Synaptic Localization of GABA\textsubscript{A} Receptor Subunits in the Striatum of the Rat

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ABSTRACT

The inhibitory amino acid \textgreek{gamma}-aminobutyric acid (GABA) is widely distributed in the basal ganglia. It plays a critical role in the functioning of the striatum as it is the transmitter of projection neurons and sub-populations of interneurons, as well as afferents from the globus pallidus. Some of the factors controlling GABA transmission are the type(s) of GABA receptor expressed at the site of transmission, their subunit composition, and their location in relation to GABA release sites. To address these issues, we examined the sub-cellular localization of subunits of the GABA\textsubscript{A} receptor in the striatum of the rat. Sections of freeze-substituted, Lowicryl-embedded striatum were immunolabelled by the post-embedding immunogold technique with antibodies specific for subunits of the GABA\textsubscript{A} receptor. Immunolabelling for \textalpha1, \textbeta2/3, and \textgamma2 GABA\textsubscript{A} receptor subunits was primarily located at symmetrical synapses on perikarya, dendrites, and spines. Quantitative analysis of the distribution of immunolabelling for the \textbeta2/3 subunits revealed that the majority of membrane associated immunogold particles were at synapses and that, on average for the whole population, they were evenly distributed across the synapse. Double labelling for the \textbeta2/3 subunits and for GABA itself revealed that receptor-positive synapses were formed by at least two populations of terminals. One population (59.3\%) of terminals forming receptor-positive synapses was positive for GABA, whereas the other (40.7\%) had low or undetectable levels of GABA. Furthermore, the post-synaptic neurons were characterised on neurochemical and morphological grounds as both medium spiny neurons and GABA interneurons. Triple immunolabelling revealed the co-localization of \textalpha1, \textbeta2/3, and \textgamma2 subunits at some symmetrical axodendritic synapse. It is concluded that fast GABA\textsubscript{A}-mediated transmission occurs primarily at symmetrical synapses within the striatum, that the populations of boutons giving rise to receptor-positive synapses are heterogeneous, and that previously reported co-existence of different subunits of the GABA\textsubscript{A} receptor at the cellular level also occurs at the level of individual synapses. J. Comp. Neurol. 416:158–172, 2000.

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The inhibitory amino acid \textgreek{gamma}-aminobutyric acid (GABA) plays a critical role in the neuronal networks of the basal ganglia. Most of the major classes of neurons in the basal ganglia utilize GABA as a neurotransmitter. Within the striatum, medium size densely spiny neurons, which are the major projection neurons of the striatum and give rise to extensive local axon collaterals, are GABAergic (Smith and Bolam, 1990). The striatum also contains populations of GABAergic interneurons (Bolam et al., 1983, 1985) that express different calcium binding proteins (Cowan et al., 1990; Kita et al., 1990; Kubota et al., 1993; Clarke and Bolam, 1997) or synthesize nitric oxide (Kubota et al., 1993). Furthermore, the striatum receives GABAergic afferents from the globus pallidus (Bevan et al., 1998; Smith et al., 1998) and possibly the substantia nigra (van der Kooy et al., 1981).

The effects of GABA are mediated by three sub-populations of GABA receptors, the ionotropic GABA\textsubscript{A} and

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GABA<sub>a</sub> receptors and the metabotropic GABA<sub>b</sub> receptors. Most of the effects in the striatum are mediated through GABA<sub>a</sub> receptors, which cause an increase in Cl<sup>-</sup> conductance that underlies early inhibitory postsynaptic potentials. Molecular studies have identified that the GABA<sub>a</sub> receptor, in keeping with other ionotropic receptors, consists of a combination of receptor subunits in a pentameric structure (Backus et al., 1993; Smith and Olsen, 1995; Stephenson, 1995; McKernan and Whiting, 1996). At least 15 subunits have been identified and, on the basis of both in situ hybridization and immunocytochemistry, the most frequently occurring combination of subunits in the rat brain is α1, β2/3, and γ2 (Fritschy and Mohler, 1995; McKernan and Whiting, 1996). Recent reports suggest that different cell types in the same brain area may contain distinct complements of subunits (Persohn et al., 1992; Wisden et al., 1992; Gao et al., 1993; Fritschy and Mohler, 1995; Gao et al., 1995) that give rise to different pharmacological profiles (McKernan et al., 1991; Smith and Olsen, 1995; Stephenson, 1995) and may mediate different forms of inhibition (Brickley et al., 1996; Wall and Usovic, 1997). Furthermore, differential localization of GABA receptor subunits in relation to synaptic specializations has been proposed to underlie phasic and tonic inhibition in the cerebellum (Nusser et al., 1998).

In situ hybridization studies have identified a variety of GABA<sub>a</sub> receptor subunits in the striatum (Zhang et al., 1991; Araki et al., 1992; Persohn et al., 1992; Wisden et al., 1992; Fritschy and Mohler, 1995). Immunocytochemical studies using antibodies that recognize different subunits of the GABA<sub>a</sub> receptor have identified α, β, and γ subunits that are differentially distributed within the striatum. For instance, several small sub-populations of morphologically distinct striatal neurons are strongly immunoreactive for α1 subunits, whereas antibodies against α2 or β2/3 subunits label larger populations of neurons that are widely distributed in the striatum (Fritschy and Mohler, 1995; Hartig et al., 1995; Caruncho et al., 1996, 1997; Waldvogel et al., 1997, 1998; Riedel et al., 1998). The α1 subunit-positive neurons also express β2/3 and γ2 subunits and glutamate decarboxylase and are thus GABAergic. Two of the populations have light and electron microscopic features of interneurons, whereas the third is similar in morphology and neurochemistry to a type of projection neuron (Waldvogel et al., 1997, 1998; Riedel et al., 1998) that probably represents ectopic pallidal neurons (Bolam et al., 1981, 1985; Penny et al., 1988; Bennett and Bolam, 1994a). Electron microscopic analysis of the α1 and β2/3 subunit-immunolabelled tissue in rat and baboon has revealed the presence of receptor-positive synapses of the symmetrical type associated with the immunoreactive neurons (Waldvogel et al., 1997, 1998). In addition, receptor immunolabelling was also detected at asymmetrical synapses, i.e., the type usually associated with excitatory transmission, and it was also detected at non-synaptic sites (Waldvogel et al., 1997, 1998). These analyses were carried out using immunoperoxidase techniques to define antigenic sites precisely at the subcellular or subsynaptic level, it is necessary to complement this approach with immunolabelling techniques in which the antigenic sites are identified by non-diffusible markers (Baude et al., 1993, 1994; Nusser et al., 1994; Ottersen and Landsend, 1997).

In view of the widespread distribution of GABA and its receptors in the striatum and its critical role in the function in the striatum, and the basal ganglia in general, it is important to characterize the position and composition of GABA receptors in relation to the synaptic circuitry of the striatum. In this study we examine the localization of subunits of the GABA<sub>a</sub> receptor in the striatum of the rat. The primary objectives were threefold: first, to determine the sub-cellular localization of subunits of the GABA<sub>a</sub> receptor, in particular to determine their spatial relationship to synaptic specializations; second, to attempt to characterize the axon terminals presynaptic to the GABA<sub>a</sub> receptor-positive synapses by GABA immunolabelling; and third, to determine whether different subunits of the GABA<sub>a</sub> receptor that have been shown to be co-expressed at the cellular level are co-expressed at individual synapses. These issues were addressed by using the post-embedding immunogold technique on freeze-substituted tissue.

**MATERIALS AND METHODS**

**Preparation of tissue**

The tissue was obtained from four female Wistar rats (Charles River, Margate, Kent; 200–250 g) maintained on a 12 hour light/12 hour dark cycle with free access to food and water. 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 and also the European Communities Council Directive (80/609/EEC).

The animals were anesthetized with pentobarbitone (Sagatal, 200 mg/kg; Rhône Mérieux, Tallaght, Dublin, Ireland) and then perfused through the heart with 100 ml phosphate-buffered saline (PBS; 0.01 M phosphate, pH 7.4) or saline (0.9% NaCl) over 1–2 minutes and then with 300 ml of 0.025–0.5% glutaraldehyde and 3% paraformaldehyde (with or without 0.2% picric acid), made up in phosphate buffer (PB: 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4), at a concentration of 1 ml per 100 g body weight. The brain was removed from the cranium, divided into 5-mm-thick coronal slices, and stored in PBS at 4°C prior to further processing. From each 5-mm slice, coronal sections (500 µm) through the striatum were taken by using a vibrating microtome. The sections were collected and washed several times in PBS.

**Freeze-substitution and Lowicryl embedding**

A similar procedure was used as described earlier (Baude et al., 1993). Small blocks of the striatum were trimmed from the 500-µm sections. After washing in PB, they were placed in 0.5 M sucrose in PB for 15 minutes followed by 1 M sucrose in PB for 2 hours for cryoprotection. They were then washed on a polished copper block cooled with liquid nitrogen (Reichert M.M 80E). The slammmed blocks of tissue were transferred to a Leica CS Auto at −90°C where freeze-substitution and embedding in Lowicryl HM20 (Agar Scientific, Stansted, UK) was carried out as described before (Clarke and Bolam, 1998). Once the resin was polymerized and the blocks elevated to room temperature, they were removed for trimming and sectioning. Ultrathin sections (70 nm) were cut on a Reichert-Jung Ultracut-E ultramicrotome and collected on gold or nickel single-slot grids coated with pioloform (Agar Scientific) or on adhesive-coated (Coat-quick “G” medium; Daido Sangyo, Japan) gold or nickel mesh grids.

**Post-embedding immunogold labelling**

The sections were then immunolabelled by the post-embedding immunogold method essentially as described.
TABLE 1. Details of Antibody Preparations

<table>
<thead>
<tr>
<th>Antibody directed against</th>
<th>Species of origin</th>
<th>Dilution or concentration</th>
<th>Source and/or characterization</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA</td>
<td>Rabbit</td>
<td>1:5,000</td>
<td>Somogyi et al. (1985); Somogyi and Hodgson (1985)</td>
</tr>
<tr>
<td>GABA_a1 subunit</td>
<td>Rabbit</td>
<td>11 µg/ml</td>
<td>Figure 1, Haring et al. (1985)</td>
</tr>
<tr>
<td>GABA_a2 subunit</td>
<td>Guinea pig</td>
<td>10 µg/ml</td>
<td>Inc.</td>
</tr>
<tr>
<td>Rabbit IgG conjugated to 20-nm gold particles</td>
<td>Goat</td>
<td>1:60</td>
<td>British BioCell Int.</td>
</tr>
<tr>
<td>Rabbit IgG conjugated to 15-nm gold particles</td>
<td>Goat</td>
<td>1:80</td>
<td>British BioCell Int.</td>
</tr>
<tr>
<td>Mouse IgG conjugated to 10-nm gold particles</td>
<td>Goat</td>
<td>1:50</td>
<td>British BioCell Int.</td>
</tr>
<tr>
<td>Guinea pig IgG conjugated to 5-nm gold particles</td>
<td>Goat</td>
<td>1:40</td>
<td>British BioCell Int.</td>
</tr>
</tbody>
</table>

Antibody preparations

Three antibodies were used in this study:

1. A monoclonal antibody that recognizes extracellular domains on both the β2 and β3 subunits of the GABA_A receptor. This antibody has been extensively characterized (Härtig et al., 1985; Schoch et al., 1985; Ewart et al., 1990) and used extensively in immunocytochemical studies (see for instance Fritschy and Mohler, 1995; Somogyi et al., 1996; Waldvogel et al., 1998).

2. A polyclonal antibody raised in guinea pigs against a synthetic peptide corresponding to the N-terminal 1–29 amino acids of the rat γ2 subunit that was conjugated to keyhole limpet hemocyanin. Detailed characterization of the antibody has been described elsewhere (Benke et al., 1996; Somogyi et al., 1996).

3. A rabbit antibody raised against the N-terminal 1–14 amino acids of the rat α1 subunit. Affinity-purified anti-α1 1–14 Cys peptide antibodies were prepared following the method of Stephenson and Duggan (1991). The peptide QPSQDELKNTTFC, which corresponds to the rat GABA_A receptor α1 1–14 subunit sequence, with a C-terminal cysteine was coupled to the carrier protein, thyroglobulin, via the maleimido benzoc acid N-hydroxysuccinimide ester method. Rabbits were injected with the peptide-carry conjugate emulsified with Freund’s complete adjuvant at two sites intramuscularly (0.1 µmol peptide/site). Subsequent immunizations were in Freund’s incomplete adjuvant again at two sites intramuscularly. Animals were bled at 7 days following the second and subsequent immunizations. The anti-α1 1–14 Cys peptide antibodies were affinity-purified by a 1–14 Cys peptide affinity chromatography where the peptide was coupled to Activated Thiol Sepharose 4B via the terminal cysteine. Affinity-purified antibodies were stored at 4°C in the presence of 0.02% NaN₃ until use. The specificity of the affinity-purified anti-α1 1–14 Cys antibodies were assessed by immunobLOTS (Fig. 1).

Analysis of material

The immunolabeled sections were examined in a Philips CM 10 transmission electron microscope. Immunoreactive sites were identified by the presence of the colloidal gold particles that were attached to the secondary antibodies. The distribution of immunogold particles coding for the β2/3 subunits was determined by systematic examination of the adjacent photomicrographs at a final magnification of 33,000 (366 gold particles; 138 µm²) or by systematic (non-overlapping) scans of sections on mesh grids (2,760 gold particles; approximate area: 8,980 µm²). The location of each gold particle was noted. To determine the association between GABAergic terminals and β2/3 subunits associated photomicrographs (area: 2,723 µm²) were examined or systematic, non-overlapping, scans of sections on mesh grids (approximate area: 7,480 µm²) were made. A synapse was considered positive by the presence of two or more immunogold particles along the synaptic membrane. Each receptor-positive synapse and the level of immunogold labelling for GABA in the presynaptic bouton was assessed. A bouton was considered to be GABA negative or to have low levels of GABA if the number of gold particles was two or less.

Quantitative analysis of the distribution of immunogold particles for the β2/3 subunits along the synaptic membrane specialization of striatal synapses was performed on electron micrographs. A total of 155 β2/3 subunit-positive synapses (746 gold particles) from two animals were analyzed. All synapses in series of adjacent photomicro-
graphs of well-preserved strips of ultrathin sections were analysed. In addition, micrographs containing synapses that had been selected for other parts of the current study were also analysed. Since the data from the random and non-random analyses were essentially similar, they were pooled. The distance of each immunoparticle from the synaptic specialization formed from the whole thickness of it. Of the membrane-associated particles, 55.5% were localized at symmetrical synapses (see below) and probably includes both specific labelling associated with the synthetic and transport machinery of the receptor as well as non-specific labelling. Forty-four percent of gold particles were associated with membranes, i.e., either touching the membrane or within 1 diameter of it. Of the membrane-associated particles, 55.5% were localized at symmetrical synapses where gold particles lined up along the half width of the synapse (see insets in Figs. 2, 3D,E, 4A–D). The antibody used recognized the N-terminal sequence of the β2 and β3 subunits; thus extracellular labeling is consistent with the predicted topography of the receptor subunits. However, it is not possible to localize an antigen unequivocally to the internal or external surface of the membrane, nor to the pre- or post-synaptic structure using post-embedding techniques because of the distortion between the image of the membrane specialization formed from the whole thickness of it. Of the membrane-associated particles, 55.5% were localized at symmetrical synapses where gold particles lined up along the half width of the synapse (see insets in Figs. 2, 3D,E, 4A–D). The antibody used recognized the N-terminal sequence of the β2 and β3 subunits; thus extracellular labeling is consistent with the predicted topography of the receptor subunits. 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of the section and the most superficial layer of the section available for the antibody. This is exemplified by the tangentially cut synapses where there is an accumulation of immunogold particles but no apparent synaptic membranes (Fig. 2D). The labelling associated with membranes at extrasynaptic sites consisted mainly of isolated gold particles (Fig. 3A), although clusters of two or more particles were sometimes seen (Fig. 2C).

Synaptology of synapses positive for the β2/3 subunits

The post-synaptic structures at synapses positive for the β2/3 subunits of the GABA<sub>A</sub> receptor included dendrites (Figs. 2B–E, 3A,B,D,E, 4A,C,D,E, 5), spines (Fig. 2D,F), and perikarya (Figs. 2A, 3C, 4B). In keeping with the known data concerning symmetrical synapses in the striatum (Ingham et al., 1998), the majority of receptor-positive synapses were axodendritic (Figs. 2B–E, 3A,B,D,E, 4A,C,D,E, 5). Synapses with both small (presumably distal) and large diameter dendrites, as well as proximal dendrites emerging from perikarya, were positive for the GABA<sub>A</sub> receptor subunits. At least two types of dendrites were identified: first, dendrites with the characteristics of medium size densely spiny neurons (Fig. 3E), i.e., they gave rise to dendritic spines and possessed morphological features of the dendrites of spiny neurons (Somogyi and Smith, 1979; Wilson and Groves, 1980); and second, in the double-labelled material, dendrites were identified that displayed immunolabelling for GABA (Fig. 3A,B,D; see below), which is indicative of the GABA interneurons of the striatum (Bolam et al., 1983; Kawaguchi et al., 1995; Kawaguchi, 1997).

Dendritic spines were the second most frequently observed post-synaptic structure at synapses positive for the β2/3 subunits (Fig. 2D,F). The synapses generally occurred on the neck of the spine (when visible), and the spine was often post-synaptic to another terminal that formed an asymmetric synapse (Fig. 2D,E).

Synapses positive for the β2/3 subunits of the GABA<sub>A</sub> receptor were observed on neuronal perikarya that had the ultrastructural characteristics of medium size densely spiny neurons, i.e., large non-indented nucleus and a relatively small volume of cytoplasm that was poor in organelles (Somogyi and Smith, 1979; Dimova et al., 1980; Wilson and Groves, 1980) (Figs. 2A, 4B). Multiple receptor-positive synapses were often detected on spiny neuron perikarya (Fig. 2A). Receptor-positive synapses were also formed on the perikarya of neurons characterised as GABA interneurons on the basis of both morphology and neurochemistry (Fig. 3C; see below).

GABA labelling of presynaptic boutons at synapses positive for the β2/3 subunits

To gain insight into the nature of the terminals that form synapses positive for the β2/3 subunits, sections of the striatum were double immunostained to reveal both GABA and the β2/3 subunits. The immunolabelling for GABA (20-nm gold particles) was similar to that described previously for the striatum and other regions of the basal ganglia (Clarke and Bolam, 1997; Smith et al., 1998). The GABA immunolabelling was widely distributed in the striatum with marked accumulations of immunogold particles over a subset of axons, axon terminals, dendrites, and perikarya (Figs. 2–4). Systematic scans of double-labelled sections revealed that most (59.3%) of the boutons forming synapses that were positive for the β2/3 subunits were also positive for GABA (Figs. 2, 3, 4A,C–E). The boutons were of variable size and made symmetrical synaptic contacts with dendrites, spines, and perikarya. They contained from 0 to 4 mitochondria and sometimes formed synapses with more than one structure in the same plane (Figs. 2C,E). In addition to the GABA-positive boutons, 40.7% of receptor-positive synapses were formed by boutons that possessed low or undetectable levels of GABA (Fig. 4). These synaptic boutons were identified in synapses in which structures that were strongly labelled for GABA were identified in the close vicinity. Postsynaptic targets included spines (not shown), dendrites (Figs. 4A,C–E), and perikarya (Fig. 4B). A similar analysis of the boutons in the entopeduncular nucleus, the substantia nigra pars reticulata, and pars compacta revealed that only 9.3%, 4.1%, and 3.8% of boutons forming receptor-positive synapses, respectively, possessed low or undetectable levels of GABA (unpublished observations).

The double-labelled sections also enabled the chemical characterization of the post-synaptic structures involved in receptor-positive synapses. Thus, some of the dendrites and perikarya were themselves immunopositive for GABA (Fig. 3). The GABA-positive perikarya (Fig. 3C) possessed indentations in the nuclear membrane and a relatively large volume of cytoplasm (compared with spiny neuron perikarya) that was rich in organelles. These morphological features and the presence of GABA immunolabelling are characteristics of the GABA interneuron of the striatum that has been characterized on the basis of the uptake

Localization of $\alpha_1$ and $\gamma_2$ subunits

Sections immunolabelled to reveal $\alpha_1$ and $\gamma_2$ subunits of the GABA<sub>Ã</sub> receptor revealed labelling for both subunits, although the labelling was not as robust as that obtained with the antibodies against the $\beta_2/3$ subunits. Immunogold particles were observed both on membranes and at intracellular sites, but the most prominent labelling, in the form of groups of immunogold particles, occurred at symmetrical synapses (Fig. 5). Labelling for each of the subunits was observed at symmetrical synapses involving spines, perikarya, and, most frequently, dendritic shafts (Fig. 5).

Triple-labelling experiments for the $\alpha_1$, $\beta_2/3$, and $\gamma_2$ subunits with three different sizes of gold particles revealed the co-localization of the GABA<sub>Ã</sub> Receptor subunits at individual symmetrical synapses (Fig. 5). In a systematic analysis of receptor-positive synapses, 42%, 92%, and 26% were positive for $\alpha_1$, $\beta_2/3$, and $\gamma_2$ subunits, respectively. Co-localization for all three subunits occurred at 12% of the synapses, co-localization of $\alpha_1$ and $\beta_2/3$ subunits at 22%, and co-localization of $\beta_2/3$ and $\gamma_2$ subunits at 14%. Synapses were detected that exhibited labelling for only $\alpha_1$ (18%) or only $\beta_2/3$ (44%) subunits. In this analysis, synapses with only single gold particles coding for a particular subunit were considered positive.

Distribution of immunolabelling for $\beta_2/3$ subunits across synapses

The quantitative analysis in the striatum revealed that the immunoparticles labelling $\beta_2/3$ subunits were, on average, evenly distributed across the width of the synapti specializations, although there was a tendency for reduced levels toward the edge (Fig. 6). Very few particles fell apparently outside the synaptic specialization at perisynaptic sites. The exact location of immunoparticles at the edge of the synapse, however, is difficult to judge because of steric distortion between the image of the membrane specialization formed from the whole thickness of the section and the most superficial layer of the section available for the antibody and the difficulty of identifying the edge of symmetrical synapses in freeze-substituted tissue where the preservation is not optimal.

DISCUSSION

The results of the present study provide a detailed analysis of the distribution of subunits of the GABA<sub>Ã</sub> receptor in relation to synaptic specializations in the striatum of the rat. They demonstrate first that subunits of the GABA<sub>Ã</sub> receptor are widely distributed in the striatum, that most of the immunolabelling is associated with the plasma membrane, and that almost half of this is associated with symmetrical synaptic specializations. The labelling for the $\beta_2/3$ subunits, when considered as an average of the whole population, is evenly distributed across the synaptic specialization. Second, the receptor-positive synapses are heterogeneous with respect to both the pre- and post-synaptic structures. The post-synaptic neurons included medium spiny neurons, identified on the basis of morphological characteristics, and GABA interneurons, identified on the basis of both morphological and neurochemical characteristics (Bolam and Bennett, 1995; Kawaguchi et al., 1995). About 60% of the pre-synaptic boutons forming $\beta_2/3$ subunit-positive synapses are GABA-positive; the remainder are formed by boutons that have low or undetectable levels of GABA. Finally, the present results demonstrate the colocalization of $\alpha_1$, $\beta_2/3$, and $\gamma_2$ subunits of the GABA<sub>Ã</sub> receptor at individual symmetrical synapses. These findings suggest therefore that fast GABA transmission mediated by GABA<sub>Ã</sub> receptors containing $\alpha_1$, $\beta_2/3$, and $\gamma_2$ subunits occurs primarily at synapses within the striatum, that the boutons giving rise to receptor-positive synapses are neurochemically heterogeneous, and that previously reported co-existence of different subunits of the GABA<sub>Ã</sub> receptor at the cellular level also occurs at the level of individual synapses.

Subcellular distribution of GABA<sub>Ã</sub> Receptor subunit immunolabelling

The present findings demonstrate that there is a selective association of immunogold particles coding for subunits of the GABA<sub>Ã</sub> receptor with symmetrical synapses in the striatum. Although data is not available for the striatum, it has been estimated that in the hippocampus only 1–2% of neuronal membrane is occupied by symmetric synapses and that the area of membrane occupied by symmetrical synapses is negligible (Rusakov et al., 1998). In the striatum about one-fifth of synapses are of the symmetrical type (Ingham et al., 1998). If the overall proportion of membrane synapses occupied by synapses in the striatum is similar to that in the hippocampus, then 0.2–0.4% of neuronal membrane is occupied by symmetrical synapses. The finding of about 25% of total gold particles associated with symmetrical synapses represents...
a 125–250-fold enrichment at synapses. When considering the number of gold particles at synapses as a proportion of membrane-associated particles, then our results reveal a 220–440-fold enrichment at synapses. These findings are consistent with those observed in other regions of the brain including the globus pallidus, cerebellum, and hippocampus, where at least some of the subunits of the GABA<sub>A</sub> receptor are preferentially localized at synapses (Nusser et al., 1995a,b, 1996a,b, 1997, 1998; Somogyi et al., 1996; Nusser and Somogyi, 1997; but see below). Furthermore, it seems to be a general principle that subunits of fast ionotropic receptors are preferentially localized at synapses, as ionotropic glutamate receptors (both AMPA and NMDA) in the basal ganglia (Bernard et al., 1997; Bernard and Bolam, 1998; Clarke and Bolam, 1998) as well as in other regions of the brain (Baude et al., 1994, 1995; Nusser et al., 1994; Kharazia et al., 1996; Kharazia and Weinberg, 1997; Ottersen and Landsend, 1997; Popratiloff et al., 1998) are selectively associated with synapses whereas at least some metabotropic receptors are preferentially located at perisynaptic sites (Baude et al., 1993; Lujan et al., 1996; Ottersen and Landsend, 1997). The nature of the immunolabelling outside of synapses or at intracellular sites remains to be established. The membrane-associated receptors may represent true “extrasynaptic receptors” that will only be exposed to GABA that has diffused from the release site in the synapse; alternatively, they may be non-functional or receptors that are in the process of being transported to the synapse. Similarly, intracellular label may represent receptors undergoing synthesis, transport, degradation, or recycling.

The detection of immunolabelling at symmetrical synapses is consistent with previous findings in the rat and baboon (Waldvogel et al., 1997, 1998). However, in these studies the postsynaptic density of some asymmetrical synapses were reported to be immunopositive for α1 and β2/3 subunits, an observation not made in the present study. The most likely explanation for this discrepancy is technical, relating to the techniques that were used. It is well recognized that peroxidase reaction products are diffusible and readily adhere to membranes and to postsynaptic densities and can thus give false-positive labelling. Colloidal gold when attached to the secondary antibody may represent receptors undergoing synthesis, transport, degradation, or recycling.

The quantitative analysis revealed that, when considered as a population, immunolabelling for the β2/3 subunits of the GABA<sub>A</sub> receptor was evenly distributed across the width of the synapse. Labeling became negligible within a few nanometers of the edge of the synaptic specialization. This indicates that GABA transmission mediated through GABA<sub>A</sub> receptors that possess β2 and/or β3 subunits is likely to occur almost exclusively within the synapse and that there is a homogeneous distribution of receptors in the postsynaptic membrane. These findings are consistent with previous findings of the distribution of GABA<sub>A</sub> receptors subunits in the hippocampus (Nusser et al., 1995a, 1996a) and subunits of ionotropic glutamate receptors in the striatum (Bernard et al., 1997; Bernard and Bolam, 1998), entopeduncular nucleus, and subthalamus (Clarke and Bolam, 1998). It must be noted, however, that GABAergic synapses in the striatum are heterogeneous with respect to their origin; the average data that we generated may obscure any variations in the distribution of immunolabelling at sub-populations of synapses.

It is not possible to determine whether immunogold labelling, using the freeze-substitution, post-embedding method, is associated with the pre- or post-synaptic membrane because of steric distortion between the image of the membrane specialization formed from the whole thickness of the section and the most superficial layer available for the antibody. On the basis of in situ hybridization and immunocytochemical studies, the weight of evidence is that most, if not all, of the immunolabelling that we observed is associated with the post-synaptic element (Wisden et al., 1992; Fritschy and Mohler, 1995; Hartig et al., 1995; Caruncho et al., 1996, 1997; Liste et al., 1997; Waldvogel et al., 1997, 1998; Kultas-Illinsky et al., 1998).

**Identity of boutons forming receptor-positive synapses**

At least two classes of axon terminals were identified that formed GABA<sub>A</sub> receptor-positive synapses in the striatum, those that were associated with a high density of GABA immunogold particles and those with low or undetectable levels. There are at least four possible origins of the terminals with high levels of GABA:

1. The medium spiny projection neuron, which accounts for the majority of striatal neurons and gives rise to

![Fig. 4. Synapses positive for the β2/3 subunits of the GABA<sub>A</sub> receptor formed by boutons with low or undetectable levels of GABA.](image-url)
Figure 4
local axon collaterals that form symmetrical synapses (Wilson and Groves, 1980; Somogyi et al., 1981; Yung et al., 1996), are GABAergic neurons (see Smith and Bolam, 1990). Their axon terminals in the globus pallidus, entopeduncular nucleus, and substantia nigra have been shown to be enriched in GABA (see Smith et al., 1998).

2. The population of GABA interneurons identified on the basis of the uptake of [3H]GABA (Bolam et al., 1983) and by GAD immunolabeling (Bolam et al., 1985; Kita and Kitai, 1988). These neurons stain more strongly for GAD than do the medium spiny neurons (Bolam et al., 1985; Kita and Kitai, 1988) and express immunoreactivity for parvalbumin (Cowan et al., 1990; Kita et al., 1990); their axon terminals within the striatum have been shown to form symmetrical synapses (Cowan et al., 1990; Kita et al., 1990; Bennett and Bolam, 1994a, 1994b) and are GABA-positive (Kubota et al., 1993; Bennett and Bolam, 1997).

3. The terminals formed by striatal interneurons that express nitric oxide synthase and those expressing the calcium binding protein calretinin, form symmetrical synapses (Bennett and Bolam, 1993; Morello et al., 1997)) and have been reported to be GABA positive (Kubota et al., 1993; Clarke and Bolam, 1997).

4. A sub-population of neurons in the globus pallidus gives rise to a projection to the striatum (Staines et al., 1981; Beckstead, 1983; J ayaraman, 1983; Staines and Fibiger, 1984; Shu and Peterson, 1988; Walker et al., 1989; Kita and Kitai, 1991; Rajakumar et al., 1994; Spooren et al., 1996; Nambu and Llinas, 1997; Bevan et al., 1998). These neurons are GABAergic (Smith et al., 1998); they give rise to symmetrical synapses in the striatum and selectively innervate striatal interneurons (Bevan et al., 1998).

The postsynaptic neurons included both medium spiny and GABA interneurons identified on the basis of both morphological and neurochemical criteria. From the known synaptology of the striatum (Bolam and Bennett, 1995), we can conclude that the GABA sub1 receptor-positive synapses on spiny neurons are formed by the collaterals of spiny neurons themselves (Wilson and Groves, 1980; Somogyi et al., 1981; Yung et al., 1996; but see below) and/or the terminals of GABA interneurons (Kita et al., 1990; Kita, 1993; Bennett and Bolam, 1994a,b) but not those of pallidostriatal neurons (Bevan et al., 1998). Those GABA-positive terminals forming receptor-positive synapses with GABA-positive neurons are likely to be derived from the globus pallidus (Bevan et al., 1998) and possibly other GABA interneurons (Bolam et al., 1985).

There are several possible explanations for the presence of the second population of axon terminals that formed GABA sub1 receptor-positive synapses, i.e., those possessing low or undetectable levels of GABA. First, it is possible that the low or undetectable levels of GABA are a technical artifact caused by the failure to maintain the antigenicity for GABA within those boutons. This, however, is unlikely, as other boutons that were strongly positive for GABA (and formed receptor-positive synapses) were found in the vicinity of the boutons with the low levels of GABA. Furthermore, similar analyses in other regions of the basal ganglia (entopeduncular nucleus, substantia nigra pars reticulata and compacta) revealed the presence of much smaller proportions of boutons with low or undetectable levels of GABA-forming receptor-positive synapses (unpublished observations). This suggests that the higher proportion of GABA-poor boutons that formed receptor-positive synapses in the striatum is a characteristic of the striatum and not the technicalities of the procedures. A second possibility is that the terminals with low or undetectable levels of GABA truly represent a population of terminals that are GABAergic but are at the lower end of the spectrum in terms of their content of GABA and are simply below the level of detection in this tissue. There is evidence, albeit indirect, that different populations of GABAergic terminals in the striatum are derived from neurons that express different levels of GABA. Thus the parvalbumin-expressing GABA interneurons stain more strongly for GAD than striatal spiny neurons (Bolam et al., 1985; Kita and Kitai, 1988; Kawaguchi et al., 1995). Similarly, the terminals of globus pallidus neurons in the entopeduncular nucleus or the substantia nigra have higher levels of GABA immunoreactivity than do the terminals of spiny projection neurons in these regions (Smith et al., 1998). It is possible, therefore, that in the striatum the terminals of GABA interneurons and the terminals of globus pallidus neurons are those terminals with high levels of GABA and that the terminals with low or undetectable levels of GABA are the local axon terminals of the spiny projection neurons.

A third possible explanation is that the terminals are indeed non-GABAergic but form synapses that are positive for GABA receptor subunits. Mismatches between the putative transmitter of synaptic terminals and the receptor located within the synapse have been reported for GABA and for other receptors. In the subthalamic nucleus, a population of terminals that have low levels of glutamate immunoreactivity and high levels of GABA forms synapses that are positive for the NR1 subunit of the NMDA receptor and the GluR2/3 subunits of the AMPA receptor (Clarke and Bolam, 1998). Furthermore, the γ2, β2/3, and α6 subunits of the GABAA receptor have been shown to be concentrated in some glutamatergic mossy fibre synapses in the cerebellum (Nusser et al., 1996b, 1998). In the striatum, non-GABAAergic terminals that form symmetrical synapses include dopaminergic axon terminals derived from the substantia nigra pars compacta (Bouyer et al., 1984; Freund et al., 1984; Smith et al., 1994; Hanley and Bolam, 1997) and cholinergic terminals derived from cholinergic interneurons (Wainer et al., 1984; Izzo and Bolam, 1997) and Kitai, 1990). The possibility of the presence of GABAA receptors at synapses formed by these classes of terminals, as yet, be excluded.
The triple-labelling study revealed the presence of symmetrical synapses that were positive for various combinations of the \( \alpha \), \( \beta \), and \( \gamma \) subunits. Thus synapses were observed that were labelled by one of the three antibodies, two out of the three antibodies and synapses that were positive for all three. Although negative findings are difficult to interpret, the present study indicates that \( \alpha_1 \), \( \beta_2/3 \), and \( \gamma_2 \) receptor subunits of the GABA \(_A\) receptor co-localize at individual synapses. This finding corroborates previous radioligand-binding, in situ hybridization, and immunohistochemical studies indicating the co-localization of GABA\(_A\) receptor subunits in the striatum at the regional and cellular levels (Fritschy and Mohler, 1995; Caruncho et al., 1996, 1997; McKernan and Whiting, 1996; Waldvogel et al., 1997, 1998; Riedel et al., 1998). In fact the \( \alpha_1 \), \( \beta_2/3 \), and \( \gamma_2 \) receptor subunit configuration has been proposed as the most common for GABA\(_A\) receptors in the mammalian brain. From pre-embedding immunocytochemical and in situ hybridisation studies (Fritschy and Mohler, 1995; Caruncho et al., 1996, 1997; McKernan and Whiting, 1996; Liste et al., 1997; Waldvogel et al., 1997, 1998; Riedel et al., 1998), it is evident that the \( \alpha_1 \) subunit in not expressed, or expressed at low levels, by medium spiny neurons but is expressed by small populations of striatal neurons, which include GABA interneurons and a large type of projection neuron (Caruncho et al., 1996; Waldvogel et al., 1997, 1998). It is thus likely that the synapses that were positive for the \( \alpha_1 \) subunits with or without the \( \beta_2/3 \) and \( \gamma_2 \) subunits are formed by these latter populations of neurons. Those synapses that were negative for the \( \alpha_1 \) subunits may express some other \( \alpha \) subunit, possibly \( \alpha_2 \), and may thus represent the synapses of spiny neurons. It is thus evident that there are differences in the GABA\(_A\) receptor subunit profiles at synapses on medium-sized projection spiny neurons and those on interneurons in the striatum.

**CONCLUSIONS**

The present findings demonstrate the precise localization of subunits of the GABA\(_A\) receptor in relation to symmetrical synaptic specializations in the rat striatum. The main conclusions that we can draw from this study are that GABA\(_A\) receptor are primarily located at symmetrical synapses formed by boutons that are heterogeneous with respect to their morphology and neurochemistry and that different subunits colocalize at the level of individual synapses. The findings represent the first step in the elucidation of the chemical anatomy of the GABA-mediated synaptic circuits of the striatum in which the anatomical connections, transmitter neurochemistry, and transmitter receptors are localized. Experiments are in progress to identify the origin of the synaptic boutons involved in these circuits.

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**LITERATURE CITED**


Bennett BD, Bolam JP. 1994b. Synaptic input and output of parvalbumin-
Hanley JJ, Bolam JP. 1997. Synaptology of the nigrostriatal projection in 
Caruncho HJ, Liste I, Labandeira-García JL. 1996. GABA A receptor 
Cowan RL, Wilson CJ, Emson PC, Heizmann CW. 1990. Parvalbumin-
Fritschy J-M, Mohler H. 1995. GABAA-receptor heterogeneity in the adult 
Bevan MD, Booth PAC, Eaton SA, Bolam JP. 1998. Selective innervation of 
Freund TF, Powell J, Smith AD. 1984. Tyrosine hydroxylase-immunoreac-
Kultas-Ilinsky K, Leontiev V, Whiting PJ. 1998. Expression of 10 GABA A 
Kita H, Kitai ST. 1988. Glutamate decarboxylase-immunoreactive neurons in 
Kita H, Kitai ST. 1994. The morphology of globus pallidus projection neurons in 
Luscher NA, Nusser Z, Roberts J, Shigemoto R, Somogyi P. 1996. Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. Eur J Neurosci 8:1488–1500.


Nusser Z, Sieghart W, Stephenson FA, Somogyi P. 1996b. The\textalpha{6} subunit of the GABA\textsubscript{A} receptor is concentrated in both inhibitory and excitatory synapses on cerebellar granule cells. J Neurosci 16:103–114.


Poland S, Thompson CL, Stephenson FA. 1995. Quantitative characterisation of the \textalpha{6} subunit of the GABA\textsubscript{A} receptor of adult cerebellum demonstrates two\textalpha{1} subunits per receptor oligomer. J Biol Chem 270:21285–21290.


Waldvogel HJ, Kubota Y, Trelavay SC, Kawaguchi Y, Fritschy J-M, Mohler H, Faull RLM. 1997. The morphological and chemical characteristics of striatal neurons immunoreactive for the \textalpha{1} subunit of the GABA\textsubscript{A} receptor in the rat. Neuroscience 80:775–792.

Waldvogel HJ, Fritschy JM, Mohler H, Faull RLM. 1998. GABA\textsubscript{A} receptors in the primate basal ganglia: an autoradiographic and a light and electron microscopic immunohistochemical study of the \textalpha{1} and \textbeta{3} subunits in the baboon brain. J Comp Neurol 397:297–325.


