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$GABA_B$ receptor upregulates fragile X mental retardation protein expression in neurons

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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 impairment[3]. 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 3β/Akt[6] 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 system[9]. 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 $GABA_{B2}$ subunits[9]. Only $GABA_{B1}$ subunit can bind agonists, whereas $GABA_{B2}$ subunit is responsible for G protein coupling[11]. Positive allosteric modulators bind within the $GABA_{B2}$

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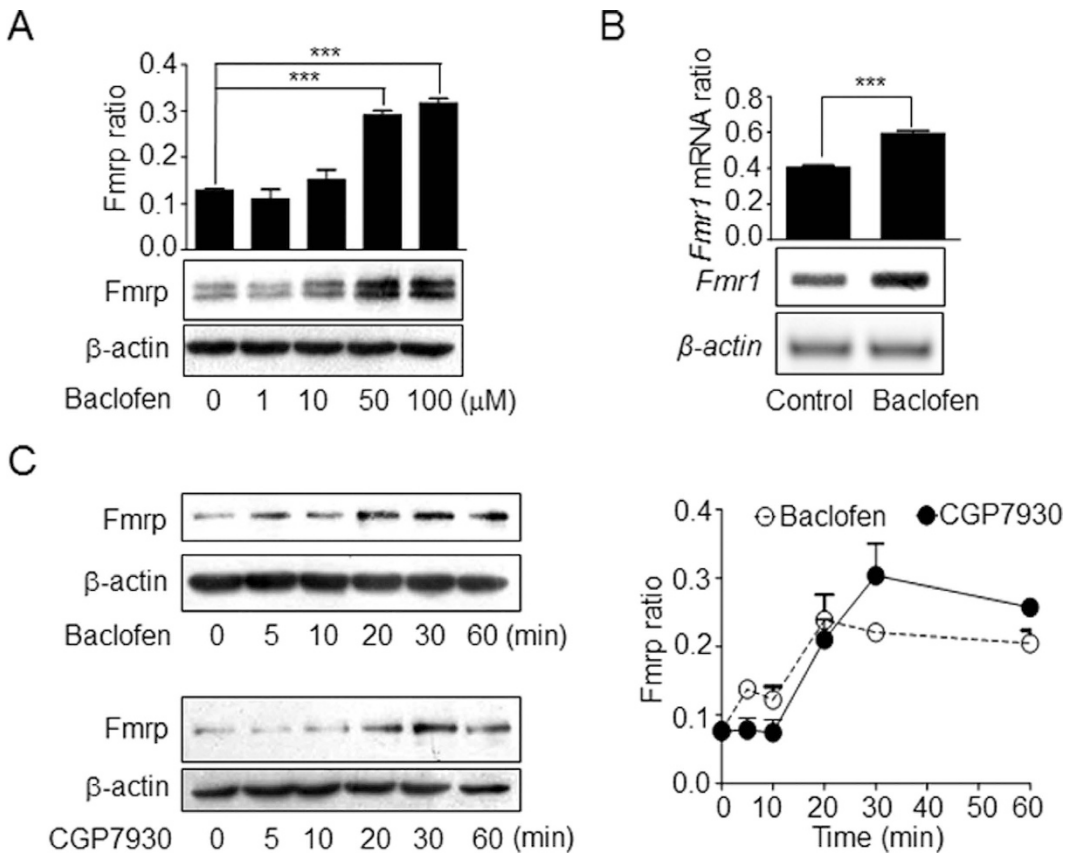

Figure 1. Activation of $GABA_B$ receptor upregulates Fmrp expression in CGNs. (A) Fmrp expression in CGNs treated with indicated concentrations of baclofen. ***$P < 0.001$ vs. basal levels. **(B)** *Fmr1* mRNA expression upon treatment with baclofen. **(C)** Time course of Fmrp expression induced by 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 agonists[12]. Presynaptic $GABA_B$ receptor activation inhibits neurotransmitter release through the depression of voltage-gated Ca^{2+} channels, whereas activated postsynaptic $GABA_B$ receptors open K^+ channels to induce neuronal hyperpolarisation[13]. $GABA_B$ receptor activation induces the $ERK_{1/2}$/cAMP response element-binding protein (CREB) signaling pathway, which is dependent on $G_{i/o}$ protein[14]. $GABA_B$ 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 baclofen[17]; 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, PLCβ 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 (PLCβ 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, PLCβ, 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 treatment[15]. 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 Ca^{2+} -sensitive PKC isotypes[26]. 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 PKCα or PKCβ 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 Ca^{2+} -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-1R[16] 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,

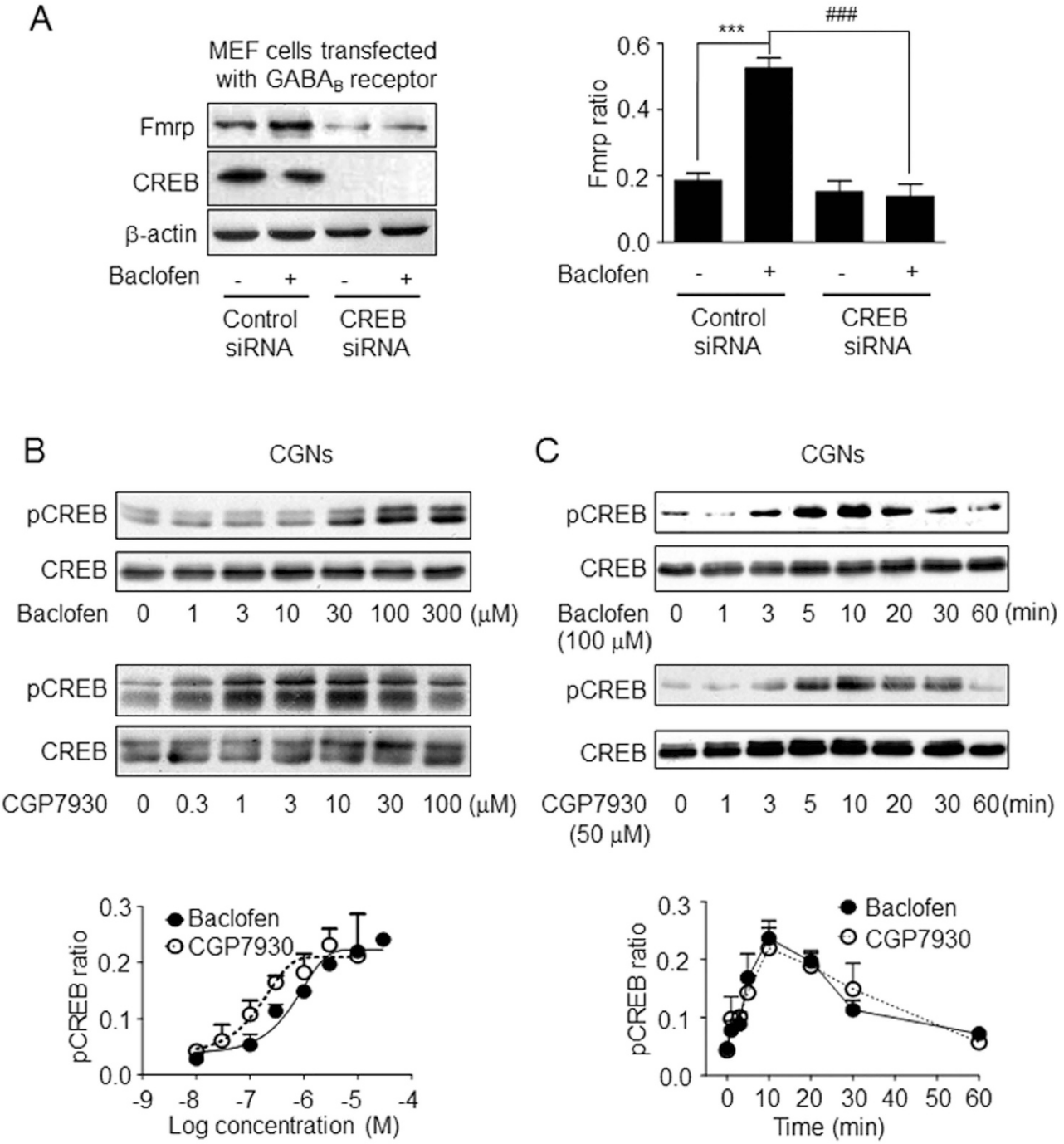

Figure 2. GABA$_B$ receptor-induced CREB signaling is required for Fmrp upregulation. (A) Effect of siRNA-mediated knockdown of CREB on baclofen-induced Fmrp expression in MEFs co-transfected with $GABA_{B1}$ and $GABA_{B2}$. Representative western blots are shown. CREB phosphorylation level in control 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.

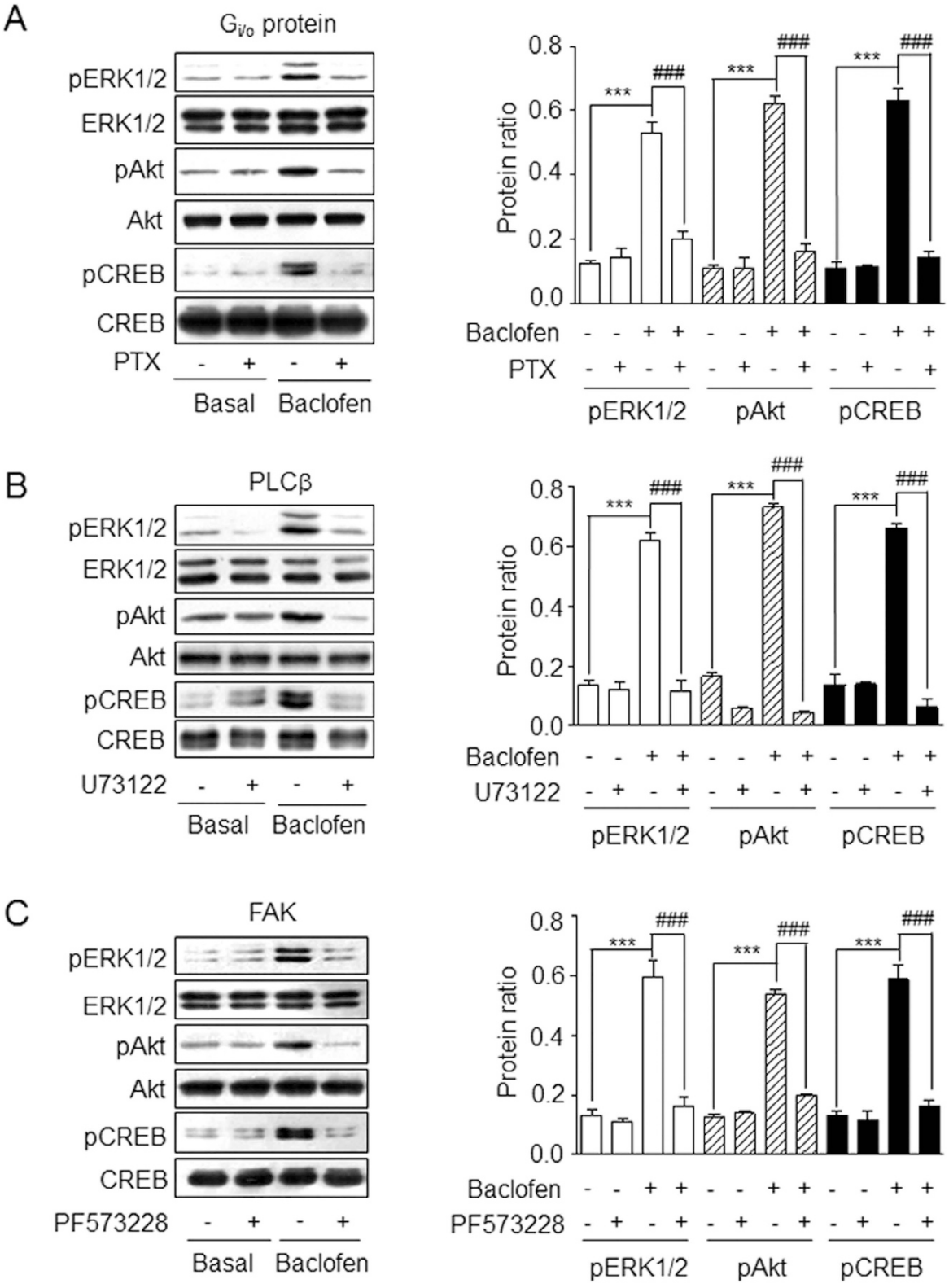

Figure 3. $GABA_B$ receptor-mediated CREB phosphorylation is dependent on $G_{i/o}$ protein, PLCβ, and FAK. CGNs were pretreated with PTX **(A)**, U73122 **(B)**, or PF573228 **(C)** before baclofen-stimulation. CREB, $ERK_{1/2}$ and Akt phosphorylation was detected by western blotting. Protein ratio 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 ± 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.

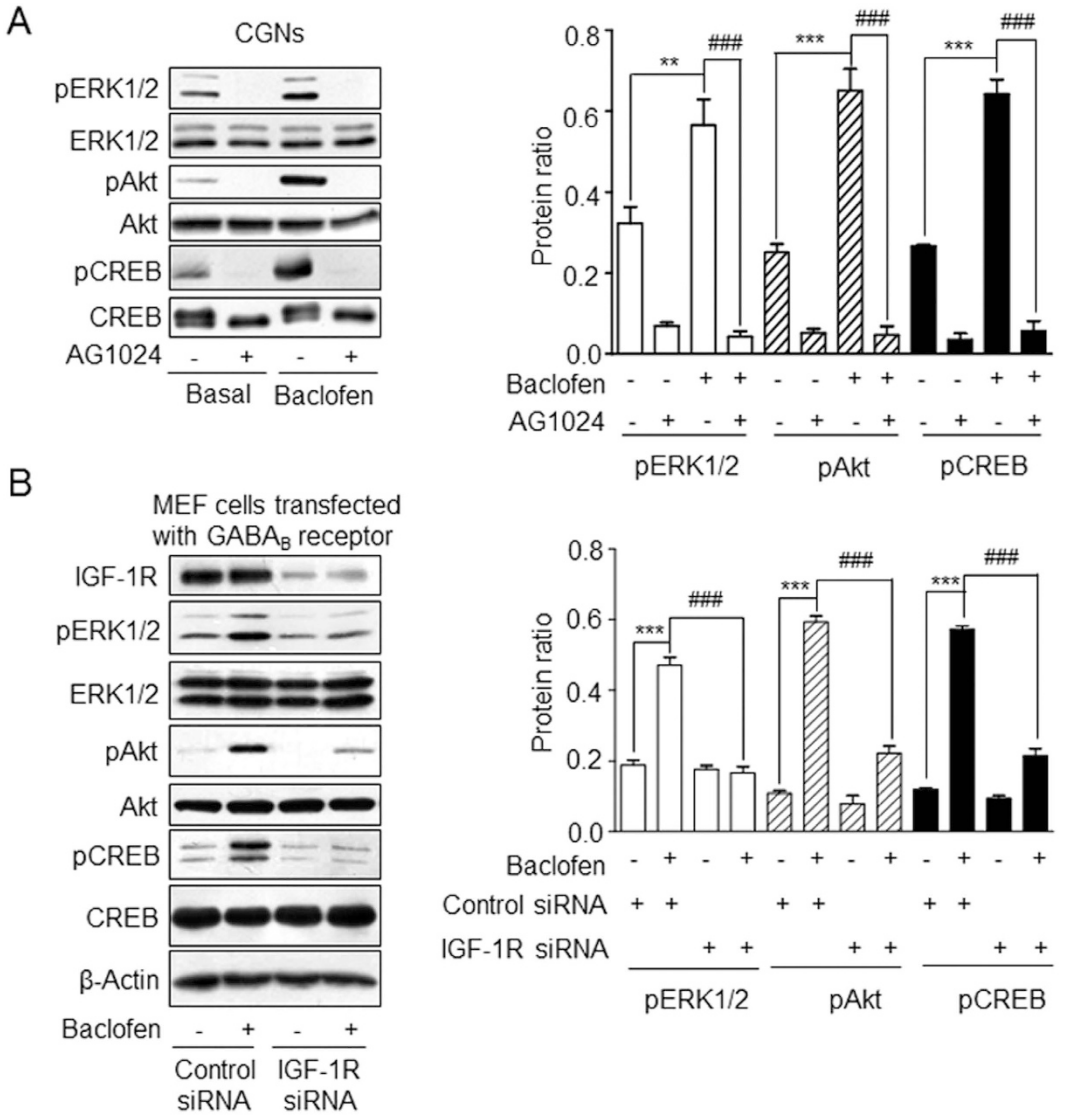

Figure 4. GABA$_B$ receptor-mediated transactivation of IGF-1R is required for CREB phosphorylation. **(A)** CGNs were pre-treated with the IGF-1R inhibitor AG1024 followed by baclofen. Expression levels of pERK$_{1/2}$, pAkt and pCREB were quantified by western blotting. Data represent the mean ± SEM 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

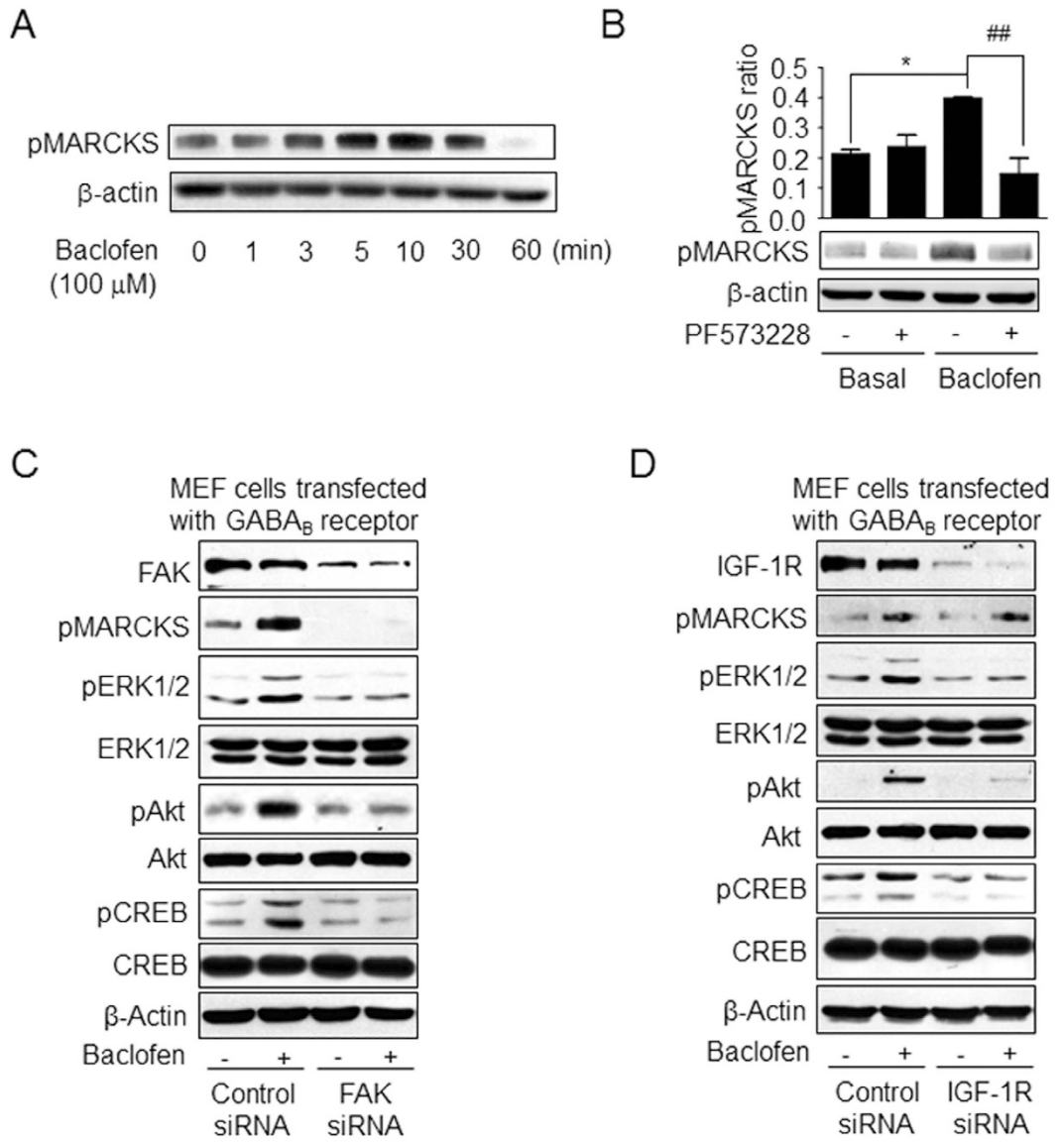

Figure 5. PKC activated by GABA$_B$ receptor acts downstream of FAK and is independent of IGF-1R signaling. (A) Time course of phosphorylation of the PKC substrate MARCKS induced by baclofen in CGNs. **(B)** Effect of the FAK inhibitor PF573228 on MARCKS phosphorylation in CGNs. pMARCKS ratio was defined as the ratio between the density of each band and the sum of the densities of all the bands in a given blot. Values represent the mean ± SEM from three independent experiments. $^{*}P < 0.05$ vs. basal level. $^{\#\#}P < 0.01$ vs. baclofen-treated group. **(C,D)** Effect of siRNA-mediated knockdown of FAK or IGF-1R on baclofen-induced phosphorylation of MARCKS, ERK$_{1/2}$, Akt, and CREB in MEFs co-transfected with GABA$_{B1}$ and GABA$_{B2}$. Full-size blots are shown in **Supplementary Figure S8** and the band of interest is indicated by a red box.

is deleted or expression is blocked[4,30]. One possible explanation to reconcile these different findings may be through the activation of CREB by the GABA$_B$ receptor. CREB is a transcription factor involved in the activation of many genes[25]; CREB phosphorylation at serine 133 promotes its binding to the CRE site and leads to gene transcription[25] and regulates learning and memory[23,31]. CREB-targeted genes may facilitate memory formation through the induction of long-term potentiation or long-term depression of synaptic plasticity[32,33], the growth and formation of new synaptic spines and connections[33,34], or new protein synthesis participating in memory reconstruction[35] which might help to improve cognition in FXS.

Our study suggests a novel physiological role for Frmp in neurons. Indeed, Fmrp is wildly expressed in neurons and participates in a number of intracellular processes involving mRNAs metabolism related to synaptic function and maturation[2,36]. Several reports also implied that Fmrp played a role in neuronal survival and apoptosis[37,38]; the GABA$_B$ receptor was also found to transactivate IGF-1R and protect

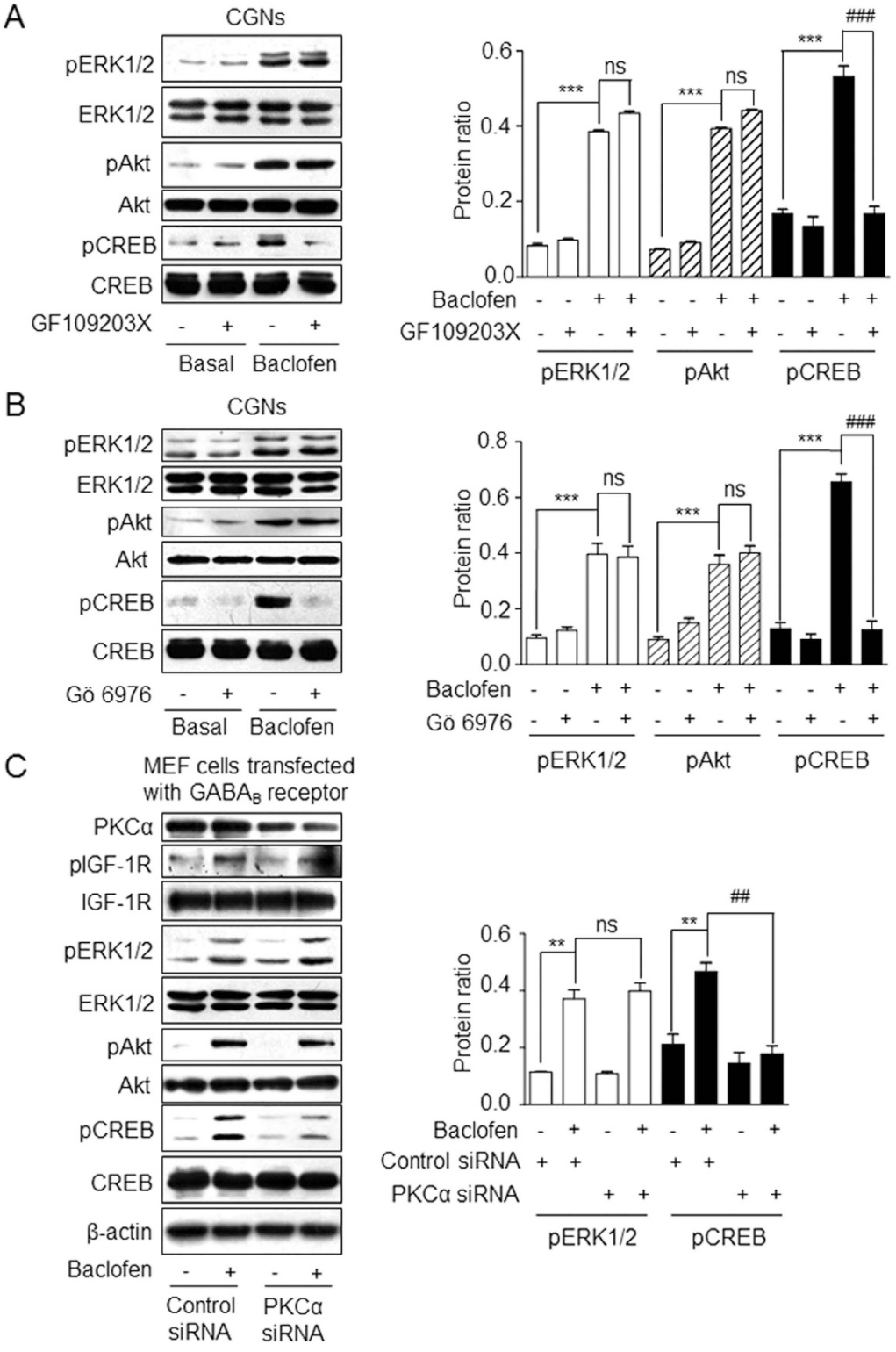

Figure 6. PKC is required for GABA$_B$ receptor-induced CREB activation. (A, B) CGNs were pre-treated with GF109203x (A) or Gö-6976 (B) followed by treatment with baclofen. ERK$_{1/2}$, Akt, and CREB phosphorylation was quantified by western blotting and the protein ratio was defined as in Fig. 3. $^{***}P < 0.001$, 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 PKCα 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 ± 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.

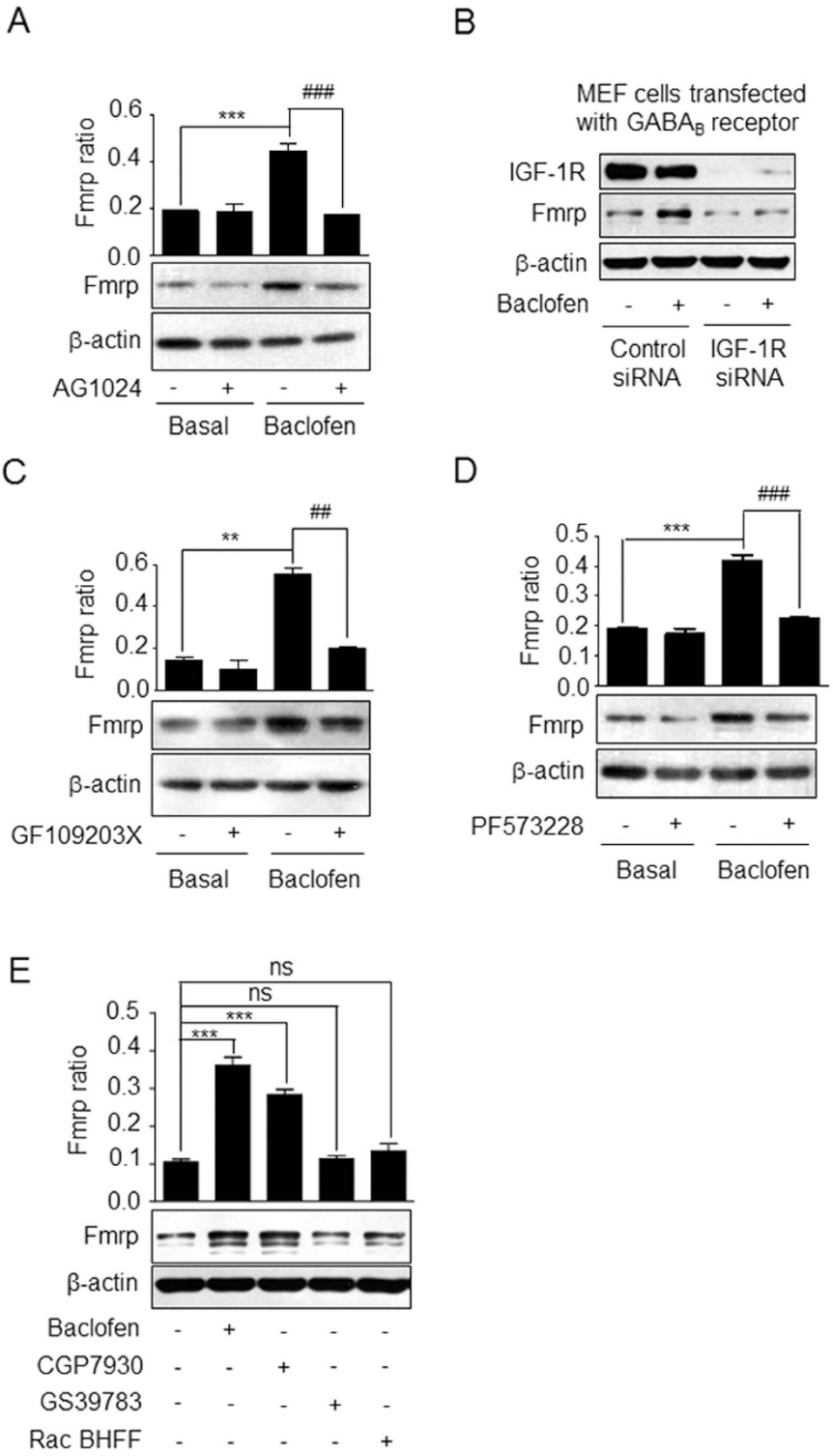

Figure 7. IGF-1R and PKC are involved in GABA$_B$ receptor-mediated Fmrp synthesis. (A) CGNs were pretreated with AG1024 followed by treatment with baclofen. Fmrp levels were detected by western blotting and Fmrp ratio was defined as in Fig. 1A. Data represent the mean ± SEM from three independent experiments. **(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 ± 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 ± 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.

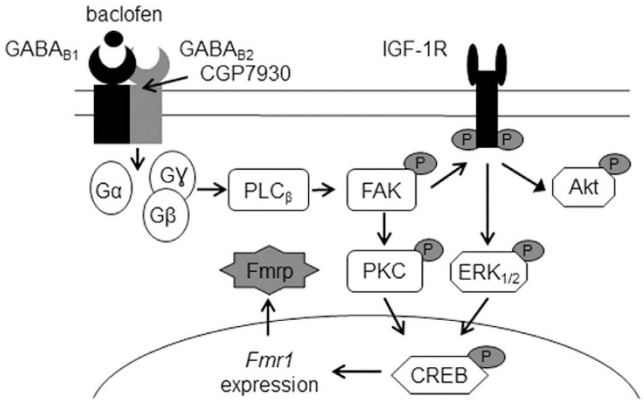

Figure 8. Schematic representation of the signaling pathway mediated by $GABA_B$ receptor leading to CREB activation and Fmrp upregulation in CGNs. Agonist (baclofen) or PAM (CGP7930) activates the $GABA_B$ receptor, leading to $G_{i/o}$ protein/PLCβ/FAK activation, which in turn transactivates the IGF-1R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

neurons against apoptosis[15], 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 neuroprotection[16]. FAK also has high affinity for PI3K and PLCγ[39]. PKC and its substrate MARCKS were reported to be activated after FAK phosphorylation[40], 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 desensitization[42], 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 profiles[44]. Among them, GS39783 was used to treat FXS mice and showed no significant improvement in an audiogenic seizure test[43], consistent with our observation that GS39783 had no effect on CREB activation and Fmrp expression. CGP7930 and Rac BHFF, both of which exhibit PAM agonist activity[27], 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 BHFF, 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-1Rβ, β-actin, and Fmrp, as well as horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology (Shanghai, China). Antibodies against PKCα and PKCβ 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 described[14]. 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-1Rα/β (sc-35638), PKCα (sc-208), PKCβII (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_2PO_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 μM, 1 h), PTX (200 ng/ml, 14–16 h), U73122 (5 μM, 1 h), PF573228 (10 μM, 1 h), GF109203x (10 μM, 1 h) and Gö-6976 (1 μM, 1 h). Baclofen (100 μM) and CGP7930 (50 μM) were applied in time-course experiments. Baclofen (100 μM) and CGP7930 (50 μM) were applied for 10 min to detect $ERK_{1/2}$, Akt, CREB, IGF-1R, and MARCKS phosphorylation; baclofen (100 μM), CGP7930 (50 μM), GS39783 (50 μM), or Rac BHFF (50 μM) 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 μg) 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 μg 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 ± 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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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

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