Published in final edited form as:

Hippocampus. 2013 December ; 23(12): 1231–1245. doi:10.1002/hipo.22161.

Association of Rgs7/Gβ5 complexes with Girk channels and GABA_B receptors in hippocampal CA1 pyramidal neurons

Ana Fajardo-Serrano¹, Nicole Wydeven², Daniele Young², Masahiko Watanabe³, Ryuichi Shigemoto⁴, Kirill A. Martemyanov⁵, Kevin Wickman², and Rafael Luján¹

¹Instituto de Investigación en Discapacidades Neurológicas (IDINE), Dept. Ciencias Médicas, Facultad de Medicina, Universidad Castilla-La Mancha, Campus Biosanitario, C/ Almansa 14, 02006 Albacete, Spain

²Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455, USA

³Department of Anatomy, Hokkaido University School of Medicine, Sapporo 060-8638, Japan

⁴Division of Cerebral Structure, National Institute for Physiological Sciences, Okazaki 444-8787, Japan

⁵Department of Neuroscience, The Scripps Research Institute, 130 Scripps Way, 3C2, Jupiter, FL 33458, USA

Abstract

In the hippocampus, signalling through G protein-coupled receptors is modulated by Regulators of G protein Signalling (Rgs) proteins, which act to stimulate the rate of GTP hydrolysis, and consequently, G protein inactivation. The R7-Rgs subfamily selectively deactivates the G_{i/o}-class of Gα subunits that mediate the action of several GPCRs. Here, we used co-immunoprecipitation, electrophysiology and immunoelectron microscopy techniques to investigate the formation of macromolecular complexes and spatial relationship of Rgs7/Gβ5 complexes and its prototypical signalling partners, the GABA_B receptor and Girk channel. Co-expression of recombinant GABA_B receptors and Girk channels in combination with co-immunoprecipitation experiments established that the Rgs7/Gβ5 forms complexes with GABA_B receptors or Girk channels. Using electrophysiological experiments, we found that GABA_B-Girk current deactivation kinetics was markedly faster in cells co-expressing Rgs7/Gβ5. At the electron microscopic level, immunolabelling for Rgs7 and Gβ5 proteins was found primarily in the dendritic layers of the hippocampus and showed similar distribution patterns. Immunoreactivity was mostly localized along the extrasynaptic plasma membrane of dendritic shafts and spines of pyramidal cells and, to a lesser extent, to that of presynaptic terminals. Quantitative analysis of immunogold particles for Rgs7 and Gβ5 revealed an enrichment of the two proteins around excitatory synapses on dendritic spines, virtually identical to that of Girk2 and GABA_{B1}. These data support the existence of macromolecular complexes composed of GABA_B receptor-G protein-Rgs7-Girk channels, in which Rgs7 and Gβ5 proteins may preferentially modulate GABA_B receptor signalling through the deactivation of Girk channels on dendritic spines. In contrast, Rgs7 and Girk2 were associated

Correspondence to: Dr. Rafael Luján, Instituto de Investigación en Discapacidades Neurológicas (IDINE), Dept. Ciencias Médicas, Facultad de Medicina, Universidad Castilla-La Mancha, Campus Biosanitario, C/Almansa 14, 02006 Albacete, Spain. Tel. + 34 967 599200 (Ext. 2176); Fax. + 34 967 599327; Rafael.Lujan@uclm.es.

but mainly segregated from GABA_{B1} in dendritic shafts, where Rgs7/Gβ5 signalling complexes might modulate Girk-dependent signalling via a different metabotropic receptor(s).

Introduction

G protein-coupled signalling is a major cellular mechanism for controlling excitability in central neurons. Signalling is initiated upon binding of ligand to a G protein-coupled receptor (GPCR) that catalyzes GDP/GTP exchange on the heterotrimeric G protein, leading to dissociation of Gα-GTP and Gβγ subunits and their subsequent modulation of enzymes or ion channels (Smrcka, 2008). An integral part of G protein-coupled signalling pathway are the regulator of G-protein signalling (Rgs) proteins, which accelerate rates of G-protein deactivation by acting as GTPase-activating proteins (GAPs) for G protein α-subunits, resulting in faster response termination of GPCR signal transmission (Ross and Wilkie, 2000; Hollinger and Hepler, 2002; Anderson et al., 2009).

Rgs proteins represent a diverse family of over 30 members that are classified into six subfamilies (Zheng et al. 1999; Anderson et al., 2009). The R7-Rgs subfamily consists of four highly-homologous proteins (Rgs6, 7, 9 and 11) which are predominantly expressed in the nervous system (Gold et al., 1997), where they play a key role in synaptic transmission, light perception, neuronal development, and sensitivity to addictive drugs by regulating several GPCR pathways (Anderson et al., 2009; Slepak, 2009). Therefore, alteration of the expression or function of these proteins is expected to have a significant effect on neuronal function.

The R7-Rgs subfamily is unique in its ability to heterodimerize with the type 5 G protein beta (Gβ5) subunit (Cabrera et al. 1998; Makino et al. 1999). This association is essential for the folding, stability and expression of R7-Rgs proteins (He et al., 2000; Witherow et al., 2000) and accordingly, ablation of the Gβ5 gene in mice results in functional elimination of the entire R7 family (Chen et al., 2003). R7-Rgs/Gβ5 complexes selectively deactivate the G_{i/o}-class of Gα subunits that mediate the diverse actions of several GPCRs (Posner et al., 1999; Rose et al., 2000; Hooks et al., 2003), including GABA_B receptors.

One of the best characterised effectors modulated by GABA_B receptors is the G protein-gated inwardly rectifying K⁺ (Girk or K_{IR3}) channel (North, 1989). Neurons in the hippocampus express high levels of Girk channels (Karschin et al., 1996), GABA_B receptors (Kaupmann et al., 1998; Kulik et al., 2003), Rgs7 (Gold et al., 1997) and Gβ5 proteins (Zhang et al., 2000). Recently, we demonstrated that GABA_B-Girk signalling in hippocampal pyramidal neurons is negatively-modulated by Rgs/Gβ5 (Xie et al., 2010). It is not clear, however, whether Girk channels and cognate GABA_B receptors function within macromolecular assemblies that include regulatory Rgs proteins *in vivo*. Here, we probed for the possible functional macromolecular complexes between Rgs7, Gβ5 and GABA_B receptors or Girk channels using co-immunoprecipitation and electrophysiology. In addition, we studied the subcellular localization of Rgs7, Gβ5, GABA_{B1} and Girk2 and determined their compartment-dependent localization in pyramidal cells by using quantitative immunohistochemical techniques. Our findings indicate that the four proteins are similarly

distributed in dendritic spines adjacent to excitatory synapses, while there is a segregation of Rgs7/G β 5-Girk channels and GABA_B receptors in dendritic shafts.

Material and Methods

Tissue Preparation

Six young adult (P30) OF-1 mice obtained from the Animal House Facility at the School of Medicine of the University of Castilla-La Mancha were used in immunohistochemical experiments at the light and electron microscopic level. The mice were housed on a 12-h light/dark cycle with *ad libitum* access to food and water. The care and handling of animals prior to and during the experimental procedures was done in accordance with Spanish (RD 1201/2005) and European Union regulations (86/609/EC), and the protocols were approved by the University's Animal Care and Use Committee.

For immunohistochemistry, animals were deeply anaesthetized by intraperitoneal injection of ketamine-xylazine 1:1 (0.1 mL/kg) and transcardially-perfused with ice-cold fixative containing 4% paraformaldehyde, with or without 0.05% glutaraldehyde, and 15% (v/v) saturated picric acid made up in 0.1 M phosphate buffer (PB, pH 7.4). After perfusion, brains were removed and immersed in the same fixative for 2 h or overnight at 4°C. Tissue blocks were washed thoroughly in 0.1 M PB. Coronal sections (60 μ m) were cut on a Vibratome (Leica V1000).

Antibodies and chemicals

Affinity-purified rabbit anti-Rgs7 and rabbit anti-G β 5 were generated and characterized previously (Cao et al., 2008; Xie et al., 2012). The characteristics and specificity of the affinity-purified polyclonal antibody against GABA_{B1} (B17) have been described elsewhere (Kulik et al., 2003; Luján and Shigemoto, 2006). The detailed characterization of the polyclonal Girk2 antibody has been described elsewhere (Aguado et al., 2008; Fernández-Alacid et al., 2009). Rabbit anti-AU5 polyclonal (Bethyl Laboratories, Inc.; Montgomery, TX, USA), and mouse anti-myc monoclonal antibodies were obtained from Bethyl Laboratories, Inc. (Montgomery, TX, USA) and Roche Diagnostics Corporation (Indianapolis, IN), respectively. IRDye 800 donkey anti-mouse and IRDye 680 donkey anti-rabbit secondary antibodies were obtained from Li-COR Biosciences (Lincoln, NE).

Molecular biology

pcDNA3-based expression constructs harbouring coding sequences for carboxyl-terminal AU5-tagged rat Girk1 (NM_031610) and c-myc-tagged mouse Girk2A (NM_001025584) were kindly provided by Dr. David Clapham. pcDNA3-based expression constructs harbouring rat GABA_{B1a} (A-splice variant; Y10369) and GABA_{B2} (NM_031802) receptors were kindly provided by Dr. Paul Slesinger. pcDNA3-based expression constructs for mouse Rgs7 (AF011360) and G β 5 (short variant; NM_138719) were used previously (Xie et al., 2010).

Cell culture and transfections

HEK293 and 293FT cells were obtained from ATCC (Manassas, VA) and Invitrogen (Carlsbad, CA), respectively, and cultured according to supplier's recommendations. For whole-cell recordings, transfections of HEK293 cells were performed on 8-mm coverslips (Warner Instruments; Hamden, CT) in 48-well plates using Lipofectamine LTX reagent (Invitrogen Corporation; Carlsbad, CA). For each well, 250 ng of total DNA (40 ng eGFP expression construct as a transfection marker and 35 ng each of GABA_BR1, GABA_BR2, Girk1, Girk2, Rgs7 and Gβ5 expression constructs, or filler/empty plasmids as appropriate) was added to Optimem (Invitrogen; 40 μL) and mixed by gentle vortexing. Lipofectamine LTX (0.4 μL/well) was then added to the DNA/Optimem mix and allowed to incubate at room temperature (20–22° C) for 30 min. Coverslips were pre-treated with 200 μL of poly-L-lysine (0.0005%) incubated at room temperature for 30 min, and then washed with PBS. The DNA/Optimem/Lipofectamine LTX mixture was added to each coverslip (40 μL), followed by 20,000 HEK 293 cells. Cells were incubated overnight at 37°C and 5% CO₂ prior to electrophysiological characterization. Some cells were treated with pertussis toxin (0.1 ng/ml; Tocris Bioscience; Bristol, UK) for 12–18 h prior to electrophysiological characterization.

For co-immunoprecipitation studies, 293FT cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 1 mM sodium pyruvate, 200 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl (Glutamax, Invitrogen), 100 U/mL streptomycin, 100 μg/mL penicillin and 10% (v/v) fetal bovine serum at 37°C and in an atmosphere of 5 % CO₂. Cells growing in six-well plates were transiently-transfected with the DNA (3.5 μg total DNA/well; 500 ng of an eGFP expression construct as a transfection marker and 500 ng each of GABA_BR1, GABA_BR2, Girk1, Girk2, Rgs7 and Gβ5 expression constructs, or filler/empty plasmids as appropriate) encoding the specified proteins by the calcium phosphate precipitation technique (Jordan, Schallhorn, and Wurm 1996). The cells were harvested 48 h after transfection.

Immunoprecipitation and immunoblotting

For immunoprecipitation, transfected 293FT cells were solubilized in ice-cold buffer containing (in mM): PBS (137 NaCl, 2.7 KCl, 4.3 Na₃PO₄, 1.4 K₃PO₄, pH 7.4), 150 NaCl, 1% Triton X-100, and a mixture of protease inhibitors (10 μg/mL aprotinin, 10 μg/mL pepstatin A, 10 μg/mL PMSF, and 1 μg/mL Leupeptin). Samples were sonicated and incubated at 4°C for 30 min. The solubilized preparation was centrifuged at 14,000xg for 15 min. 90% of supernatant was incubated with anti-myc Ig IP Beads (Abcam, Cambridge, UK) for 1 h at 4°C under constant rotation, while the remaining 10% was saved and run as input. Subsequently, the beads were washed three times with ice-cold buffer. Finally, ice-cold buffer was added (v/v), 50 μL of 2X SDS sample buffer (1 M Tris pH6.8, 50% glycerol, 10% SDS, 0.5% bromophenol blue, β-mercaptoethanol) and 10 μL 1 M DTT. Immune complexes were dissociated at 90°C for 5 min.

For immunoblotting, 3 μL 1M DTT was added to 10 μL of sample and the mixture was incubated at 90°C for 10 min. Samples were resolved by SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide gels. Proteins were transferred to nitrocellulose

membranes using a dry transfer system (iBlot, Invitrogen) and immunoblotted with the indicated primary antibody and corresponding secondary antibody. The visualization was performed using the Odyssey Imaging System (LI-COR Biosciences).

Electrophysiology

Coverslips containing transfected HEK293 cells were transferred to a chamber containing a low- K^+ bath solution (in mM): 145 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5.5 D-glucose, 5 HEPES/NaOH (pH 7.4). Transfected cells were visualized using an Olympus IX-70 microscope with epifluorescence detection. Whole-cell currents were measured with hardware (Axopatch-200B amplifier, Digidata 1320) and software (pCLAMP v. 9.0) from Molecular Devices, Inc. (Sunnyvale, CA). Borosilicate patch pipettes (3–5 M Ω) were filled with (in mM): 130 KCl, 10 NaCl, 1 EGTA/KOH (pH 7.2), 0.5 $MgCl_2$, 10 HEPES/KOH (pH 7.2), 2 Na_2ATP , 5 phosphocreatine, 0.3 GTP. Basal and baclofen-induced whole-cell currents were measured at a holding potential of -70 mV, at room temperature, in a high- K^+ bath solution (in mM): 120 NaCl, 25 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5.5 D-glucose, 5 HEPES/NaOH (pH 7.4). The high- K^+ bath solution (± 100 μM baclofen) was applied directly to the cell with an SF-77B rapid perfusion system (Warner Instruments, Inc.; Hamden, CT), which permits complete solution exchange in <20 ms. Baclofen (*R*-(+)-b-(aminomethyl)-4-chlorobenzenepropanoic acid hydrochloride) was purchased from Sigma (St. Louis, MO). Baclofen was dissolved as a high concentration (100 mM) stock solution and divided into single-use aliquots stored at $-20^\circ C$.

Currents were low-pass filtered at 1 kHz, sampled at 2 kHz, and stored on computer hard disk for subsequent analysis. Basal currents were defined as the component of the holding current measured in high- K^+ bath solution that was blocked by 0.3 mM Ba^{2+} . Peak and steady-state baclofen-induced current densities were measured for each experiment (Posokhova et al., 2010). Only those experiments in which the access resistances were stable and low (<15 M Ω) were included in the final analysis. Baclofen-induced current activation rates were extracted from a single exponential fit (standard Boltzmann equation) of the current trace corresponding to the onset of drug effect and the peak evoked current, while deactivation rates were extracted from an exponential fit of the trace corresponding to the return of current to baseline following removal of drug (Posokhova et al., 2010).

Immunohistochemistry for light microscopy

Immunohistochemical reactions at the light microscopic level were carried out using the immunoperoxidase method as described (Luján et al., 1996). Briefly, sections were incubated in 10% normal goat serum (NGS) diluted in 50 mM Tris buffer (pH 7.4) containing 0.9% NaCl (TBS), with 0.2% Triton X-100, for 1 h. Sections were incubated in anti-Rgs7 (1 $\mu g/mL$), anti-G β 5 (2 $\mu g/mL$), anti-GABA $_B$ 1 (1 $\mu g/mL$) or anti-Girk2 (1 $\mu g/mL$) antibodies diluted in TBS containing 1% NGS, followed by incubation in biotinylated goat anti-rabbit or goat anti-guinea pig IgG (Vector Laboratories, Burlingame, CA, USA) in TBS containing 1% NGS. Sections were then transferred into avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories). Bound peroxidase enzyme activity was revealed using 3, 3'-diaminobenzidine tetrahydrochloride (DAB; 0.05% in TB, pH 7.4) as the chromogen and 0.01% H_2O_2 as the substrate. Finally, sections were air-dried and coverslipped prior to

observation in a Nikon photomicroscope (Nikon, Eclipse 80i) equipped with differential interference contrast optics and a digital imaging camera.

Immunohistochemistry for electron microscopy

Immunohistochemical reactions at the electron microscopic level were carried out using the pre-embedding immunogold method as described (Luján et al., 1996). Briefly, free-floating sections were incubated in 10% NGS diluted in TBS. Sections were then incubated in anti-Rgs7 (2 µg/mL), anti-Gβ5 (5 µg/mL), anti-GABA_{B1} (2 µg/mL) or anti-Girk2 (2 µg/mL) antibodies diluted in TBS containing 1% NGS, followed by incubation in goat anti-rabbit or goat anti-guinea pig IgG coupled to 1.4 nm gold (Nanoprobes Inc., Stony Brook, NY, USA). Sections were post-fixed in 1% glutaraldehyde and washed in double distilled water, followed by silver enhancement of the gold particles with a HQ Silver kit (Nanoprobes Inc.). In co-labelling experiments, Girk2 immunoreactivity was visualized by the immunoperoxidase reaction, and Rgs7 or Gβ5 immunoreactivity was revealed with the silver-intensified immunogold reaction. Sections were then treated with osmium tetroxide (1% in 0.1 M PB), block-stained with uranyl acetate, dehydrated in graded series of ethanol and flat-embedded on glass slides in Durcupan (Fluka) resin. Regions of interest were cut at 70–90 nm on an ultramicrotome (Reichert Ultracut E, Leica, Austria) and collected on 200-mesh copper grids. Staining was performed on drops of 1% aqueous uranyl acetate followed by Reynolds's lead citrate. Ultrastructural analyses were performed in a Jeol-1010 electron microscope (Jeol, Tokyo, Japan).

Quantification of immunogold labelling

To establish the relative frequency of Rgs7, Gβ5, Girk2 and GABA_{B1} immunoreactivity in pyramidal cells, we used 60-µm coronal slices processed for pre-embedding immunogold immunohistochemistry. The procedures were similar to those used previously (Luján et al., 1996; Koyrakh et al., 2005). Briefly, for each of three young adult (P30) animals, three samples of tissue were obtained for preparation of embedding blocks, totalling $n =$ nine blocks. To minimize false negatives, electron microscopic serial ultrathin sections were cut close to the surface of each block, as immunoreactivity decreased with depth. We estimated the quality of immunolabelling by always selecting areas with optimal gold labelling at approximately the same distance from the cutting surface, which was defined within 5 to 10 µm from the surface. Randomly selected areas were then photographed from the selected ultrathin sections and printed with a final magnification of 50,000X. Quantification of immunolabelling was carried out in four different ways:

1. *Percentage of immunoparticles.* To determine the frequency of Rgs7, Gβ5, Girk2 and GABA_{B1} in different compartment of pyramidal cells, Quantification of immunogold labelling was carried out in reference areas totalling ~2,000 µm² for each protein. Immunoparticles identified in each reference area and present in different subcellular compartments (dendritic spines, dendritic shafts and axon terminals) were counted. Data were expressed as percentage of immunoparticles in each subcellular compartment (Table 1).
2. *Distribution of immunoparticles in dendritic spines.* To establish the relative frequency of Rgs7, Gβ5, Girk2 and GABA_{B1} in dendritic spines of CA1 pyramidal

cells, immunoparticles identified in this compartment were counted and the perimeter of each spine was measured in reference areas totalling $\sim 2,000 \mu\text{m}^2$ for each protein. All spines establishing synapses with putative glutamatergic axon terminals were counted and assessed for the presence of immunoparticles. Only the heads of spines were analyzed because spine necks were rarely connected to spine heads in single sections. In the few cases when the neck of a spine was present in the measured section, the natural curvature of the membrane was assumed as the continuation of the spine head, cutting off the neck (*see scheme in Fig. 7*). Because differences in the distribution of gold particles among samples were not statistically significant ($P > 0.57$, Kolmogorov-Smirnov non-parametric test), data were pooled. The length of the synaptic membrane specialization and the extrasynaptic membrane from all immunopositive spines was measured using a digitizing tablet and appropriate software (Image J). The extrasynaptic spine membrane was divided into 60 nm bins. The distance between the closest edge of the postsynaptic density and the centre of the immunoparticles was measured along the spine membrane ($n = 156$ for Rgs7; $n = 102$ for G β 5; $n = 82$ for Girk2; and $n = 89$ for GABA $_B$; Table 2). To obtain a normalized value of the relative frequency of Rgs7, G β 5, Girk2 and GABA $_B$ along the spine, the number of gold particles was expressed as relative frequency in bins corresponding to 60 nm membrane segments of plasma membrane. The data expressed in this way show the change in density of the four proteins as a function of distance from the synapse.

3. *Distribution of immunoparticles in dendritic shafts.* To establish the distribution of Rgs7, Girk2 and GABA $_B$ in dendritic shafts of CA1 pyramidal cells, immunoparticles identified in this compartment were counted and the perimeter of each shaft was measured in reference areas totalling $\sim 1,500 \mu\text{m}^2$ for each protein. In order to have a reference point, only dendritic shafts attached to a visible spine through its neck were counted and assessed for the presence of immunoparticles. The natural curvature of the membrane was assumed as the continuation of the dendritic shaft, cutting off the neck (*see scheme in Fig. 7*). The length of the extrasynaptic membrane from all immunopositive shafts was measured starting from the edge of the spine neck using a digitizing tablet and appropriate software (Image J). The distance between the edge of the spine neck and the centre of the immunoparticles was measured along the shaft membrane ($n = 271$ for Rgs7; $n = 232$ for Girk2; and $n = 247$ for GABA $_B$; Table 2). To obtain a normalized value of the relative frequency of Rgs7, Girk2 and GABA $_B$ along the shaft, the number of gold particles was expressed as relative frequency in bins corresponding to 60 nm membrane segments of plasma membrane. Although dendritic shafts are much larger in perimeter than spines, in order to establish comparisons between the two compartments, we only plotted the distribution of immunoparticles within similar a similar distance (720 nm) and then pooled data from 720 to 1000 nm. Still, this is an underestimation of the distribution of immunoparticles though the entire shaft, but data expressed in this way clearly show the change in density of the three proteins as a function of distance.

4. *Distribution of immunoparticles in axon terminals.* To establish the relative frequency of Rgs7, G β 5, Girk2 and GABA_{B1} in axon terminals, presumably from Schaffer collaterals, immunoparticles identified in this compartment were counted. The perimeter of each axon terminal was measured in reference areas totalling ~2,000 μm^2 for each protein. All axon terminals establishing excitatory synapses with dendritic spines of CA1 pyramidal cells were counted and assessed from single ultrathin sections. The lengths of the active zone and the extrasynaptic membrane surrounding the active zone were measured using a digitizing tablet and appropriate software (Image J). The distances of the immunoparticles ($n = 116$ for Rgs7; $n = 109$ for G β 5; $n = 142$ for Girk2; and $n = 151$ for GABA_{B1}; Table 2) from the edge of the active zone and the centre of the immunoparticles was measured (see scheme in Fig. 8). To obtain a normalized value of the relative frequency of Rgs7, G β 5, Girk2 and GABA_{B1} along the spine, the number of gold particles was expressed as relative frequency in bins corresponding to 60 nm membrane segments of plasma membrane. The data expressed in this way show the change in density of the four proteins as a function of distance along the axon terminal.

Three-dimensional reconstructions

The three-dimensional (3D) reconstruction of CA1 pyramidal cell dendritic spines and shafts and interneurons immunoreactive for either the RGS7 or G β 5 proteins was performed from 18 to 25 serial ultrathin sections obtained from pre-embedding material. Randomly selected areas from the very surface ($<5 \mu\text{m}$) of blocks containing the *stratum radiatum* of CA1 were captured at a final magnification of 50,000 X, and serially sectioned images were imported into the Reconstruct software (Fiala, 2005) and the image stacks were aligned. Plasma membranes of individual dendrites and spine heads were manually traced.

Controls

To test method specificity in the procedures for both light and electron microscopy, the primary antibody was either omitted or replaced with 5% (v/v) normal serum of the species of the primary antibody. When double-labelling for electron microscopy was carried out, some sections were always incubated with only one primary antibody and the full complement of secondary antibodies to test for any cross-reactivity. Other sections were incubated with two primary antibodies and one secondary antibody, followed by the full sequence of signal detection. Under these conditions, no selective labelling was observed. Similarly, labelling patterns observed by electron microscopy were compared with that of Calretinin; only the antibodies against Rgs7, G β 5, Girk2 and GABA_{B1} consistently labelled the plasma membrane. The specificity of primary antibodies is referred above in Antibodies and chemicals section.

Statistical analysis

Statistical analyses for all data ($n=4-5$ experiments) are presented as the mean $\pm$ SEM. The unpaired two-tailed Student's *t*-test and one-way ANOVA were used to compare electrophysiological parameters and were performed using Prism 5 software (GraphPad Software, Inc.; La Jolla, CA). In all cases, the threshold for statistical significance was set at

$P < 0.05$. For electron microscopic data ($n = 9$ samples), statistical analyses were performed using SigmaStat Pro (Jandel Scientific) and statistical significance in the distribution of gold particles among samples, defined as $P < 0.05$, was assessed with the Kolmogorov–Smirnov non-parametric test.

Results

Evidence for macromolecular complexes of Rgs7/G β 5, GABA_B receptors and Girk channel

We began by asking whether Rgs7 and G β 5 proteins interact with GABA_B receptors and Girk channels using a co-immunoprecipitation approach. First, 293FT cells were transfected with epitope-tagged constructs for Girk1 (AU5), Girk2 (myc), G β 5 and Rgs7. Girk2 was immunoprecipitated from whole-cell extracts using an anti-myc antibody (Fig. 1; left panel); Girk1, Rgs7, and G β 5 all reliably co-immunoprecipitated with Girk2. Importantly, in control cells where Girk2 was not expressed, no co-immunoprecipitation of Girk1, Rgs7, or G β 5 was observed. Next, we transfected cells with Girk1-AU5, Girk2-myc, G β 5, Rgs7, GABA_{B1a}, and GABA_{B2} and repeated the Girk2 immunoprecipitation. Girk1, Rgs7, G β 5, and GABA_{B1a} all reliably and selectively co-immunoprecipitated with Girk2 (Fig. 1; right panel). These results suggest that Girk1, Girk2, G β 5, Rgs7 and GABA_B receptors can co-exist in a macromolecular complex.

Impact of Rgs7/G β 5 on GABA_B-Girk signalling

We next measured the impact of Rgs7/G β 5 co-expression on GABA_B-Girk signalling in transfected HEK293 cells using the whole-cell voltage-clamp technique. Cells expressing GABA_B receptor and Girk1/Girk2 exhibited substantial basal (0.3 mM Ba²⁺-sensitive) currents, and robust inward currents triggered by a saturating concentration of baclofen (100 μ M; Fig. 2A, B). Currents exhibited modest acute desensitization over a 20-s drug exposure, and activation and deactivation kinetics were slower than reported previously for GABA_B-Girk signalling in cultured hippocampal neurons (Xie et al, 2010) (Fig. 2C). No baclofen-induced inward currents were observed if either GABA_B receptors or Girk1/2 were excluded from the transfection mixture (not shown), or if transfected cells were pre-incubated with pertussis toxin (Fig. 2B). While current densities were slightly smaller in cells expressing GABA_B receptor, Girk1/Girk2, and Rgs7/G β 5, there was no significant impact of Rgs7/G β 5 co-expression on basal current, or on peak and steady-state baclofen-induced currents (Fig. 2B). Consistent with the impact of G β 5 ablation on GABA_B receptor-Girk signalling kinetics in cultured hippocampal neurons (Xie et al, 2010), baclofen-induced current deactivation kinetics were markedly faster in cells co-expressing Rgs7/G β 5 (Fig. 2A, C). Current activation rate was also faster in Rgs7/G β 5-expressing cells, though this difference did not reach the level of statistical significance. These data show that the impact of Rgs7/G β 5 on the kinetics of GABA_B-Girk signalling reported previously in hippocampal neurons (Xie et al, 2010) can be recapitulated in an expression system.

Rgs7 and G β 5 localization in dendritic layers of the hippocampus

The cellular and subcellular localization of Girk1/Girk2 and GABA_{B1} are well-established in the hippocampus (Kulik et al., 2003; López-Bendito et al., 2004; Koyrakh et al., 2005; Fernández-Alacid et al., 2011), but no information is available about Rgs7 and G β 5 proteins.

To study the distribution patterns of Rgs7 and G β 5, we carried out immunohistochemical studies at the light microscopic level (Fig. 3). Immunoreactivity for both Rgs7 and G β 5 was widely distributed in the hippocampus, with moderate to strong labelling in dendritic layers showing patterns of distribution that strongly overlapped in the CA areas and dentate gyrus (Fig. 3A, C). In the CA1 region, immunolabelling for Rgs7 and G β 5 was generally moderate to strong, with the *strata oriens* and *radiatum* showing the highest, and the *stratum lacunosum-moleculare* showing the weakest, immunoreactivity (Fig. 3B, C). The CA3 region exhibited similar Rgs7 and G β 5 immunoreactivities as the CA1 region, with the only exception being the *stratum lucidum*, which showed the strongest immunoreactivity for both proteins within the hippocampus (Fig. 3A, C). In the dentate gyrus, immunolabelling was strong in the molecular layer and highest in the hilus (Fig. 3A, C). In the pyramidal and granule cell layers, no labelling was observed. Despite a strong correlation in the distribution pattern for both Rgs7 and G β 5 protein, their sub-regional labelling densities differed, likely due to different sensitivities of the antibodies used.

Rgs7 and G β 5 are preferentially localised to the extrasynaptic membrane of spines

To determine the subcellular localization of Rgs7 and G β 5 in dendritic layers, we used a pre-embedding immunogold approach and quantitative analysis on tissue blocks taken from the distal part of the *stratum radiatum* of the CA1 area (Figs. 4 and 5). Immunoreactivity for Rgs7 and G β 5 was primarily found along the extrasynaptic plasma membrane (44% of Rgs7; 25% of G β 5) and also associated with the endoplasmic reticulum cisterna of dendritic shafts and the spine apparatus (intracellular labelling: 56% of Rgs7; 75% of G β 5) (Figs. 4A–I and 5A–G; Table 1). Of the immunoparticles found in the plasma membrane, most (95% of Rgs7; 95% of G β 5) were found in post-synaptic compartments, mainly in dendritic spines of CA1 pyramidal cells where they were distributed along the extrasynaptic plasma membrane or at the edge of asymmetrical synapses established by putative Schaffer collaterals with CA1 pyramidal cell spines (Figs. 4A–F and 5A, C, D, E; Table 1). Immunoparticles also appeared occasionally at the edge of asymmetrical synapses on dendritic spines (Figs. 4E, F and 5A, C, E). In addition to pyramidal cells, immunoreactivity for Rgs7 and G β 5 was also detected on the extrasynaptic plasma membrane of interneuron dendrites (Figs. 4H, I and 5B), identified by the lack of dendritic spines and the convergence of symmetrical and asymmetrical synapses on the dendritic shaft. Presynaptically, weak immunoreactivity for Rgs7 (5%) and G β 5 (5%) was occasionally detected in axon terminals making asymmetrical synapses with dendritic spines throughout all dendritic layers (Figs. 4B, CF, G and 5D, F, G; Table 1). Immunoparticles were localized either to the extrasynaptic plasma membrane (Figs. 4C, F, G and 5DG) or to the active zone of axon terminals (Figs. 4G and 5F, G). The pattern of the subcellular distribution of Rgs7 and G β 5 proteins described above, particularly the strong labelling on dendritic spines of hippocampal pyramidal cells, is very similar to that observed and described for Girk2 and GABA $_B$ 1 (Supplementary Figure 1).

To get insight into the spatial relationship of the Rgs7 and G β 5 on pyramidal cells in relation to excitatory and inhibitory synapses, three-dimensional (3D) reconstructions were made from serial ultrathin sections (Figs. 4J–L and 5H–J). On dendritic spines, Rgs7 and G β 5 were found to be preferentially localized close to excitatory synapses. In contrast, on dendritic shafts, the two proteins showed a more uniform distribution and no association to

inhibitory synapses (Figs. 4J–K and 5H–J). In interneurons, Rgs7 showed no association to excitatory or inhibitory synapses (Figs. 4L).

Overlap between Rgs7, Gβ5, GABA_{B1}, and Girk2 in pyramidal cell spines

To directly compare the distribution of Rgs7 and Gβ5 with that of GABA_{B1} and Girk2 on dendrites of pyramidal cells in relation to glutamatergic synapses, the distances of the immunoparticles from the edge of asymmetrical synaptic specializations were measured (Fig. 6; Table 2). This approach revealed that on dendritic spines, the four proteins (Rgs7, Gβ5, Girk2 and GABA_{B1}) were preferentially localized around asymmetrical synapses. The distribution of Rgs7 and Gβ5 showed a peak between 0 and 300 nm from the synapses (Fig. 6B); 74% of the particles were found within this area. Similarly, the distribution of Girk2 and GABA_{B1} also showed a peak between 0 and 300 nm from the synapses (Fig. 6B) (64% of the particles), consistent with previous data obtained from the CA1 area (Kulik et al., 2003; Koyrakh et al., 2005). Therefore, all four proteins were mainly located within a distance of 300 nm from the edge of asymmetrical synapses, indicating an enrichment of the molecules in the vicinity of excitatory synapses on dendritic spines.

We next investigated the spatial relationship of Rgs7, Girk2 and GABA_{B1} on dendritic shafts of pyramidal cells (Fig. 6D; Table 2) in the same sampling area as for spines. This analysis revealed not only that the three proteins are more evenly distributed along the shafts, but also that Rgs7 and Girk2 clustered together, whereas they are mainly segregated from GABA_B receptors (Fig. 6D).

Co-localization of Rgs7, Gβ5 and Girk2

The similar labelling pattern detected at the light microscopic level and the parallel distribution observed by electron microscopy suggest that Rgs7 and Gβ5 proteins co-localize with Girk channels in the same subcellular compartments. To confirm this directly, double-labelling immunohistochemistry was performed. We found extensive co-localization of immunoreactivity for Rgs7 (Fig. 7A, C) and Gβ5 (Fig. 7B, D) proteins with Girk2 in the same presynaptic and postsynaptic compartment in the hippocampus.

We next investigated the spatial relationship of Rgs7, Gβ5, Girk2 and GABA_{B1} on excitatory axon terminals, presumably from Schaffer collaterals (Fig. 7E, F; Table 2). This analysis demonstrated that the four proteins are found both in the active zone and extrasynaptic plasma membrane of the axon terminals (Fig. 7E, F). Interestingly, Rgs7, Gβ5 and Girk2 clustered together throughout the axon terminal. GABA_B receptors overlap mostly along the 240 nm of extrasynaptic membrane surrounding the active zone (Fig. 7E, F).

Discussion

The present study shows that Rgs7 and Gβ5 proteins can exist as part of a stable protein complex with Girk channels and GABA_B receptors. This molecular association appears to be functionally relevant, as baclofen-induced current deactivation kinetics were markedly faster in cells co-expressing Rgs7/Gβ5. In addition, the present study describes the subcellular localization of Rgs7 and Gβ5 and its spatial relationship to GABA_B receptor and

the Girk2 subunit of the GIRK channel in the adult mouse hippocampus. Rgs7 and G β 5 proteins were primarily found postsynaptically and localized to dendritic shafts and dendritic spines of pyramidal cells. The observed close spatial relationship of Rgs7, G β 5, Girk2 and GABA_{B1} in dendritic spines likely reflects their functional and molecular association in this subcellular compartment. On dendritic shafts, Rgs7, G β 5, and Girk2 are also closely associated but mostly segregated from GABA_B receptors. Finally, in the presynaptic compartment, Rgs7, G β 5 and Girk2 are also closely associated. Collectively, the data reported here show that GABA_B receptor-signalling can be regulated by Rgs7/G β 5 complexes in dendritic spines of pyramidal cells, where they are likely to play a role in determining the temporal properties of Girk-dependent inhibitory postsynaptic potentials.

Rgs7 and G β 5 form stable protein complexes with Girk channels and GABA_B receptors

The newly emerging model of GPCR signalling suggests that receptors can form multi-protein complexes with many proteins with both regulatory functions and established signalling roles. Similarly, the function of the R7 Rgs proteins depends on the formation of the macromolecular complexes with other proteins that allows achieving signalling specificity (Anderson et al., 2009). Indeed, all members of the R7 subfamily exist as complexes with the G β 5 subunit (Anderson et al., 2009). Rgs7/G β 5 complexes co-immunoprecipitate with Girk channels (Xie et al., 2010), and they are also known to associate with several GPCRs and ion channels (Anderson et al., 2009). For instance, Rgs7 is thought to negatively modulate G protein signalling downstream from the μ -opioid (Sánchez-Blázquez et al., 2003; López-Fando et al., 2005) and GABA_B receptors (Xie et al., 2010). In addition, Rgs7 inhibited G α_q -promoted Ca²⁺ responses downstream of M3 receptors and 5-HT_{2c} receptors (DiBello et al., 1998; Shuey et al., 1998; Sandiford et al., 2010) and inhibited Girk channel activity in a G β 5-dependent manner (Kovoor et al., 2000).

Here, the results obtained in heterologous expression systems suggest that Rgs7 and G β 5 proteins are part of a stable protein complex with GABA_B receptors and Girk channels. This is consistent with our recent data demonstrating that both GABA_{B1} and GABA_{B2} subunit co-immunoprecipitate with Girk channel subunits (Ciruela et al., 2010). These interactions are not exclusive for the receptor, but rather they include several proteins from the GABA_B receptor-postsynaptic scaffold, as documented by parallel Rgs7-G β 5 and Rgs7-Girk channel interactions. In addition, our functional analysis showed that baclofen-induced current deactivation kinetics were markedly faster in cells co-expressing Rgs7/G β 5, indicating that macromolecular complexes formed by Rgs7/G β 5-Girk channels-GABA_B receptors exist and are functional. This might be critical and physiologically relevant as the existence of these macromolecular complexes can ensure rapid and specific receptor signalling.

Rgs7 and G β 5 are preferentially localized to dendritic shafts and spines in pyramidal cells

At the light microscopic level, our immunohistochemical findings indicate a widespread and overlapping distribution of both Rgs7 and G β 5 throughout the hippocampus. The immunoreactivity for both proteins was observed primarily in the dendritic layers, showing the most intense labelling in the *stratum radiatum* of the CA1 and CA3, as well as in the *stratum lucidum* of CA3. This distribution pattern is consistent with the results of *in situ* hybridization studies (Gold et al., 1997; Shuey et al., 1998), and expands on previous

immunohistochemical observations for Rgs7 and Gβ5 protein distribution in brain (Gold et al., 1997; Liang et al., 1998, 2000; Shuey et al., 1998; Khawaja et al., 1999). The similarity in the pattern of immunolabelling for the two proteins supports their close association throughout many of these dendritic networks.

Our immunohistochemical analysis in the hippocampus has revealed diverse patterns of Rgs7 and Gβ5 immunoreactivity. Both approaches indicated that Rgs7 and Gβ5 are expressed in pyramidal cells and interneurons but not glia. Immunoelectron microscopy showed that Rgs7 and Gβ5 labelling is readily detected in neuronal dendrites, spines and axon terminals but not in the nucleus of cells (*but see* Zhang et al., 2001), myelinated and unmyelinated axons, although this does not exclude its presence. Another notable finding of our ultrastructural studies is the presence of the two proteins within the same subcellular compartments; the majority of immunoparticles for Rgs7 and Gβ5 were observed on the extrasynaptic membrane of dendritic shafts and spines of pyramidal cells. This preferential dendritic localization offers an optimal position for the association the Rgs7/Gβ5 complex with Girk channels and different GPCRs residing in dendritic compartments (Lujan et al., 1997; Kulik et al., 2003).

Preferential colocalization of Rgs7 and Gβ5 with Girk2 and GABA_{B1} in dendritic spines

In the hippocampus, Girk channels are post-synaptic effectors for GABA_B receptors (Lüscher et al., 1997; Koyrakh et al., 2005). GABA_B receptors are heterodimers of GABA_{B1} and GABA_{B2} subunits (Kaupmann et al., 1998), both of which are highly co-expressed in pyramidal cells and localize both pre- and postsynaptically (Kulik et al., 2003), showing the same subcellular localization as Girk1 and Girk2 (Koyrakh et al., 2005; Fernández-Alacid et al., 2011). The results obtained using immunoelectron microscopy and quantitative analysis demonstrated that on dendritic spines, where glutamatergic synapses are located, Rgs7/Gβ5, Girk2 and GABA_B receptors show a close association. On dendritic shafts, both Rgs7 and Girk2 also associate but they are segregated from GABA_B receptors. This raises the questions as to how segregated complexes are activated or what are their cognate associated proteins. On one hand, it is possible that Rgs7/Gβ5/Girk channels in dendritic shafts are still associated with GABA_B receptors, but the coupling is less efficient and reliable given the spatial separation. It is also possible that Rgs7/Gβ5/Girk channels in dendritic shafts couple to other GPCRs, such as adenosine A1, 5-HT_{1A}, and D₂ receptors (Liao et al., 1996; Lüscher et al., 1997; Takigawa and Alzheimer, 1999). This possibility is supported by the results of functional studies showing that different metabotropic receptors could elicit Girk-mediated currents (Takigawa and Alzheimer, 1999; Chen and Johnston, 2005). On the other hand, GABA_B receptors expressed in dendritic shafts might couple to other effectors, such as voltage-dependent Ca²⁺ channels (Bettler et al., 2004), which are present in this subcellular compartment (Kulik et al., 2004).

Association of Rgs7 and Gβ5 with Girk2 in axon terminals

In addition to the strong postsynaptic labelling, a low but consistent presynaptic immunoreactivity for Rgs7 and Gβ5 was detected. Similar results were obtained for these proteins in other brain regions in previous immunohistochemical studies (Anderson et al., 2009; Xie et al., 2012). Although the function of the presynaptic Rgs7 and Gβ5 remains

unknown, their proximity to the axonal active zones strongly suggests an involvement in the modulation of GPCR-ion channel signalling involved in the regulation of neurotransmitter release. This mechanism is expected given that G β 5-Rgs proteins act as selective negative regulators for inhibitory G i/o proteins (Hooks et al. 2003) that mediate pre-synaptic inhibition (Blackmer et al. 2001).

At the ultrastructural level, one important finding is that Rgs7/G β 5 proteins tightly associate at the extrasynaptic membrane and presynaptic active zone of axon terminals establishing excitatory synapses with CA1 of the pyramidal cells. Interestingly, they also co-localise and follow the same subcellular pattern as Girk2. Indeed, presynaptic localization of Girk1, Girk2, and Girk3 has been reported in the brain (Koyrakh et al., 2005; Kulik et al., 2006; Aguado et al., 2008). Furthermore, GABA $_B$ receptors are present on excitatory terminals, consistent with their role as heteroreceptors regulating glutamate release (Bettler et al., 2004). Pre-synaptic GABA $_B$ receptors reduce neurotransmitter release by inhibiting Ca $^{2+}$ channels (Bettler et al., 2004). Although electrophysiological data do not support pre-synaptic Girk channel activation as a primary mechanism by which GABA $_B$ receptors modulate neurotransmitter release (Lüscher et al. 1997), our laboratory described a Girk channel-mediated inhibition of glutamate release through GABA $_B$ receptors at cerebrocortical and cerebellar nerve terminals. GABA $_B$ receptors reduces glutamate release and the Ca $^{2+}$ influx mediated by N-type Ca $^{2+}$ channels in a mode insensitive to Girk channels, but in a subpopulation of axon terminals, GABA $_B$ receptors also reduce glutamate release mediated by P/Q-type Ca $^{2+}$ channels; these responses are reversed by the Girk channel blocker tertiapin-Q (Ladera et al. 2008; Fernández-Alacid et al., 2009).

Interestingly, it has been shown recently that presynaptic GABA $_B$ receptors inhibit glutamate release through macromolecular complexes containing Ca v 2.2 and G $\alpha_o\beta$ 1 γ 2 (Laviv et al., 2011). The co-localization of Rgs7/G β 5 and Girk2 in axon terminals, as revealed here, suggests their molecular and functional association at presynaptic compartments. In addition, although the distribution pattern of GABA $_B$ receptors is mostly different from that of Rgs7/G β 5/Girk2, there is a strong overlap within an annulus of 240 nm surrounding the active zone. Therefore, it is possible that molecular and functional interaction between Rgs7/G β 5/Girk2/GABA $_B$ proteins is taking place this sub-compartment of axon terminals, thus explaining that only happens in a subpopulation of axon terminals (Ladera et al. 2008; Fernández-Alacid et al., 2009).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank to Mrs. Mercedes Gil for the excellent technical assistance. This work was supported by grants from the Spanish Ministry of Education and Science (BFU-2012-38348) and CONSOLIDER (CSD2008-00005) to R.L.; and by grants RO1 MH061933 to K.W., DA021743 and DA026405 to K.A.M. and RO1 HL105550 to K.W. and K.A.M.

References

- Aguado C, Colón J, Ciruela F, Schlaudraff F, Cabañero MJ, Perry C, Watanabe M, Liss B, Wickman K, Luján R. Cell type-specific subunit composition of G protein-gated potassium channels in the cerebellum. *J Neurochem*. 2008; 105:497–511. [PubMed: 18088366]
- Anderson GR, Luján R, Martemyanov KA. Changes in striatal signaling induce remodeling of RGS complexes containing Gbeta5 and R7BP subunits. *Mol Cell Biol*. 2009; 29:3033–44. [PubMed: 19332565]
- Anderson GR, Posokhova E, Martemyanov KA. The R7 RGS protein family: multi-subunit regulators of neuronal G protein signaling. *Cell Biochem Biophys*. 2009; 54:33–46. [PubMed: 19521673]
- Bettler B, Kaupmann K, Mosbacher J, Gassmann M. Molecular structure and physiological functions of GABA(B) receptors. *Physiol Rev*. 2004; 84:835–67. [PubMed: 15269338]
- Blackmer T, Larsen EC, Takahashi M, Martin TF, Alford S, Hamm HE. G protein betagamma subunit-mediated presynaptic inhibition: regulation of exocytotic fusion downstream of Ca²⁺ entry. *Science*. 2001; 292:293–7. [PubMed: 11303105]
- Cabrera JL, De Freitas F, Satpaev DK, Slepak VZ. Identification of the G₅-RGS7 protein complex in the retina. *Biochem Biophys Res Commun*. 1998; 249:898–902. [PubMed: 9731233]
- Cao Y, Song H, Okawa H, Sampath AP, Sokolov M, Martemyanov KA. Targeting of RGS7/Gβ5 to the Dendritic Tips of ON-bipolar Cells Is Independent of Its Association with Membrane Anchor R7BP. *J Neurosci*. 2008; 28:10443–10449. [PubMed: 18842904]
- Chen CK, Eversole-Cire P, Zhang HK, Mancino V, Chen YJ, He W, Wensel TG, Simon MI. Instability of GGL domain-containing RGS proteins in mice lacking the G protein betasubunit Gb5. *Proc Natl Acad Sci USA*. 2003; 100:6604–6609. [PubMed: 12738888]
- Chen X, Johnston D. Constitutively active G-protein-gated inwardly rectifying K⁺ channels in dendrites of hippocampal CA1 pyramidal neurons. *J Neurosci*. 2005; 25:3787–3792. [PubMed: 15829630]
- Ciruela F, Fernández-Dueñas V, Sahlholm K, Fernández-Alacid L, Nicolau JC, Watanabe M, Luján R. Evidence for oligomerization between GABAB receptors and GIRK channels containing the GIRK1 and GIRK3 subunits. *Eur J Neurosci*. 2010; 32:1265–77. [PubMed: 20846323]
- DiBello PR, Garrison TR, Apanovitch DM, Hoffman G, Shuey DJ, Mason K, Cockett MI, Dohlman HG. Selective uncoupling of RGS action by a single point mutation in the G protein alpha-subunit. *J Biol Chem*. 1998; 273:5780–4. [PubMed: 9488712]
- Fernández-Alacid L, Aguado C, Ciruela F, Martín R, Colón J, Cabañero MJ, Gassmann M, Watanabe M, Shigemoto R, Wickman K, Bettler B, Sánchez-Prieto J, Luján R. Subcellular compartment-specific molecular diversity of pre- and post-synaptic GABA-activated GIRK channels in Purkinje cells. *J Neurochem*. 2009; 110:1363–1376. [PubMed: 19558451]
- Fernández-Alacid L, Watanabe M, Molnár E, Wickman K, Luján R. Developmental regulation of G protein-gated inwardly-rectifying K⁺ (GIRK/Kir3) channel subunits in the brain. *Eur J Neurosci*. 2011; 34:1724–36. [PubMed: 22098295]
- Fiala JC. Reconstruct: a free editor for serial section microscopy. *J Microsc*. 2005; 218:52–61. [PubMed: 15817063]
- Gold SJ, Ni YG, Dohlman HG, Nestler EJ. Regulators of G-protein signalling (RGS) proteins: region-specific expression of nine subtypes in rat brain. *J Neurosci*. 1997; 17:8024–8037. [PubMed: 9315921]
- He W, Lu LS, Zhang X, El Hodiri HM, Chen CK, Slep KC, Simon MI, Jamrich M, Wensel TG. Modules in the photoreceptor RGS9-1/Gb5L GTPase-accelerating protein complex control effector coupling, GTPase acceleration, protein folding, and stability. *J Biol Chem*. 2000; 275:37093–37100. [PubMed: 10978345]
- Hollinger S, Hepler JR. Cellular regulation of RGS proteins: modulators and integrators of G protein signalling. *Pharmacol Rev*. 2002; 54:527–559. [PubMed: 12223533]
- Hooks SB, Waldo GL, Corbitt J, Bodor ET, Krumins AM, Harden TK. RGS6, RGS7, RGS9, and RGS11 stimulate GTPase activity of Gi family G-proteins with differential selectivity and maximal activity. *J Biol Chem*. 2003; 278:10087–93. [PubMed: 12531899]

- Jordan M, Schallhorn A, Wurm FM. Transfecting Mammalian Cells: Optimization of Critical Parameters Affecting Calcium-phosphate Precipitate Formation. *Nucleic Acids Research*. 1996; 24:596–601. [PubMed: 8604299]
- Khawaja XZ, Liang JJ, Saugstad JA, Jones PG, Harnish S, Conn PJ, Cockett MI. Immunohistochemical distribution of RGS7 protein and cellular selectivity in colocalizing with Galphaq proteins in the adult rat brain. *J Neurochem*. 1999; 72:174–84. [PubMed: 9886068]
- Karschin C, Dissmann E, Stuhmer W, Karschin A. IRK(1–3) and GIRK(1–4) inwardly rectifying K⁺ channel mRNAs are differentially expressed in the adult rat brain. *J Neurosci*. 1996; 16:3559–3570. [PubMed: 8642402]
- Kaupmann K, Schuler V, Mosbacher J, Bischoff S, Bittiger H, Heid J, Froestl W, Leonhard S, Pfaff T, Karschin A, Bettler B. Human g-aminobutyric acid type B receptors are differentially expressed and regulate inwardly rectifying K⁺ channels. *Proc Natl Acad Sci USA*. 1998; 95:14991–14996. [PubMed: 9844003]
- Kovoor A, Chen CK, He W, Wensel TG, Simon MI, Lester HA. Co-expression of Gbeta5 enhances the function of two Ggamma subunit-like domain-containing regulators of G protein signaling proteins. *J Biol Chem*. 2000; 275:3397–402. [PubMed: 10652332]
- Koyrakh L, Luján R, Colon J, Karschin C, Kurachi Y, Karschin A, Wickman K. Molecular and cellular diversity of neuronal G-protein gated potassium channels. *J Neurosci*. 2005; 25:11468–11478. [PubMed: 16339040]
- Krapivinsky GB, Gordon EA, Wickman K, Velimirovic B, Krapivinsky L, Clapham DE. The G protein gated atrial K⁺ channel I_{KACH} is a heteromultimer of two inwardly rectifying K⁺-channel proteins. *Nature*. 1995; 374:135–141. [PubMed: 7877685]
- Kulik A, Vida I, Lujan R, Haas CA, Lopez-Bendito G, Shigemoto R, Frotscher M. Subcellular localization of metabotropic GABAB receptor subunits GABAB1a/b and GABAB2 in the rat hippocampus. *J Neurosci*. 2003; 23:11026–11035. [PubMed: 14657159]
- Kulik A, Nakadate K, Hagiwara A, Fukazawa Y, Luján R, Saito H, Suzuki N, Futatsugi A, Mikoshiba K, Frotscher M, Shigemoto R. Immunocytochemical localization of the alpha 1A subunit of the P/Q-type calcium channel in the rat cerebellum. *Eur J Neurosci*. 2004; 19:2169–78. [PubMed: 15090043]
- Kulik A, Vida I, Fukazawa Y, Guetg N, Kasugai Y, Marker CL, Rigato F, Bettler B, Wickman K, Frotscher M, Shigemoto R. Compartment dependent colocalization of Kir3.2-containing K⁺-channels and GABAB receptors in hippocampal pyramidal cells. *J Neurosci*. 2006; 26:4289–4297. [PubMed: 16624949]
- Ladera C, del Carmen Godino M, José Cabañero M, Torres M, Watanabe M, Luján R, Sánchez-Prieto J. Pre-synaptic GABA receptors inhibit glutamate release through GIRK channels in rat cerebral cortex. *J Neurochem*. 2008; 107:1506–17. [PubMed: 19094055]
- Laviv T, Vertkin I, Berdichevsky Y, Fogel H, Riven I, Bettler B, Slesinger PA, Slutsky I. Compartmentalization of the GABAB receptor signaling complex is required for presynaptic inhibition at hippocampal synapses. *J Neurosci*. 2011; 31:12523–32. [PubMed: 21880914]
- Liang JJ, Cockett M, Khawaja XZ. Immunohistochemical localization of G protein beta1, beta2, beta3, beta4, beta5, and gamma3 subunits in the adult rat brain. *J Neurochem*. 1999; 71:345–55. [PubMed: 9648884]
- Liang JJ, Chen HH, Jones PG, Khawaja XZ. RGS7 complex formation and colocalization with the Gbeta5 subunit in the adult rat brain and influence on Gbeta5gamma2-mediated PLCbeta signaling. *J Neurosci Res*. 2000; 60:58–64. [PubMed: 10723068]
- Liao YJ, Jan YN, Jan LY. Heteromultimerization of G-protein-gated inwardly rectifying K⁺ channel proteins GIRK1 and GIRK2 and their altered expression in weaver brain. *J Neurosci*. 1996; 16:7137–7150. [PubMed: 8929423]
- López-Bendito G, Shigemoto R, Kulik A, Vida I, Fairén A, Luján R. Distribution of metabotropic GABA receptor subunits GABAB1a/b and GABAB2 in the rat hippocampus during prenatal and postnatal development. *Hippocampus*. 2004; 14:836–48. [PubMed: 15382254]
- López-Fando A, Rodríguez-Muñoz M, Sánchez-Blázquez P, Garzón J. Expression of neural RGS-R7 and Gbeta5 Proteins in Response to Acute and Chronic Morphine. *Neuropsychopharmacology*. 2005; 30:99–110. [PubMed: 15199376]

- Luján R, Nusser Z, Roberts JD, Shigemoto R, Somogyi P. Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. *Eur J Neurosci.* 1996; 8:1488–1500. [PubMed: 8758956]
- Luján R, Roberts JD, Shigemoto R, Ohishi H, Somogyi P. Differential plasma membrane distribution of metabotropic glutamate receptors mGluR1 α , mGluR2 and mGluR5, relative to neurotransmitter release sites. *J Chem Neuroanat.* 1997; 13:219–41. [PubMed: 9412905]
- Luján R, Shigemoto R. Localization of Metabotropic GABA Receptor Subunits GABAB1 and GABAB2 Relative to Synaptic Sites in the Rat Developing Cerebellum. *Eur J Neurosci.* 2006; 23:1479–1490. [PubMed: 16553611]
- Lüscher C, Jan LY, Stoffel M, et al. G protein-coupled inwardly rectifying K⁺ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. *Neuron.* 1997; 19:687–695. [PubMed: 9331358]
- Makino ER, Handy JW, Li TS, Arshavsky VY. The GTPase activating factor for transducin in rod photoreceptors is the complex between RGS9 and type 5 G protein β subunit. *Proc Natl Acad Sci USA.* 1999; 96:1947–1952. [PubMed: 10051575]
- North A. Drug receptors and the inhibition of nerve cells. *Br J Pharmacol.* 1989; 98:13–28. [PubMed: 2679954]
- Posner BA, Gilman AG, Harris BA. Regulators of G protein signaling 6 and 7. Purification of complexes with gbeta5 and assessment of their effects on g protein-mediated signaling pathways. *J Biol Chem.* 1999; 274:31087–93. [PubMed: 10521509]
- Rose JJ, Taylor JB, Shi J, Cockett MI, Jones PG, Hepler JR. RGS7 is palmitoylated and exists as biochemically distinct forms. *J Neurochem.* 2000; 75:2103–12. [PubMed: 11032900]
- Ross EM, Wilkie TM. GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu Rev Biochem.* 2000; 69:795–827. [PubMed: 10966476]
- Sánchez-Blázquez P, Rodríguez-Díaz M, López-Fando A, Rodríguez-Muñoz M, Garzón J. The GBeta5 subunit that associates with the R7 subfamily of RGS proteins regulates mu-opioid effects. *Neuropharmacology.* 2003; 45:82–95. [PubMed: 12814661]
- Sandiford SL, Wang Q, Levay K, Buchwald P, Slepak VZ. Molecular organization of the complex between the muscarinic M3 receptor and the regulator of G protein signaling, Gbeta(5)-RGS7. *Biochemistry.* 2010; 49:4998–5006. [PubMed: 20443543]
- Shuey DJ, Betty M, Jones PG, Khawaja XZ, Cockett MI. RGS7 attenuates signal transduction through the G(α q) family of heterotrimeric G proteins in mammalian cells. *J Neurochem.* 1998; 70:1964–72. [PubMed: 9572280]
- Slepak VZ. Structure, function, and localization of G β 5-RGS complexes. *Prog Mol Biol Transl Sci.* 2009; 86:157–203. [PubMed: 20374716]
- Smrcka AV. G protein $\beta\gamma$ subunits: central mediators of G protein-coupled receptor signalling. *Cell Mol Life Sci.* 2008; 65:2191–214. [PubMed: 18488142]
- Takigawa T, Alzheimer C. G protein-activated inwardly rectifying K⁺ (GIRK) currents in dendrites of rat neocortical pyramidal cells. *J Physiol (Lond).* 1999; 517:385–390. [PubMed: 10332089]
- Vigot R, Barbieri S, Bräuner-Osborne H, Turecek R, Shigemoto R, Zhang Y-P, Luján R, et al. Differential Compartmentalization and Distinct Functions of GABAB Receptor Variants. *Neuron.* 2006; 50:589–601. [PubMed: 16701209]
- Witherow DS, Wang Q, Levay K, Cabrera JL, Chen J, Willars GB, Slepak VZ. Complexes of the G protein subunit G β 5 with the regulators of G protein signalling RGS7 and RGS9: characterization in native tissues and in transfected cells. *J Biol Chem.* 2000; 275:24872–24880. [PubMed: 10840031]
- Xie K, Allen KL, Kourrich S, Colon-Saez J, Thomas MJ, Wickman K, Martemyanov KA. Gbeta5 recruits R7 RGS proteins to GIRK channels to regulate the timing of neuronal inhibitory signalling. *Nat Neurosci.* 2010; 13:661–663. [PubMed: 20453851]
- Xie K, Ge S, Collins VE, Haynes CL, Renner KJ, Meisel RL, Luján R, Martemyanov KA. G β 5-RGS Complexes Are Gatekeepers of Hyperactivity Involved in Control of Multiple Neurotransmitter Systems. *Psychopharmacology.* 2012; 219:823–834. [PubMed: 21766168]

- Zhang JH, Lai ZN, Simonds WF. Differential expression of the G protein $\alpha 5$ gene: analysis of mouse brain, peripheral tissues, and cultured cell lines. *J Neurochem.* 2000; 75:393–403. [PubMed: 10854285]
- Zhang JH, Barr VA, Mo Y, Rojkova AM, Liu S, Simonds WF. Nuclear localization of G protein $\beta 5$ and regulator of G protein signaling 7 in neurons and brain. *J Biol Chem.* 2001; 276:10284–9. [PubMed: 11152459]
- Zheng B, De Vries L, Gist Farquhar M. Divergence of RGS proteins: evidence for the existence of six mammalian RGS subfamilies. *Trends Biochem Sci.* 1999; 24(11):411–4. [PubMed: 10542401]

Figure 1. Interaction between Girk channels, Rgs7/Gβ5, and GABA_B receptors

The series of blots on the left are representative of co-immunoprecipitation experiments performed in 293FT cells involving the prototypical neuronal Girk channel (Girk1/Girk2) and Rgs7/Gβ5. Cells were transfected with expression constructs for Girk1 (Girk1-AU5; the doublet at 56–58 kDa corresponds to core and core-glycosylated forms of Girk1, while diffuse labelling between 65–76 kDa reflects heavily-glycosylated Girk1 (Krapivinsky et al., 1995), Girk2 (Girk2-myc; 45 kDa), Gβ5 (39 kDa), and/or Rgs7 (57 kDa), as indicated above the blot. Input samples on the left confirm the viability of the constructs and specificity of the immunoblotting antibodies. Immunoprecipitation (IP) samples on the right show that Girk1-AU5, Gβ5, and Rgs7 all co-immunoprecipitate in selective fashion with Girk2-myc (n=4 separate experiments). The series of blots on the right are representative of co-immunoprecipitation studies involving Girk1/Girk2, Rgs7/Gβ5, and GABA_B receptor (GABA_{B1a}/GABA_{B2}). GABA_{B1a} (108 kDa) was detected in the eluate, along with Girk1, Gβ5, and Rgs7, following immunoprecipitation of Girk2-myc.

Figure 2. Impact of Rgs7/Gβ5 on GABA_B-Girk signalling

A) Representative currents triggered by baclofen (100 μ M) in HEK293 cells transfected with GABA_{B1a}, GABA_{B2}, Girk1, and Girk2, either with (gray) or without (control, black) Rgs7/Gβ5. Peak currents were normalized to highlight the difference in deactivation kinetics between the two experiments. The black line above the trace denotes the duration of baclofen exposure. B) Summary of current densities measured in cells expressing GABA_{B1}, GABA_{B2}, Girk1, and Girk2, in the absence (black, control; n=27) or presence (gray; n=8) of Rgs7/Gβ5, and in cells expressing GABA_BR and Girk1/Girk2 that were pre-treated with pertussis toxin (PTX) prior to recordings (white; n=5). While no significant difference in basal current density was observed across groups ($F_{2,36}=0.7$, $P=0.52$), differences were observed for baclofen-induced peak ($F_{2,36}=7.4$, $P<0.01$) and steady-state (s-s; $F_{2,36}=3.7$, $P<0.05$) current densities, attributable to the negligible currents measured in PTX-treated cells. C) Summary of activation and deactivation kinetics in cells expressing GABA_{B1a}, GABA_{B2}, Girk1, and Girk2, in the absence (black, control) or presence (gray) of Rgs7/Gβ5. Deactivation ($t(33)=3.5$, $P<0.01$) rate, but not activation rate ($t(33)=1.3$, $P=0.21$), was significantly accelerated in cells co-expressing Rgs7/Gβ5. Symbols: ** $P<0.01$ vs. control.

Figure 3. Distribution of immunoreactivity for Rgs7 and Gβ5 in the hippocampus (A–D) At the light microscopic level, immunoreactivity for both Rgs7 and Gβ5 was widely distributed and overlapping in the hippocampus. In the CA1 region, immunolabelling for Rgs7 and Gβ5 was generally moderate-to-strong, with the *strata oriens* (so) and *radiatum* (sr) showing the highest and the *stratum lacunosum-moleculare* (slm) showing the weakest immunoreactivity (Fig. 2A, B). Similar immunoreactivities were observed in the CA3 region, with the exception of the *stratum lucidum* that showed the strongest immunoreactivity for both Rgs7 and Gβ5 (Fig. 2A, B). In the dentate gyrus, immunolabelling was strong in the molecular layer (ml) and the highest in the hilus (h) (Fig. 2A, B). In the pyramidal (sp) and granule cell layers, no labelling was observed. Scale bars: A, C, 500 μ m; B, D, 150 μ m.

Figure 4. Subcellular localization of Rgs7 in the hippocampus

Electron micrographs of the *stratum radiatum* of the hippocampal CA1 region showing immunoparticles for Rgs7, as detected using a pre-embedding immunogold method. (A–G) Immunoparticles for Rgs7 were abundant on the extrasynaptic plasma membrane (arrows) of dendritic spines (s) of pyramidal cells (s) contacted by terminals (at) of presumed CA3 pyramidal cells, as well as at intracellular sites (crossed arrows). They also appeared at the edge of postsynaptic densities (double arrowhead in *panels E–F*) of dendritic spines (s). Immunoparticles for Rgs7 were seen along the extrasynaptic plasma membrane (arrows) of dendritic shafts (Den) of pyramidal cells. Presynaptically, immunogold particles (arrowheads) were localized to the extrasynaptic plasma membrane and to the active zone of axon terminals (at) establishing asymmetrical synapses with spines (s), and at intracellular sites (double arrowhead). (H, I), Immunolabelling for Rgs7 was also detected along the plasma membrane (arrows) and intracellularly (crossed arrows) in dendritic shafts of presumed interneurons (Den) establishing asymmetrical synapses with axon terminals (at), as confirmed in serial sections. (J–L) Three-dimensional reconstruction of labelled spines and dendrites. In spines (s) and shafts (Den) of pyramidal cells, PSDs are represented by red and axon terminal in green; in interneuron dendrite (Den, *panel L*) excitatory synapses are represented in purple. Small dots in orange represent immunoparticles in the surface and

those in purple at intracellular sites. Note that in spines, the RGS7 immunoparticles were distributed close to the synaptic site, but instead distributed randomly over the dendritic plasma membrane without any noticeable preference to the synaptic sites. Scale bar: A–G, J–L, 0.2 μm ; H, I, 0.5 μm .

Figure 5. Subcellular localization of Gβ5 in the hippocampus

Electron micrographs of the *stratum radiatum* of the hippocampal CA1 region showing immunoparticles for Gβ5, as detected using a pre-embedding immunogold method. (A–G) Immunoparticles for Gβ5 were localised to the extrasynaptic plasma membrane (arrows) of dendritic spines (s) of pyramidal cells (s) contacted by terminals (at) of presumed CA3 pyramidal cells, as well as at intracellular sites (crossed arrows). They also appeared at the edge of postsynaptic densities (double arrowhead in panels A, C, E) of dendritic spines (s). Immunoparticles for Gβ5 were seen along the extrasynaptic plasma membrane (arrows) of dendritic shafts (Den) of pyramidal cells. Presynaptically, immunogold particles (arrowheads) were localized to the extrasynaptic plasma membrane and to the active zone of axon terminals (at) establishing asymmetrical synapses with spines (s), and at intracellular sites (double arrowhead). Immunolabelling for Gβ5 was also detected along the plasma membrane (arrows) and intracellularly (crossed arrows) in dendritic shafts of interneurons (Den) establishing synaptic contacts with axon terminals (at). (H–J) Three-dimensional reconstruction of labelled spines (s) and dendrites (Den). PSDs are represented by red and axon terminals in green. Small dots in orange represent immunoparticles in the surface and those in purple at intracellular sites. Note that in spines, the Gβ5 immunoparticles were distributed close to the synaptic site, but instead distributed randomly over the dendritic plasma membrane. Scale bar: A–I, 0.2 μm; J, 0.5 μm.

Figure 6. Dendritic compartmentalization of Rgs7, Gβ5, Girk2 and GABA_{B1} in CA1 pyramidal cells

Quantitative analysis of the distribution of the different proteins in dendritic spines (A–B) and shafts (C–D) of pyramidal cells, as assessed by pre-embedding immunogold labelling. (A) Schematic diagram showing the quantification procedure in dendritic spines. Distances of immunogold particles were measured ($n = 156$ for Rgs7; $n = 102$ for Gβ5; $n = 182$ for Girk2; and $n = 189$ for GABA_{B1}) from the closest edge of the PSD along the surface of dendritic spines. Values were allocated to 60-nm-wide bins and expressed as relative frequencies. (B) Histogram showing the spatial distribution of immunoparticles for Rgs7, Gβ5, Girk2 and GABA_{B1} around asymmetrical synapses on dendritic spines. These data show an enrichment of all four proteins in the proximity of asymmetrical synapses on dendritic spines. (C) Schematic diagram showing the quantification procedure in dendritic shafts. Distances of immunogold particles were measured ($n = 271$ for Rgs7; $n = 232$ for Girk2; and $n = 247$ for GABA_{B1}) from the edge of spine necks and values were allocated to 60-nm-wide bins and expressed as relative frequencies. (D) Histogram showing the spatial distribution of immunoparticles for Rgs7, Girk2 and GABA_{B1} on dendritic dendritic shafts. These data show that there is association of Rgs7 and Girk2 but no association of Rgs7/Girk2 and GABA_{B1} on dendritic shafts.

Figure 7. Colocalization of Rgs7, Gβ5 and Girk2 in the hippocampus

(A, D) Electron micrographs of the distal part of the *stratum radiatum* of the hippocampal CA1 region showing colocalization for Rgs7 or Gβ5 with Girk2, as detected using a pre-embedding immunogold method. Labelling is shown with immunogold for the Rgs7 or Gβ5, and with immunoperoxidase for the Girk2 subunit. Postsynaptically, Rgs7 (panel A) or Gβ5 (panel B) were seen in dendritic spines (s) of presumed pyramidal cells immunopositive for Girk2. Presynaptically, Rgs7 (panel C) or Gβ5 (panel D) were detected in Girk2-positive axon terminal (at) establishing asymmetrical contact with a dendritic spine (s). Scale bar: A–D, 0.2 μ m. (E) Schematic diagram showing the quantification procedure. Distances of immunogold particles ($n = 116$ for Rgs7; $n = 109$ for Gβ5; $n = 142$ for Girk2; and $n = 151$ for GABA_{B1}) were measured from the edge of the active zone (AZ) along the extrasynaptic membrane surrounding the active zone (SZ). Values were allocated to 60-nm-wide bins and expressed as relative frequencies. (F) Histogram showing the spatial distribution of immunoparticles for Rgs7, Gβ5, Girk2 and GABA_{B1} on excitatory axon terminals. These

data show that there is association of Rgs7/G β 5 complexes and Girk2 thought the axon terminal and their possible association with GABA_{B1} at the edge of the active zone.

Table 1

Summary of immunogold labelling for Rgs7 and G β 5 in CA1 pyramidal cells.

Antibody	PM gold	% PM	Intra gold	% Intra
<i>Rgs7 (total)</i>	2260	44	2908	56
<i>Gβ5 (total)</i>	763	25	2269	75
	Postsynaptic gold	% Postsynaptic	Presynaptic gold	% Presynaptic
<i>Rgs7 (PM)</i>	2150	95	110	5
<i>Gβ5 (PM)</i>	722	95	41	5

Table 2

Summary of immunogold labelling for Rgs7, G β 5, Girk2 and GABA_{B1} in different compartment of CA1 pyramidal cells.

Dendritic spines	Number spines	Gold
<i>Rgs7</i>	97	156
<i>Gβ5</i>	75	102
<i>Girk2</i>	63	82
<i>GABA_{B1}</i>	71	89
Dendritic shafts	Number dendrites	Gold
<i>Rgs7</i>	25	271
<i>Gβ5</i>	ns	ns
<i>Girk2</i>	25	232
<i>GABA_{B1}</i>	25	247
Axon terminals	Number terminals	Gold
<i>Rgs7</i>	100	116
<i>Gβ5</i>	100	109
<i>Girk2</i>	100	142
<i>GABA_{B1}</i>	100	151

ns: not studied