European Journal of Neuroscience, Vol. 18, pp. 3279–3293, 2003

The subcellular localization of $GABA_B$ receptor subunits in the rat substantia nigra

Justin Boyes and J. Paul Bolam
MRC Anatomical Neuropharmacology Unit, University of Oxford, Oxford OX1 3TH, UK

Keywords: metabotropic GABA receptor, pallidonigral, pre-embedding immunogold, presynaptic, striatonigral

Abstract

The inhibitory effects of GABA within the substantia nigra (SN) are mediated in part by metabotropic $GABA_B$ receptors. To better understand the mechanisms underlying these effects, we have examined the subcellular localization of the $GABA_B$ receptor subunits, $GABA_{B1}$ and $GABA_{B2}$, in SN neurons and afferents using pre-embedding immunocytochemistry combined with anterograde or retrograde labelling. In both the SN pars compacta (SNc) and pars reticulata (SNr), $GABA_{B1}$ and $GABA_{B2}$ showed overlapping, but distinct, patterns of immunolabelling. $GABA_{B1}$ was more strongly expressed by putative dopaminergic neurons in the SNc than by SNr projection neurons, whereas $GABA_{B2}$ was mainly expressed in the neuropil of both regions. Immunogold labelling for $GABA_{B1}$ and $GABA_{B2}$ was localized in presynaptic and postsynaptic elements throughout the SN. The majority of labelling was intracellular or was associated with extrasynaptic sites on the plasma membrane. In addition, labelling for both subunits was found on the presynaptic and postsynaptic membranes at symmetric, putative GABAergic synapses, including those formed by anterogradely labelled striatonigral and pallidonigral terminals. Labelling was also observed on the presynaptic membrane and at the edge of the postsynaptic density at asymmetric, putative excitatory synapses. Double immunolabelling, using the vesicular glutamate transporter 2, revealed the glutamatergic nature of many of the immunogold-labelled asymmetric synapses. The widespread distribution of $GABA_B$ subunits in the SNc and SNr suggests that $GABA_B$-mediated effects in these regions are likely to be more complex than previously described, involving presynaptic autoreceptors and heteroreceptors, and postsynaptic receptors on different populations of SN neurons.

Introduction

The substantia nigra (SN), one of the major divisions of the basal ganglia, comprises two functionally distinct regions, the dorsal pars compacta (SNc) and the ventral pars reticulata (SNr). The SNc consists primarily of a group of dopaminergic neurons that project rostrally and modulate the flow of information through the basal ganglia, whereas the SNr contains GABAergic projection neurons, which together with neurons of the entopeduncular nucleus (the internal segment of the globus pallidus in primates) provide the major output of the basal ganglia. Neurons in the SNc and SNr receive glutamatergic inputs from the subthalamic nucleus (STN), the pedunculopontine nucleus and the prefrontal cortex (Kita & Kitai, 1987; Lavoie & Parent, 1994b; Naito & Kita, 1994; Charara *et al*., 1996). However, the majority of the afferents to the SN are GABAergic, originating in the striatum and, to a lesser extent, the globus pallidus (GP) (Grofova, 1975; Ribak *et al*., 1980; Smith & Bolam, 1990). An additional source of GABAergic input to SN neurons is the local axon collaterals of SNr projection neurons (Deniau *et al*., 1982).

The actions of GABA in the brain are mediated by two classes of receptor, the ionotropic ($GABA_A$) and the metabotropic ($GABA_B$) receptors. Unlike the $GABA_A$ receptors, which are ligand-gated chloride channels, $GABA_B$ receptors are coupled to G-proteins and mediate their effects via multiple intracellular pathways (Bowery, 1993). Expression cloning has identified two receptor subunits, designated $GABA_{B1}$ and $GABA_{B2}$, with co-expression of the two resulting in the formation of the functional receptor (Kaupmann *et al*., 1997, 1998; Jones *et al*., 1998; White *et al*., 1998; Kuner *et al*., 1999). Thus, $GABA_B$ receptors function as heterodimers, in which the $GABA_{B1}$ subunit is responsible for binding GABA, and the $GABA_{B2}$ subunit is responsible for G-protein coupling and signalling (Marshall *et al*., 1999; Galvez *et al*., 2001). When activated, presynaptic $GABA_B$ receptors inhibit the release of GABA and other neurotransmitters through a decrease in membrane Ca^{2+} conductance, whereas postsynaptic receptors induce membrane hyperpolarization through an increase in K^+ conductance (Bowery, 1993).

Electrophysiological studies indicate that $GABA_B$ receptors are involved in the modulation of the activity of dopaminergic and GABAergic SN neurons by both presynaptic and postsynaptic mechanisms (Pinnock, 1984; Engberg *et al*., 1993; Lacey, 1993; Hausser & Yung, 1994; Shen & Johnson, 1997; Chan *et al*., 1998; Erhardt *et al*., 1999; Paladini & Tepper, 1999). In order to understand fully the effects of $GABA_B$ activation in the SN, however, it is necessary to know the location of the receptors within the circuits of the SN. Recent immunocytochemical studies in the rat indicate that $GABA_{B1}$ and $GABA_{B2}$ are expressed by neurons in both the SNc and SNr (Ng & Yung, 2000, 2001b). In addition, the subcellular distribution of $GABA_{B1}$ has been described in monkeys using the immunoperoxidase method (Charara *et al*., 2000). However, the precise localization of the two $GABA_B$ subunits in SN neurons and their association with nigral afferents has not been established. In order to address this, we used the pre-embedding immunogold method to determine the subcellular localization of $GABA_{B1}$ and $GABA_{B2}$ in the rat SNc and SNr. In addition, we investigated the existence of presynaptic $GABA_B$ autoreceptors on identified GABAergic afferent

Correspondence: J. P. Bolam, as above.
E-mail: paul.bolam@pharm.ox.ac.uk

Received 10 July 2003, revised 7 October 2003, accepted 13 October 2003

doi:10.1046/j.1460-9568.2003.03076.x

terminals in the SN by combined $GABA_B$ receptor immunolabelling and anterograde labelling from the striatum and the GP. Finally, we tested the hypothesis that presynaptic $GABA_B$ heteroreceptors are present on glutamatergic terminals in the SN.

Materials and methods

Animals and tissue preparation

Adult male Sprague–Dawley rats (180–300 g; Charles River, Margate, Kent, UK) were used in the study. Environmental conditions for housing of the rats and all procedures that were performed on them were in accordance with the Animals (Scientific Procedures) Act, 1986 (UK) and with the Society for Neuroscience policy on the use of animals in research.

Animals were deeply anaesthetized with sodium pentobarbitone (200 mg/kg; Sagatal, Rhône Mérieux, Tallaght, Dublin, Ireland) and perfused transcardially with 50–100 mL of phosphate-buffered saline (PBS; 0.01 M phosphate, pH 7.4) followed by 300 mL of fixative containing 3% paraformaldehde and 0.1% glutaraldehyde in phosphate buffer (PB; 0.1 M, pH 7.4) over a period of 20 min. The animals were then postperfused with ≈100 mL PBS. Following fixation, the brain was quickly removed from the skull, and coronal sections (70 μm) through the midbrain were cut on a vibrating microtome and collected in PBS prior to further processing.

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 from Chemicon International, Harrow, UK). Both antibodies have been previously characterized by immunoblot analysis (Yung *et al*., 1999; Ng & Yung, 2001a). In addition, the specificity of the $GABA_{B1}$ antibody has been established in $GABA_{B1}$ subunit knockout mice (Prosser *et al*., 2001).

Pre-embedding immunocytochemistry

To enhance the tissue penetration of immunoreagents, all sections of SN were equilibrated in a cryoprotectant solution (0.05 M PB, pH 7.4, containing 25% sucrose and 10% glycerol) and 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) in PBS for 2 h. All further incubation steps were carried out in PBS containing 2% NGS (PBS-NGS) and sections were washed three or four times between steps. The sections were incubated with the primary antibodies ($GABA_{B1}$, diluted 1 : 1000 to 1 : 2000; or $GABA_{B2}$, diluted 1 : 2000 to 1 : 4000) for 48 h at 4 °C and then in goat anti-guinea-pig IgG conjugated to 1.4 nm gold particles (1 : 100; Nanoprobes, Stony Brook, NY, USA) for 2 h at room temperature. The sections were postfixed in 1% glutaraldehyde in PBS for 10 min, before the gold particles were enhanced by silver intensification using the HQ Silver kit (Nanoprobes).

For double immunolabelling, sections from three animals were incubated in a primary antibody mixture containing either $GABA_{B1}$ or $GABA_{B2}$ antibodies (diluted as before) plus a rabbit antibody against the vesicular glutamate transporter 2 (VGLUT2; diluted 1 : 2000; Synaptic Systems, Göttingen, Germany), a marker for glutamatergic nerve terminals (Fremeau *et al*., 2001; Herzog *et al*., 2001), for 48 h at 4 °C. The sections were first processed to reveal $GABA_{B1}$ or $GABA_{B2}$ immunogold labelling by the method described above (with the omission of the postfixation step in glutaraldehyde). Subsequently, the sections were incubated in biotinylated goat anti-rabbit IgG (1 : 200; Vector) for 2 h at room temperature, followed by avidin–biotin-peroxidase complex (1 : 100 in PBS; Vector) for 1 h at room temperature. After equilibrating in Tris buffer (0.05 M, pH 7.6), the bound peroxidase was revealed by incubation in 0.025% diaminobenzidine (DAB; in Tris buffer; Sigma, Dorset, UK) in the presence of 0.01% H_2O_2. The reaction was stopped after 6–8 min by several washes in Tris buffer.

Anterograde labelling of striatonigral and pallidonigral afferents

To study the expression of $GABA_B$ receptors in relation to GABAergic inputs to the SN from the striatum and GP, immunogold labelling for $GABA_{B1}$ and/or $GABA_{B2}$ was combined with anterograde labelling of afferents. Twelve rats were anaesthetized with a mixture of fentanyl/fluanisone (0.135 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 the head secured in a stereotaxic frame. The animals received unilateral or bilateral deposits of wheatgerm agglutinin conjugated to horseradish peroxidase ($n = 7$) (WGA–HRP; 50 nL; Vector) or *Phaseolus vulgaris* leucoagglutinin ($n = 5$) (PHA-L; 2.5% in 0.01 M PB, pH 8.0; Vector) into either the striatum or the GP, using stereotaxic co-ordinates derived from the atlas of Paxinos & Watson (1986). Two or three deposits along a single penetration were made in the striatum, whereas a single deposit was made in the GP. The WGA–HRP was delivered by pressure injection, using glass micropipettes of 40- to 70-μm tip diameter, and the PHA-L by iontophoresis via glass micropipettes of 10- to 15-μm tip diameter using a pulsed (7 s on, 7 s off) positive current (9 μA) for 10 min. Following the injection of the tracer, the glass micropipettes were left *in situ* for at least 10 min to minimize leakage along the injection tract. After a survival time of 24 h (for the WGA–HRP-injected animals) or 7 days (for the PHA-L-injected animals), all animals were perfused as described above, and 70-μm thick coronal sections were cut through the injection sites and the midbrain.

Visualization of tracers combined with immunogold labelling

Sections of SN were first processed to reveal $GABA_{B1}$ or $GABA_{B2}$ immunogold labelling and subsequently processed to reveal the transported WGA–HRP or PHA-L using peroxidase reactions. The method for the pre-embedding immunogold was as described above with the omission of the postfixation step in glutaraldehyde.

In sections from animals injected with WGA–HRP, the transported tracer was revealed using the tetramethylbenzidine (TMB)/tungstate method (Weinberg & van Eyck, 1991). In brief, the sections were washed in PBS followed by PB (pH 6.0), and then pre-incubated in a reaction mixture consisting of 2.3 mL of PB (pH 6.0), 115 μL of 1% ammonium paratungstate, 29 μL of 0.2% TMB (in ethanol; Sigma), 23 μL of 0.4% ammonium chloride and 23 μL of 20% β D-glucose. The reaction was initiated by the addition of 2.5 μL of glucose oxidase (in 0.1 M sodium acetate buffer, pH 4.0; Sigma) and stopped after 3–5 min by several washes in PB (pH 6.0). The TMB/tungstate reaction product was then stabilized by incubating the sections in a mixture containing 2.5 mL of PB (pH 7.4), 2.5 mg of DAB, 25 μL of 0.4% ammonium chloride, 25 μL of 20% β D-glucose, 50 μL of 1% cobalt chloride and 2.5 μL of glucose oxidase for 10 min, before washing several times in PB. For animals injected with PHA-L, sections of the SN were incubated in rabbit anti-PHA-L antibody (diluted 1 : 1000 in PBS-NGS; Vector) for 48 h at 4 °C, followed by biotinylated goat anti-rabbit IgG (1 : 200 in PBS-NGS; Vector) for 2 h at room temperature. The PHA-L was then revealed by the peroxidase reaction with DAB as described above.

To confirm the location of the injection sites, sections through the striatum and GP (from all injected animals) were processed to reveal the injected tracer using DAB for the peroxidase reactions, with the addition of 0.3% Triton X-100 to each of the reagents. The sections were mounted on glass microscope slides and processed for light microscopic examination.

Processing for electron microscopy

All single- and double-labelled sections of SN were washed in PB and postfixed in 1% osmium tetroxide (in PB; Oxkem, UK) for 7 min. After several washes in PB, the sections were dehydrated through a graded series of dilutions of ethanol, with 1% uranyl acetate included in the 70% ethanol solution to increase contrast in the electron microscope. Following the absolute ethanol, the sections were treated with propylene oxide before being embedded in resin overnight (Durcupan, ACM; Fluka, Dorset, UK). The sections were then mounted on glass slides, a coverslip applied and the resin polymerized at 60 °C for 48 h. After examination in the light microscope, selected regions of the SNc and SNr 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 Pioloform-coated single-slot copper grids. Ultrathin sections were then contrasted with lead citrate for 3–4 min and examined in a Philips CM10 or CM100 electron microscope.

Control experiments

To control for the specificity of the $GABA_B$ and VGLUT2 antibodies in the pre-embedding protocol, sections of SN and cerebellum were incubated with the omission of one of the primary antibodies. Under these conditions, there was a complete lack of immunolabelling for the respective antigens. To further test the specificity of the $GABA_B$ antibodies, we used antibodies against $GABA_{B1}$ and $GABA_{B2}$ that were raised in sheep (a gift from Dr P. C. Emson, Babraham Institute, Babraham, UK). These resulted in a similar pattern of labelling at both the light and electron microscopic level (Billinton *et al*., 2001; Boyes *et al*., 2002).

Analysis of material

For the analysis of the subcellular distribution of immunogold labelling for $GABA_{B1}$ and $GABA_{B2}$, blocks were selected from the SN from three to five animals. In order to prevent overlap between SNr and SNc elements, SNc blocks were taken from the dorsomedial part of the SN, whereas SNr blocks were taken from the ventral part of the SN. In the electron microscope, areas of SNc and SNr at the interface between the tissue and empty resin, i.e. at the surface of the tissue, were selected at random and a series of 10 adjacent electron micrographs were taken parallel to the interface at least 5 μm into the tissue at a magnification of 15 500×, giving a total sampled area of tissue per animal of 597 μm^2 for each region. To quantify the overall distribution of labelling for $GABA_{B1}$ and $GABA_{B2}$ within identified presynaptic and postsynaptic elements, every immunogold particle in each micrograph was counted and categorized as either intracellular or associated with the plasma membrane, i.e. those gold particles touching the plasma membrane. The membrane-associated gold particles were further divided into three categories based on their localization relative to synapses: (i) synaptic, if they were located within the pre or postsynaptic specialization at symmetric or asymmetric synapses; (ii) perisynaptic, if they were at the edge of the presynaptic or postsynaptic specialization; or (iii) extrasynaptic, if they were associated with parts of the nonsynaptic membrane. To assess the expression of $GABA_B$ receptors on putative inhibitory and putative excitatory axon terminals in the SN, the proportions of labelled and unlabelled boutons forming either symmetric or asymmetric synapses was calculated in sections immunolabelled for $GABA_{B2}$. In the same random series of micrographs used for the above analysis, every bouton making a clear synaptic contact was classified as immunolabelled or unlabelled and the type of synaptic contact noted. To avoid false-positive data in this analysis, only those terminals containing two or more gold particles were classified as immunolabelled for $GABA_{B2}$. As such, the analysis generated minimum values for the proportion of terminals expressing $GABA_B$ receptors. In addition, the proportion of synaptic versus extrasynaptic membrane-associated gold particles was calculated in immunolabelled boutons.

Results

Light microscopic observations

At the light microscope level, the pattern of immunolabelling for the two $GABA_B$ receptor subunits in the SN was similar to that previously described (Margeta-Mitrovic *et al*., 1999; Charara *et al*., 2000; Charles *et al*., 2001). In both the SNc and SNr, $GABA_{B1}$ and $GABA_{B2}$ showed distinct patterns of immunolabelling. In the SNc, intense $GABA_{B1}$ labelling was observed in numerous large, densely packed neurons (Fig. 1A). Neurons of similar morphology in the adjacent ventral tegmental area (VTA) also displayed strong labelling for $GABA_{B1}$. In contrast, labelling for $GABA_{B2}$ in the SNc was largely restricted to the neuropil, with only light labelling of perikarya (Fig. 1B). In the SNr, less intense labelling for $GABA_{B1}$ was observed (Fig. 1C). However, large strongly labelled perikarya, which probably represent ventrally displaced dopaminergic neurons, were visible in the caudoventral SNr. Labelling for $GABA_{B2}$ in the SNr was similar to that in the SNc, with labelling predominantly observed in the neuropil (Fig. 1D).

Subcellular localization of $GABA_{B1}$ and $GABA_{B2}$ in the substantia nigra pars compacta

In the electron microscope, examination of immunogold-labelled sections of SNc revealed a wide distribution of immunolabelling for $GABA_{B1}$ and $GABA_{B2}$ (Fig. 2). Labelling for both $GABA_{B1}$ and $GABA_{B2}$ was present in putative dopaminergic neuronal perikarya and dendritic processes (Fig. 2A–E and G), as well as in axons and axon terminals (Fig. 2A and C–F). In agreement with light microscopic observations, labelling for $GABA_{B1}$ was especially prominent in perikarya and dendrites, where immunogold particles were commonly associated with the endoplasmic reticulum and other intracellular organelles (Figs 2A–C and 7). A less intense labelling of perikarya and dendrites was observed for $GABA_{B2}$ (Fig. 2E and G). In dendrites, almost three-quarters of $GABA_{B1}$ labelling was associated with intracellular sites (Figs 2A and C, and 3). In contrast, the proportion of intracellular labelling for $GABA_{B2}$ was considerably lower than for $GABA_{B1}$, with the majority of gold particles associated with the plasma membrane (Figs 2G and 3). For both $GABA_{B1}$ and $GABA_{B2}$, the vast majority of membrane-associated gold particles were extrasynaptic (Figs 2B, E and G, and 4). In addition, about one-tenth of membrane-associated gold particles for $GABA_{B1}$ and $GABA_{B2}$ were found at symmetric synapses formed by putative GABAergic terminals (Figs 2B–D and G, and 4). Postynaptic labelling for either subunit was not found in the main body of asymmetric synapses, although gold particles were commonly observed at the edge of the postsynaptic membrane specialization (Figs 2G and 4).

In presynaptic elements, immunogold labelling for $GABA_{B1}$ and $GABA_{B2}$ was found in preterminal portions of axons as well as in axon terminals forming both symmetric and asymmetric synapses (Fig. 2A and C–F). Labelled terminals forming symmetric, putative

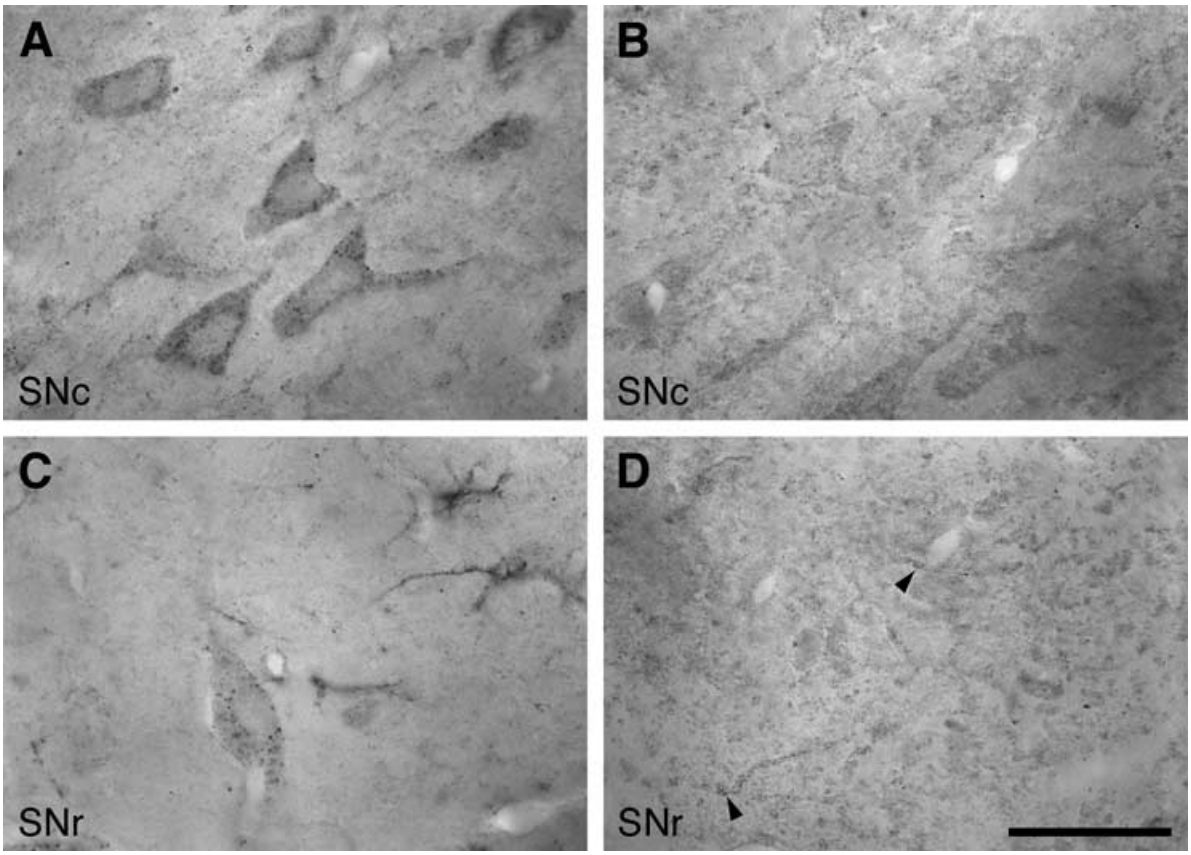

FIG. 1. Distribution of immunolabelling for $GABA_{B1}$ (A and C) and $GABA_{B2}$ (B and D) in the substantia nigra. (A and B) Strong $GABA_{B1}$ labelling is present in numerous large perikarya and proximal dendrites in the SNc, whereas labelling for $GABA_{B2}$ is weaker. (C and D) Similarly, in the SNr, $GABA_{B1}$ is stronger in perikarya, whereas $GABA_{B2}$ is predominantly in the neuropil (arrowheads). Scale bar, 25 μm.

GABAergic synapses were more numerous, as GABAergic afferents have been estimated to comprise between 60% and 90% of inputs to the SN (Ribak *et al*., 1976; Nitsch & Riesenberg, 1988; Bolam & Smith, 1990). The majority of these had the morphological features of terminals derived from the striatum, i.e. they were medium sized, rarely contained mitochondria and were often interdigitated with other structures (Fig. 2D and E) (Smith & Bolam, 1991). A second, less common type of labelled terminal forming symmetric synapses was considerably larger, usually contained several mitochondria and often formed more than one active zone, features consistent with those of terminals derived from the GP (Smith & Bolam, 1990). $GABA_{B1}$- and $GABA_{B2}$-labelled terminal boutons forming asymmetric synapses were of varying size, contained numerous synaptic vesicles and often mitochondria (Fig. 2A, C, D and F), and the postsynaptic density was sometimes associated with subjunctional dense bodies (Fig. 2F).

Immunogold labelling in presynaptic elements occurred intracellularly (Figs 2C and D, and 3) and on the plasma membrane (Figs 2A and C–F, and 3). For both $GABA_{B1}$ and $GABA_{B2}$, the majority of gold particles associated with the membrane were extrasynaptic (Fig. 4). Of the remainder, a substantial proportion were localized at the presynaptic specialization at symmetric synapses (Figs 2C–E and 4). Labelling within, and at the edge of, the presynaptic specialization at asymmetric synapses was also prominent (Figs 2C, D and F, and 4). The postsynaptic targets of labelled terminals were usually dendritic shafts, many of which were also labelled for the respective $GABA_B$ subunit (Fig. 2A and C–E). Occasionally, gold particles were localized on both the pre- and postsynaptic membranes at individual symmetric synapses (Fig. 2C and D).

Further analysis of $GABA_{B2}$ immunogold labelling in axon terminals revealed that approximately half the terminals forming either symmetric or asymmetric synapses in the SNc were immunopositive for $GABA_{B2}$ (Table 1). The distribution of membrane-associated gold particles within labelled boutons revealed an enrichment of $GABA_{B2}$ at neurotransmitter release sites in both symmetric and, to a greater extent, asymmetric synapses (Table 1).

Subcellular localization of $GABA_{B1}$ and $GABA_{B2}$ in the substantia nigra pars reticulata

Overall, the patterns of immunogold labelling for $GABA_{B1}$ and $GABA_{B2}$ in the SNr were similar to those observed in the SNc (Fig. 5). Labelling for both $GABA_{B1}$ and $GABA_{B2}$ was present in perikarya and dendritic processes (Fig. 5), likely to represent both GABAergic SNr neurons and dopaminergic SNr neurons, as well as the dendrites of dopaminergic SNc neurons that extend ventrally into the SNr. For the quantitative analysis of immunolabelling in the SNr, no distinction was made between dopaminergic and nondopaminergic postsynaptic elements. Quantitative analysis of immunogold labelling for $GABA_{B1}$ and $GABA_{B2}$ in dendritic processes in the SNr revealed a similar distribution to that in the SNc (Fig. 3). For $GABA_{B1}$, about two-thirds of immunogold particles were associated with intracellular sites (Figs 3 and 5A and C), whereas with the majority of labelling for $GABA_{B2}$ was associated with the plasma membrane (Figs 3 and 5E and F). For both $GABA_{B1}$ and $GABA_{B2}$, a high proportion of the membrane-associated gold particles were localized at the postsynaptic membrane of symmetric synapses (Figs 4 and 5F–H). Gold particles labelling for $GABA_{B1}$ and $GABA_{B2}$ were often observed at the edge of

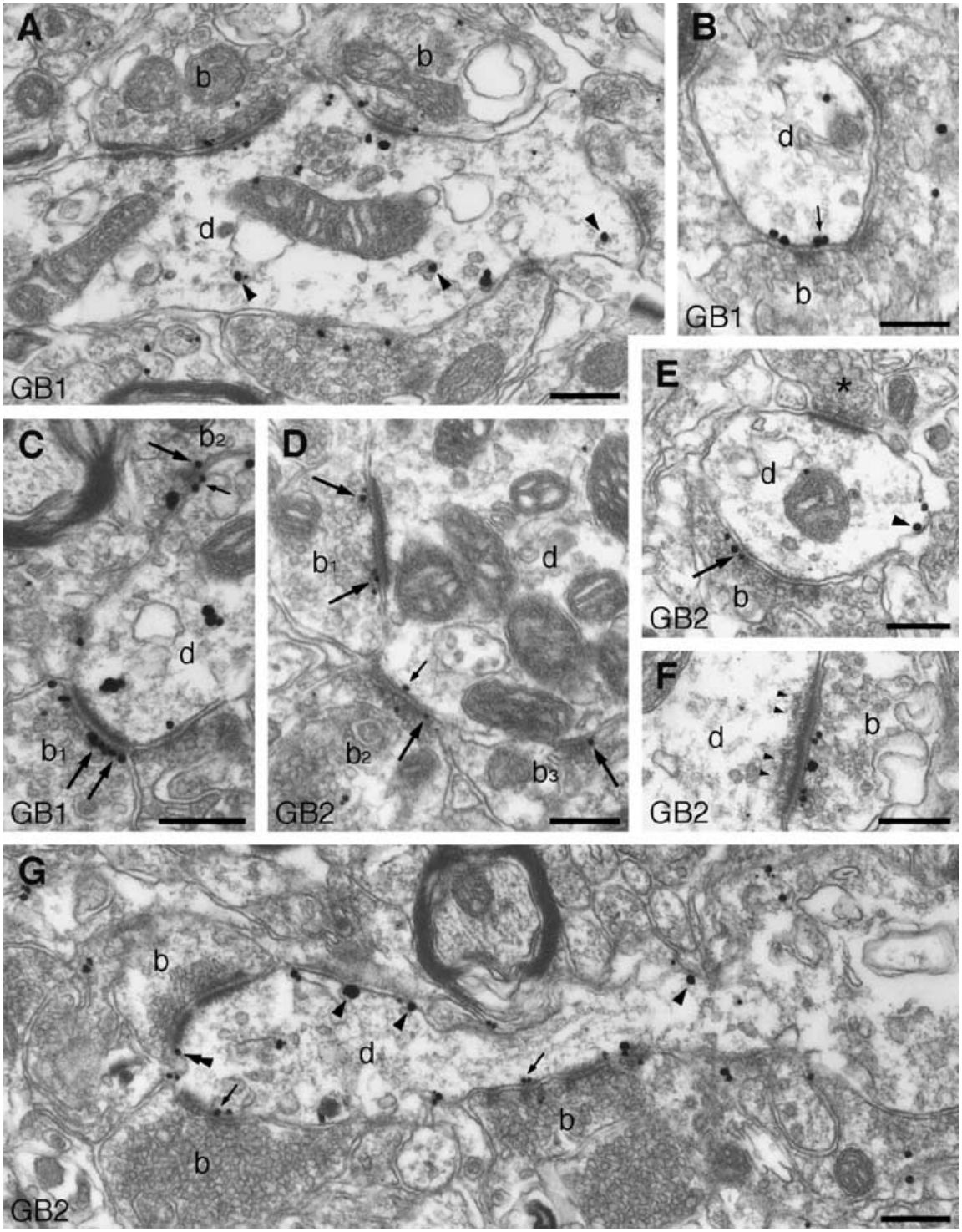

FIG. 2. Localization of immunogold labelling for $GABA_{B1}$ (GB1; A–C) and $GABA_{B2}$ (GB2; D–G) in the substantia nigra *pars compacta*. (A) Labelling for $GABA_{B1}$ in two boutons (b) forming asymmetric synapses with a $GABA_{B1}$-labelled dendrite (d). Immunogold particles in the dendrite are localized within the cytoplasm (arrowheads). An additional bouton on the opposite side of the dendrite is also labelled. (B) Labelling for $GABA_{B1}$ on the postsynaptic membrane at a symmetric synapse (small arrow). (C) Labelling for $GABA_{B1}$ on the presynaptic membrane of a bouton forming an asymmetric synapse (large arrows in b_1). A second bouton (b_2), making symmetric synaptic contact with the same dendrite (d), is labelled on both the presynaptic and postsynaptic membranes (large and small arrow, respectively). (D) Presynaptic labelling for $GABA_{B2}$ in one bouton forming an asymmetric synapse (b_1, large arrows) and two boutons forming symmetric synapses (b_2 and b_3, large arrows) with the same dendrite (d). The synapse formed by bouton b_2 is also labelled on the postsynaptic membrane (small arrow). (E) Labelling for $GABA_{B2}$ on the presynaptic membrane (large arrow) in a bouton (b) making symmetric synaptic contact with a $GABA_{B2}$-labelled dendrite (d). Gold particles in the dendrite are localized extrasynaptically (arrowhead). A bouton forming an asymmetric synapse with the same dendrite (*) is immunonegative. (F) Gold particles labelling for $GABA_{B2}$ concentrated at the presynaptic specialization of an asymmetric synapse. The small arrowheads indicate subjunctional dense bodies. (G) Postsynaptic labelling for $GABA_{B2}$ associated with symmetric synaptic (small arrows) and extrasynaptic sites (arrowheads) on a dendrite (d). The double arrowhead indicates labelling at the edge of an asymmetric synapse. Abbreviations: b, bouton; d, dendrite. Scale bars, 0.25 μm.

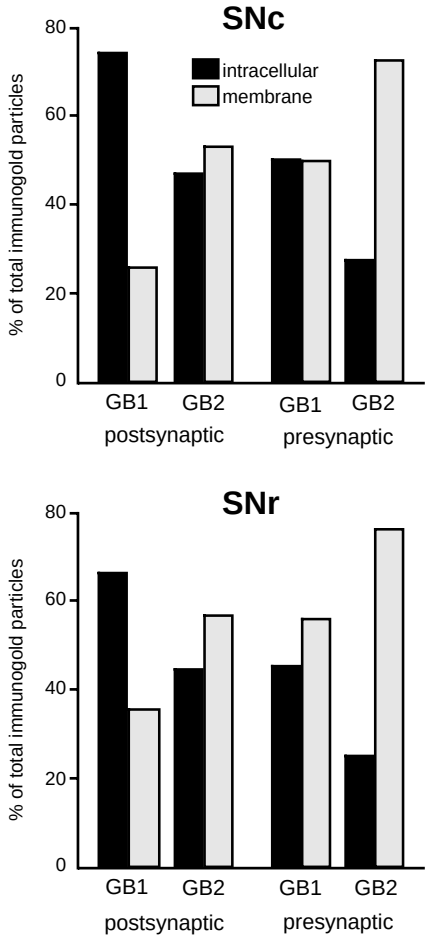

FIG. 3. Proportion of intracellular versus plasma membrane-associated immunogold particles for $GABA_{B1}$ and $GABA_{B2}$ in the SNc and SNr. The analysis was carried out on tissue from three animals for $GABA_{B1}$ and four animals for $GABA_{B2}$. The total number of gold particles counted in each region were: SNc, 1007 for $GABA_{B1}$ and 1149 for $GABA_{B2}$; SNr, 770 for $GABA_{B1}$ and 1636 for $GABA_{B2}$. For each region, data are divided into gold particles located in postsynaptic structures (dendrites) and those located in presynaptic structures (preterminal axons and axon terminals).

the postsynaptic specialization at asymmetric synapses (Figs 4 and 5C and E), but never in the main body of the synapse.

Strong immunolabelling, particularly for $GABA_{B2}$, was observed in many axons and axon terminals in the SNr (Fig. 5). Labelled terminals formed both symmetric and asymmetric synapses, primarily with dendritic shafts. $GABA_{B1}$- and $GABA_{B2}$-labelled terminals forming symmetric synapses were commonly seen surrounding dendritic shafts in a rosette-like arrangement (Fig. 5D), characteristic of the pattern of dendritic innervation by striatal terminals in the SNr (Grofova & Rinvik, 1970; Smith & Bolam, 1991). Immunogold particles associated with the axonal membrane accounted for over half of the total gold labelling for $GABA_{B1}$ (Figs 3 and 5A–C) and more than three-quarters for $GABA_{B2}$ (Figs 3 and 5D–H). However, in common with the SNc, the proportion of membrane labelling associated with symmetric and asymmetric synapses was greater for $GABA_{B2}$ than $GABA_{B1}$ (Fig. 4); $GABA_{B2}$-immunolabelled synapses frequently possessed two or more gold particles in the active zone (Fig. 5E–H). The postsynaptic targets of $GABA_{B1}$ and $GABA_{B2}$-labelled terminals in the SNr included both large and small-diameter dendritic shafts, many of which were also labelled (Fig. 5A, B and D–H).

$GABA_{B2}$-immunopositive terminals accounted for about half the terminals forming either symmetric or asymmetric synapses in the SNr (Table 1). Membrane-associated gold particles in terminals forming asymmetric synapses were concentrated at the synapse, whereas those in terminals forming symmetric synapses were distributed equally between synaptic and extrasynaptic sites on the membrane (Table 1).

$GABA_B$ receptors on striatonigral and pallidonigral afferent terminals

To investigate the origin of $GABA_B$-immunolabelled terminals forming symmetric synapses in the SN, injections of WGA–HRP ($n = 7$) or PHA-L ($n = 5$) were made in the striatum or GP. For both tracers, striatal and pallidal injections resulted in the anterograde labelling of dense terminal fields in the SNr and more discreet labelling in the SNc. In addition, striatal injections of WGA–HRP gave rise to retrograde labelling of neurons in the SNc and the SNr (see below), and in other regions that project to the striatum. Because of the possibility of the labelling of axon collaterals of retrogradely labelled neurons, only the results from the PHA-L experiments will be discussed here. Consistent with previous observations (Smith & Bolam, 1990, 1991), striatal injections of PHA-L gave rise to many labelled boutons of medium-size that formed symmetric synapses predominantly with small dendritic shafts (Fig. 6A and B), whereas pallidal injections resulted in the labelling of a population of large boutons, which formed symmetric synapses mainly with perikarya and large-diameter proximal dendritic shafts (Fig. 6C), frequently with two or more labelled boutons surrounding a single dendrite (Fig. 6D). Double immunolabelling revealed that the anterogradely labelled boutons derived from both the striatum and the GP displayed immunogold labelling for $GABA_{B2}$ on the presynaptic membrane and at extrasynaptic sites (Fig. 6A and C). Striatonigral and pallidonigral terminals formed symmetric synapses with both $GABA_{B2}$-labelled and unlabelled neurons in the SNr. At synapses between PHA-L-labelled striatal or pallidal boutons and $GABA_{B2}$-immunolabelled dendrites, immunogold labelling was often observed at the postsynaptic membrane specialization (Fig. 6B and D) as well as at extrasynaptic sites.

$GABA_B$ receptors on glutamatergic terminals

Immunolabelling for VGLUT2 was widespread in the SNc and SNr, with numerous VGLUT2-positive puncta throughout the neuropil. At the electron microscope level, VGLUT2-labelled axon terminals formed asymmetric synapses, mainly with dendritic shafts (Fig. 7). The double labelling revealed that VGLUT2-labelled terminals were often also immunolabelled for either $GABA_{B1}$ or $GABA_{B2}$. Immunogold particles for $GABA_{B1}$ in VGLUT2-labelled terminals were localized intracellularly, as well as at extracellular sites on the membrane and, less frequently, at the presynaptic membrane specialization (Fig. 7A and B). For $GABA_{B2}$, gold particles were more commonly associated with the membrane and were frequently localized at, or close to, the presynaptic specialization (Fig. 7C and E). In dendritic shafts, gold labelling for both $GABA_{B1}$ and $GABA_{B2}$ was sometimes located at the edge of the postsynaptic specialization at VGLUT2-positive synapses (Fig. 7D and F). In addition, presynaptic and postsynaptic labelling was observed at asymmetric synapses formed by VGLUT2-immunonegative terminals in both the SNc and SNr (Fig. 7B).

$GABA_B$ receptors on nigrostriatal neurons

As indicated above, injections of WGA–HRP into the striatum resulted in numerous large, retrogradely labelled cell bodies in the SNc and, to a much lesser extent, in the SNr. At the electron microscope level, crystals of the TMB reaction product, revealing the WGA–HRP, were

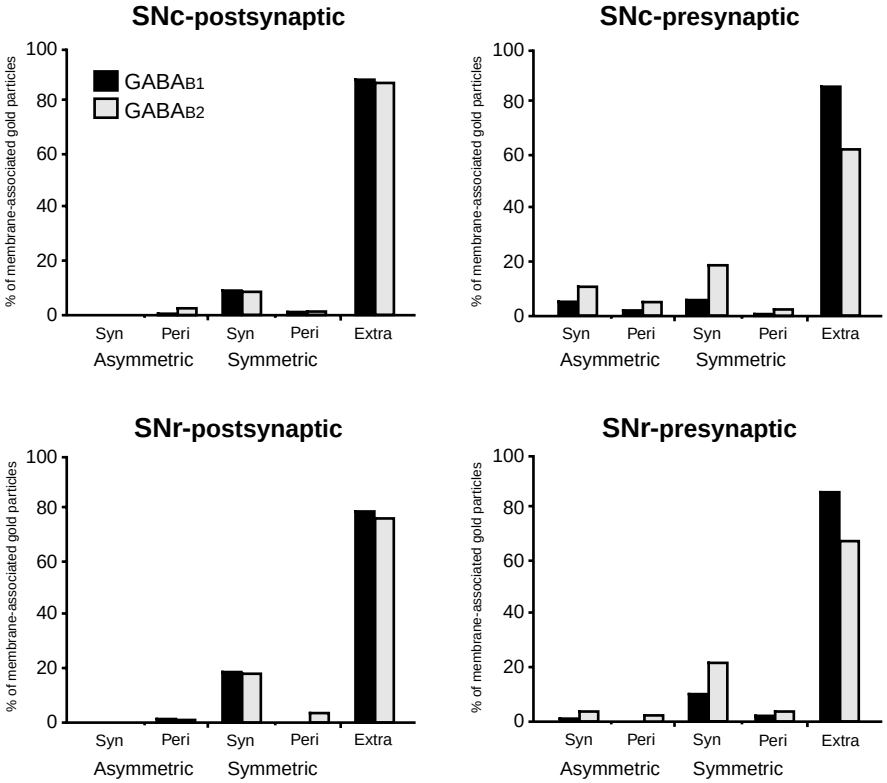

FIG. 4. Subcellular distribution of plasma membrane-associated immunogold particles for $GABA_{B1}$ and $GABA_{B2}$ in the SNc and SNr. Each gold particle was categorized as being synaptic (Syn) or perisynaptic (Peri) in relation to asymmetric and symmetric synapses, or at extrasynaptic (Extra) sites on the membrane. For each region, data are divided into gold particles located in postsynaptic structures (dendrites) and those located in presynaptic structures (preterminal axons and axon terminals).

TABLE 1. Immunogold labelling for $GABA_{B2}$ in terminals forming asymmetric and symmetric synapses in the substantia nigra

	n	Boutons labelled for $GABA_{B2}$ (%)*	Membrane labelling (%)†	
			Synaptic	Extrasynaptic
SNc				
Asym	55	54.5	73.5	26.5
Sym	97	54.6	59.6	40.4
SNr				
Asym	40	55.0	63.2	36.8
Sym	202	48.5	49.4	50.6

*The proportion of terminals forming asymmetric (Asym) and symmetric (Sym) synapses that were immunolabelled for $GABA_{B2}$.
†The subcellular distribution of immunogold particles associated with the membrane in immunolabelled terminals, in the SNc and SNr. Data were obtained from four animals for each region (*n* = total number of asymmetric or symmetric synapses).

visible within the cytoplasm of retrogradely labelled perikarya and dendrites in both the SNc and the SNr (Fig. 8). Many retrogradely labelled perikarya and dendrites in the SNc and SNr were also labelled for either $GABA_{B1}$ or $GABA_{B2}$ (Fig. 8, inset), indicating that $GABA_B$ receptors are expressed by nigrostriatal neurons. In agreement with our single immunolabelling data, labelling for $GABA_{B1}$ in retrogradely labelled neurons was stronger than for $GABA_{B2}$. In addition, immunolabelling for $GABA_{B1}$ and $GABA_{B2}$ was observed in many perikarya and dendrites in the SNc and SNr that were not retrogradely labelled with HRP-WGA.

Discussion

The findings of the present study demonstrate that $GABA_B$ receptor subunits are widely distributed at both presynaptic and postsynaptic sites in the SNc and SNr. In both regions, labelling for $GABA_{B1}$ and $GABA_{B2}$ in dendrites was associated with intracellular sites and with the membrane, predominantly at extrasynaptic sites, but also on the postsynaptic membrane at symmetric, putative GABAergic synapses. In addition, both $GABA_{B1}$ and $GABA_{B2}$ were located in preterminal axons and on the presynaptic membrane in axon terminals forming symmetric and asymmetric synapses, some of which were identified as glutamatergic by double immunolabelling for VGLUT2. Anterograde labelling from the striatum or the GP revealed that both striatonigral and pallidonigral terminals express presynaptic $GABA_B$ receptors. These findings provide an anatomical substrate for observed effects of $GABA_B$ receptor activation on GABAergic and glutamatergic neurotransmission in the SN.

Localization of $GABA_{B1}$ and $GABA_{B2}$ in the substantia nigra

The similar subcellular localization of the two $GABA_B$ receptor subunits in presynaptic and postsynaptic neuronal elements in the SN provides support for the view that $GABA_B$ receptors are heterodimers of $GABA_{B1}$ and $GABA_{B2}$ (Jones *et al*., 1998; Kaupmann *et al*., 1998; White *et al*., 1998; Kuner *et al*., 1999). However, despite the largely overlapping distributions of $GABA_{B1}$ and $GABA_{B2}$, some differences were evident. For example, intracellular labelling in SN neurons accounted for more than two-thirds of the labelling for $GABA_{B1}$, compared with less than half of that for $GABA_{B2}$. This difference may relate to the dynamic nature of the interaction between

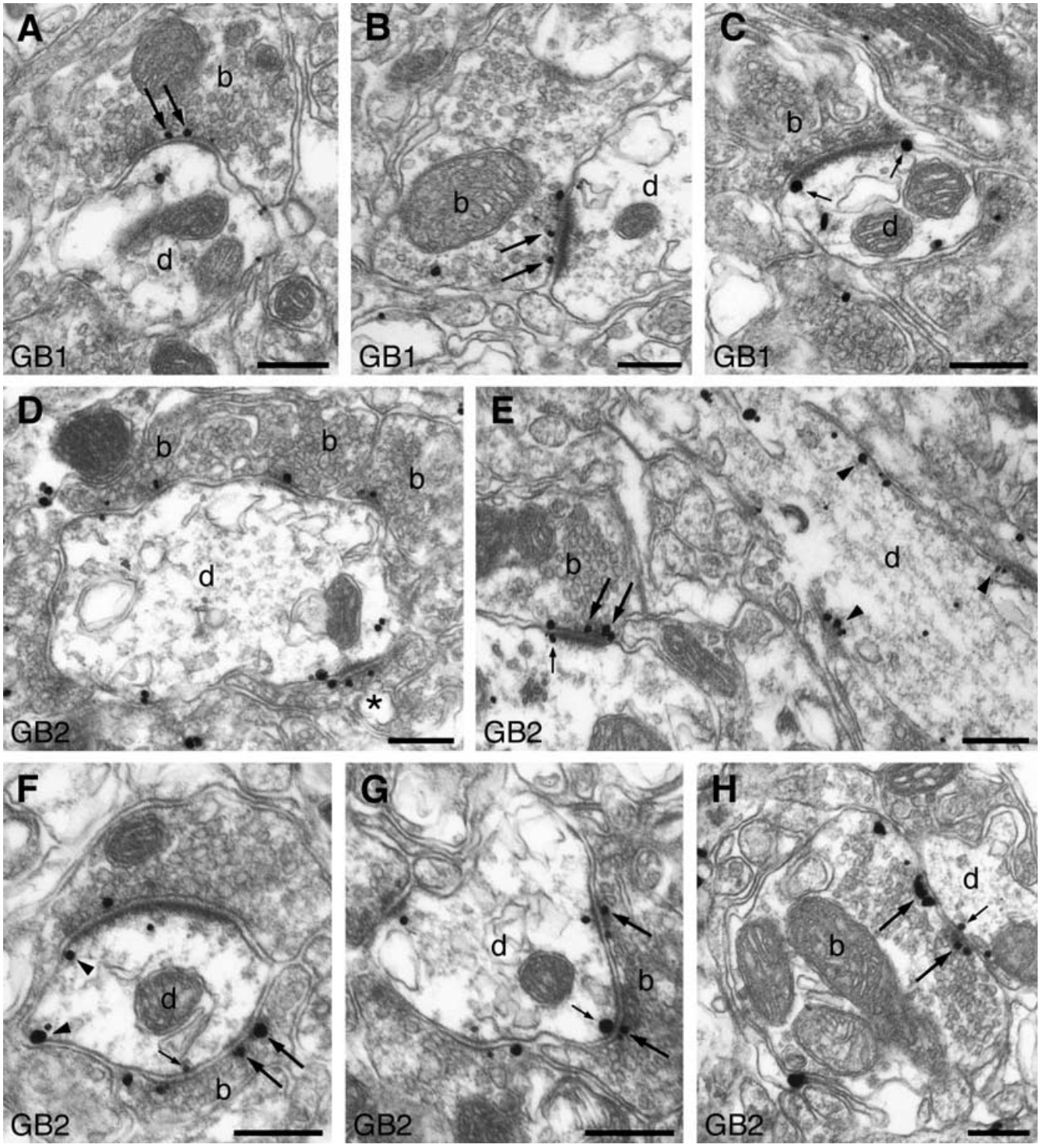

FIG. 5. Localization of immunogold labelling for $GABA_{B1}$ (GB1; A–C) and $GABA_{B2}$ (GB2; D–H) in the substantia nigra pars reticulata. (A and B) Labelling for $GABA_{B1}$ (large arrows) on the presynaptic membrane at symmetric (A) and asymmetric (B) synapses. Note that the dendrites are also labelled. (C) Postsynaptic labelling for $GABA_{B1}$ at the edges of an asymmetric synapse (small arrows). (D) A dendrite (d) apposed by several striatal-like boutons (b) that form symmetrical synapses and are labelled for $GABA_{B2}$ on the presynaptic membrane. Another bouton (*) in synaptic contact with the same dendrite is also labelled. (E) Labelling for $GABA_{B2}$ on the presynaptic membrane (large arrows) and at the edge of the postsynaptic density (small arrow) at an asymmetric axodendritic synapse. Extrasynaptic labelling (arrowheads) is present along the membrane of a second dendrite (d). (F) Labelling for $GABA_{B2}$ on the presynaptic membrane (large arrows) at a symmetric axodendritic synapse. Gold particles are also located at postsynaptic sites on the dendritic membrane, both at the synapse (small arrow) and at extrasynaptic sites (arrowheads). (G) Labelling for $GABA_{B2}$ associated with the presynaptic and postsynaptic specializations at the same symmetric synapse (large and small arrows, respectively). (H) Presynaptic $GABA_{B2}$ labelling in a GP-like bouton (large arrows). The postsynaptic dendritic membrane is also labelled (small arrow). Scale bars, 0.25 μm.

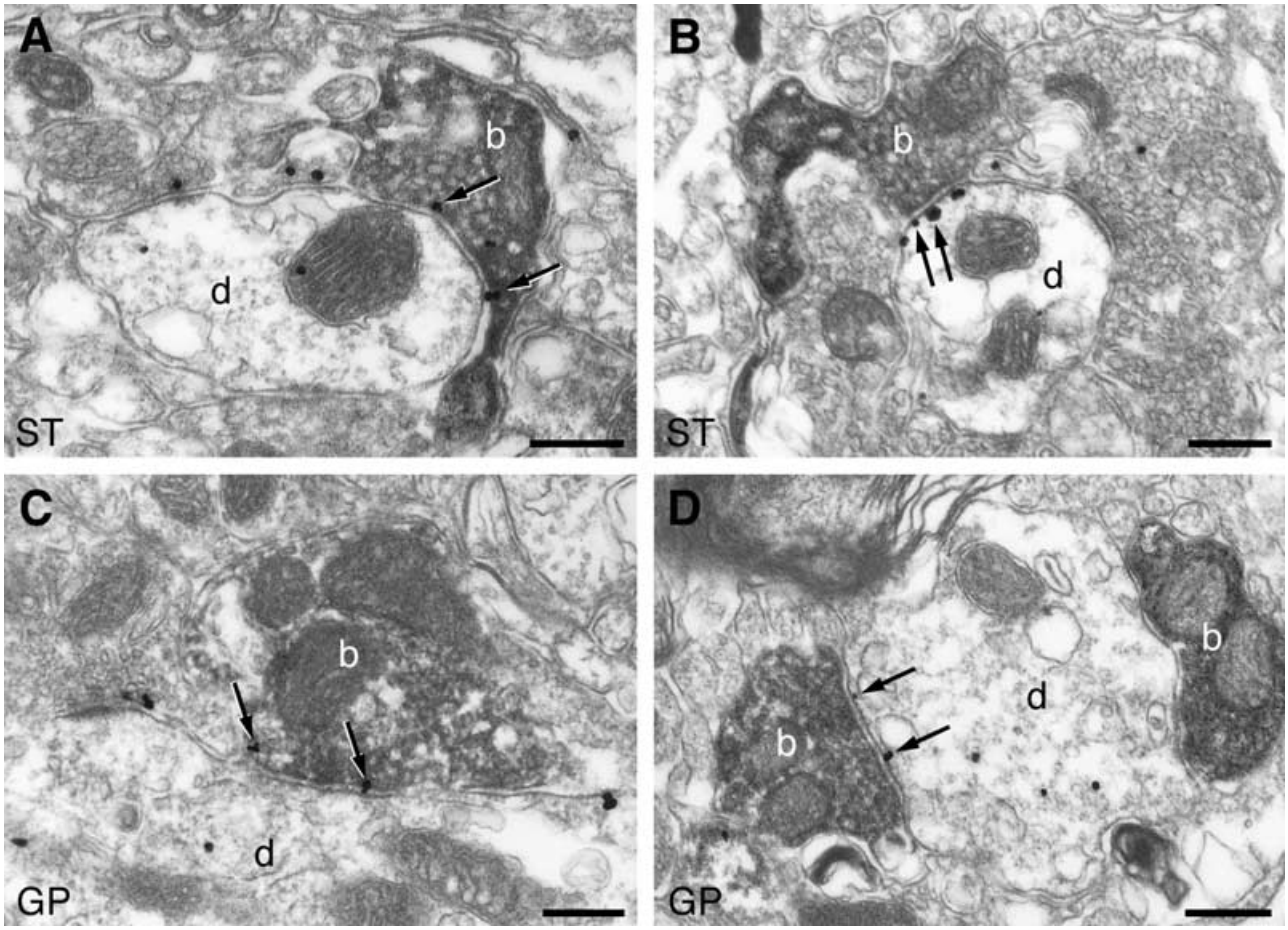

FIG. 6. $GABA_{B2}$ immunogold labelling at synapses in the substantia nigra *pars reticulata* formed by terminals anterogradely labelled from the striatum (ST; A and B) and globus pallidus (GP; C and D). The anterogradely transported PHA-L was revealed by the immunoperoxidase method. (A) Presynaptic labelling for $GABA_{B2}$ in an anterogradely labelled striatal bouton (b). Gold particles are associated with the membrane (arrows) close to the synaptic specialization. (B) Labelling for $GABA_{B2}$ on the postsynaptic membrane (arrows; d, dendrite) at a symmetric synapse formed by a striatal bouton (b). (C) Labelling for $GABA_{B2}$ on the presynaptic membrane (arrows) in a large anterogradely labelled pallidal bouton (b) making symmetric synaptic contact with a proximal dendrite (d). (D) Two pallidal boutons (b) making synaptic contact with the same dendrite (d), which is labelled for $GABA_{B2}$. Gold particles are associated with the postsynaptic membrane at the symmetric synapse formed by one of the boutons (arrows). Scale bars, 0.25 μm.

the two subunits. It has been shown that cell-surface expression of $GABA_{B1}$ requires that the subunit heterodimerizes with $GABA_{B2}$ (White *et al*., 1998) and that when expressed alone, $GABA_{B1}$ is retained on the endoplasmic reticulum (Couve *et al*., 1998). Although $GABA_{B2}$ is transported to the cell surface independently of $GABA_{B1}$ *in vitro* (Martin *et al*., 1999), given the marked reduction in the expression of $GABA_{B2}$ protein observed in $GABA_{B1}$ knockout mice (Prosser *et al*., 2001; Schuler *et al*., 2001), it seems likely that *in vivo*, $GABA_{B1}$ increases stable $GABA_{B2}$ expression and that the majority of $GABA_{B2}$ exists as a heterodimer with $GABA_{B1}$. Thus, the intracellular labelling probably reflects both $GABA_{B1/2}$ heterodimers trafficking to the membrane and $GABA_{B1}$ monomers awaiting heterodimerization.

The most striking disparity between the subcellular distributions of the two subunits was the comparatively higher proportion of presynaptic immunolabelling for $GABA_{B2}$ associated with synaptic specializations at symmetric and asymmetric synapses in both the SNc and SNr. Given that neither subunit forms homodimers and that the $GABA_{B2}$ subunit is incapable of ligand binding (Jones *et al*., 1998; Kaupmann *et al*., 1998; White *et al*., 1998), these results suggest that nonfunctional receptors may be present at synapses in the SN. Alternatively, this mismatch between the two subunits may indicate the existence of other, as yet unidentified, $GABA_B$ subunits. However, such a finding must be interpreted with caution, as the properties of the two antibodies may differ and it is unlikely that every antigenic site will be labelled by the pre-embedding method. Finally, it is interesting to note that immunolabelling for $GABA_{B1}$, but not $GABA_{B2}$, was observed in glial cells in the SN. A similar mismatch has been described for $GABA_{B1}$ and $GABA_{B2}$ mRNAs in glial cells throughout the brain (Clark *et al*., 2000). However, the functional significance of this finding has yet to be determined.

$GABA_B$ autoreceptors on striatonigral and pallidonigral afferents

Our finding of both $GABA_{B1}$ and $GABA_{B2}$ labelling in axon terminals forming symmetric synapses strongly suggests the existence of functional $GABA_B$ receptors on GABAergic nigral afferents. This further implies that GABA can regulate its own release via presynaptic autoreceptors in the SN. In support of this hypothesis, electrophysiological studies have demonstrated that the $GABA_B$ receptor agonist, baclofen, modulates the release of GABA in the SNr and SNc (Floran *et al*., 1988; Giralt *et al*., 1990) and significantly reduces $GABA_A$ inhibitory postsynaptic currents in both SNc and SNr neurons *in vitro*,

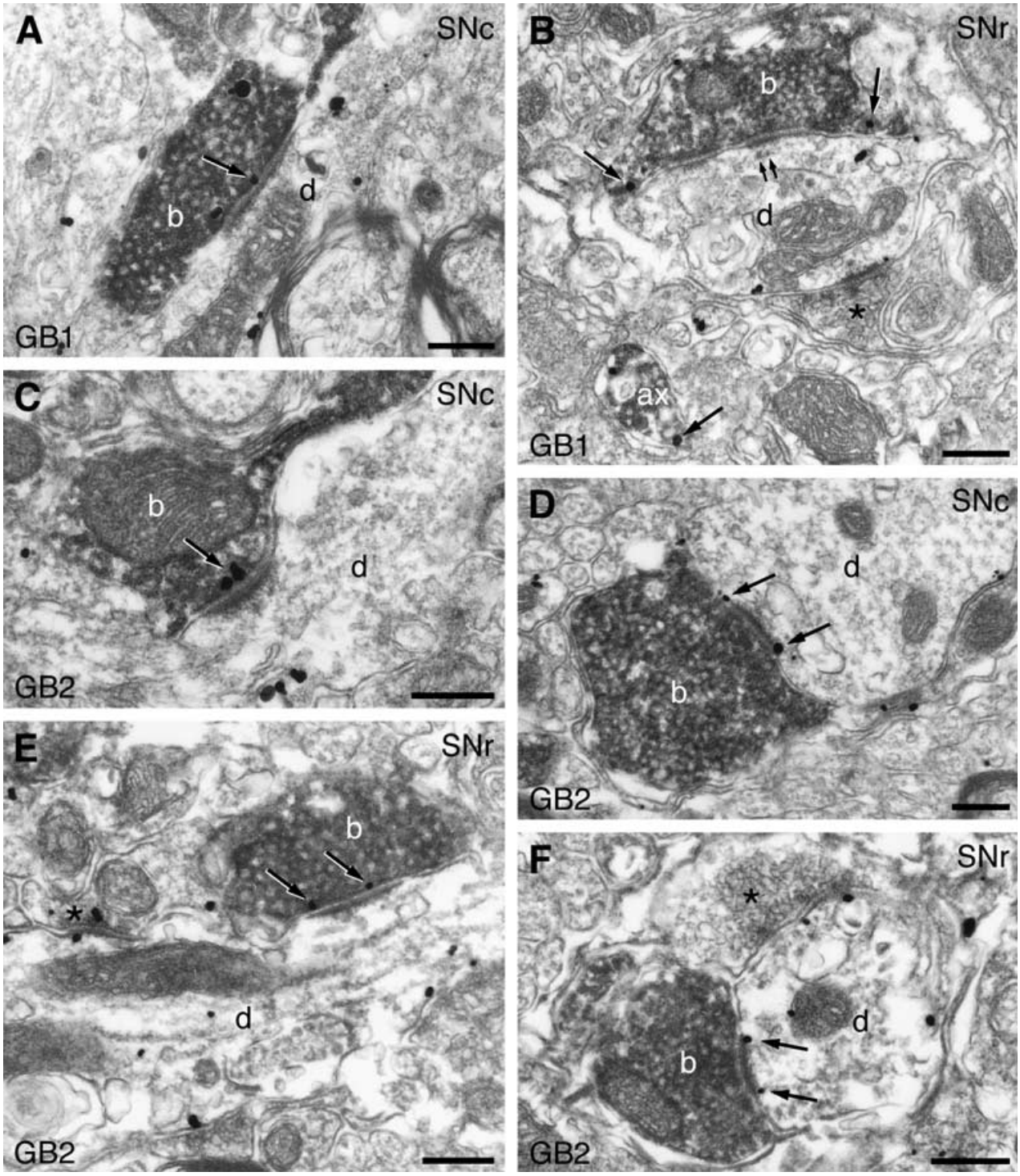

FIG. 7. Immunogold labelling for $GABA_{B1}$ (GB1; A,B) and $GABA_{B2}$ (GB2; C–F) relative to VGLUT2-labelled synapses (immunoperoxidase) in the SNc and SNr. (A) Presynaptic $GABA_{B1}$ labelling (arrow) in a VGLUT2-labelled bouton (b) in the SNc. (B) Labelling for $GABA_{B1}$ (large arrows) in the preterminal portion of an axon (ax) and a bouton (b) in the SNr, both of which are also labelled for VGLUT2. The postsynaptic density is not prominent in this plane of section, but subjunctional dense bodies are visible (small arrows). An unlabelled bouton (*) is also forming an asymmetric synapse with the dendrite. (C and D) Labelling for $GABA_{B2}$ at the presynaptic specialization (arrow in C) and at the edges of the postsynaptic specialization (arrows in D) of asymmetric synapses formed by VGLUT2-labelled boutons (b) in the SNc. (E) Labelling for $GABA_{B2}$ (arrows) at the presynaptic specialization of a VGLUT2-labelled asymmetric synapse in the SNr. An adjacent symmetric synapse is also labelled for $GABA_{B2}$ at both pre- and postsynaptic sites. (F) Postsynaptic labelling for $GABA_{B2}$ (arrows) at the edges of a VGLUT2-labelled asymmetric synapse in the SNr. The symmetric synapse formed by an adjacent unlabelled bouton (*) is labelled for $GABA_{B2}$ on the postsynaptic membrane. Scale bars, 0.25 μm.

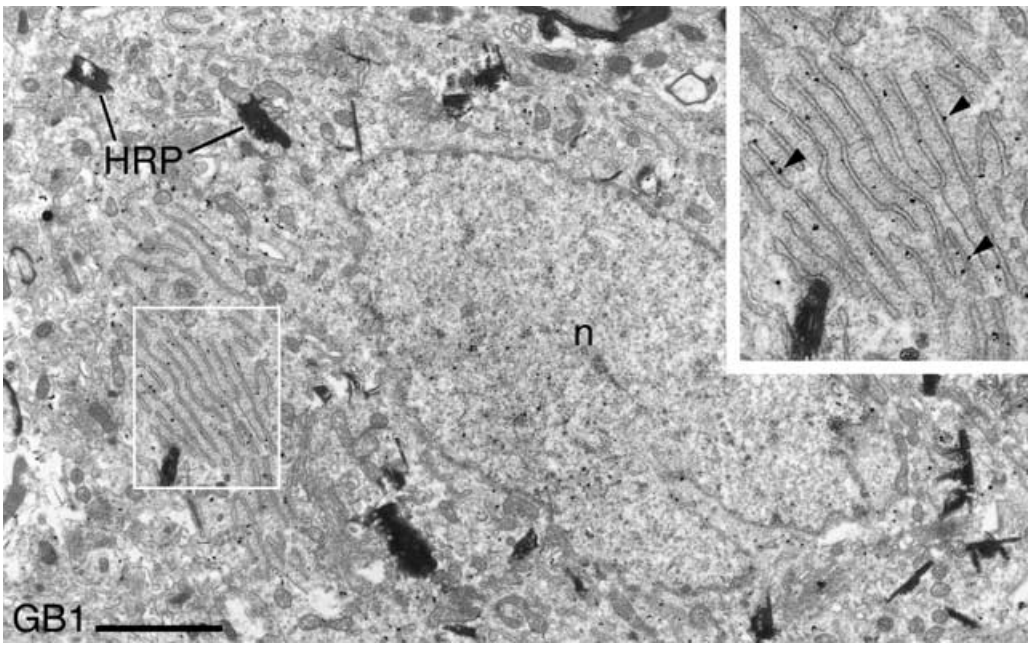

FIG. 8. A low power electron micrograph of a retrogradely labelled nigrostriatal neuron in the SNc after injection of WGA–HRP into the striatum. Electron-dense crystals of the TMB reaction product (HRP) are visible in the cytoplasm of the perikaryon (n, nucleus). The neuron is also labelled for $GABA_{B1}$, by the immunogold method, and numerous gold particles are visible at intracellular sites throughout the cytoplasm, including the surface of the endoplasmic reticulum (inset, arrowheads). Scale bar, 2 μm.

by a presynaptic mechanism involving inhibition of Ca^{2+} channels (Hausser & Yung, 1994; Shen & Johnson, 1997; Chan *et al*., 1998). Similarly, local application of $GABA_B$ antagonists *in vivo* regulates the firing pattern of dopaminergic SNc neurons by blocking presynaptic $GABA_B$ receptors on GABAergic afferents (Paladini & Tepper, 1999).

The majority of GABAergic afferents to the SN originate in the striatum and the GP (Ribak *et al*., 1980; Nitsch & Riesenberg, 1988). Our results demonstrate that presynaptic $GABA_B$ receptors are present on both striatonigral and pallidonigral afferents. The fact that inhibitory responses to single pulse stimulation of the striatum or the GP are not attenuated by $GABA_B$ antagonists (Paladini *et al*., 1999) suggests that these receptors are only activated under certain conditions. In support of this hypothesis, activation of presynaptic $GABA_B$ autoreceptors in the VTA has been linked with increased GABA levels following ethanol exposure (Melis *et al*., 2002). Thus, presynaptic autoreceptors on striatonigral and pallidonigral terminals probably represent a negative feedback mechanism (Davies *et al*., 1990), preventing overinhibition of postsynaptic targets in the SN. Although not addressed in the present study, $GABA_B$ autoreceptors may also be located on the terminals of the local axon collaterals of GABAergic SNr neurons (Deniau *et al*., 1982; Grofova *et al*., 1982), which form symmetric synaptic contacts with neurons in the SN (Damlama *et al*., 1993). However, electrophysiological data indicate that inhibition of SNc and SNr neurons arising from the SNr collaterals is mediated primarily, if not exclusively, by $GABA_A$ receptors (Hausser & Yung, 1994; Rick & Lacey, 1994; Tepper *et al*., 1995).

Although immunoperoxidase data from monkeys has suggested that $GABA_B$ autoreceptors in the SN are expressed in the preterminal segment of axons rather than in the terminals themselves (Charara *et al*., 2000), our results reveal that $GABA_{B1}$ and $GABA_{B2}$ are localized at the presynaptic membrane specialization of symmetric synapses, as well as at extrasynaptic sites. Interestingly, a small proportion of symmetric synapses in the present study were labelled for $GABA_B$ receptor subunits at both the presynaptic and postsynaptic specialization. Although several studies have suggested that presynaptic and postsynaptic $GABA_B$ receptors may differ in terms of their pharmacological properties (Dutar & Nicoll, 1988; Deisz *et al*., 1993, 1997), evidence for the existence of pharmacological distinguishable subtypes remains inconclusive.

$GABA_B$ heteroreceptors on glutamatergic afferents

In addition to demonstrating presynaptic $GABA_B$ receptors on inhibitory afferents, our results show that $GABA_B$ receptor subunits are present on terminals forming asymmetric, putative excitatory, synapses in both the SNc and SNr. Furthermore, our double-labelling results demonstrate that some of the terminals forming asymmetric synapses that express $GABA_B$ receptor subunits also express VGLUT2, and are thus likely to be glutamatergic (Fremeau *et al*., 2001; Herzog *et al*., 2001). It is probable that most of the VGLUT2-labelled terminals in the SN are derived from glutamatergic afferents from the STN (Rinvik & Ottersen, 1993). In support of this, STN neurons express high levels of VGLUT2 mRNA (Hisano *et al*., 2000), and VGLUT2 immunolabelling is also abundant in the GP, the other basal ganglia structure known to receive STN input (Kaneko *et al*., 2002; Varoqui *et al*., 2002). The SN also receives lesser glutamatergic inputs from the pedunculopontine nucleus (Lavoie & Parent, 1994a; Charara *et al*., 1996), and possibly the prefrontal cortex (Naito & Kita, 1994) and thalamus (Sadikot *et al*., 1992), each of which may also express presynaptic $GABA_B$ receptors. Thus, activation of presynaptic $GABA_B$ heteroreceptors may inhibit transmitter release from subthalamic and other glutamatergic terminals in the SN. Indeed, baclofen acts presynaptically to reduce glutamate-mediated excitatory postsynaptic currents in the SNr *in vitro* (Shen & Johnson, 1997), and intranigral administration reverses reserpine-induced akinesia (Johnston & Duty, 2003). We also observed $GABA_B$ labelling in terminals forming asymmetric synapses in the SN that were not labelled for VGLUT2. These terminals may arise from serotonergic afferents from the raphe (Corvaja *et al*., 1993; Moukhles *et al*., 1997) and/or

cholinergic afferents (Bolam *et al*., 1991), probably from the pedunculopontine nucleus (Futami *et al*., 1995), both of which give rise to terminals forming asymmetric synapses in the SN.

The distribution of $GABA_B$ subunit labelling in glutamatergic terminals revealed an enrichment at the presynaptic membrane specialization, suggesting a close association between $GABA_B$ receptors and glutamate release sites in the SN. Presynaptic labelling for $GABA_B$ receptors in putative glutamatergic terminals has also been demonstrated in both the SNc and SNr in monkeys (Charara *et al*., 2000; Smith *et al*., 2001), and has been described elsewhere in the basal ganglia (Boyes *et al*., 2001; Smith *et al*., 2001) and in other brain regions (Gonchar *et al*., 2001; Kulik *et al*., 2002). These findings raise the question as to the source of the GABA that may activate these receptors. One possibility is that GABA is co-released with glutamate at synapses. Simultaneous glutamatergic and GABAergic transmission has been observed at mossy fibre synapses following prolonged stimulation and seizures (Gutierrez, 2000, 2002). Under these conditions, GABA co-released at mossy fibre synapses may act on presynaptic $GABA_B$ receptors on mossy fibre terminals to depress subsequent glutamate release (Vogt & Nicoll, 1999). It is tempting to speculate that such a mechanism also exists in the SN, with GABA being released from subthalamonigral terminals. However, although STN neurons actively accumulate [^{3}H]GABA (Nauta & Cuenod, 1982) and despite the fact that $GABA_A$ receptor subunits have been observed at asymmetric synapses in the SN (Fujiyama *et al*., 2002), there is no direct evidence that GABA is actually released from STN terminals. Perhaps a more plausible explanation for the existence of presynaptic $GABA_B$ receptors on excitatory terminals in the SN is that these receptors are activated by spillover of GABA from nearby GABAergic synapses. $GABA_B$-mediated heterosynaptic depression of excitatory transmission has been demonstrated in the hippocampus and cerebellum following repetitive stimulation of GABAergic fibres (Isaacson *et al*., 1993; Dittman & Regehr, 1997). Whether such a mechanism exists in the SN has yet to be determined, but it is likely to depend on a number of factors, including the local concentration of GABA (Isaacson *et al*., 1993) and the density of GABAergic terminals relative to excitatory terminals. Given that GABAergic inputs to the SNc and SNr have been estimated to outnumber excitatory inputs by more than 2 : 1 (Nitsch & Riesenberg, 1988; Bolam & Smith, 1990; Smith *et al*., 1996), the concomitant release of GABA from multiple GABAergic terminals may result in sufficient accumulation of extrasynaptic GABA to activate presynaptic $GABA_B$ heteroreceptors, thereby inhibiting the release of glutamate in the SN. Such a reduction in transmitter release may lower the level of excitation in the SN, thereby increasing the functional efficacy of inhibitory inputs from the striatum and GP.

Postsynaptic $GABA_B$ receptors in the substantia nigra

Several studies have demonstrated the postsynaptic inhibitory effects of $GABA_B$ receptor activation in the SN. For example, baclofen causes hyperpolarization of dopaminergic neurons *in vitro* in both the SNc (Pinnock, 1984; Lacey *et al*., 1988) and the SNr (Chan *et al*., 1998), through the activation of an inwardly rectifying K^+ conductance (Lacey *et al*., 1988; Kim *et al*., 1997). *In vivo*, baclofen induces a regularization of the firing pattern and a decrease in burst firing of midbrain dopaminergic neurons (Engberg *et al*., 1993; Erhardt *et al*., 2002), indicating that postsynaptic $GABA_B$ receptors on dopaminergic neurons may be involved in the control of dopamine release (see Overton & Clark, 1997). In support of this, intranigral administration of baclofen is associated with a decrease in striatal dopamine release (Santiago & Westerink, 1992; Westerink *et al*., 1994). Interestingly, in a more recent study, this effect was observed following infusion of baclofen into the SNr, and not the SNc, suggesting that presynaptic and/or postsynaptic $GABA_B$ receptors on nondopaminergic SN neurons may be involved (Balon *et al*., 2002). Consistent with these findings, and *in situ* hybridization studies (Bischoff *et al*., 1999; Lu *et al*., 1999; Liang *et al*., 2000), we observed strong immunolabelling for $GABA_{B1}$ in neurons in the SNc, including retrogradely labelled nigrostriatal neurons. Labelling of neurons in the SNr was much less intense, with the exception of a small population of strongly labelled neurons in the ventral part of the SNr. This is in agreement with previous observations (Charara *et al*., 2000; Charles *et al*., 2001), suggesting that postsynaptic $GABA_B$ receptors are more highly expressed by dopaminergic neurons than by SNr output neurons. In support of this, GABAergic SNr neurons express only a weak postsynaptic $GABA_B$ response to baclofen *in vitro* compared with putative dopaminergic neurons in the same region (Shen & Johnson, 1997; Chan *et al*., 1998). However, the presence of $GABA_B$ labelling in SNr neurons that were not retrogradely labelled from the striatum, together with data showing that both $GABA_{B1}$ and $GABA_{B2}$ colocalize with parvalbumin-immunoreactive neurons in the SNr (Ng & Yung, 2000, 2001b), indicates that GABAergic SNr output neurons do express postsynaptic $GABA_B$ receptors, albeit at a lower level than nigrostriatal neurons.

Based on electrophysiological data, it has been suggested that postsynaptic $GABA_B$ receptors do not colocalize with $GABA_A$ receptors at central synapses, but that they are located at extrasynaptic sites on neurons (Mody *et al*., 1994; Scanziani, 2000). Our results revealed that the majority of $GABA_B$ receptor labelling associated with dendritic membranes in the SN was localized extrasynaptically. However, consistent with observed $GABA_B$-mediated slow inhibitory postsynaptic potentials in SNc dopamine neurons (Hausser & Yung, 1994), a common finding was that a large proportion of the postsynaptic labelling for each subunit was localized at symmetric synapses in the SN, where $GABA_A$ receptors are primarily localized (Fujiyama *et al*., 2002). Indeed, nearly a quarter of the immunogold particles associated with dendritic membranes in the SNr were associated with the postsynaptic membrane at symmetric synapses, compared with less than half this number in the SNc, most likely reflecting the higher frequency of symmetric synaptic contacts onto dendritic shafts in the SNr (Smith *et al*., 1998). In contrast, labelling for $GABA_{B1}$ or $GABA_{B2}$ at asymmetric, glutamatergic synapses was commonly observed at the edge of the postsynaptic density, suggesting that these receptors may modulate synaptic transmission through different mechanisms than at GABAergic synapses. It should be noted that Kulik *et al*. (2002) found that the proportion of labelling for $GABA_{B1}$ in the main body of synapses in the cerebellum with the postembedding immunogold method was approximately twice that with the pre-embedding method. Thus, the figures in the present study are likely to be underestimates of the true proportions of immunolabelling at symmetric and/or asymmetric synapses. Nevertheless, it is clear that $GABA_B$ receptors are localized at both synaptic and extrasynaptic compartments on the postsynaptic membrane of SN neurons.

The existence of postsynaptic $GABA_B$ receptors close to glutamatergic synapses raises the question of their function at these synapses. One possibility is that $GABA_B$ receptors interact with glutamate receptors to modulate their function. Such an interaction has been demonstrated at excitatory parallel fibre-Purkinje cell synapses in the cerebellum, where activation of postsynaptic $GABA_B$ receptors, by both baclofen and endogenous GABA, enhanced type 1 metabotropic glutamate receptor (mGluR1)-mediated excitatory postsynaptic currents (Hirono *et al*., 2001). Interestingly, the subcellular distribution of

mGluR1a in SN neurons is very similar to that of $GABA_B$ receptors observed in the present study (Hubert *et al*., 2001), indicating that a similar interaction may also occur in the SN.

Acknowledgements

The work was funded by the Medical Research Council UK. J. B. is in receipt of a Medical Research Council studentship. We thank Drs P. J. Magill and C. Blondeau for their comments on the manuscript, Dr P. C. Emson for kindly supplying us with the $GABA_B$ antibodies used in the pilot studies, and Caroline Francis, Liz Norman, Paul Jays and Ben Micklem for technical assistance and advice.

Abbreviations

DAB, diaminobenzidine; GABA, γ-aminobutyric acid; $GABA_A$, γ-aminobutyric acid type A receptor; $GABA_B$, γ-aminobutyric acid type B receptor; $GABA_{B1}$, γ-aminobutyric acid type B receptor, subunit 1; $GABA_{B2}$, γ-aminobutyric acid type B receptor, subunit 2; GP, globus pallidus; mGluR, metabotropic glutamate receptor; NGS, normal goat serum; PB, phosphate buffer; PBS, phosphate-buffered saline; PBS-NGS, phosphate-buffered saline containing 2% normal goat serum; PHA-L, *Phaseolus vulgaris* leucoagglutinin; SN, substantia nigra; SNc, substantia nigra *pars compacta*; SNr, substantia nigra *pars reticulata*; STN, subthalamic nucleus; TMB, tetramethylbenzidine; VGLUT2, vesicular glutamate transporter 2; WGA–HRP, wheatgerm agglutinin conjugated to horseradish peroxidase.

References

Balon, N., Kriem, B., Weiss, M. & Rostain, J.C. (2002) Indirect presynaptic modulation of striatal dopamine release by $GABA_B$ receptors in the rat substantia nigra. *Neurosci. Lett.*, **325**, 33–36.

Billinton, A., Ige, A.O., Bolam, J.P., White, J.H., Marshall, F.H. & Emson, P.C. (2001) Advances in the molecular understanding of $GABA_B$ receptors. *Trends Neurosci.*, **24**, 277–282.

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

Bolam, J.P., Francis, C.M. & Henderson, Z. (1991) Cholinergic input to dopaminergic neurons in the substantia nigra: a double immunocytochemical study. *Neuroscience*, **41**, 483–494.

Bolam, J.P. & Smith, Y. (1990) The GABA and substance P input to dopaminergic neurones in the substantia nigra of the rat. *Brain Res.*, **529**, 57–78.

Bowery, N.G. (1993) $GABA_B$ receptor pharmacology. *Annu. Rev. Pharmacol. Toxicol.*, **33**, 109–147.

Boyes, J., Ige, A.O., Emson, P.C. & Bolam, J.P. (2001) The localisation of R1 and R2 subtypes of the $GABA_B$ receptor in the rat striatum. *Br. Neurosci. Assoc. Abstr.*, **16**, P101.

Boyes, J., Ige, A.O., Emson, P.C. & Bolam, J.P. (2002) The precise localisation of $GABA_B$ receptors in the substantia nigra of the rat. *FENS Abstr.*, **1**, A056.5.

Chan, P.K., Leung, C.K. & Yung, W.H. (1998) Differential expression of pre- and postsynaptic $GABA_B$ receptors in rat substantia nigra pars reticulata neurones. *Eur. J. Pharmacol.*, **349**, 187–197.

Charara, A., Heilman, T.C., Levey, A.I. & Smith, Y. (2000) Pre- and postsynaptic localization of $GABA_B$ receptors in the basal ganglia in monkeys. *Neuroscience*, **95**, 127–140.

Charara, A., Smith, Y. & Parent, A. (1996) Glutamatergic inputs from the pedunculopontine nucleus to midbrain dopaminergic neurons in primates: Phaseolus vulgaris-leucoagglutinin anterograde labeling combined with postembedding glutamate and GABA immunohistochemistry. *J. Comp. Neurol.*, **364**, 254–266.

Charles, K.J., Evans, M.L., Robbins, M.J., Calver, A.R., Leslie, R.A. & Pangalos, M.N. (2001) Comparative immunohistochemical localisation of $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ subunits in rat brain, spinal cord and dorsal root ganglion. *Neuroscience*, **106**, 447–467.

Clark, J.A., Mezey, E., Lam, A.S. & Bonner, T.I. (2000) Distribution of the $GABA_B$ receptor subunit gb2 in rat CNS. *Brain Res.*, **860**, 41–52.

Corvaja, N., Doucet, G. & Bolam, J.P. (1993) Ultrastructure and synaptic targets of the raphe-nigral projection in the rat. *Neuroscience*, **55**, 417–427.

Couve, A., Filippov, A.K., Connolly, C.N., Bettler, B., Brown, D.A. & Moss, S.J. (1998) Intracellular retention of recombinant $GABA_B$ receptors. *J. Biol. Chem.*, **273**, 26361–26367.

Damlama, M., Bolam, J.P. & Tepper, J.M. (1993) Axon collaterals of pars reticulata projection neurons contact nigral dopaminergic neurons. *Soc. Neurosci. Abstr.*, **19**, 1432.

Davies, C.H., Davies, S.N. & Collingridge, G.L. (1990) Paired-pulse depression of monosynaptic GABA-mediated inhibitory postsynaptic responses in rat hippocampus. *J. Physiol. (Lond.)*, **424**, 513–531.

Deisz, R.A., Billard, J.M. & Zieglgansberger, W. (1993) Pre- and postsynaptic $GABA_B$ receptors of rat neocortical neurons differ in their pharmacological properties. *Neurosci. Lett.*, **154**, 209–212.

Deisz, R.A., Billard, J.M. & Zieglgansberger, W. (1997) Presynaptic and postsynaptic $GABA_B$ receptors of neocortical neurons of the rat in vitro: differences in pharmacology and ionic mechanisms. *Synapse*, **25**, 62–72.

Deniau, J.M., Kitai, S.T., Donoghue, J.P. & Grofova, I. (1982) Neuronal interactions in the substantia nigra pars reticulata through axon collaterals of the projection neurons. *Exp. Brain Res.*, **47**, 105–113.

Dittman, J.S. & Regehr, W.G. (1997) Mechanism and kinetics of heterosynaptic depression at a cerebellar synapse. *J. Neurosci.*, **17**, 9048–9059.

Dutar, P. & Nicoll, R.A. (1988) Pre- and postsynaptic $GABA_B$ receptors in the hippocampus have different pharmacological properties. *Neuron*, **1**, 585–591.

Engberg, G., Kling-Petersen, T. & Nissbrandt, H. (1993) $GABA_B$-receptor activation alters the firing pattern of dopamine neurons in the rat substantia nigra. *Synapse*, **15**, 229–238.

Erhardt, S., Mathe, J.M., Chergui, K., Engberg, G. & Svensson, T.H. (2002) $GABA_B$ receptor-mediated modulation of the firing pattern of ventral tegmental area dopamine neurons *in vivo*. *Naunyn Schmiedebergs Arch. Pharmacol.*, **365**, 173–180.

Erhardt, S., Nissbrandt, H. & Engberg, G. (1999) Activation of nigral dopamine neurons by the selective $GABA_B$-receptor antagonist SCH 50911. *J. Neural Transm*, **106**, 383–394.

Floran, B., Silva, I., Nava, C. & Aceves, J. (1988) Presynaptic modulation of the release of GABA by $GABA_A$ receptors in pars compacta and by $GABA_B$ receptors in pars reticulata of the rat substantia nigra. *Eur. J. Pharmacol.*, **150**, 277–286.

Fremeau, R.T. Jr, Troyer, M.D., Pahner, I., Nygaard, G.O., Tran, C.H., Reimer, R.J., Bellocchio, E.E., Fortin, D., Storm-Mathisen, J. & Edwards, R.H. (2001) The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron*, **31**, 247–260.

Fujiyama, F., Stephenson, F.A. & Bolam, J.P. (2002) Synaptic localization of $GABA_A$ receptor subunits in the substantia nigra of the rat: effects of quinolinic acid lesions of the striatum. *Eur. J. Neurosci.*, **15**, 1961–1975.

Futami, T., Takakusaki, K. & Kitai, S.T. (1995) Glutamatergic and cholinergic inputs from the pedunculopontine tegmental nucleus to dopamine neurons in the substantia nigra pars compacta. *Neurosci. Res.*, **21**, 331–342.

Galvez, T., Duthey, B., Kniazeff, J., Blahos, J., Rovelli, G., Bettler, B., Prezeau, L. & Pin, J.P. (2001) Allosteric interactions between GB1 and GB2 subunits are required for optimal $GABA_B$ receptor function. *EMBO J.*, **20**, 2152–2159.

Giralt, M.T., Bonanno, G. & Raiteri, M. (1990) GABA terminal autoreceptors in the pars compacta and in the pars reticulata of the rat substantia nigra are $GABA_B$. *Eur. J. Pharmacol.*, **175**, 137–144.

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

Grofova, I. (1975) The identification of striatal and pallidal neurons projecting to substantia nigra. An experimental study by means of retrograde axonal transport of horseradish peroxidase. *Brain Res.*, **91**, 286–291.

Grofova, I., Deniau, J.M. & Kitai, S.T. (1982) Morphology of the substantia nigra pars reticulata projection neurons intracellularly labeled with HRP. *J. Comp. Neurol.*, **208**, 352–368.

Grofova, I. & Rinvik, E. (1970) An experimental electron microscopic study on the striatonigral projection in the cat. *Expl. Brain Res.*, **11**, 249–262.

Gutierrez, R. (2000) Seizures induce simultaneous GABAergic and glutamatergic transmission in the dentate gyrus-CA3 system. *J. Neurophysiol.*, **84**, 3088–3090.

Gutierrez, R. (2002) Activity-dependent expression of simultaneous glutamatergic and GABAergic neurotransmission from the mossy fibers in vitro. *J. Neurophysiol.*, **87**, 2562–2570.

Hausser, M.A. & Yung, W.H. (1994) Inhibitory synaptic potentials in guinea-pig substantia nigra dopamine neurones *in vitro*. *J. Physiol. (Lond.)*, **479**, 401–422.

Herzog, E., Bellenchi, G.C., Gras, C., Bernard, V., Ravassard, P., Bedet, C., Gasnier, B., Giros, B. & El Mestikawy, S. (2001) The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. *J. Neurosci.*, **21**, RC181.

Hirono, M., Yoshioka, T. & Konishi, S. (2001) $GABA_B$ receptor activation enhances mGluR-mediated responses at cerebellar excitatory synapses. *Nat. Neurosci.*, **4**, 1207–1216.

Hisano, S., Hoshi, K., Ikeda, Y., Maruyama, D., Kanemoto, M., Ichijo, H., Kojima, I., Takeda, J. & Nogami, H. (2000) Regional expression of a gene encoding a neuron-specific Na^+-dependent inorganic phosphate cotransporter (DNPI) in the rat forebrain. *Brain Res. Mol. Brain Res.*, **83**, 34–43.

Hubert, G.W., Paquet, M. & Smith, Y. (2001) Differential subcellular localization of mGluR1a and mGluR5 in the rat and monkey substantia nigra. *J. Neurosci.*, **21**, 1838–1847.

Isaacson, J.S., Solis, J.M. & Nicoll, R.A. (1993) Local and diffuse synaptic actions of GABA in the hippocampus. *Neuron*, **10**, 165–175.

Johnston, T. & Duty, S. (2003) $GABA_B$ receptor agonists reverse akinesia following intranigral or intracerebroventricular injection in the reserpine-treated rat. *Br. J. Pharamacol.*, **139**, 1480–1486.

Jones, K.A., Borowsky, B., Tamm, J.A., Craig, D.A., Durkin, M.M., Dai, M., Yao, W.J., Johnson, M., Gunwaldsen, C., Huang, L.Y., Tang, C., Shen, Q., Salon, J.A., Morse, K., Laz, T., Smith, K.E., Nagarathnam, D., Noble, S.A., Branchek, T.A. & Gerald, C. (1998) $GABA_B$ receptors function as a heteromeric assembly of the subunits $GABA_B$R1 and $GABA_B$R2. *Nature*, **396**, 674–679.

Kaneko, T., Fujiyama, F. & Hioki, H. (2002) Immunohistochemical localization of candidates for vesicular glutamate transporters in the rat brain. *J. Comp. Neurol.*, **444**, 39–62.

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

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

Kim, K.-M., Nakajima, S. & Nakajima, Y. (1997) Dopamine and GABA receptors in cultured substantia nigra neurons: correlation of electrophysiology and immunocytochemistry. *Neuroscience*, **78**, 759–769.

Kita, H. & Kitai, S.T. (1987) Efferent projections of the subthalamic nucleus in the rat: light and electron microscopic analysis with the PHA-L method. *J. Comp. Neurol.*, **260**, 435–452.

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

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

Lacey, M.G. (1993) Neurotransmitter receptors and ionic conductances regulating the activity of neurones in substantia nigra pars compacta and ventral tegmental area. *Prog. Brain Res.*, **99**, 251–276.

Lacey, M.G., Mercuri, N.B. & North, R.A. (1988) On the potassium conductance increase activated by $GABA_B$ and dopamine D_2 receptors in rat substantia nigra neurones. *J. Physiol. (Lond.)*, **401**, 437–453.

Lavoie, B. & Parent, A. (1994a) Pedunculopontine nucleus in the squirrel monkey: cholinergic and glutamatergic projections to the substantia nigra. *J. Comp. Neurol.*, **344**, 232–241.

Lavoie, B. & Parent, A. (1994b) Pedunculopontine nucleus in the squirrel monkey: projections to the basal ganglia as revealed by anterograde tract-tracing methods. *J. Comp. Neurol.*, **344**, 210–231.

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

Lu, X.Y., Ghasemzadeh, M.B. & Kalivas, P.W. (1999) Regional distribution and cellular localization of gamma-aminobutyric acid subtype 1 receptor mRNA in the rat brain. *J. Comp. Neurol.*, **407**, 166–182.

Margeta-Mitrovic, M., Mitrovic, I., Riley, R.C., Jan, L.Y. & Basbaum, A.I. (1999) Immunohistochemical localization of $GABA_B$ receptors in the rat central nervous system. *J. Comp. Neurol.*, **405**, 299–321.

Marshall, F.H., Jones, K.A., Kaupmann, K. & Bettler, B. (1999) $GABA_B$ receptors – the first 7TM heterodimers. *Trends Pharmacol. Sci.*, **20**, 396–399.

Martin, S.C., Russek, S.J. & Farb, D.H. (1999) Molecular identification of the human $GABA_B$R2: cell surface expression and coupling to adenylyl cyclase in the absence of $GABA_B$R1. *Mol. Cell. Neurosci.*, **13**, 180–191.

Melis, M., Camarini, R., Ungless, M.A. & Bonci, A. (2002) Long-lasting potentiation of GABAergic synapses in dopamine neurons after a single *in vivo* ethanol exposure. *J. Neurosci.*, **22**, 2074–2082.

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

Moukhles, H., Bosler, O., Bolam, J.P., Vallee, A., Umbriaco, D., Geffard, M. & Doucet, G. (1997) Quantitative and morphometric data indicate precise cellular interactions between serotonin terminals and postsynaptic targets in rat substantia nigra. *Neuroscience*, **76**, 1159–1171.

Naito, A. & Kita, H. (1994) The cortico-nigral projection in the rat: an anterograde tracing study with biotinylated dextran amine. *Brain Res.*, **637**, 317–322.

Nauta, H.J. & Cuenod, M. (1982) Perikaryal cell labeling in the subthalamic nucleus following the injection of ^{3}H-gamma-aminobutyric acid into the pallidal complex: an autoradiographic study in cat. *Neuroscience*, **7**, 2725–2734.

Ng, T.K. & Yung, K.K. (2000) Distinct cellular distribution of $GABA_B$R1 and $GABA_A\alpha$1 receptor immunoreactivity in the rat substantia nigra. *Neuroscience*, **99**, 65–76.

Ng, T.K. & Yung, K.K. (2001a) Differential expression of $GABA_B$R1 and $GABA_B$R2 receptor immunoreactivity in neurochemically identified neurons of the rat neostriatum. *J. Comp. Neurol.*, **433**, 458–470.

Ng, T.K. & Yung, K.K. (2001b) Subpopulations of neurons in rat substantia nigra display $GABA_B$R2 receptor immunoreactivity. *Brain Res.*, **920**, 210–216.

Nitsch, C. & Riesenberg, R. (1988) Immunocytochemical demonstration of GABAergic synaptic connections in rat substantia nigra after different lesions of the striatonigral projection. *Brain Res.*, **461**, 127–142.

Overton, P.G. & Clark, D. (1997) Burst firing in midbrain dopaminergic neurons. *Brain Res. Brain Res. Rev.*, **25**, 312–334.

Paladini, C.A., Celada, P. & Tepper, J.M. (1999) Striatal, pallidal, and pars reticulata evoked inhibition of nigrostriatal dopaminergic neurons is mediated by $GABA_A$ receptors *in vivo*. *Neuroscience*, **89**, 799–812.

Paladini, C.A. & Tepper, J.M. (1999) $GABA_A$ and $GABA_B$ antagonists differentially affect the firing pattern of substantia nigra dopaminergic neurons *in vivo*. *Synapse*, **32**, 165–176.

Paxinos, G. & Watson, C. (1986) *The Rat Brain in Sterotaxic Coordinates*. Academic Press, Sydney.

Pinnock, R.D. (1984) Hyperpolarizing action of baclofen on neurons in the rat substantia nigra slice. *Brain Res.*, **322**, 337–340.

Prosser, H.M., Gill, C.H., Hirst, W.D., Grau, E., Robbins, M., Calver, A., Soffin, E.M., Farmer, C.E., Lanneau, C., Gray, J., Schenck, E., Warmerdam, B.S., Clapham, C., Reavill, C., Rogers, D.C., Stean, T., Upton, N., Humphreys, K., Randall, A., Geppert, M., Davies, C.H. & Pangalos, M.N. (2001) Epileptogenesis and enhanced prepulse inhibition in $GABA_{B1}$-deficient mice. *Mol. Cell. Neurosci.*, **17**, 1059–1070.

Ribak, C.E., Vaughn, J.E. & Roberts, E. (1980) GABAergic nerve terminals decrease in the substantia nigra following hemitransections of the striatonigral and pallidonigral pathways. *Brain Res.*, **192**, 413–420.

Ribak, C.E., Vaughn, J.E., Saito, K., Barber, R. & Roberts, E. (1976) Immunocytochemical localization of glutamate decarboxylase in rat substantia nigra. *Brain Res.*, **116**, 287–298.

Rick, C.E. & Lacey, M.G. (1994) Rat substantia nigra pars reticulata neurones are tonically inhibited via $GABA_A$, but not $GABA_B$, receptors *in vitro*. *Brain Res.*, **659**, 133–137.

Rinvik, E. & Ottersen, O.P. (1993) Terminals of subthalamonigral fibres are enriched with glutamate-like immunoreactivity: an electron microscopic, immunogold analysis in the cat. *J. Chem. Neuroanat.*, **6**, 19–30.

Sadikot, A.F., Parent, A. & Francois, C. (1992) Efferent connections of the centromedian and parafascicular thalamic nuclei in the squirrel monkey: a PHA-L study of subcortical projections. *J. Comp. Neurol.*, **315**, 137–159.

Santiago, M. & Westerink, B.H. (1992) The role of GABA receptors in the control of nigrostriatal dopaminergic neurons: dual-probe microdialysis study in awake rats. *Eur. J. Pharmacol.*, **219**, 175–181.

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

Schuler, V., Luscher, C., Blanchet, C., Klix, N., Sansig, G., Klebs, K., Schmutz, M., Heid, J., Gentry, C., Urban, L., Fox, A., Spooren, W., Jaton, A.L., Vigouret, J., Pozza, M., Kelly, P.H., Mosbacher, J., Froestl, W., Kaslin, E., Korn, R., Bischoff, S., Kaupmann, K., van der Putten, H. &

Bettler, B. (2001) Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic $GABA_B$ responses in mice lacking $GABA_{B(1)}$. *Neuron*, **31**, 47–58.

Shen, K.Z. & Johnson, S.W. (1997) Presynaptic $GABA_B$ and adenosine A_1 receptors regulate synaptic transmission to rat substantia nigra reticulata neurones. *J. Physiol. (Lond.)*, **505**, 153–163.

Smith, Y., Bevan, M.D., Shink, E. & Bolam, J.P. (1998) Microcircuitry of the direct and indirect pathways of the basal ganglia. *Neuroscience*, **86**, 353–387.

Smith, Y. & Bolam, J.P. (1990) The output neurones and the dopaminergic neurones of the substantia nigra receive a GABA-containing input from the globus pallidus in the rat. *J. Comp. Neurol.*, **296**, 47–64.

Smith, Y. & Bolam, J.P. (1991) Convergence of synaptic inputs from the striatum and the globus pallidus onto identified nigrocollicular cells in the rat: a double anterograde labelling study. *Neuroscience*, **44**, 45–73.

Smith, Y., Charara, A., Paquet, M., Kieval, J.Z., Pare, J.F., Hanson, J.E., Hubert, G.W., Kuwajima, M. & Levey, A.I. (2001) Ionotropic and metabotropic GABA and glutamate receptors in primate basal ganglia. *J. Chem. Neuroanat.*, **22**, 13–42.

Smith, Y., Charara, A. & Parent, A. (1996) Synaptic innervation of midbrain dopaminergic neurons by glutamate-enriched terminals in the squirrel monkey. *J. Comp. Neurol.*, **364**, 231–253.

Tepper, J.M., Martin, L.P. & Anderson, D.R. (1995) $GABA_A$ receptor-mediated inhibition of rat substantia nigra dopaminergic neurons by pars reticulata projection neurons. *J. Neurosci.*, **15**, 3092–3103.

Varoqui, H., Schafer, M.K., Zhu, H., Weihe, E. & Erickson, J.D. (2002) Identification of the differentiation-associated Na^+/PI transporter as a novel vesicular glutamate transporter expressed in a distinct set of glutamatergic synapses. *J. Neurosci.*, **22**, 142–155.

Vogt, K.E. & Nicoll, R.A. (1999) Glutamate and gamma-aminobutyric acid mediate a heterosynaptic depression at mossy fiber synapses in the hippocampus. *Proc. Natl Acad. Sci. USA*, **96**, 1118–1122.

Weinberg, R.J. & van Eyck, S.L. (1991) A tetramethylbenzidine/tungstate reaction for horseradish peroxidase histochemistry. *J. Histochem. Cytochem.*, **39**, 1143–1148.

Westerink, B.H., de Boer, P., Santiago, M. & De Vries, J.B. (1994) Do nerve terminals and cell bodies of nigrostriatal dopaminergic neurons of the rat contain similar receptors? *Neurosci. Lett.*, **167**, 109–112.

White, J.H., Wise, A., Main, M.J., Green, A., Fraser, N.J., Disney, G.H., Barnes, A.A., Emson, P., Foord, S.M. & Marshall, F.H. (1998) Heterodimerization is required for the formation of a functional $GABA_B$ receptor. *Nature*, **396**, 679–682.

Yung, K.K., Ng, T.K. & Wong, C.K. (1999) Subpopulations of neurons in the rat neostriatum display $GABA_B$R1 receptor immunoreactivity. *Brain Res.*, **830**, 345–352.