Cellular/Molecular

Subcellular Localization of Metabotropic $GABA_B$ Receptor Subunits $GABA_{B1a/b}$ and $GABA_{B2}$ in the Rat Hippocampus

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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 on 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 spillover 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 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).

In the hippocampus, electrophysiological and pharmacological studies have provided a detailed characterization of presynaptic and postsynaptic inhibitory actions of $GABA_B$ receptors (Dutar and Nicoll, 1988a,b; Mody et al., 1994; Misgeld et al., 1995; 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, 1988b; Olpe et al., 1993; Otis et al., 1993). Activation of both presynaptic and postsynaptic receptors is assumed to be dependent on the spilled-over GABA from inhibitory synapses (Isaacson et al., 1993; Vogt and Nicoll, 1999; Scanziani, 2000).

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

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of $GABA_B$ 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; Fritschy et al., 1999; Lu et al., 1999; Margeta-Mitrovic et al., 1999). Here we determine the precise cellular and subcellular localization of the two $GABA_B$ receptor subunits by using preembedding immunoelectron microscopy in combination with quantification of $GABA_{B1a/b}$ 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 microscopic immunohistochemistry; and (3) 12 rats were perfused with a solution containing 0.05% 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 *Eco*RI site of *pBluescript S*$^-$ vector (Stratagene, La Jolla, CA). The plasmids were linearized by restriction digest with *Sal*I 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 mM NaH_2PO_4, 250 mg/ml heat-denatured salmon sperm DNA, 100 mg/ml tRNA, 5% dextransulfate, 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 M 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 ($GABA_{B1a/b}$, 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 terahydrochloride (DAB) (0.05% in TB, pH 7.4) as a chromogen and 0.01% H_2O_2 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 $GABA_{B1a/b}$ protein (recognizing 1a and 1b splice variants of $GABA_{B1}$ subunit) and two others against $GABA_{B2}$ protein. Antibody against $GABA_{B1a/b}$ was raised in rabbits (B17), and its characteristics and specificity have been described previously (Kulik et al., 2002). One antibody against $GABA_{B2}$ (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 $GABA_{B1a/b}$ and $GABA_{B2}$ subunits, was raised in guinea pigs (Kulik et al., 2002). The B32 and B2T1 antibodies were raised against different epitopes of the $GABA_{B2}$ 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-$GABA_{B1a/b}$ or anti-$GABA_{B2}$ 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_2O_2 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 reaction and the second one by 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 (Nanoprobes, 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 (Nanoprobes) 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_2O_2 as substrate for 5–10 min. The sections were treated with 1% OsO_4 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-immunoelecron 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 peroxisade (HRP) end product, and no metal particles were detected. Using the same sequence, but only silver intensification without HRP reaction, resulted in silver grains but no amorphous HRP end product. Under these conditions, only infrequent small patches of HRP end product were detected, and the patches were not associated selectively with any particular cellular profile.

Quantification of $GABA_{B1a/b}$ immunoreactivity on pyramidal cells

Samples were taken from the stratum radiatum of CA1 and CA3 immunolabeled for either $GABA_{B1a/b}$ subunit (immunogold; for an analysis of pyramidal cell dendritic spines) or $GABA_{B1a/b}$ 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 $GABA_{B1a/b}$-labeled material) and dendritic shafts ($n = 10$ shafts in the $GABA_{B1a/b}$–GAD-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.

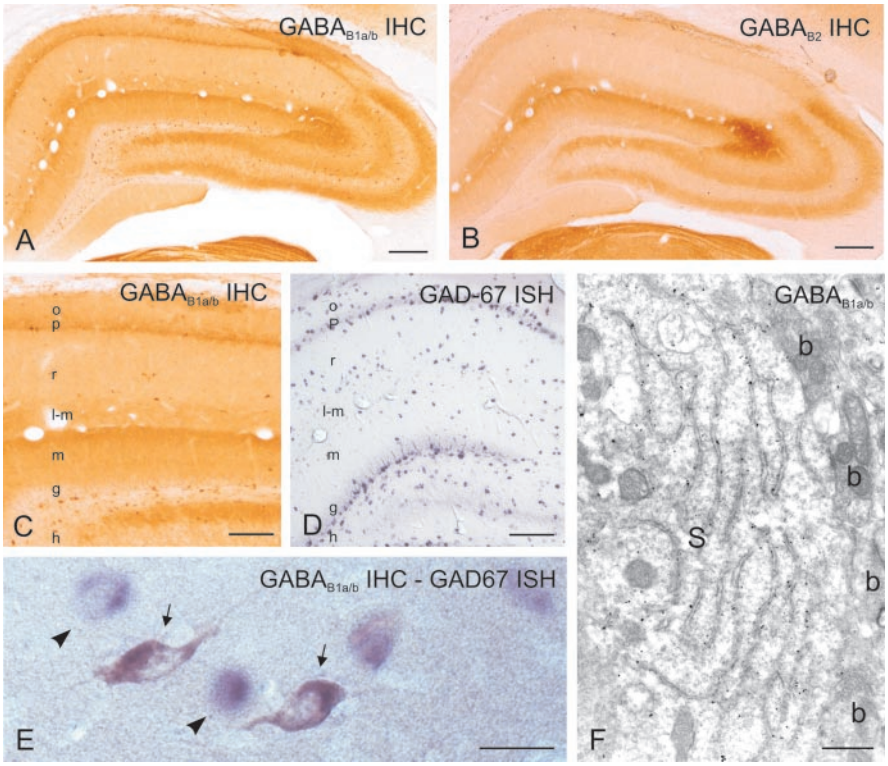


Figure 1. Distribution of immunoreactivity for $GABA_B$ receptor subunits in the hippocampus. *A*, *B*, The patterns of $GABA_{B1a/b}$ (*A*) and $GABA_{B2}$ (*B*) distribution strongly overlap. *C*, Strong immunoreactivity for $GABA_{B1a/b}$, but not for $GABA_{B2}$, was detected in the somata of CA1 pyramidal cells and nonpyramidal neurons scattered throughout the hippocampus. *D*, Detection of GAD67 mRNA by *in situ* hybridization (ISH) to localize GABAergic neurons. *E*, Colocalization of $GABA_{B1a/b}$ subunit and GAD67 mRNA in nonpyramidal cells as assessed by immunohistochemistry for $GABA_{B1a/b}$ (IHC) in combination with *in situ* hybridization for GAD67 mRNA. A subset of GABAergic cells show immunoreactivity for $GABA_{B1a/b}$ in their somata (arrows), whereas other cells were only stained for GAD67 mRNA (arrowheads). *F*, Electron micrograph of an interneuron soma (S) showing the localization of $GABA_{B1a/b}$ protein at the endoplasmic reticulum. Note that neither somatic plasma membrane nor boutons (b) in contact with the cell body are immunoreactive for $GABA_{B1a/b}$. o, Stratum oriens; p, stratum pyramidale; r, stratum radiatum; l-m, stratum lacunosum-moleculare; m, stratum moleculare; g, stratum granulosum; h, hilus. Scale bars: *A–D*, 200 μm; *E*, 50 μm; *F*, 0.5 μm.

Results

Cellular distribution of immunoreactivity for $GABA_B$ receptor subunits

At the light microscopic level, the patterns of $GABA_{B1a/b}$ and $GABA_{B2}$ distribution strongly overlapped in the CA areas and dentate gyrus (Fig. 1*A*,*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 $GABA_{B1a/b}$ and $GABA_{B2}$ 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 $GABA_{B1a/b}$ in somata of CA1 pyramidal cells and intense staining in somata of some nonpyramidal cells scattered throughout the hippocampus (Fig. 1*A*,*C*). However, these neurons did not show a similar strong somatic labeling for the $GABA_{B2}$ protein. To confirm the identity of these nonpyramidal cells, we performed immunoperoxidase labeling for $GABA_{B1a/b}$ in combination with *in situ* hybridization for GAD67 mRNA (Fig. 1*D*). This approach revealed that virtually all of these cells contained GAD67 mRNA (Fig. 1*E*). In contrast, we observed many GAD67 mRNA-expressing cells that were immunonegative for $GABA_{B1a/b}$ (Fig. 1*E*). 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 $GABA_{B1a/b}$ staining represent a subpopulation of hippocampal GABAergic interneurons. Electron microscopic investigation further revealed that the somatic immunoreactivity for the $GABA_{B1a/b}$ subunit, detected in CA1 pyramidal cells and GABAergic neurons at the light microscopic

level, could be ascribed to the abundance of the receptor in the endoplasmic reticulum (Fig. 1*F*).

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. 2*A*,*C–F*, 3*A*,*C*,*D*). Immunogold particles were localized to either the extrasynaptic plasma membrane of boutons (Figs. 2*D*,*Fd*, 3*C*,*D*) or the presynaptic membrane specialization (Figs. 2*C*,*E*,*Fa–Fc*, 3*C*,*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. 2*I*, 3*G*,*H*).

Postsynaptically, peroxidase reaction end product for both subunits was found in dendritic spines (Figs. 2*A*,*B*, 3*A*,*B*) and dendritic shafts of pyramidal cells and interneurons (Figs. 2*G*, 3*E*). Immunoparticles for $GABA_{B1a/b}$ and $GABA_{B2}$ were most abundant at the extrasynaptic plasma membrane of pyramidal cell spines (Figs. 2*C–E*,*I*, 3*C*,*D*,*F*). In spines, immunogold particles also appeared at the edge of asymmetrical synapses (Figs. 2*D*, 3*C*,*D*) and occasionally over the postsynaptic specializations (Figs. 2*C*, 3*C*). 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. 2*I*, 3*F–H*) and interneurons (Figs. 2*H*,*J*,*K*, 3*F*,*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. 5*A–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. 5*A*) or symmetrical synapses (Fig. 5*C*).

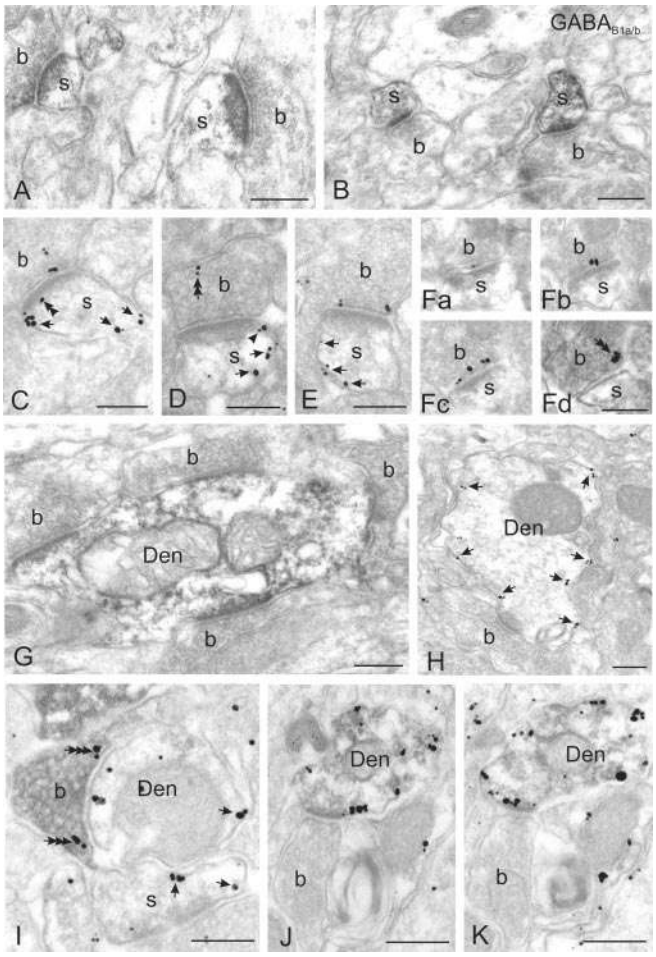


Figure 2. Electron micrographs showing immunoreactivity for the $GABA_{B1a/b}$ subunit in the strata oriens and radiatum of CA1 and CA3 areas. *A*, Peroxidase labeling was weak in presumed excitatory boutons (b) establishing asymmetrical synapses with pyramidal cell spines (s). *B*, Postsynaptically, peroxidase reaction end product for $GABA_{B1a/b}$ was accumulated in pyramidal cell spines (s) postsynaptic to presumed excitatory boutons (b). *C–E*, In the terminals (b), immunogold particles were localized to the extrasynaptic plasma membrane (double arrows) and to the presynaptic membrane specialization. *Fa–Fd*, Consecutive electron micrographs of a presumed excitatory terminal showing extrasynaptic (double arrow in *Fd*) and synaptic localization of $GABA_{B1a/b}$. *C–E*, In dendritic spines (s), immunoparticles were mainly found along the extrasynaptic plasma membrane (arrows) and occasionally over the postsynaptic specialization (double arrowhead in *C*) and at the edge of asymmetrical synapses (arrowhead in *D*). *G*, *H*, Immunolabeling was also visible in the dendritic shafts of presumed interneurons (Den) establishing asymmetrical synapses with presynaptic boutons (b). Peroxidase reaction product filled the dendrite, whereas immunoparticles were organized in clusters along the extrasynaptic plasma membrane (arrows in *H*). *I*, An axon terminal (b), immunoreactive for GAD (peroxidase reaction end product) and $GABA_{B1a/b}$ (immunoparticles; triple arrows) establishing a symmetrical synapse with a dendritic shaft of a presumed pyramidal cell (Den). Note that the dendritic shaft and the neighboring spine are also immunoreactive for $GABA_{B1a/b}$ (immunoparticles; arrows). *J*, *K*, Consecutive electron micrographs showing immunoreactivity for the $GABA_{B1a/b}$ subunit (immunoparticles) in the dendritic shaft of a GAD-immunoreactive interneuron (Den). Immunogold particles were localized to the extrasynaptic plasma membrane of the dendritic shaft. Scale bars, 0.2 μm.

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-

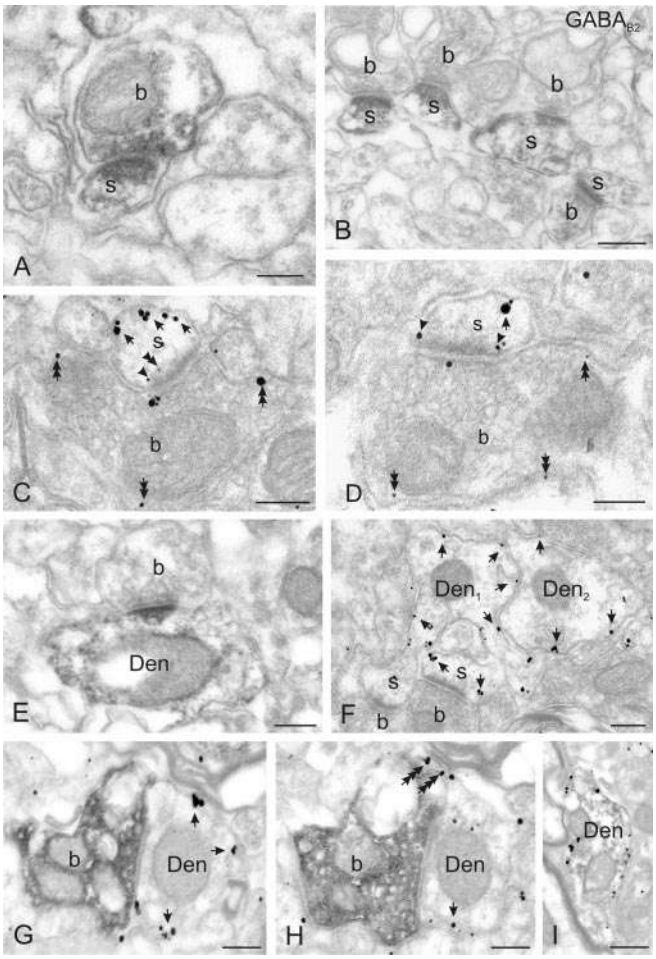


Figure 3. Electron micrographs showing immunoreactivity for the $GABA_{B2}$ subunit in the strata oriens and radiatum of CA1 and CA3 areas. *A*, Presynaptically, peroxidase reaction end product was seen in a presumed excitatory bouton (b) making asymmetrical synaptic contact with a pyramidal cell spine (s). *B*, Postsynaptically, peroxidase labeling was found in pyramidal cell spines (s) establishing asymmetrical synapses with presumed excitatory boutons (b). *C*, *D*, Immunogold particles for $GABA_{B2}$ were observed on presynaptic and postsynaptic elements: in presynaptic boutons (b), they were localized to the extrasynaptic plasma membrane (double arrows) and to the presynaptic membrane specialization, whereas postsynaptically, immunoparticles were mainly found at the extrasynaptic plasma membrane of pyramidal cell spines (arrows) and occasionally perisynaptically (arrowheads) and over the asymmetrical synaptic specialization (double arrowhead in *C*). *E*, Immunostaining (peroxidase reaction end product) in a dendritic shaft of a presumed interneuron establishing asymmetrical synapse with a bouton (b). *F*, Immunogold particles were localized to the extrasynaptic plasma membrane of dendritic shafts of presumed pyramidal cells (Den_1) and interneurons (Den_2) (arrows). Den_2 was identified as belonging to an interneuron because of the lack of spines and the presence of asymmetric shaft synapses in serial sections. *G*, *H*, Consecutive electron micrographs showing a GAD-immunoreactive (peroxidase labeled) bouton (b) that was also immunostained for $GABA_{B2}$ (immunoparticles; triple arrows). This bouton establishes a symmetrical synapse with a presumed pyramidal cell dendritic shaft (Den) also showing immunoreactivity for $GABA_{B2}$ protein (arrows). *I*, Immunoreactivity for the $GABA_{B2}$ subunit (immunoparticles) in a GAD-immunoreactive (peroxidase labeled) dendritic shaft. Note that particles were organized in clusters along the extrasynaptic plasma membrane. Scale bars, 0.2 μm.

units was predominantly present in pyramidal cell spines postsynaptic to mossy fiber terminals and occasionally also in dendritic shafts (Fig. 6*A*,*D*). The majority of immunogold particles were found on the extrasynaptic plasma membrane of pyramidal cell spines and dendritic shafts (Fig. 6*B*,*C*,*E*). They were also present at the edge of asymmetrical synapses between mossy fiber terminals and pyramidal cell spines (Fig. 6*E*). Occasionally, immunoparticles were localized to the active zone of the presynaptic plasma membrane in mossy fiber boutons (Fig. 6*C*,*E*).

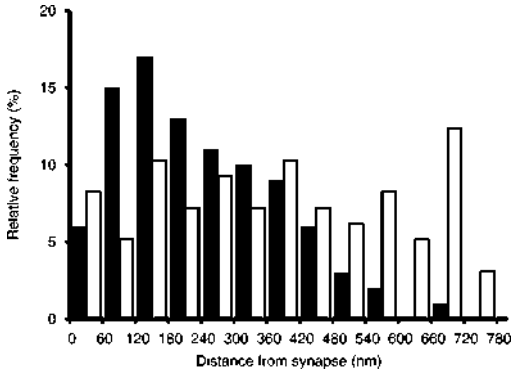


Figure 4. Graph showing the distribution of immunoparticles for the $GABA_{B1a/b}$ subunit on pyramidal cell spines relative to asymmetrical, putative glutamatergic synapses (filled columns) and on dendritic shafts of pyramidal cells relative to GAD-positive synapses (open columns) as assessed by preembedding immunogold labeling. Immunoparticles ($n = 685$ for asymmetrical synapses and $n = 379$ for GAD-positive symmetrical synapses) were collected from serial sections of spines ($n = 124$) and dendritic shafts of pyramidal cells ($n = 10$), and the distances between immunoparticles and the closest edge of synapses were measured. Immunoparticles were allocated to 60-nm-wide bins, and their relative frequency was calculated in each bin (Luján et al., 1996). The measurements revealed an enrichment of the $GABA_{B1a/b}$ subunit around putative glutamatergic synapses on spines and an even distribution on dendrites contacted by GAD-immunoreactive boutons.

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 $GABA_{B1a/b}$ and $GABA_{B2}$ proteins. Peroxidase reaction product was observed in spines and also in dendritic shafts of granule cells (Fig. 6*F*,*G*,*I*). Similarly, immunogold particles for both receptor subunits were localized to the extrasynaptic plasma membrane of dendritic spines and dendrites (Fig. 6*H*,*J*). Weak immunostaining for $GABA_B$ 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 $GABA_B$ receptor subunits

The similar staining pattern detected at the light microscopic level and the parallel distribution observed by electron microscopy suggest that $GABA_{B1a/b}$ and $GABA_{B2}$ subunits are colocalized in the same subcellular compartments. To confirm this directly, double-labeling immunocytochemistry was performed. We found extensive colocalization of immunoreactivity for $GABA_{B1a/b}$ and $GABA_{B2}$ 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 $GABA_B$ receptor subunits,

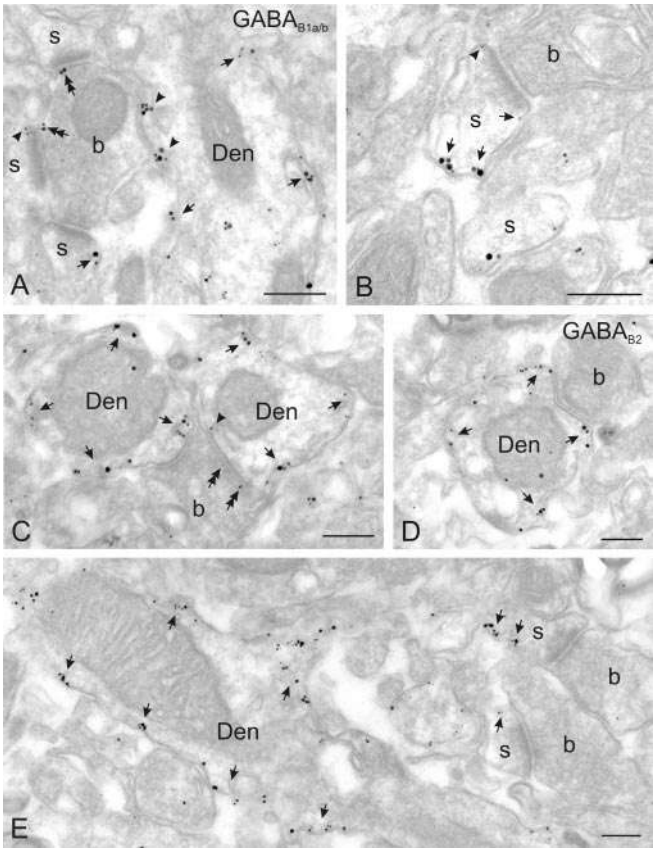


Figure 5. Electron micrographs showing immunoreactivity for GABA$_{B1a/b}$ and GABA$_{B2}$ subunits in the stratum lacunosum-moleculare of CA1 (*A*, *B*) and CA3 (*C–E*). *A–C*, Immunogold particles for GABA$_{B1a/b}$ were localized to the presynaptic membrane specialization of boutons (b, double arrows) 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*, *E*, 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.

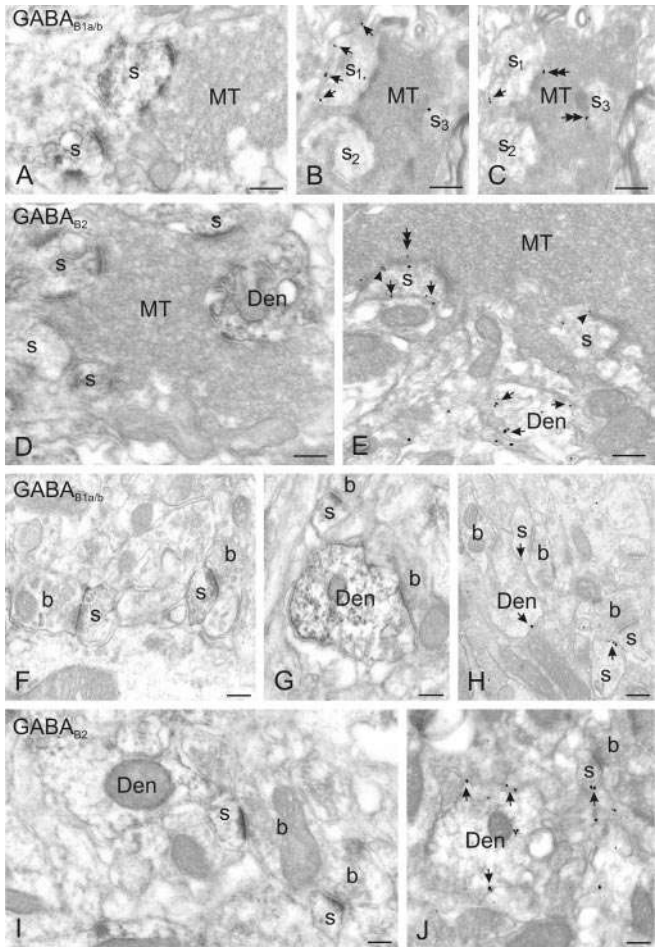


Figure 6. Electron micrographs showing immunoreactivity for GABA$_{B1a/b}$ and GABA$_{B2}$ subunits in the stratum lucidum of CA3 and in the dentate molecular layer. *A*, In the stratum lucidum of CA3, peroxidase reaction end product for GABA$_{B1a/b}$ protein was observed in dendritic spines (s) of 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$_1$–s$_3$, arrows) and over the presynaptic membrane specialization of a mossy fiber terminal (MT, double arrows). *D*, Immunostaining for GABA$_{B2}$ was detected in spines (s) and a dendritic shaft of a presumed pyramidal 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 mossy fiber terminals (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 located on the extrasynaptic membrane of spines (s) and dendritic shafts (Den) (arrows). *I*, 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.

GABA$_{B1a/b}$ and GABA$_{B2}$, in the adult rat hippocampus. Using light microscopy, we show that GABA$_{B1a/b}$ 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 and synaptic plasma membrane of excitatory terminals and, to a lesser extent, on inhibitory terminals. Postsynaptic GABA$_B$ receptor subunits 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$_B$ receptor subunits are in principal cells and GABAergic neurons

At the light microscopic level, GABA$_{B1a/b}$ and GABA$_{B2}$ were 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 (Kaupmann 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; Margeta-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$_{B1a/b}$ and GABA$_{B2}$ are colocalized and likely to form heterodimerized functional GABA$_B$ receptors in hippocampal neurons (Jones et al., 1998; Kaupmann et al., 1998a; White et al., 1998).

Besides the staining of the neuropil, somata of CA1 pyramidal cells and GABAergic neurons, scattered throughout the hippocampus, appeared intensely immunoreactive for GABA$_{B1a/b}$

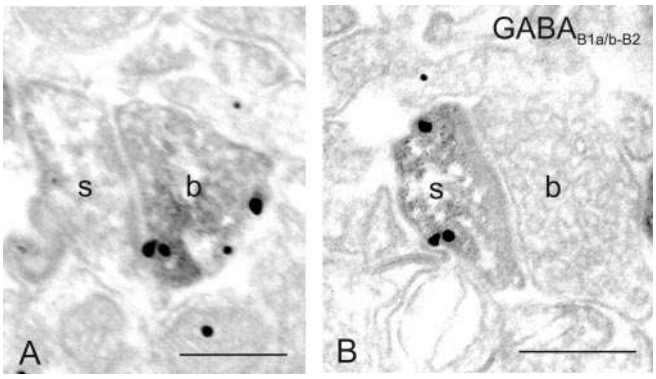


Figure 7. Colocalization of $GABA_{B1a/b}$ and $GABA_{B2}$ subunits in presynaptic and postsynaptic elements in the CA1 stratum radiatum. Labeling is shown with immunogold for the $GABA_{B1a/b}$ subunit and with immunoperoxidase for the $GABA_{B2}$ protein. *A*, Presynaptically, the two subunits were colocalized in an axon terminal (b) establishing asymmetrical contact with a dendritic spine (s). *B*, Postsynaptically, $GABA_{B1a/b}$ and $GABA_{B2}$ were seen in dendritic spines (s) of presumed pyramidal cells. Scale bars, 0.2 μm.

but not for $GABA_{B2}$. The strong somatic labeling for $GABA_{B1a/b}$ 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 $GABA_{B2}$ subunit was not detected in the somata of these cells suggests that these receptors are nonfunctional. The $GABA_{B2}$ subunit has been shown to be essential for the translocation of $GABA_{B1}$ 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 $GABA_{B2}$ subunits is responsible for the accumulation of $GABA_{B1a/b}$ protein. Consistently, in CA3, in which the level of $GABA_{B2}$ subunit mRNA is higher (Durkin et al., 1999), we did not observe an accumulation of $GABA_{B1a/b}$ 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 $GABA_B$ receptor subunits are on inhibitory and excitatory terminals

At the ultrastructural level, presynaptic $GABA_B$ 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 $GABA_B$ 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 $GABA_B$ receptors at glutamatergic terminals are likely to serve as heteroreceptors regulating glutamate release. The $GABA_B$ 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 $GABA_B$ receptors localized to excitatory terminals impinging on CA1 pyramidal cells and interneurons are tonically activated by ambient levels of GABA (Jensen et al., 2003). $GABA_B$ receptor-mediated presynaptic depression at both inhibitory and excitatory synapses is primarily attributable to the suppression of high voltage-activated Ca^{2+} channels (Misgeld et al., 1995; Wu and Saggau, 1995; Poncer et al., 2000; Lei and McBain, 2003).

Dendritic localization of $GABA_B$ receptor subunits

The majority of postsynaptic subunits were localized to the extrasynaptic membrane of dendritic spines and shafts of principal cells and interneurons. Postsynaptic $GABA_B$ receptors activating inwardly rectifying K^+ 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 (Lacaille, 1991; Khazipov et al., 1995). The prevalent localization of $GABA_B$ receptor subunits in dendrites, as revealed here, is in good agreement with electrophysiological observations showing that the extracellular stimulation or iontophoretic application of GABA or baclofen elicits $GABA_B$ receptor-mediated responses on the dendrites but not on the somata of CA1 pyramidal cells (Newberry and Nicoll, 1984, 1985).

The most intense labeling for $GABA_B$ 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, $GABA_{B1a/b}$ 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, $GABA_B$ 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 $GABA_B$ receptors. Elimination of GABA transporter-1, responsible for the uptake of GABA, however, does not result in activation of postsynaptic $GABA_B$ 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 $GABA_B$ receptor subunits (Fig. 8). First, an asso-

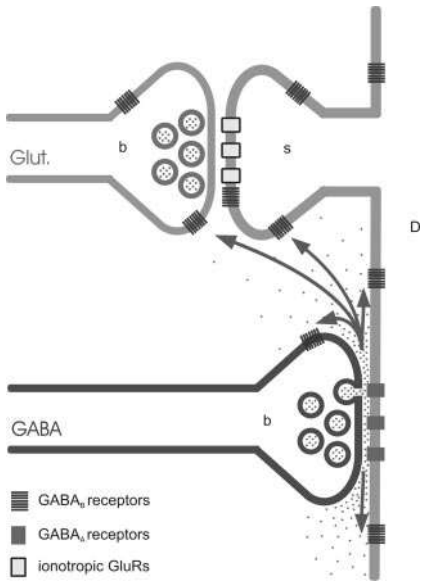


Figure 8. Presumed mechanism of $GABA_B$ receptor activation in the adult hippocampus. Metabotropic $GABA_B$ receptors (stripped boxes), localized to the extrasynaptic plasma membrane of GABAergic and glutamatergic terminals (b), spines (s), and dendritic shafts (*D*), are activated by spilled-over GABA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

ciation of the subunits with putative glutamatergic synapses was observed at both presynaptic and postsynaptic sides. Conceivably, this reflects the role of $GABA_B$ receptors in the modulation of glutamatergic synaptic transmission. Although there is ample evidence for the regulation of glutamate release by presynaptic $GABA_B$ 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 $GABA_B$ 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 (Baude et al., 1993; Luján et al., 1996, 1997; Shigemoto et al., 1996, 1997). Thus, the activation of mGluRs appears to be tightly coupled to the synaptic release of glutamate, ensuring the spatially and temporally precise transmission of information. Activation of $GABA_B$ 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

Bartos M, Vida I, Frotscher M, Geiger JR, Jonas P (2001) Rapid signaling at inhibitory synapses in a dentate gyrus interneuron network. J Neurosci 21:2687–2698.

Baude A, Nusser Z, Roberts JD, Mulvihill E, McIlhinney RA, Somogyi P (1993) The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron 11:771–787.

Bischoff S, Leonhard S, Reymann N, Schuler V, Shigemoto R, Kaupmann K, Bettler B (1999) Spatial distribution of $GABA_B$R1 receptor mRNA and binding sites in the rat brain. J Comp Neurol 412:1–16.

Bowery NG, Brown DA (1997) The cloning of $GABA_B$ receptors. Nature 386:223–224.

Bowery NG, Hudson AL, Price GW (1987) $GABA_A$ and $GABA_B$ receptor site distribution in the rat central nervous system. Neuroscience 20:365–383.

Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI, Enna SJ (2002) International Union of Pharmacology XXXIII. Mammalian gamma-aminobutyric acid(B) receptors: structure and function. Pharmacol Rev 54:247–264.

Bragin A, Jando G, Nadasdy Z, Hetke J, Wise K, Buzsaki G (1995) Gamma (40–100 Hz) oscillation in the hippocampus of the behaving rat. J Neurosci 15:47–60.

Buhl EH, Halasy K, Somogyi P (1994) Diverse sources of hippocampal unitary inhibitory postsynaptic potentials and the number of synaptic release sites. Nature 368:823–828.

Chu DCM, Albin RL, Young AB, Penney JB (1990) Distribution and kinetics of $GABA_B$ binding sites in rat central nervous system: a quantitative autoradiographic study. Neuroscience 34:341–357.

Clark JA, Mezey E, Lam AS, Bonner TI (2000) Distribution of the GABA(B) receptor subunit gb2 in the rat CNS. Brain Res 860:41–52.

Davies CH, Starkey SJ, Pozza MF, Collingridge GL (1991) $GABA_B$ autoreceptors regulate the induction of LTP. Nature 349:609–611.

Durkin MM, Gunwaldsen CA, Borowsky B, Jones KA, Branchek TA (1999) An in situ hydridization study of the distribution of the $GABA_B$2 protein mRNA in the rat CNS. Mol Brain Res 71:185–200.

Dutar P, Nicoll RA (1988a) A physiological role for $GABA_B$ receptors in the central nervous system. Nature 332:156–158.

Dutar P, Nicoll RA (1988b) Pre- and postsynaptic $GABA_B$ receptors in the hippocampus have different pharmacological properties. Neuron 1:585–591.

Erlander MG, Tillakaratne NJ, Feldblum S, Patel N, Tobin AJ (1991) Two genes encode distinct glutamate decarboxylases. Neuron 1:91–100.

Fritschy J-M, Meskenaite V, Weinmann O, Honer M, Benke D, Mohler H (1999) $GABA_B$-receptor splice variants GB1a and GB1b in rat brain: developmental regulation, cellular distribution and extrasynaptic localization. Eur J Neurosci 11:761–768.

Gähwiler BH, Brown DA (1985) $GABA_B$-receptor-activated K^+ current in voltage-clamped CA3 pyramidal cells in hippocampal cultures. Proc Natl Acad Sci USA 82:1558–1562.

Gonchar Y, Pang L, Malitschek B, Bettler B, Burkhalter A (2001) Subcellular localization of GABA(B) receptor subunits in rat visual cortex. J Comp Neurol 431:182–197.

Haas CA, Rauch U, Thon N, Merten T, Deller T (1999) Entorhinal cortex lesion in adult rats induces the expression of the neuronal chondroitin sulfate proteoglycan neurocan in reactive astrocytes. J Neurosci 19:9953–9963.

Han ZS, Buhl EH, Lorinczi Z, Somogyi P (1993) A high degree of spatial selectivity in the axonal and dendritic domains of physiologically identified local-circuit neurons in the dentate gyrus of the rat hippocampus. Eur J Neurosci 5:395–410.

Hirata K, Sawada S, Yamamoto C (1992) Quantal analysis of suppressing action of baclofen on mossy fiber synapses in guinea pig hippocampus. Brain Res 578:33–40.

Ige AO, Bolam JP, Billinton A, White JH, Marshall FH, Emson PC (2000) Cellular and subcellular localisation of $GABA_{B1}$ and $GABA_{B2}$ receptor proteins in the rat cerebellum. Mol Brain Res 83:72–80.

Isaacson JS, Solis JM, Nicoll RA (1993) Local and diffuse synaptic actions of GABA in the hippocampus. Neuron 10:165–175.

Isomoto S, Kaibara M, Sakurai-Yamashita Y, Nagayama Y, Uezono Y, Yano K, Taniyama K (1998) Cloning and tissue distribution of novel splice

variants of the rat $GABA_B$ receptor. Biochem Biophys Res Commun 253: 10–15.

Jensen K, Chiu C-S, Sokolova I, Lester HA, Mody I (2003) GABA transporter-1 (GAT1)-deficient mice: differential tonic activation of $GABA_A$ versus $GABA_B$ receptors in the hippocampus. J Neurophysiol 90:2690–2701.

Johnston GA (1996) $GABA_C$ receptors: relatively simple transmitter-gated ion channels? Trends Pharmacol Sci 17:319–323.

Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwaldsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA, Gerald C (1998) $GABA_B$ receptors function as a heteromeric assembly of the subunits $GABA_BR1$ and $GABA_BR2$. Nature 396:674–679.

Kaupmann K, Huggel K, Heid J, Flor PJ, Bischoff S, Mickel SJ, McMaster G, Angst C, Bittiger H, Froestl W, Bettler B (1997) Expression cloning of $GABA_B$ receptors uncovers similarity to metabotropic glutamate receptors. Nature 386:239–246.

Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B (1998a) $GABA_B$-receptor subtypes assemble into functional heteromeric complexes. Nature 396:683–687.

Kaupmann K, Schuler V, Mosbacher J, Bischoff S, Bittiger H, Heid J, Froestl W, Leonhard S, Pfaff T, Karschin A, Bettler B (1998b) Human γ-aminobutyric acid type B receptors are differentially expressed and regulate inwardly rectifying K^+ channels. Proc Natl Acad Sci USA 95:14991–14996.

Khazipov R, Congar P, Ben-Ari Y (1995) Hippocampal CA1 lacunosum-moleculare interneurons: modulation of monosynaptic GABAergic IPSCs by presynaptic $GABA_B$ receptors. J Neurophysiol 74:2126–2137.

Kulik A, Nakadate K, Nyiri G, Notomi T, Malitschek B, Bettler B, Shigemoto R (2002) Distinct localization of $GABA_B$ receptors relative to synaptic sites in the rat cerebellum and ventrobasal thalamus. Eur J Neurosci 15:291–307.

Kuner R, Kohr G, Grunewald S, Eisenhardt G, Bach A, Kornau HC (1999) Role of heteromer formation in $GABA_B$ receptor function. Science 283:74–77.

Lacaille JC (1991) Postsynaptic potentials mediated by excitatory and inhibitory amino acids in interneurons of stratum pyramidale of the CA1 region of rat hippocampal slices in vitro. J Neurophysiol 66:1441–1454.

Lambert NA, Wilson WA (1993) Heterogeneity in presynaptic regulation of GABA release from hippocampal inhibitory neurons. Neuron 11:1057–1067.

Lei S, McBain CJ (2003) $GABA_B$ receptor modulation of excitatory and inhibitory synaptic transmission onto rat CA3 hippocampal interneurons. J Physiol (Lond) 546:439–453.

Li JL, Shigemoto R, Kulik A, Chen P, Nomura S, Kaneko T, Mizuno N (2001) Immunocytochemical localization of GABA(B) receptors in mesencephalic trigeminal nucleus neurons in the rat. Neurosci Lett 315:93–97.

Liang F, Hatanaka Y, Saito H, Yamamori T, Hashikawa T (2000) Differential expression of γ-aminobutyric acid type receptor-1a and -1b mRNA variants in GABA and non-GABAergic neurons of the rat brain. J Comp Neurol 416:475–495.

López-Bendito G, Shigemoto R, Kulik A, Paulsen O, Fairen A, Luján R (2002) Expression and distribution of metabotropic GABA receptor subtypes $GABA_BR1$ and $GABA_BR2$ during rat neocortical development. Eur J Neurosci 15:1766–1778.

López-Bendito G, Shigemoto R, Kulik A, Vida I, Fairen A, Lujan R (2003) Distribution of metabotropic GABA receptor subunits $GABA_{B1}$ and $GABA_{B2}$ in the rat hippocampus during prenatal and postnatal development. Hippocampus, in press.

Lu XY, Ghasemzadeh MB, Kalivas PW (1999) Regional distribution and cellular localization of gamma-aminobutyric acid subtype 1 receptor mRNA in the rat brain. J Comp Neurol 407:166–182.

Luján R, Nusser Z, Roberts JDB, Shigemoto R, Somogyi P (1996) Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. Eur J Neurosci 8:1488–1500.

Luján R, Roberts JDB, Shigemoto R, Somogyi P (1997) Differential plasma membrane distribution of metabotropic glutamate receptors mGluR1α, mGluR2 and mGluR5, relative to neurotransmitter release sites. J Chem Neuroanat 13:219–241.

Lüscher C, Jan LY, Stoffel M, Malenka RC, Nicoll RA (1997) G protein-coupled inwardly rectifying K^+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. Neuron 19:687–695.

MacDonald RL, Olsen RW (1994) $GABA_A$ receptor channels. Annu Rev Neurosci 17:569–602.

Margeta-Mitrovic M, Mitrovic I, Riley RC, Yan LY, Basbaum AI (1999) Immunohistochemical localization of $GABA_B$ receptors in the rat central nervous system. J Comp Neurol 405:299–321.

Margeta-Mitrovic M, Jan YN, Jan LY (2000) A trafficking checkpoint controls GABA(B) receptor heterodimerization. Neuron 27:97–106.

Margeta-Mitrovic M, Jan YN, Jan LY (2001) Function of GB1 and GB2 subunits in G protein coupling of GABA(B) receptors. Proc Natl Acad Sci USA 98:14649–14654.

Miles R (1990) Variation in strength of inhibitory synapses in the CA3 region of guinea-pig hippocampus in vitro. J Physiol (Lond) 431:659–676.

Misgeld U, Bijak M, Jarolimek W (1995) A physiological role for $GABA_B$ receptors and the effects of baclofen in the mammalian central nervous system. Prog Neurobiol 46:423–462.

Mody I, De Koninck Y, Otis TS, Soltesz I (1994) Bridging the cleft at GABA synapses in the brain. Trends Neurosci 17:517–525.

Mott DD, Lewis DV (1991) Facilitation of the induction of long-term potentiation by $GABA_B$ receptors. Science 252:1718–1720.

Newberry NR, Nicoll RA (1984) A bicucullin-resistent inhibitory postsynaptic potential in rat hippocampal pyramidal cells *in vitro.* J Physiol (Lond) 348:239–254.

Newberry NR, Nicoll RA (1985) Comparison of the action of baclofen with γ-aminobutyric acid on rat hippocampal pyramidal cells in vitro. J Physiol (Lond) 360:161–185.

Olpe HR, Worner W, Ferrat T (1993) Stimulation parameters determine role of $GABA_B$ receptors in long-term potentiation. Experientia 49:542–546.

Otis TS, De Koninck Y, Mody I (1993) Characterization of synaptically elicited $GABA_B$ responses using patch-clamp recordings in rat hippocampal slices. J Physiol (Lond) 463:391–407.

Pagano A, Rovelli G, Mosbacher J, Lohmann T, Duthey B, Stauffer D, Ristig D, Schuler V, Meigel I, Lampert C, Stain T, Prézeau L, Blahos J, Pin J-P, Froestl W, Kuhn R, Heid J, Kaupmann K, Bettler B (2001) C-terminal interaction is essential for surface trafficking but not for heteromeric assembly of $GABA_B$ receptors. J Neurosci 21:1189–1202.

Poncer JC, McKinney RA, Gähwiler BH, Thompson SM (2000) Differential control of GABA release at synapses from distinct interneurons in rat hippocampus. J Physiol (Lond) 528:123–130.

Pozza MF, Manuel NA, Steinmann M, Froestl W, Davies CH (1999) Comparison of antagonist potencies at pre- and post-synaptic GABA(B) receptors at inhibitory synapses in the CA1 region of the rat hippocampus. Br J Pharmacol 127:211–219.

Pfaff T, Malitschek B, Kaupmann K, Prezeau L, Pin J-P, Bettler B, Karschin A (1999) Alternative splicing generates a novel isoform of the rat metabotropic $GABA_BR1$. Eur J Neurosci 11:2874–2882.

Pfrieger FW, Gottmann K, Lux HD (1994) Kinetics of $GABA_B$ receptor-mediated inhibition of calcium currents and excitatory synaptic transmission in hippocampal neurons in vivo. Neuron 12:97–107.

Scanziani M (2000) GABA spillover activates postsynaptic $GABA_B$ receptors to control rhythmic hippocampal activity. Neuron 25:673–681.

Scholz KP, Miller RJ (1991) $GABA_B$ receptor-mediated inhibition of Ca^{2+} currents and synaptic transmission in cultured rat hippocampal neurones. J Physiol (Lond) 444:669–686.

Schwarz DA, Barry G, Eliasof SD, Petroski RE, Conlon PJ, Maki RA (2000) Characterization of γ-aminobutyric acid receptor $GABA_{B(1e)}$, a $GABA_{B(1)}$ splice variant encoding a truncated receptor. J Biol Chem 275:32174–32181.

Shigemoto R, Kulik A, Roberts JD, Ohishi H, Nusser Z, Kaneko T, Somogyi P (1996) Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. Nature 381:523–525.

Shigemoto R, Kinoshita A, Wada E, Nomura S, Ohishi H, Takada M, Flor PJ, Neki A, Abe T, Nakanishi S, Mizuno N (1997) Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. J Neurosci 17:7503–7522.

Sloviter RS, Ali-Akbarian L, Elliott RC, Bowery BJ, Bowery NG (1999) Localization of GABA(B) (R1) receptors in the rat hippocampus by immunocytochemistry and high resolution autoradiography, with specific reference to its localization in identified hippocampal interneuron subpopulations. Neuropharmacology 38:1707–1721.

Sodickson DL, Bean BP (1996) $GABA_B$ receptor-activated inwardly rectify-

ing potassium current in dissociated hippocampal CA3 neurons. J Neurosci 16:6374–6385.

Turgeon SM, Albin RL (1994) Postnatal ontogeny of $GABA_B$ binding in rat brain. Neuroscience 62:601–613.

Vida I, Frotscher M (2000) A hippocampal interneuron associated with the mossy fiber system. Proc Natl Acad Sci USA 97:1275–1280.

Vida I, Halasy K, Szinyei C, Somogyi P, Buhl EH (1998) Unitary IPSPs evoked by interneurons at the stratum radiatum-stratum lacunosum-moleculare border in the CA1 area of the rat hippocampus in vitro. J Physiol (Lond) 506:755–773.

Vogt KE, Nicoll RA (1999) Glutamate and γ-aminobutyric acid mediate a heterosynaptic depression at mossy fiber synapses in the hippocampus. Proc Natl Acad Sci USA 96:1118–1122.

Wang X, Lambert NA (2000) GABA(B) receptors couple to potassium and calcium channels on identified lateral perforant pathway projection neurons. J Neurophysiol 83:1073–1078.

White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM, Marshall FH (1998) Heterodimerization is required for the formation of a functional $GABA_B$ receptor. Nature 396:679–682.

Wu LG, Saggau P (1995) $GABA_B$ receptor-mediated presynaptic inhibition in guinea-pig hippocampus is caused by reduction of presynaptic Ca^{2+} influx. J Physiol (Lond) 485:649–657.

Ylinen A, Soltesz I, Bragin A, Penttonen M, Sik A, Buzsaki G (1995) Intracellular correlates of hippocampal theta rhythm in identified pyramidal cells, granule cells, and basket cells. Hippocampus 5:78–90.

Yoon KW, Rothman SM (1991) The modulation of rat hippocampal synaptic conductances by baclofen and gamma-aminobutyric acid. J Physiol (Lond) 442:377–390.