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HAL Authorization

One-sentence summary: Distinct G protein-mediated pathways mediate neuroprotective GABA<sub>B</sub> receptor signaling.

**Editor's summary:**

**Another G for GABA<sub>B</sub>**

Activation of GABA<sub>B</sub>R, the G protein-coupled receptor for the inhibitory neurotransmitter GABA, is thought to be neuroprotective through exclusive engagement of G<sub>i/o</sub> to transactivate receptor IGF1R. However, Wang *et al.* found that GABA<sub>B</sub>R also engaged G<sub>13</sub> to differentially activate the MAPK pathway kinase JNK. In cultured cerebellar granule neurons, this G<sub>13</sub>-mediated pathway increased the abundance of the postsynaptic scaffolding protein PSD95 and enhanced neuronal survival under low-potassium conditions. The authors further uncovered biological synergy between the two G protein-mediated pathways, with different kinetics in agonist responses. The findings reveal how GABA can mediate neuroprotection through multiple synergistic pathways that depend on distinct G proteins.

## **The GABA<sub>B</sub> receptor mediates neuroprotection by coupling to G<sub>13</sub>**

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### **Abstract**

G protein-coupled receptors (GPCRs) activate various mitogen-activated protein kinase (MAPK) pathways to regulate critical cell functions.  $\beta$ -arrestins mediate this mechanism for most GPCRs but not the GABA<sub>B</sub> receptor (GABA<sub>B</sub>R). When coupled to the G protein G<sub>i/o</sub>, GABA<sub>B</sub>R phosphorylates the kinases ERK1 and ERK2. Here, we uncovered a distinct  $\beta$ -arrestin-independent mechanism of MAPK pathway activation by GABA<sub>B</sub>R. We found that GABA<sub>B</sub>R also phosphorylated the kinase JNK downstream of activation of the small guanosine triphosphatases (GTPases) RhoA and Rac1 in primary mouse neurons. However, instead of G<sub>i/o</sub> proteins, activation of this RhoA/Rac1-JNK pathway was mediated by G<sub>13</sub>. This pathway promoted the phosphorylation and accumulation of the postsynaptic scaffolding protein PSD95 and GABA<sub>B</sub>R-mediated neuroprotection in granule neurons. In addition, this pathway synergized with the previously reported GABA<sub>B</sub>R-mediated neuroprotection mediated by a G<sub>i/o</sub>-dependent mechanism through the MAPK pathway-activating protein IGF1R. GABA<sub>B</sub>R agonists activated G<sub>13</sub> with slower kinetics and lower potency than with which they activated G<sub>i/o</sub>. Our findings reveal distinct,  $\beta$ -arrestin independent, context-specific synergistic mechanisms of MAPK activation by G protein-mediated GPCR signaling.

## Introduction

Most G protein-coupled receptors (GPCRs) generate intracellular signals by activating heterotrimeric G proteins and  $\beta$ -arrestins to regulate cell function (1). These include the mitogen-activated protein kinases (MAPKs), like ERK1/2, p38, and c-Jun N-terminal kinase (JNK) (2, 3) (Fig. 1A). The discovery that  $\beta$ -arrestins can lead to MAPK activation has been essential in establishing a signaling role for this protein in inhibiting G protein signaling (4). Indeed,  $\beta$ -arrestins are shown to promote ERK1/2 and JNK phosphorylation (3, 5, 6). However, not every GPCR can recruit and activate  $\beta$ -arrestins. Among these are the metabotropic receptors for glutamate (mGluRs) and  $\gamma$ -aminobutyric acid (GABA) (GABA<sub>B</sub> receptor), which do not directly couple to  $\beta$ -arrestins (7-9), raising the question of how these receptors activate different MAPKs, if they do.

The GABA<sub>B</sub> receptor is abundantly expressed throughout the brain in both pre- and post-synaptic elements where it maintains the slow and prolonged inhibitory synaptic activity of GABA to modulate various brain functions (10, 11). It is the target of baclofen (Lioresal®), a drug used to treat spasticity associated with multiple sclerosis (12), as well as alcohol abuse disorder (13). The dysfunction of GABA<sub>B</sub> receptor is related to many other mental disorders such as epilepsy, depression, anxiety, chronic pain, schizophrenia and mental retardation (12). Studies have shown a strong connection between the GABA<sub>B</sub> receptor and Alzheimer's disease (14, 15). In addition, the discovery of genetic mutations linked to Rett-like phenotypes (16-18) and to auto-antibodies in encephalitis (19) has highlighted the GABA<sub>B</sub> receptor as an important and potentially promising drug target.

The GABA<sub>B</sub> receptor belongs to the class C GPCR family that is thought to act exclusively through G<sub>i/o</sub> proteins (11, 20). It is composed of two subunits, GABA<sub>B1</sub> (GB1) and GABA<sub>B2</sub> (GB2) (21). Agonists (GABA, baclofen) and antagonists (CGP54626) bind to the extracellular domain of GB1 to control G protein activation by the 7-transmembrane (7TM) domain of GB2 (22). The GB2 subunit is also important for exporting GB1 to the plasma membrane and is the target of positive allosteric modulators (PAMs). Some PAMs, such as CGP7930, also display agonist activity that leads to G<sub>i/o</sub> protein activation (23, 24). Our previously reported work has also illustrated the Cryo-EM structures of the agonist/PAM-bound active state of the GABA<sub>B</sub> receptor complexed with heterotrimeric G<sub>i1</sub> protein (25). Through activation of G<sub>i/o</sub> protein, the GABA<sub>B</sub> receptor induces

the phosphorylation of various downstream signaling molecules, including ERK1/2 (24) as well as insulin-like growth factor 1 receptor (IGF-1R) (26), CREB (27), FAK (26, 28), Src (28), Akt (26, 28), PKC (27), and CaMKII (29), which are critical for GABA<sub>B</sub> receptor-mediated neuroprotection (26), and up-regulation of FMRP and long-term plasticity (27). Activation of the GABA<sub>B</sub> receptor decreases adenylyl cyclase activity (26) and induces Rap-1 activation through G $\alpha_{i/o}$  (30), whereas it inhibits voltage-sensitive calcium channels and activates inwardly rectifying potassium channels through G $\beta\gamma$ , which further weakens presynaptic neurotransmitter release and postsynaptic excitability (10, 12).

JNKs can be activated by a wide range of stimuli including neurotransmitters, hormones, developmental cues, inflammatory cytokines, and extracellular/intracellular pathogens (31). GPCRs and different G proteins can regulate JNK phosphorylation. In the central nervous system, JNKs are important mediators for neuronal development, survival and degeneration (32, 33). JNKs are reported to be key regulators in determining cell fate decisions, such as apoptosis and survival (34). Phosphorylated JNKs can also lead to the phosphorylation of PSD95 which in turn modulates post-synaptic neuronal activity and structure (35). In this study, we investigated whether and how the GABA<sub>B</sub> receptor triggers the activation of JNK.

## Results

### The GABA<sub>B</sub> receptor activates JNK in a G<sub>i/o</sub>-independent manner

The GABA<sub>B</sub> receptor is well-studied as a G<sub>i/o</sub>-coupled receptor for downstream signaling, including ERK1/2 phosphorylation (11, 24). However, whether GABA<sub>B</sub> receptor can activate other MAPKs (such as JNKs) is currently unclear (Fig. 1B). To investigate the role of GABA<sub>B</sub> receptor on JNK phosphorylation, we used CGNs as a cell model. CGNs constitute the largest homogeneous neuronal population in the mammalian brain and expresses high levels of native GABA<sub>B</sub> receptor (12, 36). JNKs are generated by alternative splicing of three genes: *JNK1*, *JNK2* and *JNK3*. They can be phosphorylated on both the Thr<sup>183</sup> and Tyr<sup>185</sup> residues (31). Here, we used antibodies to recognize the total or phosphorylated JNK1, JNK2 and JNK3 proteins. Our data shows that specific GABA<sub>B</sub> receptor agonist baclofen caused a rapid and transient increase of JNK phosphorylation, whereas there was no change in total JNK protein expression (Fig. 1C). JNK phosphorylation peaked at 5 min and then decreased to basal levels at 60 min. A similar activation of JNK was found in human embryonic kidney (HEK293) cells and mouse embryonic fibroblasts

(MEFs) transfected with GABA<sub>B</sub> receptor (fig. S1A). The GABA<sub>B</sub> receptor antagonist CGP54626 blocked baclofen-induced JNK phosphorylation (Fig. 1D), which confirmed the specificity of GABA<sub>B</sub> receptor-mediated JNK activation.

In the GABA<sub>B</sub> heterodimer, the GB2 subunit is mainly responsible for the downstream signaling through G protein activation (9). Allosteric modulators can bind to the 7TM of the GB2 subunit to enhance the agonist effect (23). Meanwhile, some PAMs like CGP7930, can directly activate the GB2 subunit due to their intrinsic agonist activity; they are named ago-PAMs (23, 28). This ago-PAM of the GABA<sub>B</sub> receptor also induced transient JNK phosphorylation (fig. S1B), which further confirmed the involvement of the GB2 subunit in the activation of JNK.

It is well known that the GABA<sub>B</sub> receptor couples to G<sub>i/o</sub> proteins for ERK1/2 phosphorylation (11, 24), which was illustrated by the inhibition of baclofen or CGP7930-induced phosphorylation of ERK1/2 by treatment with pertussis toxin (PTX) (Fig. 1E, fig. S1C). Notably, baclofen or CGP7930 could still activate JNK phosphorylation in PTX-treated CGNs (Fig. 1E, fig. S1C). Similar results were obtained in HEK293 cells transfected with GABA<sub>B</sub> receptor (fig. S1D). These results indicated that the GABA<sub>B</sub> receptor-mediated phosphorylation of JNK involved a G<sub>i/o</sub> protein-independent pathway.

### **G<sub>13</sub> was required for GABA<sub>B</sub> receptor-induced JNK phosphorylation**

The G $\alpha$  subunits of heterotrimeric G proteins are classified into four subfamilies based on their homology: G<sub>s</sub>, G<sub>i</sub>, G<sub>q</sub>, and G<sub>12/13</sub> (37, 38). Our previous data showed that the activation of GABA<sub>B</sub> receptor by baclofen alone was not capable to increase cAMP levels in CGNs, which suggested the GABA<sub>B</sub> receptor cannot activate the G<sub>s</sub> pathway (26). Moreover, baclofen did not induce Ca<sup>2+</sup> signals in CGNs, in contrast to glutamate used as a positive control, indicating that the GABA<sub>B</sub> receptor cannot activate the G<sub>q</sub> type of G proteins (fig. S2A).

We thus examined the possible involvement of G<sub>13</sub> in GABA<sub>B</sub> receptor-mediated JNK activation. First, we used the G $\alpha$ <sub>13</sub> RNA interference (G $\alpha$ <sub>13</sub> siRNA) to decrease the expression of G $\alpha$ <sub>13</sub> in CGNs. Our results showed that reduction of G $\alpha$ <sub>13</sub> expression abolished the baclofen-induced JNK phosphorylation but had no effect on baclofen-activated ERK1/2 phosphorylation (Fig. 2A). Then, we applied the synthetic carboxyl terminal peptide of G $\alpha$ <sub>13</sub> (G $\alpha$ <sub>13</sub>-CT) combined with TAT peptide which make the peptide permeable into CGNs. The G $\alpha$ <sub>13</sub>-CT is reported to inhibit G $\alpha$ <sub>13</sub> downstream signaling transduction (39, 40). Our data showed that the incubation with

$G\alpha_{13}$ -CT peptide, inhibited baclofen-stimulated JNK phosphorylation in CGNs (fig. S2B). Similarly, in HEK293 cells expressing  $GABA_B$  receptor, we knocked down  $G\alpha_{13}$  using siRNA (fig. S2C) and, separately, co-transfected cells with  $G\alpha_{13}$ -CT cDNA (fig. S2D). The results showed that  $GABA_B$  receptor-activated JNK phosphorylation was blocked upon inhibition of  $G\alpha_{13}$  activity.

Next, we used wild-type (WT) MEFs and  $G\alpha_{12/13}$ -deficient MEFs transfected with the  $GABA_B$  receptor to further confirm the effect of  $G\alpha_{13}$ . Our data showed that baclofen stimulation could induce JNK phosphorylation in WT MEFs expressing the  $GABA_B$  receptor (GB1+GB2), but no activation of JNK was observed in  $G\alpha_{12/13}$ -deficient MEFs expressing the  $GABA_B$  receptor (Fig. 2B). Furthermore, overexpression of  $G\alpha_{13}$  in  $G\alpha_{12/13}$ -deficient cells expressing the  $GABA_B$  receptor showed the recovery of JNK activation mediated by the  $GABA_B$  receptor (Fig. 2C). Whereas ERK1/2 activation induced by baclofen was not changed in WT MEFs,  $G\alpha_{12/13}$ -deficient cells expressing the  $GABA_B$  receptor co-transfected without or with  $G\alpha_{13}$ . Together, these results indicated that  $G\alpha_{13}$  was necessary for  $GABA_B$  receptor-mediated JNK phosphorylation. The recovery of JNK activation was also observed during overexpression of  $G\alpha_{12}$  (fig. S2E). Because  $G\alpha_{12}$  and  $G\alpha_{13}$  have similar functions (41), we focused here on the  $G\alpha_{13}$  protein.

### **The $GABA_B$ receptor activated $G_{13}$ protein**

To ensure that  $G\alpha_{13}$  protein could be directly activated by the  $GABA_B$  receptor, we performed a BRET assay based on the rearrangement or the dissociation of  $G\alpha_{13}$  and  $G_{\beta\gamma}$  in HEK293 cells, leading to a decrease in the BRET ratio (Fig. 3A) (42, 43). First, we showed that the activation of  $GABA_B$  receptor induced a conformational change between the  $G\alpha_o$  and  $G_{\beta\gamma}$  subunits leading to a decreased BRET ratio (Fig. 3A), as previously reported (23). We then constructed the  $G\alpha_{13}$  BRET sensor composed of  $G\alpha_{13}$ -Nanoluc (Nluc) and  $G\gamma 2$ -venus, based on a previously reported BRET sensor for the  $G_{13}$  protein (43). Our data showed that baclofen treatment induced a decrease in the BRET ratio for the  $G\alpha_{13}$  BRET sensor, in contrast to the PBS control (Fig. 3B). Notably, pretreatment with PTX completely inhibited the BRET ratio of the  $G\alpha_o$  BRET sensor signal, whereas it resulted in an increase of the baclofen-induced BRET ratio of the  $G\alpha_{13}$  sensor (Fig. 3B). It is also notable that the activation of  $G\alpha_{13}$  was significantly slower than that of  $G\alpha_i$  or  $G\alpha_o$  (fig. S3A). Baclofen-induced  $GABA_B$  receptor- $G\alpha_{13}$  activation was concentration dependent; higher doses of agonist were necessary compared to the signal generated by the  $G\alpha_o$  sensor. The GABA  $EC_{50}$  for activating  $G\alpha_{13}$  was 10-fold higher than that measured with the  $G\alpha_o$

sensor (Fig. 3C). The baclofen-induced change in the  $G\alpha_{13}$  sensor could be reversed by the  $GABA_B$  receptor antagonist CGP54626 (fig. S3B). Together, these data demonstrated that the  $GABA_B$  receptor could activate  $G_{13}$  proteins.

We further analyzed if  $GABA_B$  receptor could activate endogenous  $G\alpha_{13}$  both in  $GABA_B$  receptor-expressing HEK293 cells and in CGNs endogenously expressing this receptor by using a  $G\alpha_{13}$  activation assay kit (44). The activation of  $G\alpha_{13}$  was expected to increase the proportion of  $G\alpha_{13}$  proteins loaded with GTP. In HEK293 cells transfected with the  $GABA_B$  receptor and CGNs, baclofen strongly increased the levels of  $G\alpha_{13}$ -GTP (Fig. 3, D and E, respectively). Such an effect was also obtained by CGP7930 in CGNs (fig. S3C). Activation of  $G\alpha_{13}$  by adding  $GTP\gamma S$  in the assay was used as a positive control, as part of the  $G\alpha_{13}$  protein was expected to bind this GTPase-resistant version of GTP. GDP was used as a negative control to promote the  $G\alpha_{13}$  protein in the GDP form. These results demonstrated that activation of the  $GABA_B$  receptor induced endogenous  $G\alpha_{13}$  protein activation.

We also found that  $G\alpha_{13}$  activation was much slower than that of  $G\alpha_{i/o}$  (Fig. 3B). We found that the  $G\alpha_i$  protein could be co-immunoprecipitated with the  $GABA_B$  receptor, although to lesser extent after 5 min of baclofen stimulation (Fig. 3F), which was consistent with previous results (45). In contrast,  $G\alpha_{13}$  could be immunoprecipitated with the  $GABA_B$  receptor only after 5 min of activation with baclofen (Fig. 3F). These results were consistent with  $G\alpha_{i/o}$  being pre-assembled with the  $GABA_B$  receptor, whereas  $G\alpha_{13}$  was likely recruited to this receptor after activation.

### **$GABA_B$ receptor activated RhoA and Rac1 through $G_{13}$ protein coupling**

The small Rho GTPase including RhoA, Rac1 and Cdc42, are reported as downstream effector of  $G_{12/13}$  family (46-48). First, we evaluated the effect of  $GABA_B$  receptor activation on RhoA GTPases activity. We used the RhoA-binding domain of the RhoA effector, rhotekin, to precipitate active RhoA (RhoA-GTP). Results showed that baclofen induced transient RhoA activation (Fig. 4A). Next, we pre-treated the CGNs with RhoA inhibitor, which abolished baclofen induced JNK activation. As a control, it showed no effect on baclofen-induced ERK1/2 phosphorylation, which is  $G\alpha_{i/o}$  protein dependent (Fig. 4B). These results verified that  $GABA_B$  receptor activated  $G\alpha_{13}$ , further to RhoA and JNK activation.

Rac1 is another member of Rho GTPase family, which is also reported to be a downstream effector of  $G\alpha_{13}$  proteins (47-49). Rac1 activation can also result in JNK phosphorylation (50). We

then analyzed whether Rac1 could be an intermediate in the GABA<sub>B</sub> receptor activation of JNK in neurons using a Rac1-GTP pulldown assay (51). This assay is based on the recombinant protein glutathione S-transferase (GST) fused to PAK Rac1 binding domain (PBD). The PBD protein can bind to the GTP form of Rac1 and then pulldown the Rac1-GTP attached to glutathione-sepharose 4B beads through GST. Both baclofen and CGP7930 stimulation induced Rac1 activation in CGNs. Baclofen induced a transient Rac1 activation with a peak at 3 min (Fig. 4C), whereas CGP7930 induced a more sustained Rac1 activation from 3 to 10 min (fig. S4A). Meanwhile, we pretreated in CGNs with NSC23766, a Rac1 specific inhibitor that prevents Rac1 GEF interaction with Rac1 (52). We found that NSC23766 blocked baclofen-induced activation of JNK (Fig. 4D), whereas there was no effect on baclofen-increased ERK1/2 phosphorylation. These data indicated that GABA<sub>B</sub> receptor-induced JNK phosphorylation was also dependent on Rac1.

Meanwhile, we confirmed that baclofen can also induce Rac1 activation in HEK293 cells (fig. S4B). Co-transfection of a dominant-negative mutant for Rac1 (Rac1N17) with GABA<sub>B</sub> receptor in HEK293 cells resulted in the inhibition of baclofen-induced JNK activation (fig. S4C). These data further demonstrated that GABA<sub>B</sub> receptor activate Rac1 to induce JNK phosphorylation.

We further confirmed that role of G $\alpha$ <sub>13</sub> in GABA<sub>B</sub> receptor-induced RhoA and Rac1 activation in CGNs. First, we decreased G $\alpha$ <sub>13</sub> expression in CGNs by RNAi experiment. We found that G $\alpha$ <sub>13</sub> siRNA abolished baclofen-induced RhoA-GTP increase, as well as the positive control AngII (Fig. 4E). Baclofen-increased Rac1 activation was also inhibited by G $\alpha$ <sub>13</sub> siRNA (Fig. 4F) or pre-treatment of G<sub>13</sub>-CT peptide (fig. S4D). Next, we pre-incubated the neurons with PTX, which inhibits G $\alpha$ <sub>i/o</sub> protein activity. PTX failed to decrease baclofen-induced Rac1-GTP in CGNs (fig. S 4E). These data thus supported that GABA<sub>B</sub> receptor led to RhoA and Rac1 activation through G $\alpha$ <sub>13</sub> proteins.

### **GABA<sub>B</sub> receptor increased PSD95 phosphorylation and synaptic accumulation through JNK**

We then examined the possible consequences of JNK phosphorylation by the GABA<sub>B</sub> receptor in CGNs. Both Rac1 and JNK are reported to be involved in the dynamics of synapses and morphogenesis (53-56). Enhancement of PSD95 expression and synaptic plasticity is accompanied with an increased expression of postsynaptic GABA<sub>B</sub> receptor (57). We then investigated if GABA<sub>B</sub> receptor activation regulated PSD95 through JNK in CGNs. Our data showed that GABA<sub>B</sub> receptor activation increased the phosphorylation of PSD95 at Ser<sup>295</sup> (Fig.

5A). Phosphorylation of PSD95 was fast, reached a maximum level at 5 min, persisted for at least 60 min and was inhibited by either CGP54626 (fig. S5A) or the JNK inhibitor SP600125 (Fig. 5B), or  $G\alpha_{13}$  siRNA (Fig. 5C), but not by PTX (Fig. 5D) or an ERK1/2 inhibitor (fig. S5B). The long-time stimulation of CGNs with baclofen increased the accumulation of PSD95, an effect significantly decreased by the  $GABA_B$  receptor antagonist CGP54626 or by the JNK inhibitor SP600125 (Fig. 5E). Next, we used two  $G\alpha_{13}$  shRNA lentivirus to decrease the expression of  $G\alpha_{13}$  in CGNs. The shRNA lentivirus-infected neurons were evident through the expression of GFP (fig. S5C). These neurons showed significant impairment of baclofen-induced PSD95 accumulation in dendrites (Fig. 5F, fig. S5D). These results indicated that  $GABA_B$  receptor activation increased PSD95 phosphorylation and its synaptic accumulation through JNK.

### **$GABA_B$ receptor activation protected neurons from apoptosis through JNK**

JNKs are key regulators of cell fate decisions, such as apoptosis and survival (34). CGN is a widely used model to study the mechanisms of neuronal apoptosis or survival (36). CGNs survival can be maintained in high (30 mM) potassium-containing media, whereas reducing the potassium concentration to 5 mM leads to rapid neuronal cell death. We have previously shown that  $GABA_B$  receptor activation protects CGNs from apoptosis through the  $G_{i/o}$ /IGF-1R/Akt signal axis (26). Thus, we determined if the  $GABA_B$  receptor also protected neurons from apoptosis through the  $G_{13}$ /JNK pathway. Baclofen significantly decreased caspase-3 cleavage in induced low potassium (5 mM) conditions (Fig. 6A), whereas the effect of baclofen was blocked both by the JNK inhibitor SP600125 and by the IGF-1R inhibitor AG1024. Furthermore, baclofen failed to show the neuroprotective effect when  $G\alpha_{13}$  expression was decreased by  $G\alpha_{13}$  shRNA lentivirus in CGNs (fig. S6). These data indicated that the  $GABA_B$  receptor protected CGNs from apoptosis through the activation of both the  $G_{i/o}$  and  $G_{13}$  signaling pathways.

### **Discussion**

This study revealed that the  $GABA_B$  receptor—which was assumed to exclusively couple to  $G_{i/o}$  protein—also activated  $G_{13}$  in both transfected cells and primary neurons. These data add the  $GABA_B$  receptor to the list of GPCRs demonstrated to couple to this G protein type (58). Moreover, we showed that this previously unknown signaling mechanism of the  $GABA_B$  receptor leads to the activation of RhoA and Rac1, and the subsequent phosphorylation of JNK. Such a  $GABA_B$  receptor-mediated pathway phosphorylated PSD95 and increased its expression in

dendrites and was essential for GABA<sub>B</sub> receptor-induced neuroprotection (Fig. 6B). Considering that GABA<sub>B</sub> receptor can also activate ERK1/2 through G<sub>i/o</sub> activation, these results showed that two different pathways of a given GPCR can lead to the activation of two types of MAPKs.

The GABA<sub>B</sub> receptor is well recognized as being coupled to G<sub>i/o</sub> protein (11, 20, 59), but our data clearly established that the GABA<sub>B</sub> receptor can also activate G<sub>13</sub>, though with different kinetics. Indeed, whereas the G<sub>i/o</sub> protein was activated within a few seconds, the G<sub>13</sub> activation occurred with 5-10 times slower kinetics. This was reminiscent to what was previously reported for the thrombin receptor (the protease activating receptor 1, PAR1) coupling to G<sub>i</sub> and G<sub>12</sub>. In that case, it was shown that G<sub>i</sub> was pre-coupled to PAR1, whereas G<sub>12</sub> had to be recruited to the receptor (60). It is possible that the same also occurs for the GABA<sub>B</sub> receptor. Indeed, the analysis of the GABA<sub>B</sub> receptor proteome revealed that both G<sub>i</sub> and G<sub>o</sub> are part of the native GABA<sub>B</sub> receptor complex, but not G<sub>13</sub> (45). However, more work is needed to demonstrate this is responsible for the difference in activation kinetics. Such a difference in G protein activation kinetics is in accordance with their downstream effects, as G<sub>i</sub> and G<sub>o</sub> coupling leads to relatively rapid responses such as regulation of Ca<sup>2+</sup> or K<sup>+</sup> channels (10), whereas G<sub>13</sub>-mediated responses, such as PSD95 regulation or inhibition of apoptosis, do not need to be activated as fast.

A second major difference between G<sub>i/o</sub> and G<sub>13</sub> coupling is the potency at which agonists activate these two pathways, a ten-fold lower agonist potency was measured with the G<sub>13</sub> BRET sensor, than with the G<sub>i</sub> or G<sub>o</sub> sensors. It is recognized that the activated G protein-GPCR complex displays a high affinity for agonists. Because this is the GPCR-G protein complex that is at the origin of signaling, agonist potency is close to the agonist affinity to this complex (61, 62). This is particularly the case when directly measuring G protein activation rather than a downstream signal that may be amplified leading to a further increase in agonist potency. As such, one may propose that G<sub>13</sub> stabilized the GABA<sub>B</sub> receptor in a lower agonist affinity state than G<sub>i</sub> or G<sub>o</sub>.

The GABA<sub>B</sub> receptor is the best characterized heterodimeric GPCR (9). Activation of the receptor leads to a major change of the GB1-GB2 interfaces at the level of the 7TM domains (63). Such a rearrangement results in the full activation of the GB2 7TM domain that is then responsible for G protein activation (64)(Chen et al., 2021). However, although the GB1 7TM domain does not appear to change conformation during this process (Chen et al., 2021), it is required for optimal GB2-7TM activation (65). These conclusions were made based on G<sub>i/o</sub> coupling analyses, it will

be of interest to examine whether the GB2 subunit is also responsible for G<sub>13</sub> coupling. Because the GABA<sub>B</sub> receptor ago-PAM CGP7930 also activated G<sub>13</sub>, and because this compound is likely binding at the GB1-GB2 7TM interface, as the other GABA<sub>B</sub> receptor PAMs (several ref), this suggests that the GB2 subunit may also be responsible for G<sub>13</sub> activation. More work is needed to clarify this issue, but this will reveal the importance of each subunit in a receptor heterodimer in the coupling to different G proteins.

GABA<sub>B</sub> receptor belongs to the class C GPCRs, and most of the receptors in this family form constitutive dimers (67). Though a large proportion of G<sub>12/13</sub>-coupled class A and class B GPCRs have been identified (68), GABA<sub>B</sub> receptor is the second class C GPCR able to activate G<sub>13</sub>, as the calcium-sensing receptor (CaSR) has also been reported to activate this G protein (69). Whether other G<sub>i/o</sub>-coupled class C GPCRs can activate G<sub>13</sub> will be interesting to investigate.

The MAPK network can be regulated by different G protein subtypes (2). Here, we demonstrated that GABA<sub>B</sub> receptor-activated G<sub>13</sub> activated JNK and G<sub>i/o</sub> activated ERK1/2, respectively. This differential regulation of MAPK signaling is consistent with observations in other GPCRs (2). In the present study, we further revealed specific functional consequences following different G protein activation by GABA<sub>B</sub> receptor. First, GABA<sub>B</sub> receptor activated G<sub>13</sub> and JNK leading to the phosphorylation of synaptic protein PSD95 and then likely to the related synaptic strength, whereas the G<sub>i/o</sub> and ERK1/2 pathways were not involved, which indicated that the GABA<sub>B</sub> receptor activated the G<sub>13</sub>/JNK pathway to modulate neuronal functions. Second, both GABA<sub>B</sub> receptor-induced G<sub>13</sub>/JNK and G<sub>i/o</sub>/IGF-1R/Akt activation were required for the anti-apoptotic effect. Inhibition of JNK or IGF-1R alone blocked the anti-apoptotic effect of the GABA<sub>B</sub> receptor, which suggested that neither the G<sub>i/o</sub> nor G<sub>13</sub> pathways were sufficient. The synergy of G<sub>13</sub> and G<sub>i/o</sub> signals was required for the GABA<sub>B</sub> receptor-induced neuroprotective effect and highlighted the importance of the G<sub>13</sub>/JNK pathway to control GABA<sub>B</sub> receptor function.

RhoA is well-accepted as the downstream of G<sub>12</sub> and G<sub>13</sub> family. Rac1 can also be activated by G<sub>13</sub> protein as reported in many papers (46-48, 70, 71). Both of them can lead to the activation of JNK (47, 70-74). Here, we found that GABA<sub>B</sub> receptor activation can induce both RhoA and Rac activation, through G<sub>α13</sub>, and in turn induce JNK activation, which is consistent with previous findings in other GPCRs. RhoA and Rac1 can show similar effect on the regulation of the dendrite

number, the synaptic activity and the improvement of learning and memory (53). However, different roles of RhoA and Rac1 have also been indicated in other function such as neurite outgrowth (75, 76). Here we confirmed the role of  $G_{13}$  in the  $GABA_B$  receptor-regulated synaptic activity and neuroprotection. Whether the RhoA and Rac1 display respective roles in the  $GABA_B$  receptor/ $G_{13}$ -mediated neuronal function, remains to be investigated later.

In conclusion, our findings uncovered that the  $G_{i/o}$ -coupled  $GABA_B$  receptor uses the  $G_{13}/Rac1/JNK$  signaling axis to regulate neuronal functions, such as PSD95-related post-synaptic strength and neuroprotection.  $GABA_B$  receptor-induced  $G_{13}$  signaling may provide clues for a better understanding of the multiple effects of  $GABA_B$  receptor in the brain, opening new possibilities for drug development. Moreover, our data revealed that a GPCR can activate different MAPK pathways by activating different G proteins, a double signaling capacity that appears to be necessary for some of the GPCR functions, as illustrated here with the anti-apoptotic effect resulting from  $GABA_B$  receptor activation.

## Materials and Methods

### Reagents

GABA was purchased from Sigma. Baclofen (#0796), CGP54626 (#1088), CGP7930 (#1513) and NSC23766 (#2161) were purchased from Tocris Biosciences. Pertussis toxin (PTX, #516560) and SP600125 (#420119) were bought from Merck Millipore.

Antibodies to phospho-P42/44 (pERK1/2, Thr<sup>202</sup>/Tyr<sup>204</sup>; #9101), P42/44 (ERK1/2; #9102), phospho-JNK (Thr<sup>183</sup>/Tyr<sup>185</sup>; #9251), JNK (#9252), phospho-PSD95 (Ser<sup>295</sup>; A8F8Z, #45737), PSD95 (D27E11; #3450), Rac1/Cdc42 (#4651S), caspase-3 (#9662),  $\beta$ -actin (#4967), mouse IgG (#7076, HRP-linked), and rabbit IgG (#7074, HRP-linked) were purchased from Cell Signaling Technology. The  $G\alpha_{13}$  (A-20) polyclonal antibody (SC-410), mouse  $G\alpha_{13}$  siRNA (#sc-35428), human  $G\alpha_{13}$  siRNA (#sc-35427) and control siRNA (#sc-37007) were purchased from Santa Cruz Biotechnology. The  $G\alpha_{13}$  activation kit (#80401) was purchased from Neweast Bioscience. The Cy3-conjugated antibody to rabbit IgG- (#111-165-003) was purchased from Jackson ImmunoResearch.  $G\alpha_{13}$  shRNA (shRNA1: CCGAGAGAAGCTTCATATT; shRNA2: GGAGACAAGTTGATGGCAT) and control shRNA (TTCTCCGAACGTGTACAGT) Lentivirus were constructed by Obio Technology. Cell-permeable RhoA inhibitor (#CT04-B) was purchased from Cytoskeleton.

Basal medium Eagle (BME, #21010-046), fetal bovine serum (FBS, #10437028), penicillin-streptomycin (#15140-122), Versene (#15040-066), Lipofectamine 2000 (#11668019), Pierce<sup>TM</sup> BCA protein assay kit (#23225), and Pierce<sup>TM</sup> enhanced chemiluminescence reagents (#37074) were purchased from Thermo Fisher Scientific. Poly-L-ornithine (#P4638) was bought from Sigma. Proteinase cocktail inhibitor (#04693159001) was bought from Roche. Fluor-save reagent (#AR1109) was purchased from Boster Bio. The  $G\alpha_{13}$ -CT peptide from the amino acids 366-377 of  $G\alpha_{13}$  with the TAT sequence (YGRKKRRQRRRGILHDNLKQLMLQ) was synthesized with 95% purity in Qiang Yao Biotechnology Company. Glutathione-sepharose 4B beads (#17-0756-01) were purchased from GE Healthcare Life Sciences. Nitrocellulose membranes (#HATF00010) were from Millipore. Coelenterazine H (#S2011) and Furimazine (#N1110) were obtained from Promega.

### Cerebellar granule neuronal cultures, transfection, and RNA interference

CGNs were cultured as described previously (26). All animal experiments were approved by the Animal Experimentation Ethics Committee of the College 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. Briefly, 7-day-old KunMing mice pups obtained from the Hubei Provincial Research Institute of Experimental Animals were decapitated, and the cerebellums were dissected. The cerebellums were dissociated with Versene and gently triturated using a 1-ml pipette. The homogenate was centrifuged at 1000 rpm for 5 min. The pellet was re-suspended and seeded in dishes pre-coated with poly-L-ornithine. CGNs were cultured in BME medium, supplemented with 10% FBS, 2 mM glutamine and 30 mM KCl for 4 to 5 days before drug treatment. The cultures contained about  $1.25 \times 10^5$  cells/cm<sup>2</sup>.

For the RNAi experiment in CGNs, the mixtures of the siRNA and lipofectamine 2000 (Thermo Fisher, 11668019) in a ratio of 1:3, in Opti-MEM medium (Gibco, ref 31985070) were prepared. The pellet was re-suspended with culture medium and seeded into dishes previously coated with poly-L-ornithine (Sigma). Then, the mixture of DNA and lipofectamine 2000 was added. The CGN were treated with drugs 3 days after the transfection.

For the shRNA Lentivirus infection, CGNs were infected with G $\alpha_{13}$  shRNA (1:1000) and control shRNA (1:1000) Lentivirus 24 hours after seeding. Then CGNs were stimulated with baclofen for 16 hours after 48 hours infection and fixed for immunofluorescence.

### **Drug treatments**

Cells were washed once with Ca<sup>2+</sup>-free HEPES-buffered solution (HBS, containing 10 mM HEPES, 140 mM NaCl, 4 mM KCl, 2 mM MgSO<sub>4</sub>, and 1 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) and pre-incubated at 37 °C with or without the indicated inhibitors dissolved in HBS. The concentration and treatment time of drugs were as follows: CGP54626 (20  $\mu$ M, 30 min), SP600125 (20  $\mu$ M, 30 min), PTX (250 ng/mL, 16 to 18 hours), G $\alpha_{13}$ -CT peptide (100  $\mu$ g/mL, 1 hour), RhoA inhibitor (1 $\mu$ g / ml, 4 hours) and NSC23766 (50  $\mu$ M, 30 min). At the end of the treatment, cells were washed quickly with ice-cold PBS and lysed with 200  $\mu$ L of RIPA lysis buffer supplemented with proteinase cocktail inhibitor.

### **Western blotting analysis**

Cell lysates were sonicated, and protein concentrations were determined using the BCA protein assay kit. Equal amounts of protein (20  $\mu$ g) were separated by SDS-PAGE on 10-12% gels. Proteins were transferred to nitrocellulose membranes and washed in blocking buffer (5% nonfat dry milk in TBS and 0.1% Tween 20) for 2 hours at room temperature. The blots were incubated with primary antibodies at the relevant dilution overnight at 4 °C, and then incubated with horseradish peroxidase-linked secondary antibodies for 2 hours at room temperature. Immunoblots were detected using Pierce<sup>TM</sup> enhanced chemiluminescence reagents and visualized by X-ray film or the Tanon 5200 western blot Chemiluminescence imager (Tianneng Biotechnology). The density of immunoreactive bands was measured by Image J software. All bands were normalized to the basal control.

### **G $\alpha$ <sub>13</sub>-CT construction**

To generate a construct encoding the C-terminal G $\alpha$ <sub>13</sub> (amino acids 322 to 378), short complimentary oligonucleotides encoding the peptide sequences were obtained from the G $\alpha$ <sub>13</sub> cDNA, which was generously donated by Philippe Rondard (Institut de Génomique Fonctionnelle, France). Primers for PCR are as follows: forward primer: CGGGATCCACCATGGTCCAAAATTCCTGGTG; reverse primer: CGCGAATTCTCAC TGTAGCATAAGCTGC. The C-terminal of G $\alpha$ <sub>13</sub> was inserted into the pcDNA3.1 vector and verified by DNA sequencing.

### **Ca<sup>2+</sup> release assay in CGNs.**

CGNs were seeded and cultured into 96-well, black-walled microtiter plates for four days. Then, measurements of intracellular calcium release were performed as described previously (1). Briefly, CGNs were washed with 0.1% BSA Hank's balanced salt solution (HBSS) and loaded with the calcium-sensitive dye Fluo-4 AM (1 mM; Molecular Probes). After 1 hour of incubation at 37 °C, the cells were washed and 50  $\mu$ L of 0.1% BSA HBSS was added. An application plate was prepared with different concentrations of baclofen or glutamate, and twice-concentrated drug solution (50  $\mu$ L) was added into each well 20 s after the start of the recording. Changes in fluorescence were monitored by FLIPR Tetra (Molecular Devices) over a period of 60 s following excitation at a wavelength of 488 nm and detection at 510 to 560 nm. Dose-response experiments were analyzed using nonlinear curve fitting for the log (agonist) versus response (three parameters) curves using GraphPad Prism software (GraphPad Software Inc.).

### **Bioluminescence resonance energy transfer (BRET) assay**

HEK293 cells were transfected with HA-GABA<sub>B1</sub>, Flag-GABA<sub>B2</sub>, G<sub>β1</sub>, Venus-G<sub>γ2</sub> and Gα<sub>13</sub>-Nluc or Gα<sub>o</sub>-Rluc8 by lipofectamine 2000 and split into 96-well flat-bottomed white microplates. For PTX pre-treatment group, the PTX (150 ng/ml) was added 6h after the transfection and treated for 14 to 16 hours. After 24 hours transfection, cells were washed and starved in PBS at 37 °C for 1 hour. BRET measurements were performed using the Mithras LB 940 (Berthold Technologies, German). The signals emitted by the donor (460–500 nm band-pass filter, Em 480) and the acceptor entity (510–550 nm band-pass filter, Em 530) were recorded after the addition of 10 μM Furimazine or 5 μM Coelenterazine H. All measurements were performed at 37 °C. The BRET signal was determined by calculating the ratio of the Em 530 and Em 480. The net BRET ratio was defined as the experimental BRET signal values with the baseline subtracted (basal BRET ratio), which was recorded before the stimulation of cells. For the kinetics of the drug response, BRET was measured in real time, with a counting time of 0.1 s. The drug (baclofen 100 μM) or PBS were injected by the Mithras LB 940 injector at the indicated time point. For dose-response experiments, data were analyzed using nonlinear curve fitting for the log (agonist) versus response (three parameters) curves in GraphPad Prism software.

### **Endogenous Gα<sub>13</sub> activation assay**

A Gα<sub>13</sub> activation assay was performed using a Gα<sub>13</sub> activation assay kit. Following the instructions of the kit, cells cultured in 60 mm dishes were treated with or without drugs and lysed with 1 mL ice-cold 1 × assay buffer for 30 min. Cells were scraped into a 1.5 mL tube and centrifuged at 4 °C (12000 rpm, 10 min). The supernatants were transferred into new tubes. For negative and positive controls, GDP (1 mM) and GTPγS (100 μM) were added respectively and incubated at 30 °C for 90 min with agitation. Then, 1 μL anti-active Gα<sub>13</sub> (Gα<sub>13</sub>-GTP) monoclonal antibody and 20 μL of resuspended protein A/G beads slurry were added to each tube. The mix was incubated at 4 °C for 2 hours with gentle agitation and precipitated by centrifugation for 1 min at 5000 × g. The beads were washed 3 times with 0.5 mL 1× Lysis buffer and resuspended with 20 μL 2 × reducing SDS-PAGE sample buffer. The samples were boiled for 10 minutes at 100 °C before detection by Western blotting.

### **Co-immunoprecipitation**

Total protein from HEK293 cells transfected with GABA<sub>B</sub> receptor (HA-GB1 and Flag-GB2) or PRK6 for 24 hours was isolated. Cultured cells were treated with or without baclofen (100 μM) and then lysed with IP cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, with complete proteinase inhibitors) on ice for 30 min. Cells were scraped into a microcentrifuge tube and centrifuged at 12,000 rpm for 10 min at 4 °C. To prepare the total cell lysate sample (input), equal amounts of protein from cell lysates were transferred to another tube and mixed with 4 × SDS sample loading buffer. The rest of the supernatant was then transferred to a new tube and incubated with anti-HA antibody (#11867423001, clone 3F10, Roche) overnight at 4 °C with gentle rocking. Protein G agarose beads (60 μL of a 50% bead slurry from Millipore) were added to the sample and incubated for 2 hours at 4 °C. The pellet was washed four times with 600 μL IP cell lysis buffer and resuspended with 2 × SDS sample buffer. Samples from co-IP enrichment and total cell lysates were boiled and subjected to SDS-PAGE for Western blotting analysis.

#### **Purification of the recombinant PAK Rac1 binding domain (GST-PBD) protein and rhotekin RhoA binding domain (GST-RhoA BD)**

The purification of the recombinant protein glutathione S-transferase (GST) fused to the PAK Rac1 binding domain (GST-PBD) and rhotekin RhoA binding domain (GST-RhoA BD) were conducted as in a previous study (51). The GST-PBD and GST-RhoA BD plasmids were constructed by PCR to obtain the PBD and RhoA BD using the following primers: forward primer for PBD: CTGGGATCCAAGAAGGAGAAGGAGCGACCAG; reverse primer for PBD: GGACTCGAGTTAAGCTGACTTATCTGTAAAACCTC. Forward primer for RhoABD: GGTGGATCCATCCTGGAGGAC; reverse primer for RhoABD: ATCTCGAGCTAGCCTGTCTTCTC. The PBD or RhoABD sequence was inserted into the pGEX6P1 vector using BamHI and XhoI. Expression of GST-PBD and GST-RhoA BD in BL21 (DE3) competent *E. coli* were induced with 200 mM isopropyl β-D-thiogalactoside (IPTG) for 3 hours at 37 °C. Bacteria were then sonicated in PBS supplemented with 0.5 mM dithiothreitol (DTT), 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 10 mM NaF. After sonication, 1% Triton X-100 was added to permeabilize the bacteria. The supernatants were collected after centrifugation and incubated with glutathione-sepharose 4B beads at 4 °C for 2 hours. Beads were washed with PBS supplemented with 1% Triton X-100 four times and proteins

were eluted with elution buffer (50 mM Tris-HCl, pH 7.6, 3.1 mg/mL reduced glutathione). Protein concentrations were measured using the BCA assay kit. The purified protein was aliquoted, snap frozen with liquid nitrogen and stored at -80 °C.

### **RhoA-GTP and Rac1-GTP pull-down assay**

The RhoA-GTP and Rac1-GTP were pulled down by GST-RhoA BD and GST-PBD pulldown assay and detected with RhoA or Rac1 antibody as described previously (51). First, glutathione-sepharose beads were recovered and washed four times with PBD buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 2 µg/mL leupeptin, 2 µg/mL aprotinin). GST-RhoA BD and GST-PBD fusion proteins were immobilized on glutathione-sepharose beads by incubating at 4 °C for 1 hour. Then cell lysates in PBD buffer were incubated with glutathione-sepharose beads conjugated to GST-PBD for 2 hour at 4 °C by gentle agitation. After washing four times with PBD buffer, the complex was resuspended in 2 × reducing SDS-PAGE sample buffer. Samples were boiled at 100 °C for 10 min, and then detected by western blotting with the RhoA or Rac1 antibody.

### **Immunofluorescence**

CGNs treated with indicated drugs were fixed with 4% formaldehyde and blocked with 2% BSA and 0.1% Triton X-100 in PBS. The cells were incubated with primary mouse monoclonal Gα<sub>13</sub> antibody (1:100) or rabbit polyclonal PSD95 antibody (1:100) at 4°C overnight. After washing three times with PBS, cells were incubated with secondary anti-mouse-Cy3 or anti-rabbit-Cy3 antibody at room temperature for 2 hours. Cells were washed with PBS and mounted with fluor-save reagent. Images were obtained with Olympus FV1000 Laser Scanning Confocal Microscope (60 × objective), equipped with appropriate epifluorescence and filters (Cy3: 543/620 nm; DAPI: 405/449 nm; GFP: 488/519nm). The settings of the confocal microscope were kept the same for all images when fluorescence intensity was compared. The intensity of PSD95 was quantified by using Image J software.

### **Induction of apoptosis in CGNs**

After four days of culture in vitro, CGNs were induced to undergo apoptosis. The culture cells were switched from high K<sup>+</sup> (BME, 30 mM KCl, 2 mM glutamine, and 0.5% penicillin/streptomycin) to low K<sup>+</sup> medium (BME, 5 mM KCl, 2 mM glutamine, and 0.5%

penicillin/streptomycin). Apoptosis was induced for the indicated treatment time and cells were lysed for detection by Western blotting.

### Statistical analysis

Histograms were created using the GraphPad Prism software. Data are presented as mean  $\pm$  SEM of at least three independent experiments. Statistical analyses were performed by one-way ANOVA (Ordinary) followed by Sidak's multiple comparisons post-hoc test for more than 2 different treatments in the data and unpaired t-test for only 2 different treatments in the data in GraphPad Prism software. Values with  $P < 0.05$  were considered statistically significant.

### Supplementary Materials:

Figures S1 – S6

### References and Notes:

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**Competing interests:** The authors declare that they have no competing interests.

**Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

## Figure legends

**Fig. 1. GABA<sub>B</sub> receptor induces JNK phosphorylation via the G<sub>i/o</sub> protein-independent pathway. (A)** Schematic representation of GPCR-regulated downstream G protein and  $\beta$ -arrestin signaling to MAPKs (including ERK1/2, JNKs, and p38) and cell functions modulated. **(B)** Schematic representation of heteromeric GABA<sub>B</sub> receptor and downstream signaling. GABA<sub>B</sub>

receptor is comprised of the GB1 and GB2 subunits. GB1 binds agonist (baclofen, GABA) or antagonist (CGP54626). GB2 binds PAMs, such as CGP7930. GABA<sub>B</sub> receptor is coupled to G<sub>i/o</sub> proteins and increase ERK1/2 phosphorylation, which is sensitive to PTX. The mechanism of GABA<sub>B</sub> receptor-mediated activation of JNK phosphorylation is unclear. **(C and D)** Western blotting analysis of JNK phosphorylation in CGNs with baclofen (C; 100 μM for a time course), or GABA<sub>B</sub> receptor antagonist CGP54626 (D; 20 μM, pre-incubated 30 min) and baclofen (D; 100 μM for 10 min). **(E)** Western blotting analysis of JNK and ERK1/2 phosphorylation in CGNs treated with PTX (250 ng/mL for 16 to 18 hours) and baclofen (100 μM for 10 min). Blots are representative of at least 3 independent experiments (C, *n* = 6; D, *n* = 6; E, *n* = 4). Data are mean ± SEM analyzed with one-way ordinary ANOVA followed by Sidak's multiple comparisons post-hoc test. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 versus basal levels with no drug treatment (C, D, E); ##*P* < 0.01, ###*P* < 0.001, and ns: non-significant versus treatment with the baclofen treated group (D, E).

**Fig. 2. G<sub>13</sub> proteins are required for GABA<sub>B</sub> receptor-induced JNK phosphorylation.** **(A)** Western blotting analysis of JNK phosphorylation in CGNs with Gα<sub>13</sub> siRNA and baclofen stimulation (100 μM, 10 min). Blots are representative of 6 independent experiments. **(B and C)** Western blotting analysis of JNK and ERK1/2 phosphorylation in response to baclofen for the indicated time in wild-type (WT) MEFs and Gα<sub>12/13</sub>-deficient MEFs overexpressing GABA<sub>B</sub> receptor. Blots are representative of 8 independent experiments. In (A to C), data are means ± SEM analyzed with one-way ordinary ANOVA followed by Sidak's multiple comparisons post-hoc test: \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001, versus basal levels; and ###*P* < 0.001, versus treatment with the baclofen treated group. ns: non-significance, versus Gα<sub>13</sub> siRNA basal level.

**Fig. 3. Activation of the GABA<sub>B</sub> receptor leads to G<sub>13</sub> protein activation.** **(A)** Illustration of the BRET-based G protein sensor. Upon receptor activation, rearrangement of the Gα subunit from Gβγ causes the BRET signal to decrease. **(B)** BRET experiment for Gα<sub>13</sub> and Gα<sub>o</sub> activation analysis in HEK293 cells transfected with GABA<sub>B</sub> receptor and G protein sensor. Data are mean ± SEM of 3 independent experiments. **(C)** Dose-response curve of GABA-induced BRET changes in the Gα<sub>13</sub> and Gα<sub>o</sub> sensors. Data are mean ± SEM of 3 independent experiments. **(D and E)** Gα<sub>13</sub>-

GTP pulldown analysis of the endogenous  $G\alpha_{13}$  activation. Blots are representative of 4 independent experiments. **(F)** Co-IP analysis of  $GABA_B$  receptor and  $G\alpha_{13}$  and  $G\alpha_o$  interaction. Blots are representative of at least 5 independent experiments. All data are means  $\pm$  SEM, analyzed with one-way ordinary ANOVA followed by Sidak's multiple comparisons post-hoc test:  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ , and  $****P < 0.0001$ , versus the basal level.

**Fig. 4. The RhoA and Rac1 are involved in  $GABA_B$  receptor-mediated JNK activation.** **(A)** RhoA-GTP pulldown and Western blotting analysis for RhoA activation induced by baclofen for the indicated time in CGNs. Blots are representative of 6 independent experiments. **(B)** Effect of RhoA inhibitor ( $1\mu\text{g} / \text{ml}$  for 4 hours) on baclofen-induced JNK and ERK1/2 activation detected by Western blotting. Blots are representative of 4 independent experiments. **(C)** Rac1-GTP pulldown and western blotting analysis for Rac1 activation induced by baclofen for indicated time in CGNs. Blots are representative of 5 independent experiments. **(D)** Effect of Rac1 inhibitor NSC23766 ( $50\mu\text{M}$ , 30 min) on baclofen-induced ( $100\mu\text{M}$ , 10 min) Rac1 activation detected by Western blotting. Blots are representative of 4 independent experiments. **(E and F)**  $G\alpha_{13}$  siRNA experiment and RhoA-GTP or Rac1-GTP detection in CGNs treated with indicated drugs. Blots are representative of 4 (E) and 3 (F) independent experiments. All data are means  $\pm$  SEM, analyzed with one-way ordinary ANOVA followed by Sidak's multiple comparisons post-hoc test:  $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$ , versus basal levels;  $\#P < 0.05$  and  $\#\#P < 0.01$ , versus cells treated with baclofen; and ns: non-significant, versus cells treated with baclofen or versus  $G\alpha_{13}$  siRNA basal level.

**Fig. 5.  $GABA_B$  receptor increases PSD95 phosphorylation and synaptic accumulation.** **(A to D)** Western blotting analysis of PSD95 phosphorylation in CGNs treated with baclofen (A;  $100\mu\text{M}$ , representative of  $n = 5$  independent experiments), JNK inhibitor SP600125 (B;  $20\mu\text{M}$  for 30 min,  $n = 3$ ),  $G\alpha_{13}$  siRNA (C,  $n = 5$ ), or PTX (D;  $250\text{ ng/mL}$  for 16 to 18 hours,  $n = 4$ ). **(E)** Dendritic PSD95 accumulation detected by immunofluorescence in CGNs treated with baclofen ( $100\mu\text{M}$ ), with or without CGP54626 ( $20\mu\text{M}$ ) or SP600125 ( $20\mu\text{M}$ ) for 16 hours after 3 days culture in vitro. Images are representative (scale bar:  $20\mu\text{m}$ ) and data are mean  $\pm$  SEM from 20-28 microscope fields for each group. **(F)** The dendritic PSD95 accumulation detected by

immunofluorescence in CGNs with  $G\alpha_{13}$  shRNA. Representative pictures are shown here (scale bar: 20  $\mu\text{m}$ ); full images are shown in fig. S5D. Quantified fluorescence intensity, as mean  $\pm$  SEM from 10-17 microscope fields for each group, are shown in the bar graph. In all panels, data were analyzed with one-way ordinary ANOVA followed by Sidak's multiple comparisons post-hoc test: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ , versus basal levels; # $P < 0.05$ , ## $P < 0.01$ , #### $P < 0.0001$ , versus the baclofen treated group.

**Fig. 6.  $G\alpha_{13}$ -JNK is involved in  $GABA_B$  receptor-mediated neuronal protection.** (A) Western blotting of caspase-3 cleavage in CGNs with indicated treatment. CGNs were treated for 2 or 12 hours with 5 or 30 mM  $K^+$  (K5 or K30, respectively), or with 5 mM  $K^+$  and baclofen alone (100  $\mu\text{M}$ ), baclofen and SP600125 (20  $\mu\text{M}$ ), or baclofen and AG1024. Blots are representative of at least 3 independent experiments. Data are means  $\pm$  SEM from 4 biological replicates, analyzed with one-way ordinary ANOVA followed by Sidak's multiple comparisons post-hoc test: \*\*\*\*  $P < 0.001$  versus K5; ##  $P < 0.01$  and ###  $P < 0.001$  versus K5 with the baclofen-treated group. (B) Schematic representation of the  $GABA_B$  receptor-mediated G protein signaling and function. Agonists (GABA and baclofen) or PAM (CGP7930) activate the  $GABA_B$  receptor to induce  $G\alpha_{13}$  and  $G\alpha_{i/o}$  activation.  $GABA_B$  receptor-activated  $G\alpha_{13}$  proteins promote RhoA and Rac1 to the GTP form and increase JNK phosphorylation, further increasing the PSD95 Ser<sup>295</sup> phosphorylation and synaptic accumulation, or inhibiting caspase-3 activity for neuroprotection.  $GABA_B$  receptor can activate ERK1/2 or transactivate IGF-1R through the  $G\alpha_{i/o}$  proteins. The  $GABA_B$  receptor-transactivated IGF-1R can increase Akt phosphorylation for neuroprotection.

Figure 1

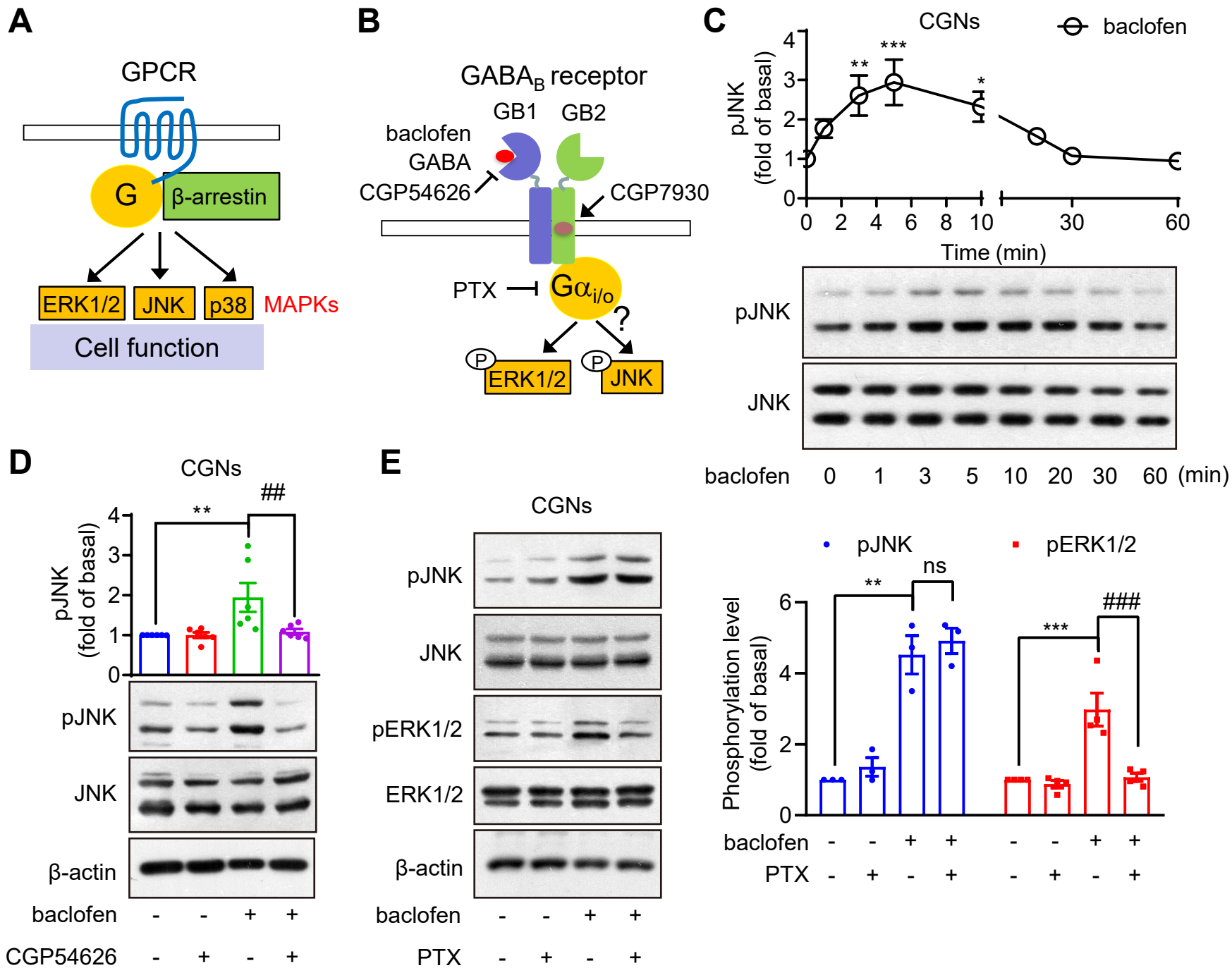
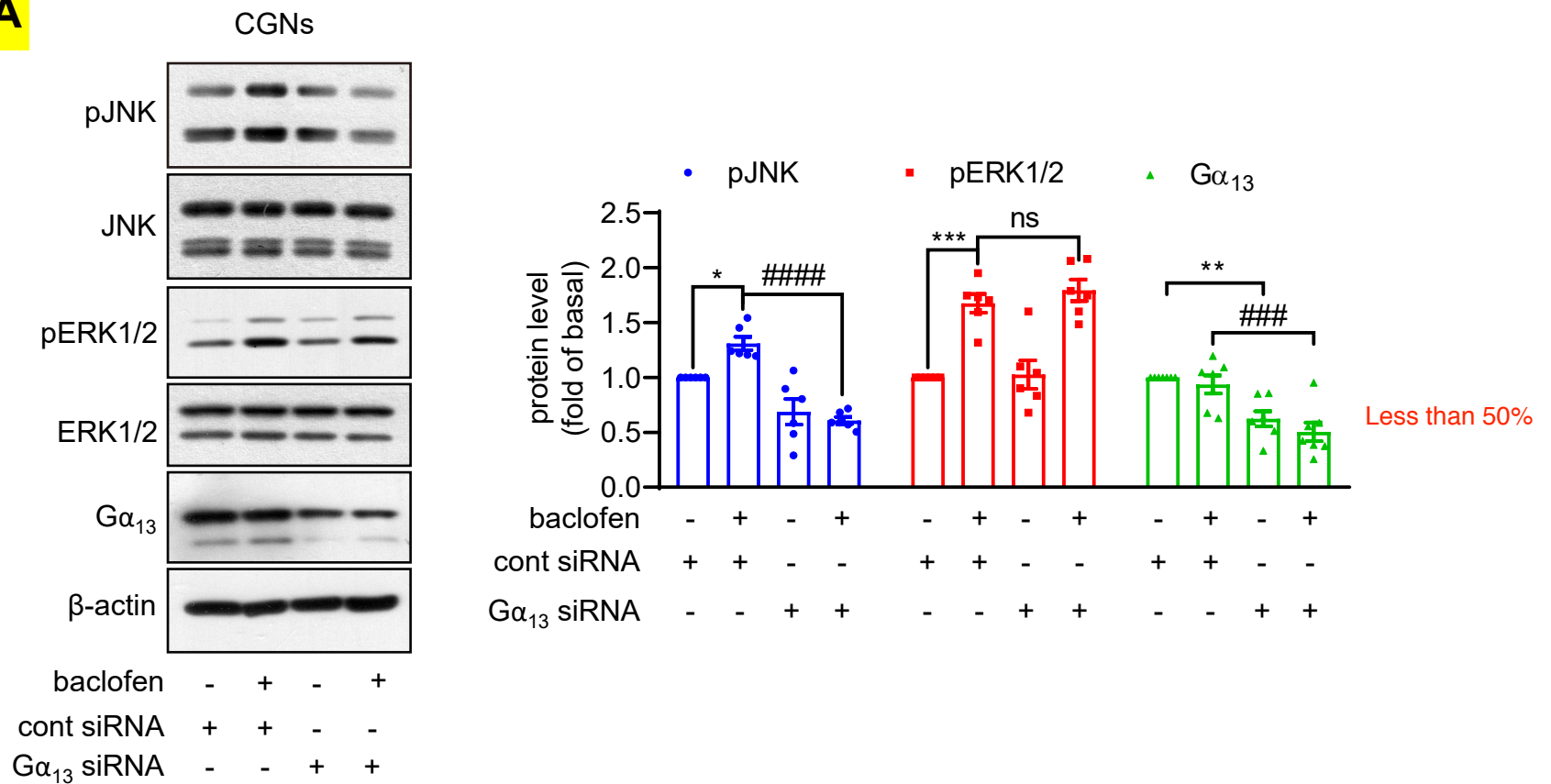
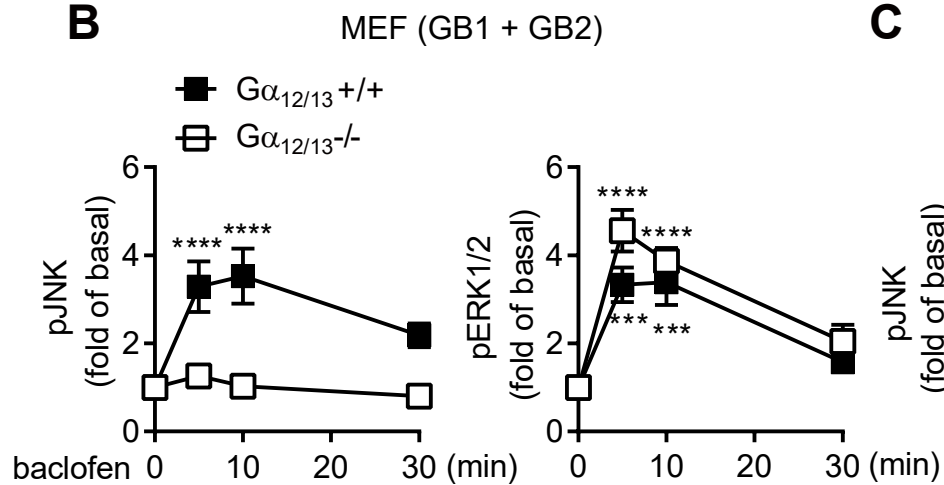


Figure 2

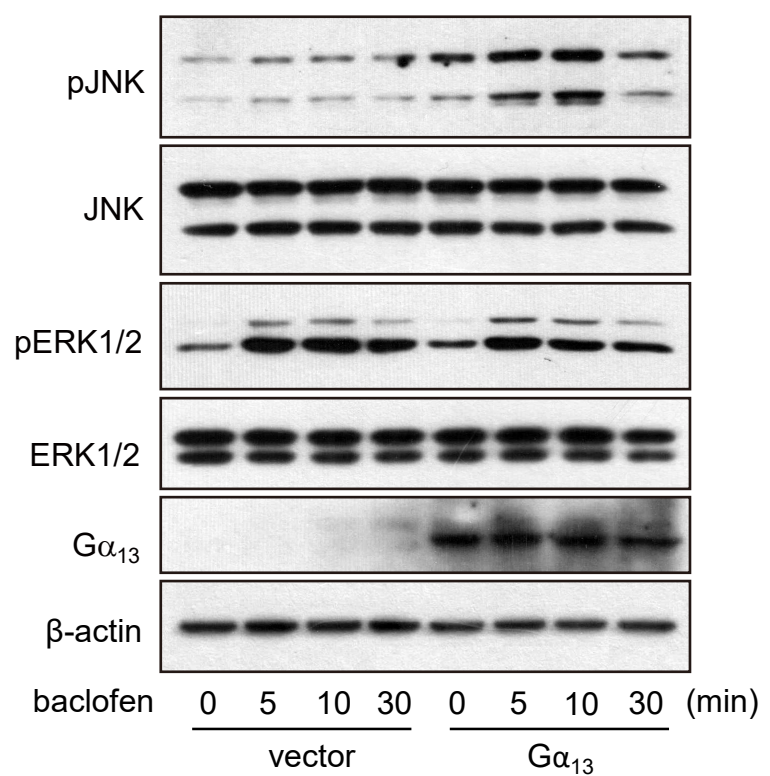
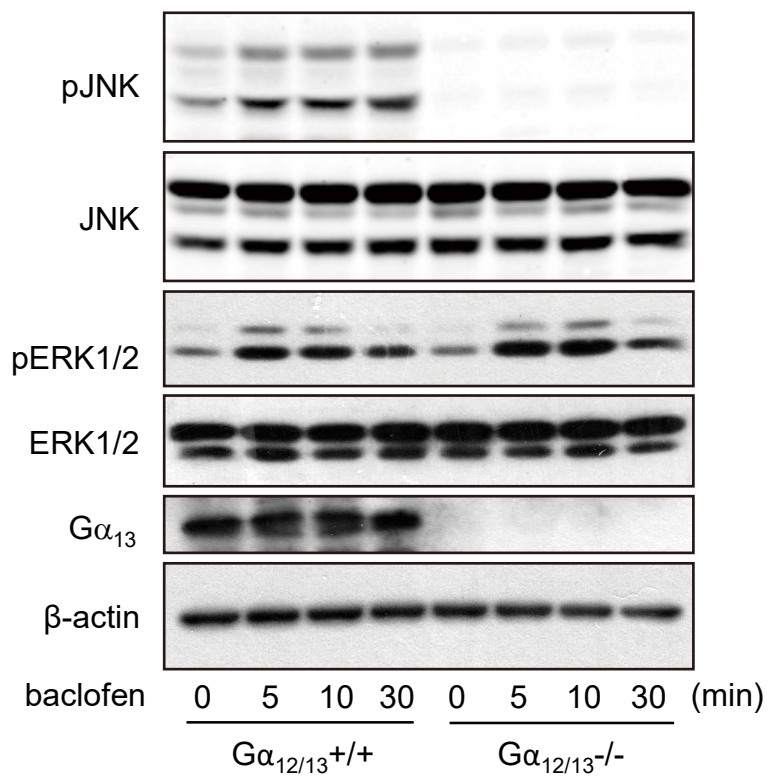
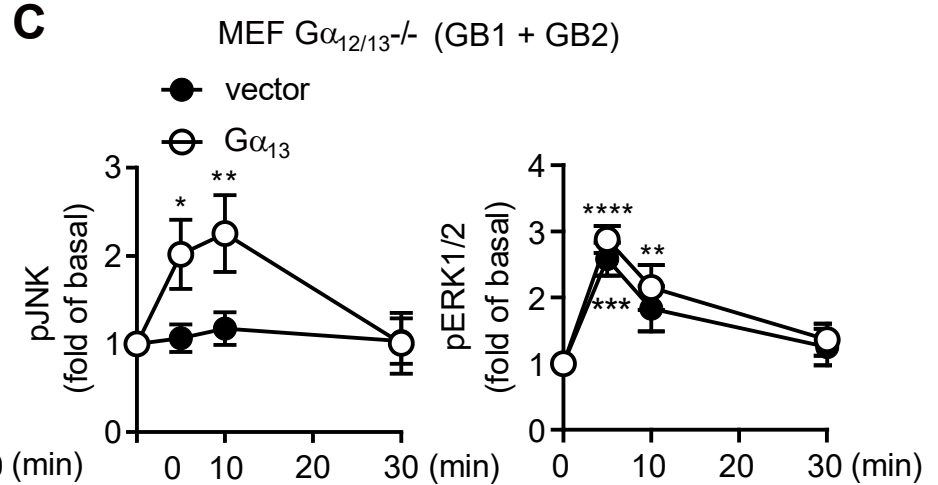
**A**



**B**



**C**



**Figure 3**

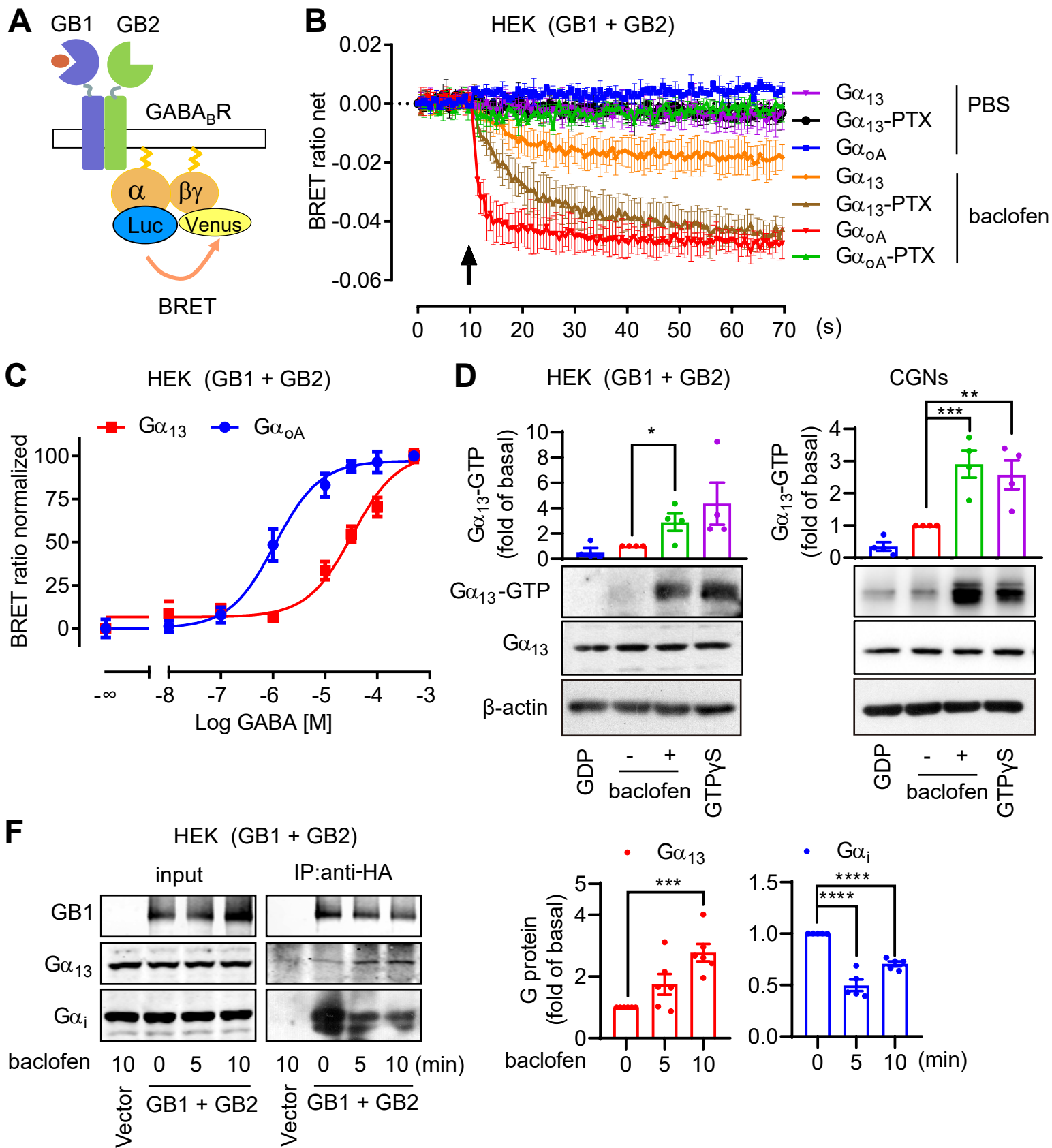


Figure 4

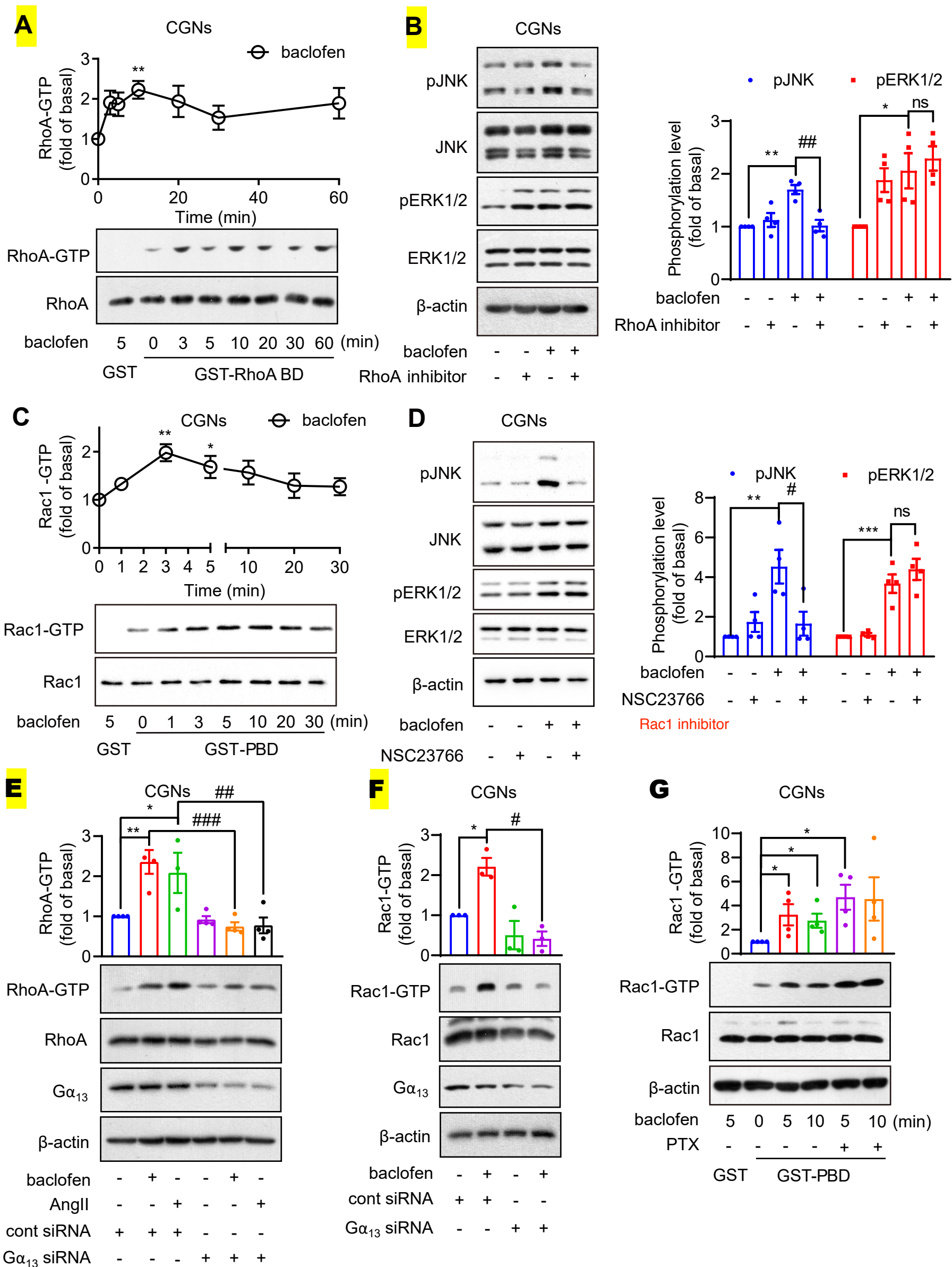


Figure 5

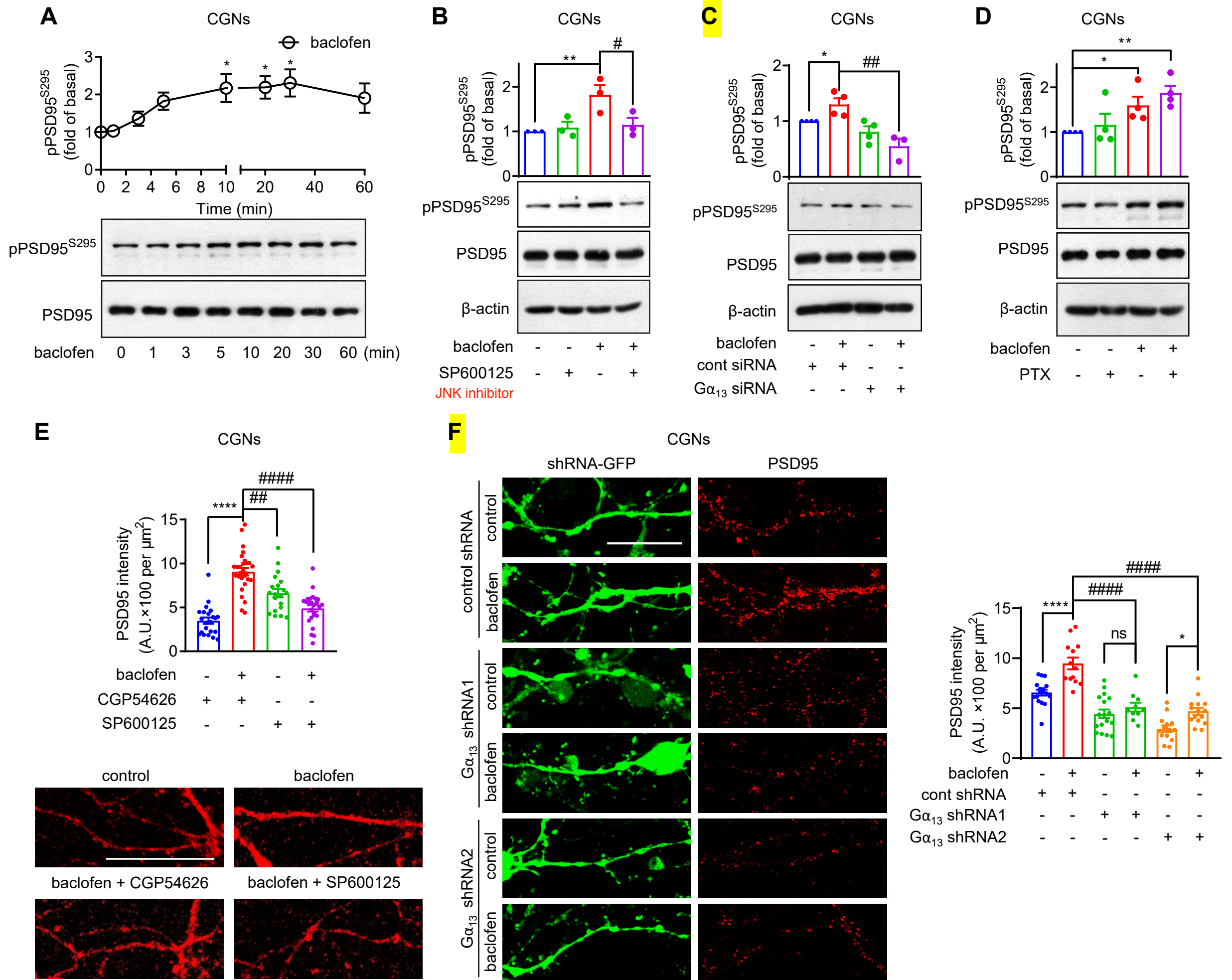


Figure 6

