

GABA_B Receptors in the Centromedian/ Parafascicular Thalamic Nuclear Complex: An Ultrastructural Analysis of GABA_BR1 and GABA_BR2 in the Monkey Thalamus

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ABSTRACT

Strong γ -aminobutyric acid type B (GABA_B) receptor binding has been shown throughout the thalamus, but the distribution of the two GABA_B receptor subunits, GABA_B receptor subunit 1 (GABA_BR1) and GABA_B receptor subunit 2 (GABA_BR2), remains poorly characterized. In primates, the caudal intralaminar nuclei, centromedian and parafascicular (CM/PF), are an integral part of basal ganglia circuits and a main source of inputs to the striatum. In this study, we analyzed the subcellular and subsynaptic distribution of GABA_B receptor subunits by using light and electron microscopic immunocytochemical techniques. Quantitative immunoperoxidase and immunogold analysis showed that both subunits display a similar pattern of distribution in CM/PF, being expressed largely at extrasynaptic and perisynaptic sites in neuronal cell bodies, dendrites, and axon-like processes and less abundantly in axon terminals. Postsynaptic GABA_BR1 labeling was found mostly on the plasma membrane (70–80%), whereas GABA_BR2 was more evenly distributed between the plasma membrane and intracellular compartments of CM/PF neurons. A few axon terminals forming symmetric and asymmetric synapses were also labeled for GABA_BR1 and GABA_BR2, but the bulk of presynaptic labeling was expressed in small axon-like processes. About 20% of presynaptic vesicle-containing dendrites of local circuit neurons displayed GABA_BR1/R2 immunoreactivity. Vesicular glutamate transporters (vGluT1)-containing terminals forming asymmetric synapses expressed GABA_BR1 and/or displayed postsynaptic GABA_BR1 at the edges of their asymmetric specialization. Overall, these findings provide evidence for multiple sites where GABA_B receptors could modulate GABAergic and glutamatergic transmission in the primate CM/PF complex. *J. Comp. Neurol.* 496:269–287, 2006. © 2006 Wiley-Liss, Inc.

Indexing terms: immunocytochemistry; GABA_BR1; GABA_BR2; vGluT1; intralaminar thalamic nuclei; Parkinson's disease

γ -Aminobutyric acid (GABA)-ergic neurotransmission is mediated by three distinct receptor subtypes, GABA_A, GABA_B, and GABA_C, characterized by their pharmacological and electrophysiological properties. GABA_A and GABA_C receptors are ligand-gated chloride channels that mediate fast inhibitory postsynaptic currents by triggering the opening of integral chloride channels (Olsen and Tobin, 1990; McDonald and Olsen, 1994). GABA_B receptors belong to the family of seven transmembrane domain receptors (Marshall et al., 1999) and are coupled to Ca²⁺ and K⁺ channels via G proteins and second messenger systems (Couve et al., 2000; Margeta-Mitrovic et al., 2000;

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Billinton et al., 2001). Functional GABA_B receptors were first identified on presynaptic terminals, where they act as auto- or heteroreceptors to influence transmitter release by suppressing Ca²⁺ conductance (Bowery, 1993; Bowery et al., 2002). Subsequent studies showed the existence of postsynaptic GABA_B receptors whose activation produces an increase in membrane K⁺ conductance and associated neuronal hyperpolarization (for reviews see Bowery et al., 2002; Bettler et al., 2004; Cryan and Kaupmann, 2005). Thus, the synaptic release of GABA in a particular brain region can mediate distinct and complex electrophysiological effects depending on the relative abundance and distribution of the different subtypes of GABA receptors in relation to the release sites of transmitter.

So far, two GABA_B receptor subunits have been identified, GABA_B receptor subunit 1 (GABA_BR1) and GABA_B receptor subunit 2 (GABA_BR2), which assemble into heterodimers to form functional GABA_B receptors (Bettler et al., 1998; Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999; Makoff, 1999; Martin et al., 1999; Bowery and Enna, 2000). The GABA_BR1 subunit is responsible for binding GABA, whereas GABA_BR2 is required for cell-surface expression (Bormann, 2000; Calver et al., 2001; Pagano et al., 2001) and G-protein coupling (Galvez et al., 2001; Margeta-Mitrovic et al., 2001a,b; Robbins et al., 2001; Havlickova et al., 2002) of GABA_B receptors.

In situ hybridization and immunocytochemical studies revealed that GABA_BR1 and GABA_BR2 expression shows considerable overlap in many areas of the central nervous system (CNS; Margeta-Mitrovic et al., 1999; Clark et al., 2000). GABA_B receptor binding sites, GABA_B receptor subtype mRNAs, and GABA_B receptor protein are strongly expressed in the rat and monkey thalamus (Chu et al., 1990; Muñoz et al., 1998; Ambardekar et al., 1999; Bowery et al., 1999; Princivalle et al., 2000, 2001), but their exact location and function remain controversial. Electrophysiological evidence indicates that GABA_B may act as pre- or postsynaptic receptors at GABAergic and glutamatergic synapses in the rat and mouse thalamus (Banerjee and Snead, 1995; Ambardekar et al., 1999; Chen and Regehr, 2003). So far, a single electron microscopic analysis of the rat ventrobasal nucleus has examined the cellular and subcellular localization of GABA_B receptor in the rat thalamus (Kulik et al., 2002).

In primates, the intralaminar nuclei, centromedian (CM) and parafascicular (PF), are an integral part of basal

ganglia circuits (Smith et al., 2004). This nuclear group is a major source of thalamic afferents to the striatum (Cowan and Powell, 1956; Parent and DeBellefeuille, 1983; Smith and Parent, 1986) and receives functionally segregated GABAergic inputs from basal ganglia output nuclei (Smith et al., 2004). Despite this substantial GABAergic innervation, very little is known about the location and function of GABA_B receptors in the caudal intralaminar complex.

To address this issue further, we undertook a detailed high-resolution electron microscopic study of the localization of GABA_BR1 and GABA_BR2 subunits in the monkey CM/PF. Findings of this study have been presented in part in abstract form (Villalba and Smith, 2004).

MATERIALS AND METHODS

Animals and tissue preparation

In total, seven rhesus monkeys (*Macaca mulatta*; Yerkes National Primate Research Center colony) and four Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used in this study. The experiments were performed according to the National Institutes of Health *Guide for the care and use of laboratory animals*. All efforts were made to minimize animal suffering and reduce the number of animals used.

For immunocytochemistry experiments, tissue was obtained from six adult monkeys and two rats. Animals were deeply anesthetized with an overdose of pentobarbital and perfused transcardially with cold, oxygenated Ringer's solution, followed by fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (PB; 0.1 M, pH 7.4). After fixative perfusion, the brains were washed with PB, taken out from the skull, and cut into 10-mm-thick blocks in the frontal plane. Tissue sections (60 μm thick) were obtained with a Vibratome, collected in cold phosphate-buffered saline (PBS; 0.01 M, pH 7.4), and treated with sodium borohydride (1% in PBS) for 20 minutes.

For Western immunoblots, brain tissue from one monkey and two rats was used. The monkey was overdosed with pentobarbital (100 mg/kg, i.v.), followed by rapid removal of the brain from the skull and dissection. The rodents were rapidly decapitated, and the brain was removed from the skull and dissected on ice.

Primary antibodies

GABA_B receptor antibodies. Two polyclonal antibodies against GABA_B receptors were used in this study. A polyclonal rabbit antiserum was generated against the intracellular carboxyl-terminal domain of the GABA_BR1 common to the two splice variants GABA_BR1a and GABA_BR1b. This polyclonal antibody was generated and biochemically characterized previously by Charara et al. (2000). For this antibody, the sequence of the peptide used for immunization was NH₂-RGPEPPDRLSCDGSRVH-LLYK-COOH. An N-terminal lysine was added to the peptide to facilitate coupling to the carrier protein. The specificity of the antibodies was evaluated by immunoblotting of monkey brain and liver membranes (100 μg). Western blot analysis for GABA_BR1 revealed that this antiserum selectively recognizes proteins with ~130 kDa and ~100 kDa molecular weights, corresponding, respectively, to the molecular weights of the two splice variants

Abbreviations

asym	asymmetric
at	axon terminal
ax	preterminal axon-like
CM	centromedian thalamic nucleus
den	dendrite
Extrasyn	extrasynaptic
LCN	local circuit neuron
Nu	nucleus
pa	puncta adherens-like contact
Peri	perisynaptic
PF	parafascicular thalamic nucleus
PN	projection neuron
sym	symmetric
syn	synaptic
vcd	vesicle-containing dendrite

GABA_BR1a and GABA_BR1b (Kaupmann et al., 1998) in the monkey brain. No band was seen for liver membrane proteins. Immunoreactivity was completely abolished when antibodies were preadsorbed with the synthetic GABA_BR1 peptide (10 µg/ml) prior to immunoblotting, whereas preadsorption of the antibodies with the GABA_BR2 peptide (Kuner et al., 1999) had no effect on the distribution and intensity of GABA_BR1 immunostaining (for details see Charara et al., 2000).

The GABA_BR2 polyclonal guinea pig antiserum (catalog No. AB5394, lot No. 23040494; Chemicon, Temecula, CA) was raised against a synthetic peptide corresponding to the C-terminal amino acids of the rat and human GABA_BR2 receptor protein, which is not present in the GABA_BR1 protein (Kaupman et al., 1998; White et al., 1998; Jones et al., 1998; Martin et al., 1999). Western blot analysis with membranes prepared from cell lines expressing GABA_BR2 or human and rat brain tissue revealed a single band labeling at approximately 120 kDa (Waldvogel et al., 2004) that corresponds to the molecular weight of GABA_BR2 (Kaupman et al., 1998; White et al., 1998; Jones et al., 1998; Martin et al., 1999). The specificity of GABA_BR1 and GABA_BR2 antibodies in monkey brain tissue has been previously shown (Charara et al., 2000, 2004, 2005; Smith et al., 2000, 2001; Galvan et al., 2004).

Vesicular glutamate transporter antibodies. Two polyclonal antibodies were used, a commercially available guinea pig polyclonal anti-vGluT1 directed against a 19-amino acid (aa) peptide sequence in the C-terminal region of the rat vGluT1 (catalog No. AB5905, lot No. 22090813; Chemicon) and a new rabbit anti-rat vesicular glutamate transporter 1 (vGluT1) antibody (Mab Technologies, Atlanta, GA).

To generate rabbit anti-vGluT1 antibodies (Mab Technologies), a peptide was generated to the COOH terminus of the rat vGluT1, corresponding to aa 543–560 (C-ATHSTVQPPRPPPPVVRDY). This epitope is highly conserved in the mouse vGluT1 sequence and has one site of divergence in the human sequence (underlined above). A cysteine was added to aid in conjugation to the protein carrier keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL). Antiserum was obtained from rabbits (Covance) immunized with the conjugated peptide, and the IgG fraction was recovered by ammonium sulfate precipitation as follows. Serum was first treated with 25% ammonium sulfate to remove any proteins that might precipitate at low ionic concentrations and incubated with stirring overnight at 4°C. After a 3,000g × 30 minutes spin, the supernatant was removed and transferred to a clean tube. Ammonium sulfate was added to a final concentration of 50% saturation. After another overnight incubation at 4°C, the IgG fraction was isolated in the pellet by centrifugation at 3,000g × 30 minutes. The pellet was resuspended in PBS and dialyzed overnight with three buffer changes.

The specificity of the rabbit anti-vGluT1 antiserum (Mab Technologies) in monkey tissue was determined by Western immunoblots and light microscopy analysis of striatal tissue, one of the richest vGluT1-containing structures in the brain (see Fig. 1). Results of the preadsorption immunocytochemical data also apply to the thalamus and other brain regions.

The specificity of the guinea pig vGluT1 antiserum was assessed by the following observations. 1) The overall pattern of labeling obtained with this antiserum was the same as that generated with the rabbit anti-vGluT1 anti-

serum. 2) At electron microscopic levels, both antibodies labeled exclusively axon terminals forming asymmetric synapses. 3) vGluT1-labeled terminals with this antiserum are segregated from vGluT2-immunoreactive boutons in different brain regions (Todd et al., 2003; Alvarez et al., 2004). 4) Preadsorption of the antiserum with the rat vGluT1 C-terminal peptide (cATHSTVQPPRPPPPVVRDY; 0.2 µg/ml) abolished completely specific immunostaining, whereas incubation with the C-terminal vGluT2 peptide (cAQDAYSYKDRDDYS; 2.0 µg/ml) had no effect on the quality of immunostaining (Raju and Smith, 2005).

Western immunoblots

All brain membrane samples were prepared at 4°C. After dissection, the striatal tissue was completely homogenized with a sonicator in an ice-cold buffer solution (20 mM HEPES, 10 mM EDTA, 2 mM Na₃VO₄). The homogenate was centrifuged for 5 minutes at 2,000 rpm to remove tissue debris, and membranes were isolated from the supernatant by subsequent centrifugation for 30 minutes at 14,000 rpm. The resulting pellet was then solubilized in a buffer solution (20 mM HEPES and 0.1 mM EDTA) before total protein concentration was measured by using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). The homogenates were then centrifuged for 30 minutes at 14,000 rpm, and the pellet was solubilized in a lysis buffer [10 mM HEPES, 50 mM NaCl, 0.1 mM EDTA, 1 mM benzamidine, 1.0% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), protease inhibitor cocktail (1 tablet per 50 ml; Roche Diagnostics GmbH, Mannheim, Germany)]. The lysates (5–30 µg protein) were then eluted with 1× SDS-PAGE sample buffer. The samples of brain tissue were resolved by SDS-PAGE and subjected to Western blot analysis with anti-vGluT1 (0.2 µg/ml) antibody. Immunoreactive bands were detected with the enhanced chemiluminescence detection system (Pierce) with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:4,000; Amersham Biosciences, Little Chalfont, United Kingdom).

Localization of GABA_BR1 and GABA_BR2 immunoreactivity at the light microscopic level

The immunoperoxidase localization of GABA_BR1 and GABA_BR2 receptor subtypes was performed by using the avidin-biotin complex (ABC) method (Hsu et al., 1981). After blocking of nonspecific sites with 10% normal goat serum (NGS) and 1% bovine serum albumin (BSA) in PBS with 0.1% Triton X-100 for 1 hour at room temperature (RT), the sections were incubated for 2 days at 4°C in the primary antibody solution. The dilutions were 1:200 for anti-GABA_BR1 antibody and 1:500 for anti-GABA_BR2 antibody. The sections were then washed in PBS and finally incubated for 90 minutes at RT in biotinylated goat anti-rabbit and anti-guinea pig IgGs (1:200; Vector, Burlingame, CA), rinsed again in PBS, and finally incubated for an additional 90 minutes at RT in the ABC solution (1:100; Vectastain Standard Kit; Vector). All immunoreagents were diluted in PBS containing 1% NGS, 1% BSA, and 0.1% Triton X-100. Sections were then rinsed in PBS and Tris buffer (0.05 M, pH 7.6) before being placed in a solution containing 0.025% 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO), 0.01 M imidazole (Fisher Scientific, Norcross, GA), and 0.006% H₂O₂ for 10 minutes. The reaction was stopped by repeated washes in

PBS. Finally, the sections were mounted on gelatin-coated slides, dehydrated in alcohol, and immersed in toluene; then, a coverslip was applied with Permount. Selected fields of CM/PF nuclei were photographed with a Leica DC 500 digital camera.

Localization of GABA_BR1 and GABA_BR2 immunoreactivity at the electron microscopic level

For electron microscopic studies, sections were placed in a cryoprotectant solution (PB, 0.05 M, pH 7.4, containing 25% sucrose and 10% glycerol) for 20 minutes, frozen at -80°C for 20 minutes, thawed, and returned to a graded series of cryoprotectant (100%, 70%, 50%, 30%) diluted in PBS. They were then washed in PBS before being processed for immunocytochemistry.

Immunoperoxidase labeling. Sections were immersed in PBS with 10% NGS and 1% BSA to block nonspecific sites, then incubated in the primary antibodies against GABA_BR1 (dilution 1:200) and GABA_BR2 (dilution 1:500). They were next processed for the visualization of GABA_B receptors according to the protocol described above, except that Triton X-100 was not included in the solutions.

Immunogold labeling. For this protocol, sections were preincubated in PBS containing 5% nonfat dry milk and then washed in Tris-buffered saline (TBS)-gelatin buffer (0.02 M Tris, 0.15 M NaCl, 1 $\mu\text{l/ml}$ fish gelatin, pH 7.6) to block the nonspecific sites. Primary and secondary antibodies were diluted in TBS-gelatin buffer containing 1% nonfat dry milk. The dilutions of GABA_BR1 and GABA_BR2 antibodies were the same as those used for immunoperoxidase. After incubation in the primary antibodies (48 hours, 4°C), sections were rinsed in TBS-gelatin and incubated for 2 hours at RT in the appropriate gold-conjugated secondary IgGs (dilution 1:100; Nanogold; Nanoprobes Inc.), and gold particles (1.4 nm) were silver enhanced with the HQ Silver kit (Nanoprobes Inc.). As controls for the specificity of the immunolabeling, omission of the primary antibodies from incubation solutions completely abolished the immunostaining for the corresponding antigens.

Double immunolabeling. For double immunolabeling, sections were incubated overnight at RT with a cocktail of rabbit anti-GABA_BR1 antibodies (dilution 1:200) and guinea pig anti-vGluT1 antibodies (dilution 1:5,000; Chemicon) or with guinea pig anti-GABA_BR2 antibodies plus rabbit anti-vGluT1 antibodies (dilution 1:5,000; MAB Technologies). For this reaction, all antibodies were diluted in TBS-gelatin solution with 1% nonfat dry milk. After rinses with TBS-gelatin, the sections were incubated for 2 hours at RT with goat anti-rabbit IgGs conjugated with 1.4-nm gold particles (1:100; Nanoprobes Inc.) and biotinylated goat anti-guinea pig antibodies (1:200 dilution; Vector) or with goat anti-guinea pig IgGs conjugated with 1.4-nm gold particles (1:200 dilution; Vector) and biotinylated goat anti-rabbit IgGs (1:200 dilution; Vector). After washes with TBS-gelatin, sections were incubated for 90 minutes at RT in an ABC solution (1:100; Vector). Sections were rinsed with TBS-gelatin and then transferred to a 1% aqueous sodium acetate solution before the silver intensification of the gold particles with HQ silver kit (Nanoprobes Inc.) for 5–6 minutes. The silver reaction was stopped in sodium acetate, and sections were washed

in PBS and Tris buffer (0.005 M, pH 7.6) before being processed with DAB as described above. Sections were then processed for electron microscopy.

A series of control sections was incubated with primary antibodies against GABA_BR1 or GABA_BR2 with vGluT1. This was followed by incubation in biotinylated secondary antibodies and ABC. Then, sections were processed for silver intensification and the DAB reaction. In both cases, only immunoperoxidase-positive boutons were detected. On the contrary, when the biotinylated secondary antibodies were omitted and the tissue was processed only with secondary gold-conjugated antibodies, single immunogold labeling for GABA_BR1 or GABA_BR2 was visualized.

A second series of control sections was incubated with both secondary antibodies and processed for immunoperoxidase or immunogold methods as described above, but the primary antibodies for either GABA_B receptor subtypes or vGluT1 were omitted in turn from the incubation solutions. These reactions led to peroxidase labeling for vGluT1 only following omission of the GABA_B receptor antibodies, whereas only the gold particle labeling for either GABA_B receptor subtypes could be seen in sections processed without the vGluT1 antibodies.

Processing for electron microscopy

After immunostaining, sections were washed in PB (0.1 M, pH 7.4) and postfixed in osmium tetroxide (1–0.5% in PB). This was followed by rinses in PB (0.1 M, pH 7.4) and dehydration in a graded series of ethanol and propylene oxide. Uranyl acetate (1%) was added to the 70% ethanol to improve the contrast in the electron microscope. The sections were then embedded in resin (Durcupan ACM; Fluka, Fort Washington, PA), mounted on microscope slides, and placed in the oven for 48 hours at 60°C . Areas of interest were selected, cut from the slides, and glued on top of resin blocks. In some cases, areas of interest were photographed in the light microscope before being cut out and glued on blocks. From these blocks, correlated semithin sections ($\sim 1\ \mu\text{m}$) were obtained on the ultramicrotome (Leica Ultracut T2), and counterstained with 0.1% toluidine blue (see Fig. 2A,B).

The CM and PF nuclei were identified in the resin-embedded sections as follows. Serial adjacent sections of monkey thalamus were either processed for electron microscopy or Nissl stained with cresyl violet. Although the nuclear outlines are not clear in the osmicated section, the Nissl-stained serial section was used as a reference to differentiate PF from CM. In Nissl-stained material, both nuclei are easily differentiated; the PF is identified as the medialmost region of the complex that contains densely packed, small, round perikarya, which can be easily differentiated from the CM, located more laterally with a lower density of cells, which appear paler in Nissl-stained material (for further details see Sidibe et al., 2002).

Serial ultrathin sections were obtained on the ultramicrotome (Leica Ultracut T2), collected onto Pioloform-coated single-slot copper grids, stained with lead citrate (Reynolds, 1963), and examined with the electron microscope (Zeiss EM 10C). The electron micrographs were acquired with a CCD camera (DualView 300W; Gatan, Pleasanton, CA) controlled by DigitalMicrograph software (version 3.8.1; Gatan).

Photomicrograph production

Some digitally acquired micrographs were adjusted only for brightness and contrast, while maintaining the pixel dimension contrast, with either DigitalMicrograph or Photoshop 7.0 (Adobe System, Inc., San Jose, CA) to optimize the quality of the images for analysis.

Analysis of the material

For quantitative measurements of the relative abundance of the different immunoreactive elements for each receptor subtype in CM/PF, only those monkeys with the best ultrastructural preservation were used. For the peroxidase analysis, the animals were MR73 and MR75 for GABA_BR1 and MR72 and MR74 for GABA_BR2. For the preembedding immunogold analysis, the animals were MR75 and MR87 for GABA_BR1 and MR75 and MR100 for GABA_BR2.

Taking into consideration the limited access of antibodies to antigenic sites deep in the sections, and to minimize false negatives, electron microscopic ultrathin sections were obtained from the surface of each block, and areas with optimal immunolabeling and good ultrastructural preservation were photographed. Furthermore, considering that GABA_BR1 and GABA_BR2 antibodies may display different degrees of sensitivity for their antigenic sites, we compared the distribution of GABA_BR1 and GABA_BR2 labeling based on the relative percentages of immunoreactive elements for either receptor subtype. By using this approach, we avoid misinterpretation of false-negative labeling resulting from technical issues, because the ground for comparison relies exclusively on labeled structures. A similar approach was used previously in our laboratory to quantify GABA_B receptors in other nuclei (Charara et al., 2004; Galvan et al., 2004) or other receptor subtypes throughout the basal ganglia (Hanson and Smith, 1999; Hubert et al., 2001; Kuwajima et al., 2004).

Immunoperoxidase labeling. Ultrathin sections from the most superficial sections of blocks were scanned at $\times 20,000$, and all immunoreactive elements randomly encountered were photographed. The total surface of tissue analyzed for GABA_BR1 in CM and PF was 2,567 μm^2 and 5,890 μm^2 , respectively, and, for GABA_BR2, 2,898 μm^2 of CM tissue and 5,969 μm^2 of PF were examined. The total number of blocks of tissue/animal analyzed was, for GABA_BR1, CM: two blocks/monkey MR73 and two blocks/monkey MR75; PF: one block/monkey MR73 and three blocks/monkey MR75; and, for GABA_BR2, CM and PF: two blocks/monkey MR72 and two blocks/monkey MR74.

The labeled elements were categorized as somatic profiles, dendrites, vesicle-containing dendrites, axon-like processes, and axon terminals based on ultrastructural criteria defined by Peters et al. (1991). Although the presence of DAB deposit complicated the identification of small-diameter processes, these were categorized as axon-like processes based on their small diameter ($<0.3 \mu\text{m}$), absence of mitochondria, lack of synaptic input, and occasional presence of neurofilaments and synaptic vesicles. The relative proportion of each element was calculated and expressed as a percentage of total number of labeled elements for each receptor subtype in each nucleus. Statistical differences in the pattern of distribution of the two GABA_B receptor subunits were assessed with nonparametric chi-square analysis (StatView 5.0).

Preembedding immunogold labeling. Ultrathin sections from the surface of the blocks were examined under the electron microscope, and elements containing gold particles were photographed at $\times 20,000$ – $31,500$. The immunogold particles for GABA_BR1 and GABA_BR2 were counted and categorized as intracellular or bound to the plasma membrane. To determine the location of GABA_BR1 and GABA_BR2 relative to synapses, the membrane-associated gold particles were categorized as 1) synaptic, when they were in the main body of symmetric or asymmetric synaptic specialization (pre- or postsynaptic); 2) perisynaptic, when they were at the edges of symmetric or asymmetric synaptic specializations; 3) extrasynaptic-postsynaptic, when they were laying along the nonsynaptic portion of the plasma membrane; and 4) extrasynaptic-presynaptic, when they were associated with nonsynaptic membrane or the intracellular compartment of the terminal. To avoid misinterpretations resulting from nonspecific light background labeling, immunoreactive elements had to contain a minimum of three gold particles to be considered in this analysis. The results for quantification were expressed as relative percentages of total gold particle labeling, and nonparametric chi-square analysis (StatView 5) was used to compare the distribution of the two GABA_B receptor subunits.

Double immunolabeling. For double labeling, data were collected in the same way as previously described, but in this case only immunogold-labeled elements making synaptic contact with immunoperoxidase-labeled boutons were photographed. To avoid false-negative resulting from the poor penetration of gold particles, analysis was restricted to the most superficial sections where both the peroxidase- and the gold-conjugated antibodies had access to their antigenic sites.

RESULTS

Immunoblotting

Antibody specificity was examined by using Western blot analysis and preadsorption tests on monkey and rat striatal tissue for a recently generated antibody against vGluT1 (Mab Technologies). Immunoblot analysis of monkey and rat striatal tissue showed that the anti-vGluT1 antibody recognized a single band at the molecular weight predicted for vGluT1 ($\sim 60 \text{ kDa}$; Fig. 1A). Immunoperoxidase staining of monkey and rat (data not shown) striatal tissue showed immunoreactivity in the neuropil (Fig. 1B), as previously reported for rat striatum (Fremeau et al., 2001; Herzog et al., 2001; Varoqui et al., 2002). Furthermore, this immunoreactivity was abolished when the anti-vGluT1 antibody was preadsorbed with the synthetic peptide of antigenic sequence against which it was generated (Fig. 1C). However, the immunoreactivity was maintained when the anti-vGluT1 antibody was preadsorbed with the synthetic peptide for vesicular glutamate transporter 2 (vGluT2; Fig. 1D). The tissue was devoid of immunoreactivity when the primary antibodies were omitted from the incubation solution (Fig. 1E).

At the ultrastructural level, the anti-vGluT1 antibodies labeled exclusively axon terminals forming asymmetric synapses mostly with dendritic spines and distal axons containing synaptic vesicles (Raju and Smith, 2005), as previously reported in the rat striatum using other specific vGluT1 antisera (Fremeau et al., 2001; Herzog et al., 2001; Varoqui et al., 2002).

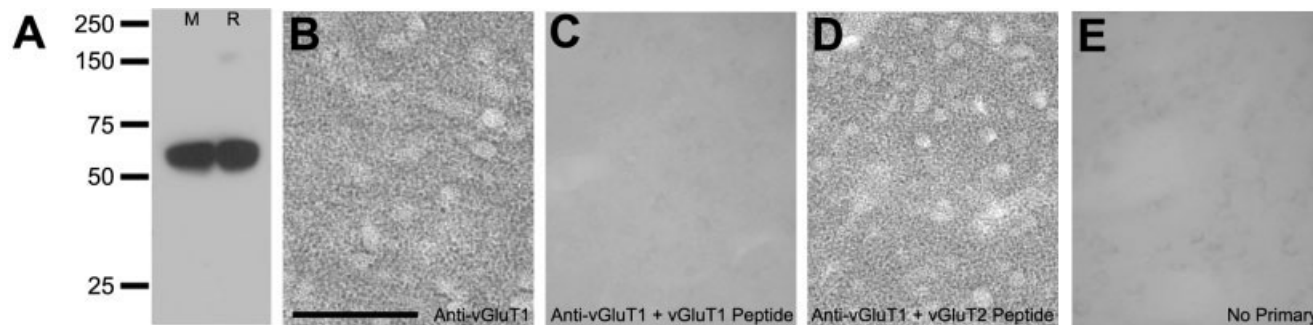


Fig. 1. Rabbit anti-vGluT1 antibody specificity (Mab Technologies, Atlanta, GA). **A:** Western blot analysis with anti-vGluT1 antibody shows bands in both monkey and rat striatal tissue at ~60 kDa, corresponding to the molecular weight predicted for vGluT1. **B:** Light microscopic examination of monkey striatal tissue with anti-vGluT1 antibodies demonstrates neuropil immunoreactivity. **C,D:** Immunolabeling in monkey striatum is abolished when antibodies are pread-

sorbed with their corresponding peptides (C) but is preserved when antibodies are preadsorbed with similar but nonidentical synthetic peptides (D). **E:** Immunostaining of monkey striatal tissue without primary antibodies is shown for reference. Molecular weight standards are indicated at left (kDa). Scale bar = 200 μ m in B (applies to B–E).

General distribution of GABA_BR1 and GABA_BR2 in the CM/PF complex

At the light microscopic level, moderate to strong immunostaining for GABA_BR1 and GABA_BR2 was found throughout the monkey thalamus. Overall, dorsal thalamic nuclei showed a higher level of immunostaining than ventral thalamic nuclei, except for the intralaminar complex, in which the labeling for both receptor subtypes was intense but lower than in the surrounding nuclei, including the mediodorsal, periventricular, pulvinar, and ventroposterior. This pattern of immunostaining is consistent with the expression of GABA_B receptor gene transcripts (Muñoz et al., 1998) and GABA_B receptor binding sites (Bowery et al., 1999) in the monkey thalamus.

A similar pattern of immunoreactivity was found for GABA_BR1 and GABA_BR2 in CM/PF in DAB-stained (Fig. 2A,B) or gold-stained (Fig. 2C,D) sections. In DAB-processed material, strong immunoreactivity was observed in both large and small cell bodies and primary dendrites (Fig. 2A,B). The DAB reaction product was diffusely distributed but also formed small aggregates within the cytoplasm of labeled neurons (Fig. 2B). In general, perikarya and proximal dendrites displayed a lighter level of immunoreactivity in tissue sections processed for immunogold, but abundant immunoreactive processes and punctate elements for both subunits formed a dense neuropil meshwork in both nuclei (Fig. 2C,D).

Subcellular localization of GABA_BR1 and GABA_BR2 in CM/PF

To study the overall cellular and subcellular distribution of the GABA_B receptor subunits in CM/PF, we used the ABC electron microscopy immunoperoxidase method. In the electron microscope, the DAB-labeled antigenic sites were recognized by the presence of amorphous electron-dense deposit (Figs. 3, 4A–C). Immunoreactivity for GABA_BR1 (Figs. 3A–C, 4A) and GABA_BR2 (Fig. 3D–F, 4B,C) was observed predominantly in dendritic processes (Fig. 3A,B,D,E), although small, unmyelinated, axon-like processes (Fig. 3A,B,D,E) and rare axon terminals (Fig. 3C,F) were also immunolabeled in CM and PF nuclei.

In both nuclei, neuronal soma of large projection neurons (PN) or small local circuit neurons (LCN) were im-

munoreactive for GABA_BR1 and GABA_BR2 (Fig. 4A,B). In general, the immunoperoxidase deposit was diffusely distributed into the labeled cell bodies, with occasional intense patches of staining mainly associated with the rough endoplasmic reticulum (see immunogold data in Fig. 6). In labeled dendrites of PN, the immunoperoxidase deposit was associated mainly with microtubules (Fig. 3A,B,D,E), whereas, in vesicle-containing dendrites (vcd) of LCN and axon terminals, the labeling was often associated with the external surface of synaptic vesicles (Figs. 3C,F, 4C).

To assess further the proportion of LCN dendrites expressing GABA_BR1 or GABA_BR2, labeled and unlabeled vesicle-containing dendrites (vcd) for either receptor subtypes were counted in sections of CM/PF. This series of experiments revealed that 17% and 15% of LCN dendrites were immunoreactive for GABA_BR1 in CM and PF, respectively, whereas 15% and 14% were labeled for GABA_BR2 in these nuclei (Fig. 4D).

Because GABA_BR1 and GABA_BR2 form functional heterodimers, one would expect these two receptor subtypes to display a similar pattern of subcellular distribution. For both subunits in both thalamic nuclei, almost 80% of the total labeling was found in dendrites, and the remaining labeled elements were accounted for by small, unmyelinated, axon-like processes (CM: 19% and 20% for GABA_BR1 and GABA_BR2, and PF: 23% and 25% for GABA_BR1 and GABA_BR2, respectively). Less than 5% of labeling was found in axon terminals, and less than 1% was associated with LCN dendrites (Fig. 5). No significant differences were found in the pattern of GABA_BR1 and GABA_BR2 labeling in CM and PF, except for the percentage of GABA_BR2-immunoreactive terminals in PF, which was significantly higher than for GABA_BR1 (chi-square analysis, $P < 0.01$; Fig. 5B).

Subsynaptic localization of GABA_BR1 and GABA_BR2

The detailed subcellular and subsynaptic localization of GABA_BR1 and GABA_BR2 in the caudal intralaminar thalamic nuclei was studied by using the preembedding immunogold method at the electron microscopic level. Although this method is less sensitive than the immunoperoxidase technique, the main advantage of the

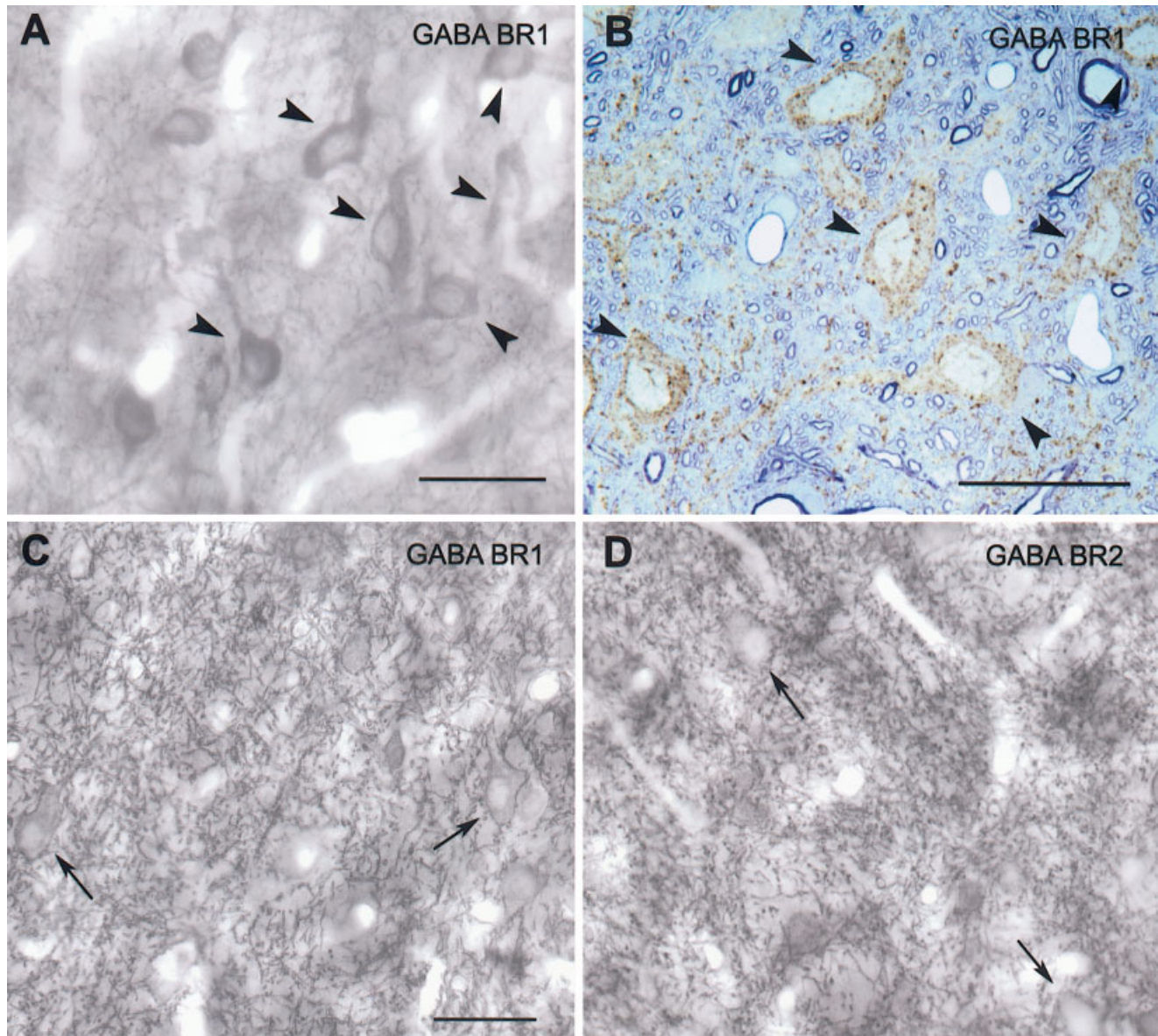


Fig. 2. Light photomicrographs of DAB (A,B)- and gold (C,D)-immunolabeled sections for GABA_BR1 and GABA_BR2 in CM/PF. **A:** DAB-immunostained neurons for GABA_BR1 in CM. **B:** Semithin (~1 μm) resin section counterstained with 1% toluidine blue. This section was obtained from the same area photographed in A and shows the distribution of the DAB reaction product within the cytoplasm of immunostained neurons. Arrowheads indicate the same

labeled neurons in A and B. **C,D:** Immunogold labeling for GABA_BR1 (C) and GABA_BR2 (D) in CM and PF. Light immunolabeling is found in perikarya and proximal dendrites (arrows), but the neuropil of both nuclei is enriched in strongly labeled dendrite-like processes and small punctate elements. Scale bars = 50 μm in A,B; 50 μm in C (applies to C,D).

immunogold method is the higher level of spatial resolution of the gold particles compared with the diffusible DAB reaction product, allowing a more accurate assessment of the spatial localization of GABA_B receptor sites. Despite the technical limitation inherent to the sensitivity of the preembedding immunogold technique to assess the extent of synaptic labeling, this method provides a detailed map of extrasynaptic and perisynaptic GABA_B receptors. Additional postembedding labeling will be necessary to confirm the precise extent of GABA_B synaptic receptors (Figs. 6, 7). However, it is noteworthy that a previous study that used pre-

and postembedding methods to examine GABA_BR1/R2 distribution in the rat thalamus did not report major differences between the degree of synaptic labeling generated with these two immunogold methods (Kulik et al., 2002).

Consistently with the peroxidase observations, the immunogold method resulted in strong labeling in both thalamic nuclei. The subcellular distribution of GABA_BR1 and GABA_BR2 immunogold labeling in CM and PF was determined by counting the number of intracellular and plasma membrane-bound gold particles as described in Materials and Methods.

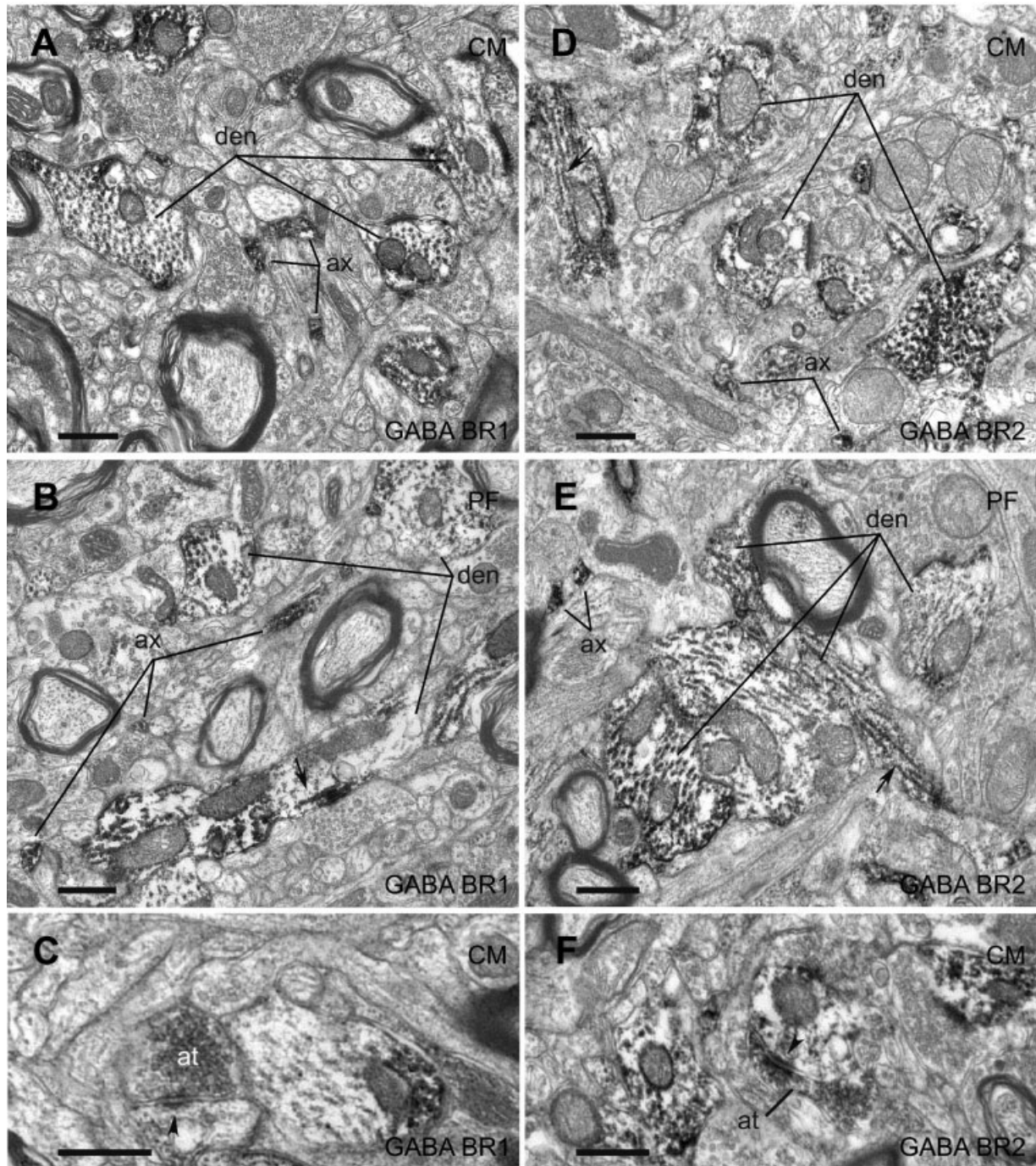


Fig. 3. Electron micrographs showing immunoperoxidase staining for GABA_BR1 and GABA_BR2 in CM and PF. **A–C:** GABA_BR1 immunostaining in CM (A,C) and PF (B). **D–F:** GABA_BR2 immunostaining in CM (D,F) and PF (E). **A,B:** GABA_BR1 immunostaining is found mainly in dendrites (den) and small axon-like processes (ax) in CM and PF. **C:** A GABA_BR1-immunolabeled axon terminal (at) forming an axodendritic asymmetric synapse (arrowhead) with a nonimmunoreactive dendrite in CM. **D,E:** A similar pattern of immunostaining is

found for GABA_BR2 in CM and PF, with abundant labeling in dendrites (den) and axon-like processes (ax). **F:** A GABA_BR2-immunoreactive axon terminal (at) forming an asymmetric synapse (arrowhead) with an immunolabeled dendrite (den) in CM. Note the peroxidase labeling associated mainly with microtubules in GABA_BR1- and GABA_BR2-immunostained dendrites (arrows). Scale bars = 0.5 μ m.

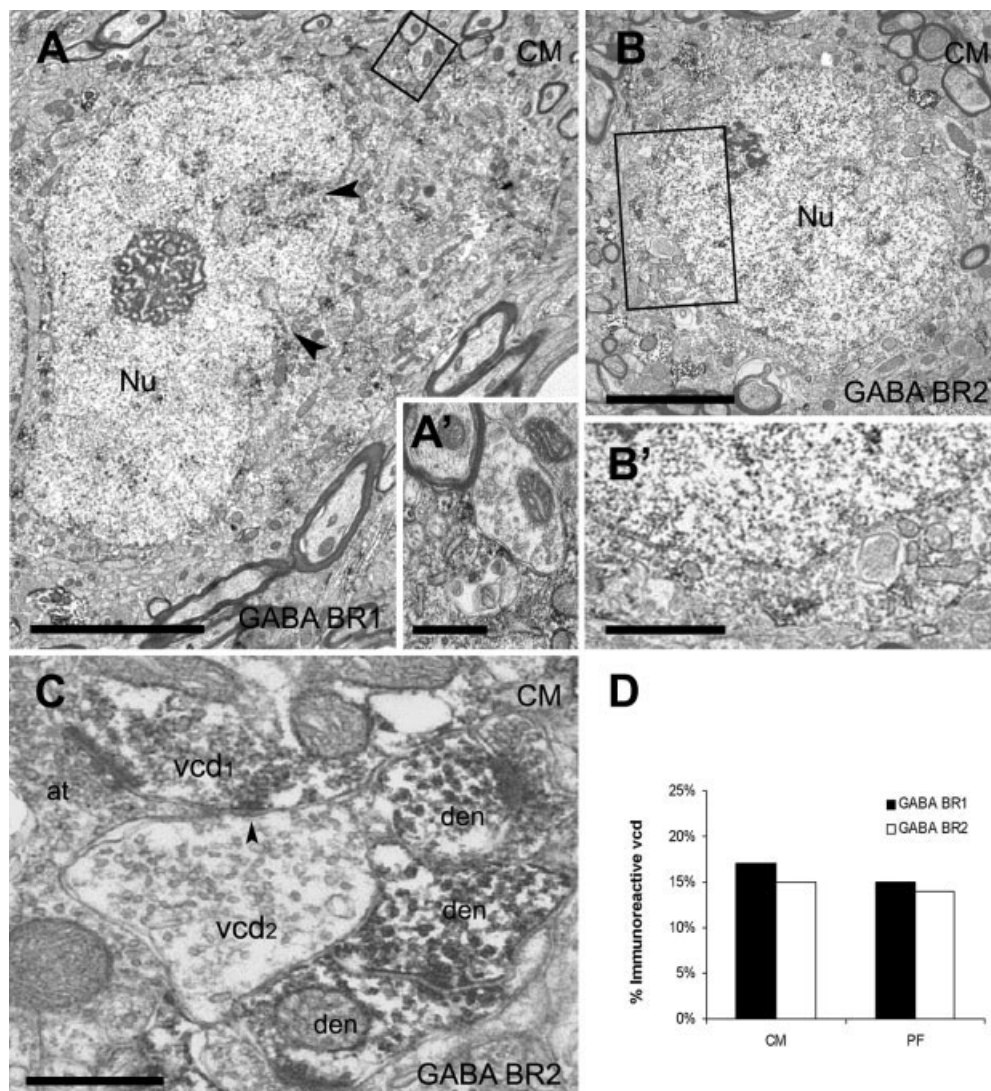


Fig. 4. Electron micrographs of neuronal perikarya (A,B) and vesicle-containing dendrites (vcd) of LCN (C,D) labeled for GABA_BR1 and GABA_BR2 in the CM/PF thalamic nuclei. **A**: Example of large projection neuron (PN) immunoreactive for GABA_BR1 in CM. Note the eccentric location of the nucleus (Nu) and the indented nuclear envelope (arrowheads). **A'**: Boxed area in A, showing an unlabeled vcd tightly apposed to the membrane of the immunoreactive PN. **B**: Low-power micrograph of a local circuit neuron (LCN) immunostained for GABA_BR2 in CM. The soma is mainly occupied by the nucleus (Nu), with only a thin rim of cytoplasm. **B'**: Enlargement of the boxed area in B, showing the diffuse deposit of DAB reaction product and the

small number of organelles in the cytoplasm, except for mitochondria. **C,D**: Examples of vcds forming different synaptic arrangements in the neuropil of CM (C) and PF (D). **C**: A vcd immunoreactive for GABA_BR2 (vcd₁) forming a dendrodendritic symmetric synapse (arrowhead) with a nonlabeled vcd (vcd₂) in CM. Note that vcd₁ receives an asymmetric axodendritic synapse from an unlabeled terminal (at). Many immunoreactive dendrites are also identified (den). **D**: Histogram comparing the relative proportion of GABA_BR1- and GABA_BR2-immunolabeled vcds in CM and PF. For GABA_BR1 the total number of vcd examined was 30 in CM and 32 in PF and for GABA_BR2 20 in CM and 21 in PF. Scale bars = 5 μm in A,B; 0.5 μm in A',C; 2 μm in B'.

GABA_BR1 and GABA_BR2 immunolabeling was observed in the soma of both PN and LCN in CM and PF (Fig. 6). For both subunits, and in both cell types, quantitative analysis showed that under 20% of the immunogold particles were bound to the plasma membrane (Fig. 6D). The majority of labeling (>80%) was located in the cytoplasm (Fig. 6A,A',D), where it was associated mainly with the membranes of the rough endoplasmic reticulum (Fig. 6A'). Plasma membrane-bound cell body labeling for GABA_BR1 and GABA_BR2 in PN and LCN was all extrasynaptic in both nuclei. In some instances, GABA_B recep-

tor labeling was associated with close appositions between immunoreactive cell bodies and LCN dendrites or large axon terminals (Fig. 6B,C).

Immunolabeling for both GABA_B receptor subunits was also strongly expressed in dendrites of PN in CM and PF (Fig. 7A–D). For GABA_BR1, most gold particles (69% in CM and 78% in PF) were associated with the plasma membrane, whereas, for GABA_BR2, the proportion of gold particles bound to the plasma membrane (55% in CM and 60% in PF) was only slightly higher than the proportion of intracellular labeling (Fig. 7E). These intracellular gold

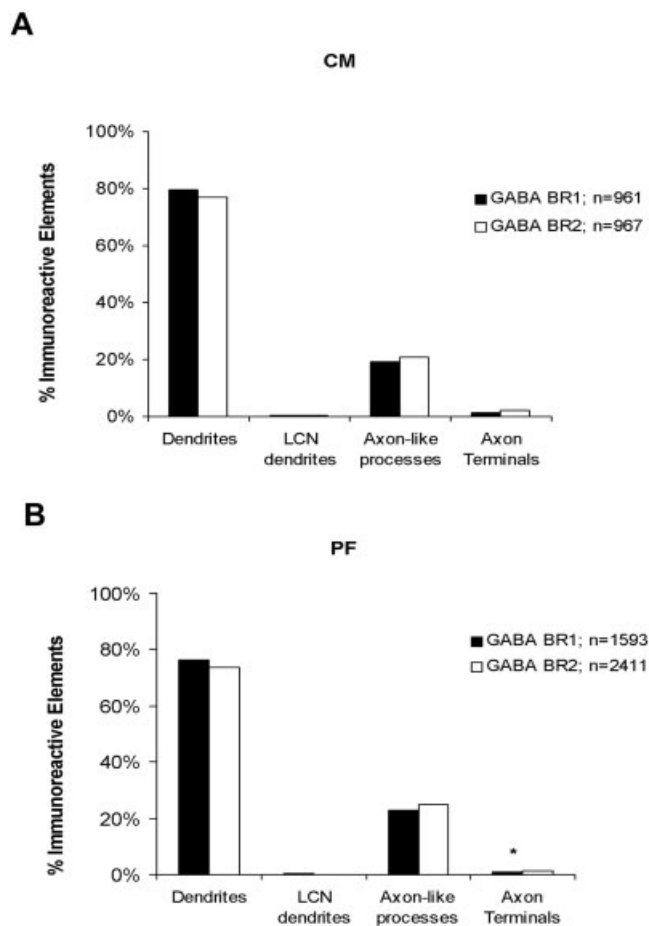


Fig. 5. Relative proportion of GABA_BR1- and GABA_BR2-immunoreactive elements in the CM/PF as revealed by the immunoperoxidase method. **A,B:** Histograms comparing the relative proportion of GABA_BR1- and GABA_BR2-immunolabeled elements in CM (A) and PF (B). For this analysis, we used four animals, two for GABA_BR1 and two for GABA_BR2. The statistical analysis (Chi-square analysis) showed no significant difference in the distribution of immunoreactive elements for both subunits, except for GABA_BR2-immunoreactive terminals in PF (*, $P < 0.01$). No significant interindividual differences in the pattern of labeling between animals (Chi-square analysis) were observed. n, Total number of immunolabeled elements.

particles were associated with vesicular organelles or bound to the smooth endoplasmic reticulum and microtubules (Fig. 7A–D). Statistical analysis (chi-square) showed that the intracellular labeling for GABA_BR2 was significantly higher than for GABA_BR1 in both CM and PF (Chi-square: 87.7, $P < 0.0001$ in CM; 53.9, *, $P < 0.0001$ in PF; Fig. 7E).

The comparative analysis of the distribution of synaptic and extrasynaptic immunogold particles for GABA_BR1 and GABA_BR2 in dendritic profiles in CM and PF (Fig. 7F) showed that the plasma membrane immunolabeling for both subunits was found mainly at extrasynaptic sites (93% for GABA_BR1 and GABA_BR2 in CM, 92.5% for GABA_BR1 and 98% for GABA_BR2 in PF). Immunogold particles labeling for GABA_BR1 associated with synapses (7.5%) was significantly higher than for GABA_BR2 (2%) synaptic labeling in PF (chi-square: 16.67, *, $P < 0.0001$).

Quantitative analysis of immunogold particles located at synaptic sites revealed that GABA_BR1 and GABA_BR2 immunolabeling was found in the main body or at the edges of symmetric synapses (Fig. 8A). Although there was no difference in the overall pattern of distribution between the two receptor subunits at symmetric synapses in CM, the proportion of synaptic labeling for GABA_BR1 and GABA_BR2 was twice more common than the level of perisynaptic labeling in PF (Fig. 8A).

Extrasynaptic labeling for GABA_BR1 and GABA_BR2 (Fig. 8B) was observed in axon terminals forming either symmetric or asymmetric synapses with dendritic shafts in CM and PF. In both thalamic nuclei, presynaptic immunogold particles labeling for GABA_BR1 (67% in CM and 73% in PF) and GABA_BR2 (71% in CM and PF) were mainly intracellular or extrasynaptic. It was located inside the terminal or associated with nonsynaptic sites along the terminal plasma membrane (Fig. 7C,D). Only ~10–20% of the presynaptic labeling was associated with the active zone of symmetric and asymmetric synapses (Figs. 7B, 8B). In both types of synapses, immunogold particles were found in the core or at the edge of presynaptic specializations (Figs. 7B–D, 8B).

Double immunolabeling

To characterize further the neurotransmitter content of terminals associated with GABA_B receptors labeling, we used a double immunocytochemical approach to localize GABA_BR1/R2 labeling with vGluT1 immunoreactivity. In this material, vGluT1 immunolabeling was localized with DAB, whereas GABA_BR1/GABA_BR2 immunoreactivities were detected with immunogold (Fig. 9). Results of these experiments confirm and extend our single-labeling data showing that postsynaptic GABA_BR1/GABA_BR2 immunoreactivity is expressed at the edges of asymmetric synapses formed by vGluT1-containing terminals in the CM/PF complex (Fig. 9A,C,D). Gold particles were never seen in the core of asymmetric synapses. Although much less frequently encountered, presynaptic immunoreactivity for GABA_BR1 or GABA_BR2 was expressed in vGluT1-labeled terminals, either extrasynaptically or in the active zones (Fig. 9B).

DISCUSSION

The present study provides the first description of the subcellular and subsynaptic distribution of GABA_BR1 and GABA_BR2 in the monkey caudal intralaminar nuclear complex. The following conclusions can be drawn from our observations. 1) There is no major difference in the pattern of subcellular and subsynaptic distribution of either GABA_B receptor subunit between CM and PF. 2) Both subunits are heavily expressed postsynaptically and largely confined to dendrites and axon-like processes but are rarely found in axon terminals. 3) About 20% of vesicle-containing dendrites of LCN neurons express GABA_BR1 and GABA_BR2 immunoreactivity in both nuclei. 4) Postsynaptic GABA_B receptor labeling is largely associated with extrasynaptic and perisynaptic sites at symmetric and asymmetric axodendritic and axosomatic synapses. 5) There is a significant difference in the pattern of subcellular distribution of GABA_BR1 and GABA_BR2 in CM and PF. Seventy to eighty percent of GABA_BR1 labeling is found on the plasma membrane, whereas GABA_BR2 immunoreactivity is more evenly distributed between the

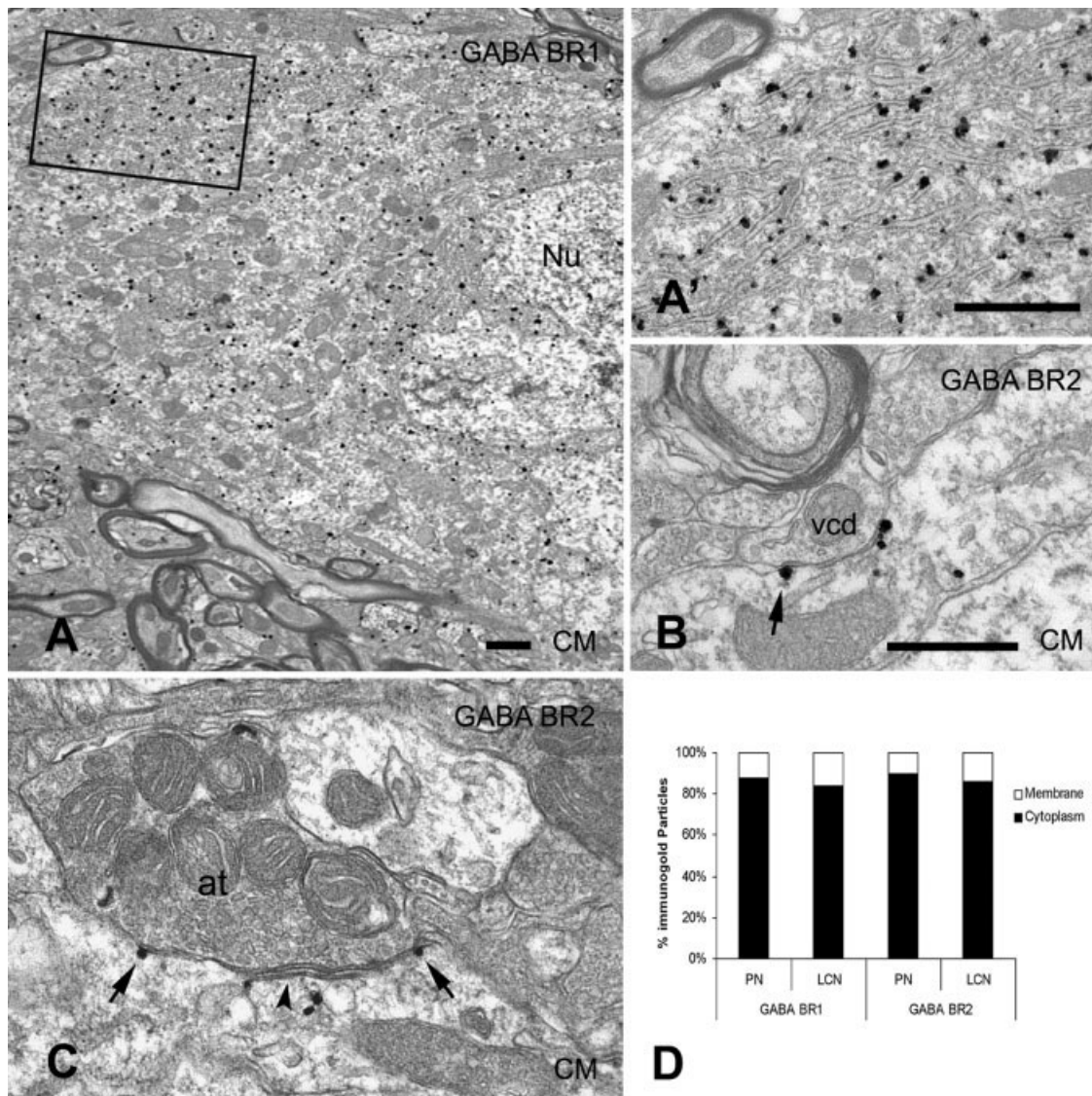


Fig. 6. Subsynaptic distribution of GABA_BR1 and GABA_BR2 immunolabeling in PN and local circuit neurons LCN in CM/PF. **A,A'**: GABA_BR1 cytoplasmic labeling in a PN in CM. The boxed area in A is shown at a higher magnification in A' to illustrate the association of the immunogold particles with the rough endoplasmic reticulum. **B**: GABA_BR2 labeling (arrow) at a site of apposition between an unlabelled vesicle-containing dendrite (vcd) and the cell body of a PN in CM. **C**: A large terminal with numerous mitochondria and pleomorphic vesicles closely apposed to a GABA_BR2-labeled LCN in CM.

Note the GABA_BR2 immunogold labeling at a site of apposition between the terminal and the cell body (arrows). Intracellular labeling close to subsurface cisterns was occasionally associated with synapses formed by large terminals (arrowhead). **D**: Histogram showing the distribution of the subcellular labeling for GABA_BR1 and GABA_BR2 in the soma of PN and LCN. In total, 19 neuronal cell bodies were analyzed for GABA_BR1 (14 PN and 5 LCN) and 17 for GABA_BR2 (13 PN and 4 LCN). Scale bars = 1 μ m in A,A'; 0.5 μ m in B (applies to B,C).

plasma membrane and the intracellular compartment. 6) Some of the GABA_BR1- and GABA_BR2-containing terminal boutons forming asymmetric synapses express vGluT1 immunoreactivity. These findings provide a solid framework whereby GABA_B receptors could be involved in regulating extrinsic and intrinsic GABAergic and glutamatergic inputs to the CM/PF complex in primates.

Subcellular localization of GABA_B receptors in CM/PF

Intense postsynaptic immunolabeling for GABA_BR1 and GABA_BR2 was found in CM/PF, being mostly associ-

ated with dendritic elements. It is important to note that the pattern of subcellular distribution of GABA_BR1 and GABA_BR2 varies substantially between brain regions. For instance, the pattern of labeling in the rat ventrobasal thalamic nucleus, cerebellum, and hippocampus (Kulik et al., 2002, 2003; López-Bendito et al., 2004) resembles that described here for CM/PF, i.e., dendritic immunolabeling for either GABA_B receptor subunits being mostly associated with the plasma membrane. On the other hand, GABA_BR1 subunit labeling is largely expressed intracellularly in various basal ganglia nuclei in rats and monkeys (Smith et al., 2000, 2001; Boyes and Bolam, 2003;

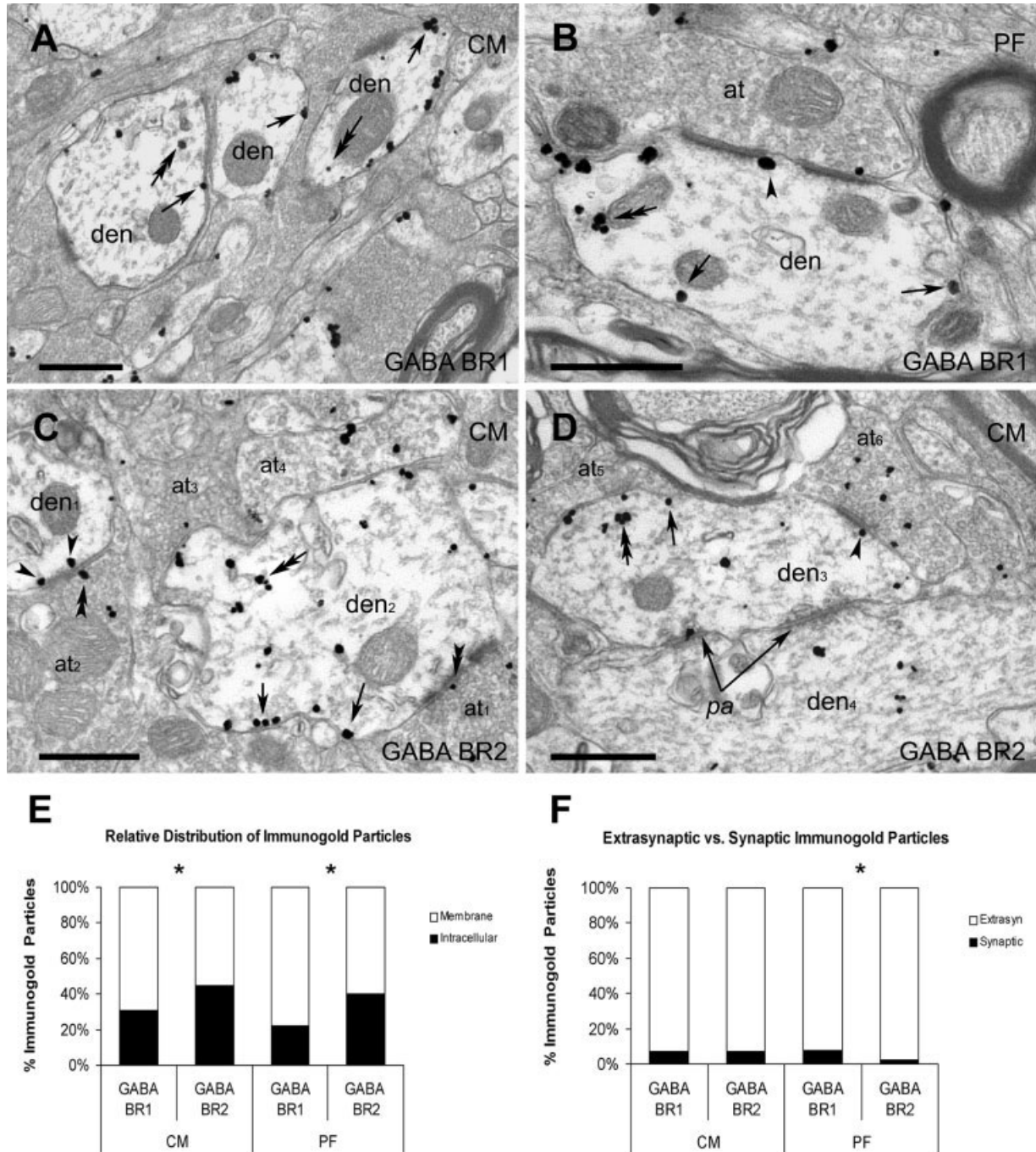


Fig. 7. Ultrastructural localization of immunogold labeling for GABA_BR1 and GABA_BR2 in CM and PF. **A:** Labeling for GABA_BR1 is found in numerous dendrites (den) in CM. Gold particles are associated mainly with the internal surface of the plasma membrane (single arrows) but are also found in intracellular sites (double arrows). **B:** Postsynaptic labeling for GABA_BR1 in PF. In the dendrite (den), gold particles are mainly attached to extrasynaptic sites (arrows) along the plasma membrane. Note also a gold particle at the edge of the postsynaptic density (arrowhead). **C,D:** Immunogold labeling for GABA_BR2 in CM. Immunogold particles are present mostly along extrasynaptic sites on the dendritic plasma membrane (single arrows in C and D) and occasionally at the edge of asymmetric synapses (single arrowheads in den₁ and den₃). Note the presence of numerous intracellular gold particles (double arrows in den₂ and den₃). Some immunogold particles are observed on the presynaptic membrane (double arrowheads in at₁ and at₂) or intracellularly (at₂ and at₆) in axon terminals forming asymmetric synapses. Note also a gold parti-

cle associated with a puncta adherens-like contact (pa) between den₃ and den₄ in D. **E,F:** Histograms showing the relative proportion and distribution of GABA_BR1 and GABA_BR2 immunogold particles labeling in CM and PF. **E:** Plasma membrane-bound immunogold particles vs. intracellular labeling. The percentage of GABA_BR1 immunogold labeling associated with the plasma membrane is significantly higher than the proportion of plasma membrane-bound GABA_BR2 labeling (chi-square analysis, *, $P < 0.0001$). **F:** Comparative distribution of synaptic and extrasynaptic immunogold particles for GABA_BR1 and GABA_BR2. For both receptor subunits, immunolabeling was found mainly extrasynaptically (90–95%). Statistical analysis demonstrated that the proportion of synaptic GABA_BR1 immunogold particles was significantly higher than for GABA_BR2 in PF (*, $P < 0.0001$). The number of gold particles/dendrites sampled in CM and PF was 2,501/315 for GABA_BR1 in CM, 617/100 for GABA_BR1 in PF, 1,829/230 for GABA_BR2 in CM, and 887/110 for GABA_BR2 in PF. Scale bars = 0.5 μ m.

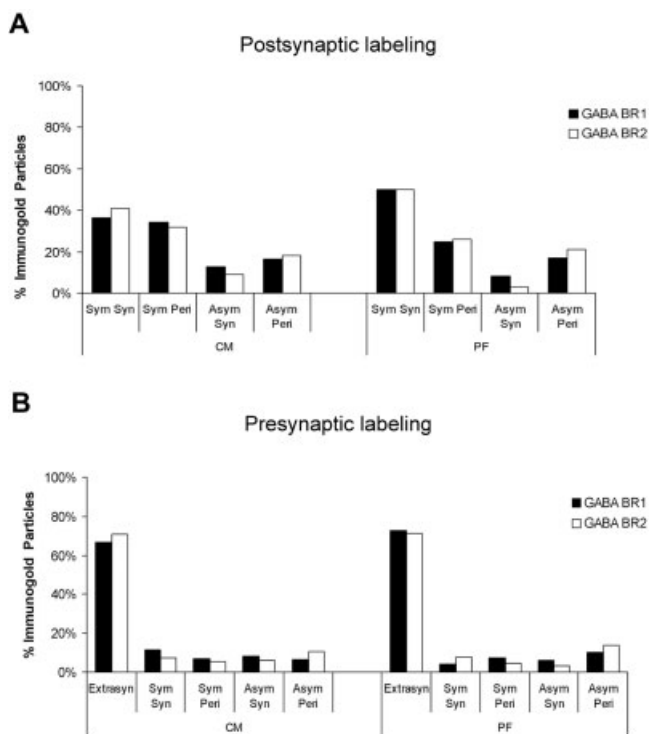


Fig. 8. Subsynaptic distribution of postsynaptic (A) and presynaptic (B) immunogold particles labeling for GABA_BR1 and GABA_BR2 in CM and PF. **A:** Relative distribution of immunogold particles associated with the postsynaptic plasma membrane of symmetric (sym) and asymmetric (asym) synapses. One hundred percent is the total number of gold particles associated with synaptic sites on the plasma membrane. **B:** Relative distribution of presynaptic GABA_BR1 and GABA_BR2 immunogold particles located in axon terminals that form symmetric and asymmetric synapses in CM and PF. One hundred percent is the total number of gold particles in the immunolabeled axon terminals. Each gold particle is categorized as extrasynaptic (Extrasyn), synaptic (Syn), or perisynaptic (Peri). Total number of gold particles/elements sampled postsynaptic in CM: 126/70 dendrites for GABA_BR1 and 70/45 dendrites for GABA_BR2, postsynaptic in PF: 36/25 for GABA_BR1 and 38/20 for GABA_BR2; presynaptic in CM: 156/35 terminals for GABA_BR1 and 150/36 terminals for GABA_BR2, presynaptic in PF: 70/25 terminals for GABA_BR1 and 66/20 terminals for GABA_BR2.

Chen et al., 2004; Galvan et al., 2004; Charara et al., 2005), suggesting the possibility of different trafficking and synaptic targeting mechanisms to regulate GABA_B receptor plasma membrane expression and functions in different brain regions.

In a functional GABA_B receptor, GABA_BR1 is the subunit responsible for the binding of GABA, and GABA_BR2 is required to bring GABA_BR1 to the cell surface (White et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999). It is believed that association of GABA_BR1 and GABA_BR2 is necessary to form functional GABA_B receptors (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Benke et al., 1999; Kuner et al., 1999; Bowery and Enna, 2000; Calver et al., 2001; Bowery et al., 2002; Bettler et al., 2004; Cryan and Kaupmann, 2005). When expressed alone, GABA_BR1 is retained in the endoplasmic reticulum as a quality-control mechanism preventing incorrectly folded GABA_B receptors from reaching the cell surface

(Couve et al., 1998; Bettler et al., 2004). Expression of GABA_BR1 on the cell surface or its retention in the cytoplasm is an obvious regulatory mechanism of GABAergic neurotransmission. Taking into account that the GABA_BR2 subunit is also necessary for proper G-protein signaling in response to agonist stimulation (Couve et al., 2000; Jones et al., 2000; Calver et al., 2001; Margeta-Mitrovic et al., 2001a; Robbins et al., 2001; Bettler et al., 2004; Pin et al., 2004), a similar pattern of subcellular plasma membrane expression for both subunits would be expected. Therefore, the significant differences between the intracellular and the plasma membrane expression of GABA_BR1 and GABA_BR2 subunits that were found in the monkey CM/PF complex suggest that some of the plasma membrane immunolabeling for GABA_BR1 likely corresponds to nonfunctional GABA_B receptors, in that GABA_BR1 is incapable of G-protein activation (Pin et al., 2004). Another possibility is that GABA_B receptor trafficking to the plasma membrane occurs by heterooligomerization of GABA_BR1 subunit with other receptor subunits, such as the GABA_A receptor $\gamma 2$, as recently shown in vitro (Balasubramanian et al., 2004), or other GABA_B subunits that have not yet been identified. It is interesting to note that a new positive allosteric regulator of the GABA_BR2 subunit, CGP7930, has recently been described (Binet et al., 2004; Pin et al., 2004), opening up new possibilities for therapeutic regulation and/or activation of GABA_B receptors under pathological conditions. It is noteworthy that correlation between GABA_BR1 and GABA_BR2 distributions is a common, but not a general, phenomenon in the CNS (Charara et al., 2000, 2004; Clark et al., 2000; Ng and Yung, 2001), suggesting that other molecular mechanisms and/or additional GABA_B receptor subunits may be involved in the formation of native GABA_B receptors in some brain regions (Kaupmann et al., 1998; Durkin et al., 1999; Calver et al., 2000, 2003; Mezler et al., 2001; Charara et al., 2004).

GABA_B receptor are expressed mostly at extrasynaptic sites

The immunogold labeling for GABA_BR1 and GABA_BR2 was found mainly at extrasynaptic sites in dendrites and axon terminals, a pattern of distribution reminiscent of that described in other brain regions, including the rat ventrobasal thalamus (Fritschy et al., 1999; Smith et al., 2000, 2001; Kulik et al., 2002; Boyes and Bolam, 2003; Chen et al., 2004; Galvan et al., 2004; Charara et al., 2005). This extrasynaptic localization of GABA_B receptors is consistent with electrophysiological data showing that prolonged and high-frequency burst discharge of inhibitory neurons are required to activate GABA_B receptors (Mody et al., 1994; Kim et al., 1997), indicating a neuro-modulatory role for these receptors. If most of the GABA_B receptor effects occur at extrasynaptic sites, this raises various issues regarding the sources of GABA and regulatory mechanisms of these receptors activation. Considering the high sensitivity of GABA_B receptors to GABA (Sodickson and Bean, 1996), spillover and extrasynaptic diffusion of GABA from adjacent GABAergic synapses have been suggested as mechanisms by which extrasynaptic receptors are activated (Atwell et al., 1993; Vogt and Nicoll, 1999; Scanziani, 2000). Consistently with this hypothesis, a recent in vivo analysis demonstrated that local application of GABA transporter (GAT) blockers increases

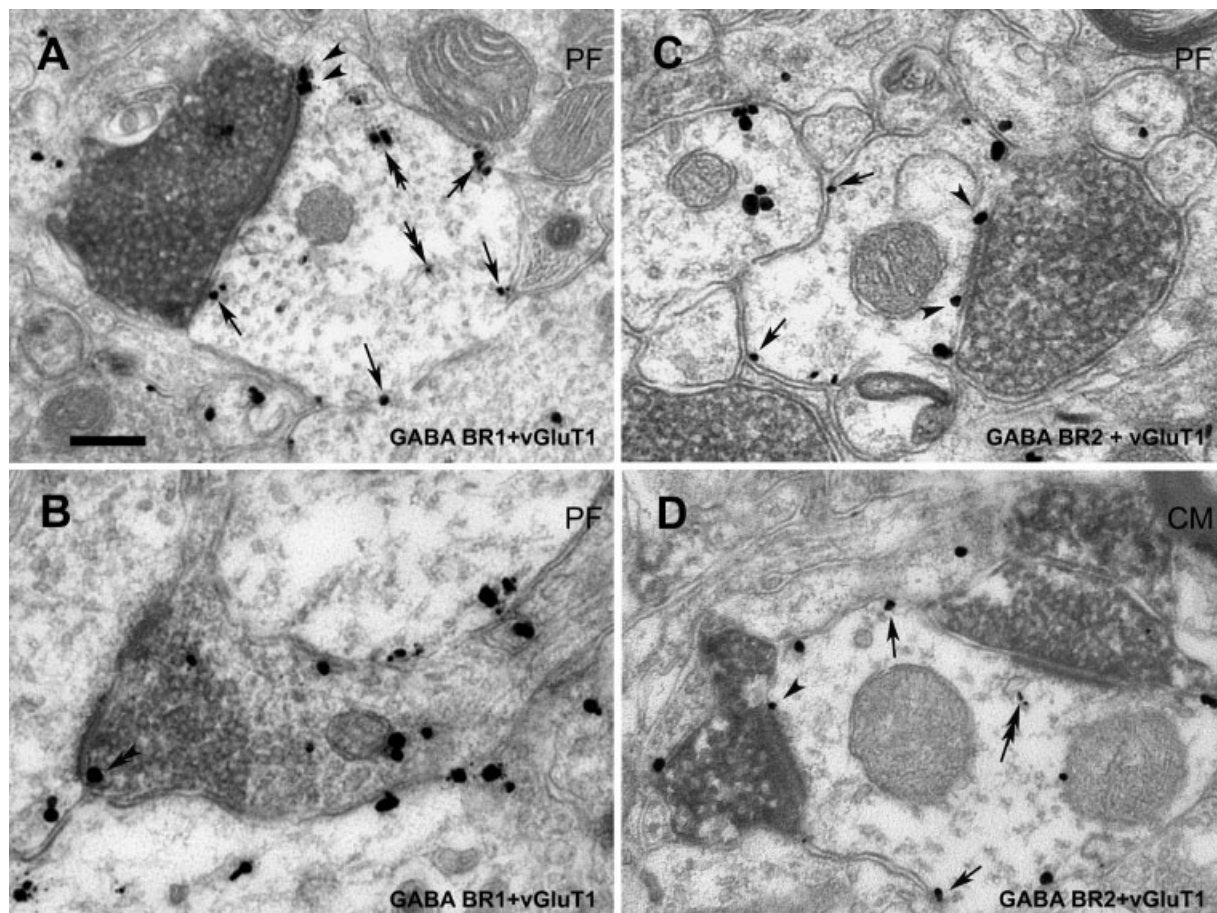


Fig. 9. Double immunolabeling for GABA_BR1 or GABA_BR2 (immunogold) and vGluT1 (immunoperoxidase) in CM/PF. **A,B:** GABA_BR1 and vGluT1 immunolabeling. **A:** Postsynaptic labeling for GABA_BR1 (arrowheads) at the edge of the postsynaptic density of an asymmetric synapse formed by a vGluT1-immunoreactive terminal forming an asymmetric synapse in PF. Note the presence of extrasynaptic (single arrows) and intracellular (double arrows) gold particles in the

postsynaptic dendrite. **B:** Presynaptic GABA_BR1 labeling (double arrowhead) in a vGluT1-immunolabeled terminal in PF. **C,D:** GABA_BR2 and vGluT1 labeling. Perisynaptic GABA_BR2 labeling (arrowheads) at asymmetric synapses formed by vGluT1-immunolabeled terminals. Gold particles are also associated with extrasynaptic sites on the plasma membrane (single arrows) and with intracellular organelles (double arrow). Scale bar = 0.5 μm.

GABA-mediated effects and GABA levels in the monkey globus pallidus (GP; Galvan et al., 2005).

Our quantitative analysis showed that ~20% of immunoperoxidase labeling for GABA_BR1 and GABA_BR2 was found in small axon-like processes in CM/PF. Axonal localization has also been described for several neurotransmitter receptors (5-HT_{1B} serotonin receptors, A1 adenosine receptors, D1 dopamine receptors, group II mGluRs, GluR6/7, KARs subunits, etc.) in the CNS (Swanson et al., 1995, 1998; Yung et al., 1995; Luján et al., 1997; Shigemoto et al., 1997; Charara et al., 1999; Riad et al., 2000; Tamaru et al., 2001; Kane-Jackson and Smith, 2003; Poisik et al., 2005). The fact that immunolabeling for GABA_B subunits in axon terminals was rather scarce in CM/PF suggests that GABA_B receptors in terminals have a fast turnover and that axonal labeling represents receptor subunits traveling to the axon terminal. Another possibility is that axonal GABA_B receptors are functional receptors that modulate axonal conduction and presynaptically regulate neurotransmitter release, as shown for other G protein-coupled receptor subtypes (Radnikow and Misgeld, 1998; Swanson et al., 1998; Bradley et al., 2000; Capogna, 2004; Poisik et al., 2005).

Although the exact mechanisms of activation of these axonal receptors remain poorly understood, they likely rely on neurotransmitter spillover in the extracellular space, insofar as axoaxonic synapses are rarely seen in brain regions endowed with axonal receptors. It is noteworthy that other presynaptic receptors are tightly linked with the presynaptic plasma membrane of terminals close to the site of release of neurotransmitter (Shigemoto et al., 1997; Bradley et al., 2000). These observations, therefore, suggest that the exact location of presynaptic receptors might be a critical factor that sets their physiological characteristics and modulatory functions of neurotransmission in the CNS. To understand better the significance of GABA_B receptor axonal labeling in the thalamus, the sources of immunoreactive axons must be determined.

Synaptic expression of GABA_B receptors in CM/PF

Electrophysiological evidence indicates that GABA_B receptors might act as pre- or postsynaptic receptors in the thalamus (Banerjee and Snead, 1995; Pape and McCormick, 1995; Williams et al., 1996; Soltesz and Crunelli,

1992; Bal et al., 1995; Emri et al., 1996; Kim et al., 1997; Sanchez-Vives et al., 1997; Ambardekar et al., 1999). Activation of postsynaptic GABA_B receptors produces only weak or no responses in interneurons of relay nuclei in the cat thalamus (Pape and McCormick, 1995; Williams et al., 1996), whereas presynaptic GABA_B receptor-mediated effects on GABA and glutamate release have been demonstrated in the ventral posterior and dorsal lateral geniculate nuclei (Crunelli and Leresche, 1991; Soltesz and Crunelli, 1992; Banerjee and Snead, 1995; Emri et al., 1996; Ulrich and Huguenard, 1996; Sanchez-Vives et al., 1997).

A small subset of GABA_B receptors was found in the main body or at the edges of symmetric, putative GABAergic, synapses in the monkey CM/PF. A similar postsynaptic localization for GABA_B receptors has been described in the rat ventrobasal thalamic nucleus (Kulik et al., 2002) and various basal ganglia nuclei in both rats and monkeys (Fritschy et al., 1999; Smith et al., 2000, 2001; Gonchar et al., 2001; Kulik et al., 2002, 2003; Boyes and Bolam, 2003; Chen et al., 2004; Galvan et al., 2004; Charara et al., 2005).

This rather scarce synaptic labeling of GABAergic synapses is strikingly different from GABA_A receptor subunits, which are almost exclusively expressed in the core of GABAergic synapses in some brain regions (Somogyi et al., 1996; Kultas-Ilinsky et al., 1998; Galvan et al., 2004; Charara et al., 2005). This obvious difference in the localization of the two main subtypes of GABA receptors provides further support for complex and heterogeneous mechanisms whereby GABAergic transmission could be mediated in the CNS.

In addition, postsynaptic GABA_B receptors were found at the edges of asymmetric synapses (putative excitatory) in CM/PF, a pattern described in different areas of the CNS (Fritschy et al., 1999; Gonchar et al., 2001; Kulik et al., 2002; Boyes and Bolam, 2003; Chen et al., 2004; Galvan et al., 2004; Luján et al., 2004, 2005; Charara et al., 2005). Some of the glutamatergic synapses endowed with pre- and postsynaptic GABA_B receptor labeling are formed by vGluT1-containing boutons. Taking into consideration that vGluT1-containing terminals in the thalamus most likely arise from the cerebral cortex (Fremeau et al., 2001, 2004), activation of GABA_B heteroreceptors in these glutamatergic terminals may regulate transmitter release from corticothalamic afferents. On the other hand, the presence of GABA_B receptors at the edges of asymmetric synapses formed for vGluT1-positive terminals suggests the possible interaction between GABAergic and metabotropic glutamate receptors (mGluRs) to regulate excitatory synaptic transmission in CM/PF. Such functional interactions between GABA_B receptors and mGluR1 have been shown in the cerebellum, where excitatory mGluR1-mediated postsynaptic currents were enhanced by activation of postsynaptic GABA_B receptors (Hirono et al., 2001). On the basis of the similarity between the pattern of group I mGluRs and the GABA_B receptor distribution throughout the CNS (Smith et al., 2000, 2001; Marino et al., 2001; Muly et al., 2003), one might expect functional receptor-receptor interactions to be an important mechanism whereby these receptors modulate their effects in the brain.

Local GABAergic interneurons, the reticular nucleus and basal ganglia afferents from the internal globus pallidus (GPi) and the substantia nigra pars reticulata (SNr),

are the main sources of GABAergic terminals to the CM/PF complex (Smith et al., 1987; Sidibé et al., 1997, 2002; Smith and Sidibé, 2003). The expression of presynaptic GABA_B receptors at symmetric synapses (putative GABAergic) and in vesicle-filled dendrites of LCN dendrites in CM/PF suggests that activation of GABA_B autoreceptors may reduce GABAergic transmission in this nuclear complex, a well-established physiological role for GABA_B receptor in other thalamic nuclei (Soltesz and Crunelli, 1992; Bal et al., 1995; Banerjee and Snead, 1995; Pape and McCormick, 1995; Williams et al., 1996; Emri et al., 1996; Kim et al., 1997; Sanchez-Vives et al., 1997; Ambardekar et al., 1999). Further studies are needed to characterize the exact source of the GABA_B-containing GABAergic terminals in CM/PF.

Presynaptic labeling for GABA_B receptors in glutamatergic terminals has been demonstrated in various brain regions, including the monkey basal ganglia (Charara et al., 2000, 2004, 2005; Smith et al., 2001; Galvan et al., 2004). Our findings revealed that such is also the case in monkey CM/PF, which is in line with electrophysiological observations that GABA_B receptor activation presynaptically modulates glutamatergic transmission in the rat and mouse thalamus (Banerjee and Snead, 1995; Chen and Regehr, 2003). The high sensitivity of GABA_B receptors for GABA (Sodickson and Bean, 1996) suggests that GABA spillover from GABAergic synapses (Atwell et al., 1993; Vogt and Nicoll, 1999; Scanziani, 2000) is the most likely mechanism of activation of these receptors.

Other sources of vGluT1-containing terminals in CM/PF that may be associated with GABA receptors are those from the brainstem pedunculo-pontine and the laterodorsal tegmental nuclei. Both of these nuclei provide substantial glutamatergic innervation to the monkey CM/PF (Sidibé et al., 2002; Smith and Sidibé, 2003). Additionally, there is good evidence that a large subset of glutamatergic inputs from these regions coexpresses vGluT1 and choline acetyltransferase (Smith and Paré, 2004), suggesting the potential corelease of glutamate and acetylcholine (ACh) at these synapses (Gutierrez, 2000, 2002). It is important to consider that functional interactions between GABA_B and ACh muscarinic receptors have previously been reported for different CNS regions (Wichmann et al., 1987; Curro Dossi et al., 1992; Libri et al., 1998; Scanziani, 2000; Morton et al., 2001; Rowell et al., 2003).

GABA_B receptors, CM/PF, and CNS pathologies

An increased GABAergic basal ganglia outflow from GPi and SNr to the thalamus is a cardinal feature in Parkinson's disease (PD) pathophysiology (Palombo et al., 1990; Gao et al., 1997; Samuel et al., 1997; Bennazouz et al., 2000; Fukuda et al., 2001). The possibility that thalamic GABA_B receptors play a role in PD is supported by results showing that the GABA_B agonist baclofen induces akinesia when administered locally in the ventromedial (VM) nucleus of the thalamus (DiChiara et al., 1979; Klockgether et al., 1986; Wullner et al., 1987). However, blockade of GABA_B receptors in the VM of unilaterally 6-hydroxydopamine-lesioned rats does not have any effects on rotational behavior (de Groote et al., 1999), and binding studies could not find any significant changes in the number of GABA_B binding sites in the thalamic ventral motor nuclei of MPTP-treated parkinsonian monkeys (Calon et al., 2000). Modulation of glutamatergic neuro-

transmission has beneficial effects in PD. In fact, systemic administration of glutamate antagonists in combination with subthreshold dose of L-DOPA has proved highly effective in reducing parkinsonian symptoms in animal models of PD (Loschmann et al., 1991; Greenamyre et al., 1994; Karcz-Kubicha et al., 1999). Results of this study and other studies demonstrate that GABA_B receptors are expressed in glutamatergic terminals at various sites along the basal ganglia-thalamostriatal circuitry. Knowing that increased glutamatergic tone on striatofugal or subthalamofugal neurons is a key factor that underlies the pathophysiology of PD, one might consider GABA_B receptor activation as a potential venue for the development of novel pharmacotherapies in PD (Calon et al., 2000; Johnston and Duty, 2003).

Tourette's syndrome (TS) is a chronic neurological disorder with an onset in early childhood that is characterized by different tics (sudden, brief, intermittent, involuntary, or semivoluntary movements or sounds). Although the cause of TS is unknown, and the precise neurobiological abnormality remains speculative (Mink, 2001; Singer and Minzer, 2003), bilateral coagulations (Hassler and Dieckmann, 1973) and chronic bilateral stimulation (Visser-Vandewalle, 2003) of the intralaminar thalamic nuclei produce a partial relief of tics in patients with TS, suggesting that overactivity of the intralaminar nuclei plays a major role in the TS. So far, drug treatment for TS consists of neuroleptics and other agents interacting with the dopaminergic system (Lavenstein, 2003). In fact, benzodiazepines, which enhance the inhibitory effect of GABA, have some efficacy in tic suppression (Gonce and Barbeau, 1977). However, these treatments have different side effects, such as depression, drowsiness, weight gain, and extrapyramidal symptoms. The strong expression of GABA_B receptors in CM/PF combined with their modulatory functions in the CNS opens up the possibility for new GABA_B-mediated pharmacotherapies for TS.

CONCLUSIONS

In conclusion, our results provide a solid framework for the involvement of GABA_B receptors in the pre- and postsynaptic regulation of GABAergic and glutamatergic transmission in the monkey caudal intralaminar thalamic complex. Given that the main target of CM/PF neurons is the striatum, our findings strongly suggest that GABA_B receptors play a major role in modulatory activity of the thalamostriatal system in primates. Future studies are needed to dissect out the mechanisms by which GABA_B receptor activation modulates excitatory and inhibitory synaptic transmission in CM/PF.

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