

Published in final edited form as:

J Neurochem. 2007 February ; 100(4): 1003–1017. doi:10.1111/j.1471-4159.2006.04271.x.

GISP: a novel brain-specific protein that promotes surface expression and function of GABA_B receptors

Sriharsha Kantamneni, Sônia A. L. Corrêa, Gina K. Hodgkinson, Guido Meyer¹, Ngoc Nga Vinh, Jeremy M. Henley², and Atsushi Nishimune²

MRC Centre for Synaptic Plasticity, Department of Anatomy, School of Medical Sciences, University of Bristol, Bristol, UK

Abstract

Synaptic transmission depends on the regulated surface expression of neurotransmitter receptors, but many of the cellular processes required to achieve this remain poorly understood. To better define specific mechanisms for the GABA_B receptor (GABA_BR) trafficking, we screened for proteins that bind to the carboxy-terminus of the GABA_{B1} subunit. We report the identification and characterization of a novel 130-kDa protein, GPCR interacting scaffolding protein (GISP), that interacts directly with the GABA_{B1} subunit via a coiled-coil domain. GISP co-fractionates with GABA_BR and with the postsynaptic density and co-immunoprecipitates with GABA_{B1} and GABA_{B2} from rat brain. In cultured hippocampal neurons, GISP displays a punctate dendritic distribution and has an overlapping localization with GABA_BRs. When co-expressed with GABA_BRs in human embryonic kidney cells, GISP promotes GABA_BR surface expression and enhances both baclofen-evoked extracellular signal-regulated kinase (ERK) phosphorylation and G-protein inwardly rectifying potassium channel (GIRK) currents. These results suggest that GISP is involved in the forward trafficking and stabilization of functional GABA_BRs.

© 2007 International Society for Neurochemistry

Address correspondence and reprint requests to Professor Jeremy Henley or Dr Atsushi Nishimune, MRC Centre for Synaptic Plasticity, Department of Anatomy, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK. j.m.henley@bris.ac.uk or a.nishimune@bris.ac.uk.

¹The present address of Guido Meyer is the Max-Planck-Institute for Experimental Medicine, Hermann-Rein-Str. 3, D-37075 Göttingen, Germany.

²These authors contributed equally to this work.

Supplementary Material The following material is available for this paper online.

Figure S1 Comparison of the molecular mass of recombinant (M1) and native GISP. HEK cell, cultured neuron and brain extracts were resolved on a 5% gel and blotted with GISP antibody. The M1 GISP construct migrates at a slightly larger Mr than native GISP. Note also that minor higher molecular mass bands are detected in brain extract that correspond to the expected sizes of rat AKAP450, AKAP350 and the hypothetical protein product of transcript XM_347223. See text for details.

Figure S2 Determination of the GISP start codon. *A*, Met 1 indicates the first methionine of predicted open reading frame. Met 2, Met 3 and Met 4 indicate the successive methionines in the GISP sequence. Immunogen is the protein used to raise the anti-GISP antibody. *B*, Deletion mutant GISP constructs starting from the different potential start codons indicated in *A* were transfected into HEK cells resolved on 5% gel and analysed by immunoblotting with anti-GISP antibody. M1 to M4, methionine 1 to 4; BE, rat brain extract. *C*, Mapping the GISP antibody recognition site. GST fusion proteins of GISP peptides encompassing the antigen sequence used to raise the GISP antibody. *D*, The purified peptides shown in *C* were tested for blockade of GISP antibody binding to the M1 protein illustrated in *A* and brain extract (BE). The preincubation with the 47-103 peptide prevented GISP antibody binding.

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1471-4159.2006.04271.x>.

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Keywords

A-kinase anchoring protein; cultured neurons; GABA_B receptor; GPCR interacting scaffolding protein; hippocampus; receptor trafficking

GABA is the main inhibitory neurotransmitter in the mammalian brain. Metabotropic GABA_BRs are present at both presynaptic and postsynaptic membranes and mediate the late phase of GABAergic inhibitory transmission. Presynaptic GABA_BRs suppress neurotransmitter release by inhibiting voltage-sensitive P, N, and L-type Ca²⁺ channels, whereas postsynaptic GABA_BRs inhibit adenylate cyclase, leading to a decrease in Ca²⁺ and an increase in G-protein inwardly rectifying potassium channel (GIRK)-mediated K⁺ conductances (for reviews see Marshall *et al.* 1999; Couve *et al.* 2000; Bowery *et al.* 2002; Calver *et al.* 2002).

Functional GABA_BRs comprise heterodimers of GABA_{B1} and GABA_{B2} subunits (Marshall *et al.* 1999). GABA_{B1} contains the ligand-binding domain (Malitschek *et al.* 1999), whereas GABA_{B2} couples to the G-protein (Robbins *et al.* 2001). Studies using recombinant GABA_{B1} and GABA_{B2} showed that the individual subunits are functionally inert unless co-expressed. In addition to these *in vitro* data, GABA_{B1}^{-/-} knockout mice show no pre- or postsynaptic GABA_BR-mediated responses (Pagano *et al.* 2001; Prosser *et al.* 2001), demonstrating that GABA_{B1} is essential for functional GABA_BRs.

It is well established that GABA_{B2} is effectively expressed at the plasma membrane in the absence of GABA_{B1} (Couve *et al.* 1998; Marshall *et al.* 1999; Pagano *et al.* 2001). However, to form functional receptors, the two subunits associate, in part, via coiled-coil domains in their cytoplasmic C termini. This assembly is necessary to overcome GABA_{B1} retention in the endoplasmic reticulum via an RSRR motif, proximal to the coiled-coil domain of GABA_{B1} (Margeta-Mitrovic *et al.* 2000; Pagano *et al.* 2001). Intriguingly, in some tissues it has also been reported that GABA_{B1} can be surface expressed in the absence of GABA_{B2} (Calver *et al.* 2000), suggesting that other protein partner(s) can combine with GABA_{B1} to occlude the ER retention/retrieval motifs.

To determine what proteins interact with GABA_BRs, and to find possible additional subunits, chaperones and/or trafficking/scaffolding/anchoring proteins, several groups have performed yeast two-hybrid screens using the C-terminal domain of GABA_{B1} (Ige *et al.* 2000; Nehring *et al.* 2000; Couve *et al.* 2001; Vernon *et al.* 2001). Among these interactors, ATF4 and 14-3-3 proteins were shown to have inhibitory effects on the assembly of the GABA_BR subunits. However, none so far identified have positive effects on the forward trafficking of the GABA_BR. Here, we report a novel protein, GPCR interacting scaffolding protein (GISP), that binds to the intracellular C-terminal domain of GABA_{B1}. We show that GISP interacts with both GABA_{B1} and the GABA_{B1}/GABA_{B2} heterodimer complex *in vivo*. Furthermore, co-expression of GISP promotes the surface expression of both GABA_B receptors in heterologous cells. We also show that co-expression of GISP with GABA_{B1} and GABA_{B2} in human embryonic kidney (HEK) cells increases both baclofen-evoked mitogen-activated protein (MAP) kinase activation and GIRK channels responses. These results suggest that GISP is involved in GABA_BR forward trafficking and may reduce receptor desensitization and/or degradation, resulting in increased levels of GABA_B receptors and the stabilization of functional GABA_BR complexes.

Materials and methods

Yeast two-hybrid screening and analysis of GABA_{B1}–GISP interaction

The cytosolic C-terminal domain of GABA_{B1} (residues Arg841–Lys944; Fig. 1a) was subcloned into the pBTM-116ADE vector and was used to screen an adult rat brain cDNA library (Clontech, Palo Alto, CA, USA) in *Saccharomyces cerevisiae* L-40 reporter strain as described previously (Nishimune *et al.* 1996, 1998).

GISP cDNA cloning

The original rat GISP cDNA fragment from two-hybrid screening was used as a hybridization probe to obtain full-length cDNA from adult rat hippocampal cDNA iZAPII phage library (Stratagene, La Jolla, CA, USA). The full-length GISP cDNA was cloned into pBluescriptII (Stratagene) by assembling two large overlapping fragments. This full-length nucleotide sequence was deposited in the database (DDBJ/EMBL/GenBank accession no. DQ228948).

GST pull-down

Truncated mutants of GISP shown in Fig. 1(b) were cloned into pGEX-4T-1 (Amersham Biosciences, Uppsala, Sweden). Glutathione S-transferase (GST) fusion proteins were purified and dialysed against phosphate-buffered saline (PBS) and then used for pull-down experiments. GST pull-downs were performed as previously described (Hirbec *et al.* 2002). Anti-GABA_{B1} antibody (Chemicon, Temecula, CA, USA) was used at 1 µg/mL for the immunoblotting.

Antibodies

Anti-GISP antisera were raised by immunizing rabbits with a hexahistidine (His6) tagged fusion protein incorporating residues 1–102 (as shown in Fig. 1b). GISP-specific antibodies were affinity-purified on a HiTrap NHS-activated SepharoseTM HP column (Amersham Biosciences) coupled to the original His6-tagged immunogen. The eluates containing specific antibodies were pooled, the buffer was exchanged to PBS and the purified antibodies were stored in PBS with 30% glycerol, 0.1% bovine serum albumin and 0.1% NaN₃ at –20°C or –80°C. Other primary antibodies used were: anti-GABA_{B1a,b} antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), guinea pig anti-GABA_{B1a,b} antibody and anti-GABA_{B2} (Chemicon), mouse anti-synaptotagmin (clone 41; Pharmingen BD Biosciences, Palo Alto, CA, USA), mouse monoclonal anti-extracellular signal-regulated kinase (ERK)1/2 (Sigma, St Louis, MO, USA), mouse monoclonal anti-postsynaptic density (PSD)95 (Upstate Biotechnology, Lake Placid, NY, USA), mouse monoclonal anti-β-actin (Sigma) and mouse monoclonal anti-calreticulin (BD Transduction Laboratories, Lexington, KY, USA). Horseradish peroxidase-conjugated secondary antibodies used were goat anti-rabbit IgG, goat anti-mouse IgG or goat anti-rabbit IgG (all from Sigma). Fluorochrome-conjugated secondary antibodies are anti-rabbit Alexa 488 (green) goat, anti-guinea pig Alexa 568 (red) goat and anti-mouse Alexa 568 (red) goat (Molecular Probes, Eugene, OR, USA).

Expression constructs and transfection

A GISP expression construct was created by subcloning the full-length GISP cDNA into the *NheI* site (5′) and the *XhoI* site (3′) of the mammalian expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA). HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen). GABA_BR expression constructs *pmyc*-GABA_{B1a}, *pHA*-GABA_{B2}, were a gift from Steve Moss and Benny Bettler, respectively. The empty vector pcDNA3.1(+) (Invitrogen) was used to keep the amount of DNA for transfection constant at 5 mg per 6-cm

dish. pEGFP-C1 (Clontech) was used to express non-interacting green fluorescent protein (GFP) as a negative control. Cells were harvested 48 h after transfection.

Primary hippocampal cultures

Primary hippocampal cultures were prepared from embryonic day-18 rats exactly as previously described (Perestenko and Henley 2003).

Immunocytochemistry and confocal microscopy

Immunocytochemistry was performed as previously described (Corrêa *et al.* 2004). For double staining, the concentration of the antibodies used were as follows; rabbit anti-GISP (15 $\mu\text{g}/\text{mL}$), guinea pig anti-GABAB1a,b (Chemicon; 10 $\mu\text{g}/\text{mL}$) or anti-GABA_{B2} (Chemicon, 30 $\mu\text{g}/\text{mL}$) and mouse anti-synaptotagmin (10 $\mu\text{g}/\text{mL}$). Secondary antibodies were used at 10 $\mu\text{g}/\text{mL}$. Negative control staining with pre-blocked primary antibody or staining only using the secondary antibodies was included for every experiment. Images were obtained on a Zeiss LSM510 Meta confocal microscope (Obelkochen, Germany). There was no bleed through between the channels under the conditions used and the same physical parameters were used for all images. Images were processed using Photoshop 5.0 (Adobe Systems, San Jose, CA, USA) and Corel-DRAW 11.0 (Corel, Ottawa, ON, Canada). Quantification of the co-localization of GISP and GABA_B receptors or presynaptic markers was carried out by counting the number of the puncta per 100- μm length of dendrites for each antibodies within given fields.

Subcellular fractionation and immunoprecipitation

Fractions were obtained by differential centrifugation (Gray and Whittaker 1962). The PSD fractions were prepared as described previously (Carlin *et al.* 1980). For the microsomal fraction, S2 was centrifuged at 201 800 g for 1 h at 4°C to obtain S3 supernatant, the pellet was detergent extracted and the solubilized fractions recovered by centrifugation at 201 800 g for 1 h at 4°C. Protein concentrations were determined by the bicinchoninic acid method (Smith *et al.* 1985) to ensure that equal amounts of protein were used for immunoblotting. GABA_B receptors were solubilized by incubating with extraction buffer (1% Triton X-100, 250 mM NaCl, protease inhibitors cocktail, 20 mM Tris-HCl) for 1 h and soluble fractions recovered as supernatant by centrifugation at 20 000 g for 15 min. The resulting supernatants from these extracts were diluted five times and used in subsequent experiments. For immunoprecipitation, 2.5 $\mu\text{g}/\text{mL}$ anti-GISP was incubated by rotation with 250 μg of protein extracts at 4°C for 1 h and then with protein A–Sepharose beads (Sigma) overnight. Beads were washed three times with 5 \times diluted solubilization buffer and proteins were eluted from beads using Laemmli's sample buffer (Harlow and Lane 1988). Total proteins (input) were resolved in parallel as a control.

Cell-surface biotinylation

Living cells were biotinylated using the membrane impermeable and cleavable biotinylation reagent sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (EZ-Link sulfo-NHS-SS-biotin; 0.1 mg/ mL in PBS; Pierce, Rockford, IL, USA) for 10 min on ice as described previously (Martin and Henley 2004). The integrity of the plasma membrane and cell surface specific biotinylation was confirmed using the intracellular protein β -actin as a control. Bands were quantified using ImageJ 1.30 software (Rasband 1997–2006) and normalized to the total receptor fraction. Unpaired Student's t -tests were performed with a Newman–Keuls post-test for multiple comparison data sets.

Immunoblotting

Proteins were blotted onto Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA) and probed with appropriate primary antibodies after blocking (Harlow and Lane 1988). For detection of the signal, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma; 1 : 10 000 dilution) for 60 min followed by substrate incubation with BM Chemiluminescence Blotting Substrate (POD; Roche Molecular Biochemicals, Indianapolis, IN, USA) or SuperSignal West Femto (Pierce). The chemiluminescence signal was detected on the Hyperfilm HP (Amersham Biosciences).

Histoblots

Adult rat whole brain horizontal cryostat sections (10 μ m; approximately bregma -4.60 mm) were transferred to nitrocellulose membrane (Tonnes *et al.* 1999) and probed with anti-GISP antibody (4 μ g/mL) and secondary anti-rabbit alkaline phosphatase-conjugated secondary (0.25 μ g/mL; Sigma).

Electrophysiology

HEK cells stably expressing Kir3.1/Kir3.2 channels (a gift from Trevor Smart) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1% penicillin/streptomycin and 0.5 mg/mL G418. Cells in 6-cm dishes were transiently transfected using Lipofectamine 2000 with 0.5 μ g each of *Myc-GABA_{B1a}* and *HA-GABA_{B2}*, 0.25 μ g GFP and 4 μ g of either pcDNA-GISP or empty vector. Whole cell baclofen-activated potassium currents were obtained from fluorescent cells 40–48 h after transfection. Cells were perfused with HEPES-buffered solution containing 119 mM NaCl, 5mM KCl, 25 mM HEPES, 30 mM glucose, 2 mM CaCl₂, 2 mM MgCl₂ and pipettes were filled with intracellular solution containing 110 mM potassium methanesulfonate, 10 mM NaCl, 40 mM HEPES, 0.6 mM EGTA, 4 mM Mg-ATP, 0.3 mM Na₂-GTP, pH 7.2. Baclofen (100 μ M; in HBS) was applied to the cell (held at -50 mV) via a computer-controlled sewer pipette for 4 s every 30 s for 10 min. The peak amplitude was recorded after each exposure and then normalized to the first peak. The time constants for the comparison of 50% inactivation were calculated by data fitted to single exponential.

ERK activation assay

Assays were performed essentially as described previously (Balasubramanian *et al.* 2004). Briefly, HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen) with plasmids encoding for *Myc-GABA_{B1a}*, *HA-GABA_{B2}*, and GISP. To adjust the cDNA levels between the different conditions, cells were transfected with the pcDNA3.1(+) plasmid. Cells were plated in 6-well plates and transfected with 250 ng of *Myc-GABA_{B1a}*, *HA-GABA_{B2}* and with 2.5 μ g of plasmid coding for the GISP [or pcDNA3.1(+) as a control] in the combination indicated in the figure legends. Cells were used 48 h after transfection and serum starved overnight the day before the assay. The cells were stimulated with 100 μ M baclofen at indicated times, rinsed with ice-cold PBS, and lysed in 250 μ L of sample buffer. The cell lysates were subjected to sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) and then analysed via western blotting with anti-phospho-p44/42 MAP kinase antibody (Sigma).

Results

Isolation of GISP

We used the intracellular C-terminal domain (residues Arg841–Lys944) of the GABA_{B1} subunit as 'bait' in yeast two-hybrid (Y2H) screens of a rat brain cDNA library. The

majority of positive interactors were ATF4, a cAMP-dependent transcription factor that we have reported previously (Vernon *et al.* 2001; and see also Nehring *et al.* 2000; White *et al.* 2000). In addition, we isolated partial cDNAs that, over the sequence isolated, had ~90% homology with human A-kinase anchoring protein (AKAP)450 (Witczak *et al.* 1999). A full-length clone, obtained by hybridization screening of the rat cDNA library, established that we had isolated a novel cDNA that encodes a protein we have named GISP (Fig. 2). We concluded that the GISP clone is full length because the GISP cDNA contains frame stop codons in its 5' (upstream) untranslated region; the proposed amino acid sequence is the biggest protein that can be encoded in the cloned cDNA. We also detected a genomic sequence that is identical to this 5' untranslated region sequence just upstream of the protein coding exons in the rat *akap9* gene and it is extremely unlikely that this isolated 5' untranslated region sequence is a cloning artifact.

GISP is not a degradation product because it is exclusively localized to brain, whereas other larger *akap9* gene product proteins (i.e. AKAP450/AKAP350/AKAP9) are expressed in many other tissues. Furthermore, within the brain, GISP is localized to neurons while other *akap9* gene products are also present in glia. GISP is also far more abundant than any other higher molecular weight species (which share their epitope with GISP) and we could not detect any smaller protein product than GISP. Finally, although GISP shares substantial sequence homology with AKAP9 (yotiao), as reported below, unlike AKAP9 (Lin *et al.* 1998), GISP does not bind to the NR1 subunit of NMDA receptors in the Y2H assay.

Subsequent Y2H assays using yeast transformed with GISP and plasmids encoding specific potential interacting proteins confirmed robust binding of GISP to GABA_{B1} but showed that it does not bind to GABA_{B2}. In a series of Y2H controls to assess GISP specificity, we also established that GISP does not interact with AMPA (*GluR1-4*), kainate (*GluR5* and 6), NMDA (NMDAR1) ionotropic glutamate receptor subunits, or metabotropic glutamate receptors (m*GluR1-5*, 7; data not shown). Truncation mutagenesis of GABA_{B1} defined residues Glu867–His910 as the minimal region required for GISP binding (Fig. 1a). This region comprises a coiled-coil domain that is also required for heterodimerization with the GABA_{B2} subunit (Kammerer *et al.* 1999). Corresponding truncation mutagenesis of GISP (Fig. 1b) revealed the minimal interaction domain as Ser583–Gln800. Of this region, only the N-terminal portion (Arg595–Ile650) contains a predicted coiled-coil domain. In an attempt to further define which part of this region constitutes the GABA_{B1} binding site, we constructed two truncations ($\Delta 6$ and $\Delta 7$) and tested for interaction with GABA_{B1} in the Y2H assay. Neither truncation activated the yeast reporter, indicating that the coiled-coil interaction is necessary but not sufficient for the interaction between GISP and GABA_{B1}.

Primary structure of GISP

Using standard hybridization screening, we obtained the full-length protein coding sequence of GISP from the adult rat hippocampal cDNA library. The open reading frame we obtained is 3.3 kb in length and encodes a 1115 amino acid protein (Figs 1 and 2) with a molecular weight deduced from the primary structure as a simple polypeptide (128.5 kDa). There are several ATG codons in the sequence and we initially designated the methionine that yielded the largest open reading frame as the start codon, but subsequent analysis comparing the molecular masses of recombinant to native GISP revealed that the second ATG most likely corresponds to the actual start codon *in vivo*.

We envisage that GISP is transcribed from the same *akap9* gene (AKAP) as AKAP450 (also known as CG-NAP, hyperion; Witczak *et al.* 1999), AKAP350 (Schmidt *et al.* 1999), AKAP9 (yotiao; Lin *et al.* 1998). However, GISP is distinct from these AKAP family members because it does not have a characteristic alpha-helical protein kinase A (PKA) regulatory (RII) subunit binding motif (Fig. 1c). Analysis by the Lupas algorithm (Lupas *et*

al. 1991) predicts nine coiled-coil heptad repeats throughout the protein. GISP does not have any apparent hydrophobic clusters (average hydrophobicity was calculated as -0.803859) and is predicted as a soluble protein using the SOSUI algorithm (Hirokawa *et al.* 1998). The fact that GISP contains no signal peptide or hydrophobic cores is also consistent with it being an intracellular protein. Although it does not contain either protein phosphatase 1 or PKA binding sites, GISP shares several other binding motifs with AKAP450/350, including a calmodulin (Gillingham and Munro 2000; Takahashi *et al.* 2002) and a casein kinase-binding motif (Sillibourne *et al.* 2002). Examination of the database also revealed several additional hypothetical transcripts from the *akap9* gene but none have been verified at the protein level. One of these hypothetical transcripts (XM_347223) has sequence identity to GISP although the hypothetical protein has a predicted molecular mass of 188 kDa, significantly larger than GISP, with an additional ~ 465 residues located at the N-terminal end. GISP is not a truncation of this larger protein for the reasons set out above and because expression of this 188 kDa protein, detected with our anti-GISP antibody (see below), was very low compared with GISP (Fig. S1).

GISP binds to GABA_{B1} in GST pull-down assays

We used GST pull-down assays to confirm that the binding of GISP to GABA_{B1} occurs *in vitro*. The post-nuclear fraction of rat brain was incubated with GST-GISP $\Delta 6$, GST-GISP $\Delta 7$ and GST-GISP $\Delta 18$ truncations attached to glutathione-agarose beads. As expected from the Y2H data, only GST-GISP $\Delta 18$ retained both isoforms of GABA_{B1} and not GST alone (Fig. 3a). A faint GABA_{B1} immunoreactive band is visible in GST-GISP $\Delta 6$ and GST-GISP $\Delta 7$. GST-GISP $\Delta 18$ is the minimum region of GISP required for efficient binding of GABA_{B1} receptors and truncations of GST-GISP $\Delta 18$, GST-GISP $\Delta 6$ and GST-GISP $\Delta 7$ might retain some weak capacity of binding.

Generation and characterization of a specific anti-GISP antibody

Because of the high degree of sequence identity between GISP and AKAPs, no available antibodies can specifically distinguish GISP. Furthermore, although there are commercially available anti-human AKAP450 antibodies, no antibodies directed against rat AKAP450 have been reported. We therefore made new polyclonal antibodies against the N-terminal sequence of GISP (residues 1–102; Fig. 2 and Fig. S2). The resultant affinity-purified anti-GISP antibody recognized the predominant ~ 130 -kDa band in immunoblots of cytosolic (S2) and crude membrane fractions (P2) from rat brain and a single ~ 130 kDa band in cultured hippocampal neurons. When the antibody was pre-incubated with the immunogen, the specific signal disappeared completely (Fig. 3b).

Given the sequence identity between the antigenic peptide used to raise our anti-GISP antibody and human AKAP350 and AKAP450, we tested if it detected AKAP350/450 and related proteins expressed in brain and cultured neurons. The commercial anti-human AKAP450 antibodies did not detect any bands in rat tissue, although we did detect a 450-kDa band in HEK cells (data not shown). However, using high-sensitivity western blotting techniques with our GISP antibody, we did detect higher molecular weight protein bands consistent with AKAP450, AKAP350 and the protein encoded by XM_347223 in rat brain (Fig. S1). Significantly, none of these additional bands was present in primary cultured rat cortical/hippocampal neurons. Because (i) the roles of AKAP350 and AKAP450 are strongly associated with cell division, (ii) neither protein has not been reported in neurons, (iii) the immunoreactive band at the molecular mass predicted for GISP is far more intense than any other bands in western blots of brain and, (iv) only the GISP band was detected in cultured neurons grown in absence of glial cells, we conclude that the non-GISP bands from brain correspond to proteins that are far less abundant than GISP and that these are present only in glial cells. Thus, our results indicate that AKAP350 and AKAP450 are not present in

mature neurons and, importantly for the immunocytochemistry, that our anti-GISP antibody recognizes only GISP in primary cultured hippocampal neurons.

It is also evident on close examination of western blots run in low percentage (5%) acrylamide gels that there is a small molecular weight difference between our recombinant and endogenous GISP. The molecular weight of the recombinant GISP is ~4 kDa larger than that of the endogenous GISP. This might be as a result of the translation starting at a different start codon. Therefore, we attempted to map the anti-GISP antibody epitope within the N-terminal of our recombinant antigen by splitting the antigen into two GST fusion proteins, GISP 1–46 and GISP 47–103. GISP antibody binding was not affected with pre-incubation of GST-GISP 1–46 recombinant protein, but completely blocked when pre-incubated with GST-GISP 47–103, suggesting that the GISP epitope is confined to this region (Fig. S2).

Regional distribution of GISP

Using this antibody, we first investigated the crude tissue distribution of GISP. Immunoblots of a variety of tissues revealed that GISP is present at detectable levels only in the brain (Fig. 3c). Histoblots were used to assess the pattern of GISP expression within the brain (Fig. 3d). GISP immunoreactivity was widespread throughout the structures of the brain with particularly high levels in the hippocampal formation.

Comparison of the developmental profiles of GABA_BR and GISP expression

We next determined the levels of expression of GISP, the two GABA_{B1} isoforms and GABA_{B2} in whole brain of rats at different age points (Fig. 4). GISP was present at all ages examined but expression increased with age, peaking around 2–3 weeks after birth. Consistent with a previous report (Fritschy *et al.* 1999), expression of GABA_{B1a} and GABA_{B1b} subunit isoforms showed an inverse relationship. While neither was abundant prior to birth, GABA_{B1a} was most evident at P2 and levels decreased thereafter. Interestingly, the GABA_{B2} subunit showed a different profile. Very little GABA_{B2} immunoreactivity was present prior to birth, followed by increasing levels that peaked at age 2–3 weeks. The marked differences in the expression profiles of GISP and GABA_B subunits suggests that GISP may have role(s) in addition to those involving interaction with GABA_BRs.

Subcellular localization of GISP and GABA_BR

To determine the compartmentalization of GISP in neurons, we performed subcellular fractionations. GISP immunoreactivity was present in both cytosolic and membrane fractions including the S3 where no GABA_BRs were observed (Fig. 5a). We further investigated the compartmentalization of GISP and GABA_BRs by detergent extraction of both microsomal and synaptic membrane fractions. GISP associated with microsomal membranes and showed a very similar extraction profile to both GABA_{B1} and GABA_{B2} subunits, with considerable resistance to the mild detergent conditions, only being efficiently extracted with 1% SDS (Fig. 5b). Similarly, in the synaptic membrane extracts, GISP and GABA_BRs were strongly associated with the mild detergent-resistant fraction (Fig. 5c). Calreticulin and PSD95 antibodies were used as controls for subcellular fractionation. As expected, calreticulin was enriched in the microsomal fraction and was not present in PSD fractions. PSD95 was predominantly enriched in the postsynaptic density fraction (Fig. 5), indicating that fractionation was efficient.

Co-immunoprecipitation of GABA_{B1} and GABA_{B2} with anti-GISP antibody

Anti-GISP antibody immunoprecipitates from rat brain were probed with anti-GABA_{B1} or anti-GABA_{B2} antibody (Fig. 6). GISP binds to both GABA_{B1b} and GABA_{B1a} and our blots reflect the relative abundance of the GABA_{B1b} and GABA_{B1a} isoforms in mature rat brain (Fritschy *et al.* 1999). Furthermore, as shown in Fig. 4, the two isoforms are developmentally regulated, with GABA_{B1a} more highly expressed in the developing brain. Both isoforms are present in the GISP immunoprecipitate from adult brain P2 fraction, although the GABA_{B1a} isoform is comparatively weak. One of the reasons may be the reflection from differential compartmentalization of GABA_{B1} receptor variants (1a and 1b), where GABA_{B1a} is preferentially localized at presynaptic sites and GABA_{B1b} postsynaptically (Vigot *et al.* 2006). GABA_{B1} and GABA_{B2} subunits were detected in the co-immunoprecipitates, confirming that GISP binds GABA_{B1} *in vivo* and, because GISP does not directly interact with GABA_{B2}, these results also demonstrate that GISP binds to the mature GABA_B heterodimer.

Immunolocalization of GISP

Our anti-GISP antibody recognizes a single ~130 kDa band in our glia-free hippocampal cultures. We therefore used this antibody to investigate the distribution of endogenous GISP in 21-days *in vitro* hippocampal cultures. Consistent with the immunoblot data, strong staining was detected which was completely abolished by pre-incubation of the antibody with the antigen peptide (Figs 7a–c). GISP expression was absent from the nucleus, but widespread throughout the cell soma. In dendrites, GISP was showed a defined punctate localization. To determine if the punctate dendritic staining corresponded to synapses, we investigated the extent of co-localization with the presynaptic protein synaptotagmin (Figs 7d–f and 4–6). There were 68.2 ± 4.8 synaptotagmin and 48.8 ± 5.1 GISP immunopositive puncta per 100 μm of dendrite. Quantification of co-localization revealed that $68.4 \pm 7.5\%$ GISP puncta were directly localized at synaptotagmin-labelled synapses.

Co-localization of GISP and GABA_BRs

The developmental profile of GABA_BR function and subunit localization in this culture system has been reported previously (Corrêa *et al.* 2004). In agreement with those data, highly defined punctate GABA_{B1} and GABA_{B2} immunostaining was present in 21-days *in vitro* hippocampal neurons (Fig. 8). In the cell body (excluding the nucleus), and in the proximal dendrites, there was a high level of overlapping distribution for GISP with both GABA_{B1} and GABA_{B2} subunits. We attribute this to the relatively high levels of endogenous expression and diffuse distribution of each of the proteins. In more distal dendrites, however, the co-localization of the proteins was more distinct. GISP labelled as puncta in the dendritic shaft and protuberances. In many cases, these structures were also labelled with GABA_{B1} staining, but less so by GABA_{B2} antibody. Quantitative analysis revealed that there were 38.2 ± 2.8 GABA_{B1} puncta and 31.6 ± 1.9 GABA_{B2} puncta per 100 μm of dendrite. Overall, there was $65.4 \pm 4.4\%$ co-localization of GABA_{B1} and $34.4 \pm 5.1\%$ co-localization of GABA_{B2} with GISP.

GISP promotes surface expression of GABA_B receptors in HEK cells

As reported previously (Couve *et al.* 1998), co-expression of GABA_{B1} and GABA_{B2} subunits in HEK cells results in the efficient surface expression of both subunits. GABA_{B2} expressed alone also traffics to the plasma membrane, whereas GABA_{B1} alone shows very low levels of surface expression (Couve *et al.* 1998; Marshall *et al.* 1999; Pagano *et al.* 2001). As GISP interacts at the same coiled-coil domain required for heteromeric receptor assembly, we tested if expression of GISP would have an effect on surface expression of GABA_B receptors. When GABA_{B1} and GABA_{B2} were co-expressed, efficient surface

expression was observed, but this was further increased by GISP (Figs 9a and b). There was a more than 70% increase in GABA_{B1a} expression ratio and a 25% increase in GABA_{B2} receptor expression ratio. As GISP interacts at the same coiled-coil domain required for heteromeric receptor assembly, we tested if expression of GISP would allow surface expression of GABA_{B1}. There was an increase in surface *myc*-GABA_{B1} expression when co-expressed with GISP (Fig. 9c), although the surface expression was less than that achieved on co-expression with GABA_{B2}. These results indicate that occupation of the coiled-coil domain of GABA_{B1} by GISP is required to promote the forward traffic of GABA_B receptors from the ER/Golgi complexes to the plasma membrane.

GISP enhances GABA_BR function

We first tested whether infusion of GISP in to CA1 hippocampal neurons altered synaptic GABA_BR-mediated responses. We did not detect any acute changes (monitored up to 40 min) in synaptic GABA_BR-mediated responses on infusion of purified recombinant GISPΔ18 from the patch pipette (data not shown). To assess the effects of GISP over a longer time course, we transfected HEK cells with GISP and GABA_BR subunits and used two independent functional assays, namely ERK MAP kinase (Balasubramanian *et al.* 2004) and GIRK channel activation (Couve *et al.* 2002). We detected robust responses in both physiological assay systems in cells co-expressing GABA_{B1} and GABA_{B2}. As shown in the Fig. 10, in the phosphorylated ERK (pERK) assay, the time of onset and total ERK phosphorylation following baclofen application was significantly increased in cells expressing GABA_{B1}/GABA_{B2} and GISP compared with cells expressing just GABA_{B1} and GABA_{B2}. Specifically, there was an increase in pERK activation post-baclofen stimulation measured over 10 min. Interestingly, GISP did not affect the peak activation, rather it prolonged the pERK response. Similarly, in HEK cells stably expressing GIRK (Kir3.1 and Kir3.2) K⁺ channels and transfected with GABA_{B1} + GABA_{B2} ± GISP, we found that the peak depolarization response was unaltered by GISP. However, there was a significant difference in the profile and extent of rundown in the baclofen-evoked response. The time constants for inactivation were calculated by data fitted to single exponentials. The time constants were 88.8 ± 12.2 and 146.8 ± 4.36 s, respectively, for control and GISP expressing cells (p = 0.01).

Discussion

We have identified GISP, a novel coiled-coil protein that interacts specifically with the GABA_{B1} subunit in a coiledcoil domain-dependent manner. Primary structure analysis revealed that GISP is transcribed from the *akap9* gene that encodes at least three different AKAPs identified at the protein level; AKAP450 (CG-NAP), AKAP350, and AKAP9. AKAPs are a family of proteins that contain two classes of binding sites, a 'targeting domain' which directs the subcellular localization of the PKA-AKAP complex to membranes or cellular organelles, and an anchoring motif that binds the Arg subunit of PKA (Michel and Scott 2002). There are now known to be examples of AKAP family members that, in addition to anchoring PKA, also anchor a diverse range of other proteins, including different kinases and phosphatases (Wong and Scott 2004). Thus, AKAPs can be regarded as a class of scaffolding proteins that serve as focal reaction centres for PKA and other enzymes. We therefore propose that AKAP-like molecules such as a GISP are likely to function as scaffolding proteins for enzymes other than PKA. For example, GISP shares consensus of calmodulin and casein kinase binding sites (Gillingham and Munro 2000; Sillibourne *et al.* 2002; Takahashi *et al.* 2002) with AKAP450/CG-NAP. Moreover, the microtubule targeting domain of CG-NAP (AKAP450) is also conserved in GISP, suggesting that GISP may be involved in some microtubule-associated functions (Takahashi *et al.* 2002).

Using a novel GISP antibody, we demonstrated that GISP is a brain-specific protein. Interestingly, other proteins encoded by the *akap9* gene are not expressed in a brain-specific manner and our data indicate that, of all these proteins so far characterized, only GISP is expressed in neurons. Within the brain, GISP is widely distributed with the highest levels present in the cerebellum and all regions of the hippocampus (Fig. 3d). GABA_B receptors show a similar pattern of immunoreactivity, with particularly high levels in the hippocampus and cerebellum. These data are consistent with GABA_B binding sites analysed by autoradiography (Bowery *et al.* 1987; Chu *et al.* 1990).

Although GISP is predicted as a soluble protein, it was detected as both a cytosolic protein and tightly membrane-attached forms. In particular, high levels of GISP were present in microsomal and synaptic membrane fractions. Furthermore, detergent extraction assays showed that GISP is enriched, together with GABA_{B1}, in the 1% Triton X-100 insoluble fraction. This is likely because of the high content of the coiled-coil regions in GISP. Coiled-coil domains are known to be sites of homomultimeric and heteromultimeric interactions with other coiled-coil domain-containing proteins to form stable complexes. For example, it is known that many cytoskeletal and associated proteins, such as a myosin heavy chain (Adamson *et al.* 1993), contain coiled-coil domains that participate in the formation of filamentous structures.

The developmental profile of GISP showed that it is present at all stages of GABA_BR subunit expression. GISP was relatively abundant at embryonic day 18, the earliest age point measured, and expression increased to a peak at around the third week after birth. In contrast, very low levels of GABA_{B1} and GABA_{B2} were detected before birth. One explanation may be that, in addition to the role in GABA_BR trafficking reported here, GISP may have other as yet unknown functions.

A key test for the association of native proteins *in vivo* is co-immunoprecipitation. This approach does not necessarily establish if proteins bind directly to each other, but it does determine if they assemble in a complex. As expected from their direct binding, a strong GABA_{B1} band was present in the anti-GISP immunoprecipitate. In addition, a robust GABA_{B2} signal was also detected, indicating that both GISP and GABA_{B2} can simultaneously bind at the coiled-coil domain of GABA_{B1}. These results suggest a role for GISP with the GABA_BR complex rather than specifically for the non-heterodimerized GABA_{B1} subunit.

As GISP is a newly discovered protein, we set out to define its cellular localization in cultured hippocampal neurons. Immunocytochemical localization of GISP in cultured hippocampal neurons revealed a highly punctate distribution throughout the cell processes. Comparison with the synaptic marker synaptotagmin indicated that GISP was present in synapses and that the majority of puncta were synaptic. GISP was co-localized to varying extents with both GABA_{B1} and GABA_{B2} (~65 and ~34%, respectively). The fact that there were higher levels of GISP co-localization with GABA_{B1} than GABA_{B2} suggests that GABA_{B1}-GISP complexes also exist. These data support the biochemical results by indicating that GISP is present in close proximity to GABA_BRs in physiologically relevant regions of the cells.

Coiled-coil interactions at the C-terminal of GABA_{B1} are known to play a role in the cell surface expression of GABA_BR subunits (Marshall *et al.* 1999). Coiled-coil domains are well-studied relatively complex structures that can specifically mediate the interaction of several protein partners at once (Lupas 1996). For example, our results for GISP differ from those obtained for ATF4, another protein that interacts with the coiled-coil domain of GABA_{B1}. ATF4 binds specifically to only the free GABA_{B1} subunit and not to the

GABA_BR complex (Vernon *et al.* 2001). This is relevant because the localization of the GABA_{B1} subunit when not associated with GABA_{B2}, is restricted to the ER because of an RXR(R) retention motif immediately following the C-terminal coiled-coil domain (Margeta-Mitrovic *et al.* 2000). This retention motif is masked by the dimerization of GABA_{B1} with GABA_{B2}, thus permitting the transport of the fully formed receptor to the cell surface.

As the GISP–GABA_{B1} interaction also accommodates the GABA_{B2} subunit, we wondered if GISP could have an effect on the forward traffic of GABA_B receptors from the ER. We show that expression of the GISP efficiently enhances the GABA_B receptors expression to the plasma membrane (Fig. 8). GISP is present in microsomal fraction from brain and, intriguingly, GISP enhances the surface expression of both subunits of the heteromeric receptor by promoting forward traffic of GABA_{B1} along with GABA_{B2} and allows the surface expression of the receptors. We postulate that GISP acts as a chaperone, releasing receptors from the ER to progress through the secretory pathway and therefore the GABA_B receptors are less rapidly targeted for degradation. As the GISP–GABA_{B1} interaction also accommodates the GABA_{B2} subunit, we wondered if GISP could fulfil a similar role in allowing the forward traffic of GABA_{B1} from the ER in the absence of GABA_{B2}. Expression of the GISP markedly enhanced GABA_{B1} expression at the plasma membrane, although the amount of GABA_{B1} surface expression is less on co-expression with GISP compared with GABA_{B2}. In terms of trafficking, therefore, GISP can substitute to some extent for GABA_{B2}. This is of particular interest because, in neurons in some brain areas (e.g. caudate putamen), the GABA_{B2} receptor mRNA message is virtually absent, and in some other regions, such as the septum, the preoptic area and the hypothalamus, its levels are significantly lower than that of GABA_{B1} mRNA (Jones *et al.* 1998; Margeta-Mitrovic *et al.* 1999; Clark *et al.* 2000). As shown in Fig. 3(d), GISP is very well expressed in the caudate putamen and we suggest that occupation of the coiled-coil domain on GABA_{B1} allows ER exit in the absence of GABA_{B2}.

To determine the functional effects of GISP, we used two HEK cell-based assay systems. In both the pERK and GIRK channel activation assays, GISP increased the overall baclofen-evoked responses, but did not alter peak levels of activation. Taken together, these results are consistent with GISP acting to enhance GABA_BR function by promoting the forward trafficking of the GABA_BR to the surface (more receptors leading to larger second messenger signal and a quicker onset of ERK phosphorylation and a slower rundown of GIRK channel current). In addition, the more prolonged response may also have a component attributable to decreased desensitization of the functional receptors. Because GABA_BRs do not readily internalize, one possibility is that GISP may lead to conformational changes in the receptor complex, facilitating longer G-protein activation. The effects of GISP on GABA_B receptor functional coupling and possible effects on membrane stabilization need further experimentation.

In conclusion, we have identified a new, brain-specific GABA_BR interacting protein that is involved in receptor forward traffic, receptor stability and possibly in slowing receptor desensitization. Further work will be directed to dissect the exact mechanisms for each of these roles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to the Wellcome Trust, the MRC and the EU (GRIPPANT) for financial support. We thank Abigail Woollard and Zafar Bashir for slice electrophysiology experiments and Stéphane Martin, Tristan Bouschet, David

Holman, Claire Palmer, Simon Ball and Elek Molnar for their help and advice during the course of this work. We are also grateful to Steve Moss, GSK and Novartis for providing cDNA plasmids and Trevor Smart for the HEK cells stably expressing GIRKs.

Abbreviations used

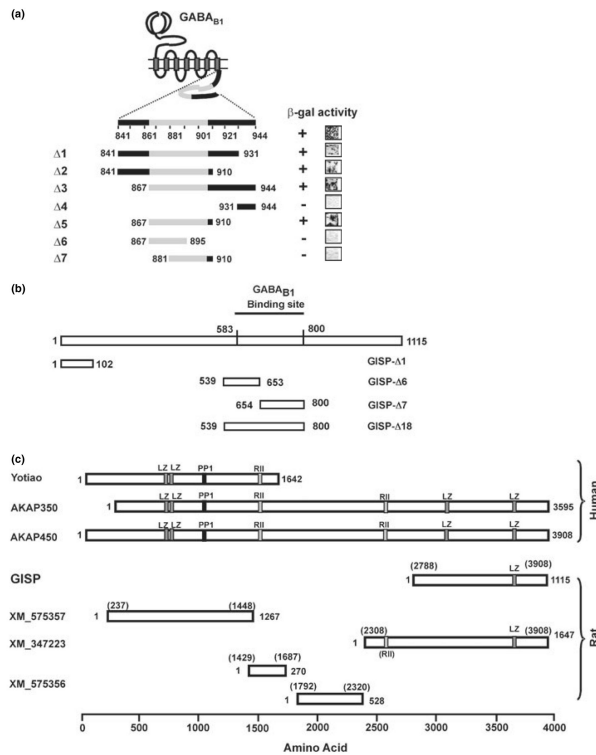
| | |
|-------------|---|
| AKAP | A-kinase anchoring protein |
| ERK | extracellular signal-regulated kinase |
| GFP | green fluorescent protein |
| GIRK | G-protein inwardly rectifying potassium channel |
| GISP | GPCR interacting scaffolding protein |
| GST | glutathione S-transferase |
| HEK | human embryonic kidney |
| MAP | mitogen-activated protein |
| PBS | phosphate-buffered saline |
| PKA | protein kinase A |
| PSD | postsynaptic density |
| SDS | sodium dodecyl sulfate |

References

- Adamson JG, Zhou NE, Hodges RS. Structure, function and application of the coiled-coil protein folding motif. *Curr. Opin. Biotechnol.* 1993; 4:428–337. [PubMed: 7763973]
- Balasubramanian S, Teissere JA, Raju DV, Hall RA. Hetero-oligomerization between GABA_A and GABA_B receptors regulates GABA_B receptor trafficking. *J. Biol. Chem.* 2004; 279:18 840–18 850.
- Bowery NG, Hudson AL, Price GW. GABA_A and GABA_B receptor site distribution in the rat central nervous system. *Neuroscience.* 1987; 20:365–383. [PubMed: 3035421]
- Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI, Enna SJ. International Union of Pharmacology. XXXIII. Mammalian γ -aminobutyric acid B receptors: structure and function. *Pharmacol. Rev.* 2002; 54:247–264.
- Calver AR, Medhurst AD, Robbins MJ, et al. The expression of GABA_{B1} and GABA_{B2} receptor subunits in the CNS differs from that in peripheral tissues. *Neuroscience.* 2000; 100:155–170. [PubMed: 10996466]
- Calver AR, Davies CH, Pangalos M. GABA_B receptors: from monogamy to promiscuity. *Neurosignals.* 2002; 11:299–314. [PubMed: 12566919]
- Carlin RK, Grab DJ, Cohen RS, Siekevitz P. Isolation and characterization of postsynaptic densities from various brain regions: enrichment of different types of postsynaptic densities. *J. Cell Biol.* 1980; 86:831–845. [PubMed: 7410481]
- Chu DC, Albin RL, Young AB, Penney JB. Distribution and kinetics of GABA_B binding sites in rat central nervous system: a quantitative autoradiographic study. *Neuroscience.* 1990; 34:341–357. [PubMed: 2159128]
- Clark JA, Mezey E, Lam AS, Bonner TI. Distribution of the GABA_B receptor subunit gb2 in rat CNS. *Brain Res.* 2000; 860:41–52. [PubMed: 10727622]
- Corrêa SA, Munton R, Nishimune A, Fitzjohn S, Henley JM. Development of GABA_B subunits and functional GABA_B receptors in rat cultured hippocampal neurons. *Neuropharmacology.* 2004; 47:475–484. [PubMed: 15380367]
- Couve A, Filippov AK, Connolly CN, Bettler B, Brown DA, Moss SJ. Intracellular retention of recombinant GABA_B receptors. *J. Biol. Chem.* 1998; 273:26 361–26 367.

- Couve A, Moss SJ, Pangalos MN. GABA_B receptors: a new paradigm in G protein signaling. *Mol. Cell. Neurosci.* 2000; 16:296–312. [PubMed: 11085869]
- Couve A, Kittler JT, Uren JM, Calver AR, Pangalos MN, Walsh FS, Moss SJ. Association of GABA_B receptors and members of the 14-3-3 family of signaling proteins. *Mol. Cell. Neurosci.* 2001; 17:317–328. [PubMed: 11178869]
- Couve A, Thomas P, Calver AR, Hirst WD, Pangalos MN, Walsh FS, Smart TG, Moss SJ. Cyclic AMP-dependent protein kinase phosphorylation facilitates GABA_B receptor-effector coupling. *Nat. Neurosci.* 2002; 5:415–424. [PubMed: 11976702]
- Fritschy JM, Meskenaite V, Weinmann O, Honer M, Benke D, Mohler H. GABA_B-receptor splice variants GB1a and GB1b in rat brain: developmental regulation, cellular distribution and extrasynaptic localization. *Eur. J. Neurosci.* 1999; 11:761–768. [PubMed: 10103070]
- Gillingham AK, Munro S. The PACT domain, a conserved centrosomal targeting motif in the coiled-coil proteins AKAP450 and pericentrin. *EMBO Rep.* 2000; 1:524–529. [PubMed: 11263498]
- Gray EG, Whittaker VP. The isolation of nerve endings from brain: an electron-microscopic study of cell fragments derived by homogenisation and centrifugation. *J. Anat.* 1962; 96:79–88. [PubMed: 13901297]
- Harlow, E.; Lane, D. *Antibodies, A Laboratory Manual.* Cold Spring Harbour Laboratory; Cold Spring Harbour, NY: 1988. p. 471-510.
- Hirbec H, Perestenko O, Nishimune A, Meyer G, Nakanishi S, Henley JM, Dev KK. The PDZ proteins PICK1, GRIP, and syntenin bind multiple glutamate receptor subtypes: analysis of PDZ binding motifs. *J. Biol. Chem.* 2002; 277:15 221–15 224.
- Hirokawa T, Boon-Chiang S, Mitaku S. SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics.* 1998; 14:378–379. [PubMed: 9632836]
- Ige AO, Bolam JP, Billinton A, White JH, Marshall FH, Emson PC. Cellular and sub-cellular localisation of GABA_{B1} and GABA_{B2} receptor proteins in the rat cerebellum. *Brain Res. Mol. Brain Res.* 2000; 83:72–80. [PubMed: 11072097]
- Jones KA, Borowsky B, Tamm JA, et al. GABA_B receptors function as a heteromeric assembly of the subunits GABA_B R1 and GABA_B R2. *Nature.* 1998; 396:674–679. [PubMed: 9872315]
- Kammerer RA, Frank S, Schulthess T, Landwehr R, Lustig A, Engel J. Heterodimerization of a functional GABA_B receptor is mediated by parallel coiled-coil α -helices. *Biochemistry.* 1999; 38:13 263–13 269.
- Lin JW, Wyszynski M, Madhavan R, Sealock R, Kim JU, Sheng M. Yotiao, a novel protein of neuromuscular junction and brain that interacts with specific splice variants of NMDA receptor subunit NR1. *J. Neurosci.* 1998; 18:2017–2027. [PubMed: 9482789]
- Lupas A. Coiled coils: new structures and new functions. *Trends Biochem. Sci.* 1996; 21:375–382. [PubMed: 8918191]
- Lupas A, Van Dyke M, Stock J. Predicting coiled coils from protein sequences. *Science.* 1991; 252:1162–1164.
- Malitschek B, Schweizer C, Keir M, et al. The N-terminal domain of c-aminobutyric acid B receptors is sufficient to specify agonist and antagonist binding. *Mol. Pharmacol.* 1999; 56:448–454. [PubMed: 10419566]
- Margeta-Mitrovic M, Mitrovic I, Riley RC, Jan LY, Basbaum AI. Immunohistochemical localization of GABA_B receptors in the rat central nervous system. *J. Comp. Neurol.* 1999; 405:299–321. [PubMed: 10076927]
- Margeta-Mitrovic M, Jan YN, Jan LY. A trafficking checkpoint controls GABA_B receptor heterodimerization. *Neuron.* 2000; 27:97–106. [PubMed: 10939334]
- Marshall FH, Jones KA, Kaupmann K, Bettler B. GABA_B receptors – the first 7TM heterodimers. *Tips.* 1999; 20:396–399. [PubMed: 10498952]
- Martin S, Henley JM. Activity-dependent endocytic sorting of kainate receptors to recycling or degradation pathways. *EMBO J.* 2004; 23:4749–4759. [PubMed: 15549132]
- Michel JJ, Scott JD. AKAP mediated signal transduction. *Annu. Rev. Pharmacol. Toxicol.* 2002; 42:235–257. [PubMed: 11807172]

- Nehring RB, Horikawa HP, El Far O, Kneussel M, Brandstatter JH, Stamm S, Wischmeyer E, Betz H, Karschin A. The metabotropic GABA_B receptor directly interacts with the activating transcription factor 4 (ATF-4). *J. Biol. Chem.* 2000; 45:35 185–35 191.
- Nishimune A, Nash SR, Nakanishi S, Henley JM. Detection of protein–protein interactions in the nervous system using the two-hybrid system. *Trends Neurosci.* 1996; 19:261–266. [PubMed: 8799967]
- Nishimune A, Isaac JT, Molnar E, Noel J, Nash SR, Tagaya M, Collingridge GL, Nakanishi S, Henley JM. NSF binding to GluR2 regulates synaptic transmission. *Neuron.* 1998; 21:87–97. [PubMed: 9697854]
- Pagano A, Rovelli G, Mosbacher J, et al. C-terminal interaction is essential for surface trafficking but not for heteromeric assembly of GABA_B receptors. *J. Neurosci.* 2001; 21:1189–1202. [PubMed: 11160389]
- Perestenko PV, Henley JM. Characterization of the intracellular transport of GluR1 and GluR2 α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunits in hippocampal neurons. *J. Biol. Chem.* 2003; 278:43 525–43 532.
- Prosser HM, Gill CH, Hirst WD, et al. Epileptogenesis and enhanced prepulse inhibition in GABA_{B1}-deficient mice. *Mol. Cell. Neurosci.* 2001; 17:1059–1070. [PubMed: 11414794]
- Rasband, W. ImageJ 1.30 software. US National Institutes of Health; Bethesda, Maryland, USA: 1997–2006. <http://rsb.info.nih.gov/ij>
- Robbins MJ, Calver AR, Filippov AK, et al. GABA_{B2} is essential for G-protein coupling of the GABA_B receptor heterodimer. *J. Neurosci.* 2001; 21:8043–8052. [PubMed: 11588177]
- Schmidt PH, Dransfield DT, Claudio JO, Hawley RG, Trotter KW, Milgram SL, Goldenring JR. AKAP350, a multiply spliced protein kinase A-anchoring protein associated with centrosomes. *J. Biol. Chem.* 1999; 274:3055–3066. [PubMed: 9915845]
- Sillibourne JE, Milne DM, Takahashi M, Ono Y, Meek DW. Centrosomal anchoring of the protein kinase CK1d mediated by attachment to the large, coiled-coil scaffolding protein CG-NAP/AKAP450. *J. Mol. Biol.* 2002; 322:785–797. [PubMed: 12270714]
- Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goetze NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 1985; 150:76–85. [PubMed: 3843705]
- Takahashi M, Yamagiwa A, Nishimura T, Mukai H, Ono Y. Centrosomal proteins CG-NAP and kendrin provide microtubule nucleation sites by anchoring c-tubulin ring complex. *Mol. Biol. Cell.* 2002; 13:3235–3245. [PubMed: 12221128]
- Tonnes J, Stierli B, Cerletti C, Behrmann JT, Molnar E, Streit P. Regional distribution and developmental changes of GluR1-flop protein revealed by monoclonal antibody in rat brain. *J. Neurochem.* 1999; 73:2195–2205. [PubMed: 10537080]
- Vernon E, Meyer G, Pickard L, Dev K, Molnar E, Collingridge GL, Henley JM. GABA_B receptors couple directly to the transcription factor ATF4. *Mol Cell. Neurosci.* 2001; 17:637–645. [PubMed: 11312600]
- Vigot R, Barbieri S, Brauner-Osborne H, et al. Differential compartmentalization and distinct functions of GABA_B receptor variants. *Neuron.* 2006; 50:589–401. [PubMed: 16701209]
- White JH, McIllhinney RA, Wise A, Ciruela F, Chan WY, Emson PC, Billinton A, Marshall FH. The GABA_B receptor interacts directly with the related transcription factors CREB2 and ATFx. *Proc. Natl Acad. Sci. USA.* 2000; 97:13 967–13 972.
- Witczak O, Skalhegg BS, Keryer G, Bornens M, Tasken K, Jahnsen T, Orstavik S. Cloning and characterisation of a cDNA encoding an A-kinase anchoring protein located in the centrosome, AKAP450. *EMBO J.* 1999; 18:1858–1868. [PubMed: 10202149]
- Wong W, Scott JD. AKAP signalling complexes: focal points in space and time. *Nat. Rev. Mol. Cell Biol.* 2004; 5:959–970. [PubMed: 15573134]
- Yap KL, Kim J, Truong K, Sherman M, Yuan T, Ikura M. Calmodulin target database. *J. Struct. Funct. Genomics.* 2000; 1:8–14. [PubMed: 12836676]

**Fig. 1.**

Isolation of GISP and characterization of binding domains. (a) Identification of GISP binding domain on GABA_{B1}. Grey region denotes the coil-coiled domain. Truncation mutagenesis of GABA_{B1} defined residues Glu867–His910 as the minimal region required for GISP binding. Representative β-galactosidase stain of yeast colonies is illustrated. (b) Schematic diagram showing the truncated mutants of GISP used in this study. When used in the yeast two-hybrid assay, only Δ18 (539–800) and the original cDNA fragment from the screening activated the β-galactosidase reporter. (c) Comparison of GISP with other AKAPs transcribed from the same gene *akap9*. Human yotiao, AKAP350 and AKAP450 have been characterized at the protein level. With the exception of GISP, the rat proteins are hypothetical from expressed sequence tag (EST) sequences. LZ, leucine zipper domain; PP1, protein phosphatase 1 consensus binding motif, RII, PKA RII consensus binding motif.

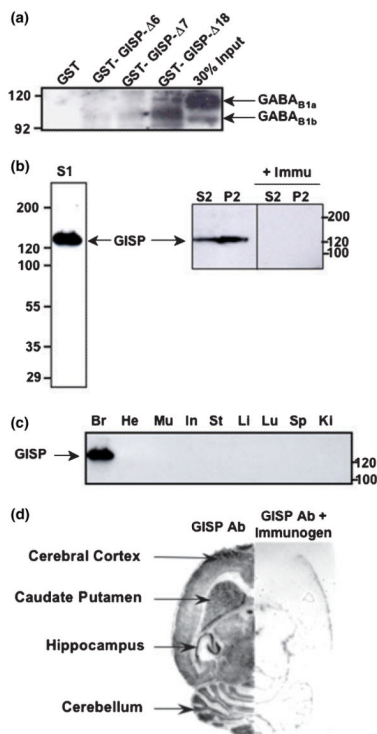
```

MERTGKVSSSNQTPQILVRHAGVQIDLQSERSQEELRDTINQFTEKMERI 50
QELHAAEILDMESRHILETESILKKEHSVAIQLLTKECETLKDMTQYLRCK 100
KGSSISDLADSVAYQSREYVSSDSESDWGSQGFDTATEGREEGETSDLF 150
PKKIKGLVKAVHSEGMQVLSLSNPLCDDGEDRSVQQLSESWLKERQTYLN 200
TISSLKDLISKMQVQRETEVYDSCQSHDSFSDWRGELLACQRVFIKERS 250
VLLATFQTELTSLSTRDADGVLNSLEQRIQEQQIEYQTAMDCLOKADRRS 300
LLTEIEDLRAQINGRKMTLEREQEIEKPSQELLDNLOQKQSHVLEMQLE 350
LGSRLDRAAELEQQLSSEKMVVAELKSELAQAKLELGTTLKAQHKLKEL 400
EAFRSEIKEKTDEIHFLSDTLAREQKKSLELQWALEKEKAKSGHHEWEK 450
EELEDLKFSLQDQRKNTQLNLLLEQQKQLLNESQOKMESQKMLHDAQLS 500
EEQGRNLGLQALLESEQVRVQEMKSTLDKERELYAQLQSRDDGGQPLPVL 550
PSEDLLKELQKQLEEKHTRIVELLSETEKYKLDSLQTRQMEKDREVHQK 600
TLQTEQEANTQGQKMQELOSKVEELORQLOEKRQVYKLDLEGKRLQGI 650
MQEFQKQELEPEEKRGSRGLVYQNLNEPASWTFDDRTRNVVLQOKMGET 700
KDTNFTKLTIEINGELDHNHDLEVIROTLQHVASKLQHVAKACNRLQFET 750
ASDDAFIWIQENIDGIILQLOKLTGQPGDEHSLAPPSSSCGSLTESLMRQ 800
NTELTRLINQLTEEKNTLRSIVVKLEELNRCHWHTGAGRDCCSRFSFID 850
ADIEAIIASEKEVWNREKLSLQKALKRAEAKVYKLAELRNDALLRSLSP 900
DSEHAALQKIYNKYLRASSFRKALIQKYYLLLLLGGFQECEDATLGVLA 950
RMGGHPALKDPKTIITSHPKGFTRFRSAVRVSIASRMKFLVRRWQQTST 1000
SSININRDGFGLSPGIEKTDPFYHSSGGLYELYGEPRHTTYRSRFDLDYPR 1050
SPLPLQNRYPGAPDLNSTSMASSQLHQYNPKSLTDYVTRLEALKRRLGA 1100
IQSGSTTQFHFGMR 1115

```

Fig. 2.

Primary structure of GISP. The deduced primary structure of GISP is shown in single-letter amino acid code. The region of GISP prepared as His₆-tagged immunogen is shown as highlighted in grey. The mapped epitope within this immunogen is shown as bold letters. Underlined regions represent predicted coiled-coil as predicted by the Lupas algorithm (Lupas *et al.* 1991). The minimum GABA_{B1} binding site is highlighted in black. Leucines in bold and larger font show the predicted leucine-zipper region. Methionine 47 is likely to be used as the start codon *in vivo*. The predicted calmodulin binding site (CRS motif; unclassified category) is shown by the dotted-underline (Yap *et al.* 2000).

**Fig. 3.**

Interaction between GISP and GABA_{B1} *in vitro* and regional distribution of GISP. (a) Verification of the interaction between GISP and GABA_{B1} by GST-pull down: GST was used as negative control and GST-GISPΔ6, GST-GISPΔ7 and GST-GISPΔ18 (GABA_{B1} interacting region in GISP). GABA_{B1} binds to GST-GISPΔ18 but not to GST. The input was 30% obtained from post-nuclear fractions of P8 rats. Eight per cent gel was used and 10 μg of GST fusion protein was used for the pull-down. The blot is a representative of three different experiments. (b) Anti-GISP antibody recognizes a predominant 130-KDa band protein band in rat brain (S1 = post-nuclear supernatant = S2 + P2; S2 = crude cytosolic fraction; P2 = crude membrane fraction). No signal was present on pre-incubation with the immunogen. Representative of two different experiments. (c) Tissue distribution of GISP. All lanes were loaded with 100 μg protein and anti-GISP antibody was used at 1 μg/mL. Br, whole brain; He, heart; Mu, skeletal muscle; In, intestine; St, stomach; Li, liver; Lu, lung; Sp, spleen; Ki, kidney. Representative of three different experiments. (d) The distribution of GISP immunoreactivity in brain was assessed by histoblot technique. Representative of three different experiments.

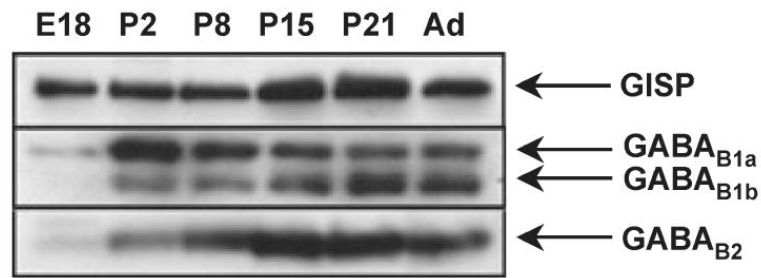


Fig. 4.

Developmental profiles of GISP and GABA_B receptor subunits in rat brain. Membrane extracts were prepared from rat whole brain taken at the ages indicated in days (E, embryonic, P, post-natal, Ad, adult) and probed for GISP, GABA_{B1} and GABA_{B2} immunoreactivity. GISP was expressed at the every stage when either GABA_{B1} or GABA_{B2} is expressed. Note that the two GABA_{B1} isoforms show a markedly different profile. Only GISP was abundant prior to birth. The data are representative of three separate experiments. β -actin blots were performed as a loading controls (data not shown).

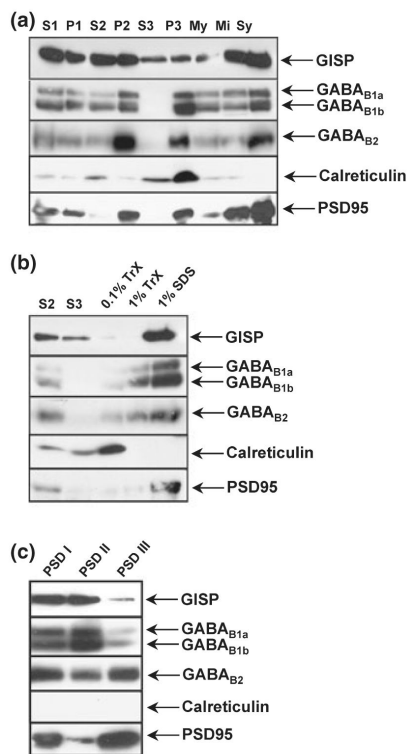


Fig. 5. Subcellular compartmentalization of GISP and GABA_BRs in rat brain extracts. (a) Distribution of GISP in subcellular fractions from rat brain. Protein (50 μ g) was loaded per lane. Purified anti-GISP antibody was used at 1 μ g/mL. The fractions are; S1, cell contents minus nuclei and debris; P1, nuclei and debris; S2, crude microsomes and cytosol; P2, crude synaptosomes; S3, cytosol; P3, enriched microsomes; My, myelin; Mi, mitochondria; Sy, enriched synaptosomes. These data are representative of three separate experiments. (b) Detergent extraction of microsomal fraction. S2, crude microsomes and cytosol; S3, cytosol; 0.1% TrX, enriched microsomes (P3) solubilized with 0.1% Triton X-100; 1% TrX, enriched microsomes (P3) solubilized with 1% Triton X-100; 1% SDS, enriched microsomes (P3) solubilized in 1% SDS. These data are representative of three separate experiments. (c) Detergent extraction of PSD fraction. PSD I, insoluble PSD fraction after 0.5% Triton X-100 extraction; PSD II, PSD fraction insoluble after 1% Triton X-100 extraction; PSD III, insoluble PSD fraction after 3% sarcosyl extraction. These data are representative of three separate experiments.

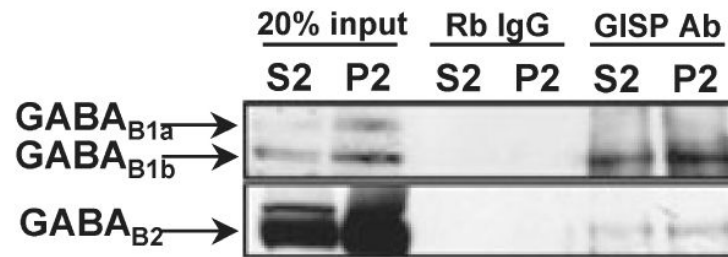


Fig. 6.

Co-immunoprecipitation of GABA_{B1} and GABA_{B2} subunits with anti-GISP antibody. Anti-GISP antibody was used to isolate GABA_B receptor complex from rat brain. GISP antibody (2.5 lg) was used for immunoprecipitation of both GABA_{B1} and GABA_{B2} subunits from crude microsomal and crude membrane fractions. Rabbit IgG was used as a negative control. The data are representative of three separate experiments.

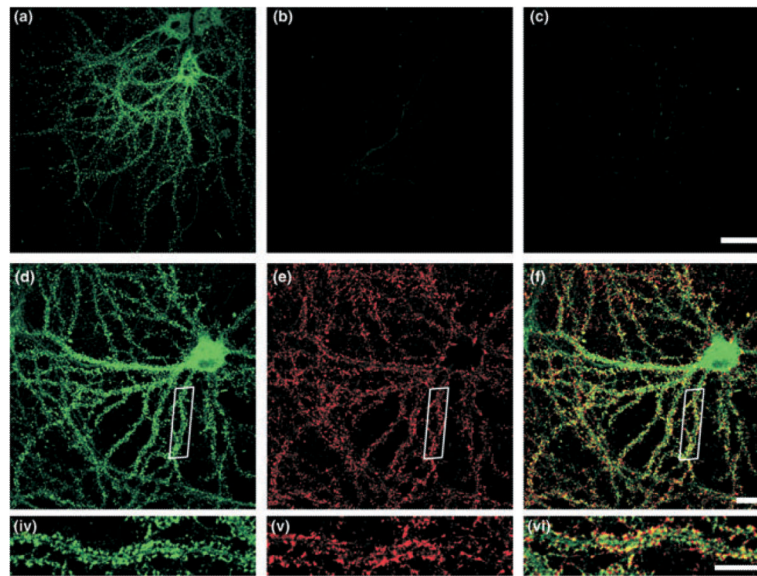


Fig. 7. Localization of GISP in neurons. Embryonic hippocampal neurons were permeabilized with 0.1% Triton X-100 and stained with anti-GISP antibody (green; 15 $\mu\text{g}/\text{mL}$). Anti-synaptotagmin antibody (red; 10 $\mu\text{g}/\text{mL}$) was used as a presynaptic marker. The images are representative of four different experiments. (a) GISP, (b) GISP antibody pre-incubated with immunogen, (c) secondary antibody only, (d) GISP, (e) synaptotagmin and (f) merged. Scale bar 10 μm .

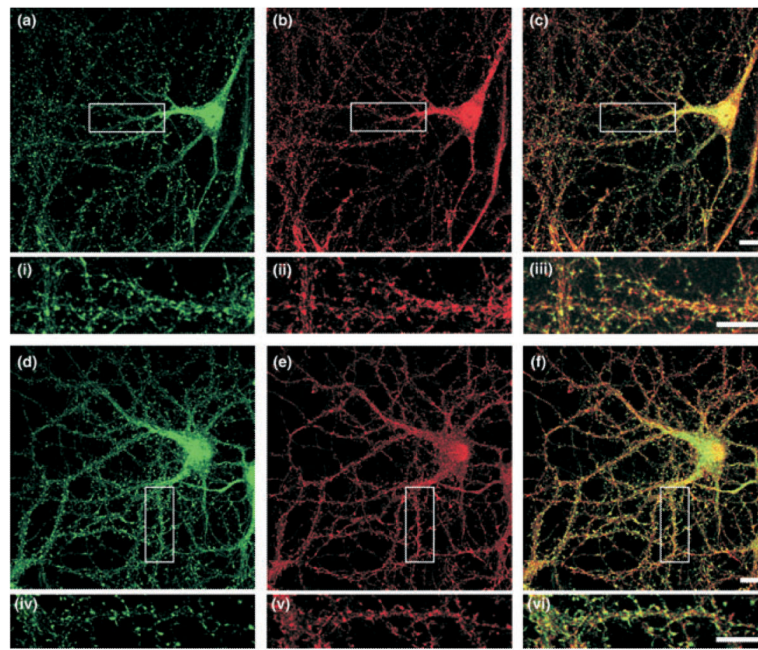
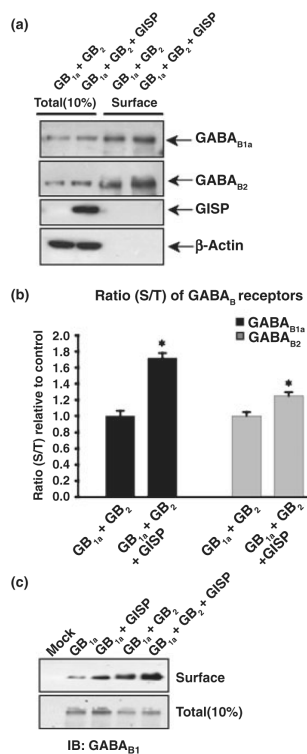


Fig. 8. Comparison of the localization of GISP and GABA_BR subunits in cultured hippocampal neurons. Embryonic hippocampal neurons were permeabilized with 0.1% Triton X-100 and stained with anti-GISP antibody (green; 15 $\mu\text{g}/\text{mL}$). Anti-GABA_{B1} antibody (red; 10 $\mu\text{g}/\text{mL}$), or anti-GABA_{B2} antibody (red; 30 $\mu\text{g}/\text{mL}$) were used. The figure is representative of four different experiments. (a) GISP, (b) GABA_{B1}, (c) merged; (d) GISP; (e) GABA_{B2}, (f) merged. Scale bar 10 μm .

**Fig. 9.**

GISP promotes surface expression of GABA_B receptors in HEK cells. (a) Effect of GISP on GABA_{B1}/GABA_{B2} complex surface expression assessed by surface biotinylation. HEK293 cells were transfected with *myc*-GABA_{B1a} and *HA*-GABA_{B2} in combination with a control vector (pcDNA3.1) or vector encoding GISP. The data are representative of three separate experiments. Forty-eight hours posttransfection, the total and cell surface GABA_B protein was determined as described in Materials and methods. Immunoblots were probed with anti-GABA_B antibodies and re-probed with anti- β -actin antibody to ensure equal loading and the specificity of surface biotinylation. (b) Histogram of densitometric analysis of the effect of GISP on GABA_{B1a} and GABA_{B2} protein surface expression ratio (surface to total) measured by biotinylation assays as illustrated in (a). The results shown are the ratios of three independent experiments. **p* < 0.001 compared with control (Students's t-test). (c) Effect of GISP on GABA_{B1} surface expression assessed by surface biotinylation. HEK293 cells were transfected with *myc*-GABA_{B1a} in combination with a control vector (pcDNA3.1) or the vector encoding GISP. Forty-eight hours post-transfection, the total and cell surface GABA_B protein was determined as described in Materials and methods. The data are representative of three separate experiments. Immunoblots were probed with anti-GABA_{B1} antibody and re-probed with anti- β -actin antibody (data not shown) to ensure equal loading and the specificity of surface biotinylation.

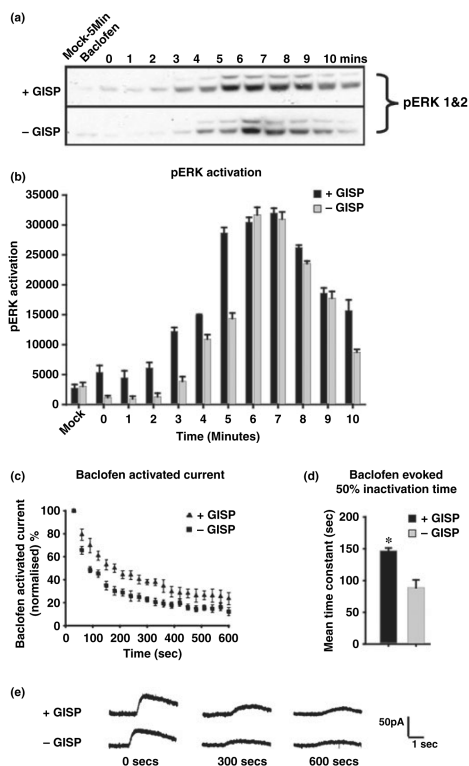


Fig. 10.

GISP increases GABA_BR function in HEK cells. (a) The effect of GISP on GABA_BR-evoked ERK phosphorylation assessed by MAP kinase assay. HEK293 cells were transfected with *myc*-GABA_{B1a} and *HA*-GABA_{B2} in combination with a control vector (pcDNA3.1) or vector encoding GISP. Forty-eight hours post-transfection, the cells were stimulated with 100 μM baclofen at the times indicated and pERK was determined as described in Materials and methods. The blots were also probed with anti-β-actin antibody to ensure equal loading (data not shown). (b) Densitometric analysis of the effect of GISP on the GABA_BR activation pERK. Cumulative histogram of the time course and extent of pERK activation from three independent experiments. (c) Repeated application of baclofen (4 s every 30 s) results in desensitization of potassium currents in HEK cells stably expressing GIRK channels (KIR3.1/3.2) transfected with GABA_{B1} and GABA_{B2}. In cells co-transfected with GISP (filled triangles; *n* = 4) the rate of desensitization is reduced compared with controls (filled squares; *n* = 7). (d) The time constants for the comparison of 50% inactivation were calculated from data fitted to a single exponential. In cells co-transfected with GISP, (black bars) the time of desensitization is longer compared with controls (grey bars). **p* = 0.01 compared with control (without GISP; Student's *t*-test). (e) Raw data from patch-clamp recordings of baclofen activated currents.