Supplementary informations

Material and methods

Materials

All reagent for cell culturing (Fetal bovin serum, G418, glutamine, F12/DMEM media and antibiotics) were purchased from Wisent. Different antibodies have been used in this study: mouse 9E10 anti-myc or 12CA5 anti-HA antibodies (house made), rat 3F10 anti-myc antibody (Roche), anti-NSF antibodies from both rabbit (Calbiochem) and mouse (Boehringer) and finally anti-GBR2 and anti-GBR1 polyclonal antibodies were both made by GSK and generated in rabbit and in sheep, respectively. For Western blot analysis, secondary mouse anti-sheep (SIGMA) and goat anti-rabbit or anti-mouse antibodies (Amersham) conjugated to the horseradish peroxydase were used. For immunofluorescence, secondary antibodies were conjugated as follow: goat anti-rat, anti-rabbit or anti-mouse antibodies coupled respectively to Alexa 488, Alexa568, Alexa633, Texas Red or Oregon green were used (all from Molecular Probes). GF109203X (GFX) was purchased from Calbiochem while phorbol 12-myristate 13-acetate (PMA), Baclofen and GABA were obtained from SIGMA. 3-(1-pyridinio)-1-propanesulfonate (NDSB) was obtained from Fluka. For deglycosylation experiments, EndoH and PNGase F enzymes were supplied by Roche.

Constructs

For YTH studies, GBR1 and GBR2 c-termini (L^{860}-K^{961} and V^{741}-L^{941}) or the indicated GBR2 c-termini truncations were generated and cloned in frame with Gal4 binding domain ($GAL4_{BD}$). $GAL4_{AD}$ fusions for NSF were expressed in pACT2 (Clontech), either isolated directly from human brain cDNA library or constructed directly by PCR. Full-length sequence of tagged myc-

$GABA_B$-R1b and HA-$GABA_B$R2 were inserted into pcDNA3.1(-) as described previously (White et al. 1998). To generate double stable cell line (HA-GBR2/myc-GBR1b), myc-GBR1b was subcloned into pCMVpuro3 plasmid (Invitrogen) between NheI and AflII. For Gluthation-S-Transferase (GST) pull down studies, GBR1 and GBR2 c-termini (containing the residues ^{861}I-^{961}K and ^{744}I-^{941}L, respectively) were amplified by PCR and cloned in frame with GST into pGEX4T2 (Amersham) between EcoRI and XhoI. His_6tagged-NSF plasmid was constructed by amplifying NSF from YTH template and inserting it into pQE81 plasmid (Qiagen) between BamHI and SalI; the NSF portionwas verified by sequencing. GST-TAT fusion proteins were generated by inserting synthesized oligonucleotides coding for the TAT-HA tag between BamHI and EcoRI sites of the pGEX4T2 plasmid. Similar method was used to fuse the coding sequence of human GBR2 derived Pep27 (^{799}RMKITELDKDLEEVTMQLQDTPEKTT^{825}Y) peptide or rat GluR2 derived pep2m (K^{844}RMKVAKNPQ^{853}) to either GST-TAT-HA or GST proteins between EcoRI and XhoI. In each case, the coding sequence of the peptide was flanked with a stop codon. The random sequence peptide (RSP) contained in GST-RSP or GST-TAT-HA-RSP proteins corresponds to an eleven amino acid peptide found in pGEX4T2 plasmid after EcoRI site.

YTH

Yeast (Saccharomyces cerevisiae Y190) expressing either a $GAL4_{BD}$–GBR1ct or a $GAL4_{BD}$–GBR2ct fusion protein were selected and transformed with a human brain Matchmaker cDNA library (HL4004AH, CLONTECH) to give a 3-fold representation of the library. Interacting clones were selected with 20 mM 3-amino-1,2,4-triazole (Sigma) followed by production of β-galactosidase as determined by a freeze-fracture assay. DNA from positive clones was recovered from yeast by using the Yeastmaker plasmid isolation kit (CLONTECH), and transformed into Escherichia coli before sequencing. Direct two-hybrid interactions were confirmed in Y190 co-transformed with pYTH16, containing the whole c-terminus of GBR1 or GBR2 or c-terminal

fragments of GBR2 fused to $GAL4_{BD}$, and pACT2, expressing NSF as $GAL4_{AD}$ fusions. Fusion constructs were generated directly through PCR or were retrieved as isolates from the library screen. Levels of interaction were determined as described before.

Protein purification

All recombinant proteins were expressed in BL21 Rosetta Blue following an induction of 4h. All purification steps were performed on ice. For His_6-tagged-NSF purification, bacteria were lysed in 50 mM HEPES/KOH pH 7.6, 250 mM KCl, 2 mM MgCl2, 2 mM ATP, 10% glycerol, and 0.5 mM DTT by mild sonication as described previously (Hanson et al. 1997). Insoluble materials were removed by a 30000g centrifugation for 30min and NSF was precipitated by binding to Ni^{2+}-agarose (Qiagen). Agarose was washed with 50mM Imidazole and eluted with 250mM imidazole. Imidazole was removed on a PD-10 columns (Biorad). The purified ATPase was then quick-frozen in liquid nitrogen.

To purify GST-GBR1ct or GST-GBR2ct, bacteria were harvested and washed in buffer A (25 mM HEPES-KOH (pH 7,4), 200 mM KCl, 2mM $MgCl_2$, 1% Triton-X100, 25 mM DTT and 1mM PMSF). Cells were disrupted by sonication and centrifuged for 30 min at 30000 g. In the case of GST-GBR1ct protein purification, supernatant were incubated with glutathione-agarose beads (Amersham) during 2h under rocking. Beads were then harvested by centrifugation, washed in the same buffer and eluted with 20mM Gluthation. Gluthation was removed on PD-10 column. Since GST-GBR2ct protein displayed insolubility, proteins had to be extracted from inclusion bodies present in the pellet. So, pellet were washed twice in 25 mM Hepes-KOH (pH 7,4), 200 mM KCl, 1% TritonX-100, 1 mM DTT, 1mM PMSF and solubilized in 25 mM Hepes-KOH (pH 7,4), 6M Guanidium-HCl for 1h and insoluble material were pelleted for 10 min at 100000g. Guanidium solubilized proteins were then quickly diluted in 1M NDSB solution and

incubated for 1h under rocking to favor folding. After an O/N dialysis in 25 mM Hepes-KOH (pH 7,4), 200 mM KCl buffer, properly folded proteins were purified on glutathione-agarose beads as described previously for GST-GBR1ct. GST-TAT-peptide proteins were purified in PBS following manufacturer's instructions. The level of purity and the amount of purified proteins were quantified by coumassie blue staining following their resolution on a SDS-PAGE, using increasing amounts of BSA protein as reference**.**

In vitro binding assay

GST-hybrid proteins (2 µg) were immobilized on glutathione-agarose beads in 25 mM Hepes-KOH (pH 7,4), 200 mM KCl, 1% TritonX-100, 10% glycerol and 1 mM DTT (buffer A). Beads were then washed twice with buffer A plus 2 mM ATP, 8 mM $MgCl_2$ and 8 mM EDTA (buffer B) or 2mM ATPγS and 8mM $MgCl_2$ in the presence of 0,2% BSA and incubated with 50 nM or the indicated amount of NSF for 1h under rocking. After four washes in buffer B without BSA, bound proteins were eluted with 20 mM glutathione and detected by western blotting. To determine the effect of the modulation of NSF-ATPase activity on its binding to GST-fusion proteins, the same procedure was performed in the presence of the indicated mix of $MgCl_2$, EDTA and nucleotide.

Cell culture and rat hippocampal slices preparation

CHO cells stably expressing either myc-GBR1b, HA-GBR2, GBR1a/GBR2 (White et al. 2000)), or myc-GBR1b/HA-GBR2 were maintained in F12/DMEM media containing 10% FBS, 2 mM L-glutamine and 250µg/mL G418. Myc-GBR1b/HA-GBR2 cell line was maintained in media containing puromycin 5µg/mL. Antibiotics were removed 48h before experiment. Transfections were performed using Fugene 6 following manufacturer's instructions.

Primary rat brain cortical cells were plated onto glass coverslips for 17-21 days and prepared for immunocytochemistry (Almeida et al. 2001).

In order to isolate hippocampi, Sprague-Dawley rat (P14-P15) were anesthetized with halothane (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and sacrificed by decapitation. Brains were quickly removed and placed in artificial cerebrospinal fluid (ACSF) solution gassed with 95% O_2 and 5% CO_2. Coranal slices were cut in ACSF solution at RT with a Leica VT1000S vibratome (Leica Microsystems, Heidelberg, Germany). Hippocampi were isolated from slices in oxygenated ACSF and treatments were performed as described below.

Immunoprecipitation

Every step was performed on ice. Cells were washed twice with PBS, harvested and centrifuged at 800g. Cells were solubilized in TNTO buffer (50mM Tris-HCl pH7.4 at 4°C, 150mM NaCl, 1% TritonX100, 60mM β-octylglucopyranoside, 1mM DTT, 5 mg/ml leupeptine, 5 mg/ml soybean trypsin inhibitor and 10 mg/ml benzamidine), under rocking for 1h. Nuclei were removed and supernatant was centrifuged at 150000g for 1h. 1mg/mL of cell lysates was incubated with the indicated antibody, 0.2% BSA and protein G-sepharose beads overnight under agitation. Immune complexes were pelleted and wash three times in TNTO buffer containing 350mM NaCl. Immunoprecipitated proteins were eluted in SDS-PAGE loading buffer containing 100mM DTT. Proteins were then submitted to western blot analysis (see details in supplementary methods).

Whole-cell phosphorylation assay

Following incubation for 60 min in phosphate-free medium containing 1% FBS, cells expressing myc-GBR1b/HA-GBR2 were incubated for 2 h in phosphate-free medium containing [^{32}P]Pi

(0.25mCi/mL) and treated or not with 1 mM GABA for 30 min. Labeled cells were then lysed for 30min on ice in RIPA buffer (50 mM TrisHCl pH 7.4, 150 mM NaCl, 2.5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1 mM DTT, 0,1% SDS, 5 mg/ml leupeptine, 5 mg/ml soybean trypsin inhibitor and 10 mg/ml benzamidine) containing 0.2 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM sodium phosphate. Lysates were centrifuged for 15min at 15000g and immunoprecipitation performed as described in material and methods in RIPA buffer.

After immunoprecipitation, as described previously, precipitated proteins were resolved on SDS-PAGE, transferred onto nitrocellulose, and [^{32}P] detected by autoradiography using BioMax MR Kodak Films. Autoradiograms were scanned with a 1200ppi resolution then analyzed with Quantity One BioRad software.

[^{35}S] GTPγS binding assay

CHO cells or hippocampal tissues were resuspended and lysed under hypotonic conditions (25 mM TrisHCl pH 7.4, 5 mM $MgCl_2$, 2 mM EDTA, 5 mg/ml leupeptin, 5 mg/ml soybean trypsin inhibitor and 10 mg/ml benzamidine) and homogenized with a polytron homogenizer (Ultra-Turrax; Janke and Kunkel) for 10 s. Homogenates were centrifuged at 500 g for 5 min at 4°C and the resulting supernatant fraction was centrifuged at 35 000 g for 20 min at 4°C. Membrane pellets were resuspended in assay buffer [50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 15 mM GDP and 0.5% BSA] and incubated in 300 pM [^{35}S]GTPγS (1250 Ci/mmol) in the presence of increasing baclofen amount for 60 min at 25°C. Binding was terminated by rapid filtration over GF/B filters using a cell harvester. Filters were washed three times in 50 mM Tris pH 7.4 containing 50 mM NaCl and 5 mM MgCl2, and counted.

Western blot analysis

Protein samples were resolved on SDS-PAGE, transferred onto nitrocellulose and subjected to immunoblotting using the indicated antibody. The nitrocellulose was then incubated with the appropriate secondary antibody conjugated to horseradish peroxidase for 30 min and developed using the Renaissance chemiluminescence kit (Perkin Elmer). Quantification analysis were performed in parallel by scanning blots with a 1200ppi resolution or detecting chemiluminescence directly with phosphoimager device (BioRad). Band intensities were then quantified with the Quantity One BioRad software.

Immunofluorescence

Appropriate treatment was performed on CHO stably expressing myc-GBR1b/HA-GBR2. Cells were then washed with cold PBS and fixed with 3% paraformaldehyde for 15 min, washed again in PBS and finally incubated in PBS containing 0.25% BSA for 30 min. Surface labeling were performed by incubating the indicated primary antibody for 30 min followed by extensive wash in PBS and a permeabilization step with PBS containing 0.15% TritonX100 and 0.25%BSA for 15min. NSF was labeled with the appropriate antibody for another 30min. Cells were washed in the same buffer and incubated with secondary antibodies coupled to fluorescent markers. The samples were analyzed by confocal laser-scanning microscopy utilizing a Leica TCS SP1 confocal microscope, and co-localisation was performed by overlay of the images using the Leica Confocal Software LCS (Heidelberg, Germany). Excitation and emission filters for the different labelled dyes were as follows: YFP (green): λex¼488 nm, λem¼540/25 nm; Texas red (red): λex¼568 nm, λem¼610/30 nm; Alexa 633 (blue): λex¼633 nm, λem¼705/45 nm.

To visualize GBR2 and NSF localizations in primary cultures of neurons, rabbit anti-GBR2 and mouse anti-NSF antibodies were used as well as the secondary antibodies Alexa 488 (λex¼488 nm, λem¼540/25 nm) conjugated anti-rabbit and Alexa 568 (λex¼568 nm, λem¼610/30 nm) conjugated anti-mouse. Images were collected using a Zeiss LSM 510 confocal microscope.

Mass spectroscopy analysis of GBR1 associated proteins

Synaptosome membranes (SPM) were prepared from adult Sprague Dawley rat brains and stored frozen at –70^{o}C until use. The membranes were solubilized in sodium deoxycholate (1%) in 50mM Tris-HCl pH 8.0 (solubilization buffer) containing protease inhibitors (Complete EDTA-free Roche Diagnostics) and 10mM iodoacetamide. The detergent to protein ratio was maintained at 10:1 with 9-10mg of SPM protein being used per isolation. The membranes were incubated on ice for 30 minutes and centrifuged at 100,000g for 1 hour. The supernatant from this step was divided into two samples and 5µg of either normal sheep IgG or affinity purified anti-GBR1 serum added. The samples were rotated at 4^{o}C for 4-6 hours before adding 40ul of a 1:1 suspension of protein-G in solubilization buffer. Rotation was continued overnight and the beads washed three times in solubilization buffer transferred to a clean eppondorf tube and washed once with 50mM Tris-HCl pH 8.0. Elution was performed with Novex 2 X SDS-sample buffer (25µl) and heating at 100^{o}C for 2 min.

The eluates were analysed on 4-12% Bis-Tris Novex gradient polyacrylamide gels using MOPS running buffer. The gels were stained using Gel Code Blue staining reagent and destained in distilled water. Slice of decreasing molecular weight were cut from both the control and specific tracks and analysed by mass spectrometry. None of the proteins found in the control (normal sheep IgG) track were considered as positive hits, nor were proteins that appeared only in one isolate. Likewise proteins yielding peptides from gel positions not corresponding to their normal

molecular weight were eliminated from the analysis, as were those giving only single peptides. Thus positive immunoprecipitating proteins were those that appeared specifically in two independent immunoprecipitations, that were isolated from the appropriate molecular weight region of the gel and that yielded more than 2 peptides.

Biotinylation, streptavidin pull-down and deglycosylation

For surface biotinylation, cells were washed with PBS on ice, and incubated at 4 °C for 30 min in Dulbecco's PBS containing 0.5 mg/ml of sulfo-NHS-biotin under gentle agitation. Excess biotin was quenched by adding Tris-HCl, pH 7.4, to a final concentration of 50 mM. Cells were then washed twice with cold Dulbecco's PBS and harvested. Biotinylated cell surface proteins were isolated using immobilized streptavidin after solubilizing the total cellular membranes as described for immunoprecipitation in TNTO buffer. 25 µl of resin was used for 1 mg of solubilized membrane proteins in presence of 0.1% BSA (w/v) for 2 h at 4 °C under rocking. Resin was then washed three times and precipitated proteins were eluted as described above. For deglycosylation, eluted streptavidin pull downed or immunoprecipitated proteins were first eluted from the resin in 1% (w/v) SDS, 50 mM sodium phosphate, pH 7.5 then diluted ten times with 50 mM sodium phosphate, pH 7.5. Deglycosylation were performed in the appropriate buffer in presence of EndoH or PNGaseF at final concentrations of 100 milliunits/ml and 0.001-20 milliunits/ml respectively, over night at 37°C.