Dominant role of GABAB2 and Gbetagamma for GABAB receptor-mediated-ERK1/2/CREB pathway in cerebellar neurons.

Haijun Tu, Philippe Rondard, Chanjuan Xu, Federica Bertaso, Fangli Cao, Xueying Zhang, Jean-Philippe Pin, Jianfeng Liu

To cite this version:
Haijun Tu, Philippe Rondard, Chanjuan Xu, Federica Bertaso, Fangli Cao, et al.. Dominant role of GABAB2 and Gbetagamma for GABAB receptor-mediated-ERK1/2/CREB pathway in cerebellar neurons.. Cellular Signalling, Elsevier, 2007, 19 (9), pp.1996-2002. 10.1016/j.cellsig.2007.05.004 . hal-00319022

HAL Id: hal-00319022
https://hal.archives-ouvertes.fr/hal-00319022
Submitted on 5 Sep 2008

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Dominant role of GABA_B2 and Gßγ for GABA_B receptor mediated-ERK1/2/CREB pathway in cerebellar neurons

Haijun Tu1,4, Philippe Rondard23, Chanjuan Xu1, Federica Bertaso2, Fangli Cao1, Xueying Zhang1, Jean-Philippe Pin2,3 and Jianfeng Liu1,*

1 Sino-France Laboratory for Drug Screening, Key Laboratory of Molecular Biophysics (Huazhong University of Science and Technology), Ministry of Education, School of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, Hubei, China.

2 Department of Molecular Pharmacology, Institute of Functional Genomics, CNRS UMR5203, Université de Montpellier, F-34094 Montpellier, France;

3 INSERM U661, F-34094 Montpellier, France;

4 Co-first authors.

Running title: GABA_B2- and Gßγ-mediated ERK1/2 activation by GABA_B.

*To whom correspondence should be addressed:

Dr Jianfeng LIU

Sino-France Laboratory for Drug Screening, Key Laboratory of Molecular Biophysics (Huazhong University of Science and Technology), Ministry of Education, School of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, Hubei, China.
1037, Luoyu Road,
430074, Wuhan, Hubei, China.
Phone: +86 27 87792031
Fax: +86 27 87792024
Email: jfliu@mail.hust.edu.cn

Keywords: GABA_B Receptor - ERK1/2 - CREB- G_i/o protein - Gßγ subunit - neurons
Abstract:

γ-aminobutyric acid type B (GABA_B) receptor is an allosteric complex made of two subunits, GABA_B1 and GABA_B2. GABA_B2 plays a major role in the coupling to G-protein whereas GABA_B1 binds GABA. It has been shown that GABA_B receptor activates ERK1/2 in neurons of the central nervous system, but the molecular mechanisms underlying this event are poorly characterized. Here, we demonstrate that activation of GABA_B receptor by either GABA or the selective agonist baclofen induces ERK1/2 phosphorylation in cultured cerebellar granule neurons. We also show that CGP7930, a positive allosteric regulator specific of GABA_B2, alone can induce the phosphorylation of ERK1/2. PTX, a G_i/o inhibitor, abolishes both baclofen and CGP7930-mediated ERK1/2 phosphorylation. Moreover, both baclofen and CGP7930 induce ERK-dependent CREB phosphorylation. Furthermore, by using LY294002, a PI-3 kinase inhibitor, and a C-term of GRK-2 that has been reported to sequester Gßγ subunits, we demonstrate the role of Gßγ in GABA_B receptor mediated-ERK1/2 phosphorylation. In conclusion, the activation of GABA_B receptor leads to ERK1/2 phosphorylation via the coupling of GABA_B2 to G_i/o and by releasing Gßγ subunits which in turn induce the activation of CREB. These findings suggest a role of GABA_B receptor in long-term change in the central nervous system.
1. Introduction

γ-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system [1, 2]. It mediates fast synaptic inhibition through GABA\textsubscript{A} and GABA\textsubscript{C} ionotropic receptors as well as slow and prolonged synaptic inhibition through the metabotropic GABA\textsubscript{B} receptor [3]. GABA\textsubscript{B} receptor mediates both presynaptic inhibition of neurotransmitter release and post-synaptic inhibition of neuronal excitability [4, 5]. Accordingly, GABA\textsubscript{B} receptor is involved in various types of epilepsy, nociception and drug addiction. Activation of the receptor also has myorelaxant activity that are commonly used to treat spasticity associated with multiple sclerosis [2, 6]

GABA\textsubscript{B} receptor belongs to the class C of G-protein coupled receptors (GPCRs), together with metabotropic glutamate (mGlu), extracellular Ca\textsuperscript{2+}-sensing, and some pheromone and taste receptors [7]. Each of these receptors is composed of an extracellular domain called the Venus flytrap domain (VFT), to which agonists bind, and a heptahelical domain (HD), which is responsible for the recognition and activation of heterotrimeric G-proteins. Whereas mGluRs and Ca\textsuperscript{2+}-sensing receptors exist as homodimers, GABA\textsubscript{B} receptor is a heterodimer composed of two homologous subunits, GABA\textsubscript{B1} and GABA\textsubscript{B2} [8-11].

Heterodimerization of GABA\textsubscript{B} receptor is a prerequisite for GABA\textsubscript{B} receptor function. The VFT domain of GABA\textsubscript{B1} is sufficient for ligand binding [7], but its assembly with GABA\textsubscript{B2} increases the affinity of GABA\textsubscript{B1} for agonists [12]. Although GABA\textsubscript{B2} does not appear to bind any natural ligand [13], GABA\textsubscript{B1} needs to be associated with GABA\textsubscript{B2} to reach the cell surface [14]. Agonist binding to the GABA\textsubscript{B1} VFT domain results in its closure and this is likely responsible for a change in the relative position of the two VFT and HD domains [15]. This movement then allows activation of G proteins mediated by the HD domains of GABA\textsubscript{B2}[16, 17]. Recently, a novel class of compounds called GABA\textsubscript{B} positive allosteric modulators (PAM) such as CGP7930, appeared to represent a better therapeutic alternative than
the classical agonist [18, 19]. Although CGP7930 alone was reported to be inactive in several assays, direct activation of the receptor by this positive allosteric modulator acting in the HD domain of GABA\textsubscript{B} have also been observed [20].

Functional GABA\textsubscript{B} receptor is predominantly coupled to heterotrimeric G\textsubscript{i/o}-type protein since most of GABA\textsubscript{B} receptor-mediated effects are inhibited by pertussis toxin (PTX) [21, 22]. Upon activation of the G protein, the G\textsubscript{βγ} complex represses Ca\textsuperscript{2+} influx by inhibiting Ca\textsuperscript{2+} channels [23] and triggers K\textsuperscript{+} channels opening [24, 25]. In the mean time, the Ga\textsubscript{i/o} subunits modulate the level of cyclic adenosine monophosphate (cAMP) by regulating adenylate cyclase activities [26].

Protein phosphorylation plays a critical role in synaptic plasticity, learning and memory in vertebrates [27]. The Extracellular signal-Regulated protein Kinases 1/2 (ERK\textsubscript{1/2}), also known as p42/44 mitogen-activated protein kinase (MAPK), signaling cascade plays important roles in the modulation of long-term potentiation in area CA1 of the hippocampus and is required for several forms of learning and memory [28]. Recently, it has been reported that GABA\textsubscript{B} receptor induces ERK\textsubscript{1/2} phosphorylation in the CA1 area of mouse hippocampus [29]. Regulation of ERK\textsubscript{1/2} signaling cascade by GPCRs is highly complex and cell type-specific [30], and the mechanism of GABA\textsubscript{B} receptor-mediated ERK\textsubscript{1/2} phosphorylation is still poorly understood. Furthermore, GABA\textsubscript{B} receptor is reported to bind to the transcription factor CREB2(cAMP responsive element binding protein-2)/ATF4(activating transcription factor 4) through coiled-coil interactions [31-33]. Nuclear translocation of CREB2 is also observed following GABA\textsubscript{B} receptor activation [31, 33]. The physiological significance for GABA\textsubscript{B} receptor activation-induced CREB2 translocation awaits elucidation.

In the current study, we examine the role of GABA\textsubscript{B} receptor in the regulation of ERK\textsubscript{1/2} and CREB phosphorylation in cultured cerebellar granule neurons. We find that selective GABA\textsubscript{B} receptor activation induces ERK\textsubscript{1/2} phosphorylation that in turn mediates CREB phosphorylation. We also show that this effect occurs via the
GABA\textsubscript{B2} coupling to G\textsubscript{i/o} proteins by releasing G\textsubscript{\beta\gamma} subunits. Interestingly, we show for the first time that selective activation of GABA\textsubscript{B2} is sufficient to induce CREB phosphorylation.
2. Materials and Methods

2.1 Materials

GABA was obtained from Sigma (Shanghai, China). Baclofen was purchased from Tocris (Fisher-Bioblock, Illkirch, France). GP54626, CGP7930, Pertussis toxin (PTX) were purchased from Calbiochem (US and Canada). Fetal bovine serum, culture medium, and other solutions used for cell culture were from Invitrogen (Shanghai, China). LY294002 and U0126 were purchased from Cell Signaling Technology (Beverly, MA). pRK5 plasmids encoding wild-type GABA_B1 and GABA_B2 with an epitope tag at their N-terminal ends under the control of a cytomegalovirus promoter were described previously [14]. The pcDNA3-c-myc-CD8-βARK plasmid, which was composed of the CD8 antigen membrane receptor and a domain containing the G-protein βγ subunit-binding site of GRK2, was a generous gift from Dr. M. De Waard.

2.2 Primary cerebellar granule neuronal cultures

Primary cultures of cerebellar cells were prepared as previously described [34]. Briefly, 1-week-old newborn mice were decapitated and cerebellum-dissected. The tissue was then gently triturated using fire-polished Pasteur pipettes, and the homogenate was centrifuged at 500rpm. The pellet was resuspended and plated in tissue culture dishes previously coated with poly-L-ornithine. Cells were maintained in a 1:1 mixture of DMEM and F-12 nutrient (Life Technologies, Gaithersburg, MD), supplemented with glucose (30mM), glutamine (2mM), sodium bicarbonate (3mM) and HEPES buffer (5mM), decomplemented fetal calf serum (10%), and 25mM KCl to improve neuronal survival. Three-days-old cultures contained 1.25 x 10^5 cells/cm^2.

2.3 Cell culture and transfection

Human embryonic kidney HEK293 were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and transfected by
electroporation as described previously [35]. Cells (10 \times 10^6) were transfected with plasmid DNA containing the cDNAs encoding GABA_B1 (4\mu g) or GABA_B2 (4\mu g), and completed to a total amount of 10\mu g of plasmid DNA with the pRK5 empty vector.

2.4 Western blot analysis

Cell lysates from cultures were sonicated, and protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories LTD, Hertfordshire, UK). Equal amounts of protein (20\mu g) were resolved by SDS-PAGE on 12\% gels. Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) and blocked in blocking buffer (5\% nonfat dry milk in TBS and 0.1\% Tween 20) for 1 hr. The blots were then incubated with the primary rabbit polyclonal antibodies against phospho-ERK_{1/2} (1:1000; Cell Signaling Technology, Beverly, MA), or with a rabbit polyclonal antibodies against the total ERK_{1/2} (1:1000; Cell Signaling Technology), overnight at 4°C. This was followed by 1 hr incubation with goat anti-rabbit horseradish peroxidase (HRP)-linked secondary antibodies (1:20000; Cell Signaling Technology). Immunoblots were revealed using the enhanced chemiluminescence reagents (Pierce, USA) and visualized using the X-Ray film. The density of immunoreactive bands was measured using NIH image software, and all bands were normalized to percentages of control values.

2.5 Drug treatment

Cultures were washed once with HBS (Ca^{2+} free) and preincubated at 37°C with HBS for 60 min. Cells were treated by adding freshly made drugs to the HBS. At the end of the treatment, cells were washed quickly with ice-cold PBS, pH 7.4 (Ca^{2+} free), lysed with 200\mu L lysis buffer and placed immediately on ice. The cell monolayer was rapidly scraped in ice-cold lysis buffer. Drugs were dissolved in HBS with or without dimethyl sulfoxide (DMSO) or/and alcohol. Whenever DMSO or/and alcohol were used, HBS containing the same concentration of DMSO or/and alcohol were used as the control vehicle.
3. Results

3.1. Activation of GABA\textsubscript{B} receptor increases ERK\textsubscript{1/2} phosphorylation in neurons

We first studied the effect of GABA and the GABA\textsubscript{B}-selective agonist baclofen, on ERK\textsubscript{1/2} phosphorylation in cultured mouse cerebellar granule neurons (CGNs). We found that GABA at 100\(\mu\)M caused a rapid and transient increase in ERK\textsubscript{1/2} phosphorylation with no changes in ERK\textsubscript{1/2} expression levels (Fig. 1A, upper panel). ERK\textsubscript{1/2} phosphorylation peaked at 10min and then decreased. Similar results were obtained with baclofen at 100\(\mu\)M (Fig. 1A, lower panel). These data showed that the activation of GABA\textsubscript{B} receptor leads to increased ERK\textsubscript{1/2} phosphorylation in CGNs.

To demonstrate that ERK\textsubscript{1/2} phosphorylation occurs via the specific activation of GABA\textsubscript{B} receptor, we evaluated the effect of the GABA\textsubscript{B} receptor selective antagonist on baclofen-induced ERK\textsubscript{1/2} phosphorylation in CGNs. Cells were pretreated for 20min with GABA\textsubscript{B} receptors antagonist, CGP54626 (10\(\mu\)M) and then stimulated with baclofen (100\(\mu\)M) for 10min. We found that this antagonist blocked baclofen-induced ERK\textsubscript{1/2} phosphorylation without to alter ERK\textsubscript{1/2} expression levels (Fig. 1B), thus demonstrating that GABA\textsubscript{B} receptor activation contributes to the baclofen-induced ERK\textsubscript{1/2} phosphorylation in CGNs.

3.2 GABA\textsubscript{B} receptor-mediated ERK\textsubscript{1/2} phosphorylation occurs through the coupling of GABA\textsubscript{B2} to G\textsubscript{i/o} protein

GABA\textsubscript{B} receptor, reported as a G\textsubscript{i/o} protein-coupled receptor activates its downstream effectors through coupling of GABA\textsubscript{B2} subunits towards G proteins [22, 23]. Recently, it was demonstrated that CGP7930, a positive allosteric modulator (PAM) of the GABA\textsubscript{B} receptor not only modulates GABA\textsubscript{B} receptor activity by directly acting on GABA\textsubscript{B2} HD domain but also activates GABA\textsubscript{B2} when expressed alone [36]. To evaluate whether GABA\textsubscript{B} receptor-mediated ERK\textsubscript{1/2} effect occurs through GABA\textsubscript{B2} coupling to G protein, we then studied the effect of CGP7930 on ERK\textsubscript{1/2} phosphorylation in CGNs. CGP7930 at a concentration of 50\(\mu\)M caused a
rapid, transient and strong increase in ERK1/2 phosphorylation in CGNs (Fig. 2A). ERK1/2 phosphorylation levels induced upon CGP7930 treatment peaked at 10 min prior to decreasing gradually to the basal level. Pretreatment of CGNs with CGP54626 even at the concentration of 100µM did not abolish CGP7930-induced ERK1/2 phosphorylation (Fig. 2A, inset panel). These results demonstrate that GABAB2 directly activates ERK1/2 pathway. In addition, the required association of GABAB1 with GABAB2 to reach the cell surface [14] indicates that GABAB receptor mediated-ERK1/2 phosphorylation in CGNs occurs through GABAB2 coupling to G protein.

We further verified that GABAB receptor mediated-ERK1/2 phosphorylation is also regulated through coupling of Gi/o protein by using pertussis toxin (PTX). Neurons were pretreated with PTX at 200ng/ml for 14-18hrs, and then stimulated for 10 min with 100µM baclofen or 50µM CGP7930 (Fig. 2B). Under those circumstances, PTX inhibited baclofen or CGP7930-induced ERK1/2 phosphorylation in CGNs (Fig. 2B), demonstrating that baclofen or CGP7930 induced ERK1/2 activation via G i/o proteins.

We confirmed the observed GABAB receptor-mediated ERK1/2 phosphorylation in HEK293 cells transfected with both GABAB1 and GABAB2, or with GABAB2 alone. GABA at 100µM caused a rapid and transient ERK1/2 phosphorylation without change in ERK1/2 levels in cells expressing both GABAB1 and GABAB2 whereas it had no effect on the mock-transfected cells (cells transfected with pRK5 empty vector) (Fig. 3A). CGP7930 alone also induced ERK1/2 phosphorylation (Fig. 3B), and this effect was not antagonized by a CGP54626 pre-treatment of the cells (Fig. 3B, inset panel). Pretreatment of the cells with PTX blocked both GABA and CGP7930-induced ERK1/2 phosphorylation (Fig. 3B). These results demonstrate that GABAB receptor-mediated ERK1/2 phosphorylation occurs via GABAB2 coupling to G i/o protein. In HEK293 cells expressing GABAB2 alone, CGP7930 induced an acute phosphorylation of ERK1/2 whereas it had no effect on mock-transfected cells (Fig.
Pretreatment of the cells with PTX efficiently reduced CGP7930-mediated ERK$_{1/2}$ phosphorylation with no change in ERK$_{1/2}$ expression levels (Fig. 4B), thus suggesting that the activation of GABA$_B_2$ by CGP7930 is sufficient to mediate ERK$_{1/2}$ phosphorylation via $G_{i/o}$ protein.

Our results in CGNs and in HEK293 cells transfected with GABA$_B_2$ alone or with both GABA$_B_1$ and GABA$_B_2$, showed that GABA$_B$ receptor induced ERK$_{1/2}$ phosphorylation via the coupling of GABA$_B_2$ to $G_{i/o}$ heterotrimeric proteins.

3.3 $G_{B\gamma}$ subunits mediate GABA$_B$ receptor-induced ERK$_{1/2}$ phosphorylation

To test whether the coupling of GABA$_B_2$ to $G_{i/o}$ protein occurs via $G_{B\gamma}$/PI-3 kinase to induce ERK$_{1/2}$ phosphorylation, we treated CGNs with the selective PI-3 kinase inhibitor, LY294002. Interestingly, the inhibition of the PI-3 kinase pathway led to a strong inhibition of both baclofen and CGP7930-induced ERK$_{1/2}$ phosphorylation in neurons (Fig. 5A), thus suggesting an important role for $G_{B\gamma}$ derived from $G_{i/o}$ proteins.

We then investigated the role of $G_{B\gamma}$ subunits in GABA$_B$ receptor-mediated ERK activation in HEK293 cells expressing the GABA$_B$ receptor. To this end, we used the previously characterized $G_{B\gamma}$-scavenger consisting of the C-terminal region of GRK2 (BARK) fused to the extracellular and transmembrane domains of CD8 which then provides a membrane anchor for BARK’s C-tail (CD8-BARK)[37] [38]. The overexpression of CD8-BARK inhibited GABA-induced ERK$_{1/2}$ phosphorylation in HEK293 cells co-expressing both GABA$_B_1$ and GABA$_B_2$ and CGP7930-induced ERK$_{1/2}$ phosphorylation in HEK293 cells expressing GABA$_B_2$ alone (Fig. 5B). Taken together, these results show that GABA$_B$ receptor mediates ERK$_{1/2}$ phosphorylation through $G_{B\gamma}$ subunits.

3.4 GABA$_B$ receptor-mediated ERK$_{1/2}$ phosphorylation induces CREB phosphorylation
Phosphorylation of ERK$_{1/2}$ plays an important role in the regulation of gene expression via phosphorylation of nuclear transcription factors [39]. We therefore tested the response of CREB to the GABA$_B$ receptor mediated-ERK$_{1/2}$ pathway. We found that baclofen at 100µM induced a rapid and transient increase in CREB phosphorylation in CGNs (Fig. 6A). Baclofen-induced CREB phosphorylation was abolished by the pretreatment of CGNs with either CGP54626 (Fig. 6B) or MEK$_{1/2}$ inhibitor U0126 (Fig. 6C). Furthermore, the pretreatment of CGNs with U0126 also inhibited CGP7930-induced CREB phosphorylation (Fig. 6C). These results show that GABA$_B$ receptor-mediated CREB phosphorylation occurs through the ERK$_{1/2}$ pathway.
4. Discussion

The main findings of the present study concern the mechanism of GABA\textsubscript{B} receptor-mediated ERK\textsubscript{1/2} phosphorylation. We show that: 1) GABA\textsubscript{B} receptor activation leads to increased ERK\textsubscript{1/2} phosphorylation in cultured cerebellar granule neurons which in turn induces CREB phosphorylation; 2) selective activation of GABA\textsubscript{B2} by CGP7930 is sufficient for ERK\textsubscript{1/2} activation in both cultured cerebellar neurons and HEK293 cells transfected with GABA\textsubscript{B2} alone or in the presence of GABA\textsubscript{B1}; 3) all these effects rely on a PTX-sensitive G\textsubscript{i/o} heterotrimeric protein-dependent pathway by releasing G\beta\gamma and by implicating PI-3 kinase pathway, and 4) both baclofen and CGP7930-mediated CREB phosphorylation is ERK\textsubscript{1/2} dependent.

Several reports have recently shown that G\textsubscript{i/o}-coupled GABA\textsubscript{B} receptor induced ERK\textsubscript{1/2} phosphorylation [29, 40-43]. However, the signaling cascades transmitting GABA\textsubscript{B} receptor signals towards ERK\textsubscript{1/2} remained unclear. Indeed, the biochemical routes linking GPCRs to ERK\textsubscript{1/2} are highly complex and cell type-specific [44]. Our results demonstrate the role of G\alpha\textsubscript{i/o} protein in selective activation of GABA\textsubscript{B} receptor to ERK\textsubscript{1/2} signaling. Furthermore, it is well established that PI3 kinase acts downstream of G\beta\gamma subunits to mediate GPCR-controlled MAPK activation [45-47]. The inhibition of the PI-3 kinase leading to a strong inhibition of baclofen-induced ERK\textsubscript{1/2} phosphorylation in neurons suggests an important role for G\beta\gamma derived from G\textsubscript{i/o} proteins for GABA\textsubscript{B} receptor-mediated ERK\textsubscript{1/2} activation.

We showed that GABA\textsubscript{B} receptor-mediated ERK\textsubscript{1/2} activation requires the presence of GABA\textsubscript{B2}. These results are compatible with a recent study where GABA\textsubscript{B1} has been reported to reach the cell surface by co-expression with GABA\textsubscript{A} receptors \(\gamma2\) subunits in heterologous cellular systems but failed to stimulate ERK\textsubscript{1/2} phosphorylation in the absence of GABA\textsubscript{B2} [43]. Furthermore, CGP7930, a positive modulator of GABA\textsubscript{B} receptor, was reported to potentiate GABA\textsubscript{B} receptor activity
by interacting with HD domain of GABA_{B2} \[36\]. However, the effect of CGP7930 alone on GABA_{B} receptor activity is controversial. Meanwhile, CGP7930 produced little or no stimulation of the GABA_{B} receptor activity in some studies \[18\], others show that CGP7930 alone can stimulate inositol phosphate accumulation in HEK293 cells co-expressing the GABA_{B} receptor and the chimeric G-protein Gqi9 \[36\]. Here, we observed CGP7930 alone displays an intrinsic agonist activity on GABA_{B} receptor mediated-ERK_{1/2} activation in both CGNs and HEK293 cells expressing GABA_{B} receptor. Competitive antagonist of GABA_{B} receptors such as CGP54626 that binds on GABA_{B1} did not block the CGP7930-mediated ERK_{1/2} phosphorylation, suggesting that GABA_{B2} activation is sufficient to induce ERK_{1/2} activation. CGP7930-mediated ERK1/2 phosphorylation in HEK-293 cells expressing GABA_{B2} alone is compatible with the effect of CGP7930 on inositol phosphate accumulation in HEK-293 expressing GABA_{B2} alone \[36\].

Several studies suggested that GABA_{B} receptor plays an important role in memory processing. For example, mice lacking the GABA_{B1} \[25\] or the GABA_{B2} subunit \[48\] suffer from severe memory impairment. Increasing evidence has shown that ERK_{1/2} plays an important role in long-term synaptic plasticity and memory through regulating protein synthesis and gene expression \[49, 50\]. Stimulation of CREB is also critical for long term potentiation \[51\] through either ERK_{1/2} or p38 pathway \[52, 53\]. In our study, we demonstrate that CREB phosphorylation is induced by selective activation of GABA_{B} receptor via an ERK_{1/2}-dependent pathway. These data suggest a role of the ERK/CREB pathway in GABA_{B} receptor-mediated long-term synaptic plasticity and memory.
Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grants No. 30530820, No.30470368) and Hi-Tech Research and Development Program of China (863 project) (Grants No. 2006AA02Z326). H. T was supported by the fund of outstanding thesis from Huazhong University of Science and Technology (D0611). We would like to thank Dr Eric Chevet and Dr Jian Zhu for helpful discussions.
Figure Legends:

**Figure 1.** Activation of GABA<sub>B</sub> receptor increases ERK<sub>1/2</sub> phosphorylation in cultured mice cerebellar granule neurons. *A*, Upper panel, time course of the endogenous ERK<sub>1/2</sub> phosphorylation after incubation of GABA (100µM). Data represent the mean ± SEM from at least five independent experiments. *Lower panel*, typical immunoblots used to quantify the phosphorylated ERK<sub>1/2</sub> (pERK<sub>1/2</sub>). *B*, Effects of the antagonist CGP54626 on baclofen-induced ERK<sub>1/2</sub> phosphorylation. CGP54626 (10µM) was incubated for 30 min before and during treatment with baclofen (10min). pERK<sub>1/2</sub> was quantified as previously in Fig. 1A, and data are the mean ± SEM from three independent experiments.

**Figure 2.** GABA<sub>B</sub> receptor-mediated ERK<sub>1/2</sub> phosphorylation occurs through the coupling of GABA<sub>B2</sub> to G<sub>i/o</sub>-protein. *A*, Effect of the CGP7930 (50µM), a PAM of GABA<sub>B2</sub> in the increase of ERK<sub>1/2</sub> phosphorylation in cultured mice CGNs. Data are the mean ± SEM from three independent experiments. *Inset*, effect of CGP54626 (100µM) on CGP7930-induced ERK<sub>1/2</sub> phosphorylation. *B*, Inhibitory effect of PTX on baclofen- and CGP7930-stimulated ERK<sub>1/2</sub> phosphorylation. Data are the mean ± SEM from three independent experiments.

**Figure 3.** GABA<sub>B</sub> receptor-mediated ERK<sub>1/2</sub> phosphorylation in HEK293 cells co-expressing both GABA<sub>B1</sub> and GABA<sub>B2</sub>. *A*, Effect of GABA (100µM) in the increase of transient ERK<sub>1/2</sub> phosphorylation. Data are the mean ± SEM from five independent experiments. *B*, Inhibitory effect of PTX on GABA- and CGP7930-stimulated ERK<sub>1/2</sub> phosphorylation. *Inset*, Effect of CGP54626 (100µM) on CGP7930-induced ERK<sub>1/2</sub> phosphorylation. Data represent the mean ± SEM from at least three independent experiments.

**Figure 4.** Activation of GABA<sub>B2</sub> increases ERK<sub>1/2</sub> phosphorylation in HEK293 cells expressing GABA<sub>B2</sub> alone. *A*, Effect of CGP7930 (50µM) in the increase of transient ERK<sub>1/2</sub> phosphorylation. *B*, Inhibitory effect of PTX on GABA- and CGP7930-
stimulated ERK$_{1/2}$ phosphorylation. Data are the mean ± SEM from at least four independent experiments.

**Figure 5.** *Gßγ* subunit mediates GABA$_B$ receptor-induced ERK$_{1/2}$ phosphorylation. 
*A,* Effect of LY294002 (20µM) on GABA$_B$ receptor-mediated ERK$_{1/2}$ phosphorylation induced by baclofen (100µM) or CGP7930 (50µM) in CGNs. *B,* Effect of over-expression of c-myc-tagged CD8-ßARK, Gßγ subunits inhibiting peptide, on GABA and CGP7930-induced ERK$_{1/2}$ phosphorylation in HEK293 cells expressing the heterodimer GABA$_B$ and GABA$_B2$ alone, respectively. Data are the mean ± SEM from at least four independent experiments.

**Figure 6.** *GABA$_B$* dependent CREB phosphorylation is mediated by ERK$_{1/2}$ phosphorylation. 
*A,* The effect of baclofen (100µM) on CREB phosphorylation (pCREB) was determined by immunoblotting, and quantified as previously for pERK$_{1/2}$ in Figure 1A. 
*B,* Effect of CGP54626 (10µM) on baclofen-induced CREB phosphorylation. 
*C,* Effects of U0126 (10µM) on baclofen- or CGP7930-mediated ERK$_{1/2}$ and CREB phosphorylation in CGNs. Data are the mean ± SEM from at least four independent experiments.
References

[1] Bettler B, Kaupmann K, Bowery N. Curr Opin Neurobiol 1998;8(3):345-350.
[2] Bowery NG. Curr Opin Pharmacol 2006;6(1):37-43.
[3] Couve A, Moss SJ, Pangalos MN. Mol Cell Neurosci 2000;16(4):296-312.
[4] Couve A, Calver AR, Fairfax B, Moss SJ, Pangalos MN. Biochem Pharmacol 2004;68(8):1527-1536.
[5] Bettler B, Tao JY. Pharmacol Ther 2006;110(3):533-543.
[6] Cryan JF, Kaupmann K. Trends Pharmacol Sci 2005;26(1):36-43.
[7] Galvez T, Prezau L, Miloti G, Franek M, Joly C, Froestl W, Bettler B, Bertrand HO, Blahos J, Pin JP. J Biol Chem 2000;275(52):41166-41174.
[8] White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM, Marshall FH. Nature 1998;396(6712):679-682.
[9] Kuner R, Kohr G, Grunewald S, Eisenhardt G, Bach A, Kornau HC. Science 1999;283(5398):74-77.
[10] Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulk A, Shigemoto R, Karschin A, Bettler B. Nature 1998;396(6712):683-687.
[11] Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwalsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagamaynam D, Noble SA, Brancheck TA, Gerald C. Nature 1998;396(6712):674-679.
[12] Liu J, Maurel D, Etzol S, Bnabet I, Ansanay H, Pin JP, Rondard P. J Biol Chem 2004;279(16):15824-15830.
[13] Kniazeff J, Galvez T, Labesse G, Pin JP. J Neurosci 2002;22(17):7352-7361.
[14] Pagano A, Rovelli G, Mosbacher J, Lohmann T, Duthey B, Stauffer D, Ristig D, Schuler V, Meigel I, Lampert C, Stein T, Prezeau L, Blahos J, Pin J, Froestl W, Kuhn R, Heid J, Kaupmann K, Bettler B. J Neurosci 2001;21(4):1189-1202.
[15] Pin JP, Kniazeff J, Binet V, Liu J, Maurel D, Galvez T, Duthey B, Havlickova M, Blahos J, Prezau L, Rondard P. Biochem Pharmacol 2004;68(8):1565-1572.
[16] Galvez T, Duthey B, Kniazeff J, Blahos J, Rovelli G, Bettler B, Prezau L, Pin JP. Embo J 2001;20(9):2152-2159.
[17] Duthey B, Caudron S, Perroy J, Bettler B, Fagni L, Pin JP, Prezeau L. J Biol Chem 2002;277(5):3236-3241.
[18] Urwyler S, Mosbacher J, Lingenhoehl K, Heid J, Ho-fettter K, Froestl W, Bettler B, Kaupmann K. Mol Pharmacol 2001;60(5):963-971.
[19] Urwyler S, Gjoni T, Koljatic J, Dupuis DS. Neuropharmacology 2005;48(3):343-353.
[20] Binet V, Goudet C, Brajon C, Le Corre L, Acher F, Pin JP, Prezau L. Biochem Soc Trans 2004;32(Pt 5):871-872.
[21] Leaney JL, Tinker A. Proc Natl Acad Sci U S A 2000;97(10):5651-5656.
[22] Odagaki Y, Koyama T. Neurosci Lett 2001;297(2):137-141.
[23] Hanayama N, Shùuya I, Tanaka K, Kabashima N, Ueto Y, Yamashita H. J Physiol 1998;509( Pt 2):371-383.
[24] Luscher C, Jan LY, Stoffel M, Malenka RC, Nicoll RA. Neuron 1997;19(3):687-695.
[25] Schuler V, Luscher C, Blanchet C, Klix N, Sanyi G, Klebs K, Schmutz M, Heid J, Gentry C, Urban L, Fox A, Spooeren W, Jaton AL, Vigouret J, Pozza M, Kelly PH, Mosbacher J, Froestl
[26] Simonds WF. Trends Pharmacol Sci 1999;20(2):66-73.
[27] Sweatt JD. Curr Biol 2001;11(10):R391-394.
[28] Sweatt JD. J Neurochem 2001;76(1):1-10.
[29] Vanhoose AM, Emery M, Jimenez L, Winder DG. J Biol Chem 2002;277(11):9049-9053.
[30] Wetzker R, Bohmer FD. Nat Rev Mol Cell Biol 2003;4(8):651-657.
[31] White JH, McIlhinney RA, Wise A, Ciruela F, Chan WY, Emson PC, Billinton A, Marshall FH. Proc Natl Acad Sci U S A 2000;97(25):13967-13972.
[32] Nehring RB, Horikawa HP, El Far O, Kneussel M, Brandstatter JH, Stamm S, Wischmeyer E, Betz H, Karschin A. J Biol Chem 2000;275(45):35185-35191.
[33] Vernon E, Meyer G, Pickard L, Dev K, Molnar E, Collingridge GL, Henley JM. Mol Cell Neurosci 2001;17(4):637-645.
[34] Van Vliet BJ, Sebben M, Dumuis A, Gabrion J, Bockaert J, Pin JP. J Neurochem 1989;52(4):1229-1239.
[35] Franck M, Pagano A, Kaupmann K, Bettler B, Pin JP, Blahos J. Neuropharmacology 1999;38(11):1657-1666.
[36] Binet V, Brajon C, Le Corre L, Acher F, Pin JP, Prezeau L. J Biol Chem 2004;279(28):29085-29091.
[37] Perroy J, Prezeau L, De Waard M, Shigemoto R, Bockaert J, Fagni L. J Neurosci 2000;20(21):7896-7904.
[38] Charest PG, Ogilvy-Longpre G, Bonin H, Azzi M, Bouvier M. Cell Signal 2006.
[39] Wang Q, Zhao J, Brady AE, Feng J, Allen PB, Lefkowitz RJ, Greengard P, Limbird LE. Science 2004;304(5679):1940-1944.
[40] Richman RW, Tombok E, Lau KK, Anantharam A, Rodriquez J, O'Bryan JP, Diverse-Pierluissi MA. J Biol Chem 2004;279(23):24649-24658.
[41] Ren X, Mody I. J Biol Chem 2003;278(43):42006-42011.
[42] Pontier SM, Lahaie N, Ginham R, St-Gelais F, Bonin H, Bell DJ, Flynn H, Trudeau LE, McIlhinney J, White JH, Bouvier M. Embo J 2006;25(12):2698-2709.
[43] Balasubramanian S, Teissere JA, Raju DV, Hall RA. J Biol Chem 2004;279(18):18840-18850.
[44] Werry TD, Sexton PM, Christopoulos A. Trends Endocrinol Metab 2005;16(1):26-33.
[45] Lopez-Ilasaca M, Crespo P, Pellici PG, Gutkind JS, Wetzker R. Science 1997;275(5298):394-397.
[46] Kim S, Jin J, Kunapuli SP. J Biol Chem 2004;279(6):4186-4195.
[47] Yart A, Roche S, Wetzker R, Laffargue M, Tonks N, Mayeux P, Chap H, Raynal P. J Biol Chem 2002;277(24):21167-21178.
[48] Gassmann M, Shaban H, Vigot R, Sansig G, Haller C, Barbieri S, Humeau Y, Schuler V, Muller M, Kinzel B, Kiehs K, Schmutz M, Froestl W, Heid J, Kelly PH, Gentry C, Jaton AL, Van der Putten H, Mombereau C, Lecourtier L, Mosbacher J, Cryan JF, Fritschey JM, Luthi A, Kaupmann K, Bettler B. J Neurosci 2004;24(27):6086-6097.
[49] Kelleher RJ, 3rd, Govindarajan A, Jung HY, Kang H, Tonegawa S. Cell 2004;116(3):467-479.
[50] Sweatt JD. Curr Opin Neurobiol 2004;14(3):311-317.
[51] Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, Storm DR. Neuron 1998;21(4):869-883.
[52] Shen CP, Tsimberg Y, Salvadore C, Meller E. BMC Neurosci 2004;5(1):36.
[53] Iordanov M, Bender K, Ade T, Schmid W, Sachsenmaier C, Engel K, Gaestel M, Rahmsdorf HJ, Herrlich P. Embo J 1997;16(5):1009-1022.
Figure 1

A

![Graph showing the effect of GABA and Baclofen on pERK1/2](image)

B

![Graph showing the effect of CGP54626 on pERK1/2](image)

C

![Bar graph showing the effect of CGP54626 on pERK1/2](image)
Figure 2

A. Time course of phosphorylated ERK1/2 (pERK1/2) following treatment with CGP7930. The graph shows the fold increase in pERK1/2 over basal levels at different time points (0, 1, 3, 5, 10, 20, 30, 45, and 60 minutes). Error bars indicate standard error of the mean.

B. Bar graph illustrating the fold increase in pERK1/2 (Fold of Basal) following treatment with various compounds in the presence or absence of PTX. The compounds include Basal, Baclofen, Basal, and CGP7930. The graph compares the levels of pERK1/2 and ERK1/2 in each treatment condition.
Figure 3

A

- Cells expressed both GABA_B1 and GABA_B2
- MOCK cells

B

- PTX - + - + - + CGP7930
- Basal GABA Basal

pERK1/2 (Fold of Basal)

- ERK1/2

- GABA

- Basal

- CGP54626

- CGP7930

- PTX

- Basal

- CGP7930

pERK1/2 (Fold of Basal)
Figure 4

A

- Cells expressed only GABA\textsubscript{B2}
- Mock cells

B

PTX

- -
- +

CGP7930

- Basal
- CGP7930

ERK1/2

pERK1/2 (Fold of Basal)

Time (min)

0 1 3 5 10 20 30 60

0

2

4

6

pERK1/2 (Fold of Basal)
Cells expressed both GABA\textsubscript{B1} and GABA\textsubscript{B2}
Cells expressed only GABA\textsubscript{B2}

LY294002
- +
Basal Baclofen
- +
Basal CGP7930

CD8-\textbetaARK
- +
Basal GABA
- +
Basal CGP7930

p\textit{ERK}1/2
(Fold of Basal)

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{}
\end{figure}
Figure 6

A

![Graph showing time course of pCREB levels](image1)

B

![Bar graph of pCREB levels](image2)

C

![Bar graph showing pCREB and pERK levels](image3)