Cellular/Molecular

$GABA_B$ Receptor Activation Protects Neurons from Apoptosis via IGF-1 Receptor Transactivation

Haijun Tu,[1]* Chanjuan Xu,[1]* Wenhua Zhang,[1] Qiuyao Liu,[1] Philippe Rondard,[2] Jean-Philippe Pin,[2] and Jianfeng Liu[1]

[1]Sino-France Laboratory for Drug Screening, Key Laboratory of Molecular Biophysics of Ministry of Education, School of Life Science and Technology, Huazhong University of Science and Technology, 430074 Wuhan, Hubei, China, and [2]Centre National de la Recherche Scientifique, UMR5203, Institut de Génomique Fonctionnelle, Inserm, U661 and Université Montpellier 1, 2, Montpellier F-34000, France

The G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) play key roles in cell–cell communication. Several studies revealed important synergisms between these two types of receptors, with some of the actions of either receptor being mediated through transactivation of the other. Among the large GPCR family, $GABA_B$ receptor is activated by the neurotransmitter GABA, and is expressed in most neurons where it mediates slow and prolonged inhibition of synaptic transmission. Here we show that this receptor is involved in the regulation of life and death decisions of cerebellar granule neurons (CGNs). We show that specific activation of $GABA_B$ receptor can protect neurons from apoptosis through a mechanism that involves transactivation of the IGF-1 receptor (IGF-1R). Further work demonstrated that this cross talk was dependent on $G_{i/o}$-protein, PLC, cytosolic Ca^{2+}, and FAK1 but independent of PKC, while IGF-1R-induced signaling involved Src kinase, PI3 kinase, and Akt activation. These results reveal a new function for this important GPCR and further highlight the importance of functional cross-talk networks between GPCRs and RTKs. Our results reveal $GABA_B$ receptor as a potential drug target for the treatment of neurodegenerative disorders.

Introduction

GABA is a major inhibitory neurotransmitter in the vertebrate CNS (Bettler et al., 1998) that mediates fast synaptic inhibition through $GABA_A$ (and $GABA_C$) ionotropic receptors as well as slow and prolonged synaptic inhibition through the metabotropic $GABA_B$ receptor (Couve et al., 2000). The $GABA_B$ receptor belongs to the class C G-protein-coupled receptors (GPCRs) and is composed of two subunits, $GABA_{B1}$ and $GABA_{B2}$ (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999; Galvez et al., 2000). $GABA_{B1}$ contains the GABA binding site, while $GABA_{B2}$ is responsible for $G_{i/o}$-protein activation (Galvez et al., 2001; Margeta-Mitrovic et al., 2001). Upon activation of the G-protein, the $G\beta\gamma$ complex represses Ca^{2+} influx by inhibiting Ca^{2+} channels at presynaptic sites, suppresses neurotransmitter release (Harayama et al., 1998), and triggers the opening of K^+ channels at the postsynaptic level (Lüscher et al., 1997; Schuler et al., 2001). $G\alpha_{i/o}$ subunits modulate the level of cAMP by regulating adenylate cyclase activities at postsynaptic sites, and inhibition of neuronal excitability (Simonds, 1999; Billinton et al., 2001). Interestingly, recent studies suggest that in addition to a role in neuronal excitability and plasticity, $GABA_B$ receptor may promote neuronal survival under conditions of metabolic stress or after ischemia (Dave et al., 2005; Kuramoto et al., 2007; Zhang et al., 2007). Accumulating evidences have shown that $GABA_B$ receptor plays an important role in anxiety and depression related disorders while the different classes of antidepressants and mood stabilizers that have been shown to prevent cell death (Cryan and Kaupmann, 2005; McKernan et al., 2009). However, the mechanisms by which $GABA_B$ receptor mediates neuroprotection remains elusive.

Insulin-like growth factor 1 (IGF-1) is essential for normal brain development (Cheng et al., 2000) and promotes neuronal survival by rescuing neurons from apoptosis (D'Mello et al., 1993). IGF-1 triggers autophosphorylation of its cognate tyrosine kinase receptor (IGF-1R) and activates the PI3 kinase/Akt signaling cascade, which in turn mediates the neuroprotective action of IGF-1 (Delcourt et al., 2007). Akt (also known as protein kinase B) is a serine/threonine kinase that functions downstream of PI3 kinase and is critical for neuronal survival (Bondy and Cheng, 2004).

In this study, by using cerebellar granule neurons (CGNs) as a cellular model of apoptosis (D'Mello et al., 1993), we further document the neuroprotective action of $GABA_B$ receptor. Most importantly, we demonstrate that this effect of the $GABA_B$ receptor results from functional transactivation of the IGF-1R, leading to Akt phosphorylation and survival signaling. Together, our results identify a novel cellular and molecular mechanism by which $GABA_B$ receptor can regulate neuronal survival.

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*H.T. and C.X. contributed equally to this work.

Correspondence should be addressed to Dr. Jianfeng Liu, Sino-France Laboratory for Drug Screening, Key Laboratory of Molecular Biophysics of Ministry of Education, School of Life Science and Technology, Huazhong University of Science and Technology, 1037 Luoyu Road, 430074 Wuhan, Hubei, China. E-mail: jfliu@mail.hust.edu.cn.

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Materials and Methods

Materials. GABA and IGF-1 from mouse were purchased from Sigma. Baclofen, CGP54626, CGP7930, FAK14, and PF573228 were purchased from Tocris Bioscience (Fisher-Bioblock). Pertussis toxin (PTX), AG1024, PP2, LY294002, wortmannin, U73122, and U73343 were purchased from Merck4Biosciences. BAPTA-AM, basal medium Eagle (BME), fetal bovine serum, and other solutions used for cell cultures were from Invitrogen. Primary antibodies including phospho-Ser^{473} Akt (193H12) antibody, Akt antibody, caspase-3 (#9662) antibody, PARP (#9542) antibody, phospho-Tyr$^{1135/1136}$ IGF-IR antibody (19H7), IGF-IR antibody (111A9), phospho-Thr$^{638/641}$ PKCα/βII (#9375) antibody, phospho-Tyr^{416} Src kinase (#2101) antibody, Src (#2108) antibody, and CREB (#9197) antibody were purchased from Cell Signaling Technology. PKCα (sc-208), PKCβII (sc-210), and FAK (sc-557) antibody were purchased from Santa Cruz Biotechnology.

Primary cerebellar granule neuronal cultures. One-week-old newborn KunMing mice obtained from Hubei Provincial Research Institute of Experimental Animals were decapitated, and the cerebella were dissected. The tissue was then gently triturated using fire-polished Pasteur pipettes, and the homogenate was centrifuged at 1000 rpm. The pellet was resuspended and plated in tissue culture dishes previously coated with poly-L-ornithine (Sigma). Cells were maintained in a 1:1 mixture of DMEM and F-12 nutrient (Invitrogen), supplemented with 30 mM glucose, 2 mM glutamine, 3 mM sodium bicarbonate, and 5 mM HEPES buffer, decomplemented 10% fetal calf serum, and 30 mM KCl to improve neuronal survival. Three-day-old cultures contained 1.25×10^5 cells/cm^2.

Induction of apoptosis, TUNEL assay, and measurement of apoptotic nuclei. After 4 d of culture, CGNs were analyzed for apoptosis. The culture cells were switched from high-K^+ (BME, 30 mM KCl, 2 mM glutamine, and 0.5% penicillin/streptomycin; K30) to low-K^+ medium (BME, 5 mM KCl, 2 mM glutamine, and 0.5% penicillin/streptomycin; K5). Cell death analyses were performed after 24 h of treatment.

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay was performed by using the DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. Briefly, CGNs were fixed with 4% paraformaldehyde in PBS and incubated with permeabilization buffer (0.1% Triton X-100 in PBS) for 5 min. The cells were then incubated with rTdT incubation buffer at 37°C for 1 h. Measuring green fluorescence of fluorescein-12-dUTP at 488 nm distinguished the nucleus of apoptotic cells only. Around 800–1200 cells in six to eight different fields were counted per coverslip for detecting TUNEL-positive cells under phase-contrast microscope. Cell numbers were calculated as a percentage of TUNEL-positive cells calculated from total cells.

For measuring apoptotic nuclei inside CGNs, cells were then washed twice with PBS containing 33 mM glucose, fixed with 4% paraformaldehyde in PBS for 30 min at 4°C, and incubated with 1 mg/ml Hoechst 33258 staining for 10 min. Nuclear DNA staining was examined by digital fluorescence imaging microscopy (Axiophot 2 microscope, Zeiss).

Drug treatments. For Akt, IGF-1R, Src, and PKCα/βII activation assays, cultures were washed once with Ca^{2+}-free HEPES-buffered solution (HBS) (containing 10 mM HEPES, pH 7.4, 140 mM NaCl, 4 mM KCl, 2 mM $MgSO_4$, and 1 mM KH_2PO_4) and preincubated at 37°C with or without indicated inhibitors dissolved in HBS for 60 min. For PTX treatment, the cultures were pretreated 14–16 h with PTX (200 ng/ml) or not. Cells were then stimulated at the indicated time by incubating with GABA, baclofen, CGP7930, or IGF-1 prepared in fresh HBS. At the end of the treatment, the cells were washed quickly with ice-cold Ca^{2+}-free PBS pH 7.4, and 200 μl of lysis buffer was added to the cells and placed immediately on ice.

For caspase-3 and PARP assays, the cultures were incubated at different times in K5 media containing the indicated drugs. The cell monolayer was immediately scraped into ice-cold lysis buffer after treatment with K5 media, and the cell lysate was used for Western blotting detection. Drugs were dissolved in HBS with or without dimethyl sulfoxide (DMSO) or alcohol. Whenever DMSO or alcohol were used, HBS containing the same concentration of DMSO and/or alcohol were used as the

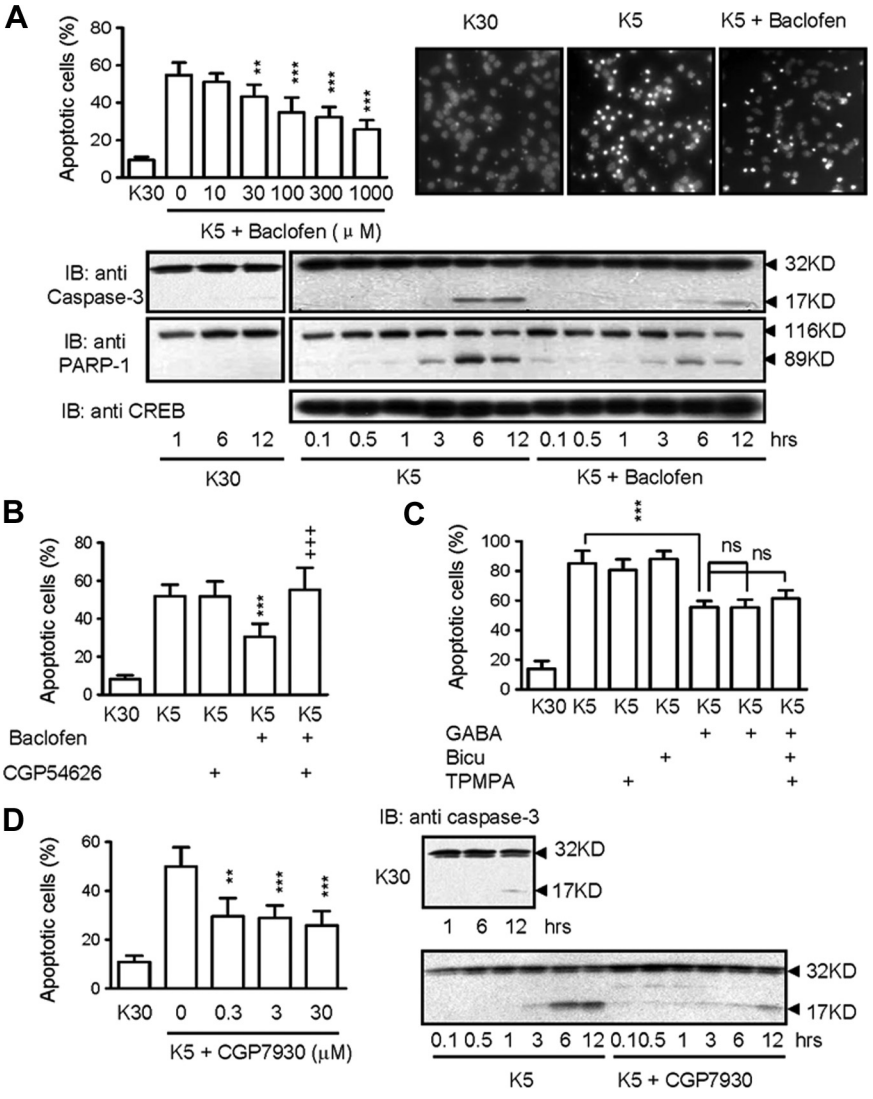

Figure 1. $GABA_B$ receptor activation protect neurons from potassium-deprivation-induced apoptosis. ***A***, Dose–response of baclofen on apoptosis when CGNs were transferred from high (30 mM K^+, K30) to low (5 mM K^+, K5) potassium concentrations for 24 h. Apoptotic cells were measured by TUNEL assay (left) and observed by Hoechst 33258 staining (right). Time course study of the effect of baclofen at 300 μM on caspase-3 activation and PARP activity were shown in the bottom panel and CREB protein expression was used as loading control. ***B***, ***C***, Effect of $GABA_A$ [bicuculline (10 μM)] and $GABA_C$ [TPMPA (10 μM)] receptor antagonists on apoptotic CGNs after GABA (100 μM) stimulation. For all TUNEL assay or Hoechst 33258 staining, cells were treated with drugs in K5 media for 18–20 h before fixing and staining. For caspase-3 activation, cells were treated with drugs in K5 media for 0.1–12 h. ***D***, Dose–response of the $GABA_B$ receptor-positive allosteric modulator CGP7930 on apoptotic CGNs (left) and the effects of CGP7930 at 30 μM on caspase-3 activation (right). $^{**}p < 0.01$, $^{***}p < 0.001$, versus K30 conditions. $^{+++}p < 0.001$; ns, no significance, versus in the absence of antagonist. The blots shown are representative of three separate experiments.

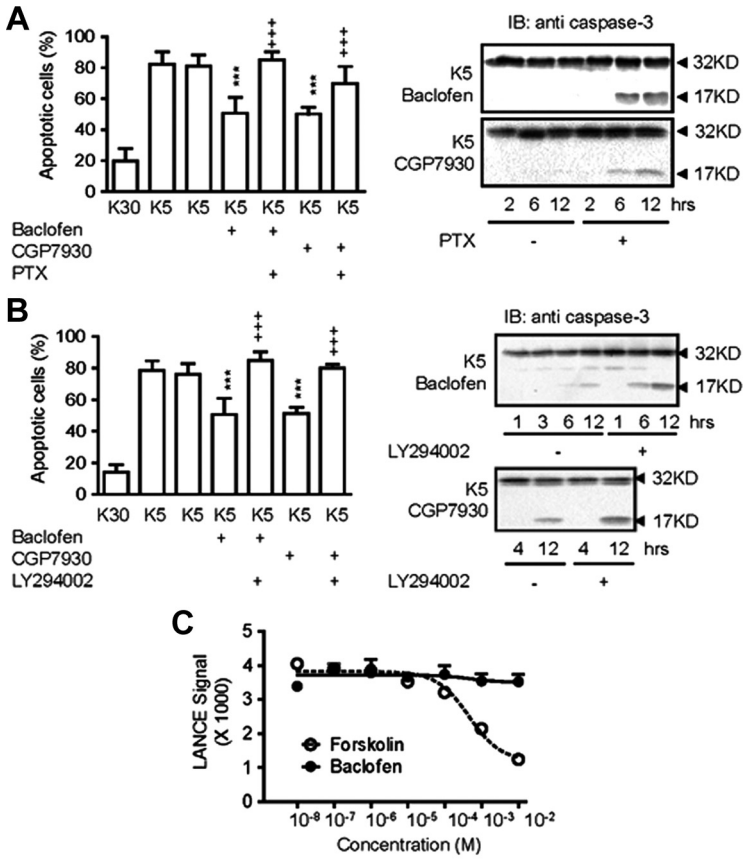

Figure 2. The neuroprotective effect of $GABA_B$ receptor is dependent on $G_{i/o}$-protein and PI3 kinase but independent of intracellular cAMP levels. ***A***, ***B***, Effect of PTX (200 ng/ml) (***A***) and LY294002 (20 μM) (***B***) on the protective effect of baclofen (300 μM) or CGP7930 (50 μM) on apoptotic CGNs (left) and caspase-3 activation (right). For TUNEL assay, cells were treated with drugs in K5 media for 18–20 h. For caspase-3 activation, cells were treated with drugs in K5 media for 1–12 h. $^{***}p < 0.001$, versus K30 conditions. $^{+++}p < 0.001$, versus the absence of PTX or LY294002. The blots shown are representative of three separate experiments. ***C***, $GABA_B$ receptor activation does not induce cAMP production in CGNs. Dose–response effect of baclofen or adenylyl cyclase activator forskolin on cAMP production.

control vehicle. All immunoblots were performed in at least three independent experiments.

RNAi transfection in MEF cells and primary cerebellar granule neurons. For IGF-R RNAi knockdown experiment in MEF cells, mammalian shRNA constructs were designed as described previously (Dong et al., 2007). First, we design the target site of small hairpin RNAs (shRNA) for IGF-1R from 3476 to 3494 cDNA (NM_000875.2). The forward DNA template was as follows: gatccccgaagatttcacagtcaacttcctgtcagattgactgtgaaatcttcggtttttg, and the reverse DNA template was as follows: aattcaaaaaccgaagatttcacagtcaatctgacaggaagttgactgtgaaatcttcggg. Synthesized shRNA template oligonucleotides were phosphorylated, annealed, and then ligated into linearized pSIH-H1-copGFP shRNA vector (System Biosciences) digested with EcoRI/BamHI. Murine embryonic fibroblasts (MEFs) were first transfected with shRNA plasmid by Lipofectamine 2000 reagent (Invitrogen) and then with $GABA_{B1}$-HA and $GABA_{B2}$-Flag plasmid after 24 h; cells were then treated with drugs. For IGF-1R RNAi knockdown experiments in CGNs, primary cerebellar granule neurons were transfected with IGF-IRα/β siRNA (sc-35638), or control siRNA-A (sc-37007) following Santa Cruz siRNA transfection protocol *in vitro* for 2 d. Cells were then treated with drugs. For PKCα, PKCβII, and FAK RNAi knockdown experiments in MEF cells, MEF cells were transfected following Santa Cruz siRNA transfection protocol *in vitro* with PKCα (sc-208), PKCβII (sc-210), and FAK siRNA (sc-35353) or control siRNA-A (sc-37007) for 2 d and then transfected with $GABA_{B1}$-HA and $GABA_{B2}$-Flag plasmid for 24 h; cells were then treated with drugs.

Western blot analysis. Cell lysates were sonicated, and protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories). Equal amounts of protein (20 μg) were resolved by SDS-PAGE on 8–12% gels. Proteins were transferred to nitrocellulose membranes (Millipore) and blocked in blocking buffer (5% nonfat dry milk in TBS and 0.1% Tween 20) for 1 h at room temperature. The blots were then incubated with primary antibodies at the relevant dilution (Cell Signaling Technology) overnight at 4°C, and with horseradish peroxidase-linked secondary antibodies (1:20,000; Cell Signaling Technology) for 2 h. Immunoblots were detected by using enhanced chemiluminescence reagents (Pierce) and visualized by using x-ray film. The density of immunoreactive bands was measured by using NIH Image software, and all bands were normalized to percentages of control values.

Immunoprecipitation. Proteins from CGNs were prepared under weakly denaturing conditions to permit the protein and protein interaction. Cultured cells were scraped into a microtube containing ice-cold 1× cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin) and homogenized by sonication. The homogenate was centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was then transferred to a new tube and incubated with $GABA_{B1}$ antibody (sc-14006, Santa Cruz Biotechnology) or normal rabbit IgG (#2729, Cell Signaling Technology) overnight at 4°C with gentle rocking. Fifty percent protein A-agarose/Sepharose beads (50 μl of 50% bead slurry, Millipore) were added to the sample and incubated to allow for binding with primary antibody for 2 h at 4°C. Tubes were centrifuged for 30 s at 4°C. The pellet was washed five times with 500 μl of 1× cell lysis buffer and kept on ice during washes. Pellets were resuspended with 3× SDS sample buffer, vortexed and boiled for 5 min, and then centrifuged for 5 min at 12,000 rpm. The boiled samples were then loaded onto SDS-PAGE gel for Western blotting.

cAMP production assay. Cultures were washed once with HBS and preincubated 2 h with HBS at 37°C after 3–4 d cultured *in vitro* and then stimulated 30 min with different concentrations of baclofen or forskolin dissolved in stimulation buffer. The assay steps were performed according to protocol of LANCE cAMP 384 Kit (PerkinElmer).

Statistical analysis. Data are presented as means ± SEM of at least three independent experiments. Statistical analysis was performed by Student's *t* test. Values with $p < 0.05$ were considered statistically significant.

Results

$GABA_B$ receptor protects cerebellar granule neurons from low-potassium-induced apoptosis

CGN survival during development involves neuronal excitation, a situation mimicked in cultures by incubating in high-potassium-containing media (30 mM, K30). By reducing the potassium concentration to 5 mM (K5), the cells show rapid neuronal cell death; thus, CGNs have been widely used as a model to study neuronal apoptosis (D'Mello et al., 1993). By measuring the number of apoptotic CGNs by TUNEL assay, we found that the selective agonist of $GABA_B$ receptor, baclofen, at 30 μM or higher concentrations significantly decreased the number of apoptotic CGNs after potassium deprivation (Fig. 1*A*, top left). Nuclear DNA staining with Hoechst 33258

confirmed that baclofen decreased the number of apoptotic CGNs (Fig. 1*A*, top right). Caspase-3 activity is commonly associated with apoptotic processes, and was found to be largely increased in CGNs after transfer to K5 media. Poly(ADP-ribose) polymerase-1 (PARP-1), which can be proteolytically cleaved by caspase-3 at the DEVD site to generate a 85 kDa and a 24 kDa fragment, is recognized as a known caspase-3 substrate (Du et al., 1997). Consistent with the anti-apoptotic effects of the $GABA_B$ receptor, baclofen decreased caspase-3 activation and PARP-1 activity (Fig. 1*A*, bottom; supplemental Fig. 4*A*,*B*, available at www.jneurosci.org as supplemental material) but have no effect on CREB protein expression, used here as loading control (Tu et al., 2007). This protective effect resulted from the activation of the $GABA_B$ receptor and was reversed by the $GABA_B$ receptor antagonist CGP54626 (Fig. 1*B*). The endogenous agonist of the $GABA_B$ receptor, GABA, significantly decreased the number of apoptotic CGNs, and this effect was not blocked by pretreatment with the $GABA_A$ and $GABA_C$ receptor antagonists, bicuculline and TPMPA, respectively (Fig. 1*C*). The positive allosteric modulator of $GABA_B$ receptor, CGP7930, also significantly decreased the number of apoptotic CGNs (Fig. 1*D*, left) and caspase-3 activity induced by potassium deprivation (Fig. 1*D*, right; supplemental Fig. 4*A*, available at www.jneurosci.org as supplemental material). Together, these results demonstrate that specific activation of $GABA_B$ receptor, but not $GABA_A$ or $GABA_C$ receptors, protects CGNs from low-potassium-induced apoptosis.

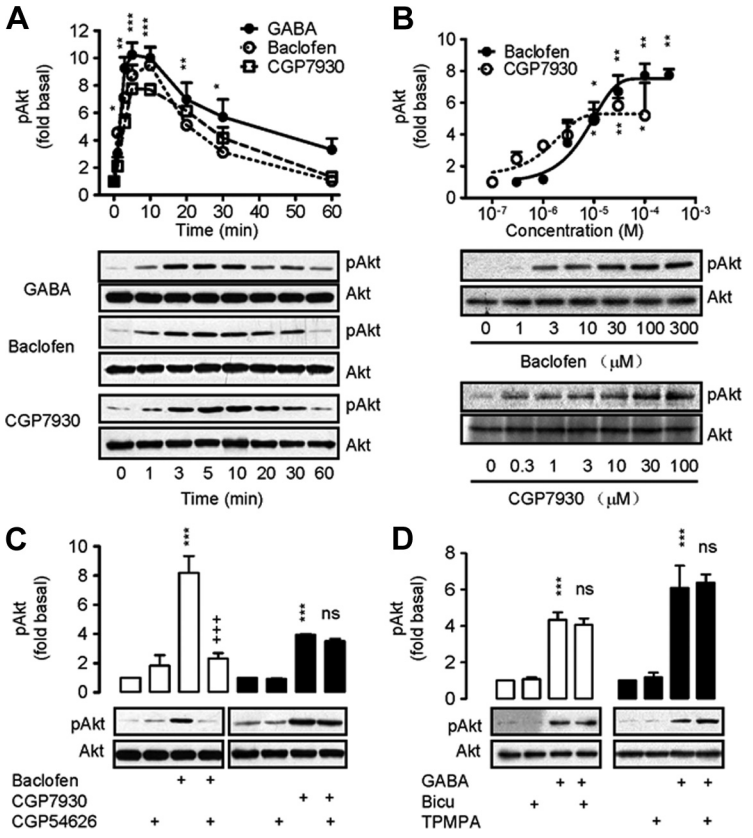

Figure 3. $GABA_B$ receptor activation can increase Akt phosphorylation. ***A***, Time course of Akt activation in CGNs after incubation with GABA (100 μM), baclofen (100 μM), or CGP7930 (50 μM). Representative immunoblots were used to quantify phosphorylated Akt levels (pAkt). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, versus basal level for baclofen dose–response. ***B***, Response of baclofen or CGP7930-induced Akt phosphorylation after 10 min incubation. Immunoblots were used to quantify pAkt levels. $^*p < 0.05$, $^{**}p < 0.01$, versus basal levels. ***C***, ***D***, Cells were treated with CGP54626, bicuculline, or TPMPA at 10 μM for 20 min before the stimulation of baclofen/GABA at 100 μM (10 min). Akt phosphorylation was measured by quantifying pAkt from immunoblots. $^{***}p < 0.001$, versus basal levels. $^{+++}p < 0.001$; ns, nonsignificance, versus treated with baclofen, GABA, or CGP7930 in the absence of antagonist. The blots shown are representative of three separate experiments.

$GABA_B$ receptor-mediated neuroprotection is via $G_{i/o}$-protein and PI3 kinase but not intracellular cAMP levels

To elucidate the downstream signaling pathways involved in $GABA_B$ receptor-induced neuroprotection, we analyzed the importance of the $G_{i/o}$-protein, which is coupled to the $GABA_B$ receptor in neurons (Mannoury la Cour et al., 2008), and PI3 kinase, which plays an important role in cell survival (Downward, 2004). Under low-potassium-induced apoptosis conditions, the $G_{i/o}$ inhibitor, PTX, reversed the protective effect of baclofen/CGP7930 on cell survival (Fig. 2*A*, left). In agreement with this, PTX suppressed the inhibitory action of baclofen and CGP7930 on caspase-3 degradation at low potassium concentrations (Fig. 2*A*, right; supplemental Fig. 4*C*, available at www.jneurosci.org as supplemental material). Interestingly, two specific inhibitors of PI3 kinase, LY294002 (Fig. 2*B*, left) and wortmannin (data not shown), abolished the neuroprotective effect of baclofen and CGP7930. LY294002 also reversed the effect of baclofen and CGP7930 on caspase-3 activity in CGNs (Fig. 2*B*, right; supplemental Fig. 4*D*, available at www.jneurosci.org as supplemental material). Apoptosis of CGNs induced by low potassium has been known to be prevented either by elevated intracellular cAMP levels (D'Mello et al., 1993; Galli et al., 1995). Although coupled to $G_{i/o}$ type of G-proteins, the $GABA_B$ receptor could well increase cAMP formation through an indirect pathway, then leading to inhibition of apoptosis. This is unlikely since no increase in cAMP production could be detected after baclofen stimulation of CGNs, whereas a nice increase of cAMP could be detected upon adenylate cyclase activation with forskolin (Fig. 2*C*).

These results demonstrate that $G_{i/o}$-protein and PI3 kinase but not intracellular cAMP are involved in $GABA_B$ receptor-mediated neuroprotection of CGNs from potassium deprivation.

$GABA_B$ receptor activates PI3 kinase/Akt via $G_{i/o}$-protein, PLC, and Src kinases

The $GABA_B$ receptor can activate Akt, a downstream PI3 kinase effector that is important for cell survival (Schlessinger, 2000), and we explore whether this signaling pathway is involved in the neuroprotective effect of this receptor. In CGNs, GABA caused a rapid and transient increase in Akt phosphorylation on Ser^{473} residue with no changes in total Akt protein expression levels (Fig. 3*A*). Similar results were obtained with baclofen or CGP7930 (Fig. 3*A*), and both baclofen and CGP7930 induced Akt phosphorylation in a dosage-dependent manner (Fig. 3*B*). CGP54626 can antagonize baclofen's effect on Akt phosphorylation (Fig. 3*C*), while CGP7930-induced Akt phosphorylation was not antagonized by CGP54626, consistent with its action at a different site on the $GABA_B$ receptor (Binet et al., 2004). In addi-

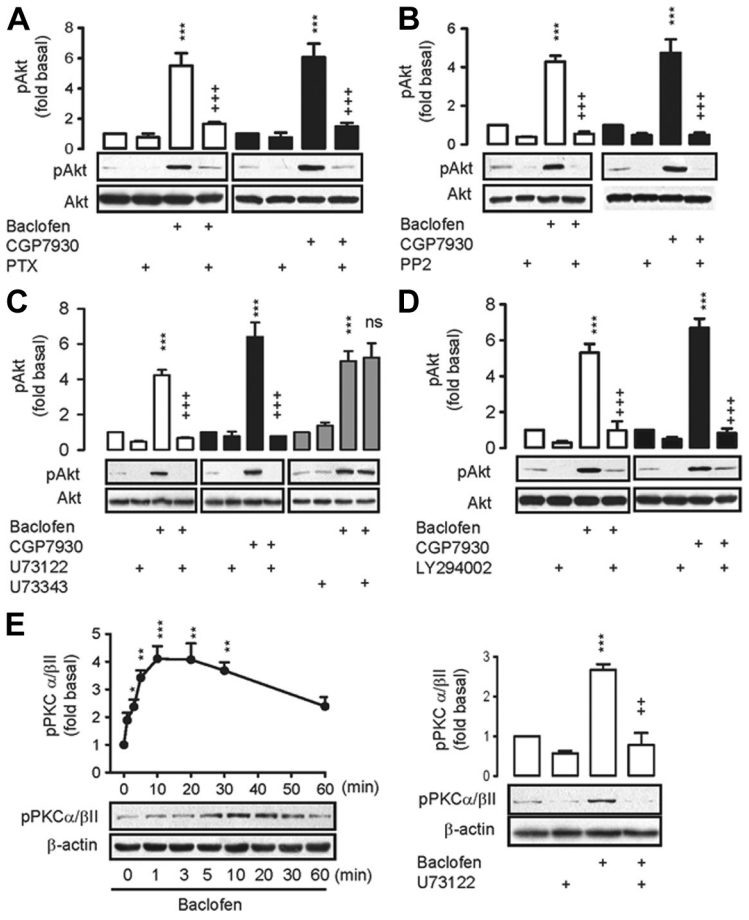

Figure 4. $G_{i/o}$-protein, Src family kinase, PLC, and PI3 kinase are required for $GABA_B$ receptor-mediated Akt phosphorylation. ***A–D***, Cells were pretreated with PTX (200 ng/ml), PP2 (5 μM), U73122 (5 μM), U73343 (5 μM), or LY294002 (10 μM) for 60 min, and Akt phosphorylation was induced by 100 μM baclofen (or 50 μM CGP7930) for 10 min. pAkt levels were quantified by immunoblotting. ***E***, Time course of the PKCα/βII phosphorylation in CGNs after incubation with baclofen at 100 μM. Cells were pretreated with U73122 at 5 μM for 60 min before baclofen stimulation (100 μM). To quantify pAkt or pPKCα/βII, immunoblots from separate experiments (mean ± SEM; $n = 3$–5) were analyzed, and a representative immunoblot is shown. For all results, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, versus basal levels. $^{++}p < 0.01$, $^{+++}p < 0.001$; ns, nonsignificance, versus treated with baclofen or CGP7930 in the absence of inhibitors.

tion, GABA-mediated Akt phosphorylation could not be blocked by pretreatment with bicuculline or TPMPA (Fig. 3*D*), and is in agreement with the specific involvement of $GABA_B$ receptor in Akt activation.

We then further analyzed the signaling pathways that lead the $GABA_B$ receptor to activate Akt. PTX treatment completely inhibited $GABA_B$ receptor-induced Akt activation, demonstrating the involvement of $G_{i/o}$-protein in the $GABA_B$ receptor-mediated pathway (Fig. 4*A*). In various cell types, $G_{i/o}$-coupled receptors have been shown to activate the Src family kinases (Satoh et al., 1992; Luttrell et al., 1996; Thodeti et al., 2000). PP2, a Src-family kinase inhibitor, completely abolished Akt phosphorylation induced by baclofen and CGP7930 (Fig. 4*B*). $G_{i/o}$-coupled receptors have been shown to enhance phospholipase C (PLC) activity and activate protein kinase C (PKC) through $G\beta\gamma$ subunits (Blank et al., 1992; Selbie and Hill, 1998). Accordingly, we showed that pretreating CGNs with U73122, an inhibitor of PLC but not its inactive analog U73343, completely abolished Akt phosphorylation (Fig. 4*C*). We showed that baclofen can induce the phosphorylation of PKCα/βII on $Thr^{638/641}$ residues, an effect that was blocked by U73122 (Fig. 4*E*). We verified that PI3 kinase is involved in $GABA_B$ receptor-mediated Akt phosphorylation by showing that LY294002 can completely block this effect (Fig. 4*D*).

Together, these results demonstrate that $G_{i/o}$-protein, Src-family kinase, PLC, and PI3 kinase are required for $GABA_B$ receptor-induced Akt phosphorylation, and may be required for the neuroprotective effect of $GABA_B$ receptor in CGNs.

IGF-1R is required for $GABA_B$ receptor-mediated Akt phosphorylation and neuroprotection

It has been shown that apoptosis induced by potassium deprivation in CGNs can be inhibited by IGF-1 (D'Mello et al., 1993). In our work, we confirmed that IGF-1 can induce a sustain phosphorylation on IGF-1R at $Tyr^{1135/1136}$ residues and Akt at Ser^{473} residue (Fig. 5*A*). This $GABA_B$ receptor-induced Akt activation was blocked by pretreatment with two different classes of IGF-IR inhibitors, AG1024 (Fig. 5*B*) and NDGA (7H) (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material) (Blecha et al., 2007).

Several lines of evidence confirmed that IGF-1R is necessary for $GABA_B$ receptor-mediated neuroprotection. First, AG1024 reversed the decrease of caspase-3 activity induced by GABA, baclofen, or CGP7930 (Fig. 5*C*; supplemental Fig. 1*E*, available at www.jneurosci.org as supplemental material). Second, AG1024 suppressed the effect of baclofen on the protection of CGNs from apoptosis (Fig. 5*D*). Third, transfection with shRNA of IGF-1R into MEF cells cotransfected with $GABA_{B1}$ and $GABA_{B2}$ could inhibit IGF-1R expression, which in turn inhibited baclofen-induced Akt phosphorylation (Fig. 5*E*). Finally, transfection with IGF-1R siRNA into CGNs could also inhibit baclofen-induced Akt phosphorylation by reducing endogenous IGF-1R expression in CGNs (supplemental Fig. 1*B*, available at www.jneurosci.org as supplemental material). Furthermore, we verified that CGP54626, a $GABA_B$ antagonist, failed to modify the effect of IGF-1 on cell signaling (supplemental Fig. 1*C*, available at www.jneurosci.org as supplemental material) and CGN neuroprotection from apoptosis (supplemental Fig. 1*D*, available at www.jneurosci.org as supplemental material).

$GABA_B$ receptor activation induces IGF-1R transactivation

It has been reported that the GPCR angiotensin II type 1 receptors (AT1Rs) can transactivate IGF-1R to mediate downstream signaling from AT1Rs to PI3 kinase (Zahradka et al., 2004). We first showed that baclofen or CGP7930 can cause a transient increase of IGF-1R phosphorylation without altering IGF-1R protein expression levels (Fig. 6*A*), and that this effect was abolished by inhibition with CGP54626 and PTX (Fig. 6*B*).

Functional cross talk between two receptors could be due to their association in the same signaling platform or through indirect interactions (Rives et al., 2009). We found that IGF-1R could be coimmunoprecipitated with $GABA_{B1}$ from CGN lysates (Fig.

6*C*), indicating that IGF-1R and $GABA_{B1}$ are part of the same signaling complex (Fig. 6*C*).

Two different mechanisms have been proposed for GPCR-induced transactivation of receptor tyrosine kinases: ligand-dependent or ligand-independent mechanisms (Shah and Catt, 2004; Delcourt et al., 2007). The αIR3 mouse monoclonal antibody against IGF-1R specifically recognizes the extracellular α-subunit of IGF-1R and inhibits its ligand-mediated effects (Kienlen Campard et al., 1997). We found that αIR3 had no effect on baclofen-induced IGF-1R phosphorylation (Fig. 6*D*), whereas it decreased IGF-1-induced IGF-1R phosphorylation.

Together, these results demonstrate that $GABA_B$ receptor activation of $G_{i/o}$-protein can induce ligand-independent IGF-1R transactivation in CGNs.

$GABA_B$ receptor-induced IGF-1R transactivation requires PLC through Ca^{2+}-dependent FAK1 pathway

The selective PLC inhibitor U73122, but not its inactive analog U73343, abolished baclofen-induced IGF-1R phosphorylation (Fig. 7*A*). However, PP2 failed to block baclofen-stimulated IGF-1R phosphorylation, whereas AG1024 completely inhibited baclofen-induced IGF-1R phosphorylation (Fig. 7*B*), indicating that Src is not involved in this pathway. Our data show that PLC is involved in $GABA_B$ receptor-induced IGF-1R phosphorylation and that Src kinases are acting downstream of IGF-1R. Note that we detected two bands using the IGF-IR antibody (111A9) from Cell Signaling Technology (Fig. 7*A*, right). We think that the fragment with a lower molecular weight corresponds to a degradation product of IGF-1R, as it can also be found in the Cell Signaling Technology product information provided for IGF-IR antibody (111A9).

Further experiments showed that baclofen can induce Src kinase phosphorylation at Tyr^{416} residue in a transient manner (Fig. 7*C*), an effect that was blocked by both U73122 and AG1024 (Fig. 7*D*), but not by U73343 (supplemental Fig. 2*A*, available at www.jneurosci.org as supplemental material). However, LY294002 did not inhibit baclofen-induced phosphorylation of either IGF-1R or Src (supplemental Fig. 2*B*, available at www.jneurosci.org as supplemental material), suggesting that Src kinase can act upstream of PI3 kinase/Akt.

Finally, we examined the possible involvement of PLC downstream effectors such as PKC, intracellular Ca^{2+}, and focal adhesion kinase (FAK1) on $GABA_B$ receptor-induced IGF-1R phosphorylation. PKC inhibitor (Gö6983 or Gö6976) failed to inhibit baclofen-induced Akt phosphorylation, whereas it inhibited baclofen-induced CREB phosphorylation in CGNs (supplemental Fig. 3*A*,*B*, available at www.jneurosci.org as supplemental material). Furthermore, RNAi-mediated knockdown experiments of PKCα and PKCβII failed to block baclofen-induced Akt phosphorylation in MEF cells coexpressing $GABA_{B1}$ and $GABA_{B2}$ (supplemental Fig. 3*C*, available at www.jneurosci.org as supplemental material). These data suggest that $GABA_B$ receptor-induced IGF-1R/Akt signaling does not need PKC activation. Group 1 metabotropic glutamate receptors (mGluR1 and mGluR5) that stimulate PLC can lead to both mobilization of intracellular Ca^{2+} and activation of PKC. However, mGluR1- and mGluR5-induced tyrosine phosphorylation of the downstream FAK1 results from the Ca^{2+}/CaM signaling and not from PKC activation

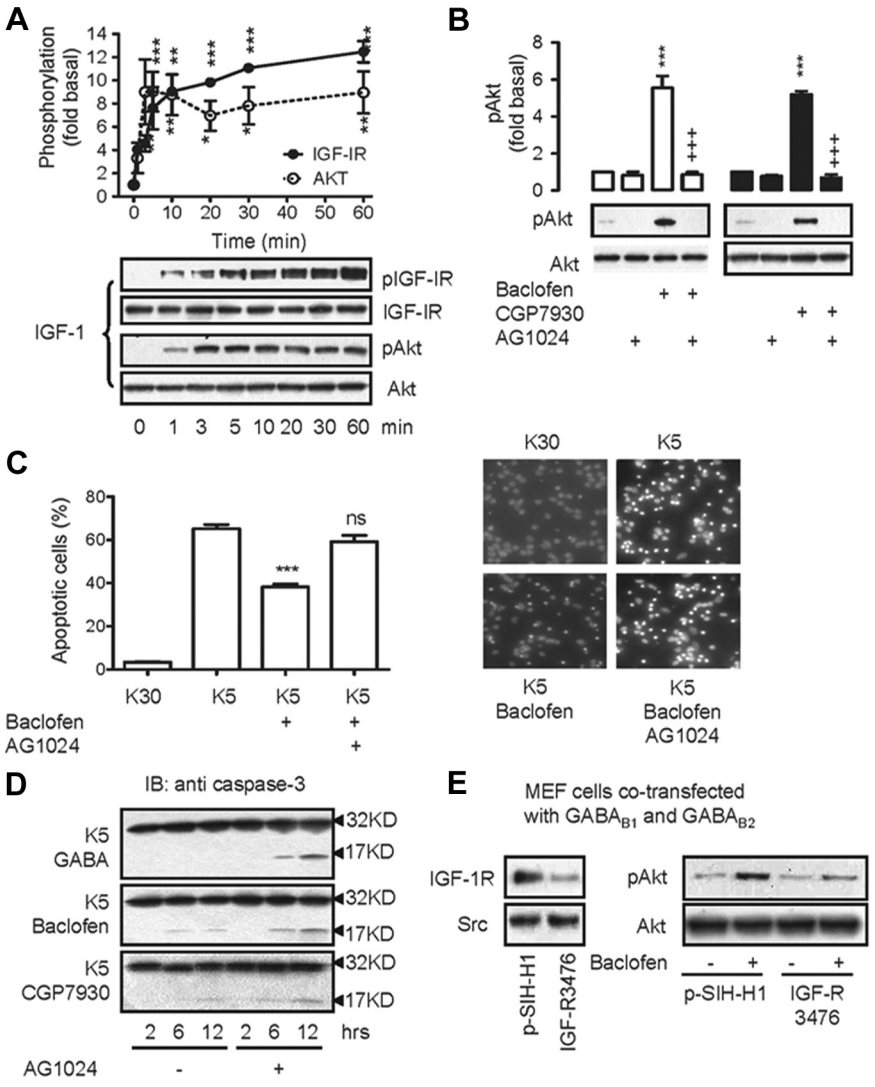

Figure 5. IGF-1R is required for $GABA_B$ receptor-mediated neuroprotection. ***A***, Time course of IGF-1 (10 ng/ml)-induced IGF-1R and Akt phosphorylation in CGNs. ***B***, Effect of pretreatment with AG1024 at 0.1 μM for 60 min on Akt phosphorylation, followed by induction by baclofen (100 μM) or CGP7930 (50 μM) for 10 min. To quantify pAkt or pIGF-1R, immunoblots from separate experiments (mean ± SEM; $n = 3$–5) were analyzed. For all results, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, versus the basal levels, and $^{+++}p < 0.001$, versus treated with baclofen or CGP7930 in the absence of AG1024. ***C***, Effect of AG1024 (0.1 μM) on apoptotic CGNs after baclofen (100 μM) stimulation. For all TUNEL assay (left) or Hoechst 33258 staining (right), cells were treated with drugs in K5 media for 18–20 h before fixing and staining. $^{***}p < 0.001$; ns, no significance, versus K5 conditions. The blots shown are representative of three separate experiments. ***D***, Effect of AG1024 (0.1 μM) on caspase-3 activity in CGNs induced by GABA (100 μM), baclofen (100 μM), or CGP7930 (50 μM). The blots shown are representative of three separate experiments. For caspase-3 activation, cells were treated with drugs in K5 media for 2–12 h. ***E***, Effect of shRNA of IGF-1R (IGF-R3467) on Akt phosphorylation induced by baclofen (100 μM) for 5 min in MEF cells cotransfected $GABA_{B1}$ and $GABA_{B2}$. Expression of IGF-R3467 decreased MEF cells endogenous IGF-1R expression but not Src kinase expression (left). The blots shown are representative of three separate experiments.

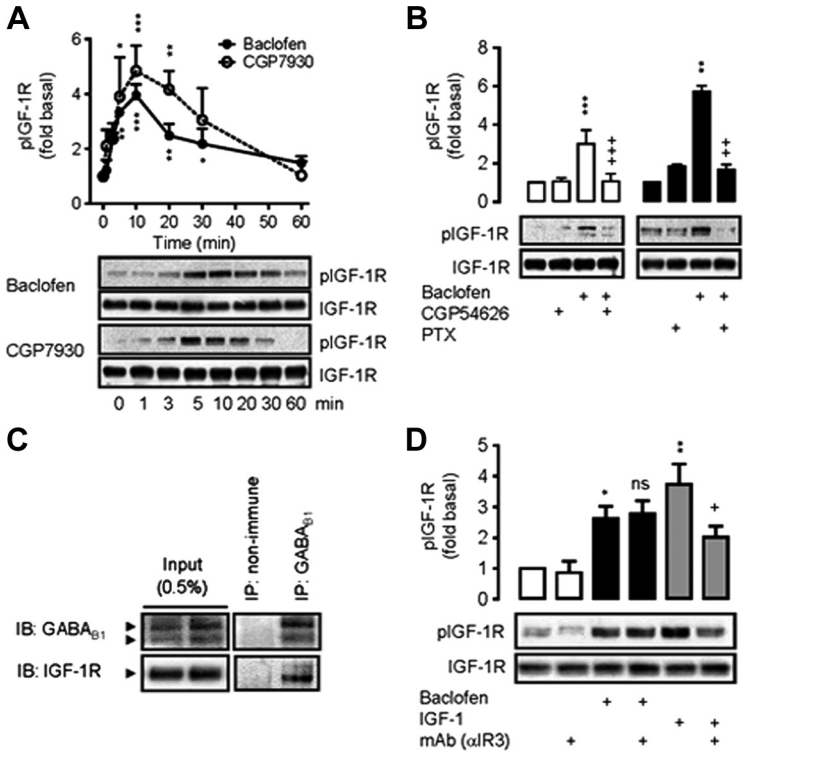

Figure 6. Activation of $GABA_B$ receptor transactivated IGF-1R. ***A***, Time course of baclofen- (100 μM) and CGP7930- (50 μM) induced IGF-1R phosphorylation in CGNs. ***B***, Effects of pretreatment with PTX (200 ng/ml) or CGP54626 (10 μM) on IGF-1R phosphorylation induced by baclofen (100 μM) or CGP7930 (50 μM) for 10 min, and representative immunoblots. ***C***, Western blot analysis of IGF-1R coimmunoprecipitated from CGNs with $GABA_{B1}$ antibody. The membrane was immunoblotted by anti-IGF-1R antibodies. The total lysate (input) was analyzed. ***D***, Effect of the neutralizing antibody anti-IGF-1R (αIR3; 2 μg/ml) on baclofen- (100 μM) or IGF-1 (10 ng/ml) induced IGF-1R phosphorylation for 10 min. For all IGF-IR phosphorylation results, representative immunoblots are shown under the quantified data of pIGF-IR analyzed from separate experiments (mean ± SEM; $n = 3$–5). For all results, $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, versus the basal levels, and $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, and ns, nonsignificance, versus treatment with baclofen or CGP7930 in the absence of CGP54626, PTX, IGF-1, or mAb αIR3.

(Shinohara et al., 2001). Here we showed that pretreatment of CGNs with either BAPTA-AM (cell membrane-permeable Ca^{2+} chelator) or FAK1 inhibitors (FAK14 or PF573228) inhibited baclofen-induced Akt phosphorylation (Fig. 7*E*). Furthermore, we showed that RNAi knockdown for FAK1 could block baclofen-induced phosphorylation of both IGF-1R and Akt in MEF cells coexpressing $GABA_{B1}$ and $GABA_{B2}$ (Fig. 7*F*). These indicated that $GABA_B$ receptor induced IGF-1R transactivation through PLC/Ca^{2+}-dependent FAK1 pathway, which in turn activated Akt.

Together, these results demonstrate that $GABA_B$ receptor-induced IGF-IR transactivation is mediated by PLC/FAK1 pathway via an intracellular Ca^{2+}-dependent but PKC-independent mechanism and that $GABA_B$ receptor can induce Src kinase phosphorylation via IGF-1R transactivation (Fig. 8).

Discussion

$GABA_B$ receptor has previously been shown to promote neuronal survival, but the mechanism of such neuroprotection remains elusive. In the present study, we elucidated the mechanism by which $GABA_B$ receptor induced anti-apoptotic effect in CGNs, a model system in which apoptosis can be induced by potassium deprivation. We demonstrated that $GABA_B$ receptor-induced neuroprotection is mediated by Akt phosphorylation through a signaling pathway that involves IGF-1R transactivation. We show that the mechanism of $GABA_B$ receptor-induced Akt activation is through $G_{i/o}$-protein and PLC/Ca^{2+}-dependent FAK1, which leads to IGF-1R transactivation and Src family kinase and PI3 kinase/Akt phosphorylation.

Apoptosis of CGNs induced by low potassium is known to be prevented either by elevated intracellular cAMP levels or by IGF-1 (D'Mello et al., 1993; Galli et al., 1995). In accordance with this, activation of pituitary adenylate cyclase-activating polypeptide (PACAP) receptor, which is known to couple to G_s, produces the anti-apoptotic effect in CGNs by increasing intracellular cAMP levels (Kienlen Campard et al., 1997; Delcourt et al., 2007). In our present work, we demonstrated that GABA can protect CGNs from apoptosis, and this effect is mediated by $GABA_B$ receptor coupling to $G_{i/o}$-protein and not by the $GABA_A$ and $GABA_C$ receptors. Interestingly, we found that this anti-apoptotic effect mediated by $GABA_B$ receptor may not require changes in intracellular cAMP levels. Works have shown that $GABA_B$ receptor from rat brain membranes can couple to both G_o- and G_i-proteins, resulting in an inhibition of adenylate cyclase activity and cAMP production, but not to G_q and G_s (Nishikawa et al., 1997; Odagaki et al., 2000; Odagaki and Koyama, 2001). Previous studies suggested that due to the low expression of G_i in comparison to G_o, $GABA_B$ receptor may be preferentially coupled to G_o in neurons (Gierschik et al., 1986). PAM effects such as CGP7930 that act on the $GABA_{B2}$ have been shown to enhance efficacy of coupling to G_o over G_i (Mannoury la Cour et al., 2008). Finally, unlike G_i, most functions of G_o can be interpreted through the actions of a common pool of $G\beta\gamma$ (Ghil et al., 2006). Together, our data suggest that the anti-apoptotic effect of $GABA_B$ receptor is mediated by $G\beta\gamma$ subunits released from $G_{i/o}$-protein and IGF-1R signaling pathway rather than by an increase in cAMP levels.

Earlier reports have shown that IGF-1 acts as a cell survival growth factor, protecting CGNs from apoptosis through the PI3 kinase/Akt pathway (Linseman et al., 2002; Subramaniam et al., 2005). Our data demonstrated that $GABA_B$ receptor can transactivate IGF-1R. Recent studies in different cell types have established the potential involvement of RTKs in transducing the growth-promoting signals of GPCRs (Shah and Catt, 2004). To date, a few GPCRs in the CNS have been investigated in terms of their ability to transactivate RTKs in neurons, although much is known for peripheral GPCRs transactivating epidermal growth factor receptor (EGFR), neurotrophin receptor (Trk), platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor (FGFR) (Peavy et al., 2001; Shah and Catt, 2004). From these studies, two different mechanisms of RTK transactivation by GPCRs have been reported. One is ligand-dependent such as gonadotropin-releasing hormone (GnRH) receptors-EGFR transactivation resulting from the induction of metalloprotease-dependent ectodomain shedding from heparin-binding EGF (Peavy et al., 2001; Shah et al., 2003; Shah and Catt, 2004). Alternatively, the receptor can promote the release of the RTK agonist as observed for the μ-opioid receptors-FGFR-1 transactivation (Belcheva et al.,

2002). The second model is ligand independent and results from a direct phosphorylation of the transactivated RTKs by a GPCR downstream tyrosine kinase. This has been well illustrated by the adenosine- and PACAP-induced Trk receptor transactivation (Lee and Chao, 2001; Lee et al., 2002) and dopamine D_4 receptor-mediated PDGFR transactivation (Heldin and Westermark, 1999). Of major importance, whereas the first mechanism leads to the RTK activation in cells surrounding the activated GPCR, leading to a diffusion of the effect, the second mechanism is exclusively mediated by intracellular events such that RTK transactivation is limited to the cells expressing the activated GPCR.

Here, we show that $GABA_B$ receptor-induced IGF-1R transactivation is independent of endogenous IGF-1, and likely results mainly from an intracellular pathway, such that only CGNs activated by $GABA_B$ receptor agonists are expected to benefit from the neuroprotective effect. Our coimmunoprecipitation experiments show that $GABA_B$ receptor and IGF-1R can be part of the same signaling complex; however, there is no evidence suggesting whether these two receptors form direct interactions, nor that these may be required for the observed cross talk. This will be the subject of future work. Our results suggest that $GABA_B$ receptor may transactivate IGF-1R through a ligand-independent mechanism that may involve downstream $GABA_B$ receptor signaling.

To date, there are limited numbers of reports demonstrating IGF-1R transactivation by GPCRs. The best characterized example is angiotensin II type 1 (AT1) receptor, which was shown to activate PI3 kinase through IGF-1R transactivation in smooth muscle cells (Zahradka et al., 2004). However, the mechanism involved is not exactly the same as those reported here. In smooth muscle cells, AT1 receptor couples to both $G_{i/o}$- and G_q-proteins and mediates IGF-1R phosphorylation through Src activation (Zahradka et al., 2004). In CGNs, we show that $GABA_B$ receptor-mediated IGF-1R phosphorylation is independent of Src family kinases activation, but Src is involved in the downstream effect of IGF-1R, in agreement with the IGF-1R action on these neurons (Delcourt et al., 2007).

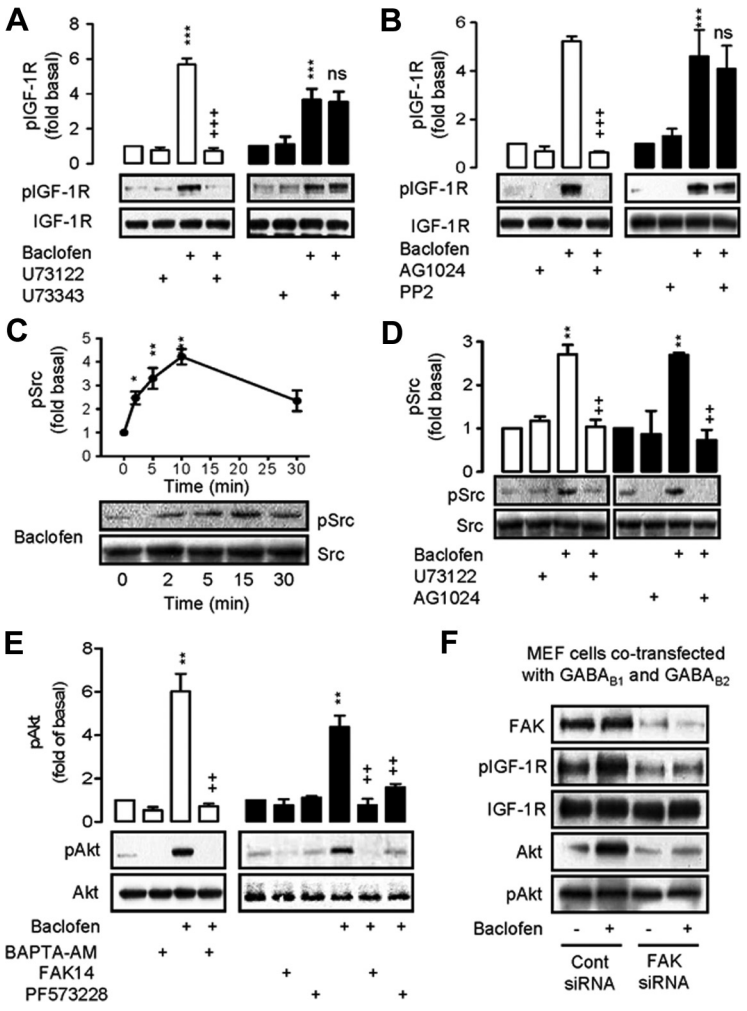

Figure 7. PLC downstream effectors FAK1 but not Src kinase is involved in $GABA_B$ receptor-mediated IGF-1R transactivation. ***A***, Cells were pretreated with U73122 (5 μM) or U73343 (5 μM) for 60 min and induced by baclofen at 100 μM for 10 min. IGF-1R phosphorylation was measured by immunoblotting. ***B***, PP2 (5 μM) was used to pretreat cells for 60 min or AG1024 (0.1 μM) before baclofen (100 μM) for 10 min. IGF-1R phosphorylation was detected by immunoblotting. ***C***, Time course of baclofen (100 μM)-induced Src kinase phosphorylation in CGNs. ***D***, Effect of pretreatment with U73122 (5 μM) or AG1024 (0.1 μM) on Src kinase phosphorylation induced by baclofen (100 μM) for 10 min. ***E***, Effect of intracellular Ca^{2+} and FAK1 on baclofen-induced Akt phosphorylation for 10 min. Cells were pretreated with cell membrane-permeable Ca^{2+} chelator BAPTA-AM (10 μM) for 30 min and FAK1 inhibitors [FAK14 (50 μM) or PF573228 (10 μM)] for 10 min before the CGNs were stimulated with baclofen (100 μM) for 10 min, respectively. Akt phosphorylation was measured by immunoblotting. For all results, representative immunoblots are shown under the quantified data of pIGF-IR, pSrc, or pAkt analyzed from separate experiments (mean ± SEM; $n = 3$–5). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, versus the basal levels, and $^{++}p < 0.01$, $^{+++}p < 0.001$, ns, no significance, versus treated with baclofen in the absence of inhibitors. ***F***, MEF cells cotransfected with $GABA_{B1}$ and $GABA_{B2}$ were pretreated with control RNAi or RNAi of FAK1 for 48 h and then stimulated with baclofen for 5 min, respectively. IGF-1R and Akt phosphorylation were measured by immunoblotting. The blots shown are representative of three separate experiments.

Given the relative abundance of $G_{i/o}$-protein in neurons, this G-protein is a likely source of $G\beta\gamma$ subunits for PLC activation (Blank et al., 1992). Accordingly, a number of GPCR agonists that activate $G_{i/o}$, including LPA and thrombin, can activate PLC through $G\beta\gamma$ (Rozengurt, 2007), then leading to a rapid increase in the intracellular concentration of Ca^{2+} (Berridge et al., 2000), and activation of protein kinase C (PKC) through DAG (Dempsey et al., 2000). We show here that activation of $GABA_B$ receptor-induced PKCα/βII phosphorylation in CGNs, in agreement with other studies (Thompson and Gahwiler, 1992; Tosetti et al., 2004; Pontier et al., 2006), consistent with $GABA_B$ receptor-mediated PLC activity through $G\beta\gamma$ subunits. However, our data show that PKC activation is not required for $GABA_B$ receptor-mediated IGF-1R transactivation and Akt phosphorylation. This is consistent with the absence of IGF-1R phosphorylation by PKC, in contrast to what has been reported for EGFR (Hunter et al., 1984).

Although PKC activation does not appear to be involved, $GABA_B$ receptor-mediated increase in intracellular Ca^{2+} concentration (New et al., 2006; Mizuta et al., 2008) plays a major role in IGF-1R transactivation. Recently, a large body of evidence suggested that a rapid increase of the tyrosine phosphorylation for a nonreceptor tyrosine kinase, FAK, is a common response to multiple GPCR agonists (Rozengurt, 2002). Interestingly, whereas

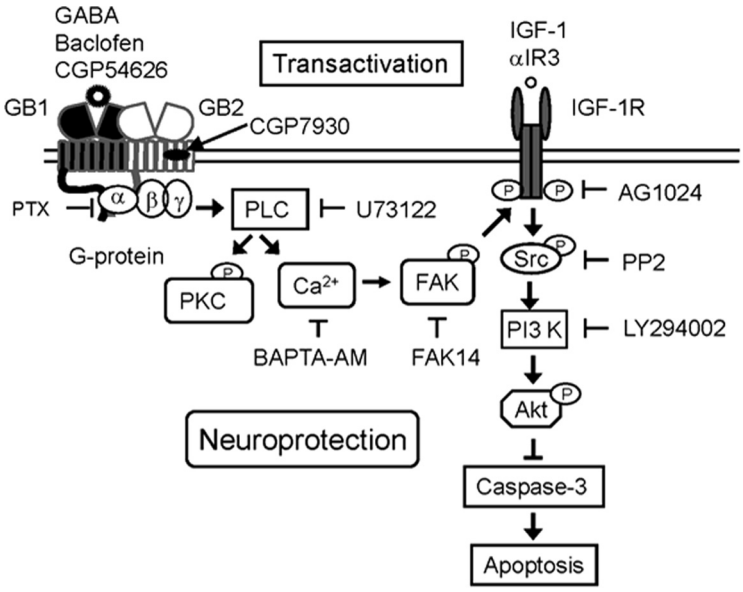

Figure 8. Schematic representation of the signaling pathway mediated by $GABA_B$ receptor in CGN neuroprotection. Activation of $GABA_B$ receptor leads to the release of $G\beta\gamma$ subunits from $G_{i/o}$-protein to activate PLC/Ca^{2+}-dependent FAK1 pathway, which in turn transactivates IGF-IR to induce Akt phosphorylation through the Src family kinases.

tyrosine phosphorylation of FAK induced by the neuropeptide bombesin is not mediated by either of the two signaling arms initiated by PLC, namely Ca^{2+} and PKC (Sinnett-Smith et al., 1993), activation of mGluR1/5 induces FAK tyrosine phosphorylation through PLC-modulated Ca^{2+}/calmodulin signaling but independent of PLC-induced PKC activation (Shinohara et al., 2001). Indeed, Ca^{2+} transient signals mediated by GPCRs were reported to induce rapid phosphorylation of FAK at serine residues (Ser^{910}, Ser^{843}) through Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), indicating that FAK is a point of integration of tyrosine and serine phosphorylation (Fan et al., 2005; Jacamo et al., 2007; Jiang et al., 2007). Our results showed that Ca^{2+} signaling and FAK1 are involved in $GABA_B$ receptor-IGF-1R transactivation, suggesting that $GABA_B$ receptor-mediated Ca^{2+} signaling may induce phosphorylation of FAK at Ser^{843}, which in turn induces FAK tyrosine kinase activity and downstream signaling. Accordingly, FAK was reported to be involved in phosphorylation of IGF-1R and may influence its downstream signaling such as Akt and ERK and receptor stability through a direct interaction (Liu et al., 2008; Watanabe et al., 2008; Andersson et al., 2009; Zheng et al., 2009). Together, we demonstrated that one GPCR could transactivate IGF-1R through $G\beta\gamma$/PLC/Ca^{2+}-dependent FAK1 pathway. Whether and how $GABA_B$-mediated $G\beta\gamma$ subunits/PLC/Ca^{2+} signaling/FAK Ser^{843} phosphorylation signaling could participate in IGF-1R transactivation through phosphorylation of FAK at tyrosine residue remains to be established.

Our results may have important significance in cerebellum development and function, as $GABA_B$ receptor has been shown to actively participate in the regulation of life and death decisions in CGNs during cerebellum development. GABA was reported to be acting as a trophic factor that can influence proliferation, migration, differentiation, synapse maturation, and cell death in immature neurons in the cerebellum (Eilers et al., 2001) through the $GABA_A$ receptors (Owens and Kriegstein, 2002; Ben-Ari et al., 2007). The developmental switch from GABA-mediated excitation to inhibition occurs after birth (Ganguly et al., 2001; Ben-Ari et al., 2007). In mature neurons, the inhibitory action of GABA is balanced by the excitatory effect of glutamate (Foster and Kemp, 2006). Overall, $GABA_B$ receptor-mediated IGF-1R pathways may be important in protecting neurons from apoptosis after $GABA_A$ receptor turn inhibitory in CGNs from postnatal mice. The anti-apoptotic effects of the $GABA_B$ receptor could be used to balance the effect of glutamate attenuating the survival properties of IGF-1 in hippocampal neurons, by decreasing IGF-1R signaling and responsiveness (Zheng and Quirion, 2009). Further studies will be required to elucidate the neuroprotective effects of $GABA_B$ receptor, and to verify whether $GABA_B$ receptor plays a similar role in other regions of the brain where this receptor is strongly expressed, such as the cortex and hippocampus.

References

Andersson S, D'Arcy P, Larsson O, Sehat B (2009) Focal adhesion kinase (FAK) activates and stabilizes IGF-1 receptor. Biochem Biophys Res Commun 387:36–41.

Belcheva MM, Haas PD, Tan Y, Heaton VM, Coscia CJ (2002) The fibroblast growth factor receptor is at the site of convergence between mu-opioid receptor and growth factor signaling pathways in rat C6 glioma cells. J Pharmacol Exp Ther 303:909–918.

Ben-Ari Y, Gaiarsa JL, Tyzio R, Khazipov R (2007) GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev 87:1215–1284.

Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signaling. Nat Rev Mol Cell Biol 1:11–21.

Bettler B, Kaupmann K, Bowery N (1998) GABAB receptors: drugs meet clones. Curr Opin Neurobiol 8:345–350.

Billinton A, Ige AO, Bolam JP, White JH, Marshall FH, Emson PC (2001) Advances in the molecular understanding of GABAB receptors. Trends Neurosci 24:277–282.

Binet V, Brajon C, Le Corre L, Acher F, Pin J-P, Prézeau L (2004) The heptahelical domain of GABAB2 is activated directly by CGP7930, a positive allosteric modulator of the GABAB receptor. J Biol Chem 279:29085–29091.

Blank JL, Brattain KA, Exton JH (1992) Activation of cytosolic phosphoinositide phospholipase C by G-protein beta gamma subunits. J Biol Chem 267:23069–23075.

Blecha JE, Anderson MO, Chow JM, Guevarra CC, Pender C, Penaranda C, Zavodovskaya M, Youngren JF, Berkman CE (2007) Inhibition of IGF-1R and lipoxygenase by nordihydroguaiaretic acid (NDGA) analogs. Bioorg Med Chem Lett 17:4026–4029.

Bondy CA, Cheng CM (2004) Signaling by insulin-like growth factor 1 in brain. Eur J Pharmacol 490:25–31.

Cheng CM, Reinhardt RR, Lee WH, Joncas G, Patel SC, Bondy CA (2000) Insulin-like growth factor 1 regulates developing brain glucose metabolism. Proc Natl Acad Sci U S A 97:10236–10241.

Couve A, Moss SJ, Pangalos MN (2000) GABAB receptors: a new paradigm in G protein signaling. Mol Cell Neurosci 16:296–312.

Cryan JF, Kaupmann K (2005) Don't worry 'B' happy!: a role for GABA(B) receptors in anxiety and depression. Trends Pharmacol Sci 26:36–43.

Dave KR, Lange-Asschenfeldt C, Raval AP, Prado R, Busto R, Saul I, Pérez-Pinzón MA (2005) Ischemic preconditioning ameliorates excitotoxicity by shifting glutamate/gamma-aminobutyric acid release and biosynthesis. J Neurosci Res 82:665–673.

Delcourt N, Thouvenot E, Chanrion B, Galéotti N, Jouin P, Bockaert J, Marin P (2007) PACAP type I receptor transactivation is essential for IGF-1 receptor signaling and antiapoptotic activity in neurons. EMBO J 26:1542–1551.

Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, Insel

PA, Messing RO (2000) Protein kinase C isozymes and the regulation of diverse cell responses. Am J Physiol Lung Cell Mol Physiol 279:L429–438.

D'Mello SR, Galli C, Ciotti T, Calissano P (1993) Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. Proc Natl Acad Sci U S A 90:10989–10993.

Dong AQ, Kong MJ, Ma ZY, Qian JF, Xu XH (2007) Down-regulation of IGF-IR using small, interfering, hairpin RNA (siRNA) inhibits growth of human lung cancer cell line A549 in vitro and in nude mice. Cell Biol Int 31:500–507.

Downward J (2004) PI 3-kinase, Akt and cell survival. Semin Cell Dev Biol 15:177–182.

Du Y, Bales KR, Dodel RC, Hamilton-Byrd E, Horn JW, Czilli DL, Simmons LK, Ni B, Paul SM (1997) Activation of a caspase 3-related cysteine protease is required for glutamate-mediated apoptosis of cultured cerebellar granule neurons. Proc Natl Acad Sci U S A 94:11657–11662.

Eilers J, Plant TD, Marandi N, Konnerth A (2001) GABA-mediated Ca2+ signaling in developing rat cerebellar Purkinje neurones. J Physiol 536:429–437.

Fan RS, Jácamo RO, Jiang X, Sinnett-Smith J, Rozengurt E (2005) G protein-coupled receptor activation rapidly stimulates focal adhesion kinase phosphorylation at Ser-843. Mediation by Ca2+, calmodulin, and Ca2+/calmodulin-dependent kinase II. J Biol Chem 280:24212–24220.

Foster AC, Kemp JA (2006) Glutamate- and GABA-based CNS therapeutics. Curr Opin Pharmacol 6:7–17.

Galli C, Meucci O, Scorziello A, Werge TM, Calissano P, Schettini G (1995) Apoptosis in cerebellar granule cells is blocked by high KCl, forskolin, and IGF-1 through distinct mechanisms of action: the involvement of intracellular calcium and RNA synthesis. J Neurosci 15:1172–1179.

Galvez T, Prezeau L, Milioti G, Franek M, Joly C, Froestl W, Bettler B, Bertrand HO, Blahos J, Pin JP (2000) Mapping the agonist-binding site of GABAB type 1 subunit sheds light on the activation process of GABAB receptors. J Biol Chem 275:41166–41174.

Galvez T, Duthey B, Kniazeff J, Blahos J, Rovelli G, Bettler B, Prézeau L, Pin JP (2001) Allosteric interactions between GB1 and GB2 subunits are required for optimal GABA(B) receptor function. EMBO J 20:2152–2159.

Ganguly K, Schinder AF, Wong ST, Poo M (2001) GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. Cell 105:521–532.

Ghil S, Choi JM, Kim SS, Lee YD, Liao Y, Birnbaumer L, Suh-Kim H (2006) Compartmentalization of protein kinase A signaling by the heterotrimeric G protein Go. Proc Natl Acad Sci U S A 103:19158–19163.

Gierschik P, Milligan G, Pines M, Goldsmith P, Codina J, Klee W, Spiegel A (1986) Use of specific antibodies to quantitate the guanine nucleotide-binding protein Go in brain. Proc Natl Acad Sci U S A 83:2258–2262.

Harayama N, Shibuya I, Tanaka K, Kabashima N, Ueta Y, Yamashita H (1998) Inhibition of N- and P/Q-type calcium channels by postsynaptic GABAB receptor activation in rat supraoptic neurones. J Physiol 509:371–383.

Heldin CH, Westermark B (1999) Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 79:1283–1316.

Hunter T, Ling N, Cooper JA (1984) Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. Nature 311:480–483.

Jacamo R, Jiang X, Lunn JA, Rozengurt E (2007) FAK phosphorylation at Ser-843 inhibits Tyr-397 phosphorylation, cell spreading and migration. J Cell Physiol 210:436–444.

Jiang X, Sinnett-Smith J, Rozengurt E (2007) Differential FAK phosphorylation at Ser-910, Ser-843 and Tyr-397 induced by angiotensin II, LPA and EGF in intestinal epithelial cells. Cell Signal 19:1000–1010.

Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao W-J, Johnson M, Gunwaldsen C, Huang L-Y, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA, Gerald C (1998) GABAB receptors function as a heteromeric assembly of the subunits GABABR1 and GABABR2. Nature 396:674–679.

Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B (1998) GABAB-receptor subtypes assemble into functional heteromeric complexes. Nature 396:683–687.

Kienlen Campard P, Crochemore C, René F, Monnier D, Koch B, Loeffler JP (1997) PACAP type I receptor activation promotes cerebellar neuron survival through the cAMP/PKA signaling pathway. DNA Cell Biol 16:323–333.

Kuner R, Köhr G, Grünewald S, Eisenhardt G, Bach A, Kornau HC (1999) Role of heteromer formation in GABAB receptor function. Science 283:74–77.

Kuramoto N, Wilkins ME, Fairfax BP, Revilla-Sanchez R, Terunuma M, Tamaki K, Iemata M, Warren N, Couve A, Calver A, Horvath Z, Freeman K, Carling D, Huang L, Gonzales C, Cooper E, Smart TG, Pangalos MN, Moss SJ (2007) Phospho-dependent functional modulation of GABAB receptors by the metabolic sensor AMP-dependent protein kinase. Neuron 53:233–247.

Lee FS, Chao MV (2001) Activation of Trk neurotrophin receptors in the absence of neurotrophins. Proc Natl Acad Sci U S A 98:3555–3560.

Lee FS, Rajagopal R, Kim AH, Chang PC, Chao MV (2002) Activation of Trk neurotrophin receptor signaling by pituitary adenylate cyclase-activating polypeptides. J Biol Chem 277:9096–9102.

Linseman DA, Phelps RA, Bouchard RJ, Le SS, Laessig TA, McClure ML, Heidenreich KA (2002) Insulin-like growth factor-I blocks Bcl-2 interacting mediator of cell death (Bim) induction and intrinsic death signaling in cerebellar granule neurons. J Neurosci 22:9287–9297.

Liu W, Bloom DA, Cance WG, Kurenova EV, Golubovskaya VM, Hochwald SN (2008) FAK and IGF-IR interact to provide survival signals in human pancreatic adenocarcinoma cells. Carcinogenesis 29:1096–1107.

Lüscher C, Jan LY, Stoffel M, Malenka RC, Nicoll RA (1997) G protein-coupled inwardly rectifying K+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. Neuron 19:687–695.

Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ, Lefkowitz RJ (1996) Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gbetagamma subunit-mediated activation of mitogen-activated protein kinases. J Biol Chem 271:19443–19450.

Mannoury la Cour C, Herbelles C, Pasteau V, de Nanteuil G, Millan MJ (2008) Influence of positive allosteric modulators on GABA(B) receptor coupling in rat brain: a scintillation proximity assay characterisation of G protein subtypes. J Neurochem 105:308–323.

Margeta-Mitrovic M, Jan YN, Jan LY (2001) Function of GB1 and GB2 subunits in G protein coupling of GABA(B) receptors. Proc Natl Acad Sci U S A 98:14649–14654.

McKernan DP, Dinan TG, Cryan JF (2009) "Killing the blues": a role for cellular suicide (apoptosis) in depression and the antidepressant response? Prog Neurobiol 88:246–263.

Mizuta K, Osawa Y, Mizuta F, Xu D, Emala CW (2008) Functional expression of GABAB receptors in airway epithelium. Am J Respir Cell Mol Biol 39:296–304.

New DC, An H, Ip NY, Wong YH (2006) GABAB heterodimeric receptors promote Ca2+ influx via store-operated channels in rat cortical neurons and transfected Chinese hamster ovary cells. Neuroscience 137:1347–1358.

Nishikawa M, Hirouchi M, Kuriyama K (1997) Functional coupling of Gi subtype with GABAB receptor/adenylyl cyclase system: analysis using a reconstituted system with purified GTP-binding protein from bovine cerebral cortex. Neurochem Int 31:21–25.

Odagaki Y, Koyama T (2001) Identification of G[alpha] subtype(s) involved in [gamma]-aminobutyric acidB receptor-mediated high-affinity guanosine triphosphatase activity in rat cerebral cortical membranes. Neurosci Lett 297:137–141.

Odagaki Y, Nishi N, Koyama T (2000) Functional coupling of GABA(B) receptors with G proteins that are sensitive to N-ethylmaleimide treatment, suramin, and benzalkonium chloride in rat cerebral cortical membranes. J Neural Transm 107:1101–1116.

Owens DF, Kriegstein AR (2002) Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3:715–727.

Peavy RD, Chang MS, Sanders-Bush E, Conn PJ (2001) Metabotropic glutamate receptor 5-induced phosphorylation of extracellular signal-regulated kinase in astrocytes depends on transactivation of the epidermal growth factor receptor. J Neurosci 21:9619–9628.

Pontier SM, Lahaie N, Ginham R, St-Gelais F, Bonin H, Bell DJ, Flynn H, Trudeau LE, McIlhinney J, White JH, Bouvier M (2006) Coordinated action of NSF and PKC regulates GABAB receptor signaling efficacy. EMBO J 25:2698–2709.

Rives M-L, Vol C, Fukazawa Y, Tinel N, Trinquet E, Ayoub MA, Shigemoto R, Pin J-P, Prézeau L (2009) Crosstalk between GABAB and mGlu1a re-

ceptors reveals new insight into GPCR signal integration. EMBO J 28:2195–2208.
Rozengurt E (2002) Neuropeptides as growth factors for normal and cancerous cells. Trends Endocrinol Metab 13:128–134.
Rozengurt E (2007) Mitogenic signaling pathways induced by G protein-coupled receptors. J Cell Physiol 213:589–602.
Satoh T, Nakafuku M, Kaziro Y (1992) Function of Ras as a molecular switch in signal transduction. J Biol Chem 267:24149–24152.
Schlessinger J (2000) New roles for Src kinases in control of cell survival and angiogenesis. Cell 100:293–296.
Schuler V, Lüscher C, Blanchet C, Klix N, Sansig G, Klebs K, Schmutz M, Heid J, Gentry C, Urban L, Fox A, Spooren W, Jaton AL, Vigouret J, Pozza M, Kelly PH, Mosbacher J, Froestl W, Käslin E, Korn R, et al. (2001) Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic GABA(B) responses in mice lacking GABA(B(1)). Neuron 31:47–58.
Selbie LA, Hill SJ (1998) G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signaling pathways. Trends Pharmacol Sci 19:87–93.
Shah BH, Catt KJ (2004) GPCR-mediated transactivation of RTKs in the CNS: mechanisms and consequences. Trends Neurosci 27:48–53.
Shah BH, Farshori MP, Jambusaria A, Catt KJ (2003) Roles of Src and epidermal growth factor receptor transactivation in transient and sustained ERK1/2 responses to gonadotropin-releasing hormone receptor activation. J Biol Chem 278:19118–19126.
Shinohara Y, Nakajima Y, Nakanishi S (2001) Glutamate induces focal adhesion kinase tyrosine phosphorylation and actin rearrangement in heterologous mGluR1-expressing CHO cells via calcium/calmodulin signaling. J Neurochem 78:365–373.
Simonds WF (1999) G protein regulation of adenylate cyclase. Trends Pharmacol Sci 20:66–73.
Sinnett-Smith J, Zachary I, Valverde AM, Rozengurt E (1993) Bombesin stimulation of p125 focal adhesion kinase tyrosine phosphorylation. Role of protein kinase C, Ca2+ mobilization, and the actin cytoskeleton. J Biol Chem 268:14261–14268.
Subramaniam S, Shahani N, Strelau J, Laliberté C, Brandt R, Kaplan D, Unsicker K (2005) Insulin-like growth factor 1 inhibits extracellular signal-regulated kinase to promote neuronal survival via the phosphatidylinositol 3-kinase/protein kinase A/c-Raf pathway. J Neurosci 25: 2838–2852.
Thodeti CK, Adolfsson J, Juhas M, Sjölander A (2000) Leukotriene D(4) triggers an association between gbetagamma subunits and phospholipase C-gamma1 in intestinal epithelial cells. J Biol Chem 275:9849–9853.
Thompson SM, Gähwiler BH (1992) Comparison of the actions of baclofen at pre- and postsynaptic receptors in the rat hippocampus in vitro. J Physiol 451:329–345.
Tosetti P, Bakels R, Colin-Le Brun I, Ferrand N, Gaiarsa JL, Caillard O (2004) Acute desensitization of presynaptic GABAB-mediated inhibition and induction of epileptiform discharges in the neonatal rat hippocampus. Eur J Neurosci 19:3227–3234.
Tu H, Rondard P, Xu C, Bertaso F, Cao F, Zhang X, Pin JP, Liu J (2007) Dominant role of GABAB2 and Gbetagamma for GABAB receptor-mediated-ERK1/2/CREB pathway in cerebellar neurons. Cell Signal 19:1996–2002.
Watanabe N, Takaoka M, Sakurama K, Tomono Y, Hatakeyama S, Ohmori O, Motoki T, Shirakawa Y, Yamatsuji T, Haisa M, Matsuoka J, Beer DG, Nagatsuka H, Tanaka N, Naomoto Y (2008) Dual tyrosine kinase inhibitor for focal adhesion kinase and insulin-like growth factor-I receptor exhibits anticancer effect in esophageal adenocarcinoma in vitro and in vivo. Clin Cancer Res 14:4631–4639.
White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM, Marshall FH (1998) Heterodimerization is required for the formation of a functional GABAB receptor. Nature 396:679–682.
Zahradka P, Litchie B, Storie B, Helwer G (2004) Transactivation of the insulin-like growth factor-I receptor by angiotensin II mediates downstream signaling from the angiotensin II type 1 receptor to phosphatidylinositol 3-kinase. Endocrinology 145:2978–2987.
Zhang F, Li C, Wang R, Han D, Zhang QG, Zhou C, Yu HM, Zhang GY (2007) Activation of GABA receptors attenuates neuronal apoptosis through inhibiting the tyrosine phosphorylation of NR2A by Src after cerebral ischemia and reperfusion. Neuroscience 150:938–949.
Zheng D, Kurenova E, Ucar D, Golubovskaya V, Magis A, Ostrov D, Cance WG, Hochwald SN (2009) Targeting of the protein interaction site between FAK and IGF-1R. Biochem Biophys Res Commun 388:301–305.
Zheng WH, Quirion R (2009) Glutamate acting on N-methyl-D-aspartate receptors attenuates insulin-like growth factor-1 receptor tyrosine phosphorylation and its survival signaling properties in rat hippocampal neurons. J Biol Chem 284:855–861.