Cellular/Molecular

Subcellular Localization of Metabotropic GABA_B Receptor Subunits GABA_B1a/b and GABA_B2 in the Rat Hippocampus

Ákos Kulik,1,4 Imre Vida,1 Rafael Luján,2 Carola A. Haas,1 Guillermina López-Bendito,1 Ryuichi Shigemoto,4,2 and Michael Frotscher1

1Department of Anatomy and Cell Biology, University of Freiburg, 79104 Freiburg, Germany, 2Centro Regional de Investigaciones Biomédicas, Facultad de Medicina, Universidad de Castilla-La Mancha, Campus Biosanitario, 02071 Albacete, Spain, 3Department of Human Anatomy and Genetics, University of Oxford, Oxford, OX1 3QX, United Kingdom, 4Division of Cerebral Structure, National Institute for Physiological Sciences, Myodaiji, Okazaki 444-8585, Japan, and 5Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Kawaguchi 332-0012, Japan

Metabotropic GABA_B receptors mediate slow inhibitory effects presynaptically and postsynaptically. Using preembedding immunohistochemical methods combined with quantitative analysis of GABA_B receptor subunit immunoreactivity, this study provides a detailed description of the cellular and subcellular localization of GABA_B1a/b and GABA_B2 in the rat hippocampus. At the light microscopic level, an overlapping distribution of GABA_B1a/b and GABA_B2 was revealed in the dendritic layers of the hippocampus. In addition, expression of the GABA_B1a/b subunit was found in somata of CA1 pyramidal cells and of a subset of GABAergic interneurons. At the electron microscopic level, immunoreactivity for both subunits was observed in presynaptic and, more abundantly, on postsynaptic elements. Presynaptically, subunits were mainly detected in the extrasynaptic membrane and occasionally over the presynaptic membrane specialization of putative glutamatergic and, to a lesser extent, GABAergic axon terminals. Postsynaptically, the majority of GABA_B receptor subunits were localized to the extrasynaptic plasma membrane of spines and dendritic shafts of principal cells and shafts of interneuron dendrites. Quantitative analysis revealed enrichment of GABA_B1a/b around putative glutamatergic synapses on spines and an even distribution on dendritic shafts of pyramidal cells contacted by GABAergic boutons. The association of GABA_B receptors with glutamatergic synapses at both presynaptic and postsynaptic sides indicates their intimate involvement in the modulation of glutamatergic neurotransmission. The dominant extrasynaptic localization of GABA_B receptor subunits suggests that their activation is dependent on spill-over of GABA requiring simultaneous activity of populations of GABAergic cells as it occurs during population oscillations or epileptic seizures.

Key words: GABA_B1; GABA_B2; G-protein-coupled receptors; immunocytochemistry labeling; electron microscopy; inhibition; spill-over

Introduction

GABA is a major inhibitory neurotransmitter in the mammalian brain, and its action is mediated by ionotropic and metabotropic receptors (MacDonald and Olsen, 1994; Misgeld et al., 1995; Johnston, 1996). Metabotropic GABA_B receptors (GABA_B receptors) are coupled to second-messenger systems through G-proteins and mediate slow and prolonged inhibitory effects (Misgeld et al., 1995). Up to date, two subunits have been identified, GABA_B1, which exists in five alternatively spliced forms, and GABA_B2 (Bowery and Brown, 1997; Kaupmann et al., 1997; Isomoto et al., 1998; Pfaff et al., 1999; Schwarz et al., 2000). For surface localization, coupling to the physiological effectors and formation of fully functional GABA_B receptors coassembly of GABA_B1 and GABA_B2 subunits is required (Jones et al., 1998; Kaupmann et al., 1998a; White et al., 1998; Kuner et al., 1999; Margeta-Mitrovic et al., 2000, 2001; Pagano et al., 2001; Bowery et al., 2002). Presynaptically located GABA_B receptors modulate neurotransmitter release by depressing Ca^{2+} influx via voltage-activated Ca^{2+} channels (Scholz and Miller, 1991; Pfrieger et al., 1994; Wu and Saggau, 1995). Such presynaptic inhibition at GABAergic terminals is involved in the induction of long-term potentiation (Davies et al., 1991). The effect of postsynaptic GABA_B receptors is primarily mediated by G-protein-coupled inwardly rectifying K^{+} channels (Kir3 channels) (Lüscher et al., 1997; Kaupmann et al., 1998b), resulting in slow IPSPs (Dutar and Nicoll, 1988a,b; Mody et al., 1994; Misgeld et al., 1995; Bowery et al., 2002). The goal of the present study was to determine the distribution of GABA_B1a/b and GABA_B2 subunits in the adult rat hippocampus. Most of the information available on the localization

Received Aug. 8, 2003; revised Oct. 6, 2003; accepted Oct. 7, 2003.

This work was supported by Deutsche Forschungsgemeinschaft Sonderforschungsbereich 505. A.K. was supported by the Alexander von Humboldt Foundation. We are grateful to Drs. Peter Jonas and Bernhard Bettler for their helpful suggestions on this manuscript. We thank Anikó Schneider, Sigrun Nestel, and Susanne Huber for their

Correspondence should be addressed to Dr. Ákos Kulik, Department of Anatomy and Cell Biology, University of Freiburg, Albertstrasse 17, D-79104 Freiburg, Germany. E-mail: akos.kulik@anat.uni-freiburg.de.

Copyright © 2003 Society for Neuroscience 0270-6474/03/2311026-10$15.00/0

1026 • The Journal of Neuroscience, December 3, 2003 • 23(35):11026 –11035
of GABA<sub>B</sub> receptors so far was obtained from autoradiographic, in situ hybridization, and light microscopic immunohistochemical studies (Bowery et al., 1987; Chu et al., 1990; Turgeon and Albin, 1994; Kaupmann et al., 1997; Bischoff et al., 1999; Fritschi et al., 1999; Lu et al., 1999; Margeta-Mitrovic et al., 1999). Here we determine the precise cellular and subcellular localization of the two GABA<sub>B1a/b</sub> receptor subunits by using preembedding immunoelectron microscopy in combination with quantification of GABA<sub>B1a/b</sub> immunoreactivity.

**Materials and Methods**

**Tissue preparation**

Twenty-three adult male Wistar rats were used in the present study. Care and handling of the animals before and during the experimental procedures followed European Union regulations and were approved by the Animal Care and Use Committees of our institutions.

Animals were deeply anesthetized by Narkodorm-n (180 mg/kg, i.p.) (Alvetra, Neumünster, Germany), and the hearts were surgically exposed for perfusion fixation. First, the vascular system was flushed by circulating 0.9% saline for 1 min. This was followed by transcardial perfusion with one of the three freshly prepared fixatives for 13 min: (1) three rats were perfused with a solution containing 4% paraformaldehyde made up in 0.1 M phosphate buffer (PB), pH 7.4, for in situ hybridization histochemistry and double-labeling in situ hybridization–immunohistochemistry; (2) eight rats were perfused with a solution containing 4% paraformaldehyde and 15% (v/v) saturated picric acid for light microscopy in combination with quantification of the GABA<sub>B1a/b</sub> neurotransmitter; and (3) 12 rats were perfused with a solution containing 0.5% glutaraldehyde, 4% paraformaldehyde, and 15% (v/v) saturated picric acid for electron microscopic immunocytochemistry. After perfusion, brains were removed from the skull, and tissue blocks containing the hippocampus were dissected and washed in 0.1 M PB.

**Preparation of cRNA probes**

Digoxigenin (DIG)-labeled cRNA probes were generated by in vitro transcription from a cDNA clone encoding rat glutamic acid decarboxylase (GAD67) mRNA (Erlander et al., 1991) inserted in both orientations into the EcoRI site of pBluescript S′ vector (Stratagene, La Jolla, CA). The plasmids were linearized by restriction digest with Sall to serve as template for T3 RNA polymerase (antisense and sense, respectively). In vitro transcription was performed as described previously (Haas et al., 1999). DIG-labeled GAD67 cRNAs (~3.2 kb) were purified by ethanol precipitation and were treated by alkaline hydrolysis to reduce their sizes to ~250 bases following standard protocols.

In situ hybridization histochemistry

In situ hybridization histochemistry was performed as described previously (Haas et al., 1999). Briefly, cryostat sections were pretreated in hybridization buffer (50% formamide, 4× SSC, 50 μM NaH<sub>2</sub>PO<sub>4</sub>, 250 mg/ml heat-denatured salmon sperm DNA, 100 mg/ml tRNA, 5% dextran sulfate, and 1% Denhardt’s solution) diluted with 2× SSC (1:1) for 15 min and prehybridized in hybridization buffer for 60 min at 45°C. Hybridization was performed in the same buffer with the addition of 50 ng/ml digoxigenin-labeled GAD67 antisense or sense cRNA probes at 45°C overnight. After hybridization, the brain sections were washed in 2× SSC (two times for 15 min each) at room temperature, 2× SSC and 50% formamide, 0.1× SSC and 50% formamide for 15 min each, and 0.1× SSC (two times for 15 min each) at 55°C. Immunological detection of DIG-labeled hybrids was performed with anti-DIG-AP (anti-digoxigenin antibody from sheep conjugated with alkaline phosphatase; Roche, Mannheim, Germany) following standard protocols.

Double-labeling in situ hybridization–immunohistochemistry

Tissue sections processed for GAD67 in situ hybridization were extensively rinsed in 0.1× Tris/HCl, pH 7.5, three times for 10 min, followed by treatment with 10% normal goat serum (NGS) in Tris buffer (TB) for 30 min. Sections were incubated with the primary antibody (GABA<sub>B1a/b</sub>; 1:250) in the presence of 1% NGS and TB for 4 hr at room temperature and 4°C overnight. After three washes with TB, sections were exposed to the secondary biotinylated anti-rabbit antibody (1:250; Vector Laboratories, Burlingame, CA) for 2 hr at room temperature. Tissue-bound antibodies were detected with the indirect immunoperoxidase method by using avidin–biotin–peroxidase complex (ABC Elite kit; Vector Laboratories) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) (0.05% in TB, pH 7.4) as a chromogen and 0.01% H<sub>2</sub>O<sub>2</sub> as substrate. All regions and layers of the hippocampus were examined at high-power magnification to count cells labeled with the immunocytochemical staining and the in situ hybridization signal. The immunocytochemical staining and the in situ hybridization signal were distinguished on the basis of color (brown versus blue, respectively) and spatial distribution (somata and proximal dendrites versus somata, respectively).

**Immunocytochemistry**

**Antibodies.** Affinity-purified polyclonal antibodies were used: one was raised against GABA<sub>B1a/b</sub> protein (recognizing 1a and 1b splice variants of GABA<sub>B1a/b</sub> subunit) and two others against GABA<sub>B2</sub> protein. Antibody against GABA<sub>B1a/b</sub> was raised in rabbits (B17), and its characteristics and specificity have been described previously (Kulik et al., 2002). One antibody against GABA<sub>B2</sub> (B32) was raised in rabbits, and its specificity was described recently (Li et al., 2001), whereas another antibody (B2T1), used only for the covisualization of the GABA<sub>B1a/b</sub> and GABA<sub>B2</sub> subunits, was raised in guinea pigs (Kulik et al., 2002). The B32 and B2T1 antibodies were raised against different epitopes of the GABA<sub>B2</sub> protein. These antibodies, as well as another from Chemicon (Temecula, CA), gave a strikingly similar staining patterns in the hippocampus (Kulik et al., 2002), further confirming the specificity of these antibodies. To identify the GABAergic neuronal elements, we used a monoclonal antibody to glutamic acid decarboxylase (GAD65), the synthesizing enzyme of GABA (Chemicon).

**Immunocytochemistry for light microscopy.** Sections were incubated in 10% NGS diluted in 50 mM TB containing 0.9% NaCl [Tris-buffered saline (TBS)] with 0.2% Triton X-100 for 1 hr. Sections were then incubated for 24 hr with affinity-purified polyclonal antibodies anti-GABA<sub>B1a/b</sub> or anti-GABA<sub>B2</sub> at a final protein concentration of 1–2 μg/ml diluted in TBS containing 1% NGS. After several washes in TBS, the sections were incubated for 2 hr in biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:100 in TBS containing 1% NGS. Then, the sections were transferred to ABC (1:100) for 2 hr at room temperature. Bound peroxidase enzyme activity was revealed using DAB as a chromogen and 0.01% H<sub>2</sub>O<sub>2</sub> as substrate. Finally, the sections were air dried and coverslipped.

**Immunocytochemistry for electron microscopy.** Sections were first incubated in 20% NGS diluted in TBS for 1 hr and then incubated in a solution of primary antibody or in a mixture of two antibodies, diluted in TBS containing 3% NGS for 24 hr. When a single primary antibody was used, it was visualized by either the immunoperoxidase method or the silver-intensified immunogold reaction. When two primary antibodies were used, one of them was visualized by the immunoperoxidase method or the silver-intensified immunogold reaction. After primary antibody incubation, the sections were incubated at 4°C overnight in one secondary antibody or in a mixture of the following secondary antibodies: goat anti-rabbit (Fab fragment, diluted 1:100) coupled to 1.4 nm gold (Nanoprobe, Stony Brook, NY) made up in TBS containing 1% NGS and biotinylated goat anti-rabbit, and biotinylated goat anti-mouse antibodies (diluted 1:100; Vector Laboratories). After washes in TBS, sections were washed in double-distilled water, followed by silver enhancement of the gold particles with an HQ Silver kit (Nanoprobe) for 4–6 min. Subsequently, the sections were incubated in the ABC complex (Vector Laboratories) made up in TBS and then washed in TB. Peroxidase was visualized with DAB (0.05% in TB, pH 7.4) using 0.01% H<sub>2</sub>O<sub>2</sub> as substrate for 5–10 min. The sections were treated with 1%OsO<sub>4</sub> in PB for 40 min, washed in PB and double-distilled water, and then contrasted in 1% uranyl acetate for 40 min. They were dehydrated in a series of ethanol and propylene oxide and flat embedded in epoxy resin (Durcupan ACM; Sigma-Aldrich, Gillingham, UK). After polymerization, sections were cut at 70–80 nm thickness using an ultramicrotome (Reichert Ultracut E; Leica, Vienna, Austria). Ultrathin sections...
were analyzed in a Philips CM100 electron microscope. For double-immunoelectron microscopy, sections were first stained by the immunogold method followed by silver enhancement and then incubated with the ABC reagent followed by a peroxidase reaction.

Controls. To test method specificity of the procedures for light and electron microscopy, the primary antibody was omitted or replaced with 5% (v/v) normal serum of the species of the primary antibody. Under these conditions, no selective labeling was observed. For electron microscopy, some sections were incubated with both gold-labeled and biotinylated secondary antibodies, followed by the ABC complex and peroxidase reaction without silver intensification. This resulted in amorphous horseradish peroxidase (HRP) end product, and no metal particles were detected. Using the same sequence, but only silver intensification without HRP reaction, resulted in silver grains without HRP reaction, resulted in silver grains.

Quantification of GABA<sub>B1a/b</sub> immunoreactivity on pyramidal cells

Samples were taken from the stratum radiatum of CA1 and CA3 immunolabeled for either GABA<sub>B1a/b</sub> subunit (immunogold; for an analysis of pyramidal cell dendritic spines) or GABA<sub>B2</sub> subunit (immunogold) and GAD (immunoperoxidase; for an analysis of pyramidal cell dendritic shafts in contact with GABAergic terminals). Serial ultrathin sections were cut from the very surface (up to 3 μm in depth) of the samples of pyramidal cell spines (n = 124 spines in the GABA<sub>B1a/b</sub>-labeled material) and dendritic shafts (n = 10 shafts in the GABA<sub>B1a/b</sub>-labeled material). Spines and portions of dendritic shafts were three-dimensionally (3D) reconstructed by using a three-dimensional reconstruction imaging software (Ratoc, Tokyo, Japan) as described previously (Kulik et al., 2002). For each asymmetrical synapse made by a bouton on a pyramidal cell spine and for each symmetrical synapse made by a GAD-immunoreactive (GAD-IR) terminal on a pyramidal cell dendritic shaft, distances between immunoparticles (n = 685 in spines; n = 379 in dendritic shafts) and the closest edge of the synapses were measured along the surface of the 3D reconstructed images. Immunoparticles were allocated to 60-nm-wide bins, and the relative frequencies were calculated.

Results

Cellular distribution of immunoreactivity for GABA<sub>B</sub> receptor subunits

At the light microscopic level, the patterns of GABA<sub>B1a/b</sub> and GABA<sub>B2</sub> distribution strongly overlapped in the CA areas and dentate gyrus (Fig. 1A, B). In CA1, the immunostaining for both subunits was generally weak to moderate, with the stratum radiatum showing the weakest and the stratum lacunosum-moleculare showing the highest immunoreactivity. The CA3 region exhibited higher GABA<sub>B1a/b</sub> and GABA<sub>B2</sub> immunoreactivities than the CA1 region. The stratum lacunosum-moleculare of CA3 showed the strongest immunoreactivity for both receptor subunits within the hippocampus. In the stratum radiatum of CA3, the intensity of immunoreactivity for both proteins was stronger in the proximal two-thirds than in the distal one-third of the layer. The stratum lucidum showed weak immunostaining throughout. In the dentate gyrus, the immunolabeling was weak in the hilus and moderate in the molecular layer, showing a gradual increase toward the hippocampal fissure.

We also observed immunoreactivity for GABA<sub>B1a/b</sub> in somata of CA1 pyramidal cells and intense staining in somata of some nonpyramidal cells scattered throughout the hippocampus (Fig. 1A, C). However, these neurons did not show a similar strong somatic labeling for the GABA<sub>B2</sub> protein. To confirm the identity of these nonpyramidal cells, we performed immunoperoxidase labeling for GABA<sub>B1a/b</sub> in combination with in situ hybridization for GAD67 mRNA (Fig. 1D). This approach revealed that virtually all of these cells contained GAD67 mRNA (Fig. 1E). In contrast, we observed many GAD67 mRNA-expressing cells that were immunonegative for GABA<sub>B1a/b</sub> (Fig. 1E). Of 553 cells labeled for GAD67 mRNA in the hippocampus, 266 cells were found to be double labeled. These results indicate that nonpyramidal cells with strong somatic GABA<sub>B1a/b</sub> staining represent a subpopulation of hippocampal GABAergic interneurons. Electron microscopic investigation further revealed that the somatic immunoreactivity for the GABA<sub>B1a/b</sub> subunit, detected in CA1 pyramidal cells and GABAergic neurons at the light microscopic...
Subcellular localization of GABA_B receptor subunits

At the electron microscopic level, immunostaining for GABA_B1a/b and GABA_B2 proteins was found primarily in the dendritic layers of the hippocampus and showed similar distribution patterns. Immunoreactivity was localized to the plasma membrane of presynaptic terminals and, to a larger extent, to that of postsynaptic elements. In the somatic layers, no immunoparticles for GABA_B receptor subunits could be detected along the plasma membrane.

Strata oriens and radiatum of CA areas

In presynaptic elements, labeling for GABA_B1a/b and GABA_B2 was found in putative pyramidal cell axon terminals establishing asymmetrical synapses with dendritic spines (Figs. 2A, C–F, 3A,C,D). Immunogold particles were localized to either the extrasynaptic plasma membrane of boutons (Figs. 2D, Fd, 3C,D) or the presynaptic membrane specialization (Figs. 2C,E,Fa–Fc, 3C,D). Furthermore, GAD-IR boutons were also found immunoreactive for GABA_B receptor subunits: immunoparticles were located on the presynaptic membrane specialization and on the extrasynaptic axonal membranes (Figs. 2I, 3G,H).

Postsynaptically, peroxidase reaction end product for both subunits was found in dendritic spines (Figs. 2A, B, 3A,B) and dendritic shafts of pyramidal cells and interneurons (Figs. 2G, 3E). Immunoparticles for GABA_B1a/b and GABA_B2 were most abundant at the extrasynaptic plasma membrane of pyramidal cell spines (Figs. 2C–E, 3C,D,F). In spines, immunogold particles also appeared at the edge of asymmetrical synapses (Figs. 2D, 3C,D) and occasionally over the postsynaptic specializations (Figs. 2C, 3C). The labeling in the postsynaptic density, however, may be underestimated because of the limited penetration of immunoreagents in the preembedding method (Kulik et al., 2002). Immunoparticles were also found extrasynaptically and perisynaptically in dendritic shafts of pyramidal cells (Figs. 2I, 3F–H) and interneurons (Figs. 2H,IK, 3F,I). To further examine the spatial relationship between the subunits and putative glutamatergic synapses on spines and GABAergic synapses on pyramidal cell dendritic shafts, quantitative analyses were performed (Fig. 4). Dendritic spines (n = 124) and dendritic shafts (n = 10) immunoreactive for GABA_B1a/b were 3D reconstructed from serial ultrathin sections, and the distances of the immunoparticles (n = 685 on spines; n = 379 on dendritic shafts) from the closest edge of the synapses were measured along the surface of three-dimensional images. In spines, the distribution of immunoparticles for GABA_B1a/b, at the plasma membrane showed a peak between 60 and 240 nm from the edge of the synapse (Fig. 4, filled bars); 46% of the particles were found within this area. In dendritic shafts, however, immunoparticles for the GABA_B1a/b subunit showed an even distribution (Fig. 4, open bars). Hence, these results indicate that, in pyramidal cells, postsynaptic GABA_B1a/b subunits are enriched around putative glutamatergic but not around GABAergic synapses.

Stratum lacunosum-moleculare of CA areas

Strong immunoreactivity for both receptor subunits was observed in spines and dendritic shafts. Immunoparticles were mainly localized to the extrasynaptic membrane of postsynaptic elements (Fig. 5) and occasionally were found at the edge of asymmetrical and symmetrical synapses (Fig. 5A–C). Presynaptic labeling was less frequent compared with postsynaptic labeling and could be detected on the presynaptic membrane specialization of axon terminals establishing either asymmetrical (Fig. 5A) or symmetrical synapses (Fig. 5C).

Stratum lucidum of CA3

In correlation with the faint staining detected at the light microscopic level, weak immunoreactivity for both subunits was seen on postsynaptic and presynaptic elements in the stratum lucidum. Peroxidase reaction end product for GABA_B receptor sub-
units was predominantly present in pyramidal cell spines postsynaptic to mossy fiber terminals and occasionally also in dendritic shafts (Fig. 6A, D). The majority of immunogold particles were found on the extrasynaptic plasma membrane of pyramidal cell spines and dendritic shafts (Fig. 6C,E). They were also present at the edge of asymmetrical synapses between mossy fiber terminals and pyramidal cell spines (Fig. 6E). Occasionally, immunoparticles were localized to the active zone of the presynaptic plasma membrane in mossy fiber boutons (Fig. 6C,E).

Dentate gyrus

In the dentate gyrus, immunostaining for the two subunits was primarily found in the molecular layer. Postsynaptic neuronal elements establishing asymmetrical synapses with presynaptic terminals were predominantly immunoreactive for GABA\textsubscript{B1a/b} and GABA\textsubscript{B2} proteins. Peroxidase reaction product was observed in spines and in dendritic shafts of granule cells (Fig. 6F,G). Similarly, immunogold particles for both receptor subunits were localized to the extrasynaptic plasma membrane of dendritic spines and dendrites (Fig. 6H,J). Weak immunostaining for GABA\textsubscript{B} receptor subunits was found on the extrasynaptic membrane of presynaptic terminals.

Hilar region

Similar to other hippocampal regions, immunoreactivity for the two subunits was predominantly localized to postsynaptic elements in the hilus. Immunogold particles were found on dendritic shafts and spines of putative mossy cells. Immunostaining was also observed on GAD-IR dendritic shafts of presumed GABAergic interneurons. Presynaptically, weak immunoreactivity for both subunits was detected on mossy fiber terminals as well as on GAD-IR axon varicosities.

Colocalization of the two GABA\textsubscript{B} receptor subunits

The similar staining pattern detected at the light microscopic level and the parallel distribution observed by electron microscopy suggest that GABA\textsubscript{B1a/b} and GABA\textsubscript{B2} subunits are colocalized in the same subcellular compartments. To confirm this directly, double-labeling immunocytochemistry was performed. We found extensive colocalization of immunoreactivity for GABA\textsubscript{B1a/b} and GABA\textsubscript{B2} subunits in the same presynaptic and postsynaptic profiles in the hippocampus (Fig. 7).

Discussion

This study provides a detailed description of the precise cellular and subcellular localization of the two GABA\textsubscript{B} receptor subunits,
GABAB1a/b and GABA B2, in the adult rat hippocampus. Using light microscopy, we show that GABAB1a/b protein is widely distributed in principal cells and nonpyramidal neurons throughout the hippocampal formation. Immunoelectron microscopy revealed weak presynaptic and abundant postsynaptic labeling. Presynaptic subunits are mainly localized on the extrasynaptic plasma membrane of excitatory terminals and, to a lesser extent, on inhibitory terminals. Postsynaptic GABAB receptors are enriched in dendritic spines around putative glutamatergic synapses, whereas they show an even distribution on dendritic shafts of pyramidal cells contacted by GABAergic boutons.

**GABA B2 receptor subunits are in principal cells and GABAergic neurons**

At the light microscopic level, GABA B2 is widely distributed in the hippocampus showing a strong overlap. The immunoreactivity for both subunits was mainly observed in the dendritic layers, showing the most intense labeling in the stratum lacunosum-moleculare of CA3. This distribution pattern is consistent with the results of autoradiographic (Chu et al., 1990; Turgeon and Albin, 1994), in situ hybridization (Kaufmann et al., 1998a; Bischoff et al., 1999; Durkin et al., 1999; Clark et al., 2000; Liang et al., 2000), and light microscopic immunocytochemical studies (Fritschy et al., 1999; Margera-Mitrovic et al., 1999). The similarity in the pattern of immunostaining for the two proteins, observed in light microscope, suggested their colocalization. Indeed, immunoelectron microscopy revealed the presence of the two subunits in the same subcellular compartment. Thus, GABA B2 is localized to the extrasynaptic plasma membrane of spines and dendritic shafts and, occasionally, at the edge of asymmetrical synapses. Immunostaining for GABA B2 was observed on spines and dendritic shafts of presumptive granule cells. Scale bars, 0.2 μm.

**Figure 5.** Electron micrographs showing immunoreactivity for GABA B1a/b and GABA B2 subunits in the stratum lacunosum-moleculare of CA1 (A–D) and CA3 (E–G). A–C, Immunogold particles for GABA B1a/b were localized to the presynaptic membrane specialization of boutons (b, double arrows) establishing asymmetrical synapses with spines (s) (A) or symmetrical synapses (C) with dendritic shafts (Den). Postsynaptically, silver-enhanced gold grains were mainly found at the extrasynaptic plasma membrane of dendritic shafts (Den) and dendritic spines (s) of presumed pyramidal cells (arrows) and occasionally at the edge of asymmetrical synapses (arrowheads). D–G, Strong immunoreactivity for GABA B2 (arrows) was seen along the extrasynaptic plasma membrane of dendritic shafts (Den) and spines (s) of presumed pyramidal cells. Scale bars, 0.2 μm.

**Figure 6.** Electron micrographs showing immunoreactivity for GABA B1a/b and GABA B2 subunits in the stratum lucidum of CA3 and in the dentate molecular layer. A, In the stratum lucidum of CA3, peroxidase reaction end product for GABA B1a/b protein was observed in dendritic spines (s) of pyramidal cells postsynaptic to mossy fiber terminals (MT). B, C, Serial ultrathin sections showing immunogold particles for GABA B1a/b at the extrasynaptic membrane of spines (s) and dendritic shafts (Den) and occasionally at the presynaptic membrane specialization of mossy fiber terminals (MT, double arrows). D, Immunostaining for GABA B1a/b was detected in dendritic shafts of a presumed granule cell (Den). E, Immunoparticles for GABA B2 were localized to the extrasynaptic plasma membrane of spines (s) and dendritic shafts (Den, arrows) and occasionally to the presynaptic membrane specialization of a mossy fiber terminal (MT, double arrows). F, G, In the dentate molecular layer, peroxidase staining for the GABA B1a/b subunit was weak in spines (s) and in a dendritic shaft of a presumed granule cell (Den). H, Immunogold particles for GABA B1a/b were localized on the extrasynaptic membrane of spines (s) and dendritic shafts (Den) and occasionally to the presynaptic membrane specialization of mossy fiber terminals (MT, double arrows). I, J, Immunostaining for GABA B2 was observed on spines (s) and dendritic shafts (Den) of presumed granule cells. J, Immunoparticles for GABA B2 were localized to extrasynaptic membranes (arrows). Scale bars, 0.2 μm.
but not for GABA<sub>B2</sub>. The strong somatic labeling for GABA<sub>B1a/b</sub> in CA1 pyramidal cells and in a subset of GABAergic interneurons has been described previously at the light microscopic level in the adult (Fritschy et al., 1999; Sloviter et al., 1999) and in the developing hippocampus (López-Bendito et al., 2003). The fact that the GABA<sub>B2</sub> subunit was not detected in the somata of these cells suggests that these receptors are nonfunctional. The GABA<sub>B2</sub> subunit has been shown to be essential for the translocation of GABA<sub>B1</sub> protein to the plasma membrane and for the formation of functional receptors (Jones et al., 1998; Kaupmann et al., 1998a; White et al., 1998; Pagano et al., 2001). Indeed, immunoelectron microscopy revealed that this strong somatic labeling is attributable to the abundance of the subunit in the endoplasmic reticulum. Thus, we can hypothesize that a lower level of synthesis of GABA<sub>B2</sub> subunits is responsible for the accumulation of GABA<sub>B1a/b</sub> protein. Consistently, in CA3, in which the level of GABA<sub>B2</sub> subunit mRNA is higher (Durkin et al., 1999), we did not observe an accumulation of GABA<sub>B1a/b</sub> in the somata of pyramidal cells but found a stronger staining for both subunits in all dendritic layers, indicating more functional receptors on the dendritic surface of these cells.

Presynaptic GABA<sub>B</sub> receptor subunits are on inhibitory and excitatory terminals

At the ultrastructural level, presynaptic GABA<sub>B</sub> receptor subunits are found to be localized to GABAergic and putative glutamatergic axon terminals. The subunits are detected at the extrasynaptic membrane but also at the presynaptic membrane specialization. The presence of GABA<sub>B</sub> receptor subunits on inhibitory terminals is consistent with their role as autoreceptors regulating GABA release (Davies et al., 1991; Mott and Lewis, 1991; Lambert and Wilson, 1993; Poncer et al., 2000). Interestingly, axon terminals establishing asymmetrical, putative glutamatergic synapses showed stronger immunoreactivity for both receptor subunits than GABAergic boutons. It has been suggested that GABA<sub>B</sub> receptors at glutamatergic terminals are likely to serve as heteroreceptors regulating glutamate release. The GABA<sub>B</sub> receptor agonist baclofen depresses excitatory neurotransmission at several hippocampal synapses (Dutar and Nicoll, 1988b; Hirata et al., 1992; Wu and Saggau, 1995; Wang and Lambert, 2000; Lei and McBain, 2003). There is also evidence that synaptically released GABA can inhibit excitatory neurotransmission at the Schaffer collateral synapses in CA1 and at the mossy fiber synapses in CA3 (Isaacson et al., 1993; Vogt and Nicoll, 1999). Furthermore, recent data indicate that presynaptic GABA<sub>B</sub> receptors localized to excitatory terminals impinging on CA1 pyramidal cells and interneurons are tonically activated by ambient levels of GABA (Jensen et al., 2003). GABA<sub>B</sub> receptor-mediated presynaptic depression at both inhibitory and excitatory synapses is primarily attributable to the suppression of high voltage-activated Ca<sup>2+</sup> channels (Misgeld et al., 1995; Wu and Saggau, 1995; Poncer et al., 2000; Lei and McBain, 2003).

Dendritic localization of GABA<sub>B</sub> receptor subunits

The majority of postsynaptic subunits were localized to the extrasynaptic membrane of dendritic spines and shafts of principal cells and interneurons. Postsynaptic GABA<sub>B</sub> receptors activating inwardly rectifying K<sup>+</sup> channels (Gähwiler and Brown, 1985; Sodickson and Bean, 1996; Lüscher et al., 1997) are responsible for the slow IPSP. Slow inhibitory responses have been observed in both principal cells (Newberry and Nicoll, 1985; Dutar and Nicoll, 1988a; Otis et al., 1993) and inhibitory interneurons (La-caille, 1991; Khazipov et al., 1995). The prevalent localization of GABA<sub>B</sub> receptor subunits in dendrites, as revealed here, is in good agreement with electrophysiological observations showing that the extrasynaptic stimulation or iontophoretic application of GABA or baclofen elicits GABA<sub>B</sub> receptor-mediated responses on the dendrites but not on the somata of CA1 pyramidal cells (Newberry and Nicoll, 1985, 1988).

The most intense labeling for GABA<sub>B</sub> receptor subunits was found in pyramidal cell spines in which they showed an association with putative glutamatergic synapses. The functional relevance of this intimate spatial relationship is unclear. Interestingly, this distribution in spines corresponds well to that of the metabotropic glutamate receptor subtype mGluR5 (Luján et al., 1997), raising the possibility of an interaction between these two proteins and/or sharing some of their effectors, resulting in a concerted control of glutamatergic transmission.

In dendritic shafts, GABA<sub>B1a/b</sub> is localized to the extrasynaptic membrane with no apparent association with inhibitory synapses. The activation of these extrasynaptic receptors requires the spillover of GABA from inhibitory synapses (Scanziani, 2000). Consistent with this hypothesis, GABA<sub>B</sub> receptor-mediated slow IPSPs are readily elicited by extracellular stimulation (Newberry and Nicoll, 1985; Dutar and Nicoll, 1988a; Otis et al., 1993) but were not detected between synaptically coupled interneuron–principal cell pairs in the hippocampus (Miles, 1990; Buhl et al., 1994; Vida et al., 1998; Bartos et al., 2001). In the presence of uptake blockers, however, a single interneuron can elicit slow IPSCs in pyramidal cells (Scanziani, 2000) indicating that, under physiological conditions, efficient uptake mechanisms control the activation of postsynaptic GABA<sub>B</sub> receptors. Elimination of GABA transporter-1, responsible for the uptake of GABA, however, does not result in activation of postsynaptic GABA<sub>B</sub> receptors, although presynaptic receptors are tonically active at ambient levels in wild-type animals (Jensen et al., 2003). Thus, an additional aspect that may be responsible for the high threshold of postsynaptic response is the lower affinity of postsynaptic receptors to GABA (Yoon and Rothman, 1991; Pozza et al., 1999; Jensen et al., 2003).

Functional implications

There are two major aspects emerging from our results on the distribution of GABA<sub>B</sub> receptor subunits (Fig. 8). First, an asso-
activation of the subunits with putative glutamatergic synapses was observed at both presynaptic and postsynaptic sides. Conceivably, this reflects the role of GABAB receptors in the modulation of glutamatergic synaptic transmission. Although there is ample evidence for the regulation of glutamate release by presynaptic GABAB receptors (Dutar and Nicoll, 1988b; Isaacson et al., 1993; Vogt and Nicoll, 1999; Wang and Lambert, 2000; Jensen et al., 2003), possible mechanisms of modulation on the postsynaptic side are not yet identified. Because several types of GABAergic interneurons show an axon coalignment with excitatory pathways (Han et al., 1993; Buhl et al., 1994; Vida et al., 1998; Vida and Frotscher, 2000), the observed distribution of GABAB receptor subunits could provide the molecular basis for the selective GABAergic control of synaptic transmission at glutamatergic inputs.

Second, metabotropic GABA receptor subunits are localized extrasynaptically, showing no association with GABAergic synapses (Fig. 8). Similar observations were made in the cortex (Gonchar et al., 2001; López-Bendito et al., 2002), the cerebellum, and ventrobasal thalamus (Ige et al., 2000; Kulik et al., 2002). Metabotropic glutamate receptors are also localized extrasynaptically but, with the exception of mGluR2/3, show enrichment around glutamatergic synapses (Baudet et al., 1993; Luján et al., 1996, 1997; Shigemoto et al., 1996, 1997). Thus, the activation of mGlRs appears to be tightly coupled to the synaptic release of glutamate, ensuring the spatially and temporally precise transmission of information. Activation of GABAB receptors, in contrast, is dependent on transients of the ambient GABA and may serve to detect enhanced and/or simultaneous activity of GABAergic interneurons. Such a mechanism may play a role in population oscillations, when many interneurons fire synchronously (Bragin et al., 1995; Ylinen et al., 1995), or during epileptic activity, in which it could serve as an emergency brake.

References


Figure 8. Presumed mechanism of GABAB receptor activation in the adult hippocampus. Metabotropic GABAB receptors (striped boxes), localized to the extrasynaptic plasma membrane of GABAergic and glutamatergic terminals (b), spines (s), and dendritic shafts (d), are activated by spilled-over GABA (dots), whereas the synaptic ionotropic GABAA receptors (gray boxes) are directly exposed to the neurotransmitter. Glut, Glutamate; GluRs, glutamate receptors.


