THE JOURNAL OF COMPARATIVE NEUROLOGY 474:340–352 (2004)

Subcellular Localization of $GABA_B$ Receptor Subunits in Rat Globus Pallidus

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ABSTRACT

The inhibitory amino acid γ-aminobutyric acid (GABA) is the major neurotransmitter in the globus pallidus. Although electrophysiological studies have indicated that functional $GABA_B$ receptors exist in rat globus pallidus, the subcellular localization of $GABA_B$ receptor subunits and their spatial relationship to glutamatergic and GABAergic synapses are unknown. Here, we use pre-embedding immunogold labeling to study the subcellular localization of $GABA_B$ receptor subunits, $GABA_{B1}$ and $GABA_{B2}$, in globus pallidus neurons and identified populations of afferent terminals. Immunolabeling for $GABA_{B1}$ and $GABA_{B2}$ was observed throughout the globus pallidus, with $GABA_{B1}$ more strongly expressed in perikarya and $GABA_{B2}$ mainly expressed in the neuropil. Electron microscopic analysis revealed that the majority of $GABA_{B1}$ labeling was localized within the cytoplasm, whereas most of $GABA_{B2}$ labeling was associated with the plasma membrane. At the subcellular level, both the $GABA_{B1}$ and $GABA_{B2}$ immunogold labeling was localized at pre- and postsynaptic sites. At asymmetric, putative excitatory, synapses, $GABA_{B1}$ and $GABA_{B2}$ immunogold labeling was found at perisynaptic sites of both pre- and postsynaptic specializations. Double immunolabeling, using the vesicular glutamate transporter 2 (VGLUT2), revealed the glutamatergic nature of most immunogold-labeled asymmetric synapses. At symmetric, putative GABAergic, synapses, including those formed by anterogradely labeled striatopallidal terminals, $GABA_{B1}$ and $GABA_{B2}$ immunogold labeling was found in the main body of both pre- and postsynaptic specializations. These results demonstrate the existence of presynaptic $GABA_B$ auto- and heteroreceptors and postsynaptic $GABA_B$ receptors, which may be involved in modulating synaptic transmission in the globus pallidus. J. Comp. Neurol. 474:340–352, 2004.

Indexing terms: basal ganglia; synapses, striatopallidal; pre-embedding immunogold; VGLUT2

The globus pallidus (the external segment of the globus pallidus in primates) occupies a critical position in the indirect pathway of the basal ganglia and, as such, plays an important role in the expression of basal ganglia behaviour. Morphological and physiological studies indicate that the globus pallidus receives inhibitory γ-aminobutyric acid (GABA)ergic inputs from the striatum and excitatory glutamatergic inputs from subthalamic nucleus, neocortex, and thalamus (Smith et al., 1990b, 1998; Kita, 1993; Parent and Hazrati, 1995; Nambu et al., 2000). In turn, the globus pallidus is in a position to influence the activity of the whole basal ganglia through its GABAergic projections to the striatum, substantia nigra, entopeduncular nucleus, and subthalamic nucleus (Smith et al., 1990a; Bolam and Smith, 1992; Kita and Kitai, 1994; Bevan et al., 1998; Smith et al., 1998; Bolam et al., 2000). It has been suggested that abnormal hypoactivity and decreased

Grant sponsor: Medical Research Council UK; Research Grants Council of Hong Kong; Grant number: CUHK 4080/00M.

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Received 1 October 2003; Revised 5 December 2003; Accepted 9 December 2003

DOI 10.1002/cne.20143

Published online in Wiley InterScience (www.interscience.wiley.com).

GABAergic output of the globus pallidus contribute to the overactivity of the indirect pathway that is associated with the akinesia and hypokinetic symptoms of Parkinson's disease (Albin et al., 1989; Filion and Tremblay, 1991; Chesselet and Delfs, 1996; Wichmann and DeLong, 1996). Furthermore, the complex interactions of the globus pallidus with the subthalamic nucleus and the cortex have been proposed to underlie oscillatory activity within this network and possibly the tremor seen in parkinsonism (Bergman et al., 1998; Plenz and Kitai, 1999; Magill et al., 2001).

The effects of GABA in the central nervous system are mediated by two types of receptors: the ionotropic, bicuculline-sensitive $GABA_A$ receptors and the metabotropic, G-protein coupled $GABA_B$ receptors, which are activated by baclofen. By inhibiting calcium influx and facilitating potassium conductance, activation of $GABA_B$ receptors produces pre- and postsynaptic inhibitory effects, respectively. The recent cloning of $GABA_B$ receptors has revealed that two subunits, $GABA_{B1}$ and $GABA_{B2}$, are assembled to form functional $GABA_B$ receptors (Jones et al., 1998; Kaupmann et al., 1997, 1998; White et al., 1998; Kuner et al., 1999). By using in situ hybridization and immunocytochemistry, the regional and cellular distribution of $GABA_B$ receptor subunits and their splice variants have been reported in the brain revealing the presence of mRNA and receptor protein in the globus pallidus (Benke et al., 1999; Bischoff et al., 1999; Fritschy et al., 1999; Charara et al., 2000; Gonchar et al., 2001; Kulik et al., 2002; López-Bendito et al., 2002). In addition, autoradiographic studies have revealed binding sites for $GABA_B$ receptors in the globus pallidus (Bowery et al., 1987; Chu et al., 1990). Furthermore, immunolabeling at the electron microscopic level has demonstrated the pre- and postsynaptic localization of one of the subunits of the $GABA_B$ receptor, $GABA_{B1}$, in the globus pallidus in monkeys (Charara et al., 2000; Smith et al., 2000).

In view of the importance of the striatopallidal pathway and the interconnections between pallidal neurons in the expression of activity in the globus pallidus, a detailed knowledge of the nature of GABAergic transmission in the globus pallidus is critical in understanding its role in normal and pathological conditions. Although fast GABA transmission in the globus pallidus occurs through $GABA_A$ receptors that are primarily located at synaptic specialisations (Somogyi et al., 1996), a role for $GABA_B$ receptors has been proposed. Thus, physiological and pharmacological studies have revealed that activation of $GABA_B$ receptors in rat globus pallidus inhibits the release of GABA and glutamate and hyperpolarizes pallidal neurons (Chan et al., 2000; Chen et al., 2002). To better understand the effects of $GABA_B$ receptor activation on inhibitory and excitatory transmission in the globus pallidus, it is important to know the receptor subunit distribution in globus pallidus neurons and their relationship to GABAergic and glutamatergic synapses. Therefore, the objectives of the present study were to determine the subcellular localization of $GABA_B$ receptor subunits in the rat globus pallidus: first, to determine the spatial distribution of $GABA_{B1}$ and $GABA_{B2}$ subunits; second, to determine the localization of $GABA_B$ receptor subunits relative to glutamatergic synapses; finally, to determine the distribution of $GABA_B$ receptor subunits at striatopallidal synapses. To this end, we used the pre-embedding immunolabeling technique to enable us to precisely localize the receptors and quantify the distribution of the immunolabeling and to enable us to perform double immunolabeling with a marker of glutamatergic terminals and an anterograde marker.

MATERIALS AND METHODS

Preparation of animals and tissue

Sprague-Dawley rats (Charles River, Margate, Kent; 200–250 g) used in the present study were maintained on a 12 hour light/dark cycle with free access to food and water. Environmental conditions for housing of the rats, and all procedures performed on them, were in accordance with the Animals (Scientific Procedures) Act 1986 (UK) and in accordance with the European Communities Council Directive (80/609/EEC). All efforts were made to minimize the suffering of the animals.

The rats were deeply anesthetized with sodium pentobarbitone (Sagatal, Rhône Mérieux, Tallaght, Dublin, Ireland; 200 mg/kg, i.p) and then perfused transcardially with 50–100 ml phosphate-buffered saline (PBS; 0.01 M phosphate, pH 7.4) over 1–2 minutes followed by 300 ml of 3% paraformaldehyde containing 0.1% glutaraldehyde in PB (0.1 M, pH 7.4) over a period of 20 minutes. The animals were then post-perfused with ~100 ml of PBS. After fixation, the brain was removed from the cranium and coronal sections of globus pallidus were cut on a vibrating microtome at 70 μm and collected in PBS. To enhance the penetration of immunoreagents, sections were equilibrated in a cryoprotectant solution (0.05 M PB, pH 7.4, containing 25% sucrose and 10% glycerol) overnight and freeze-thawed the following day by freezing in isopentane (BDH Chemicals, Letterworth, Leicestershire, UK) cooled in liquid nitrogen and then in liquid nitrogen and thawing in PBS. The sections were then washed several times in PBS. Before the pre-embedding immunocytochemical labeling, the sections were incubated with 10% normal goat serum (NGS; in PBS) for 2 hours at room temperature.

Antibodies

Two commercially available polyclonal antibodies raised in guinea pig against synthetic peptides from the C-termini of the two known $GABA_B$ receptor subunits, $GABA_{B1}$ (common to both $GABA_{B1a}$ and $GABA_{B1b}$ splice variants; Kaupmann et al., 1997) and $GABA_{B2}$, were used in the study (both Chemicon International, Harrow, UK). Both antibodies have been characterized previously by immunoblot analysis (Yung et al., 1999; Ng and Yung, 2001a). In addition, the specificity of the $GABA_{B1}$ antibody has been established in $GABA_{B1}$ subunit knockout mice (Prosser et al., 2001).

Anterograde labeling of striatopallidal afferents

Two rats were anesthetized by intraperitoneal injection of a mixture of fentanyl/fluanisone (0.135 mg/ml and 10 mg/ml, respectively; Hypnorm; Janssen-Cilag, Ltd., High Wycombe, UK) and midazolam (5 mg/ml; Hypnovel; Roche Products, Ltd., Welwyn Garden City, UK; 1:1:2 with sterile water; 2.7 ml/kg) and then fixed in a stereotaxic frame. By using coordinates derived from a stereotaxic atlas (Paxinos and Watson, 1986), deposits of *Phaseolus vulgaris* leucoagglutinin (PHA-L; 2.5% in 0.01 M PB, pH 8.0;

Vector Laboratories, Peterborough, UK) were delivered bilaterally into the striatum (three to four injections per hemisphere) by iontophoresis using glass micropipettes of 10- to 15-μm diameter and a pulsed (7 seconds on, 7 seconds off) positive current (5 μA) for 15 minutes. The glass micropipettes were then left in situ for at least 10 minutes to minimize leakage along the injection tract. After a survival time of 7 days, the rats were perfused as described above, and 70-μm-thick coronal sections were cut through the injection sites and the globus pallidus.

PHA-L injection sites in the striatum were revealed by the immunoperoxidase method using 3,3′-diaminobenzidine (DAB; Sigma, Poole, Dorset, UK) as the chromogen (see below). The sections were mounted on glass microscope slides and processed for light microscopic examination.

Immunogold labeling

The pre-embedding immunogold method was carried out as previously described (Yung et al., 1995; Boyes and Bolam, 2003). All incubation steps were carried out in PBS containing 2% NGS (PBS-NGS) and sections were washed 3 or 4 times between steps. The sections were incubated in primary antibodies against $GABA_B$ receptors: anti-$GABA_{B1}$ at 1:1,000 or anti-$GABA_{B2}$ at 1:2,000, for 48 hours at 4°C. After washing (3× in NGS-PBS), they were incubated in 1.4-nm gold-conjugated goat anti-guinea pig IgG (Nanoprobes, Stony Brook, NY) at 1:100 overnight at room temperature. The sections were then washed (3× in PBS and 2× in sodium acetate buffer, 0.1 M, pH 7.0), and the gold labeling was intensified by using a silver enhancement kit (Nanoprobes HQ Silver) for 3–5 minutes at room temperature in the dark. The sections were then washed in acetate buffer followed by PB.

Double immunolabeling

Double immunolabeling was carried out to reveal $GABA_B$ receptor subunits and the PHA-L–labeled striatopallidal terminals or VGLUT2-immunoreactive terminals in the same sections. The $GABA_B$ receptors were revealed first by the immunogold method (as described above) and then the PHA-L or VGLUT2 by the immunoperoxidase method. For animals injected with PHA-L, after silver enhancement, sections of the globus pallidus were incubated in rabbit anti–PHA-L antibody (1:5,000 in NGS-PBS; Vector Laboratories) for 48 hours at 4°C. For VGLUT2 double labeling, the sections were incubated in rabbit anti-VGLUT2 antibody (Synaptic Systems, Göttingen, Germany) at 1:2,000 in 2% NGS-PBS for 48 hours at 4°C. All the sections for double labeling were then incubated in biotinylated goat anti-rabbit IgG (1:100 in 2% NGS-PBS; Vector Laboratories) for 2 hours at room temperature. The sections were incubated in avidin-biotin-peroxidase complex (ABC; 1:100 in PBS; Vector Laboratories) for 1 hour at room temperature. After 2× PBS and 1× Tris buffer (0.05 M, pH 7.6) washes, the immunoreactive sites were revealed by incubation in 0.025% DAB (in Tris buffer) in the presence of 0.01% H_2O_2. The reaction was stopped after 6–8 minutes by several washes in Tris buffer.

Processing for electron microscopy

All sections were post-fixed in 1% osmium tetroxide (in PB; Oxkem, UK) for 7 minutes. After washing in PB (3×), the sections were dehydrated in an ascending series of dilutions of ethanol, with 1% uranyl acetate included in the 70% ethanol solution to increase contrast in the electron microscope. After the absolute ethanol, the sections were treated with propylene oxide and equilibrated in resin overnight (Durcupan, ACM, Fluka, Gillingham, Dorset, UK). The sections were then mounted on microscope slides, and the resin polymerized at 60°C for 48 hours. All the sections were first examined in the light microscope and areas of interest were cut out from the slides and glued to blank cylinders of resin. Serial ultrathin sections (approximately 70 nm) were cut on a Riechert-Jung Ultracut E ultramicrotome (Leica, Nussloch, Germany) and collected on pioloform-coated single-slot copper grids. The sections were then stained with lead citrate for 3–4 minutes and examined in a Philips CM 10 electron microscope.

Controls for specificity of the immunolabeling

The specificity of the pre-embedding immunolabeling technique was demonstrated by the absence of labeling for the respective antigens when the primary antibodies were omitted. Furthermore, sections of cerebellum were included in each incubation vial to act as a positive control for the $GABA_B$ immunolabeling and a control for the silver enhancement. Because the double labeling was performed, it was necessary to control the specificity of the secondary antibodies. Sections were incubated with either a primary antibody raised in guinea pig or rabbit and then with secondary antibody directed against immunoglobulins of rabbit or guinea pig respectively. In parallel, positive controls for each antibody were performed. In each case of negative controls, no specific labeling was observed, although the positive controls revealed specific labeling for single or double labeling.

Quantitative analysis

The subcellular distribution of immunogold labeling for $GABA_{B1}$ and $GABA_{B2}$ subunits was analyzed on immunolabeled sections from three rats for single-labeled material and two rats for double-labeled material, i.e., VGLUT2 or PHA-L. To quantify the overall distribution of immunogold labeling for $GABA_{B1}$ and $GABA_{B2}$ within pre- and postsynaptic elements, areas of globus pallidus were selected at random in the electron microscope and then systematically scanned. Each gold particle was counted and categorized as either intracellular or associated with the plasma membrane. Included in this analysis were gold particles counted in photomicrographs of immunolabeled synapses that had been used for other aspects of the analysis. As these photomicrographs were not taken in a random manner, only those particles *not* associated with the synapse that was the object of the photomicrograph were analyzed to ensure random sampling of the immunoparticles.

To determine the location of $GABA_{B1}$ and $GABA_{B2}$ relative to synapses in single- and double-labeled material, gold particles associated with the pre- or postsynaptic membrane at symmetric and asymmetric synapses were classified as being either in the main body of the synapse or perisynaptic, i.e., at the edge of the synaptic specialization. Note that, in some cases, particularly at symmetrical synapses, it was difficult to define a gold particle as being truly perisynaptic or within the main body of the synapse

but very close to the edge. The category perisynaptic, thus, will include both.

In the VGLUT2-labeled material, the proportion of terminals forming asymmetric synapses that express VGLUT2 in the globus pallidus, the proportion of those also immunolabeled for the $GABA_B$ receptors and the distribution of the $GABA_B$ immunolabeling in relation to the synapses were calculated in systematic scans in the electron microscope. For PHA-L–labeled material, sections were scanned in the electron microscope and all double-labeled terminals that were encountered were photographed. The analysis of the distribution of the immunolabeling was carried out on the photomicrographs.

Image capture and manipulation

Light microscopic analysis was carried out on a Leitz Dialux 22 or Leitz Diaplan. Images were captured using a Photometrics Coolsnap digital camera and Openlab software (Improvisions, Ltd., version 2.2.5) on a Macintosh computer. Images were manipulated (contrast, brightness, and labeling) using Adobe Photoshop. Images from the electron microscope were recorded on film. High-quality prints were then scanned and manipulated using Adobe Photoshop as above.

RESULTS

Light Microscopic Observations

At the light microscope level, the pattern of immunolabeling for $GABA_{B1}$ receptor subunits in the globus pallidus was similar to that previously described in monkeys (Charara et al., 2000). Immunogold labeling was observed in perikarya and proximal dendrites of medium-sized multipolar neurons and more weakly in the neuropil (Fig. 1A). Immunolabeling for $GABA_{B2}$ was also present in perikarya and in the neuropil. However, the perikaryal labeling was less intense than for $GABA_{B1}$, with stronger $GABA_{B2}$ immunolabeling observed in the neuropil (Fig. 1B). Immunolabeling in other regions of the basal ganglia, i.e., the striatum and substantia nigra, was consistent with that observed previously (not illustrated; Yung et al., 1999; Charara et al., 2000; Smith et al., 2000; Ng and Yung, 2001a,b; Boyes and Bolam, 2003). Furthermore, immunolabeling for both receptor subunits in the cerebellum (Fig. 1C,D) was similar to that described previously on many occasions (see for instance, Fritschy et al., 1999; Margeta-Mitrovic et al., 1999; Ige et al., 2000; Liang et al., 2000; Kulik et al., 2002).

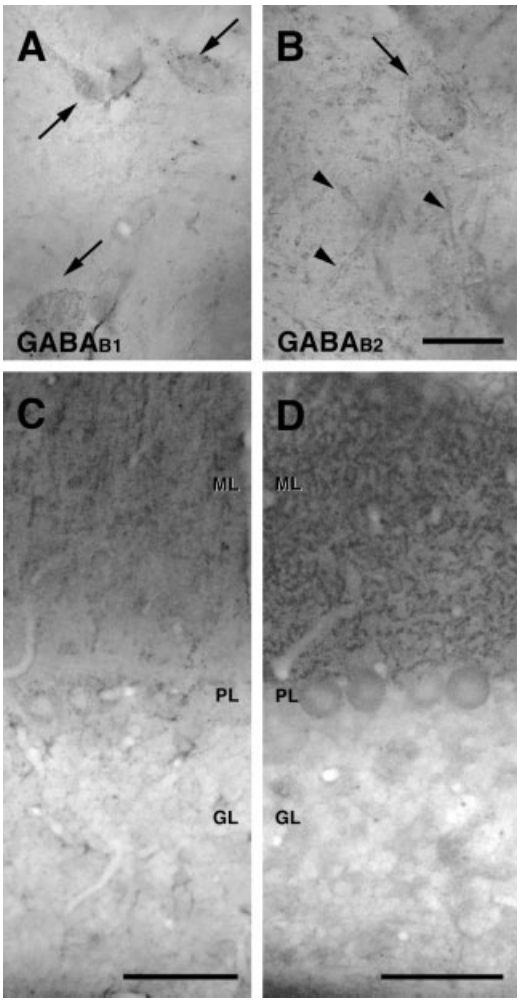

Fig. 1. A–D: Light photomicrographs of immunogold-labeled sections for γ-aminobutyric acid $(GABA)_{B1}$ and $GABA_{B2}$ in the globus pallidus (A,B) and the cerebellum (C,D). **A,B:** High-power light photomicrographs showing $GABA_{B1}$ (A) and $GABA_{B2}$ (B) immunolabeling in the globus pallidus. $GABA_{B1}$ (A) labeling is mainly observed in perikarya in the globus pallidus (arrows), whereas $GABA_{B2}$ (B) labeling is mainly present in the neuropil (arrowheads) but also in perikarya (arrow). **C,D:** Consistent with previous observations, both $GABA_{B1}$ (C) and $GABA_{B2}$ (D) immunolabeling was strong in the molecular layer (ML), was present in some Purkinje cell perikarya, and was present, albeit less intensely, in the granule cell layer (GL). PL: Purkinje cell layer. Scale bars = 20 μm in B (applies to A,B); 50 μm in C,D.

Electron microscopic observations

Localization of $GABA_{B1}$ in the globus pallidus. The $GABA_B$ receptor-immunoreactive sites were identified by the irregularly shaped, electron dense silver-enhanced immunogold particles (hereafter simply referred to as immunogold particles; Figs. 2, 5). Immunolabeling for $GABA_{B1}$ was widely distributed within the globus pallidus. Immunogold particles were detected in neuronal perikarya, dendritic shafts, axons, and axon terminals, both on the plasma membrane and at intracellular sites (Fig. 2). Consistent with light microscopic observations, $GABA_{B1}$ immunolabeling was especially prominent in perikarya and dendrites. The majority (70%) of $GABA_{B1}$ immunoparticles were located within the cytoplasm (Fig. 3), where they were associated with a variety of organelles, including endoplasmic reticulum and Golgi apparatus. The membrane-associated immunogold particles (30%) were located on the internal surface of the membrane at extrasynaptic sites, perisynaptic sites, and within synaptic specializations on pre- and/or postsynaptic membranes (Fig. 2). At asymmetric synapses, only 13% of synapse-associated $GABA_{B1}$ immunogold particles were found in the main body of the synapses (Fig. 2A). Most synapse-associated $GABA_{B1}$ immunogold particles (87%) were observed at perisynaptic sites on the presynaptic (17%) or postsynaptic (70%) membrane (Figs. 2A,B, 4A). In contrast, at symmetric synapses, most (90%) synapse-associated $GABA_{B1}$ immunogold particles were localized

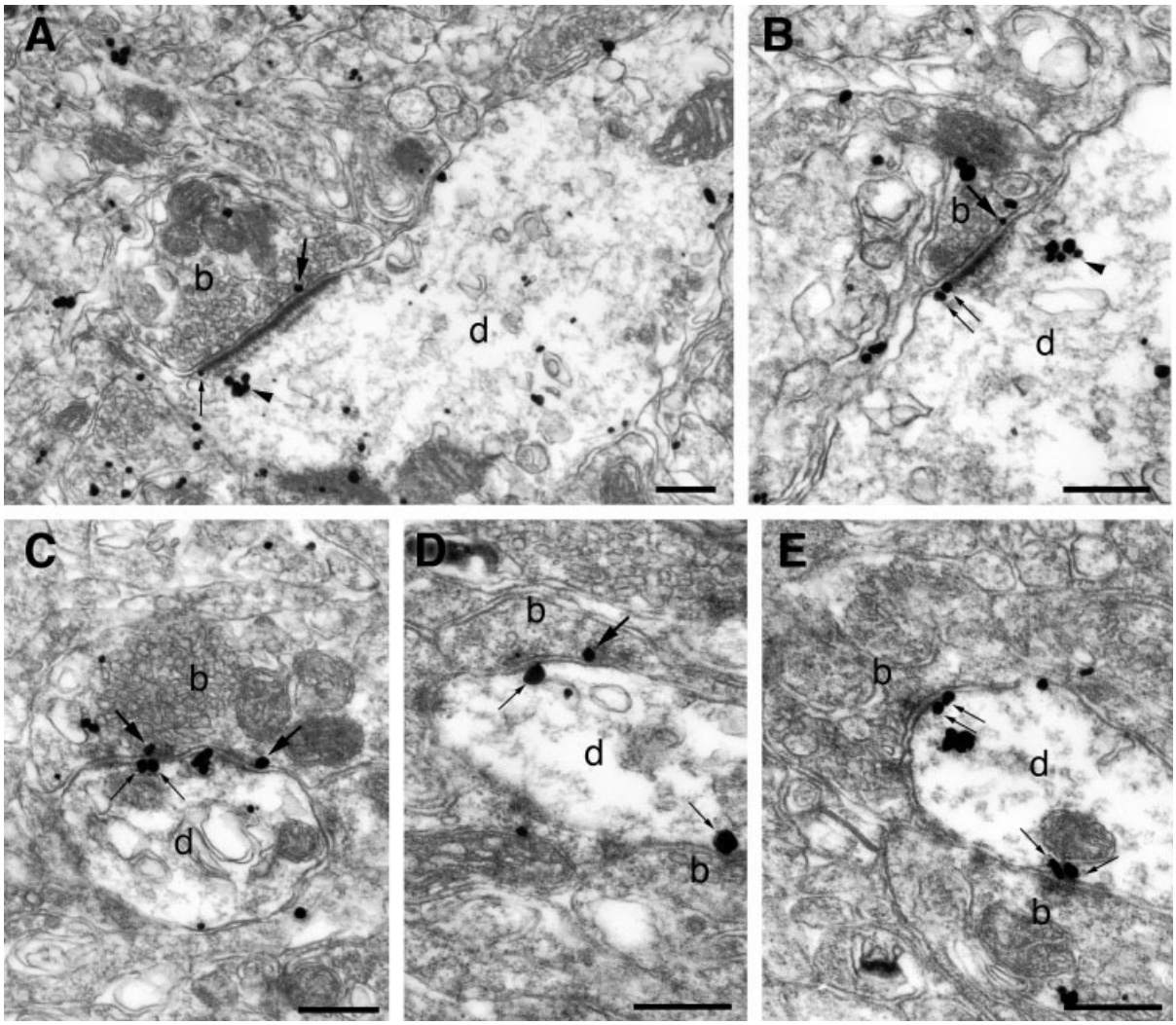

Fig. 2. Localization of γ-aminobutyric acid $(GABA)_{B1}$ immunogold labeling in the globus pallidus. A,B: Immunogold labeling associated with asymmetric synapses. **A:** A large bouton (b) forms an asymmetric synapse with a large dendritic shaft (d). Labeling is present on the presynaptic membrane (large arrow) and at the edge of the synapse on the postsynaptic membrane (small arrow). **B:** A small bouton forms an asymmetric synapse that is labeled at perisynaptic sites on both the presynaptic (large arrow) and postsynaptic (small arrows) membrane. Note the presence of intracellular labeling in the dendrites (some indicated by arrowheads). C–E: Immunolabeling for $GABA_{B1}$ at symmetric synapses. **C,D:** In C and the upper synapse in D, labeling is associated with both the presynaptic (large arrows) and postsynaptic (small arrows) membranes. **D,E:** The labeling associated with the lower synapse in D and the synapses in E is associated with the postsynaptic membrane (small arrows). b, bouton; d, dendrite. Scale bars = 0.25 μm.

in the main body of the synapse (Fig. 2C–E), accounting for 34% on presynaptic and 56% on postsynaptic membrane (Fig. 4A).

Localization of $GABA_{B2}$ in the globus pallidus. In contrast to the predominantly cytoplasmic localization of $GABA_{B1}$, the majority of $GABA_{B2}$ immunogold particles (63%) were associated with the plasma membrane (Fig. 3). The $GABA_{B2}$ immunogold particles were located at both pre- and postsynaptic membranes of symmetric and asymmetric synapses (Fig. 5). At asymmetric synapses, the majority (85%) of the synapse-associated immunogold particles were located at the edges of the synapse (Fig. 5B), accounting for 34% and 51% for pre- and postsynaptic sites, respectively (Fig. 4B), with only 15% located in the main body (Fig. 5A). However, at symmetric synapses, most (89%) $GABA_{B2}$ immunogold particles were localized in the main body of the synapses (Fig. 5C–E); 41% associated with the presynaptic membrane and 48% with the postsynaptic membrane (Fig. 4B). Some asymmetric and symmetric synapses immunolabeled for $GABA_{B2}$ were formed with the same dendritic shaft (Fig. 5A,B). In addition to synapse-associated $GABA_{B2}$ immunolabeling, immunogold particles were also located extrasynaptically in dendrites (Fig. 5A,B).

Localization of $GABA_{B1}$ and $GABA_{B2}$ at glutamatergic synapses. To test whether the $GABA_B$ receptor subunit-positive asymmetric synapses are glutamatergic, we used VGLUT2 as a marker for glutamatergic terminals (Fremeau et al., 2001). Immunolabeling for VGLUT2 was widespread in the globus pallidus. At the electron micro-

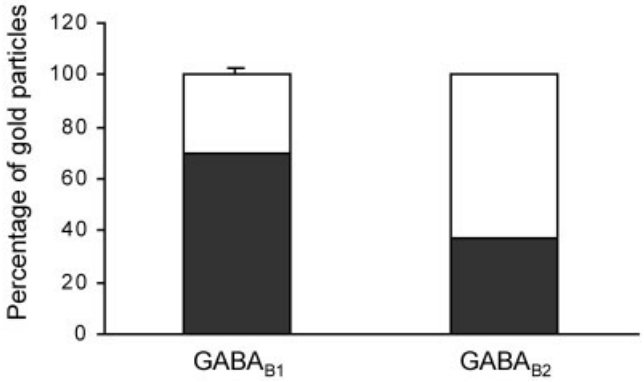

Fig. 3. Relative distribution of γ-aminobutyric acid (GABA)$_{B1}$ and GABA$_{B2}$ immunogold particles between the cytoplasm and the plasma membrane in the globus pallidus. A total of 70% of GABA$_{B1}$ immunogold particles are located within the cytoplasm (black portion of bars), whereas 63% of GABA$_{B2}$ immunogold particles are located on the plasma membrane (white portion of bars). A total of 1,864 GABA$_{B1}$ immunogold particles and 2,103 GABA$_{B2}$ immunogold particles were analyzed from three rats.

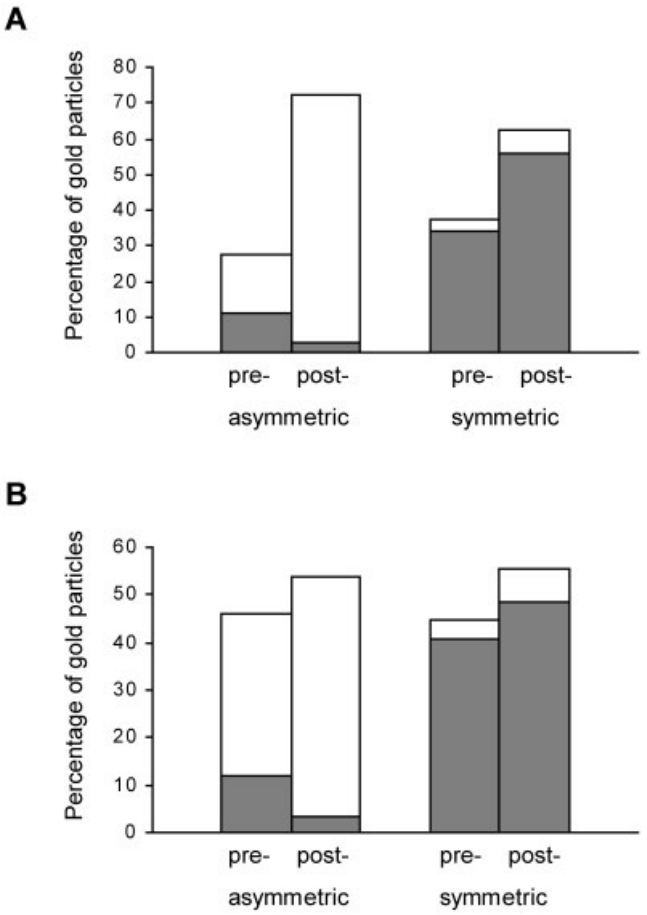

Fig. 4. **A,B:** The distribution of γ-aminobutyric acid (GABA)$_{B1}$ (A) and GABA$_{B2}$ (B) immunogold particles at synapses in the globus pallidus. The majority of immunogold particles associated with asymmetric synapses are located at perisynaptic sites (white portion of bars), whereas at symmetric synapses, gold particles are predominantly located in the main body of the synapse (black portion of bars). For GABA$_{B1}$, 70 immunogold particles were examined at asymmetric synapses (n = 54) and 165 at symmetric synapses (n = 101). For GABA$_{B2}$, 131 immunogold particles were examined at asymmetric synapses (n = 78) and 221 at symmetric synapses (n = 109).

scope level, VGLUT2-labeled axon terminals formed asymmetric synapses, mainly with dendritic shafts (Fig. 6). Quantitative analysis revealed that 92% of all asymmetric synapses in the globus pallidus (n = 692) were formed by VGLUT2-positive terminals. A large proportion of the VGLUT2-positive boutons and terminals were positive for GABA$_{B1}$ and GABA$_{B2}$. The distribution of GABA$_{B1}$ and GABA$_{B2}$ at pre- and postsynaptic membranes of synapses formed by VGLUT2-labeled terminals (Fig. 6) was similar to that observed at asymmetric synapses in the single immunogold-labeled tissue. Thus, at presynaptic sites, more synapse-related GABA$_{B1}$ immunogold particles were located at the edges of synapses (Fig. 6A,B) than were found in the main body (19% perisynaptic, 10% main body). On the postsynaptic membrane, almost all of the synapse-related GABA$_{B1}$ immunogold particles were located at the perisynaptic sites of the synapses (69% perisynaptic, 2% main body; Fig. 6A,B; 68 synapses/121 immunogold particles). A similar distribution was found for GABA$_{B2}$ synaptic immunolabeling (Fig. 6C,D), both presynaptically (27% perisynaptic, 13% main body) and postsynaptically (59% perisynaptic, 1% main body; 50 synapses/121 immunogold particles). Frequently, asymmetric synapses formed by VGLUT2-labeled terminals were close to symmetric synapses formed by VGLUT2-negative, presumably GABAergic terminals (Fig. 6A,D).

Localization of GABA$_{B1}$ and GABA$_{B2}$ at striatopallidal synapses. To determine the distribution of GABA$_B$ receptor subunits at striatopallidal synapses, we combined pre-embedding immunogold labeling for GABA$_{B1}$ and GABA$_{B2}$ subunits with anterograde labeling of striatopallidal terminals. The PHA-L injection sites were exclusively confined to the striatum, without diffusion into adjacent structures (Fig. 7A). Anterograde transport of the PHA-L resulted in the labeling of dense terminal fields within the globus pallidus (Fig. 7B). At the electron microscopic level, anterogradely labeled striatal boutons were of medium size and formed symmetric synapses with large and small dendritic shafts (Fig. 8). The morphological features of the labeled terminals were similar to those previously reported (Smith and Bolam, 1991). Double immunolabeling revealed that anterogradely labeled striatopallidal boutons were also immunolabeled for GABA$_{B1}$ (Fig. 8A) and GABA$_{B2}$ (Fig. 8C,D). At symmetric synapses formed by PHA-L–labeled boutons, GABA$_{B1}$ and GABA$_{B2}$ immunolabeling was predominantly found in the main body of the synapses on both pre- (Fig. 8A,C) and postsynaptic membranes (Fig. 8A,B,D). Quantitative analysis revealed a similar immunogold particle distribution between the presynaptic and postsynaptic specialization for GABA$_{B1}$ (pre, 45; post, 55%; 30 synapses/38 immunogold particles) and GABA$_{B2}$ (pre, 50; post, 50%; 27 synapses/41 gold particles).

DISCUSSION

The present study provides a detailed description of the subcellular localization of GABA$_{B1}$ and GABA$_{B2}$ receptor

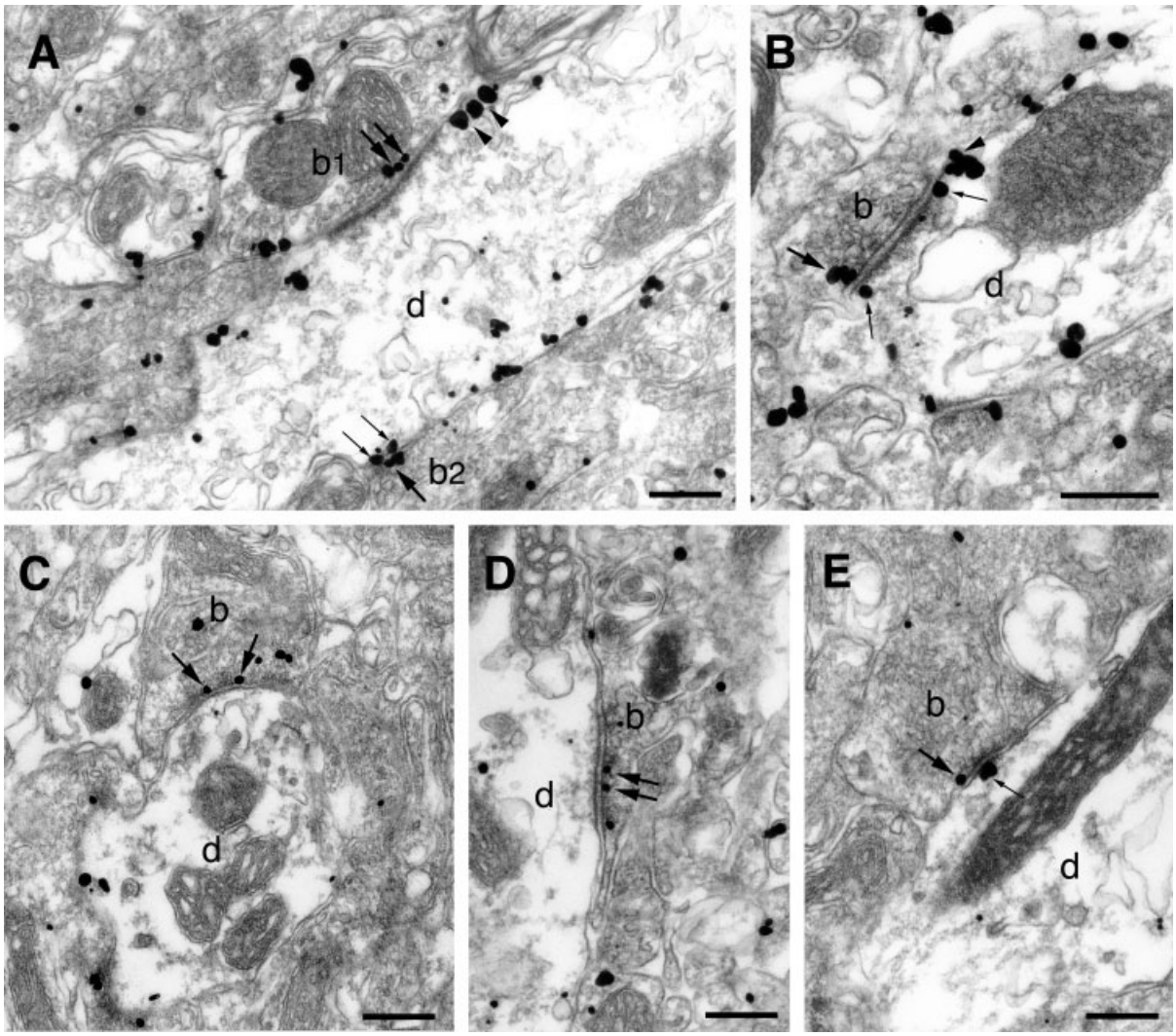

Fig. 5. Localization of γ-aminobutyric acid $(GABA)_{B2}$ immunogold labeling in the globus pallidus. **A:** Immunogold labeling for $GABA_{B2}$ in two boutons forming asymmetric (b1) and symmetric (b2) synapses with a $GABA_{B2}$ labeled dendrite (d). Presynaptic immunolabeling (large arrows) occurs in the main body of the asymmetric synapse. Presynaptic (large arrow) and postsynaptic (small arrows) immunolabeling occurs in the main body of the symmetric synapse. Immunogold particles are also localized extrasynaptically in the dendrite (arrowheads). **B:** Perisynaptic labeling at both presynaptic (large arrow) and postsynaptic (small arrows) sites at an asymmetric synapse. Immunogold particles are also present at extrasynaptic sites (arrowhead). **C,D:** Presynaptic immunolabeling (large arrows) in the main body of symmetric axodendritic synapses. **E:** Presynaptic immunolabeling (large arrow) at the edge of a symmetric synapse, which is also labeled on the postsynaptic membrane (small arrow). b, bouton; d, dendrite. Scale bars = 0.25 μm.

subunits in the rat globus pallidus. By using preembedding immunogold labeling, we demonstrate that both $GABA_{B1}$ and $GABA_{B2}$ are expressed at pre- and postsynaptic sites. At asymmetric synapses, most $GABA_{B1}$ and $GABA_{B2}$ immunogold labeling was found at the edges of synapses, i.e., at perisynaptic sites. However, at symmetric synapses, including those formed by anterogradely labeled striatopallidal terminals, most $GABA_{B1}$ and $GABA_{B2}$ immunogold labeling was found in the main body of the synapses. Furthermore, by using VGLUT2 as a marker for glutamatergic terminals, we further demonstrated that the majority of immunogold-labeled asymmetric synapses were formed by glutamatergic terminals, presumably arising from the subthalamic nucleus. This distribution of labeling is similar to that observed in both the substantia nigra *pars compacta* and *pars reticulata* (Boyes and Bolam, 2003). Furthermore, the distribution is markedly different from that found for $GABA_A$ receptors in the globus pallidus (Somogyi et al., 1996; Waldvogel et al., 1998) and in other regions of the basal ganglia (Fujiyama et al., 2000, 2002), in as much as $GABA_A$ receptors are mainly located at symmetrical synapses and rarely, if ever, occur in the presynaptic structure.

Localization of $GABA_B$ receptor subunits at symmetric, including identified striatopallidal, synapses

Electrophysiological studies have demonstrated that the selective $GABA_B$ receptor agonist, baclofen, signifi-

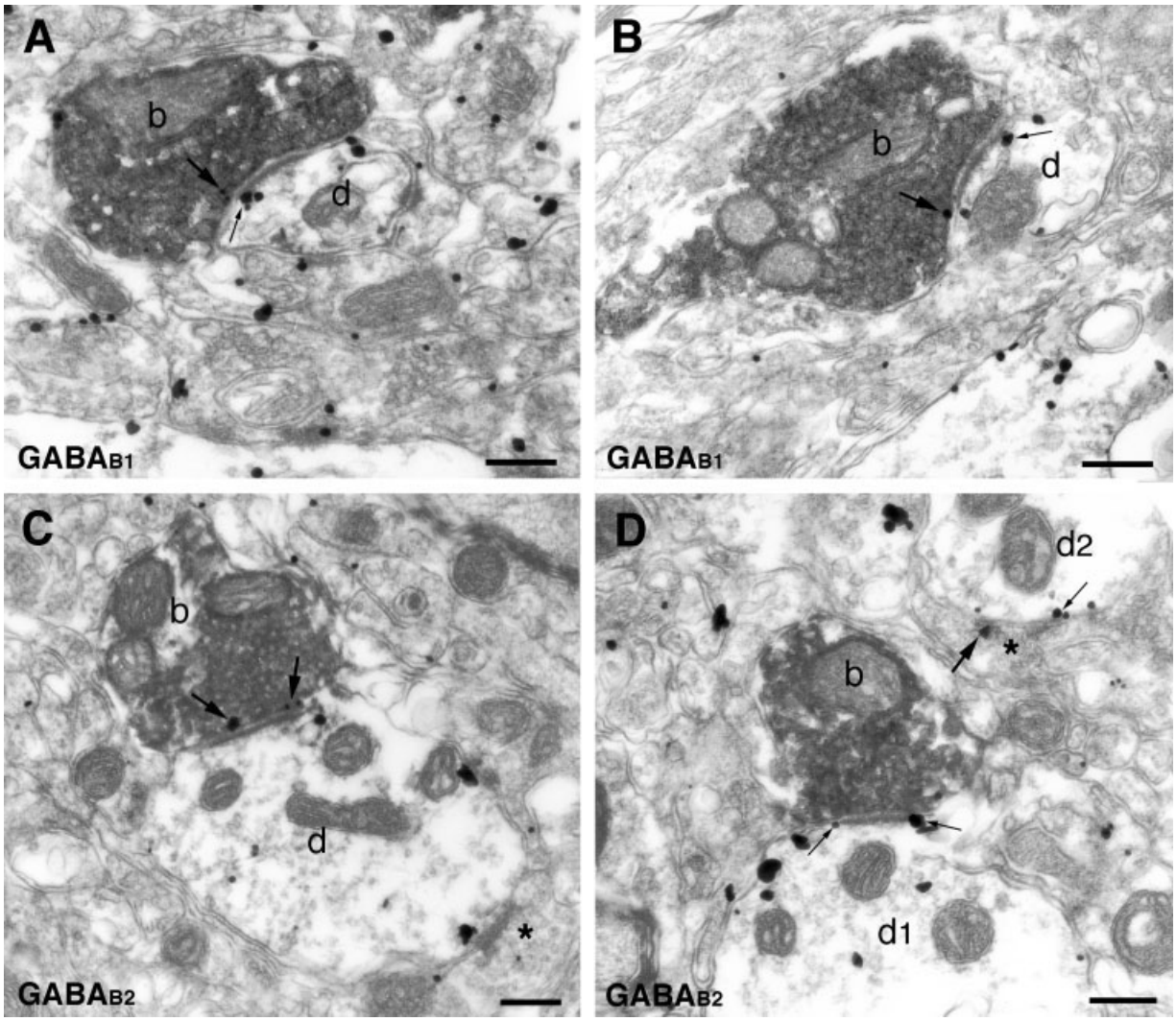

Fig. 6. Double immunolabeling for γ-aminobutyric acid $(GABA)_{B1}$ or $GABA_{B2}$ (immunogold) and VGLUT2 (immunoperoxidase) in the globus pallidus. **A,B:** Presynaptic (large arrows) and postsynaptic (small arrows) $GABA_{B1}$ immunogold particles at the edges of asymmetric synapses between VGLUT2-labeled boutons (b) and small dendritic shafts (d). **C,D:** Presynaptic (C, large arrows) and postsynaptic (D, small arrows) $GABA_{B2}$ immunolabeling at asymmetric synapses formed by VGLUT2-labeled boutons. The postsynaptic labeling is at the edge of the synapse, whereas the presynaptic labeling is within the main body. D: A second dendrite, d2, receives symmetric synaptic input from a VGLUT2-negative bouton (asterisk); both pre- (large arrow) and postsynaptic (small arrow) membranes are immunolabeled for $GABA_{B2}$. b, bouton; d, dendrite. Scale bars = 0.25 μm.

cantly reduces the frequency of $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) by activating presynaptic $GABA_B$ receptors (Chan et al., 2000). A similar effect on $GABA_A$-mediated IPSCs was also observed as a result of increasing extracellular levels of GABA by using the GABA uptake blocker, tiagabine (Chen and Yung, 2003). Our data support the presence of functional $GABA_B$ autoreceptors in the globus pallidus. We demonstrate that both $GABA_{B1}$ and $GABA_{B2}$ are expressed on the presynaptic membrane of symmetric, putative GABAergic, synapses, and it is presumably stimulation of these receptors that leads to the suppression of GABA release. Our data also reveal the existence of both subunits on the postsynaptic membrane of symmetric synapses. This observation is again consistent with electrophysiological data demonstrating that baclofen also acts postsynaptically on pallidal neurons (Stefani et al., 1999; Chan et al., 2000).

The two major sources of GABAergic terminals in the globus pallidus are the striatum and the local collaterals of pallidal projection neurons (for review, see Smith et al., 1998). Immunoperoxidase data in monkeys has revealed labeling for $GABA_{B1}$ in boutons with the ultrastructural features of striatal boutons (Charara et al., 2000). Our findings demonstrate that at least one of these populations of GABAergic terminals express the $GABA_B$ receptors. Thus, $GABA_{B1}$ and $GABA_{B2}$ subunits are expressed at both pre- and postsynaptic sites on striatopallidal afferents identified by the anterograde transport of PHA-L. It remains to be established whether the local collaterals of pallidal neurons express $GABA_B$ receptors, although anatomical (Boyes and Bolam, 2003) and physiological

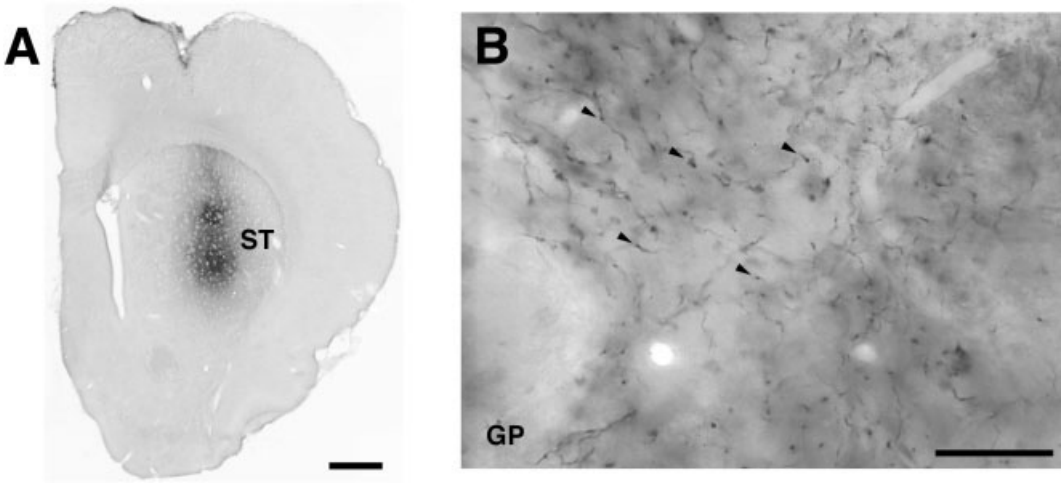

Fig. 7. **A,B:** *Phaseolus vulgaris* leucoagglutinin (PHA-L) injection sites in the striatum (A, ST) and anterogradely transported PHA-L in the globus pallidus (B, GP). High-magnification photomicrograph of anterogradely labeled striatal fibers and boutons (some of which are indicated by arrowheads) in the globus pallidus (GP). ST, striatum. Scale bars = 1 mm in A; 20 μm in B.

studies (Paladini et al., 1999) suggest that functional $GABA_B$ receptors are present on the terminals of pallidal neurons in the substantia nigra. Thus, both the morphological and electrophysiological studies strongly suggest that pre- and postsynaptic $GABA_B$ receptors are present at GABAergic synapses in the globus pallidus, including those formed by striatopallidal terminals, where they are likely to play an important role in modulating the excitability of the globus pallidus through their effects on GABA transmission.

Localization of $GABA_B$ receptor subunits at glutamatergic synapses in the globus pallidus

In addition to the presence of $GABA_{B1}$ and $GABA_{B2}$ immunolabeling at symmetric synapses, the present results also demonstrated pre- and postsynaptic $GABA_{B1}$ and $GABA_{B2}$ subunits at asymmetric, putative excitatory, synapses in the globus pallidus. To determine whether the immunolabeled asymmetric synapses were glutamatergic, we used VGLUT2 as a marker for glutamatergic axon terminals (Fremeau et al., 2001; Varoqui et al., 2002). The electron microscopic analysis revealed that 92% of asymmetric synapses in the globus pallidus are formed by VGLUT2-positive terminals, indicating that the majority of asymmetric synapses in the globus pallidus are glutamatergic. The subthalamic nucleus provides the major glutamatergic input to the globus pallidus (Parent and Smith, 1987; Smith and Parent, 1988) and in situ hybridisation studies have revealed a high level of VGLUT2 mRNA expression in subthalamic neurons (Hisano et al., 2000). We thus suggest that the VGLUT2-positive terminals are derived from the subthalamic nucleus. Immunolabeling for both $GABA_{B1}$ and $GABA_{B2}$ was observed in many VGLUT2-labeled terminals, indicating that functional $GABA_B$ receptors are likely to be expressed on glutamatergic afferents in the globus pallidus. This finding is consistent with in vitro pharmacological data showing that baclofen reduces the frequency of glutamate-mediated excitatory postsynaptic currents (Chen et al., 2002), indicating that activation of presynaptic $GABA_B$ receptors inhibits the release of glutamate in the globus pallidus. Furthermore, unilateral injection of baclofen into the globus pallidus induced ipsilateral turning (Chen et al., 2002), which has been suggested to be due to an inhibition of glutamate release by $GABA_B$ receptor stimulation.

The small proportion of VGLUT2-negative terminals forming asymmetric synapses observed in the present study may represent glutamatergic input arising from the cortex, where only a low level of VGLUT2 mRNA is expressed (Hisano et al., 2000), or the thalamus. It is also possible that they represent terminals using acetylcholine or serotonin as a neurotransmitter (Lavoie and Parent, 1990; Mesulam et al., 1992; Charara and Parent, 1994). Whatever the situation, it is interesting to note that some of the VGLUT2-negative asymmetric synapses were positive for $GABA_B$ subunits (not illustrated), suggesting that $GABA_B$ receptors are likely to influence activity of other afferent terminals.

Perisynaptic distribution of $GABA_B$ receptors at glutamatergic synapses

The immunogold labeling at asymmetric synapses and VGLUT2-positive glutamatergic synapses for both $GABA_{B1}$ and $GABA_{B2}$ was mainly located at the edge of the synapse, i.e., at perisynaptic sites. This finding is in contrast to symmetrical, putative GABAergic, synapses where the majority was found in the main body of the synapse. Perisynaptic labeling for $GABA_B$ subunits at asymmetric synapses has also been described in the primate globus pallidus (Smith et al., 2000), as well as in the substantia nigra, cerebellum, thalamus, and neocortex in rats (Kulik et al., 2002; López-Bendito et al., 2002; Boyes and Bolam, 2003). This association between $GABA_B$ receptors and glutamatergic synapses raises the question of how these receptors are activated under physiological conditions. One possibility is that, in addition to activating

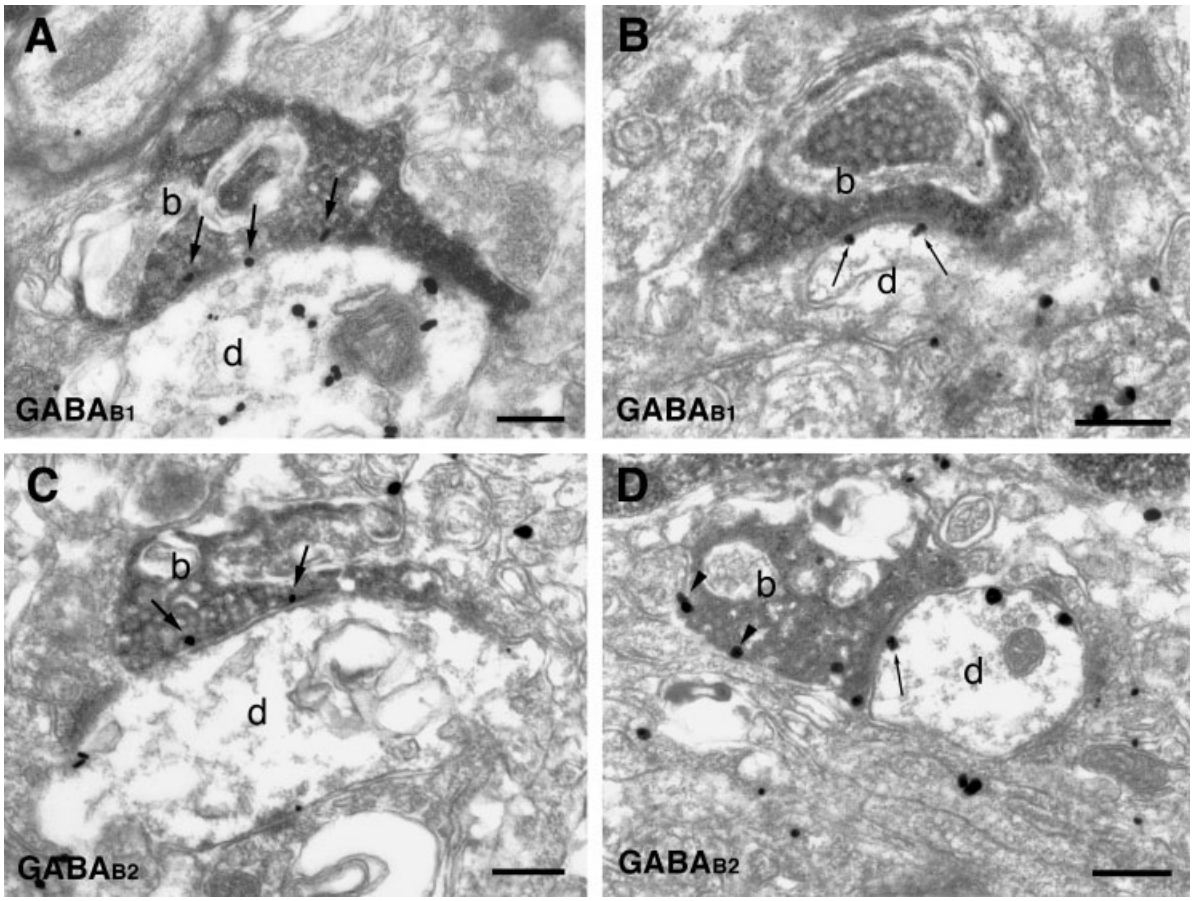

Fig. 8. Double immunolabeling for γ-aminobutyric acid (GABA)$_{B1}$ or GABA$_{B2}$ (immunogold) and *Phaseolus vulgaris* leucoagglutinin (PHA-L; immunoperoxidase) in the globus pallidus. **A,B:** Presynaptic (large arrows) and postsynaptic (small arrows) GABA$_{B1}$ immunogold particles at symmetric synapses formed by striatal boutons (b) anterogradely labeled with PHA-L. **C,D:** Presynaptic (large arrows) and postsynaptic (small arrow) GABA$_{B2}$ immunogold particles at synapses formed by PHA-L-labeled striatal boutons (b). D: Immunogold particles are also localized extrasynaptically in the striatal bouton (arrowheads). b, bouton; d, dendrite. Scale bars = 0.25 μm.

pre- and/or postsynaptic GABA$_B$ receptors at GABAergic synapses, GABA released from GABAergic synapses may spill out of the synapse to activate GABA$_B$ receptors located at the edge of neighbouring glutamatergic synapses. Indeed, we often observed presumed GABAergic synapses in close proximity to the glutamatergic synapses in the globus pallidus. It has been reported that GABA can spill out of the synaptic cleft to activate extrasynaptic GABA$_B$ receptors in the hippocampus (Scanziani, 2000), as well as act on presynaptic GABA$_B$ heteroreceptors to depress excitatory synaptic transmission in both the hippocampus and cerebellum (Isaacson et al., 1993; Dittman and Regehr, 1997). GABA$_B$ receptors positioned at the edge of the synaptic cleft presumably would be more accessible to extracellular GABA than those located in the body of the synapse. Thus, by activating presynaptic GABA$_B$ heteroreceptors located at the edge of the presynaptic active zone, GABA may inhibit the release the glutamate as indicated in the physiological analyses (Chen et al., 2002), and by activating postsynaptic GABA$_B$ receptors located perisynaptically, GABA may modulate excitatory postsynaptic currents mediated by the glutamate receptors. Although we observed a small proportion of labeling on the presynaptic membrane at glutamatergic synapses (i.e., within the main body of the synapse), it is unclear whether these receptors modulate glutamate release by means of different mechanisms from those located perisynaptically, but it should be remembered that they are strategically placed to have a profound effect on transmitter release.

The interpretation of *negative* findings with preembedding immunogold labeling, particularly in relation to the localization of labeling at synapses, should be treated with caution because the immunogold-conjugated secondary antibodies have a significant size that restricts penetration into the tissue. It has been shown that antibodies against ionotropic excitatory amino acid receptors (or the gold-conjugated secondary antibodies) have restricted access to asymmetric synapses in the preembedding immunogold method (Baude et al., 1993; Bernard et al., 1998). Thus, it may be that the low density of labeling for GABA$_B$ receptors observed within asymmetric synapses is not a reflection of the true distribution of the receptor but due to a technical limitation of the technique. Nevertheless, as mentioned above, the perisynaptic distribution of GABA$_B$ receptors described here in the globus

pallidus is consistent with observations made elsewhere in the basal ganglia (Smith et al., 2000; Boyes and Bolam, 2003) and in other regions of the brain (Kulik et al., 2002; López-Bendito et al., 2002).

Patterns of distribution of $GABA_{B1}$ and $GABA_{B2}$ immunolabeling in globus pallidus

Immunolabeling at the light microscopic level revealed that $GABA_{B1}$ was predominantly located in perikarya in the globus pallidus, whereas $GABA_{B2}$ was mainly distributed in neuropil, which is similar to observations reported in striatum and substantia nigra (Yung et al., 1999; Ng and Yung, 2001b; Boyes and Bolam, 2003). Furthermore, at the electron microscope level, most of the labeling for $GABA_{B1}$ was located in the cytoplasm of globus pallidus neurons, whereas most of the labeling for $GABA_{B2}$ was located on the membrane. It is known that the structure of the intracellular C-terminus of the $GABA_{B1}$ subunit determines its retention within the endoplasmic reticulum (Margeta-Mitrovic et al., 2000; Calver et al., 2001), and recombinant $GABA_{B1}$ subunits fail to reach the cell surface and are retained in the endoplasmic reticulum (Couve et al., 1998). An interaction between $GABA_{B1}$ and $GABA_{B2}$ subunits is necessary for the cell-surface expression of $GABA_{B1}$ and the formation of fully functional $GABA_B$ receptors (Jones et al., 1998; Kaupman et al., 1998; White et al., 1998). Thus the apparent mismatch between $GABA_{B1}$ and $GABA_{B2}$ subunits on the membrane is consistent with the molecular studies and, furthermore, suggests that a proportion of $GABA_{B2}$ subunits are located on the membrane independently of $GABA_{B1}$. Given that $GABA_{B2}$ appears unable to bind GABA, it is possible that these $GABA_{B2}$ subunits are interacting with other receptors or other, as yet unidentified, $GABA_B$ receptor subunits. It is important to note, however, that the preembedding immunogold technique reveals the relative distributions of the subunits and not the absolute numbers at different sites, and it is possible that all the $GABA_{B2}$ labeling we observed in the membrane does, in fact, represent functional heterodimers.

Pre- and postsynaptic $GABA_B$ receptors in the globus pallidus

Pharmacological studies have indicated that baclofen is more potent at presynaptic sites than at postsynaptic sites in the globus pallidus (Chan et al., 2000; Chen et al., 2002) and the hippocampus (Davies et al., 1990) and may suggest a greater number of $GABA_B$ receptors at presynaptic sites compared with postsynaptic sites. The results of the present study, however, reveal similar levels of $GABA_{B1}$ and $GABA_{B2}$ immunogold particles at pre- and postsynaptic sites of symmetric synapses, but more $GABA_{B1}$ and $GABA_{B2}$ immunogold particles located at postsynaptic sites of asymmetric synapses, as has been reported in the cerebellum (Kulik et al., 2002). This apparent discrepancy may relate to differences in the pharmacology of the receptors (Dutar and Nicoll, 1988) or differences in the pre- and postsynaptic $GABA_B$ receptor distributions during development (Madtes, 1987; Turgeon and Albin, 1994), as the physiological studies were performed on young rats, whereas the present anatomical studies were performed on adults.

CONCLUSIONS

The findings of the present study provide an anatomical substrate for the pre- and postsynaptic effects of $GABA_B$ receptor stimulation at GABAergic synapses and the presynaptic effects at glutamatergic synapses. They also predict that, through perisynaptic receptors, $GABA_B$ receptor stimulation is likely to modulate the postsynaptic responses to excitatory amino acid receptor stimulation.

ACKNOWLEDGMENTS

The authors thank Caroline Francis, Ben Micklem, and Liz Norman for technical assistance. J.B. is in receipt of an MRC Studentship.

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