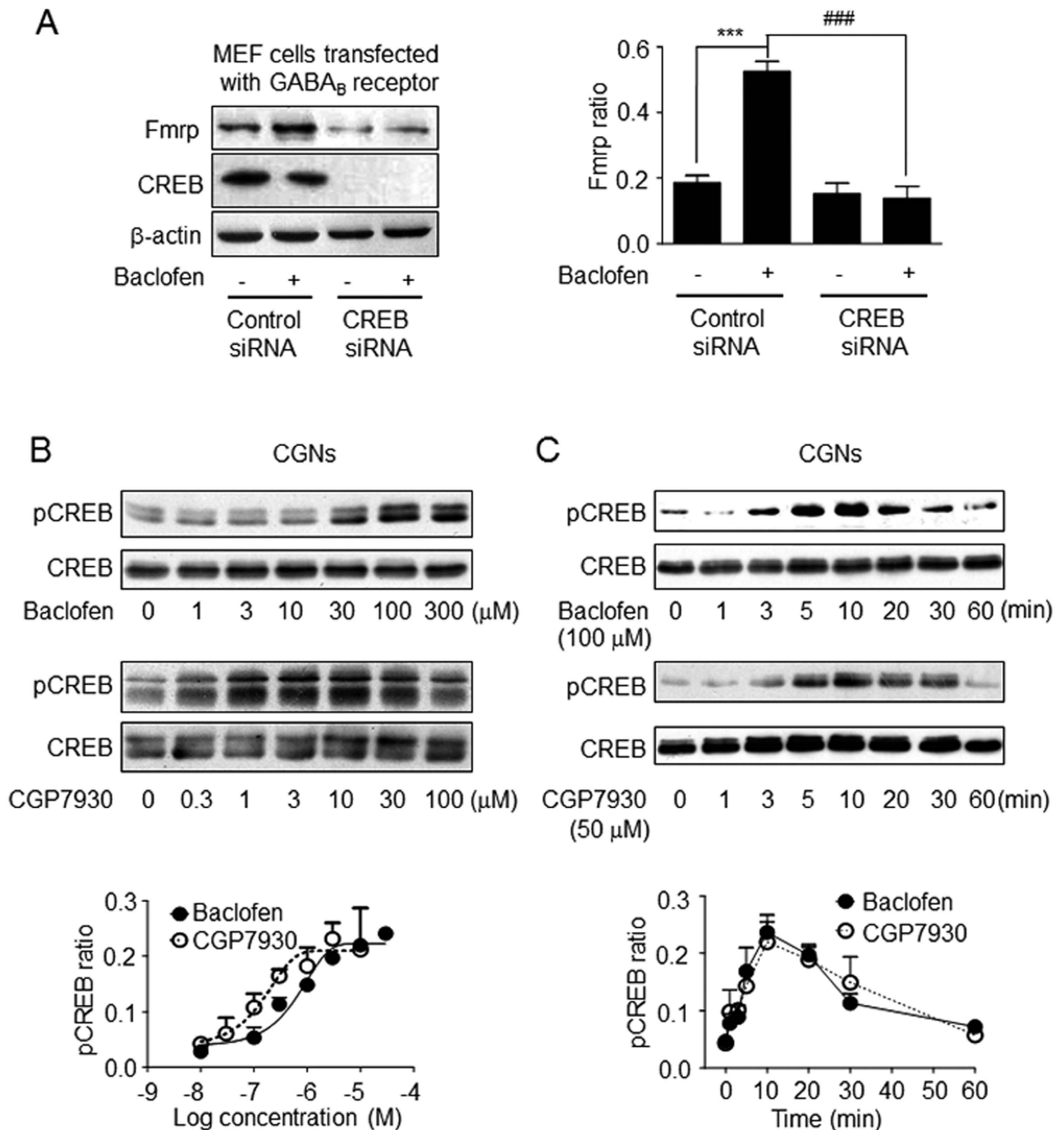


Figure 2. GABA_B receptor-induced CREB signaling is required for Fmrp upregulation. (A) Effect of siRNA-mediated knockdown of CREB on baclofen-induced Fmrp expression in MEFs co-transfected with GABA_{B1} and GABA_{B2}. Representative western blots are shown. CREB phosphorylation level in control siRNA-transfected cells. Fmrp ratio was defined as in Fig. 1A. The level in baclofen-treated cells was quantified based on three independent experiments (mean ± SEM). ***P < 0.001 vs. basal with control siRNA. ###P < 0.001 vs. baclofen-treated cells transfected with control siRNA. (B) CREB phosphorylation in CGNs induced by indicated concentrations of baclofen or CGP7930. The data were quantified from three independent experiments (mean ± SEM). (C) Time course of CREB phosphorylation induced by baclofen and CGP7930. Protein level was quantified based on three independent experiments (mean ± SEM). pCREB ratio was defined as the ratio between the density of each band and the sum of the densities of all the bands in a given blot. Full-size blots are shown in **Supplementary Figure S3** and the band of interest is indicated by a red box.

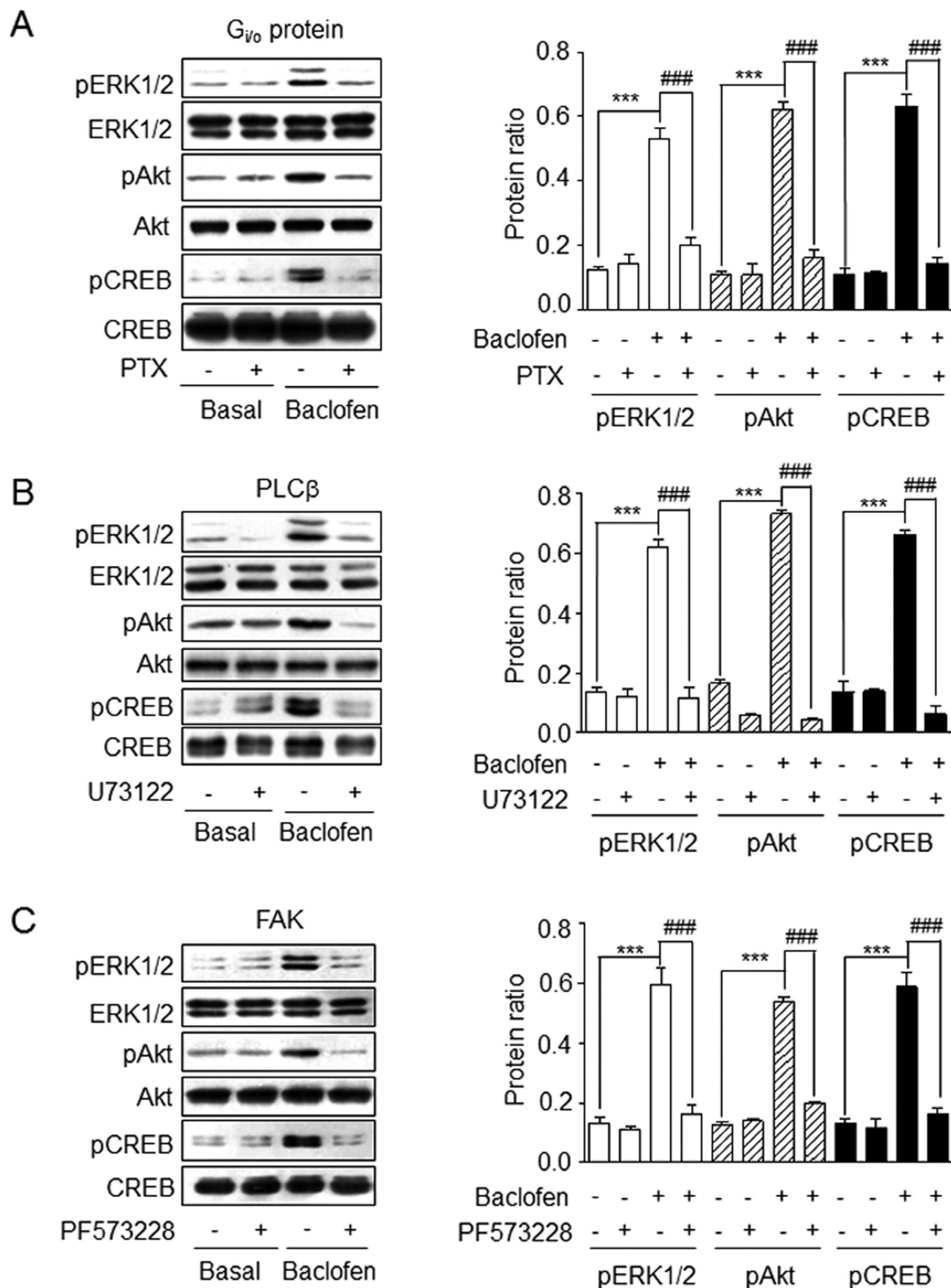


Figure 3. GABA $_B$ receptor-mediated CREB phosphorylation is dependent on $G_{i/o}$ protein, PLC β , and FAK. CGNs were pretreated with PTX (A), U73122 (B), or PF573228 (C) before baclofen-stimulation. CREB, ERK $_{1/2}$ and Akt phosphorylation was detected by western blotting. Protein ratio on the Y-axis was defined as the ratio between the density of each band and the sum of the densities of all the bands in a given blot. Data represent the mean \pm SEM from three separate sets of immunoblots. ***P < 0.001 vs. basal level. ###P < 0.001 vs. baclofen-treated group. Full-size blots are shown in **Supplementary Figure S5** and the band of interest is indicated by a red box.

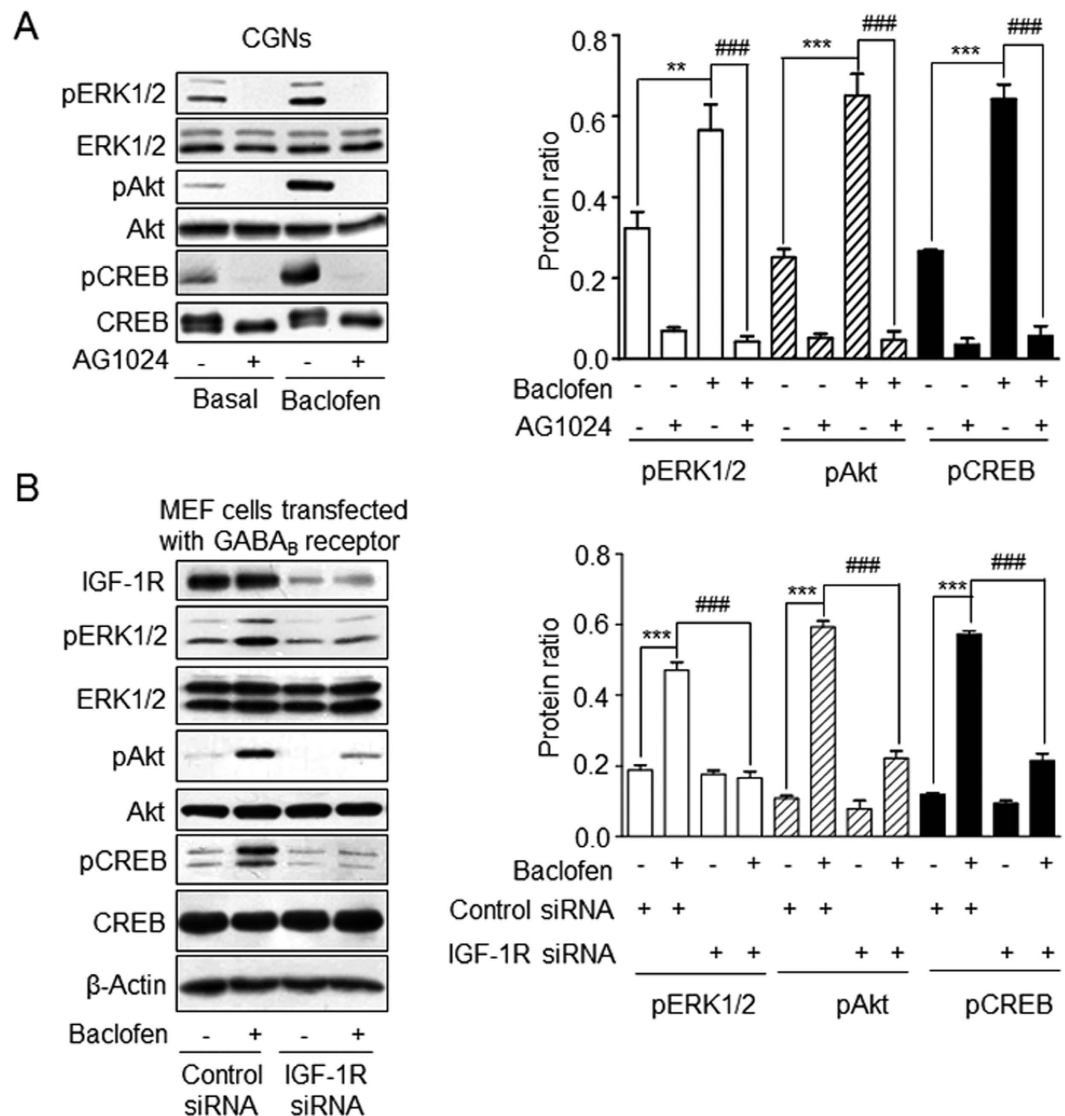


Figure 4. GABA_B receptor-mediated transactivation of IGF-1R is required for CREB phosphorylation. (A) CGNs were pre-treated with the IGF-1R inhibitor AG1024 followed by baclofen. Expression levels of pERK_{1/2}, pAkt and pCREB were quantified by western blotting. Data represent the mean \pm SEM from at least three independent experiments. ** $P < 0.01$, *** $P < 0.001$ vs. basal level. ### $P < 0.001$ vs. baclofen-treated group. (B) MEFs were transfected with GABA_{B1}, GABA_{B2}, and control or IGF-1R siRNA before treatment with baclofen. Phosphorylation of ERK_{1/2}, Akt, and CREB was quantified by western blotting. *** $P < 0.001$ vs. basal level in control siRNA-transfected cells. ### $P < 0.001$ vs. baclofen-treated cells transfected with control siRNA. Protein ratio was defined as in Fig. 3. Full-size blots are shown in **Supplementary Figure S6** and the band of interest is indicated by a red box.

and Rac BHFf), CGP7930 and Rac BHFf but not GS39783 act as PAM agonists^{18,19,27–29}. The PAMs were compared with respect to their effects on signaling events downstream of GABA_B receptor activation. Interestingly, CGP7930 but not GS39783 or Rac BHFf induced the phosphorylation of Akt and CREB and increased the level of Fmrp in a manner similar to the agonist baclofen (Fig. 7E and **Figure S12**), confirming the role of GABA_B receptor activation in the modulation of Fmrp expression.

Discussion

This study investigated the signaling events linking GABA_B receptor activation to Fmrp expression. The results demonstrate that activation of the GABA_B receptor by baclofen upregulates Fmrp synthesis via induction of CREB, which involves IGF-1R- and PKC-dependent signaling (Fig. 8). We also found that the GABA_B receptor PAM CGP7930 upregulated Fmrp expression.

Our results clarify the signaling link between GABA_B receptor and Fmrp expression. However, they cannot explain the beneficial effect of baclofen in FXS mouse model or in patients, where the *Fmr1* gene

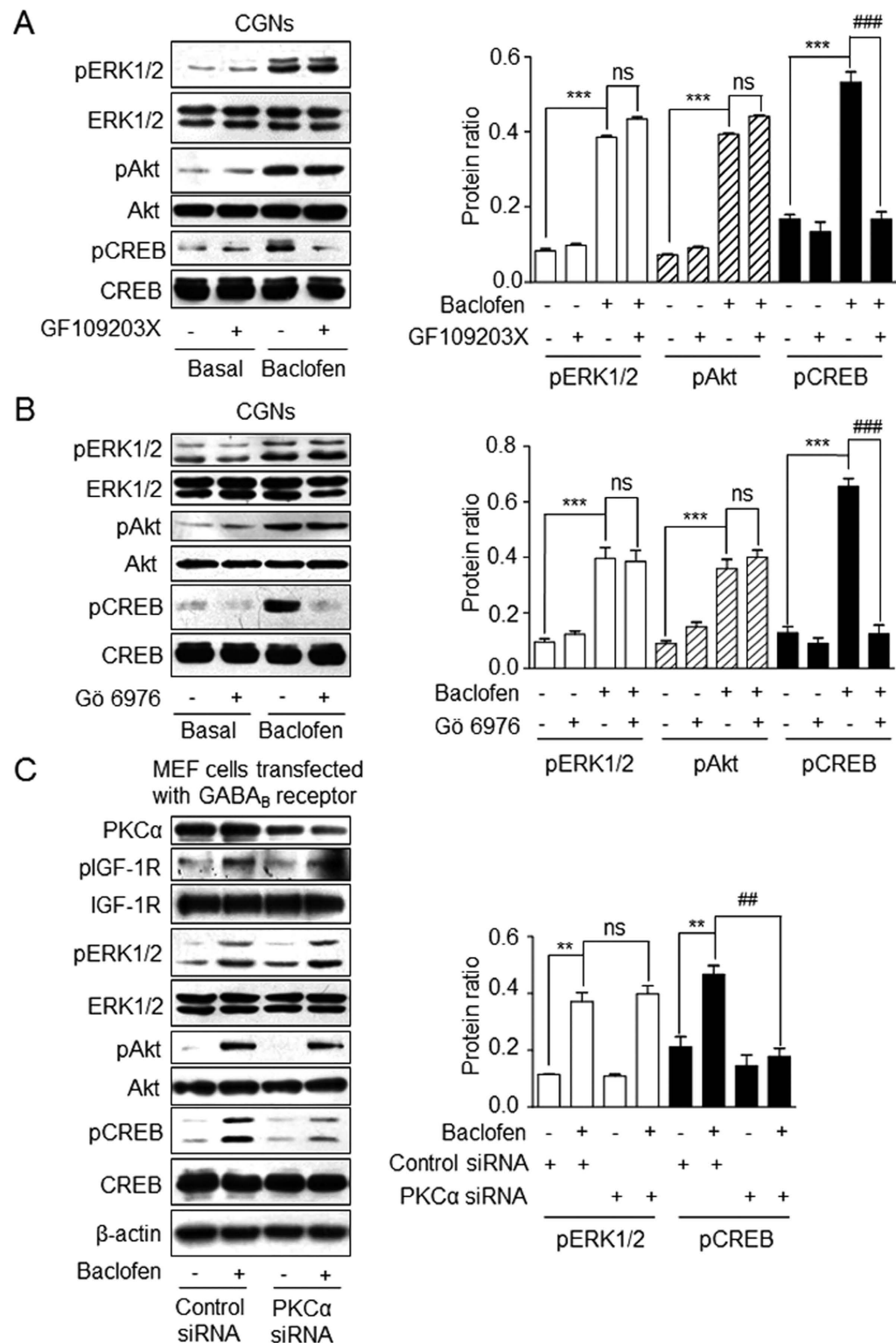


Figure 6. PKC is required for GABA_B receptor-induced CREB activation. (A, B) CGNs were pre-treated with GF109203x (A) or G6-6976 (B) followed by treatment with baclofen. ERK_{1/2}, Akt, and CREB phosphorylation was quantified by western blotting and the protein ratio was defined as in Fig. 3. ***P < 0.001, vs. basal level. ###P < 0.001, ns, not significant vs. baclofen-treated group. (C) MEFs were co-transfected with GABA_{B1}, GABA_{B2}, and control or PKC α siRNA and then treated with baclofen. pERK_{1/2} and pCREB levels were quantified by western blotting and the protein ratio was defined as in panels A and B. Data represent the mean \pm SEM from three independent experiments. **P < 0.01, vs. basal level in control siRNA-transfected cells. ##P < 0.01, ns, not significant vs. baclofen-treated cells transfected with control siRNA. Full-size blots are shown in **Supplementary Figure S9** and the band of interest is indicated by a red box.

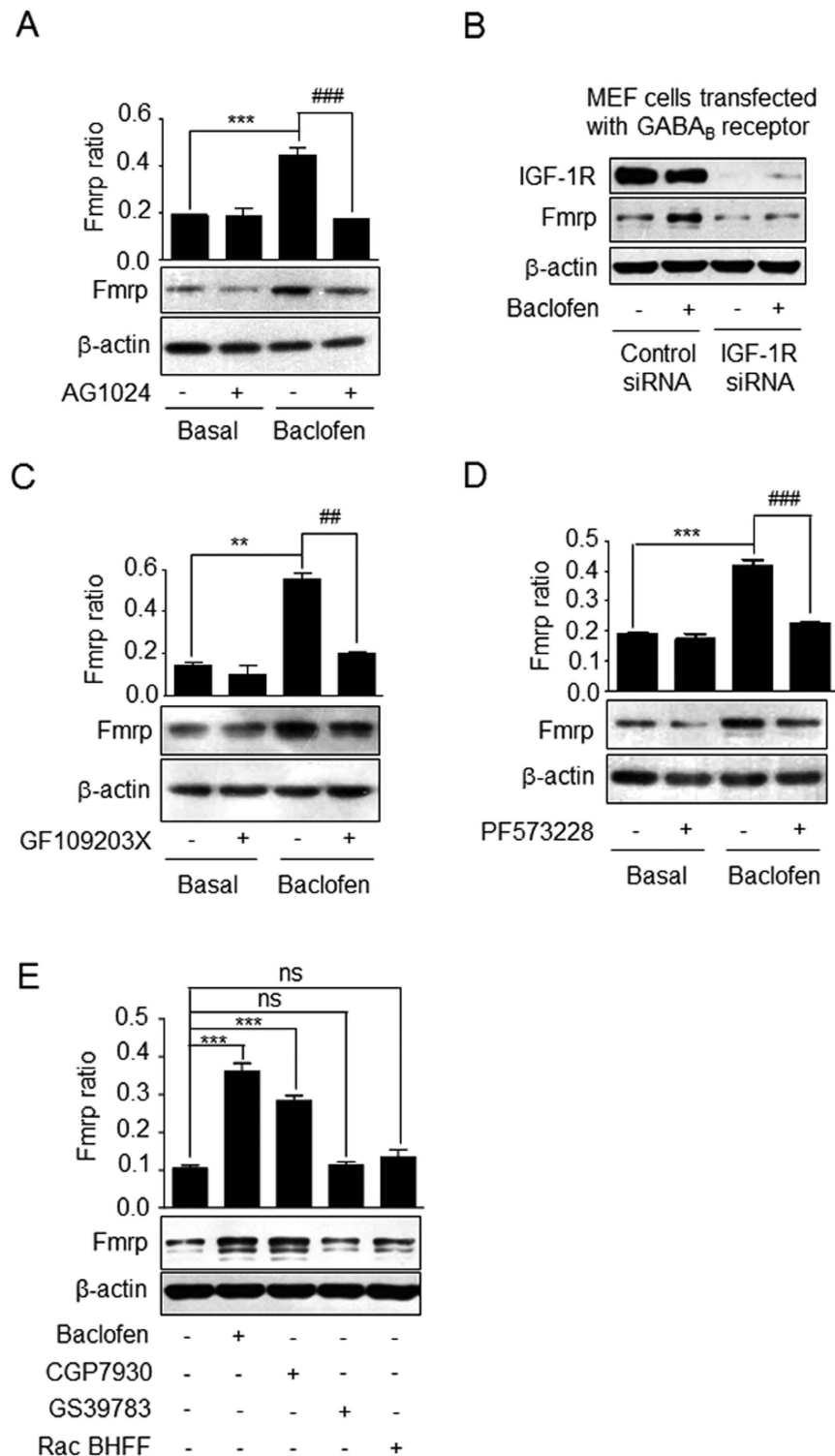


Figure 7. IGF-1R and PKC are involved in GABA_B receptor-mediated Fmrp synthesis. (A) CGNs were pretreated with AG1024 followed by treatment with baclofen. Fmrp levels were detected by western blotting and Fmrp ratio was defined as in Fig. 1A. Data represent the mean \pm SEM from three independent experiments. (B) MEFs were co-transfected with GABA_{B1}, GABA_{B2}, and control or IGF-1R siRNA and then treated with baclofen. Fmrp levels were quantified as in panel A. (C, D) CGNs were pretreated with GF109203x or PF573228, and then treated with baclofen. Fmrp levels were detected as in panel A. Data represent the mean \pm SEM from three independent experiments. For results in A, C, D, ** $P < 0.01$, *** $P < 0.001$ vs. basal level; ## $P < 0.01$, ### $P < 0.001$, vs. baclofen-treated group. (E) CGNs were treated with vehicle, baclofen, CGP7930, GS39783, or Rac BHFF and Fmrp expression level was quantified as in panel A. Data represent the mean \pm SEM from three independent experiments. *** $P < 0.001$, ns, not significant vs. basal level. Full-size blots are shown in **Supplementary Figure S11** and the band of interest is indicated by a red box.

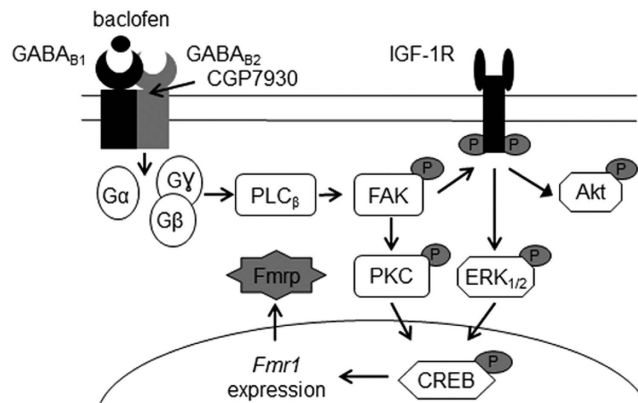


Figure 8. Schematic representation of the signaling pathway mediated by GABA_B receptor leading to CREB activation and Fmrp upregulation in CGNs. Agonist (baclofen) or PAM (CGP7930) activates the GABA_B receptor, leading to G_{i/o} protein/PLC β /FAK activation, which in turn transactivates the IGF-1R signaling pathway and induces PKC-mediated CREB phosphorylation, thereby upregulating Fmrp expression both at the mRNA and protein levels.

neurons against apoptosis¹⁵, suggesting a possible role of Fmrp in mediating the anti-apoptotic effects of GABA_B receptor.

IGF-1R and PKC act independently in GABA_B receptor/CREB/Fmrp regulation, but it is unclear how these two signaling pathways from membrane receptor and intracellular kinase integrated. We showed in our previous study that FAK serves as a platform for recruiting G protein, IGF-1R, and Akt to the activated GABA_B receptor and further regulating GABA_B receptor-induced neuroprotection¹⁶. FAK also has high affinity for PI3K and PLC γ ³⁹. PKC and its substrate MARCKS were reported to be activated after FAK phosphorylation⁴⁰, supporting our finding that FAK acts upstream of PKC. However, as the phosphorylation profiles of FAK tyrosine and serine residues are important for distinct signal transduction cascades^{39,41}, how FAK phosphorylation regulates IGF-1R and PKC is still under investigation. Meanwhile, given that PKC translocates from the cytosol to the membrane after GABA_B receptor activation to modulate desensitization⁴², the PKC pathway may play a role in controlling CREB and Fmrp activity.

PAMs bind to the GABA_B receptor at a site distinct from agonists such as R-baclofen (STX209)^{16,25,26}, which was shown to improve FXS-associated symptoms in mice and humans^{7,8,43}. Extensive data from preclinical studies on GABA_B receptor PAMs indicate that their benefits are similar to those of agonists, but with superior side effect profiles⁴⁴. Among them, GS39783 was used to treat FXS mice and showed no significant improvement in an audiogenic seizure test⁴³, consistent with our observation that GS39783 had no effect on CREB activation and Fmrp expression. CGP7930 and Rac BHHF, both of which exhibit PAM agonist activity²⁷, have not yet been tested in an FXS mouse model. In our study, only CGP7930 induced an upregulation in the level of Fmrp expression similar to baclofen. This is consistent with the finding of CGP7930 alone being able to activate ERK_{1/2} and Akt signaling^{14,15}. These results suggest that CGP7930 is a promising candidate for the treatment of FXS symptoms and useful for the development of novel drugs.

Methods

Drugs. GABA was purchased from Sigma (St. Louis, MO, USA). (R)-Baclofen, CGP54626, CGP7930, GS39783, Rac BHHF, PTX, U73122, and PF573228 were purchased from Tocris (Fisher-Bioblock, Illkirch, France). AG1024 was purchased from Santa Cruz Biotechnology (Shanghai, China). Foetal bovine serum (FBS) and other solutions used for cell culture were from Invitrogen (Shanghai, China).

Antibodies. Primary antibodies against phospho-ERK_{1/2} (rabbit monoclonal), ERK_{1/2}, phospho-Akt (Ser473) (193H12; rabbit monoclonal), Akt, phospho-CREB (rabbit), phospho-MARCKS, CREB, IGF-1R β , β -actin, and Fmrp, as well as horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology (Shanghai, China). Antibodies against PKC α and PKC β were from Santa Cruz Biotechnology.

Primary culture of CGNs. All animal experiments were approved by the Animal Experimentation Ethics Committee of the School of Life Science and Technology at Huazhong University of Science and Technology and were carried out in accordance with the approved guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). Primary CGN cultures were established as previously described¹⁴. Briefly, the cerebellum was dissected from 1-week-old

KunMing mice of either sex obtained from Hubei Provincial Center for Disease Control and Prevention. Cells were maintained in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) with F-12 nutrients (Invitrogen) supplemented with 30 mM glucose, 2 mM glutamine, 3 mM sodium bicarbonate, 5 mM HEPES buffer, 30 mM KCl, and 10% FBS.

MEFs culture and transfection. MEFs were cultured in DMEM supplemented with 10% FBS. For RNA interference experiments, MEFs were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Shanghai, China) according to the manufacturer's protocol, using siRNAs against IGF-1R α/β (sc-35638), PKC α (sc-208), PKC β II (sc-210), and FAK (sc-35353) or control siRNA-A (sc-37007) from Santa Cruz Biotechnology. One day after transfection, cells were transfected with HA-GABA $_{B1}$ and Flag-GABA $_{B2}$ plasmids for another 24 h before drug treatment.

Drug treatments. Cultures were washed once with Ca $^{2+}$ -free HEPES-buffered solution (HBS; 10 mM HEPES, 140 mM NaCl, 4 mM KCl, 2 mM MgSO $_4$, 1 mM KH $_2$ PO $_4$, pH 7.4) and pre-incubated at 37 °C in the same solution for 60 min. Drugs were freshly prepared in HBS with or without dimethyl sulfoxide (DMSO)/1 M NaOH. Inhibitor pre-treatment was as follows: AG1024 (0.1 μ M, 1 h), PTX (200 ng/ml, 14–16 h), U73122 (5 μ M, 1 h), PF573228 (10 μ M, 1 h), GF109203x (10 μ M, 1 h) and Gö-6976 (1 μ M, 1 h). Baclofen (100 μ M) and CGP7930 (50 μ M) were applied in time-course experiments. Baclofen (100 μ M) and CGP7930 (50 μ M) were applied for 10 min to detect ERK $_{1/2}$, Akt, CREB, IGF-1R, and MARCKS phosphorylation; baclofen (100 μ M), CGP7930 (50 μ M), GS39783 (50 μ M), or Rac BHF (50 μ M) were applied for 30 min before measuring Fmrp expression. At the end of the treatment, cells were quickly washed with ice-cold phosphate-buffered saline (PBS; pH 7.4) before lysis buffer was added to the cells, which were immediately placed on ice. The cell monolayer was scraped into Eppendorf tubes. HBS containing the same concentration of DMSO or NaOH was used as the vehicle control.

Western blot analysis. Lysates from cultured cells were sonicated and protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories, Hertfordshire, UK). Equal amounts of protein (20 μ g) were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA), which were incubated in blocking buffer (5% non-fat dry milk in Tris-buffered saline and 0.1% Tween 20) for 1 h, followed by incubation with primary antibodies (1:1000) overnight at 4 °C and a 2 h incubation with HRP-conjugated secondary antibodies (1:20,000). Immunoreactivity was visualized on X-ray films using the enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA). The density of the protein bands was measured using Image J software (National Institutes of Health, Bethesda, MD, USA). Protein ratio on the ordinates (Y) axis of the histograms was defined as the ratio between the density of each band and the sum of the densities of all the bands in a given blot.

Reverse transcription PCR. After drug treatment, total cellular RNA was isolated using TRIzol reagent, and reverse transcription was carried out according to the manufacturer's protocol (Invitrogen). First-strand cDNA was generated from 4 μ g total RNA using oligo-dT primer and M-MLV reverse transcriptase (Invitrogen). PCR analysis was performed using the following sense and antisense primers: *Fmr1*, 5'-CCG AAC AGA TAA TCG TCC ACG-3' and 5'-ACG CTG TCT GGC TTT TCC TTC-3' and β -actin, 5'-CCG CCC TAG GCA CCA GGG TG-3' and 5'-GGC TGG GGT GTT GAA GGT CTC AAA-3' (internal control). The mRNA ratio was defined as the ratio between the density of each band and the sum of the densities of all bands in a given gel.

Statistical analysis. Data are presented as mean \pm SEM of at least three independent experiments. Data in Fig. 1B were analysed by the student's t test and statistical analysis of other data was carried out with one-way ANOVA analysis.

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Acknowledgements

The authors thank Drs Jean-Philippe Pin (Institut de Génomique Fonctionnelle, Montpellier, France) and X.Z. Shawn Xu (University of Michigan, Ann Arbor, MI, USA) for critically reading an early version of the manuscript. This work was supported by the National Natural Science Foundation of China (NSFC grant nos. 31225011, 31130028, and 31420103909), the Ministry of Science and Technology (grant no. 2012CB518000), the Program of Introducing Talents of Discipline to the Universities of the Ministry of Education (grant no. B08029), the Program for Changjiang Scholars and Innovative Research Team in University (PCSIRT) [grant no. IRT13016], Natural Science Foundation of Hubei province [grant no. 2014CFA010], and the Mérieux Research Grants Program of Institut-Mérieux (to J.L.).

Author Contributions

J.L. conceived the project; W.Z., C.X., H.T. and J.L. designed the experiments; W.Z., C.X., Y.W., Q.S., H.P. and Y.H. performed the experiments; W.Z., C.X., P.R. and J.L. analysed the results; and C.X. wrote the manuscript with J.L. and P.R.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhang, W. *et al.* GABA_B receptor upregulates fragile X mental retardation protein expression in neurons. *Sci. Rep.* **5**, 10468; doi: 10.1038/srep10468 (2015).



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