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$GABA_B$ RECEPTORS AT GLUTAMATERGIC SYNAPSES IN THE RAT STRIATUM

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Abstract—Although multiple effects of $GABA_B$ receptor activation on synaptic transmission in the striatum have been described, the precise locations of the receptors mediating these effects have not been determined. To address this issue, we carried out pre-embedding immunogold electron microscopy in the rat using antibodies against the $GABA_B$ receptor subunits, $GABA_{B1}$ and $GABA_{B2}$. In addition, to investigate the relationship between $GABA_B$ receptors and glutamatergic striatal afferents, we used antibodies against the vesicular glutamate transporters, vesicular glutamate transporter 1 and vesicular glutamate transporter 2, as markers for glutamatergic terminals. Immunolabeling for $GABA_{B1}$ and $GABA_{B2}$ was widely and similarly distributed in the striatum, with immunogold particles localized at both presynaptic and postsynaptic sites. The most commonly labeled structures were dendritic shafts and spines, as well as terminals forming asymmetric and symmetric synapses. In postsynaptic structures, the majority of labeling associated with the plasma membrane was localized at extrasynaptic sites, although immunogold particles were also found at the postsynaptic specialization of some symmetric, putative GABAergic synapses. Labeling in axon terminals was located within, or at the edge of, the presynaptic active zone, as well as at extrasynaptic sites. Double labeling for $GABA_B$ receptor subunits and vesicular glutamate transporters revealed that labeling for both $GABA_{B1}$ and $GABA_{B2}$ was localized on glutamatergic axon terminals that expressed either vesicular glutamate transporter 1 or vesicular glutamate transporter 2. The patterns of innervation of striatal neurons by the vesicular glutamate transporter 1- and vesicular glutamate transporter 2-positive terminals suggest that they are selective markers of corticostriatal and thalamostriatal afferents, respectively. These results thus provide evidence that presynaptic $GABA_B$ heteroreceptors are in a position to modulate the two major excitatory inputs to striatal spiny projection neurons arising in the cortex and thalamus. In addition, presynaptic $GABA_B$ autoreceptors are present on the terminals of spiny projection neurons and/or striatal GABAergic interneurons. Furthermore, the data indicate that GABA may also affect the excitability of striatal neurons via postsynaptic $GABA_B$ receptors. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: basal ganglia, vesicular glutamate transporters, VGLUT1, VGLUT2, corticostriatal, thalamostriatal.

The inhibitory amino acid GABA plays a critical role in the neuronal networks of the basal ganglia. Two receptor types mediate the actions of GABA in the basal ganglia: fast-acting ionotropic $GABA_A$ receptors and slower-acting metabotropic $GABA_B$ receptors. Both postsynaptic and presynaptic $GABA_B$ receptors exert their influence on synaptic transmission through G-protein coupling and downstream intracellular effector systems (Kaupmann et al., 1997). Activation of postsynaptic $GABA_B$ receptors leads to increases in potassium conductance and hence, hyperpolarization, and activation of presynaptic $GABA_B$ receptors leads to an inhibition of transmitter release through a decrease in calcium influx (Bowery et al., 2002; Calver et al., 2002). $GABA_B$ receptors are heterodimers of two subunits, $GABA_{B1}$ and $GABA_{B2}$ (Kaupmann et al., 1997, 1998; Jones et al., 1998; White et al., 1998; Kuner et al., 1999) which are both necessary and sufficient for normal functioning *in vivo* (Prosser et al., 2001; Schuler et al., 2001; Gassmann et al., 2004). The $GABA_{B1}$ subunit is responsible for binding GABA whereas $GABA_{B2}$ is necessary for surface trafficking and G-protein activation (Galvez et al., 2000; Jones et al., 2000; Margeta-Mitrovic et al., 2000; Calver et al., 2001; Robbins et al., 2001).

The striatum is the major division of the basal ganglia and the majority of its neurons are GABAergic. These include the spiny projection neurons, which account for about 95% of the total population of striatal neurons and which form the major output of the striatum, and at least three classes of GABAergic interneurons (see Kawaguchi, 1993; Tepper et al., 2004 for reviews). In addition, the striatum receives GABAergic afferents from the globus pallidus (Kita and Kitai, 1994; Bevan et al., 1998) and possibly the substantia nigra (van der Kooy et al., 1981; Rodríguez and González-Hernández, 1999). Although the effects of GABA in the striatum are mediated principally by postsynaptic $GABA_A$ receptors (Tepper et al., 2004), several effects of $GABA_B$ receptor activation on synaptic transmission in the striatum have been described. Thus, application of the $GABA_B$ agonist, baclofen, causes a reduction in cortically evoked excitatory postsynaptic poten-

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Abbreviations: ABC, avidin–biotin–peroxidase complex; EPSP, excitatory postsynaptic potential; $GABA_{B1}$ and $GABA_{B2}$, subunits of the $GABA_B$ receptor; IPSP, inhibitory postsynaptic potential; NGS, normal goat serum; PB, phosphate buffer; PBS, phosphate-buffered saline; VGLUT, vesicular glutamate transporter.

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tials (EPSPs) in striatal neurons, including spiny projection neurons, *in vitro* (Calabresi et al., 1990, 1991; Nisenbaum et al., 1992, 1993). In support of this effect being mediated by presynaptic $GABA_B$ receptors, lesions of the corticostriatal pathway are associated with a marked reduction in $GABA_B$ binding sites in the striatum (Moratalla and Bowery, 1991). In addition, $GABA_B$ activation also reduces inhibitory postsynaptic potentials (IPSPs) following intrastriatal stimulation (Calabresi et al., 1991; Seabrook et al., 1991; Nisenbaum et al., 1992, 1993). These findings suggest that $GABA_B$ receptors are present on both glutamatergic and GABAergic inputs to striatal neurons.

The presence of functional $GABA_B$ receptors in the striatum is supported by immunocytochemical studies in rats and monkeys demonstrating that both $GABA_{B1}$ and $GABA_{B2}$ are expressed by striatal neurons (Margeta-Mitrovic et al., 1999; Yung et al., 1999; Charara et al., 2000, 2004; Charles et al., 2001; Waldvogel et al., 2004). However, this is inconsistent with *in situ* hybridization studies showing that $GABA_{B2}$ mRNA is virtually absent from the striatum (Durkin et al., 1999; Martin et al., 1999, 2004; Clark et al., 2000). In view of this apparent mismatch, one of the main objectives of the present study was to characterize the precise localization of both $GABA_B$ receptor subunits at the electron microscopic level in the rat striatum, using the pre-embedding immunogold labeling method. In monkeys, immunoperoxidase labeling for $GABA_B$ receptors has previously been detected in a population of terminals forming asymmetric, putative excitatory synapses, which is characteristic of corticostriatal terminals (Charara et al., 2000, 2004). However, in addition to the input from the cortex, the striatum receives excitatory, glutamatergic afferents from the intralaminar thalamic nuclei (see Smith et al., 2004). Glutamatergic terminals in the striatum can be characterized by their expression of vesicular glutamate transporter (VGLUT) subtypes; VGLUT1 and VGLUT2 are localized in terminals that form asymmetric synapses in the striatum (Bellocchio et al., 1998; Herzog et al., 2001; Fujiyama et al., 2004) and have been proposed to be selectively associated with corticostriatal and thalamostriatal afferents, respectively (Fremeau et al., 2001; Varoqui et al., 2002; Bacci et al., 2004; Fujiyama et al., 2004). Thus, a further objective of the study was to determine the nature of the afferent terminals that express $GABA_B$ receptors by combining immunogold labeling for $GABA_B$ receptor subunits with immunoperoxidase labeling for VGLUTs.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

The experiments were carried out on 12 male Sprague–Dawley rats (180–300 g; Charles River, Margate, Kent, UK). Environmental conditions for housing of the rats and all procedures that were performed were in accordance with the Animals (Scientific Procedures) Act of 1986 (UK). Every effort was made to use the minimum number of animals and to minimize suffering. They were deeply anesthetized with sodium pentobarbitone (200 mg/kg; i.p.; Sagatal; Rhône Mérieux, Tallaght, Dublin, Ireland) and perfused via the ascending aorta with 50–100 ml of phosphate-buffered saline (PBS; 0.01 M phosphate, pH 7.4) followed by 300 ml of 3% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (PB; 0.1 M, pH 7.4). Coronal sections (65–70 μm) of striatum were cut on a vibrating microtome and collected in PBS.

Antibodies

All antibodies used in these studies were obtained from commercial sources. For $GABA_B$ immunocytochemistry, polyclonal antibodies raised in guinea-pig against the C termini of $GABA_{B1}$ (AB1531; Chemicon, Chandlers Ford, Hampshire, UK) and $GABA_{B2}$ (AB5394; Chemicon) were used. The specificity of both antibodies has been established by immunohistochemistry on brain sections from $GABA_B$-subunit knockout mice (Prosser et al., 2001; Gassmann et al., 2004). For VGLUT immunocytochemistry, polyclonal antibodies raised in rabbit against VGLUT1 and VGLUT2 (both Synaptic Systems, Göttingen, Germany) were used. In addition, a guinea-pig polyclonal antibody against VGLUT1 (AB5905; Chemicon) was also used. The specificity of the VGLUT antibodies has been established by others (Todd et al., 2003; Montana et al., 2004).

Immunolabeling

To enhance the tissue penetration of the immunoreagents, the sections were equilibrated in a cryoprotectant solution (0.05 M PB, pH 7.4, containing 25% sucrose and 10% glycerol) for at least 3 h. The sections were then freeze-thawed by freezing in isopentane cooled in liquid nitrogen, followed by liquid nitrogen, and thawing in PBS. The sections were then washed several times in PBS and incubated in 10% normal goat serum (NGS; Vector Laboratories, Peterborough, UK) in PBS for 2 h. All further incubation steps were carried out in PBS containing 2% NGS and sections were washed three to four times between steps.

For immunogold labeling of $GABA_B$ receptors, sections were incubated with anti-$GABA_{B1}$ (diluted 1:1000–1:2000) or anti-$GABA_{B2}$ (diluted 1:2000–1:4000) for 48–72 h at 4 °C. The sections were then incubated in goat anti-guinea-pig IgG conjugated to 1.4 nm gold particles (diluted 1:100; Nanoprobes, Yaphank, Stony Brook, NY, USA), sometimes supplemented with 0.5% bovine serum albumin (Sigma, Gillingham, Dorset, UK), for 2 h at room temperature or overnight at 4 °C. The sections were post-fixed in 0.1% glutaraldehyde (in PBS) for 10 min, and then the gold particles were enhanced by silver intensification using the HQ Silver kit (Nanoprobes).

For immunoperoxidase labeling of VGLUTs, sections were incubated with guinea-pig or rabbit anti-VGLUT1 (diluted 1:50,000 or 1:2000–1:4000 respectively) or rabbit anti-VGLUT2 (diluted 1:2000) for 48–72 h at 4 °C. The sections were then incubated in either biotin-conjugated goat anti-guinea-pig IgG or biotin-conjugated goat anti-rabbit IgG (diluted 1:200; Vector) for 2 h, followed by avidin–biotin–peroxidase complex (ABC; 1:100 in PBS; Vector) for 1.5–2 h, at room temperature. After equilibrating in Tris buffer (0.05 M, pH 7.6), the peroxidase was revealed by incubation in 0.025% diaminobenzidine (Sigma) in Tris buffer in the presence of 0.01% H_2O_2. The peroxidase reaction was stopped after 6–8 min by several washes in Tris buffer. In two animals, sections were incubated in a cocktail of the rabbit primary antibodies against VGLUT1 and VGLUT2 (1:2000 dilution) and revealed by the peroxidase method.

For double immunolabeling of $GABA_B$ receptors and VGLUTs, the immunogold labeling and silver intensification were performed before the immunoperoxidase labeling in order to prevent the non-specific deposition of silver on the peroxidase reaction product. The sections were incubated in a mixture of primary antibodies: guinea-pig anti-$GABA_{B1}$ or anti-$GABA_{B2}$ (diluted 1:1000 and 1:2000, respectively) plus either rabbit anti-VGLUT1 or anti-VGLUT2 (diluted 1:2000) for 48 h at 4 °C. The sections were then incubated in gold-conjugated anti-guinea-pig IgG (di-

luted 1:100; Nanoprobes) and biotin-conjugated anti-rabbit IgG (diluted 1:200; Vector), overnight at 4 °C. $GABA_B$-immunoreactive sites were then revealed by silver intensification of the immunogold labeling. Subsequently, VGLUT-immunoreactive sites were revealed by incubation in ABC and a peroxidase reaction, as described above.

Immunofluorescence

After washing in PBS, sections were incubated in 10% NGS and 0.2% Triton X-100 (Sigma) in PBS for 2 h at room temperature. All further incubation steps were carried out in PBS containing 2% NGS and 0.2% Triton X-100 and sections were washed three to four times between steps. The sections were incubated in the guinea-pig anti-VGLUT1 (diluted 1:100,000–1:200,000) and rabbit anti-VGLUT2 (diluted 1:2000–1:5000) for 48 h at 4 °C. Subsequently, the sections were incubated in a mixture of Cy3-conjugated donkey anti-guinea-pig (for VGLUT1; diluted 1:200; Jackson Immunoresearch, West Grove, PA, USA) and Alexa Fluor 488-conjugated donkey anti-rabbit (for VGLUT2; diluted 1:1000; Molecular Probes, Eugene, OR, USA), overnight in the dark at 4 °C. The sections were then rinsed in PBS and mounted in Vectashield (Vector) under coverslips. The sections were examined using a Zeiss LSM 510/Axiovert 100 M confocal microscope (Carl Zeiss, Welwyn Garden City, Hertfordshire, UK). Three sections, ranging from rostral to caudal striatum, from each of three rats were examined and images captured using Zeiss LSM software.

Control experiments

The specificity of the $GABA_B$ and VGLUT antibodies was assessed by incubating sections of striatum through the whole immunolabeling protocols but with the omission of the primary antibodies. Under these conditions, there was a complete lack of immunolabeling for the respective agents. Sections of cerebellum were included as a positive control for the GABA receptor. To test for possible cross-reactivity of the secondary antibodies in the double immunolabeling experiments, sections were incubated through the whole double immunolabeling protocol but with omission of one of the primary antibodies. No evidence of cross-reactivity was evident under these conditions.

Processing for electron microscopy

All sections were washed several times in PB and post-fixed with 1% osmium tetroxide (in PB; Oxkem, Oxford, UK) for 7 min (for immunogold- and double-labeled sections) or 25 min (for immunoperoxidase-labeled sections). After several washes in PB, the sections were dehydrated through a graded series of dilutions of ethanol, with 1% uranyl acetate (TAAB, Berkshire, UK) added to the 70% ethanol solution. Following absolute ethanol, the sections were treated with propylene oxide (Sigma) and placed in resin overnight (Durcupan, ACM; Fluka, Dorset, UK). The sections were then mounted on glass slides, a coverslip applied and the slides were placed in an oven at 60 °C for 48 h. After examination in the light microscope, selected regions of striatum were cut out from the slides and glued onto resin blocks. Serial ultrathin sections (approximately 70 nm) were cut on a Riechert-Jung Ultracut E ultramicrotome (Leica, Nussloch, Germany) and collected on single-slot copper grids coated with pioloform (Agar Scientific, Essex, UK). Ultrathin sections were then contrasted with lead citrate for 3–4 min and examined in a Philips CM 10 or 100 electron microscope.

Analysis of material

The analysis of the subcellular distribution of immunogold labeling for $GABA_{B1}$ and $GABA_{B2}$ was performed on six blocks of tissue from dorsal striatum (two from each of three rats). Areas of striatum close to the surface of the tissue were selected at random in the electron microscope and a series of 18 adjacent electron micrographs were taken parallel to, and at least 5 μm from, the interface between 'empty resin' and the tissue at a magnification of ×15,500, giving a total sampled area of tissue of 1074 μm^2 per animal. To quantify the overall distribution of immunolabeling for $GABA_{B1}$ and $GABA_{B2}$ within presynaptic and postsynaptic structures, every immunogold particle in each photomicrograph was counted and categorized as being either within the cytoplasm or associated with the plasma membrane, i.e. those gold particles in contact with the membrane. In addition, to assess the spatial relationship between postsynaptic $GABA_B$ receptors and neurotransmitter release sites, membrane-associated gold particles in dendritic shafts and spines were classified as synaptic, if they were located within the postsynaptic specialization at asymmetric or symmetric synapses, or extrasynaptic, if they were associated with the non-synaptic parts of the membrane.

The analysis of VGLUT expression in the dorsal striatum was performed on random serial electron micrographs taken at approximately the same distance from the interface between 'empty resin' and the tissue and in systematic scans in the electron microscope (five rats for VGLUT1, seven rats for VGLUT2 and two rats for double immunolabeling). In the micrographs, each terminal forming an asymmetric synapse was categorized as being immunolabeled or unlabeled. In both the micrographs and systematic scans, the structures postsynaptic to the immunolabeled boutons, i.e. dendritic shaft or spine, were characterized. Spines were identified on the basis of their size, the absence of mitochondria and/or the presence of spine apparatus. In addition, the sizes of VGLUT1 and VGLUT2-labeled synaptic boutons were determined from the micrographs by measuring the cross-sectional area of the boutons, the diameter of the boutons and the length of the synaptic specialization, using the public-domain image-processing program, ImageJ. Data were represented as means and standard deviations and comparisons made using the Mann-Whitney U test with $P<0.05$ considered significant.

Image capture and manipulation

Images from the electron microscope were recorded on film or recorded digitally using a Gatan multiscan CCD digital camera (Gatan, Oxfordshire, UK). Qualitative and quantitative analyses were carried out on the digital images, on high quality prints or on digital images created from the prints or negative. Images were viewed and manipulated (contrast, brightness and application of lettering, arrows etc) using Adobe Photoshop on a Macintosh computer.

RESULTS

Subcellular localization of $GABA_{B1}$ and $GABA_{B2}$ in the striatum

Immunolabeling for $GABA_{B1}$ and $GABA_{B2}$ was identified by the presence of the electron dense immunogold particles. Labeled structures generally contained multiple particles and it is on this labeling that our qualitative description of the distribution is based. However, structures were observed that possessed only one immunogold particle and, although we cannot be sure that they do not represent background labeling, the profile of structures labeled was similar to those labeled by multiple particles.

The immunolabeling for $GABA_{B1}$ and $GABA_{B2}$ was widely distributed in the striatum, being detected in both presynaptic and postsynaptic structures (Fig. 1). The immunogold particles were thus present in unmyelinated ax-

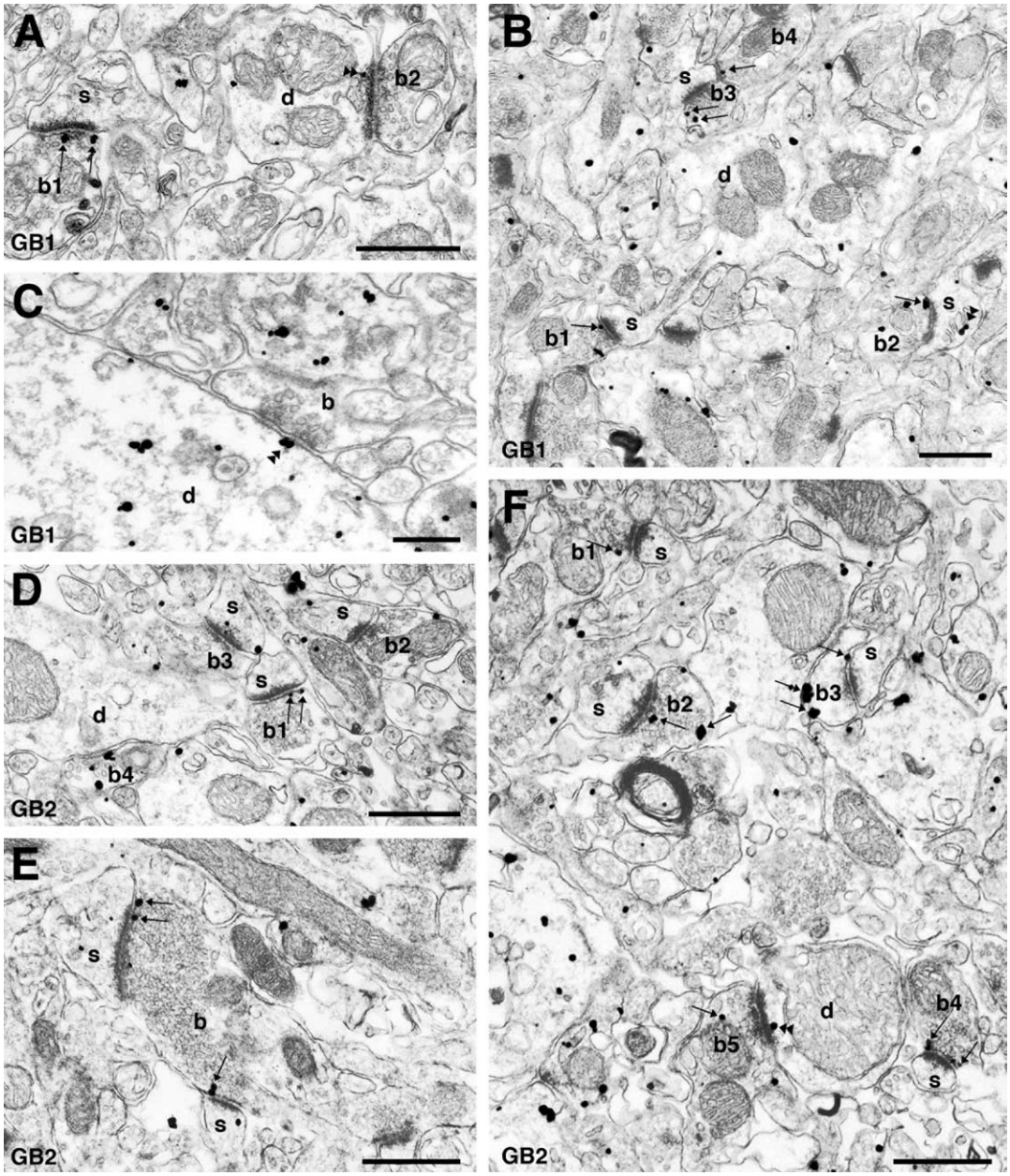

Fig. 1. Localization of immunogold labeling for $GABA_{B1}$ (GB1; A–C) and $GABA_{B2}$ (GB2; D–F) in the striatum. (A) Immunolabeling for $GABA_{B1}$ (arrows) on the presynaptic membrane of a bouton (b1) forming an asymmetric synapse with a dendritic spine (s). $GABA_{B1}$ immunolabeling (double arrowhead) is also associated with the postsynaptic density at a second asymmetric synapse between an unlabeled bouton (b2) and a dendritic shaft (d). (B) Presynaptic labeling for $GABA_{B1}$ in boutons (b1–b3) forming asymmetric synapses with spines (s). Immunogold particles (some of which are indicated by arrows) are located at synaptic (b1 and b2) and extrasynaptic sites (b1–3) on the membrane of the boutons. The spine postsynaptic to bouton b1, emerges from a dendrite (d) that is also immunolabeled for $GABA_{B1}$. Similarly, the spine postsynaptic to bouton b2, is also immunolabeled; the immunogold particles are associated with the spine apparatus (double arrowhead). The spine postsynaptic to bouton b3 receives symmetric synaptic input from a second bouton (b4) that is labeled on the presynaptic membrane (arrow). (C) A bouton (b) forms symmetrical synaptic contact with a dendrite that is immunolabeled for $GABA_{B1}$. The immunogold particles are associated with the postsynaptic membrane (double arrowhead) and intracellular sites. (D) Immunolabeling for $GABA_{B2}$ at the presynaptic specialization (arrows) of a bouton (b1) forming an asymmetric synapse with an immunonegative spine (s). Another $GABA_{B2}$-positive bouton (b2) forms an asymmetric synapse with a $GABA_{B2}$-positive spine (s). An immunonegative bouton (b3) forms an asymmetric synapse with a $GABA_{B2}$-positive spine (s). The $GABA_{B2}$-labeled bouton, b4, is in symmetrical synaptic contact with a dendrite. Note that some of the immunogold labeling in this bouton is associated with the presynaptic membrane specialization. (E) A $GABA_{B2}$-immunolabeled bouton (b) forming asymmetric synapses with two spines (s). The immunogold particles (arrows) are associated with the presynaptic specialization at each synapse and the postsynaptic spines also express $GABA_{B2}$-immunolabeling. (F) Immunogold labeling for $GABA_{B2}$ (some gold particles indicated by arrows) in boutons (b1–b5) forming asymmetric synapses with spines or a dendrite. The immunogold labeling is associated with the presynaptic specialization (b4), extrasynaptic sites on the membrane (b2, b3) and intracellular sites (b1, b2, b5). Several of the postsynaptic structures are also immunolabeled including the spine contacted by b5. In this case immunogold labeling is closely associated with the postsynaptic density (double arrowhead). Scale bars = 0.25 μm.

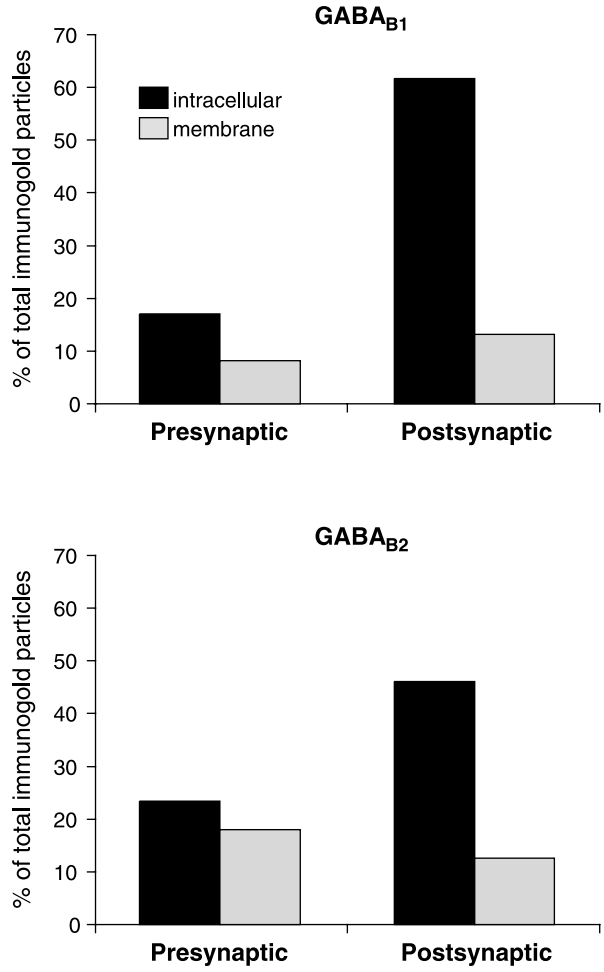

Fig. 2. Distribution of immunogold labeling for $GABA_{B1}$ and $GABA_{B2}$ in the striatum. Immunogold particles for either $GABA_{B1}$ and $GABA_{B2}$ were categorized as being associated with presynaptic structures (axons and terminals) or postsynaptic structures (dendrites and spines). Those immunogold particles touching the plasma membrane were categorized as membrane-associated and the remainder as intracellular. The bars represent the percentage of total immunogold particles counted for $GABA_{B1}$ (upper; $n=339$) and $GABA_{B2}$ (lower; $n=421$).

ons and axon terminals forming symmetrical or asymmetrical synapses, and in perikarya, dendrites and spines. A larger proportion of immunogold particles were associated with dendrites and spines (60–70%) than with presynaptic structures (30–40%) (Fig. 2). However, the labeling in axons and axon terminals was much more frequently located on the plasma membrane than in dendrites and spines. In presynaptic structures 32.5% $GABA_{B1}$ immunogold particles and 43.2% of $GABA_{B2}$ immunogold particles were associated with the membrane, whereas only 17.5% of $GABA_{B1}$ immunogold particles and 21.4% of $GABA_{B2}$ immunogold particles in postsynaptic structures were associated with the membrane.

Immunolabeling for $GABA_{B1}$ and $GABA_{B2}$ was particularly prominent in axon terminals forming asymmetric synaptic contacts (Fig. 1). For both subunits, immunogold particles associated with the membrane of the axon terminals were located at extrasynaptic sites, at the edge of the synaptic specializations and at the presynaptic active zone (Fig. 1A, B, D–F). In cases where a single terminal bouton gave rise to more than one synaptic active zone, gold particles were sometimes found at each synaptic site (Fig. 1E). Immunogold particles were also often present within the cytoplasm of labeled terminals forming asymmetric synapses (Fig. 1F). The postsynaptic targets of the $GABA_B$-immunopositive terminals forming asymmetric synapses were mostly dendritic spines of presumed medium spiny neurons (Fig. 1A, B, D–F) and occasionally dendritic shafts (Fig. 1A, F). The postsynaptic structures themselves were also often immunopositive for the $GABA_B$ receptor subunits (Fig. 1A, B, D–F).

Labeling for $GABA_{B1}$ and $GABA_{B2}$ was also observed in terminals forming symmetric synapses (Fig. 1B, D; see also Fig. 5A). As with the terminals forming asymmetric synapses, the immunolabeling was located within the cytoplasm (Fig. 1D), at extrasynaptic sites on the membrane (Fig. 1D) and at the presynaptic active zone (Fig. 1B, D). In keeping with the known data concerning symmetrical synapses in the striatum (Ingham et al., 1998), the postsynaptic targets of these terminals were more commonly dendritic shafts (Fig. 1D). However, labeled terminals were also seen in symmetrical synaptic contact with dendritic spines that also received asymmetric synaptic input (Fig. 1B). Unlabeled boutons forming symmetrical synapses were also observed to form $GABA_B$-positive synapses with immunogold particles located at the postsynaptic membrane specialization (Fig. 1C). Immunogold particles were also associated with extrasynaptic sites on the membrane as well as intracellular sites (Fig. 1C).

The majority of immunogold labeling in spines was associated with intracellular sites ($GABA_{B1}$, 67.2%; $GABA_{B2}$, 64.5%) including the spine apparatus (Fig. 1B, D; 2; see also Fig. 4B, D). About a third of immunogold particles were associated with extrasynaptic sites on the membrane ($GABA_{B1}$, 32.1%; $GABA_{B2}$, 31.6%). Similarly, in large and small dendritic shafts, labeling for $GABA_B$ subunits was mostly intracellular ($GABA_{B1}$, 84.1%; $GABA_{B2}$, 80.5%; Fig. 1A, B, F) or when associated with the membrane, was at extrasynaptic sites ($GABA_{B1}$, 15.8%; $GABA_{B2}$, 19.3%). Immunogold labeling in dendritic shafts was sometimes associated with the postsynaptic density of asymmetric synapses (Fig. 1A, F).

VGLUT1 and VGLUT2 immunolabeling in the striatum

In agreement with previous light microscopic studies (Kaneko et al., 2002; Varoqui et al., 2002), immunoreactivity for VGLUT1 and VGLUT2 in the striatum was in the form of punctate labeling that was uniformly distributed throughout the striatal neuropil (data not shown). No labeling of cell bodies was observed for either subtype.

Electron microscopy confirmed that the peroxidase-reaction end product used to label VGLUT1 or VGLUT2 was present within vesicle-containing axonal boutons of variable size and shape. All synapses formed by VGLUT-immunolabeled boutons were of the asymmetric type

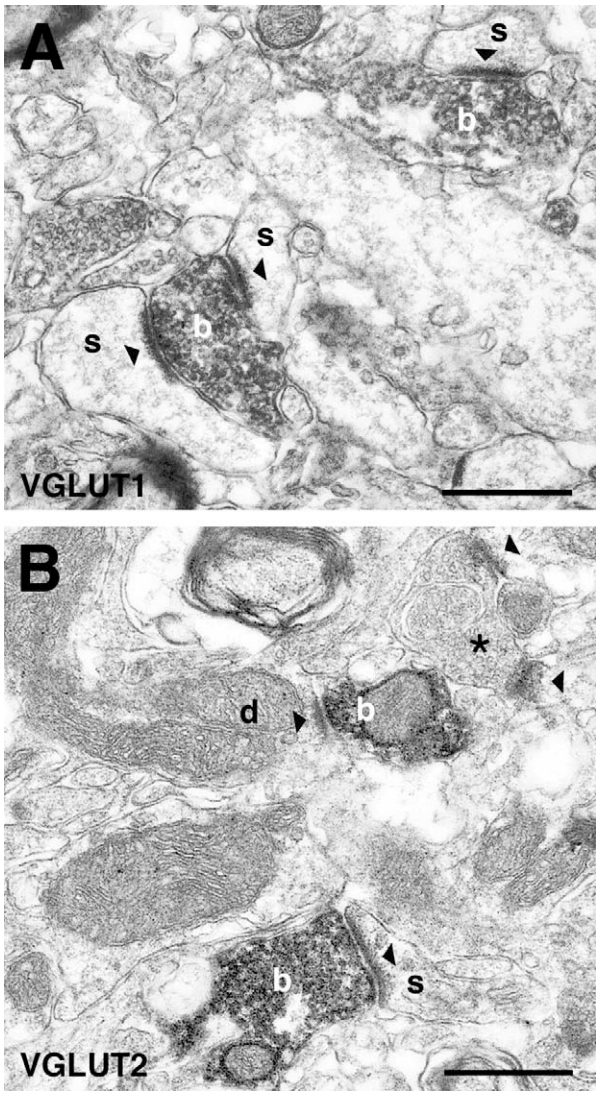

Fig. 3. Immunolabeling for VGLUT1 and VGLUT2 in the striatum. (A) VGLUT1-positive boutons (b) labeled by the immunoperoxidase method, form asymmetric synapses (arrowheads) with dendritic spines (s). Note that a single VGLUT1-positive bouton (lower left) can form synapses with more than one spine. (B) VGLUT2-positive boutons (b) form asymmetric synapses (arrowheads) with both spines (s) and dendritic shafts (d). Note the unlabeled bouton (*) forming asymmetric synapses with two spines (arrowheads). (C) Bar chart of the distributions of the postsynaptic targets of terminals immunolabeled for VGLUT1 (n=371) and VGLUT2 (n=385). Scale bars=0.25 μm.

(Figs. 3A, B; 4; 5). Approximately one third and one quarter of terminal boutons forming asymmetric synapses in the striatum were immunolabeled for VGLUT1 (34.8%; n=118/339) or VGLUT2 (24.5%; n=103/421), respectively. These data suggest that a significant proportion of terminals forming asymmetric synapses are VGLUT-negative. In order to address this point more directly we carried out double immunolabeling for both VGLUTs in the same tissue. From a total of 512 terminals forming asymmetric synapses (two blocks of dorsal striatum from each of two animals), 71.5% were immunopositive and 28.5% immunonegative. Overall, immunopositive boutons in contact with spines accounted for 64.8% of terminals forming asymmetric synapses (24.4% immunonegative) and those in contact with dendritic shafts accounted for 6.6% (4.1% immunonegative).

There were no differences (Mann-Whitney test, $P>0.05$) between VGLUT1- and VGLUT2-positive boutons in the cross-sectional area of the labeled boutons, the width of the synaptic specialization or the diameter of the synaptic bouton (Table 1). There was a marked difference, however, in the postsynaptic targets of VGLUT1- and VGLUT2-labeled boutons. Thus, VGLUT1-labeled boutons (n=371) formed asymmetric synapses almost exclusively with spines (98.9%; Figs. 3A, C; 4; 5), whereas VGLUT2-labeled boutons (n=385) formed asymmetric synapses with both dendritic shafts (28.1%) and spines (71.9%) (Figs. 3B, C; 4; 5). Occasionally, single VGLUT1- or VGLUT2-labeled boutons made synaptic contact with more than one postsynaptic structure (Fig. 3A).

Consistent with the data from the immunoperoxidase labeling, immunofluorescence labeling revealed that immunoreactivity for VGLUT1 and VGLUT2 was restricted to punctate profiles throughout the neuropil of the striatum that showed no rostro-caudal variations. Double immunofluorescence revealed, as in a previous study (Fujiyama et al., 2004), that there is no co-localization of VGLUT1 and VGLUT2 in the striatum (data not illustrated).

$GABA_B$ receptor immunolabeling associated with VGLUT-positive terminals

Immunogold labeling for $GABA_{B1}$ and $GABA_{B2}$ was identified in numerous axon terminals that were immunoreactive for either VGLUT1 (Figs. 4A, B; 5A, B) or VGLUT2 (Figs. 4C–E; 5C–F). In agreement with the single labeling studies, terminals that were double-labeled for VGLUT1 and $GABA_B$ receptor subunits formed asymmetric synapses with dendritic spines of presumed medium spiny neurons (Figs. 4A, B; 5A, B), whereas VGLUT2/$GABA_B$-labeled terminals made synaptic contact with both dendritic spines (Figs. 4C, D; 5C, E) and shafts (Figs. 4E; 5D,

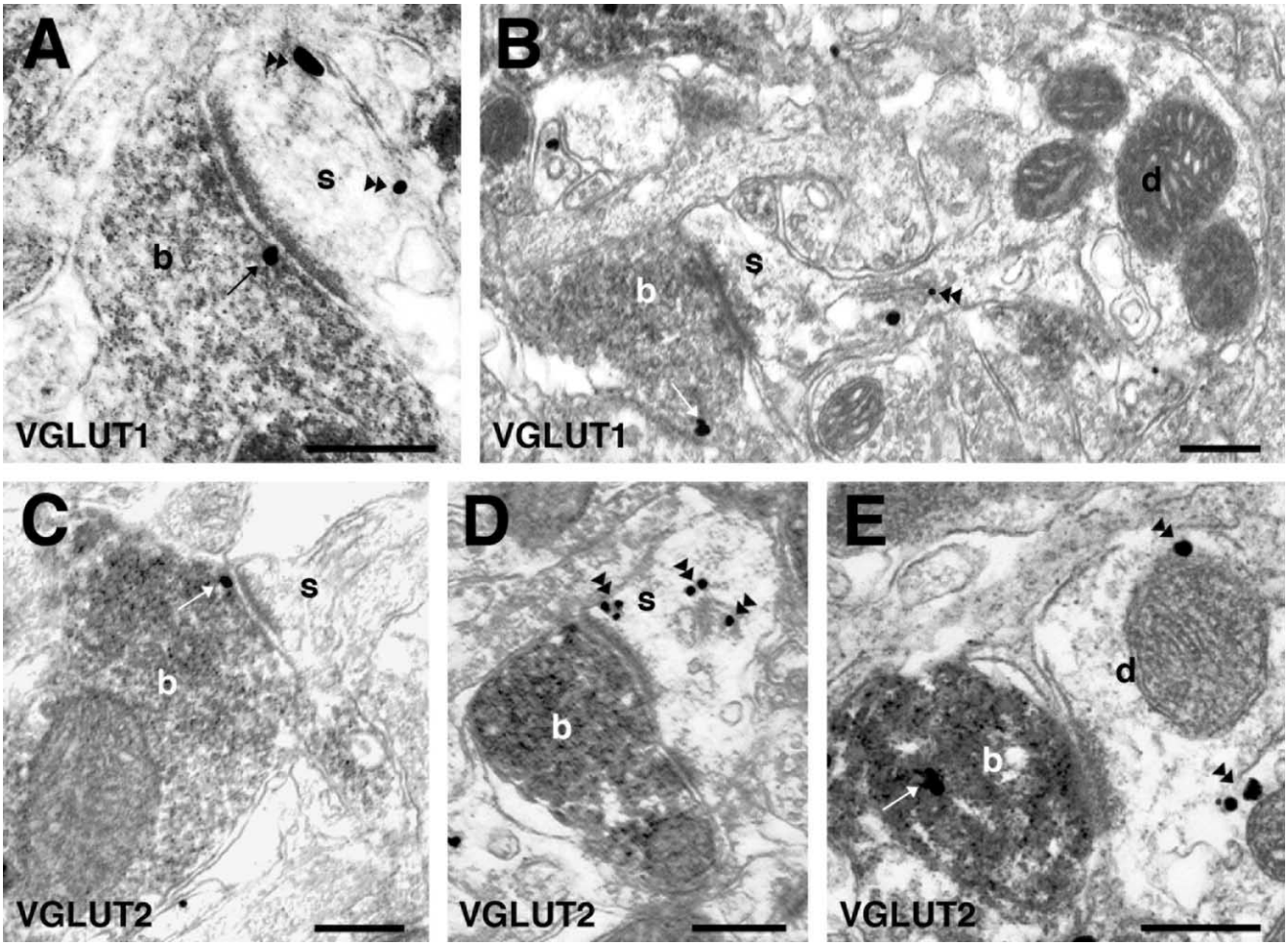

Fig. 4. $GABA_{B1}$ immunolabeling in relation to VGLUT-positive synaptic boutons in the striatum. Immunolabeling for $GABA_{B1}$ is by the immunogold method and the VGLUTs by the immunoperoxidase method. (A) Immunolabeling for $GABA_{B1}$ (arrow) at the presynaptic specialization of a VGLUT1-labeled bouton (b) forming an asymmetric synapse with a dendritic spine (s). The spine is also labeled for $GABA_{B1}$ at extrasynaptic sites (double arrowheads). (B) A spine, which is emerging from a dendritic shaft (d), in asymmetric synaptic contact with a VGLUT1-positive bouton (b). Both the presynaptic bouton (arrow) and the postsynaptic spine are immunopositive for $GABA_{B1}$; the immunolabeling in the spine is associated with the spine apparatus (double arrowhead). (C) Immunolabeling for $GABA_{B1}$ (arrow) at the presynaptic specialization of a VGLUT2-positive bouton (b) forming an asymmetric synapse with a spine. (D) A VGLUT2-positive bouton (b) in asymmetric synaptic contact with a spine that is immunolabeled for $GABA_{B1}$ (double arrowheads). The immunogold particles are located within the cytoplasm and associated with the spine apparatus. (E) Immunolabeling for $GABA_{B1}$ in the cytoplasm (arrow) of a VGLUT2-positive bouton (b) forming an asymmetric synapse with a $GABA_{B1}$-positive (double arrowheads) dendrite. Scale bars = 0.25 μm.

F). In VGLUT-labeled terminals, immunogold particles labeling $GABA_B$-positive sites were located on the presynaptic membrane (Figs. 4A, D; 5A, C, E, F), at extrasynaptic sites along the membrane (Figs. 4B; 5E) and within the cytoplasm (Figs. 4E; 5A, B, E, F). Because of the problems of false negatives in double immunolabeling studies at the electron microscopic level due to the differential penetration of immunoreagents, no attempt was made to quantify the degree of co-localization of the $GABA_B$ receptor labeling and VGLUT labeling.

The postsynaptic targets of $GABA_B$/VGLUT-labeled axon terminals were also frequently labeled for the respective $GABA_B$ subunit. Consistent with the single-labeling studies, immunogold particles in postsynaptic spines or dendrites were largely intracellular or associated with the extrasynaptic membrane (Figs. 4A, B, D, E; 5A, C–E).

DISCUSSION

Localization of $GABA_B$ receptor subunits in the striatum

Studies in knockout mice lacking either $GABA_{B1}$ or $GABA_{B2}$ have provided the most compelling evidence to date that $GABA_B$ receptors require both subunits in order to function normally *in vivo* (Prosser et al., 2001; Schuler et al., 2001; Gassmann et al., 2004). *In situ* hybridization studies, however, have demonstrated that $GABA_{B2}$ mRNA is at low or undetectable levels in rat striatum, whereas $GABA_{B1}$ is more strongly expressed in both embryonic and adult striatum (Durkin et al., 1999; Clark et al., 2000; Johnston and Duty, 2003; Martin et al., 2004). In contrast, both $GABA_{B1}$ and $GABA_{B2}$ proteins have been detected in

striatal neurons in rats, monkeys and humans using immunocytochemistry (Yung et al., 1999; Charara et al., 2000, 2004; Charles et al., 2001; Waldvogel et al., 2004). In view of this discrepancy, we used antibodies against both subunits to investigate the precise localization of $GABA_B$ receptors at the electron microscopic level. Our findings demonstrate that $GABA_B$ receptor immunoreactive sites are widespread in the rat striatum. In agreement with immunoperoxidase data from monkeys (Charara et al., 2000, 2004), the current data show that immunogold labeling for $GABA_{B1}$ and $GABA_{B2}$ is present in postsynaptic structures, including both dendritic shafts and spines. In addition, we observed labeling for both subunits in unmyelinated axons and in axon terminals, most of which formed asymmetric synapses. These findings suggest that functional $GABA_B$ receptors are expressed at both presynaptic and postsynaptic sites in the striatum. Moreover, the similarity between the subcellular distributions of $GABA_{B1}$ and $GABA_{B2}$ in the striatum is supportive of the view that $GABA_B$ receptors exist as heterodimers of the two subunits in this brain region.

Consistent with findings in other brain regions (Boyes and Bolam, 2003; Chen et al., 2004; Galvan et al., 2004; Luján et al., 2004), a large proportion of the labeling for $GABA_{B1}$ and $GABA_{B2}$ was located within the cytoplasm of dendritic and axonal processes. This intracellular pool is likely to reflect receptors or receptor subunits undergoing synthesis, assembly, trafficking, degradation and/or a reserve pool of receptors. A greater proportion of $GABA_B$ labeling was associated with postsynaptic structures than with presynaptic structures, but the proportion of labeling that was associated with the plasma membrane was much higher in the presynaptic structures. Thus a larger proportion of the presynaptic labeling is potentially in the 'active form', i.e. the form that can be exposed to the endogenous transmitter. Although the *density* of immunolabeling was not measured, it appears that presynaptic $GABA_B$ receptors are the predominant subtype of $GABA_B$ receptor expressed in the striatum, which may explain, in part, the *in vitro* electrophysiological data describing exclusively presynaptic effects of $GABA_B$ receptor activation (for references see below).

The most common type of $GABA_B$-labeled terminal formed asymmetric synapses, mainly with spines of presumed spiny projection neurons, as well as with dendritic shafts. In the rat striatum, asymmetric synapses account for up to 80% of all synapses (Ingham et al., 1998) and are formed mainly by excitatory afferents from the cortex and the thalamus. It is well documented that stimulation of presynaptic $GABA_B$ receptors can depress excitatory transmission in the CNS (Dutar and Nicoll, 1988; Isaacson, 1998) and indeed, this is also the case in the striatum (see below). In support of these findings, we and others have described $GABA_B$ receptor subunits on glutamatergic terminals in several brain regions, including the globus pallidus (Chen et al., 2004), substantia nigra (Boyes and Bolam, 2003), cerebellum and thalamus (Kulik et al., 2002). In the present study and our previous studies, membrane-associated immunogold particles in presynaptic terminals were located within the main body of the presynaptic active zone and at the edge of the active zone, as well as at extrasynaptic sites. Presynaptic $GABA_B$ receptors have been shown to regulate neurotransmitter release by both calcium-dependent and calcium-independent mechanisms, including G-protein-mediated inhibition of presynaptic Ca^{2+} channels (Isaacson, 1998) and/or direct modulation of synaptic vesicle priming (Sakaba and Neher, 2003). The different subcellular localizations of $GABA_B$ receptors raise the interesting possibility that the different $GABA_B$-mediated mechanisms regulating transmitter release are related to the spatial relationship of the receptors to the transmitter release site.

In dendrites and spines of striatal neurons, membrane-associated labeling for $GABA_{B1}$ and $GABA_{B2}$ was predominantly found at extrasynaptic sites. In addition, and in agreement with immunogold data for $GABA_{B1}$ in the monkey striatum (Smith et al., 2000), labeling for $GABA_{B1}$ and $GABA_{B2}$ was also localized at the postsynaptic membrane of some symmetric synapses. This suggests that, at least at some synapses, $GABA_B$ receptors may be co-localized with $GABA_A$ receptors, as the majority of $GABA_A$ receptors are located at synaptic specializations in the rat striatum (Fujiyama et al., 2002).

VGLUTs in the striatum

To gain further insight into the expression of $GABA_B$ heteroreceptors in striatal microcircuits, we used immunolabeling for VGLUT1 and VGLUT2 as markers of glutamatergic terminals in the striatum. It has previously been shown that VGLUT1 and VGLUT2 are associated with synaptic vesicles at excitatory synapses in the striatum (Bellocchio et al., 1998; Herzog et al., 2001; Fujiyama et al., 2004), consistent with their role in mediating vesicular glutamate uptake (see Fremeau et al., 2004). The two transporters display essentially complementary patterns of distribution in the brain (Fremeau et al., 2001; Herzog et al., 2001; Kaneko et al., 2002; Varoqui et al., 2002). However, both subtypes are expressed in the striatum and it has been proposed that VGLUT1 and VGLUT2 are selectively expressed by corticostriatal and thalamostriatal af-

Table 1. Dimensions of VGLUT-immunopositive synaptic boutons

	VGLUT1		VGLUT2	
	$n=69$		$n=58$	
Axospinous contacts	Mean	SD	Mean	SD
Area of bouton (μm^2)	0.25	0.21	0.24	0.20
Width of synapse (μm)	0.35	0.16	0.34	0.21
Bouton diameter (μm)	0.68	0.33	0.63	0.37
	$n=1$		$n=19$	
Axodendritic contacts	Mean	SD	Mean	SD
Area of bouton (μm^2)	0.19		0.25	0.14
Width of synapse (μm)	0.37		0.28	0.08
Bouton diameter (μm)	0.64		0.58	0.21

No significant differences (Mann-Whitney test; $P>0.05$) were observed between the dimensions of VGLUT1- and VGLUT2-positive boutons forming axospinous synapses nor between VGLUT2-positive boutons forming axospinous and axodendritic synapses.

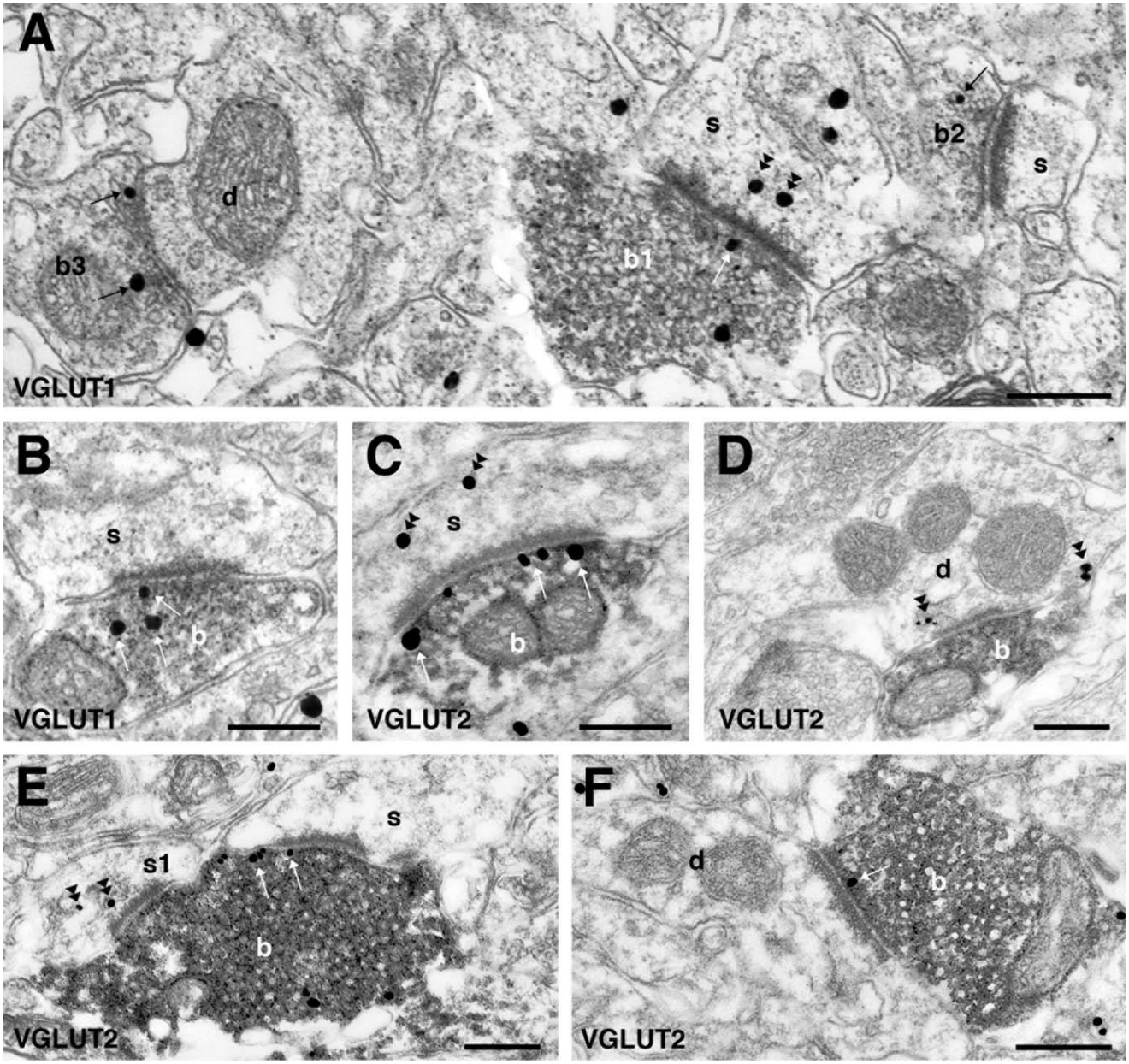

Fig. 5. $GABA_{B2}$ immunolabeling in relation to VGLUT-positive synaptic boutons in the striatum. Immunolabeling for $GABA_{B2}$ is by the immunogold method and the VGLUTs by the immunoperoxidase method. (A) $GABA_{B2}$ immunolabeling at the presynaptic specialization (arrow) of a VGLUT1-positive bouton (b1) forming an asymmetric synapse with a $GABA_{B2}$-positive dendritic spine (s) (some immunogold particles indicated by double arrowheads). The field contains two other $GABA_{B2}$-immunolabeled but VGLUT-negative (arrows) boutons: b2 forms an asymmetric synapse with a spine; b3 is in symmetrical synaptic contact with a dendritic shaft (d). (B) $GABA_{B2}$ immunolabeling of the presynaptic specialization and cytoplasm (arrows) of a VGLUT1-positive bouton (b) forming an asymmetric synapse with a spine. (C) $GABA_{B2}$ immunolabeling at the presynaptic specialization of a VGLUT2-positive bouton (b) forming an asymmetric synapse with a spine. The immunogold particles are lined up along the presynaptic membrane (some indicated by arrows). The spine is also labeled for $GABA_{B2}$ (double arrowheads). (D) $GABA_{B2}$ immunolabeling (double arrowheads) at extrasynaptic sites and in the cytoplasm of a dendrite receiving asymmetric synaptic input from a VGLUT2-positive bouton (b). (E) $GABA_{B2}$ immunolabeling (arrows) on the presynaptic membrane and at extrasynaptic sites of a VGLUT2-positive bouton (b) forming two asymmetric synapses with two spines (s, s1). One of the spines (s1) is also $GABA_{B2}$-immunolabeled (double arrowheads). (F) $GABA_{B2}$ immunolabeling (arrow) on the presynaptic membrane of a VGLUT2-immunoreactive bouton forming an asymmetric synapse with a dendrite. Scale bars = 0.25 μm.

ferents, respectively (Fremeau et al., 2001; Varoqui et al., 2002; Bacci et al., 2004; Fujiyama et al., 2004; Raju and Smith, 2005). Our electron microscopic findings are essentially in agreement with this. Although the sizes of the VGLUT1- and VGLUT2-labeled terminals were not different, the distribution of their postsynaptic targets was different. Thus, VGLUT1-labeled terminals formed synapses almost exclusively with dendritic spines of presumed spiny projection neurons, the main target of corticostriatal afferents (Kemp and Powell, 1971; Somogyi et al., 1981). In

contrast, VGLUT2-labeled terminals formed synapses with both dendritic shafts (28%) and spines (72%), which is characteristic of the more complex pattern of innervation of striatal neurons by afferents from the intralaminar thalamic nuclei (Dubé et al., 1988; Xu et al., 1991; Lapper and Bolam, 1992; Rudkin and Sadikot, 1999; Ichinohe et al., 2001; Smith et al., 2004). Furthermore, by the use of double immunofluorescence, we confirmed the recent finding of Fujiyama et al. (2004) that VGLUT1 and VGLUT2 are not co-localized in individual boutons in the striatum. Taken together, these data are further support for a differential expression of VGLUTs by cortical and thalamic afferents to the striatum. Although the functional significance of this is unclear, the expression of the two transporters may be linked with the probability of glutamate release at different synapses (Fremeau et al., 2001).

Approximately 70% of terminals forming asymmetric synapses in the striatum were immunoreactive for the VGLUTs. Although we cannot exclude the possibility of false-negatives, these data suggest that up to 30% of terminals forming asymmetric synapses do not express either VGLUT1 or VGLUT2. This implies either that a significant proportion of synaptic boutons forming asymmetric synapses are not glutamatergic or that there is a population of glutamatergic afferents to the striatum that does not express VGLUT1 or 2. One possible source of non-glutamatergic terminals forming asymmetric synapses is the serotonergic projection from the dorsal raphé (Soghomonian et al., 1989; Descarries et al., 1992). It is interesting to note that VGLUT3, a third VGLUT subtype that is expressed in populations of non-glutamatergic neurons, including neurons of the raphé nuclei (Gras et al., 2002), has been found in a small proportion of terminals forming asymmetric synapses in striatum (Fujiyama et al., 2004). The possibility that sub-populations of thalamostriatal or corticostriatal neurons do not express any of the known VGLUTs remains to be established.

$GABA_B$ receptors at glutamatergic synapses

Double immunolabeling demonstrated that $GABA_B$ receptors are expressed by VGLUT1-positive axon terminals in the striatum, which are likely to be derived from the cortex (see above). In support of this conclusion, lesions of the corticostriatal pathway are associated with a decrease in the density of $GABA_B$ binding sites in the striatum (Moratalla and Bowery, 1991). Furthermore, as indicated in the introduction, in corticostriatal slices, baclofen depresses the EPSPs in striatal neurons that are evoked by stimulation of cortical afferents or by intrastriatal stimulation (Calabresi et al., 1990, 1991; Seabrook et al., 1990; Nisenbaum et al., 1992, 1993), an effect that is absent in mice with mutations that render the $GABA_B$ receptor inactive (Thuault et al., 2004). Labeling for $GABA_B$ receptors was also expressed by terminals that were labeled for VGLUT2, which are likely to be derived from the thalamus (see above). This is consistent with *in situ* hybridization studies showing expression of $GABA_B$ subunit mRNA in the intralaminar thalamic nuclei that give rise to the thalamostriatal projections (Durkin et al., 1999; Lu et al., 1999). Our data thus predict that glutamate release at thalamostriatal synapses is subject to modulation by presynaptic $GABA_B$ receptors. Taken together, the findings suggest that presynaptic $GABA_B$ receptors regulate both of the major excitatory inputs to striatal neurons.

One of the key questions that arises concerning $GABA_B$ receptor expression by glutamatergic terminals is the source of the GABA that activates these receptors. The most plausible explanation is that these receptors are activated by extrasynaptic GABA that diffuses out of the synaptic cleft at neighboring GABAergic synapses. Cross-talk between GABAergic and glutamatergic synapses has been demonstrated in both the hippocampus and cerebellum (Isaacson et al., 1993; Vogt and Nicoll, 1999; Mitchell and Silver, 2000). Calabresi et al. (1990) demonstrated that increasing GABA levels in the striatum *in vitro* causes a decrease in spontaneous and evoked EPSPs that is mimicked by baclofen, indicating that striatal $GABA_B$ heteroreceptors are sensitive to extracellular GABA. Thus, GABA released from spiny projection neurons and/or striatal interneurons may activate presynaptic $GABA_B$ receptors located on cortical and thalamic terminals to simply inhibit transmission or may possibly regulate release to enable sustained transmission at higher frequencies, as has been observed in the avian auditory system (Brenowitz et al., 1998).

We also observed labeling for $GABA_{B1}$ and $GABA_{B2}$ in dendritic spines that were postsynaptic to terminals labeled for either VGLUT1 or VGLUT2. Although the majority of this was intracellular, labeling was also localized extrasynaptically on spine membranes. Interestingly, in several brain regions, postsynaptic $GABA_B$ receptors appear to be more closely associated with glutamatergic synapses than with GABAergic synapses (Kulik et al., 2002; Galvan et al., 2004; Luján et al., 2004). There is evidence in the cerebellum for an interaction between $GABA_B$ receptors and glutamate receptors (Hirono et al., 2001), however, this might not be the case in the striatum, as baclofen does not alter the responses of striatal neurons to exogenously applied glutamate (Calabresi et al., 1991). An intriguing possibility, recently proposed by Tabata et al. (2004), is that $GABA_B$ receptors at glutamatergic synapses might function independently of GABA through calcium-mediated mechanisms, to increase the sensitivity of metabotropic glutamate receptors to glutamate.

Presynaptic $GABA_B$ receptors on other classes of terminals in the striatum

Several studies have reported that baclofen reduces $GABA_A$-mediated IPSPs in striatal neurons *in vitro* (Calabresi et al., 1991; Seabrook et al., 1991; Nisenbaum et al., 1992, 1993). In support of the idea that this is an effect of presynaptic $GABA_B$ receptors on GABAergic terminals, we observed $GABA_{B1}$ and $GABA_{B2}$ immunolabeling of terminals forming symmetric axodendritic synapses with the typical appearance of striatal GABAergic terminals (Bolam et al., 1985). Thus, in addition to regulating glutamate release through presynaptic $GABA_B$ heteroreceptors in the

striatum, GABA may also exert a feedback control over its own release through presynaptic $GABA_B$ autoreceptors. There are several sources of GABA in the striatum, including the recurrent axon collaterals of spiny projection neurons, the axons of GABAergic striatal interneurons and extrinsic sources, and it has been proposed that different populations of GABAergic synapses may be differentially regulated by $GABA_B$ autoreceptors (Seabrook et al., 1991; Radnikow et al., 1997). It is clearly important to establish the identity of those terminals forming symmetrical synapses and expressing $GABA_B$ receptors because the selective expression of $GABA_B$ receptors will have significant implications for our understanding of GABA transmission in the striatum. Based on the ultrastructural features alone (i.e. synapses on the necks of spines that also receive asymmetric synaptic input), it is likely that at least some of the $GABA_B$-labeled terminals forming symmetric synapses are derived from dopaminergic nigrostriatal neurons. This is supported by the observation that $GABA_B$ receptors are expressed by nigrostriatal neurons (Boyes and Bolam, 2003), although the issue of presynaptic $GABA_B$ receptors on dopaminergic terminals in the striatum is somewhat controversial (Arias-Montaño et al., 1991; Santiago et al., 1993; Westerink et al., 1994; Smolders et al., 1995). This question awaits the application of double immunolabeling for a marker of dopaminergic terminals and $GABA_B$ receptors.

CONCLUSIONS

The findings of the present study provide an anatomical substrate for the presynaptic effects of $GABA_B$ receptor stimulation at glutamatergic cortical and thalamic synapses in the striatum thus underpinning the close interactions between the major inhibitory and excitatory neurotransmitter systems in this nucleus. They also demonstrate that stimulation of synaptic and extrasynaptic $GABA_B$ receptors is likely to affect GABAergic transmission at both presynaptic and postsynaptic sites.

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