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An Electron Microscope Immunocytochemical Study of $GABA_B$ R2 Receptors in the Monkey Basal Ganglia: A Comparative Analysis with $GABA_B$ R1 Receptor Distribution

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ABSTRACT

Functional γ-aminobutyric acid $(GABA)_B$ receptors are heterodimers made up of $GABA_B$ R1 and $GABA_B$ R2 subunits. The subcellular localization of $GABA_B$ R2 receptors remains poorly known in the central nervous system. Therefore, we performed an ultrastructural analysis of the localization of $GABA_B$ R2 receptor immunoreactivity in the monkey basal ganglia. Furthermore, to characterize better the neuronal sites at which $GABA_B$ R1 and $GABA_B$ R2 may interact to form functional receptors, we compared the relative distribution of immunoreactivity of the two $GABA_B$ receptors in various basal ganglia nuclei. Light to moderate $GABA_B$ R2 immunoreactivity was found in cell bodies and neuropil elements in all basal ganglia nuclei. At the electron microscope level, $GABA_B$ R2 immunoreactivity was commonly expressed postsynaptically, although immunoreactive preterminal axonal segments were also frequently encountered, particularly in the globus pallidus and substantia nigra, where they accounted for the third of the total number of $GABA_B$ R2-containing elements. A few labeled terminals that displayed the ultrastructural features of glutamatergic boutons were occasionally found in most basal ganglia nuclei, except for the subthalamic nucleus, which was devoid of $GABA_B$ R2-immunoreactive boutons. The relative distribution of $GABA_B$ R2 immunoreactivity in the monkey basal ganglia was largely consistent with that of $GABA_B$ R1, but some exceptions were found, most noticeably in the globus pallidus and substantia nigra, which contained a significantly larger proportion of presynaptic elements labeled for $GABA_B$ R1 than $GABA_B$ R2. These findings suggest the possible coexistence and heterodimerization of $GABA_B$ R1 and $GABA_B$ R2 at various pre- and postsynaptic sites, but also raise the possibility that the formation of functional $GABA_B$ receptors in specific compartments of basal ganglia neurons relies on mechanisms other than $GABA_B$ R1/R2 heterodimerization. J. Comp. Neurol. 476:65–79, 2004.

Indexing terms: striatum; globus pallidus; subthalamic nucleus; substantia nigra; $GABA_B$ R1 receptors; immunocytochemistry

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The inhibitory amino acid γ-aminobutyric acid (GABA) plays a major role in regulating neuronal activity within the basal ganglia. Studies in rodents and primates have shown that the intrinsic synaptic circuits as well as output pathways of the basal ganglia utilize GABA as a neurotransmitter (Parent and Hazrati, 1995; Smith et al., 1998). Three types of receptors mediate the effects of GABA in the central nervous system (CNS). The fast inhibitory responses result from the stimulation of the postsynaptic chloride-gated $GABA_A$ and $GABA_C$ receptor channels (Olsen and Tobin, 1990; Macdonald and Olsen, 1994), whereas the slow GABA-mediated postsynaptic hyperpolarization or presynaptic modulatory effects on neurotransmission result from the activation of G protein-coupled $GABA_B$ receptors (Bormann, 1988, 2000; Bowery, 1989; Misgeld et al., 1995; Deisz, 1997; Bettler et al., 1998).

Expression cloning initially showed that $GABA_B$ receptors consist of at least four splice variants, termed $GABA_B$ R1a, -b, -c, and -d, which differ in their N termini (Kaupmann et al., 1997; Isomoto et al., 1998; Pfaff et al., 1999). Moreover, several groups have cloned a second $GABA_B$ receptor, termed $GABA_B$ R2, which exhibits a similar molecular weight and 30–40% sequence homology to $GABA_B$ R1 (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). Unexpectedly for a G protein-coupled receptor, heteromeric assembly of $GABA_B$ R1 and $GABA_B$ R2 is necessary to form functional $GABA_B$ receptors (Marshall et al., 1999; Bowery and Enna, 2000).

In situ hybridization and immunohistochemical studies revealed that $GABA_B$ R1 and $GABA_B$ R2 mRNAs expression show considerable overlap in the rat CNS. However, in some areas like the striatum, there is very little $GABA_B$ R2 relative to $GABA_B$ R1 mRNAs (Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; Ng et al., 1999; Charles et al., 2001). This led to the suggestion that $GABA_B$ R1 can either function as a monomer in some discrete regions or interact with other partners, which have not yet been identified in the mammalian brain (Marshall et al., 1999).

$GABA_B$ receptors subserve a variety of functions within the basal ganglia, ranging from presynaptic modulation of neurotransmitter release to postsynaptic regulation of inhibitory neurotransmission (Seabrook et al., 1991; DeBoer and Westerink, 1994; Shen and Johnson, 1997, 2001; Tepper et al., 1998; Stefani et al., 1999). Localization studies have shown radioactive binding, mRNA expression, and cellular immunolabeling for $GABA_B$ R1 in various basal ganglia nuclei in rats (Bowery et al., 1987; Bischoff et al., 1999; Fritschy et al., 1999; Lu et al., 1999; Margeta-Mitrovic et al., 1999; Yung et al., 1999; Liang et al., 2000; Ng and Yung, 2001a,b; Boyes and Bolam, 2003) and monkeys (Charara et al., 2000). Furthermore, we recently showed that $GABA_B$ R1 immunoreactivity is expressed in putative glutamatergic afferents throughout the monkey basal ganglia (Charara et al., 2000). At present, very little is known about the localization of $GABA_B$ R2 receptor immunoreactivity, except for recent light microscopic studies showing the cellular distribution of this receptor subtype in the rat (Durkin et al., 1999; Clark et al., 2000; Ng and Yung et al., 2001a,b) and human (Billinton et al., 2000) CNS. Subcellular localization analyses have been restricted to the rat visual cortex, cerebellum, thalamus, and substantia nigra (Gonchar et al., 2001; Kulik et al., 2002; Boyes and Bolam, 2003).

To characterize further the potential sites where $GABA_B$ R1 and $GABA_B$ R2 may functionally interact, we performed an electron microscopic study of $GABA_B$ R2 receptor localization and compared its overall pattern of distribution with that of $GABA_B$ R1 in the monkey basal ganglia.

Abbreviations

Ax	axon
b	bouton
DEN	dendrite
GABA	γ-aminobutyric acid
$GABA_A$	GABA receptor subtype A
$GABA_C$	GABA receptor subtype C
$GABA_B$	GABA receptor subtype B
$GABA_B$ R1	$GABA_B$ receptor subunit R1
$GABA_B$ R2	$GABA_B$ receptor subunit R2
$GABA_B$ R1a	$GABA_B$ receptor R1 isoform a
$GABA_B$ R1b	$GABA_B$ receptor R1 isoform b
$GABA_B$ R1c	$GABA_B$ receptor R1 isoform c
$GABA_B$ R1d	$GABA_B$ receptor R1 isoform d
GPe	globus pallidus, external segment
GPi	globus pallidus, internal segment
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SP	dendritic spine
STN	subthalamic nucleus
VTA	ventral tegmental area

MATERIALS AND METHODS

Animals and preparation of tissue

For the immunocytochemical localization of $GABA_B$ R2 receptors, tissue was collected from three adult male rhesus monkeys (*Maccaca mulatta*, 3–5 kg; Yerkes National Primate Research Center colony). 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.

The monkeys 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 through the rostrocaudal extent of the basal ganglia were then obtained with a Vibratome (60 μm thick), 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 the Western immunoblots, one rhesus monkey (Yerkes National Primate Research Center colony) and one Sprague-Dawley rat (Charles River Laboratories, Wilmington, MA) were used. The monkey was overdosed with pentobarbital (100 mg/kg, i.v.), followed by rapid removal of the brain from the skull and dissection of regions of interest. The rat was rapidly decapitated, and the brain was removed from the skull and dissected on ice.

Immunoblot analysis

A polyclonal guinea pig antiserum raised against the C-terminal of the $GABA_B$ R2 receptor (Chemicon International, Temecula, CA) was used in the present study.

Specificity of this antiserum was determined by using immunoblots of membrane samples from transfected cells and brain tissues. Human embryonic kidney (HEK-293) cells were maintained in complete medium (minimum essential medium with 10% fetal bovine serum and 1% penicillin/streptomycin) in 10-cm culture dishes at 37°C with 5% CO_2. At ~50–80% confluency, the cells were transiently transfected with 2 μg of plasmid for $GABA_B$ R1, $GABA_B$ R2, or both, mixed with LipofectAMINE (Invitrogen, Carlsbad, CA), followed by a 4-hour incubation at 37°C with 5% CO_2. After 6 ml of complete medium was added, the cells were incubated for an additional 24 hours. The cells were then harvested and completely homogenized with a sonicator in ice-cold buffer solution containing 20 mM HEPES, 10 mM EDTA, and 2 mM sodium vanadate. The membrane samples were prepared at 4°C.

The homogenate was then centrifuged for 5 minutes at 2,000 rpm to remove tissue debris, and the membrane was isolated from the supernatant by subsequent centrifugation for 30 minutes at 14,000 rpm. The resulting pellet was then solubilized in a buffer solution containing 20 mM HEPES and 0.1 mM EDTA before total protein concentration was measured using a Bio-Rad (Hercules, CA) Protein Assay. The homogenates were then centrifuged for 30 minutes at 14,000 rpm, and the pellet was solubilized in a lysis buffer containing 10 mM HEPES, 50 mM NaCl, 0.1 mM EDTA, 1 mM benzamidine, 1.0% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail (1 tablet per 50 ml; Roche, Mannheim, Germany). The lysates (6 μg protein) were then eluted with 6X SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, resolved by SDS-PAGE, and subjected to Western blot analysis with guinea pig polyclonal antisera against $GABA_B$ R2 (1:2,000 dilution; Chemicon International). Immunoreactive bands were detected using the enhanced chemiluminescence detection system (Pierce, Rockford, IL) with horseradish peroxidase-conjugated goat anti-guinea pig secondary antibody (Chemicon International). All brain membrane samples were prepared, and 40 μg of protein was subjected to Western blot analysis in the same manner as above.

Localization of $GABA_B$R2 immunoreactivity at the light microscope level

The immunocytochemical localization of $GABA_B$ R2 receptor subtype was performed by using the avidin-biotin complex (ABC) method (Hsu et al., 1981). After blocking nonspecific sites with 10% normal goat serum (NGS) and 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature, the sections were incubated for 2 days at 4°C in the primary antibody solution (1:500 dilution; Chemicon International). The sections were then washed in PBS, incubated for 90 minutes in biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA), rinsed again in PBS, and finally incubated for an additional 90 minutes 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% H_2O_2 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.

Localization of $GABA_B$ R2 immunoreactivity at the electron microscope level

The 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 washed in PBS before being processed for immunocytochemistry. They were then processed for the visualization of $GABA_B$ R2 receptors according to the protocol described above, except that Triton X-100 was not included in solutions. After immunostaining, they were washed in PB (0.1 M, pH 7.4) and postfixed in osmium tetroxide (1% in PB) for 20 minutes. This was followed by washings in PB and dehydration in a graded series of ethanol and propylene oxide. Uranyl acetate (1%) was added to the 70% ethanol for 35 minutes to improve the contrast in the electron microscope. The sections were then embedded in resin (Durcupan ACM, Fluka, Ft. Washington, PA) on microscope slides and placed in the oven for 48 hours at 60°C. Areas of interest were selected, cut out from the slides, and glued on the top of resin blocks. Serial ultrathin sections were then cut on an ultramicrotome (Leica Ultracut T2), collected onto Pioloform-coated single copper grids, stained with lead citrate (Reynolds, 1963), and analyzed with the electron microscope (Zeiss EM 10C).

Analysis of data

Transverse sections containing basal ganglia structures were taken from each of the three monkeys for both light and electron microscope analyses. At least one block was taken from the striatum, external globus pallidus (GPe), subthalamic nucleus (STN), and substantia nigra (SN) in the three monkeys. The blocks from the striatum were all cut from levels of putamen rostral to the anterior commissure, whereas blocks from the SN were selected from the ventral part of sections corresponding to the middle third of the structure, which comprised perikarya and neuropil elements of the pars reticulata (SNr) intermingled with dendrites of pars compacta (SNc) neurons. The ultrastructural analysis was carried out on ultrathin sections collected from the surface of each block where the staining was optimal.

To compare the pattern of $GABA_B$ R2 and $GABA_B$ R1 immunolabeling, we made quantitative measurements of the relative abundance of immunoreactive elements for each receptor subtype in basal ganglia nuclei from two monkeys with the best ultrastructural preservation. For this part of the study, the $GABA_B$ R1 measurements were made from immunostained sections used in our previous study of $GABA_B$ R1 localization in monkey basal ganglia (Charara et al., 2000). Quantitative data were collected the same way in both materials, i.e., ulthrathin sections from the most superficial sections of blocks were scanned at 20,000–25,000×, and all immunoreactive elements randomly encountered were photographed. To decrease the likelihood of sampling immunoreactive elements more than once, ultrathin sections used for quantification were separated from each other by at least 0.5 μm (i.e., 8-12 ultrathin sections). The labeled elements were then categorized in various groups based on ultrastructural features (Peters et al., 1991), and their relative proportion was calculated and expressed as percent of total labeled

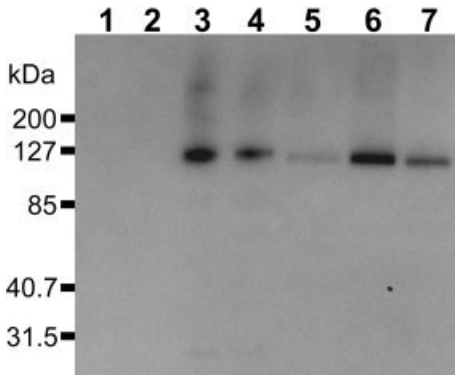

Fig. 1. Western immunoblot of HEK-293 cells transfected with no plasmid (lane 1), $GABA_B$ R1 (lane 2), $GABA_B$ R2 (lane 3), and both (lane 4) as well as membrane samples from the monkey putamen (lane 5), the monkey cerebellum (lane 6), and the rat cerebellum (lane 7). The Chemicon anti-$GABA_B$ R2 antibodies detected $GABA_B$ R2 (lane 3) but not $GABA_B$ R1 (lane 1). A band of about 120 kDa was also detected in samples from the monkey putamen, monkey cerebellum, and rat cerebellum (lanes 5–7, respectively). Note that expression of $GABA_B$ R2 is substantially lower in the monkey putamen (lane 5) compared with the cerebellum (lane 6). Omission of primary antibodies resulted in total absence of labeling (data not shown).

elements for each receptor subtype. The total surface of tissue examined in basal ganglia nuclei for each receptor subtype is given in the legend of Figure 5. Statistical differences in the pattern of distribution of the two receptors subtypes were assessed with a Chi-square analysis.

The micrographs were acquired with a 3.25 ×4-inch Kodak EM plates (printed on Ilford paper) or with a CCD camera (DualView 300W, Gatan, Pleasanton, CA) controlled by DigitalMicrograph software (version 3.7.4., Gatan). The digitally acquired micrographs were adjusted for brightness and contrast, while maintaining the image resolution constant, with Photoshop software (version 7.0, Adobe Systems, San Jose, CA).

RESULTS

Immunoblotting

Immunoblot analysis of transfected HEK-293 cell extracts showed that the anti-$GABA_B$ R2 antisera recognized $GABA_B$ R2, with no detectable cross-reaction with $GABA_B$ R1 expressed in HEK-293 cells (Fig. 1). This antibody also labeled a single band in monkey and rat brain membranes with an estimated molecular weight of 120 kDa, which corresponds to the predicted molecular weight of $GABA_B$ R2 (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998) (Fig. 1). Omission of primary antisera resulted in total absence of labeling (not shown).

$GABA_B$ R2 localization in monkey basal ganglia

In all basal ganglia nuclei examined, the pattern of $GABA_B$ R2 immunostaining was quite similar, i.e., light to moderate cellular and neuropil labeling was found at the light microscopic level, whereas dendritic processes and unmyelinated axons accounted for most labeled elements in the electron microscope. There were no significant interindividual differences in the pattern of cellular and ultrastructural labeling among the three animals used in this study. Omission of the primary $GABA_B$ R2 antiserum from incubation solutions completely abolished the immunostaining.

The striatum displayed light $GABA_B$ R2 immunoreactivity made up of faintly labeled small cell bodies among which were interspersed more darkly stained large-sized neuronal perikarya (Fig. 2A). At the light microscope level, the neuropil staining was rather discrete and largely made up of small punctate structures. There was no obvious patch/matrix pattern of $GABA_B$ R2 neuropil labeling in the caudate nucleus and putamen (Graybiel and Ragsdale, 1978; Gerfen and Wilson, 1996). At the electron microscope level, both large and small neuronal cell bodies with ultrastructural features reminiscent of those previously described for projection neurons and interneurons (DiFiglia et al., 1976; DiFiglia and Aronin, 1982; Bolam et al., 1983, 1984) contained immunoreactivity. In either case, patchy peroxidase deposits were found throughout the cytoplasm where they appeared to be associated preferentially with stalks of endoplasmic reticulum (Fig. 2B). Dendrites of various sizes (0.25–1.0 μm) accounted for more than 50% of the total number of labeled elements in the striatum, whereas almost 20% of the labeled structures were dendritic spines (Figs. 2C,D, 5). In dendrites, the labeling was mainly associated with microtubules, whereas spines contained a more diffuse deposit that often filled the labeled element (Fig. 2C,D).

Other frequently encountered (20% of total labeled elements) $GABA_B$ R2-immunoreactive structures were small unmyelinated axons that often contained synaptic vesicles (Figs. 2E, 5). In those sections that were cut in the appropriate plane, the labeled axons gave rise to terminals that formed asymmetric axospinous synapses (Fig. 2E). Although much more rarely seen (less than 5% total labeled elements), a subset of axon terminals packed with round synaptic vesicles and a few mitochondria forming asymmetric synapses with dendritic spines were also labeled (Figs. 2F, 5).

In the external globus pallidus (GPe), subthalamic nucleus (STN), and substantia nigra (SN), $GABA_B$ R2 immunoreactivity was found in cell bodies, dendrites, and small neuropil elements (Figs, 3, 4). Putative dopaminergic neuronal cell bodies in the pars compacta of the SN (SNc) and the ventral tegmental area were more strongly stained for $GABA_B$ R2 than neurons in the SN pars reticulata (SNr; Fig. 4A). At the electron microscopic level the common feature to these brain regions was the predominance of immunoreactive dendritic processes (50–70% of total labeled elements; Figs. 3F,G, 4B, 5). In all cases, the immunoreactivity was preferentially associated with microtubules, although patches of peroxidase were occasionally seen along the plasma membrane (Figs. 3F,G, 4B). A striking feature that distinguished the labeling in GPe and SN from that in STN was the abundance of immunoreactive small unmyelinated axons (30% of total labeled elements in GPe and SN; 10% in STN; Figs. 3C, 4C,D, 5). These axons were often found in aggregates, although isolated labeled elements were also seen. When the labeled axons were cut in the longitudinal plane and could be followed to their terminals, the boutons were nonimmunoreactive and always formed asymmetric axo-dendritic synapses (Figs 3D, 4D). Although they were relatively rare (1–10% total labeled structures), immunoreactive axon

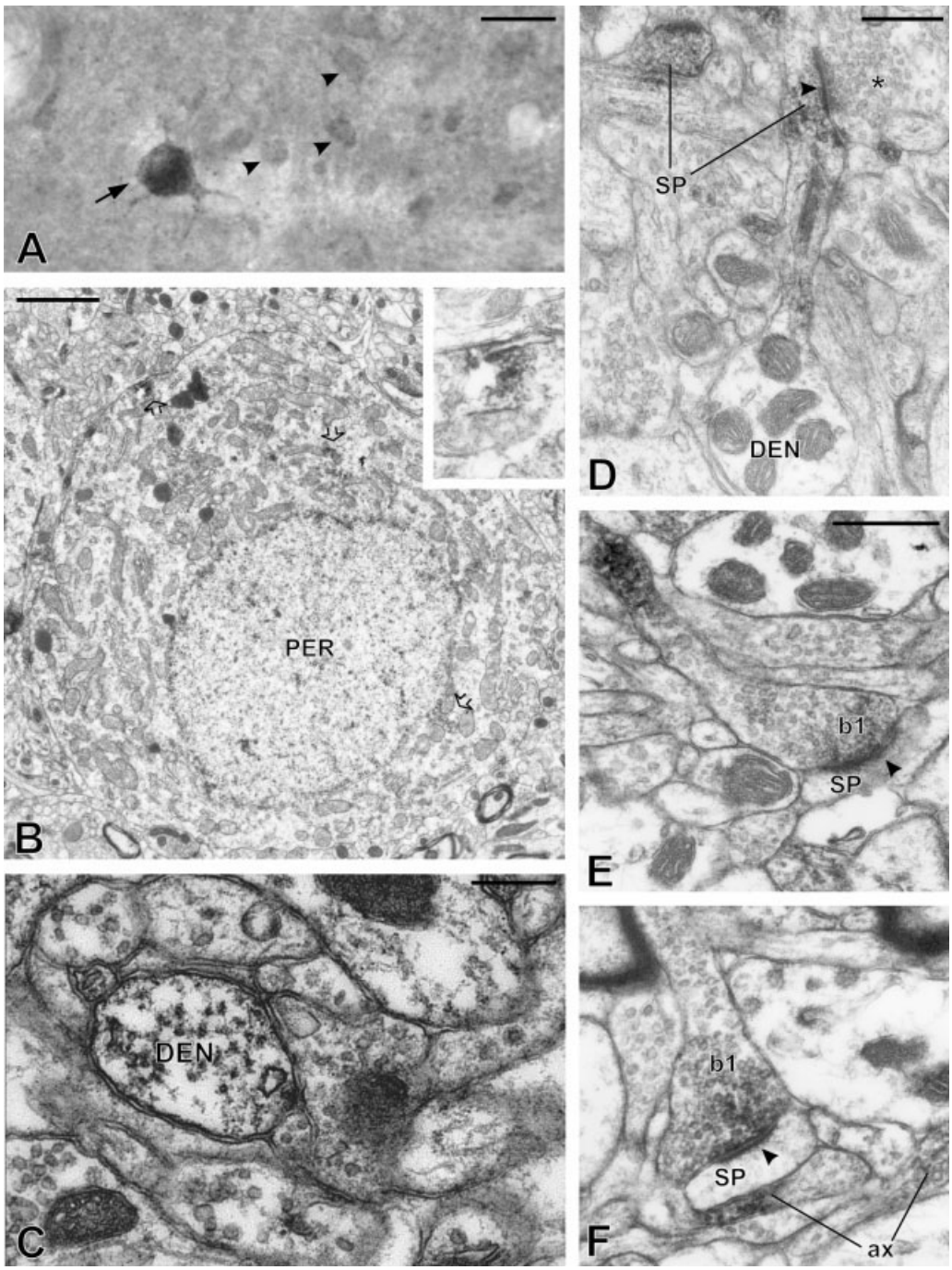

Fig. 2. GABA$_B$ R2 immunoreactivity in the striatum. **A:** Numerous lightly labeled small GABA$_B$ R2-immunoreactive perikarya (arrowheads) and a large (arrow) darkly labeled neuron in the putamen. **B:** Low-power electron micrograph of a GABA$_B$ R2-immunoreactive perikaryon (PER) in the putamen. The peroxidase deposit displays a patchy distribution (open arrows and inset) associated with organelles such as the endoplasmic reticulum and/or Golgi apparatus. No particular aggregation of peroxidase reaction product was found along the plasma membrane. **C:** A GABA$_B$ R2-immunoreactive dendrite (DEN). Note the peroxidase reaction product associated with microtubules. **D:** GABA$_B$ R2-containing dendritic spines (SP) in the putamen. Note that one of the dendritic spines emerges from a nonimmunoreactive dendrite (DEN). The asterisk (*) indicates a nonimmunoreactive terminal forming an asymmetric synapse with a labeled spine (arrowhead). **E:** Preterminal axonal segment that displays GABA$_B$ R2 immunoreactivity and gives rise to a putative glutamatergic terminal (b1) that contacts (arrowhead) a dendritic spine (SP) in the putamen. **F:** A GABA$_B$R2-immunoreactive terminal (b1) forming an asymmetric axospinous synapse (arrowhead) in the putamen. Note the presence of GABA$_B$ R2-immunoreactive vesicle-filled axons (ax). Scale bars = 25 μm in A; 2 μm in B; 0.25 μm in C; 0.5 μm in D; 0.5 μm in E (applies to E,F).

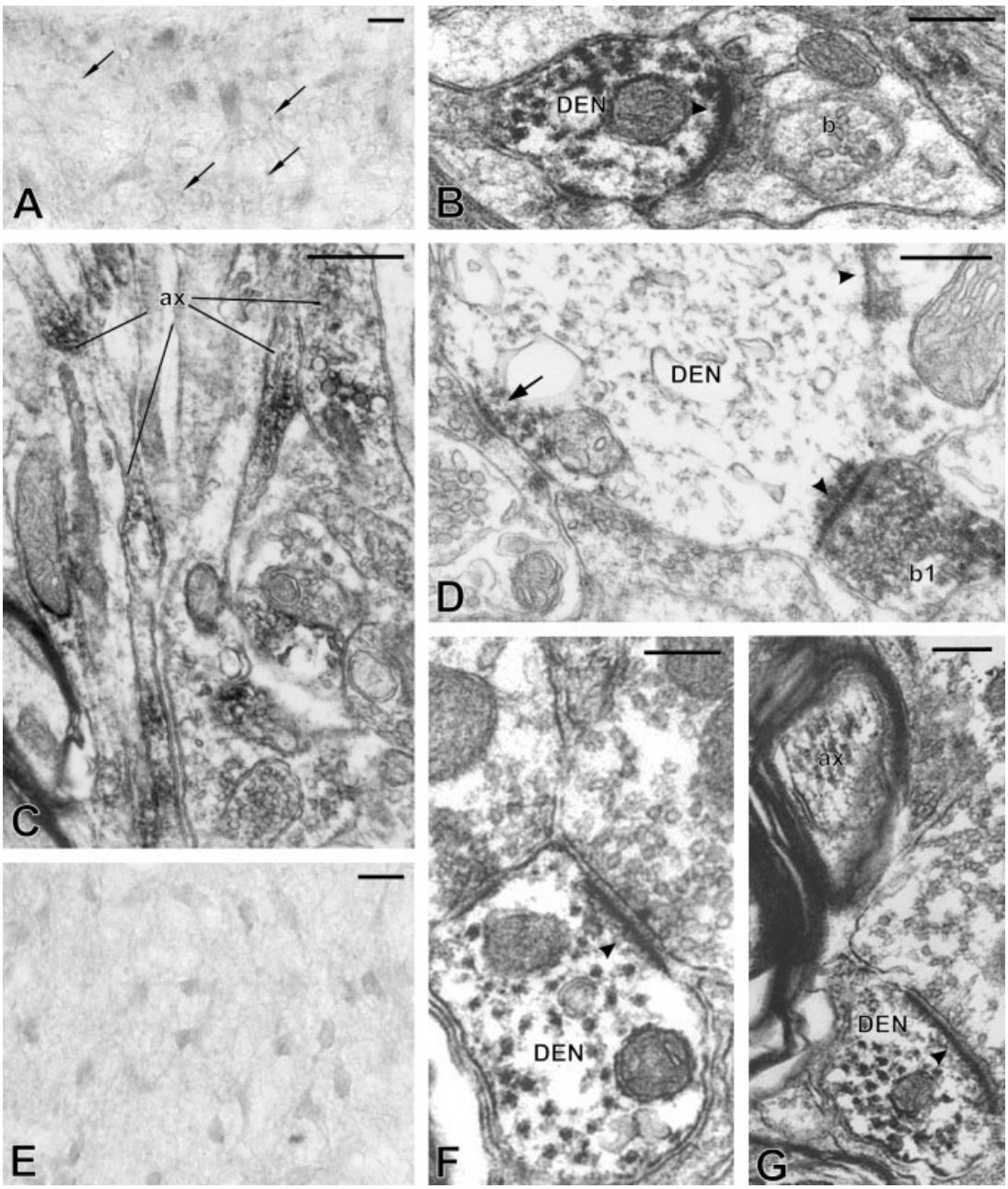

Fig. 3. $GABA_B$ R2 immunostaining in the globus pallidus (A–D) and subthalamic nucleus (E–G). **A:** A low-power micrograph showing $GABA_B$ R2-immunoreactive neurons that lay in a punctate neuropil (small arrows) in the external segment of the globus pallidus (GPe). **B:** GPe dendrite (DEN) showing immunoreactivity for $GABA_B$ R2 associated with microtubules. **C:** A large number of vesicle-filled unmyelinated axonal segments (ax) immunoreactive for $GABA_B$ R2 in GPe. **D:** A $GABA_B$ R2-immunolabeled bouton (b1) forming an asymmetric synapse (arrowhead) with a labeled dendrite (DEN, arrow) in GPe. **E:** Numerous cell bodies in the subthalamic nucleus (STN) display moderate $GABA_B$ R2 immunoreactivity. **F:** A $GABA_B$ R2-immunoreactive dendrite in the STN receiving an asymmetric synaptic contact (arrowhead). **G:** A myelinated axonal segment (ax) and a dendrite (DEN), immunoreactive for $GABA_B$ R2 in STN. The dendrite receives an asymmetric synaptic contact (arrowhead). Scale bars = 25 μm in A; 0.25 μm in B; 0.3 μm in C; 0.5 μm in D; 25 μm in E; 0.25 μm in F,G.

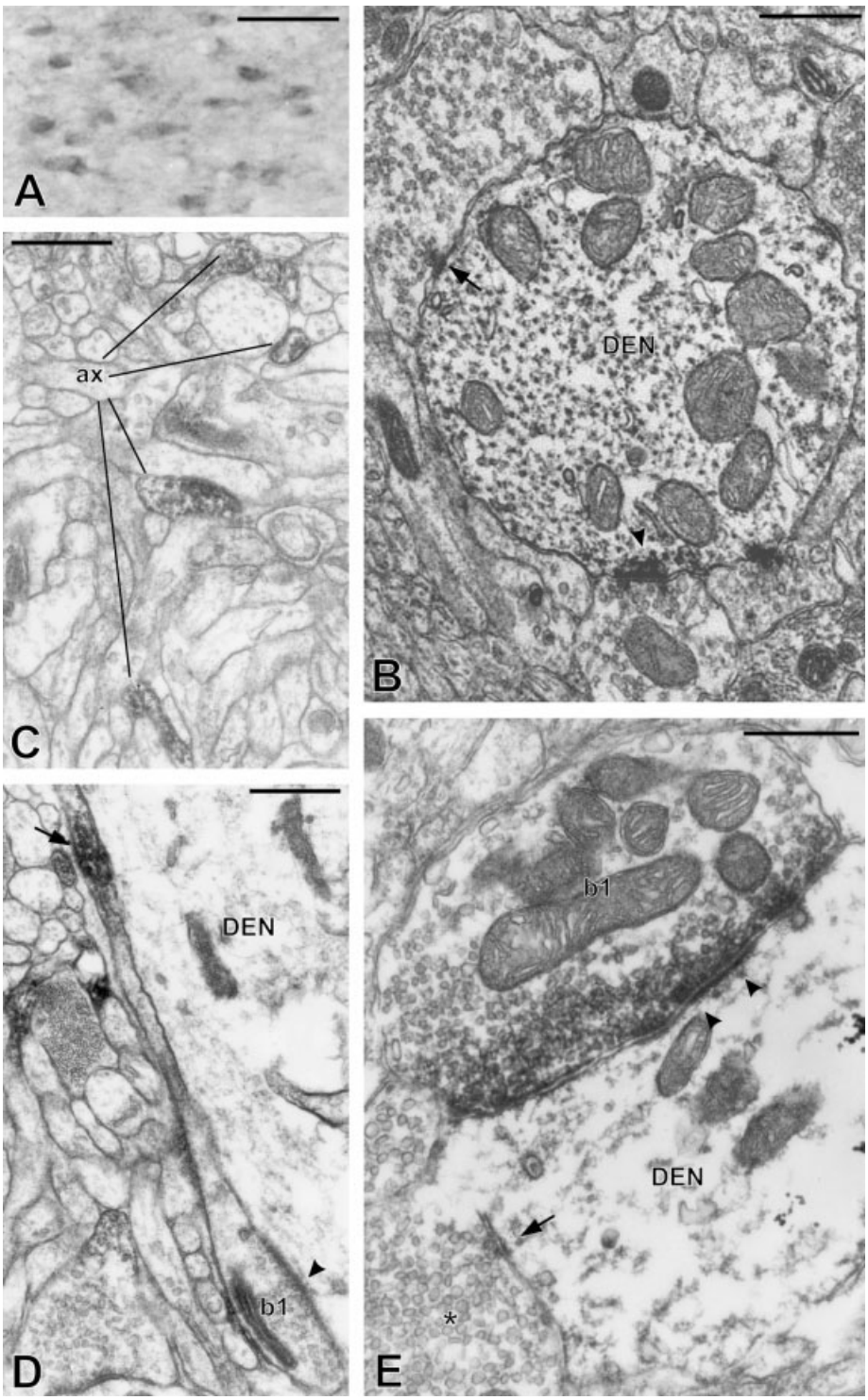

Fig. 4. GABA$_B$ R2 immunostaining in the substantia nigra. **A:** A low-power micrograph showing moderate neuronal labeling in the substantia nigra pars compacta. **B:** A large GABA$_B$ R2-labeled dendrite receives asymmetric (arrowhead) and symmetric (arrow) synaptic contacts. **C:** Many preterminal axonal segments (ax) display GABA$_B$ R2 immunoreactivity. **D:** A GABA$_B$ R2-immunoreactive preterminal axonal segment gives rise to a nonimmunoreactive terminal bouton (b1) in contact with a dendrite (DEN). **E:** A GABA$_B$ R2-labeled bouton (b1) that forms an asymmetric synapse (arrowhead) with a dendrite (DEN). The asterisk indicates a nonimmunoreactive bouton that forms a symmetric synapse (arrow) with the same dendrite. Scale bars = 50 μm in A; 0.5 μm in B–E.

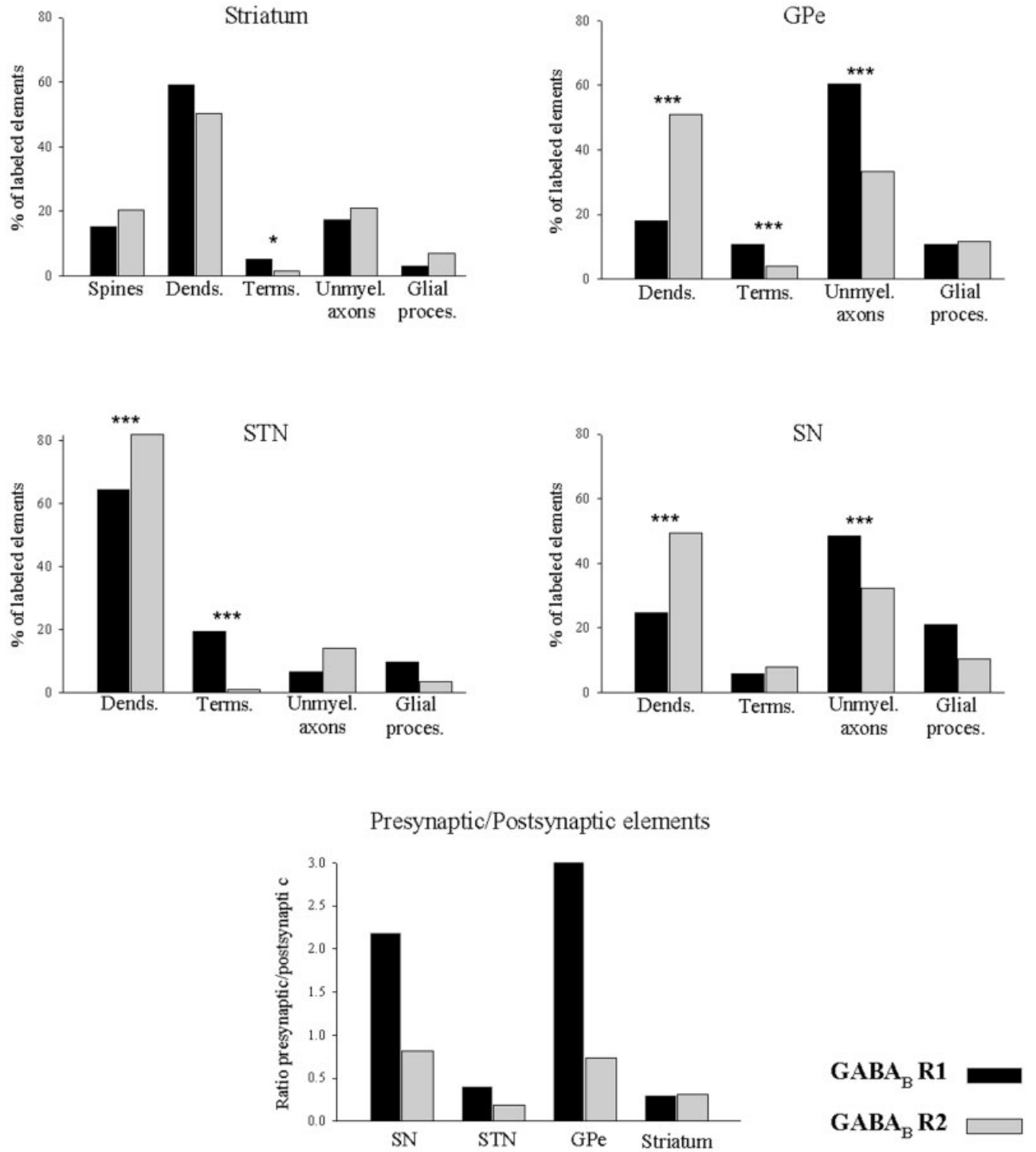

Fig. 5. Histograms comparing the relative distribution of $GABA_B$ R1- and $GABA_B$ R2-immunoreactive elements in various basal ganglia nuclei. The data are expressed as percentages of immunoreactive elements, 100% being the total number of labeled elements examined in each nucleus. The last panel shows the ratio of labeled presynaptic (terminals + unmyelinated axons) versus postsynaptic (dendrites + spines) elements for the two receptor subtypes. Data were collected from two monkeys immunostained for $GABA_B$ R1 (Charara et al., 2000) and two different monkeys immunostained for $GABA_B$ R2. Chi-square analysis showed significant differences in the relative distribution of immunoreactive dendrites (Dends.) and unmyelinated axons (Unmyel. axons) in GPe and SN. The relative proportion of $GABA_B$ R1-containing terminals (Terms.) was significantly higher than $GABA_B$ R2-immunoreactive boutons in striatum and STN (*, $P = 0.05$; ***, $P < 0.0001$). Total surface of tissue (μm^2)/total number of elements examined: $GABA_B$ R2: striatum, 1,436/201; GPe, 756/102; STN, 1,180/121; SN, 529/87. $GABA_B$ R1: striatum, 1,795/284; GPe, 977/287; STN, 500/62; SN, 1,378/388.

terminals forming asymmetric synapses were found in these basal ganglia nuclei (Figs 3D, 4E, 5).

$GABA_B$ R2 vs. $GABA_B$ R1 localization in monkey basal ganglia

Because $GABA_B$ R2 and $GABA_B$ R1 form functional heterodimers, one would expect these two receptor subtypes to display a similar pattern of distribution in CNS. To address this issue, we quantified and compared the relative abundance of $GABA_B$ R2- and $GABA_B$ R1-immunoreactive elements in basal ganglia nuclei (Fig. 5). The $GABA_B$ R1 data were collected from immunostained material used in our previous ultrastructural study of $GABA_B$ R1 localization in monkey basal ganglia (Charara et al., 2000). The total number of immunoreactive elements examined and the total surface of tissue that was scanned to collect these data are given in the legend of Figure 5. Overall, the pattern of labeling for $GABA_B$ R1 and $GABA_B$ R2 in the striatum was not significantly different, except for the relative proportion of labeled terminals, which was slightly higher in the $GABA_B$ R1- than $GABA_B$ R2-immunostained tissue (Chi-square 12.5, $P = 0.01$; Fig. 5). Differences in the distribution of labeling were more striking in GPe and SN due to the significant increase in the proportion of $GABA_B$ R1-containing unmyelinated axons in these brain regions (Chi-square 44.7, $P < 0.0001$ in GPe; 23.9, $P < 0.0001$ in SN; Fig. 5).

This shift in labeling toward presynaptic elements resulted in a reduced proportion of $GABA_B$ R1-containing dendrites in these nuclei compared with $GABA_B$ R2. The main difference between the two receptor subtypes distribution in STN was found at the level of axon terminals, which accounted for almost 20% of the total number of $GABA_B$ R1-labeled elements, compared with less than 2% of $GABA_B$ R2-immunostained structures (Chi-square 26.3, $P < 0.0001$). The abundance of presynaptic $GABA_B$ R1 labeling in GPe and SN is clearly depicted in the bottom panel of Figure 5, which compares the ratios of pre-/postsynaptic labeled elements in the four basal ganglia nuclei examined. This ratio ranged from 2.3 to 3.0 for $GABA_B$ R1 in SN and GPe, whereas it remained lower than 0.5 in STN and striatum. In contrast, all other basal ganglia nuclei contained a significantly larger proportion of $GABA_B$ R2-labeled postsynaptic elements, keeping the ratio pre/postsynaptic labeling below 1 (Fig. 5).

DISCUSSION

This study is the first description of the ultrastructural localization of $GABA_B$ R2 immunoreactivity in the primate basal ganglia. Despite its relatively low level, $GABA_B$ R2 immunostaining was found to be widely expressed pre- and postsynaptically throughout the monkey basal ganglia. Both proximal and distal dendrites accounted for the bulk of postsynaptic labeling, whereas small unmyelinated axons were the most frequently encountered presynaptic elements, particularly in GPe and SN. Although relatively rare compared with other immunoreactive elements, $GABA_B$ R2-labeled axon terminals forming asymmetric synapses were found in all basal ganglia nuclei, except the STN. A comparative analysis with $GABA_B$ R1 revealed two main differences between the distribution of the two $GABA_B$ receptor subtypes in monkey basal ganglia: 1) the relative proportion of $GABA_B$ R1-labeled presynaptic elements in GPe and SN was significantly higher than $GABA_B$ R2-containing presynaptic structures, whereas $GABA_B$ R2 immunoreactivity remained preferentially associated with postsynaptic dendrites in all basal ganglia nuclei; and 2) the relative proportion of $GABA_B$ R1-containing terminals was significantly higher than that of $GABA_B$ R2-positive boutons in STN.

Together, these findings suggest multiple pre- and postsynaptic sites at which $GABA_B$ R1 and $GABA_B$ R2 subunits may heterodimerize to form functional $GABA_B$ receptors in the primate basal ganglia. On the other hand, the differential distribution of the two subunits in some neuronal structures also highlights the possibility that other molecular mechanisms and/or additional $GABA_B$ receptor subunits may be involved in the formation of functional receptors in specific neuronal elements.

In the following account, these observations will be discussed in light of our current knowledge of $GABA_B$ receptor localization and functions in basal ganglia, and the possibility of additional $GABA_B$ receptor subtypes will be examined.

Methodological considerations

Various methodological issues must be taken into consideration when interpreting the findings presented in this study. As is the case for any immunocytochemical analysis, the validity of immunolabeling disclosed with a particular antiserum relies on the specificity of antibodies for their antigenic sites. We present various data to demonstrate that the $GABA_B$ R2 antiserum used in the present study is specific and does not cross-react with $GABA_B$ R1:

1. Immunoblotting of homogenates from cell lines expressing $GABA_B$ R2 or monkey brain tissue revealed a single band of labeling at approximately 120 kDa, which corresponds to the molecular weight of $GABA_B$ R2 (Kaupmann et al., 1998; White et al., 1998; Jones et al., 1998; Martin et al., 1999). A similar observation was made in another study using immunoblots of rat brain tissue (Ng and Yung, 2001a).
2. Immunoblotting of homogenates from cell lines expressing $GABA_B$ R1 did not reveal any band labeling, indicating the lack of $GABA_B$ R1/$GABA_B$ R2 cross-reactivity. These observations are consistent with the fact that the $GABA_B$ R2 antiserum was raised against a synthetic peptide corresponding to the C-terminal amino acids of the rat and human $GABA_B$ R2 receptor protein, which is not present in the $GABA_B$ R1 protein (Kaupmann et al., 1998; White et al., 1998; Jones et al., 1998).
3. The intensity of band labeling obtained in brain tissue immunoblots from the monkey cerebellum and striatum is in line with the differential level of mRNA or immunoreactivity expression for $GABA_B$ R2 in those two brain regions as revealed by in situ hybridization (Kaupmann et al., 1998; Kuner et al., 1999; Clark et al., 2000; Ige et al., 2000) or light microscopic immunohistochemistry using other $GABA_B$ R2 antisera (Benke et al., 1999; Billinton et al., 2000; Ige et al., 2000; Kulik et al., 2002).

Together, these findings strongly suggest that the antiserum used in the present study is highly specific for the $GABA_B$ R2 protein and does not cross-react with $GABA_B$ R1. However, we cannot rule out the possibility that this antiserum may cross-react with another 120-kDa protein

that has not yet been cloned, because a search for amino acid sequence alignment in the basic local alignment search tool (BLAST) database (Altschul et al., 1997) did not reveal any significant homology with other proteins expressed in the CNS.

Another important issue to keep in mind when discussing data obtained in this study is the relatively low level of immunoreactivity detected by the $GABA_B$ R2 antiserum in the monkey basal ganglia. In general, neuronal cell bodies and neuropil elements were weakly to moderately labeled, raising the possibility of false-negative data due to the limited level of detection offered by electron microscopic immunocytochemical approaches for low levels of protein expression. Although this concern remains valid for any immunocytochemical studies, it is noteworthy that the overall pattern of $GABA_B$ R2 labeling observed in this study is consistent with data reported in previous in situ hybridization or light microscopic immunohistochemical studies using the same or other $GABA_B$ R2 antibodies in human and rat brain tissue (Kaupmann et al., 1998; Benke et al., 1999; Durkin et al., 1999; Kuner et al., 1999; Billinton et al., 2000; Clark et al., 2000; Ige et al., 2000; Kulik et al., 2002; Boyes and Bolam, 2003).

To increase the likelihood of detecting low-level immunoreactivity, we used the avidin-biotin-peroxidase-immunoperoxidase method, which represents the most sensitive immunocytochemical approach for antigen detection in brain tissue (Hsu et al., 1981; Totterdell et al., 1992; Yi and Hersch, 1998). However, the main limitation of the immunoperoxidase technique is its poor spatial resolution, which limits considerably the use of this approach for the subsynaptic localization of receptor proteins. Findings presented in this study, therefore, do not attempt to relate the localization of $GABA_B$ R2 to specific subsets of synapses on basal ganglia neurons but rather provide an overview of the relative distribution of neuronal and glial elements that express $GABA_B$ R2 immunoreactivity throughout the monkey basal ganglia. Although high-resolution pre- and postembedding immunogold approaches have been used successfully to localize $GABA_B$ R2 immunoreactivity in a few $GABA_B$ R2-enriched brain nuclei in rats (Gonchar et al., 2001; Lopez-Bendito et al., 2002; Kulik et al., 2002; Boyes and Bolam, 2003), we could not reliably use these techniques for the localization of $GABA_B$ R2 in monkey basal ganglia because of the low signal/noise ratio of the immunogold labeling. In contrast, $GABA_B$ R1 immunoreactivity, which is significantly more abundant than $GABA_B$ R2 labeling in most basal ganglia structures, could be detected with the pre-embedding immunogold method in the monkey basal ganglia (Smith et al., 2000).

Despite this technical limitation inherent to the sensitivity of some immunocytochemical techniques for the detection of low-level proteins expression, we believe that our immunoperoxidase data provide interesting and novel observations regarding the differential distribution of $GABA_B$ R2 in specific basal ganglia nuclei, which raise important issues regarding the molecular mechanisms that underlie $GABA_B$ receptor functions in primate basal ganglia (see discussion below).

Because functional $GABA_B$ receptors are made up of $GABA_B$ R1 and $GABA_B$ R2 subunits (Kaupmann et al., 1998; Benke et al., 1999; Durkin et al., 1999; Bowery and Enna, 2000), we attempted to correlate the distribution of these two receptor subtypes in basal ganglia through quantitative measurements of the relative density of $GABA_B$ R1- and $GABA_B$ R2-containing elements. Although this approach does not provide direct evidence for receptor colocalization, one may speculate that $GABA_B$ R1 and $GABA_B$ R2 immunoreactivity should display a common pattern of distribution if they, indeed, coexist and form functional $GABA_B$ receptor heterodimers in the monkey basal ganglia. A differential expression of $GABA_B$ R1 and $GABA_B$ R2 may, therefore, be indicative of a partial segregation of the two receptor subtypes in specific neuronal elements. The low level of $GABA_B$ R2 expression in monkey basal ganglia hampered the use of double electron microscopic immunocytochemical studies to assess more directly the degree of $GABA_B$ R1 and $GABA_B$ R2 coexistence at the level of individual neuronal structures.

Quantitative measurements of immunostained profiles using pre-embedding immunocytochemical techniques must be interpreted cautiously, taking into consideration the limited access of antibodies to antigenic sites deep into the sections. Furthermore, the reliability of the comparative analysis of labeling generated by two different antibodies (i.e., $GABA_B$ R1 and $GABA_B$ R2) must be considered because of the different degree of sensitivity these antibodies may display for their antigenic sites. Taking into consideration these technical limitations, we decided to compare the distribution of $GABA_B$ R1 and $GABA_B$ R2 labeling based on the relative percentages of immunoreactive elements for either receptor subtype. Using this approach, we avoid misinterpretation of false-negative labeling due to technical issues because the ground for comparison relies exclusively on labeled structures. In addition, this method allows us to compare data collected from different sizes of areas within the same regions. Although the total number of labeled elements is likely to be significantly higher in larger areas, the relative distribution of labeling among immunoreactive structures should not be significantly changed as long as a substantial amount of tissue has been sampled for either receptor subtypes. Therefore, one could assume that a change in the relative distribution of labeling for a particular receptor subtype compared with the other suggests that a subset of elements in this particular brain region do not coexpress both receptor subtypes.

An alternative approach to address this problem would have been to sample systematically ultrathin sections, in which densitometry and stereology allow precise definition of the region of interest, and the stereological optical dissector method allows quantification of the total numbers of $GABA_B$-immunoreactive elements. Such an approach would allow us to compare the total number of immunostained profiles for $GABA_B$ R1 or $GABA_B$ R2 in the same total surface of tissue from different basal ganglia nuclei and compare the densities of immunoreactive elements for either receptor subtypes. However, because of the technical limitations discussed above regarding potential differences in the degree of sensitivity and tissue penetration of the two antibodies as well as tissue sampling used in the present study, a comparison of the absolute numbers of $GABA_B$ R1- and $GABA_B$ R2-labeled elements in a particular brain region might be hard to interpret. Thus, although comparing the relative percentages of $GABA_B$ R1- and $GABA_B$ R2-labeled structures does not provide much information on the absolute density of immunoreactive elements for either receptor subtype in a particular brain region, we consider this method a reli-

able way to compare patterns of distribution of two different antigens immunostained in separate sections.

Another important issue to consider when interpreting quantitative data collected from our material is the possibility that part of the same immunoreactive element is counted twice. Although we cannot rule out that such might indeed be the case, the fact that we analyzed grids separated from each other by at least 0.5 μm reduces the likelihood of counting twice small structures such as unmyelinated axons, glia, and spines. On the other hand, large dendrites may obviously end up being sampled more than once because their size extends far beyond 0.5 μm. However, the fact that the material immunostained for both receptor subtypes was sampled using the same approach suggests that this double counting might have occurred as frequently in the $GABA_B$ R1- as in $GABA_B$ R2-immunostained sections. Therefore, it should not have any significant effect on the comparative analysis of the distribution of labeling generated by the two antibodies. The only way to rule out this possibility completely would be to reconstruct all immunoreactive elements through serial sections, which is beyond the scope of the present study.

$GABA_B$ receptor localization and functions in basal ganglia

Striatum. As pointed out in the original cloning studies of $GABA_B$ R2, the striatum stands out among CNS structures for its low level of $GABA_B$ R2 protein and mRNA expression compared with $GABA_B$ R1 (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Benke et al., 1999; Ng et al., 1999). These earlier observations have now been confirmed in rats and humans using in situ hybridization or immunohistochemical methods with different antibodies (Durkin et al., 1999; Billinton et al., 2000; Clark et al., 2000; Ige et al., 2000; Ng and Yung , 2001a). This differential distribution of the two $GABA_B$ receptor subunits in striatal neurons raised various issues regarding the functional significance of $GABA_B$ R1/$GABA_B$ R2 heterodimerization in forming functional $GABA_B$ receptors in the striatum. The possibility that other $GABA_B$ receptor homologues may associate with $GABA_B$ R1 to produce new receptor subtypes in this region has been suggested but remains hypothetical (Calver et al., 2003). Our findings are consistent with these observations showing low levels of $GABA_B$ R2 immunoreactivity in both cellular and neuropil elements of the caudate nucleus and putamen in monkeys. However, despite this rather scarce labeling, our electron microscopy data demonstrate that the pattern of distribution of $GABA_B$ R2-labeled elements is not strikingly different from that of $GABA_B$ R1, suggesting a close association between the two receptor subtypes.

On the other hand, in vitro data from non-neuronal culture cells suggested that $GABA_B$ R1 and $GABA_B$ R2 have to be linked in a 1:1 stoichiometry ratio through coiled-coil domains at the C terminus to make functional $GABA_B$ receptors (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999). If such is indeed the case in striatal neurons, it is likely that the molecular mechanisms underlying functional $GABA_B$ receptor functions in the striatum may be more complex and possibly different from other brain regions.

Future studies should be undertaken to examine the possibility of heteromeric complexes made up of $GABA_B$ receptor subtypes and other members of the G protein-coupled receptor superfamily or additional protein-protein interactions in striatal neurons (see below for further discussion). In this regard, it is noteworthy that direct physical association between $GABA_B$ R1 and the $GABA_A$ γ2 subunit has been found in vitro, suggesting that this cross talk may be a potential mechanism to form functional $GABA_B$ receptors independent of the $GABA_B$ R2 expression level (Balasubramanian et al., 2002). Furthermore, direct functional interactions of G protein-coupled receptors with metabotropic or ionotropic receptors have been disclosed in vitro in various neuronal and non-neuronal transfected cell preparations (Jordan et al., 2001; Gines et al., 2000; Liu et al., 2000; Ciruela et al., 2001; Ferré et al., 2002; Nishi et al., 2003).

Despite this rather incomplete view of the molecular biology of $GABA_B$ receptors in striatal neurons, there is strong evidence for $GABA_B$-mediated presynaptic regulation of GABAergic (Wilson and Wilson, 1985; Chu et al., 1990) or glutamatergic (Kilpatrick et al., 1983; Calabresi et al., 1990, 1991; Seabrook et al., 1990; Moratalla and Bowery, 1991; Nisenbaum et al., 1992, 1993) transmission in the rat striatum. Our data support the existence of presynaptic $GABA_B$ heteroreceptors on glutamatergic terminals and preterminal axons in the primate striatum (Charara et al., 2000). Although we could not find clear evidence for presynaptic $GABA_B$ R2 immunoreactivity in GABAergic terminals, it is noteworthy that $GABA_B$ R1 is expressed in a subpopulation of putative GABAergic or dopaminergic boutons in the monkey striatum (Charara et al., 2000a). Recent confocal microscopic data confirmed the presynaptic localization of $GABA_B$ R2 in putative glutamatergic and dopaminergic terminals in the rat caudate-putamen (Ng and Yung, 2001a). Unmyelinated preterminal axons provide additional sites at which $GABA_B$ receptors could mediate presynaptic regulation of neurotransmitter release in the striatum.

The fact that dendrites account for the largest proportion of $GABA_B$ R1- and $GABA_B$ R2-labeled elements in the monkey putamen strongly suggests the expression of functional postsynaptic $GABA_B$ receptors in striatal neurons. The significantly larger percentages of labeled dendrites compared with spines is consistent with the fact that most GABAergic inputs form axodendritic synapses with striatal projection neurons (Smith and Bolam, 1990). However, the exact role of these receptors remains poorly known and surely deserves further consideration (Seabrook et al., 1991; Nisenbaum et al., 1992, 1993).

Globus pallidus and substantia nigra. In GPe and SN, the proportion of $GABA_B$ R1- and $GABA_B$ R2-containing unmyelinated axons was significantly higher than in other basal ganglia nuclei, suggesting a prominent role for functional presynaptic $GABA_B$ receptors in these brain regions. Although the proportion of labeled terminals was relatively low compared with other neuronal elements in GPe, the fact that both $GABA_B$ R1- and $GABA_B$ R2-containing boutons formed asymmetric synapses and displayed the ultrastructural features of putative glutamatergic terminals suggests the existence of presynaptic $GABA_B$ heteroreceptors that modulate glutamate release in the primate GPe. A preliminary report showed that, indeed, local application of the $GABA_B$ agonist baclofen decreases the efflux of glutamate in the rat GP in vivo (Singh, 1990) and reduces the excitatory postsynaptic potentials (EPSPs) mediated by glutamate in the rat SNr in vitro (Shen and Johnson, 1997). Furthermore, $GABA_B$ receptor agonists reduce the frequency of

miniature EPSCs in slices of rat GP, suggesting a presynaptic regulation of glutamatergic transmission (Chen et al., 2002). These electrophysiological observations corroborate in vivo rotational behavioral data showing that unilateral microinjection of baclofen, a $GABA_B$ receptor agonist, in the GP induces glutamate receptor-dependent ipsilateral turning in rats (Chen et al., 2002).

Interestingly, the situation appears to be different in the SN, where presynaptic $GABA_B$ autoreceptors on striatal, pallidal, and intranigral terminals have been characterized (Floran et al., 1988; Giralt et al., 1990; Hausser and Yung, 1994; Shen and Johnson, 1997; Chan et al., 1998; Tepper et al., 1998; Paladini et al., 1999; Boyes and Bolam, 2003). Although we could not detect significant $GABA_B$ R2 labeling in GABAergic terminals in the monkey basal ganglia, the abundance of labeled preterminal unmyelinated axons in GPe and SN suggests that these presynaptic autoreceptor effects might be mediated through activation of $GABA_B$ receptors expressed along incoming axons of GABAergic neurons. If such is the case, the regulation of extrasynaptic spillover of GABA released from axon terminals remains a critical factor in controlling the activation of these receptors (Attwell et al., 1993; Isaacson et al., 1993; Dittman and Regehr, 1997; Vogt and Nicoll, 1999; Mitchell and Silver, 2000; Scanziani, 2000). Our data are consistent with those recently published from the rat SNr, although a larger number of $GABA_B$ R2-containing putative GABAergic terminals was found in rats compared with monkeys (Boyes and Bolam, 2003). Whether this indicates a species difference between primates and nonprimates in the distribution of presynaptic $GABA_B$ autoreceptors or a lower sensitivity of the $GABA_B$ receptor antibodies for their antigenic sites in monkeys remains to be established.

The abundance of $GABA_B$ R1- and $GABA_B$ R2-immunoreactive dendrites in the monkey pallidum suggests the expression of postsynaptic $GABA_B$ receptors in pallidal neurons. However, the literature regarding $GABA_B$-mediated postsynaptic effects in GP is rather scarce and relies on recent in vitro patch-clamp recording studies showing postsynaptic modulation of voltage-dependent calcium currents and the induction of a weak outward current in a subset of GP neurons (Stefani et al., 1999; Chen et al., 2002). Future studies are definitely needed to better assess the exact roles played by postsynaptic $GABA_B$ receptors in pallidal functions.

Subthalamic nucleus. In the monkey STN, both $GABA_B$ R1 and $GABA_B$ R2 immunoreactivity was largely found in dendrites, which is consistent with recent in vitro (Shen and Johnson, 2001) and in vivo (Urbain et al., 2002) evidence for modest postsynaptic $GABA_B$-mediated effects in rat STN neurons. However, there was a significant difference in the relative proportion of axon terminals labeled for either receptor subtypes in this nucleus. Although almost 15% of the total number of $GABA_B$ R1-containing STN elements were accounted for by axon terminals, very little terminal labeling was found in the $GABA_B$ R2-immunostained tissue. The significance of presynaptic $GABA_B$ receptor functions in rat STN neurons was recently emphasized by Shen and Johnson (2001), who demonstrated robust presynaptic effects of the $GABA_B$ receptor agonist baclofen on GABAergic and glutamatergic transmission in slices of rat STN. Therefore, despite the low incidence of $GABA_B$ R1/$GABA_B$ R2 expression at the terminal level in monkeys, it appears that functional presynaptic $GABA_B$ receptors are expressed in the rat STN. Whether the reduced expression of $GABA_B$ R2 at the terminal level is a characteristic feature of the monkey STN remains to be established.

As discussed above for the striatum, an alternative hypothesis for the differential expression of $GABA_B$ R1 and $GABA_B$ R2 in the CNS could be that $GABA_B$ R1 functionally interacts with other $GABA_B$-related proteins in $GABA_B$ R2-negative elements (Calver et al., 2003). Although future studies are needed to better understand the molecular mechanisms that underlie presynaptic $GABA_B$ receptor-mediated effects in the STN, it is noteworthy that such receptors may have important clinical implications for the development of novel therapeutic strategies for Parkinson's disease. The functional balance between the activity of extrinsic glutamatergic and GABAergic inputs is, indeed, critical for the control of STN neurons under both normal and parkinsonian conditions (DeLong, 1990; Wichman and DeLong, 1996). Therefore, therapeutic approaches that aim at modulating transmission at these synapses using $GABA_B$-related compounds surely deserve further consideration. Along those lines, recent rodent data demonstrate a significant upregulation of $GABA_B$ R1a subunit mRNA in basal ganglia output nuclei and subthalamic nucleus following lesion of the nigrostriatal dopaminergic pathway (Johnston and Duty, 2003).

$GABA_B$ R1/R2 heterodimerization and more. . .

As discussed above, it is now well established that $GABA_B$ R1/$GABA_B$ R2 heterodimerization is essential to form functional $GABA_B$ receptors. Although $GABA_B$ R1 is responsible for the binding of GABA, $GABA_B$ R2 is needed for the correct trafficking of the receptor to the cell surface and the proper downstream G protein signaling in response to agonist stimulation (Martin et al., 1999; Filippov et al., 2000; Bowery and Enna, 2000; Couve et al., 2000, 2002; Jones et al., 2000; Kuriyama et al., 2000; Galvez et al., 2001; Margeta-Mitrovic et al., 2000; 2001; Calver et al., 2001; Pagano et al., 2001; Robbins et al., 2001). The potential functions of $GABA_B$ receptors in the CNS, including some basal ganglia nuclei, are many and comprise important physiological processes critical for the proper development, regulation, and plasticity of synaptic communication (Bowery and Enna, 2000; Couve et al., 2000; Jones et al., 2000; Kuriyama et al., 2000; Margeta-Mitrovic et al., 2000; Sakaba and Neher, 2003). In addition, therapeutic benefits of $GABA_B$-related compounds have been reported for various CNS diseases including drug addiction, spasticity, pain, epilepsy, and complex cognitive impairments (Kerr and Ong, 1995; Bowery, 1997; Couve et al., 2000). It would be surprising that such a large variety of physiological processes is mediated by a single $GABA_B$ receptor. There is, indeed, strong evidence for the existence of various $GABA_B$ receptor subtypes with different pharmacological properties in the central and peripheral nervous systems (Thompson and Gahwiler, 1992; Shoji et al., 1997; Yamada et al., 1999; Marcoli et al., 2000).

Although the exact molecular mechanisms that underlie these functional effects remain largely unknown, one may speculate that a differential subunit composition may account for such variability. As mentioned above, the striatum is a typical example for which $GABA_B$ R1/$GABA_B$ R2 heterodimerization cannot explain all $GABA_B$-mediated effects in this brain region because of the low expression levels of

GABA$_B$ R2 compared with GABA$_B$ R1. Similarly, the hypothalamus contains low levels of GABA$_B$ R2 (Kaupmann et al., 1998; Durkin et al., 1999), and all peripheral tissues studied so far appear to be devoid of GABA$_B$ R2 mRNA, whereas they contain functional GABA$_B$ receptors (Ong and Kerr, 1990; Calver et al., 2000). However, as for now, the existence of other GABA$_B$ receptor subunits remains speculative because only GABA$_B$ R1 and GABA$_B$ R2 have been cloned from mammalian systems.

On the other hand, it is noteworthy that the invertebrate *Drosophila* expresses an additional putative GABA$_B$ receptor subunit, named GABA$_B$R3 (Mezler et al., 2001). Furthermore, a novel putative seven-transmembrane G protein-coupled receptor, named GABA$_B$L, was recently identified in humans. Although the amino acid sequence and distribution of this protein strongly suggest potential functional interactions with GABA$_B$ R1 and GABA$_B$ R2, isolated expression of GABA$_B$L with either of the two GABA$_B$ receptor subunits did not result in any functional GABA$_B$ receptors (Calver et al., 2003). Therefore, if this protein is, indeed, a GABA$_B$ receptor subunit, it probably interacts with other GABA$_B$ receptor subunits that have not yet been cloned. Although research over the past 5 years has had a tremendous impact on our understanding of the molecular biology of GABA$_B$ receptors, it is clear that further cloning studies are essential to fully characterize the molecular substrate that underlies GABA$_B$ receptor functions in various areas of the central and peripheral nervous systems.

GABA$_B$ receptors: targets for GABA spillover

Another interesting aspect of GABA$_B$ receptor subunits is their pattern of subsynaptic localization. Although we could not use immunogold methods to study the subsynaptic localization of GABA$_B$ R2 in basal ganglia, due to the low level of immunoreactivity detected with our antibodies in monkey tissue, we and others have used immunogold labeling techniques to look at the subcellular and subsynaptic distribution of GABA$_B$ R1 and GABA$_B$ R2 in the basal ganglia and other CNS regions (Smith et al., 2000; Kulik et al., 2002; Boyes and Bolam, 2003). These receptors display a rather unique and surprising pattern of synaptic targeting, being mainly expressed extrasynaptically or at the edges of putative glutamatergic synapses (Fritschy et al., 1999; Smith et al., 2000; Kulik et al., 2002; Boyes and Bolam, 2003). Such a pattern of labeling raises interesting questions regarding the mechanisms of activation and functions of GABA$_B$ receptors at these non-GABAergic sites. Extrasynaptic diffusion of GABA and receptor-receptor interactions should definitely be considered when investigating the mechanisms through which GABA$_B$ receptors modulate neurotransmission in the nervous system (Attwell et al., 1993; Isaacson et al., 1993; Dittman and Regehr, 1997; Vogt and Nicoll, 1999; Mitchell and Silver, 2000; Scanziani, 2000).

Together, these findings emphasize the potential degree of complexity through which GABA$_B$ receptors could mediate their effects in the mammalian nervous system. It is clear that our current knowledge of GABA$_B$ receptor molecular biology and functions is rather limited and surely deserves further consideration. The characterization, localization, and function of novel GABA$_B$ receptor subunits should remain the cornerstone of future research if one hopes to better understand the roles of these receptors and their potential therapeutic relevance for brain diseases.

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