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Distribution of Glutamate Receptor Subunits at Neurochemically Characterized Synapses in the Entopeduncular Nucleus and Subthalamic Nucleus of the Rat

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ABSTRACT

Glutamatergic neurotransmission in the subthalamic nucleus (STN) and in the output nuclei of the basal ganglia is critical in the expression of basal ganglia function, and increased glutamate transmission in these nuclei has been implicated in the pathology of Parkinson's disease. In order to determine the precise spatial relationship of subunits of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and *N*-methyl-D-aspartate (NMDA) glutamate receptors to nerve terminals enriched in glutamate or γ-aminobutyric acid (GABA) in one of the output nuclei, the entopeduncular nucleus (EP), and the STN, postembedding immunolabelling for glutamate receptor subunits and for glutamate and GABA was carried out in the rat.

Immunolabelling for the AMPA glutamate receptor subunits 1, 2/3, and 4 (GluR1, GluR2/3, and GluR4) and the NMDA receptor subunit 1 (NR1) was localized predominantly within asymmetrical synapses in both the EP and STN. Quantitative analysis revealed that, on average for the whole population, each of the receptor subunits was evenly distributed along the synaptic specialization. Multiple AMPA receptor subunits and the GluR2/3 and NMDA (NR1) subunits were co-localized within individual synapses. The combination of immunolabelling for glutamate and GABA with the receptor immunolabelling revealed that the majority of axon terminals presynaptic to the receptor-immunoreactive synapses were enriched in glutamate immunoreactivity and were GABA-immunonegative. However, at some NR1- and GluR2/3-positive synapses, the level of glutamate immunoreactivity was low in the presynaptic terminal and, in the STN, some of them were GABA-immunopositive.

It is concluded that glutamatergic transmission at individual synapses of different origins in the EP and STN is mediated by a combination of AMPA and NMDA glutamate receptors. J. Comp. Neurol. 397:403–420, 1998.

Indexing terms: basal ganglia; excitatory amino acid receptors; AMPA; NMDA

The major output nuclei of the basal ganglia in the rat are the entopeduncular nucleus (EP) and the substantia nigra pars reticulata. The neurons in these nuclei convey the final output signal of the basal ganglia to the thalamus and/or subcortical premotor regions. Neurons of the subthalamic nucleus (STN) also play a key role in the basal ganglia by virtue of their excitatory drive to the output nuclei as well as other regions of the basal ganglia (Kita and Kitai, 1987; Nakanishi et al., 1987, 1991; Smith and Parent, 1988; Groenewegen and Berendse, 1990; Smith et al., 1990, 1994; Rinvik and Ottersen, 1993; Bevan et al., 1994a,b). The importance of the EP and STN in basal ganglia function is exemplified by the recent findings that these nuclei are overactive in Parkinson's disease or its models (Albin et al., 1989; Alexander and Crutcher, 1990; DeLong, 1990). Furthermore, lesions of the internal segment of the globus pallidus (GPi), which is the primate

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TABLE 1. Distribution of Ionotropic Glutamate Receptor Subunits Obtained From Previous In Situ Hybridization (ISH) and Pre-Embedding Immunocytochemical (Immuno) Studies in Different Species[1]

Receptor subunit	Technique	Rat EP	Monkey GPi	Human GPi	Rat STN	Reference
AMPA GluR1	ISH	0			+++	Sato et al. (1993)
	ISH/immuno	++		+		Bernard et al. (1996)
	Immuno				+++	Petralia and Wenthold (1992)
	Immuno	++			++	Martin et al. (1992)
	Immuno		0			Paquet and Smith (1996)
GluR2	ISH	++			++	Sato et al. (1993)
	ISH/immuno	++		++		Bernard et al. (1996)
GluR3	ISH	+			0	Sato et al. (1993)
	ISH/immuno	++		+		Bernard et al. (1996)
GluR2/3	Immuno				+++	Petralia and Wenthold (1992)
	Immuno		++			Paquet et al. (1996)
GluR2/3/4c	Immuno	+			0	Martin et al. (1993)
GluR4	ISH	++			++	Sato et al. (1993)
	ISH/immuno	++		+++		Bernard et al. (1996)
	Immuno				++	Petralia et al. (1992)
	Immuno	0			0	Martin et al. (1993)
NMDA NR1	ISH	+++			+++	Standaert et al. (1994)
	Immuno				++	Petralia et al. (1994)

[1]The levels of expression in the neurons of the entopeduncular nucleus (EP), the internal segment of the globus pallidus (GPi), and the subthalamic nucleus (STN) are indicated as follows: 0, none detected; +, very low levels detected; ++, low; +++, moderate; ++++, high levels.

equivalent of the EP (Kondoh et al., 1994; DeLong, 1995; Iacono et al., 1995; Lozano et al., 1995; Baron et al., 1996; Obeso et al., 1997; Tasker et al., 1997), and lesions or inactivation of the STN (Bergman et al., 1990; Aziz et al., 1991; Brotchie et al., 1991; Benazzouz et al., 1993; Guridi et al., 1996; Piallet et al., 1996) are effective treatments for relieving the motor signs and symptoms of Parkinson's disease and its models. The increased activity of neurons in the EP and STN is dependent on many factors, one of which is likely to be their excitatory inputs. The major excitatory inputs to the EP are derived from the mesopontine tegmentum (MTg; Clarke et al., 1996, 1997) and from the STN (Bevan et al., 1994b), whereas the major excitatory inputs to the STN are derived from the cortex, thalamus, and MTg (Bevan and Bolam, 1995; Bevan et al., 1995; Clarke et al., 1997). Each of these afferents has been shown to be enriched in glutamate immunoreactivity (Bevan and Bolam, 1995; Bevan et al., 1995; Clarke et al., 1996, 1997).

The effects of glutamate at synapses in the central nervous system are mediated by three types of receptors; the ionotropic *N*-methyl-D-aspartate (NMDA) receptor, the ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-kainate receptor, and metabotropic receptors (Nakanishi, 1992; Hollmann and Heinemann, 1994; Pin and Duvoisin, 1995). In addition to the metabotropic glutamate receptors, neurons of the EP and STN have been shown to express subunits of the AMPA and NMDA-type glutamate receptors at the cellular level by in situ hybridization (Sato et al., 1993; Standaert et al., 1994) and immunocytochemistry (Petralia and Wenthold, 1992; Martin et al., 1993; Petralia et al., 1994; Bernard et al., 1996, 1997b; Paquet and Smith, 1996; Table 1). Transmission at glutamatergic synapses is influenced by many mechanisms including the differential expression of receptor subunits and the distribution of receptors at the subcellular and subsynaptic level in relation to glutamate release sites (Baude et al., 1993, 1995; Nusser et al., 1994; Kharazia et al., 1996; Matsubara et al., 1996; Popratiloff et al., 1996; Bernard et al., 1997a; Landsend et al., 1997; Nusser and Somogyi, 1997; Rubio and Wenthold, 1997; Somogyi et al., 1998). The objectives of the present study were, therefore, to define the composition and precise location of receptors at glutamatergic synapses in the EP and STN: first, to determine the spatial relationship of AMPA and NMDA receptor subunits to synaptic specializations, second, to determine whether different subunits of the AMPA glutamate receptor are expressed at the same synapse and to determine whether AMPA and NMDA receptor subunits co-localize at individual synapses, and third, to characterize, on morphological and neurochemical grounds, the terminals that form receptor-positive synapses in order to gain insight as to their origin.

Some of the data reported in this manuscript have been presented in abstract form (Clarke and Bolam, 1997).

MATERIALS AND METHODS

Preparation of tissue

Tissue was obtained from three female Wistar rats (150–250 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.

The animals were anaesthetised with pentobarbitone (Sagatal, 200 mg/kg) and then perfused via the heart with

Fig. 1. Pairs of electron micrographs of serial sections of synaptic terminals in the entopeduncular nucleus (EP; A–F) and subthalamic nucleus (STN; G,H) that were immunolabelled to reveal AMPA receptor subunit–immunoreactive sites using the postembedding immunogold method. **A,B:** A terminal forming an asymmetrical synaptic contact (arrows) with a dendrite (d_1). The synapse is immunopositive for both the GluR1 (A) and GluR2/3 (B) subunits. An additional terminal forming an asymmetrical synapse (arrowhead) with a different dendrite (d_2) is immunonegative for both subunits. **C,D:** Two terminals forming asymmetrical synaptic contacts (arrows) with a dendrite (d). The synapse formed by bouton b_1 is immunopositive for both the GluR2/3 and GluR4 subunits, and the synapse formed by bouton b_2 is immunopositive for the GluR4 subunit only; however the synaptic specialization was not present in the GluR2/3-labelled section. An additional terminal is immunopositive for the GluR2/3 subunit only and forms an asymmetrical synapse (arrow/arrowhead) with a spine (s). **E,F:** A terminal forming a perforated asymmetrical synaptic contact (arrows) with a dendrite (d_1) that is immunopositive for both the GluR2/3 (E) and GluR4 (F) subunits. A second terminal forms an asymmetrical synapse (arrowhead) with an adjacent dendrite (d_2) but is immunonegative for both subunits. **G,H:** A terminal forming an asymmetrical synaptic contact (arrow) with a dendrite (d) of a neuron in the subthalamic nucleus that is immunopositive for both the GluR1 (G) and GluR2/3 (H) subunits. Scale bars = 0.5 μm in A (for A–D), 0.5 μm in E (for E–H).

100 ml phosphate-buffered saline (PBS; 0.01 M phosphate, pH 7.4) 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, pH 7.4) over 20 minutes. Following fixation, the brain was removed from the cranium, divided

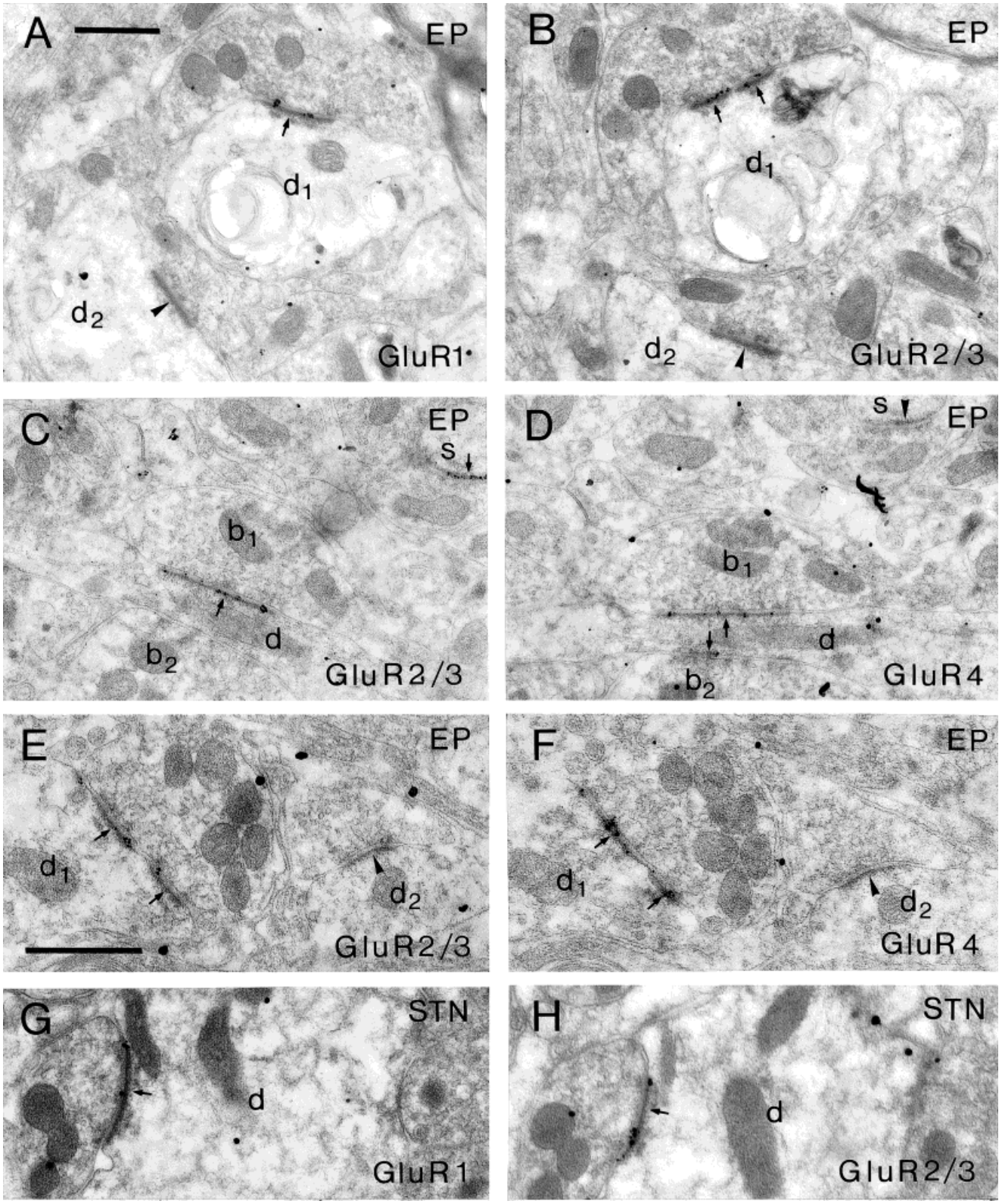

Figure 1

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 EP and the STN 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 EP and the STN were trimmed from the 500-µm sections and washed in PB. They were then placed in 0.5 M sucrose in PB for 15 minutes followed by 1 M sucrose in PB for 2 hours for cryoprotection before they were slammed on a polished copper block cooled with liquid nitrogen (Reichert MM80E). The slammed blocks of tissue were transferred to a Leica CS Auto at −90°C where freeze-substitution proceeded as follows: 0.5% uranyl acetate in dry methanol at −90°C for 30 hours; dry methanol for 15 hours; the temperature was increased by 10°C/hour to −50°C at which all the following steps were conducted; methanol/Lowicryl HM20 (Agar Scientific Ltd., Stansted, UK) 2:1 for 3 hours, 1:1 for 2 hours, 1:2 for 3 hours; pure Lowicryl for 2 hours; fresh pure Lowicryl overnight. The blocks of tissue were then placed in capsules in fresh Lowicryl and polymerized under UV light for 48 hours. The temperature was then increased at a rate of 10°C/hour to 20°C, and the blocks were removed for trimming and sectioning. Serial ultrathin sections (70 nm) of the EP and STN from the Lowicryl-embedded blocks were cut on a Reichert-Jung Ultracut-E ultramicrotome, and single or pairs of sections were collected on gold or nickel single-slot grids coated with Pioloform (Agar Scientific Ltd.).

Post-embedding immunocytochemistry

Post-embedding immunogold labelling was carried out essentially as described previously (Nusser et al., 1995; Lujan et al., 1996). Briefly, the sections were incubated on drops of blocking solution consisting of Tris-buffered saline, pH 7.6 (TBS), containing 20% normal goat serum (NGS) for 45 minutes. They were then incubated overnight at room temperature on drops of primary antibodies in a humid chamber. Two protocols were used:

1. Adjacent sections on three sequential grids were incubated in solutions of antibodies, raised in rabbits, against AMPA receptor subunits as follows: grid (i) anti-GluR1 [AB1504], grid (ii) anti-GluR2/3 [AB1506], grid (iii) anti-GluR4 [AB1508] (Chemicon International Ltd, Harrow, UK; Petralia and Wenthold, 1992; Wenthold et al., 1992; Chen et al., 1996) at a concentration of 5 µg/ml diluted in TBS containing 5% NGS.
2. Adjacent sections on four sequential grids were incubated in solutions as follows: grid (i) anti-NR1 (60021A; 17 µg/ml; raised in mice; Pharmingen, Becton Dickinson, Oxford, UK; Siegel et al., 1995), grid (ii) anti-glutamate (1:500–1,000 dilution; Arnel Products Co., NY; Hepler et al., 1988; Petrusz et al., 1990; Abdullah et al., 1992), grid (iii) anti-GluR2/3 (AB1506; 5 µg/ml; Chemicon International Ltd), grid (iv) anti-γ-aminobutyric acid (GABA; 1:2,000–10,000 dilution; code 9; Hodgson et al., 1985; Somogyi and Hodgson, 1985; Somogyi et al., 1985) raised in rabbits diluted in TBS containing 5% NGS.

After several washes in TBS, most of the sections were incubated in goat anti-rabbit IgG conjugated to gold (1.4-nm-diameter gold particles, 1:100, for the GluR1, GluR2/3, and GluR4 antibodies; 10-nm-diameter colloidal gold particles, 1:80, for the GABA and glutamate; Nanoprobes, Stony Brook, NY), except the NR1 sections, which were incubated in goat anti-mouse IgG conjugated to 1.4-nm gold (1:100; Nanoprobes) diluted in TBS containing 5% NGS. After washing in TBS, the sections that had been incubated in 10-nm colloidal gold were washed in water, dried, and then contrasted in 1% uranyl acetate in water for 30–90 minutes, washed in water, and dried again before being lead stained and examined in a Philips CM10 or CM100 electron microscope. The sections that had been incubated in 1.4-nm gold were washed in TBS and then post-fixed in 2% glutaraldehyde in TBS for 2 minutes. They were washed in water, and the labelling was silver-intensified by using a silver enhancement kit (HQ Silver, Nanoprobes) for 5 minutes. After washing in water, the sections were dried and then contrasted in 1% uranyl acetate in water for 30–90 minutes, washed in water, and dried again before being lead stained and examined in the electron microscope.

Sections derived from the neostriatum were incubated in parallel to act as a positive control (Bernard et al., 1997a). The specificity of the secondary reagents was confirmed by the different patterns of immunolabelling obtained on serial sections using different primary antibodies but the same secondary antibodies. No immunolabelling was obtained with any of the secondary antibodies when the primary antibodies were omitted.

Analysis of material

Analysis of the distribution of immunogold particles for GABA, glutamate, and the glutamate receptor subunits was carried out at the electron microscopic level. A synapse was considered immunopositive for a receptor subunit when it was associated with two or more immunoparticles (Baude et al., 1995; Popratiloff et al., 1996; Bernard et al., 1997a; Rubio and Wenthold, 1997). In addition to the small immunoparticles associated with synaptic specializations, occasional larger, irregularly shaped silver deposits that were randomly distributed over the sections were observed (e.g., Fig. 1D,E). These are an artifact caused by the silver intensification reaction and do not represent immunolabelling for the receptor subunits. Different search strategies were adopted for the two experiments:

1. For the AMPA receptor subunit–immunolabelled sections, the number of immunopositive synapses was greatest with the antibody to the GluR2/3 subunits. Consequently, when looking for colocalization of the AMPA receptor subunits, the GluR1- and GluR4-immunolabelled sections were examined initially, all immunopositive synapses were photographed, and the same synapse was then identified in the adjacent GluR2/3-immunolabelled section and photographed.
2. For the receptor- and neurotransmitter-immunolabelled sections, the number of immunopositive synapses was greatest with the antibody to the GluR2/3 subunits. Consequently, when looking for colocalization of the AMPA and NMDA receptor subunits, the NR1-immunolabelled sections were usually examined initially; all immunopositive synapses were photographed and then identified in the adjacent GluR2/3-immunolabelled section and photographed. The immunopositive synapses were then identified and photographed in the adjacent glutamate- and GABA-labelled sections, to determine the levels of fixed amino acids in the presynaptic terminals.

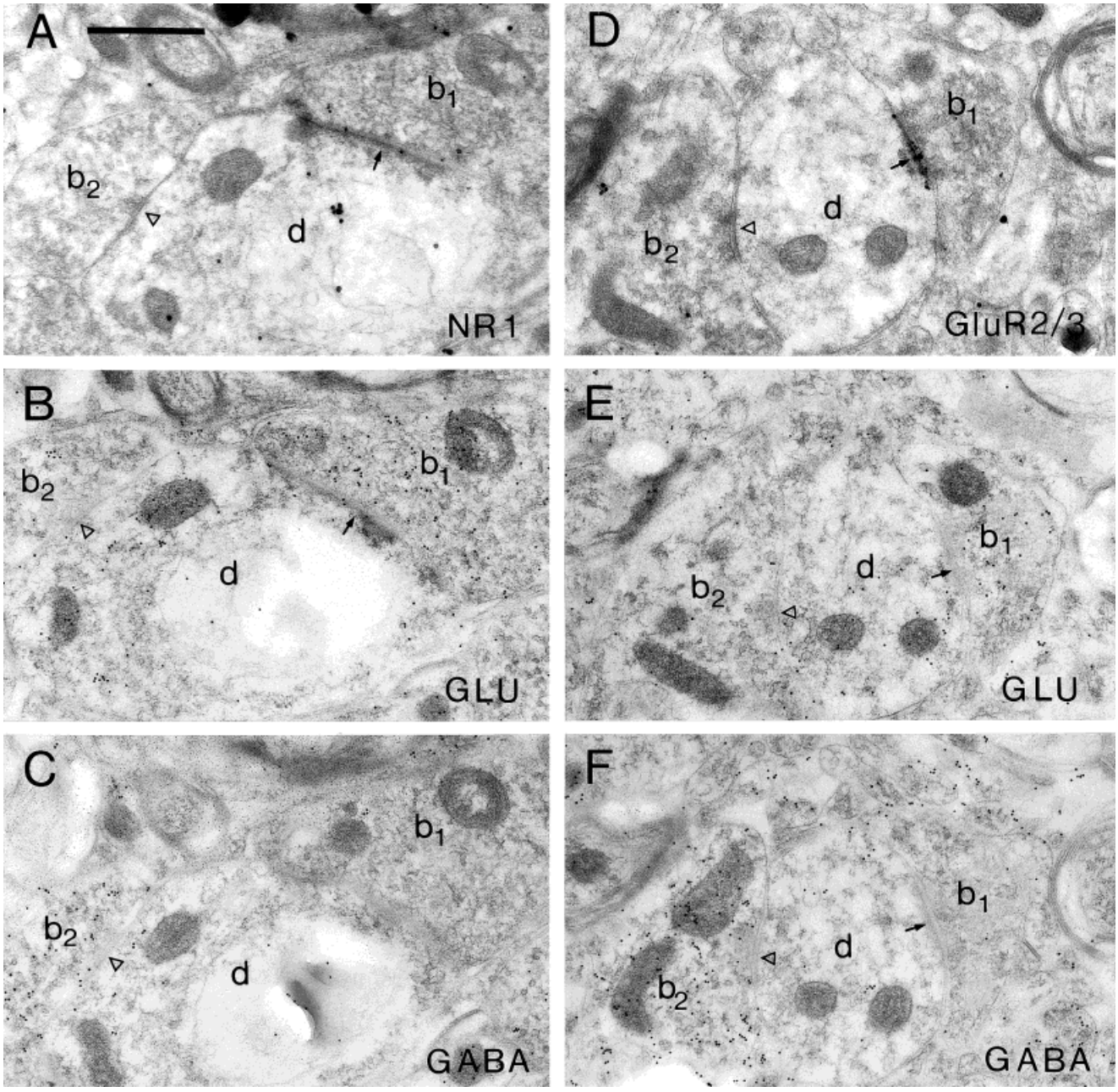

Fig. 2. Serial sections of synaptic terminals in the entopeduncular nucleus that were immunolabelled to reveal NR1-, glutamate- (GLU), GluR2/3-, or GABA-immunoreactive sites by using the postembedding immunogold method. **A–C:** Sections of a bouton (b_1) that forms an asymmetrical synapse (arrow) with a dendrite (d). The synapse is immunopositive for the NR1 subunit (A) and the terminal is associated with a relatively high level of glutamate immunoreactivity (B; index of glutamate immunoreactivity of 5.31) and is GABA immunonegative (C; index of GABA immunoreactivity of 0.21). An adjacent bouton (b_2) forms a synapse that is symmetrical (open arrowhead) with the same dendrite and has a low level of glutamate immunoreactivity (index = 0.82), but is GABA-immunopositive (index = 15.43). This bouton has the characteristics of a terminal derived from the striatum. **D–F:** Sections of a bouton (b_1) that forms an asymmetrical synapse (arrow) with a dendrite (d). The synapse is immunopositive for the GluR2/3 subunit (D), and the bouton is associated with a relatively high level of glutamate immunoreactivity (E; index = 3.13) and is GABA-immunonegative (F; index = 1.42). The same dendrite also receives synaptic input from a bouton (b_2) that forms symmetrical synaptic contact that is receptor-immunonegative (open arrowhead) and has a low level of glutamate immunoreactivity (E; index = 0.74), but is GABA-immunopositive (F; index = 19.37). This bouton has the characteristics of a terminal derived from the globus pallidus. Scale bar = 0.5 µm.

In order to quantify the immunoreactivity for glutamate and GABA in the terminals forming synapses that were immunopositive for the glutamate receptor subunits, the density (particles/µm^2) of immunogold particles overlying individual structures was calculated (Somogyi et al., 1986). This value was corrected for nonspecific binding of the antibody to tissue-free resin by subtracting the density of gold particles overlying the lumen of capillaries in the

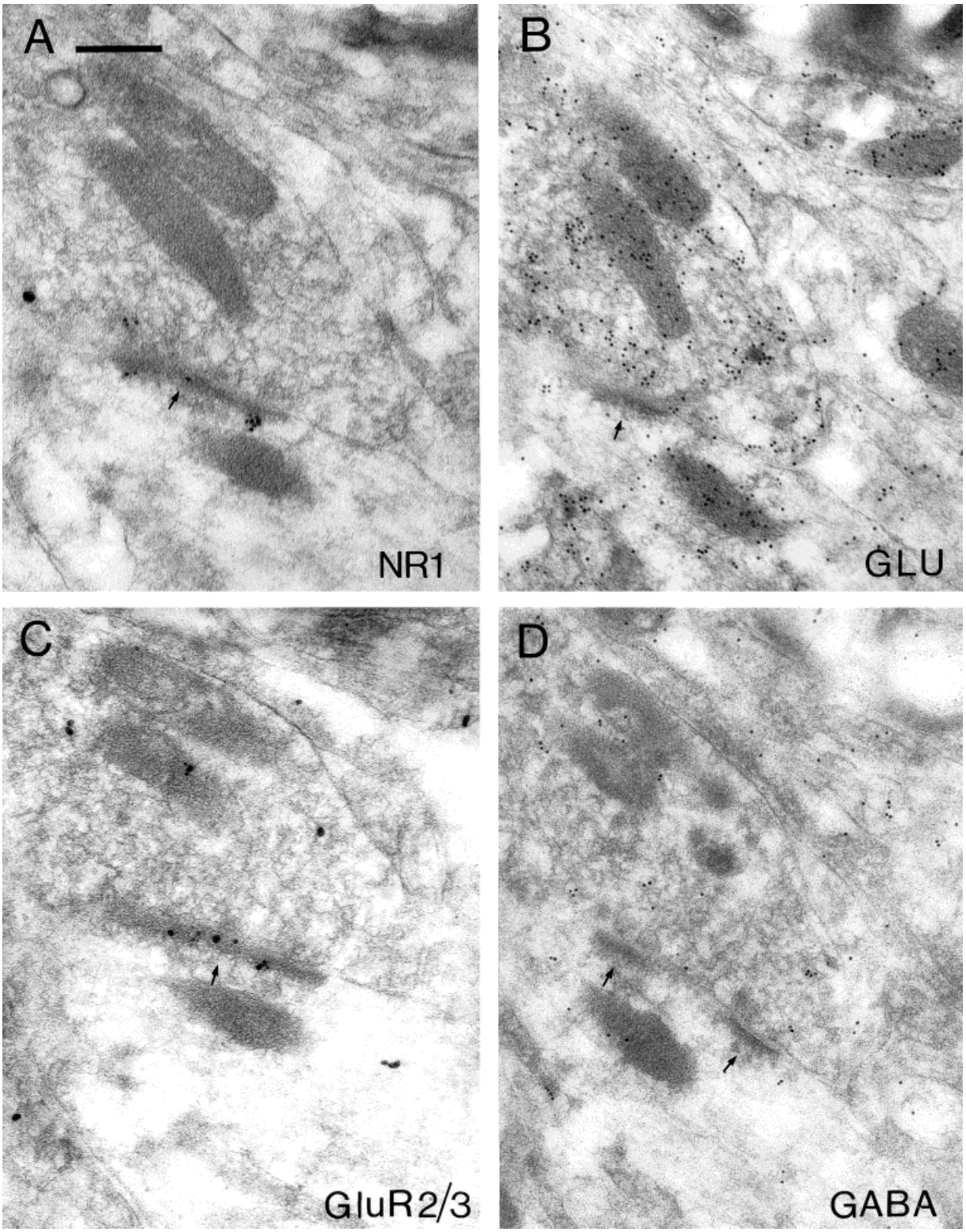

Fig. 3. Serial sections of a bouton in the entopeduncular nucleus that were immunolabelled to reveal NR1-, glutamate- (GLU), GluR2/3-, or GABA-immunoreactive sites by using the postembedding immunogold method. **A–D:** Sections through a bouton that forms an asymmetrical synapse (arrow) with a dendrite. The synapse is immunopositive for both the NR1 subunit (A) and the GluR2/3 subunit (C). The bouton is associated with a relatively high index of glutamate immunoreactivity (B; index = 5.18) but is GABA-immunonegative (D; index = 2.87). Scale bar = 0.25 μm.

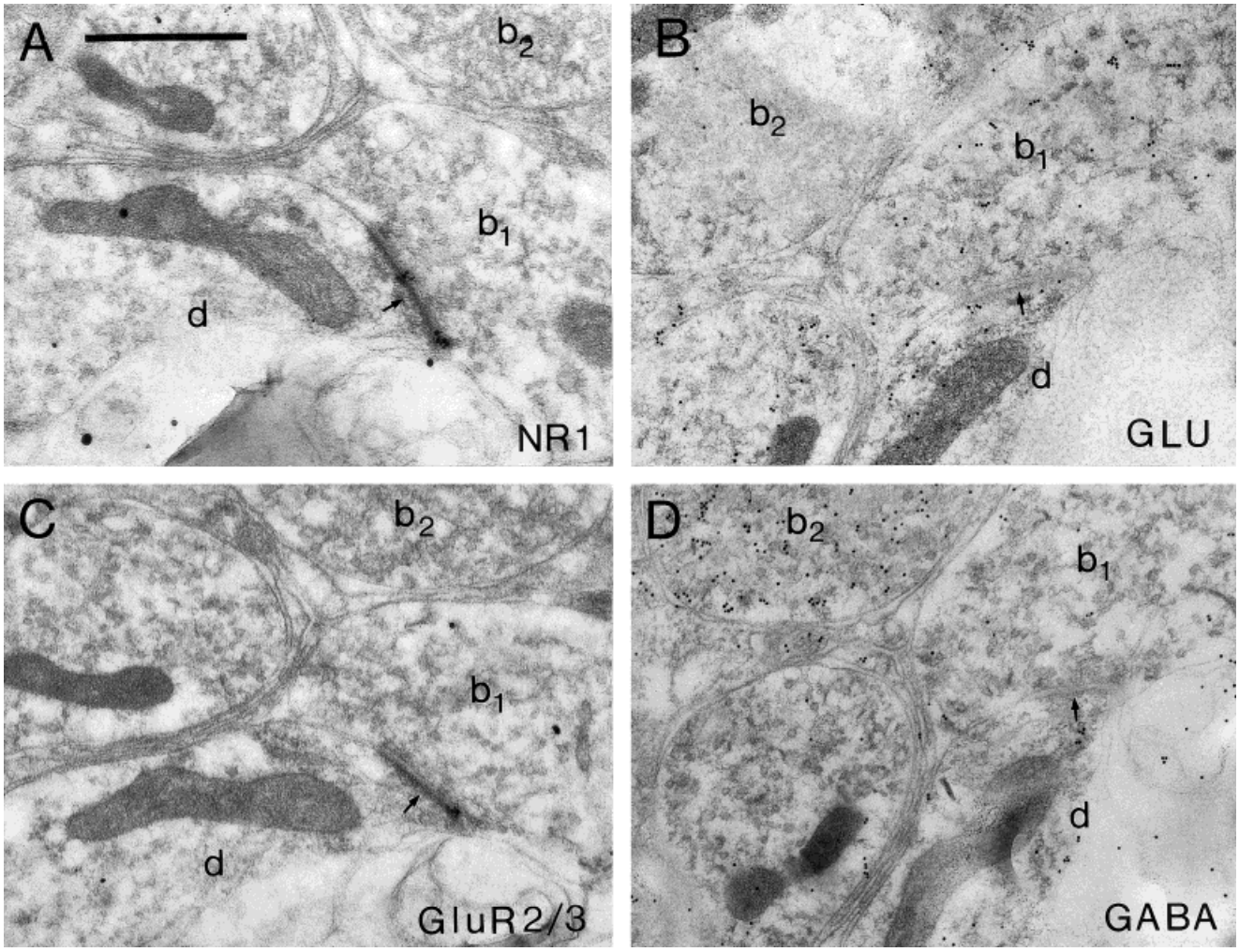

Fig. 4. Serial sections of a bouton (b1) in the entopeduncular nucleus that were immunolabelled to reveal NR1-, glutamate- (GLU), GluR2/3-, or GABA-immunoreactive sites using the postembedding immunogold method. **A–D:** The bouton (b_1) forms an asymmetrical synapse (arrow) with a dendrite (d). The synapse is immunopositive for both the NR1 subunit (A) and the GluR2/3 subunit (C). The terminal is associated with a relatively low index of glutamate immunoreactivity (B; index = 1.58) and is GABA-immunonegative (D; index = 3.81). Another bouton (b_2) does not make synaptic contact in these micrographs and has a low level of glutamate immunoreactivity (index = 0.45) but is GABA-immunopositive (index = 22.28). Note that the images in B and D are rotated in an anticlockwise direction by about 110° with respect to A and C. Scale bar = 0.5 μm.

same ultrathin section. The corrected density overlying each terminal was then normalized. This was achieved for glutamate by normalizing with respect to the labelling associated with GABA-immunopositive terminals forming symmetrical synapses on the same ultrathin section (average of 14 terminals per section from two animals [EP], 21 terminals per section from two animals [STN]) and expressed as an index of glutamate immunoreactivity. The index of GABA immunoreactivity for each terminal was similarly calculated by normalizing the corrected density with respect to the labelling associated with GABA-immunonegative terminals forming asymmetrical synapses on the same ultrathin GABA-immunolabelled section (average of eight terminals per section from two animals [EP], 13 terminals per section from two animals [STN]). A terminal with an index of GABA immunoreactivity of greater than five was considered GABA-immunopositive. The glutamate and GABA-immunoreactivity in-

populations of terminals forming synapses that were immunopositive for the glutamate receptor subunits was compared statistically using the Mann-Whitney U-test, and a value of $P < .01$ was considered significant. Issues concerning the quantification of immunogold labelling for GABA and glutamate have been discussed extensively on previous occasions (Somogyi et al., 1986; Ottersen, 1989; Bevan et al., 1995; Clarke et al., 1997).

The subsynaptic distribution of the AMPA and NMDA receptor subunits was determined by measuring the length of the postsynaptic specialization and the distance of each immunometal particle from the centre of the synapse and normalizing it to take into account the different sizes of synapses. The normalized data for the whole population were expressed as the proportion of immunoparticles in five bins along half the width of the synapse. The subsynaptic distributions of the receptor subunits was compared statistically by using the Kruskal-Wallis test, and a value

of $P < .01$ was considered significant. Although the distribution of immunolabelling at an individual synapse can only be determined by serial section analysis, the data in these analyses represent the average distribution over the whole population of synapses sampled.

RESULTS

AMPA and NMDA receptor subunit immunolabelling

Consistent with previous in situ hybridization (Sato et al., 1993; Standaert et al., 1994) and pre-embedding immunocytochemical studies (Petralia and Wenthold, 1992; Martin et al., 1993; Petralia et al., 1994; Bernard et al., 1996, 1997b; Paquet and Smith, 1996), postembedding immunolabelling of sections of the EP and the STN revealed the presence of AMPA and NMDA receptor subunit-immunoreactive sites identified by the presence of silver-intensified immunogold particles. In both regions the majority of immunoparticles were associated with neuronal membranes and most of them were located in asymmetrical synapses (EP: Figs. 1A–F, 2A,D, 3A,C, 4A,C; STN: Figs. 1G,H, 6A,D, 7A,C,E). In previous pre-embedding experiments by peroxidase methods (references as above), immunolabelling was observed in the postsynaptic neuron; therefore, the immunolabelling that we observed at synapses is likely to be associated with the postsynaptic membrane. Occasional clusters of immunoparticles were observed (Fig. 4A,C), which probably do not represent the true distribution of receptors and are likely to be due to limitations in the accessibility of antigenic sites. None of the antibodies against the AMPA receptor subunit or the NR1 receptor subunit labelled symmetrical synapses. Immunogold particles were occasionally observed within perikarya in association with the endoplasmic reticulum. In both regions, the proportion of synapses that were labelled with any of the antibodies was small; the highest frequency of labelling was obtained with the antibody against the GluR2/3 subunit of the AMPA receptor. Technical factors, including the affinity of the antibodies, the accessibility of the antigenic sites, and the differential fixation of the tissue, are likely to influence the proportions of synapses that are immunopositive. The failure of a synapse to be labelled may thus represent an artifact, the presence of receptor in amounts undetectable with this method, or a genuine lack of the antigen (Baude et al., 1995). Consequently we cannot make conclusions about the relative or absolute proportions of synapses labelled with the different antibodies. However, if we assume that conditions are equal for each synapse within an individual section, then the juxtaposition of unlabelled synapses and synapses with various levels of immunoreactivity (Fig. 1A,B,E,F) suggests that the density of antigenic sites is markedly different between different synapses on a single section.

Entopeduncular nucleus. In the EP synapses were positive for the GluR1, GluR2/3, and GluR4 subunits of the AMPA receptor and the NR1 subunit of the NMDA receptor (Figs. 1A–F, 2A,D, 3A,C, 4A,C). The majority of immunopositive synapses were axodendritic, including both large- and small-diameter dendrites (n = 190 of 207 immunopositive synapses observed). Occasional axospinous (n = 16; Fig. 1C) and axosomatic (n = 1; GluR2/3-positive) synapses were also observed. The presynaptic terminals contained large numbers of vesicles that generally accumulated around the active zone and up to four mitochondria. The terminals were occasionally associated with postjunctional dense bodies (Figs. 1A,B, 2D). In addition to the glutamate receptor subunit-immunopositive synaptic input, the dendrites or soma of EP neurons also received synaptic input from terminals that formed receptor-immunonegative synapses that were symmetrical and had the morphological characteristics of terminals derived from the striatum (n = 10; Fig. 2A–C) or globus pallidus (n = 21; Fig. 2D–F; Bolam and Smith, 1992) or were asymmetrical (n = 11). The quantitative analysis of the distribution of immunoparticles at synapses (Fig. 5) revealed that immunolabelling for each of the glutamate receptor subunits was almost exclusively confined to the synaptic specializations. All GluR4 immunolabelling was found within synapses, whereas 4%, 1.5%, and 3% of the immunoparticles near synaptic sites for GluR1, GluR2/3, and NR1, respectively, fell apparently outside of the postsynaptic specialization, within a distance of 25 nm from the edge of the synapse (12% of the width of the postsynap-

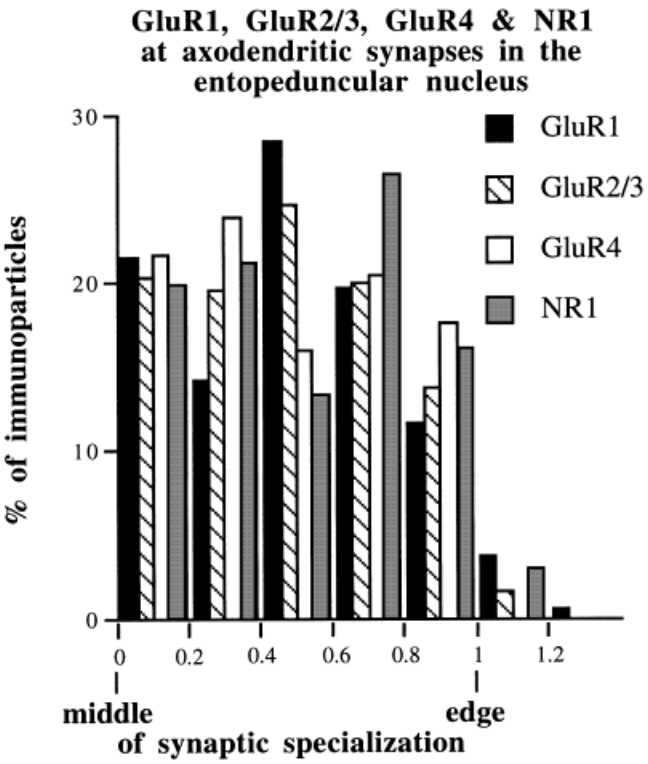

Fig. 5. The average distribution of immunoparticles for GluR1, GluR2/3, GluR4, and NR1 subunits along the synaptic membrane at axodendritic synapses in the entopeduncular nucleus labelled by the postembedding immunogold method. A similar distribution was obtained for all subunits (GluR1, 26 synapses [mean length ± SEM, 419 ± 4 nm], 162 immunometal particles; GluR2/3, 91 synapses [6 synapses examined in two serial sections; mean length ± SEM, 400 ± 3 nm], 779 immunometal particles; GluR4, 45 synapses [10 synapses examined in two serial sections; mean length ± SEM, 449 ± 5 nm], 175 immunometal particles and NR1, 28 synapses [mean length ± SEM, 363 ± 3 nm], 136 immunometal particles). On average, immunoparticles for each subunit are evenly distributed along the synaptic membrane, and the distributions for each subunit are not significantly different from one another (Kruskal-Wallis test). Only synapses labelled with two or more immunoparticles were included in the analysis.

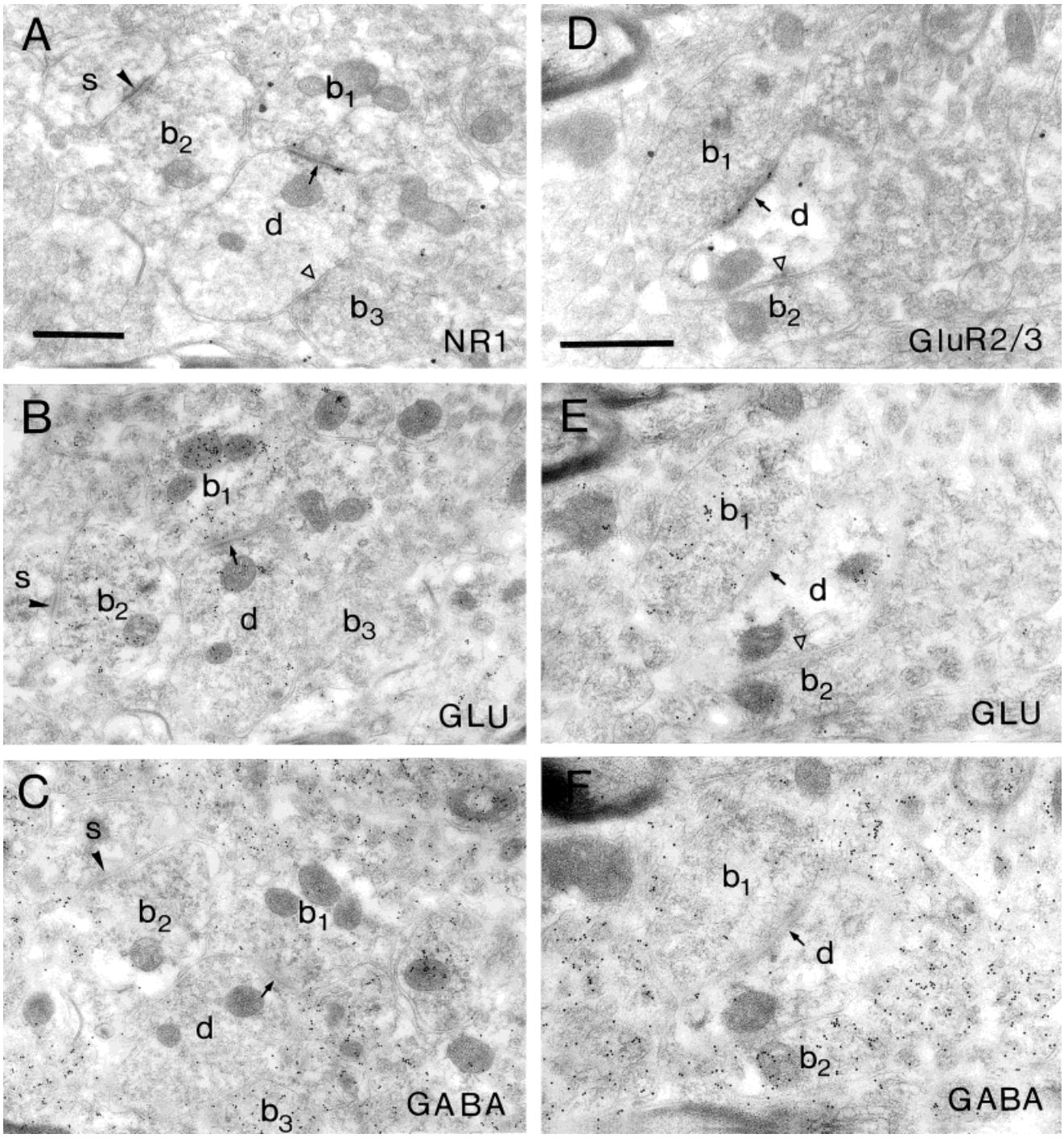

Fig. 6. Serial sections of the subthalamic nucleus that were immunolabelled to reveal NR1-, glutamate- (GLU), GluR2/3-, or GABA-immunoreactive sites by using the postembedding immunogold method. **A–C:** Sections of three boutons (b_1, b_2, and b_3) one of which (b_1) forms an asymmetrical synapse (arrow) with a dendrite (d). The synapse is immunopositive for the NR1 subunit (A), and the terminal is associated with a high index of glutamate immunoreactivity (B; index = 16.90) and is GABA-immunonegative (C; index = 1.00). Bouton b_2 makes asymmetrical synaptic contact (solid arrowhead) with a spine (s) and is also associated with a high index of glutamate immunoreactivity (B; index = 9.53) and is GABA-immunonegative (C; index = 0.87), but the third bouton (b_3) forms symmetrical synaptic contact that is receptor-immunonegative (open arrowhead), with the dendrite (d) and has undetectable levels of glutamate immunoreactivity (B), but is GABA-immunopositive (C; index = 10.30). This bouton has the characteristics of terminal derived from the globus pallidus. **D–F:** Sections of two boutons (b_1 and b_2), one of which (b_1) forms an asymmetrical synapse (arrow) with a dendrite (d). The synapse is immunopositive for the GluR2/3 subunit (D), and the bouton is associated with a relatively high index of glutamate immunoreactivity (E; index = 6.73) and is GABA-immunonegative (F; index = 1.07). The second bouton (b_2) forms symmetrical synaptic contact that is receptor-immunonegative (open arrowhead) with a dendrite (d) and has a low level of glutamate immunoreactivity (E; index = 1.05), but is GABA-immunopositive (F; index = 13.00). This bouton has the characteristics of a terminal derived from the globus pallidus. Scale bars = 0.5 μm in A (for A–C), 0.5 μm in D (for D–F).

tic membrane specialization; Fig. 5). However, this could be due to steric distortion between the image of the membrane specialization formed from the whole thickness of the section and the most superficial surface available for the antibody. On average for the whole population of synapses analysed, the immunolabelling was evenly dis-

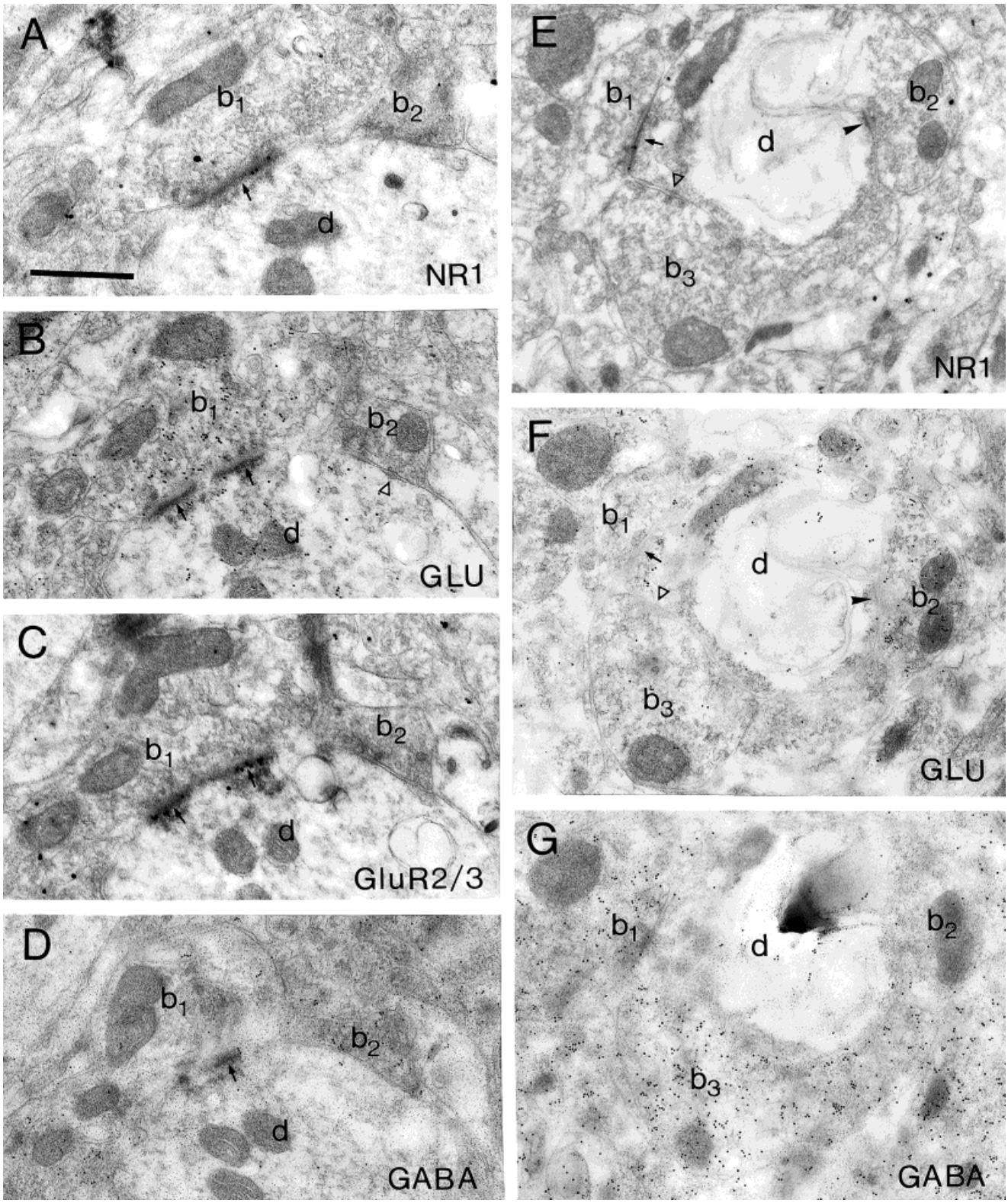

Figure 7

tributed along the synaptic specialization, and there were no significant differences between the distributions of the different subunits of the AMPA receptor or the NR1 subunit of the NMDA receptor along the synapse (Kruskal-Wallis, $P = .611$; Fig. 5).

Subthalamic nucleus. In the STN, immunolabelling for the GluR1 and GluR2/3 subunits of the AMPA receptor and NR1 subunit of the NMDA receptor was observed at asymmetrical synapses (Fig. 1G,H, 6A,D, 7A,C,E). Immunolabelling for the GluR4 subunit was not detected, which probably reflects the low density of GluR4-immunoreactive structures that has been observed in the STN in pre-embedding immunostained tissue (Petralia and Wenthold, 1992; Martin et al., 1993; Bernard et al., 1997b). The majority of positive synapses labelled with each of the antibodies were axodendritic, most of which involved small-diameter (presumably distal) dendrites or occasional large-diameter (presumably proximal) dendrites (113 of 120 immunopositive synapses observed). Additionally, a few axospinous (n = 6) and axosomatic (n = 1; NR1-positive) synapses were also labelled. The presynaptic terminals contained large numbers of vesicles, up to four mitochondria, and were also occasionally associated with postjunctional dense bodies (Figs. 1G,H, 7A–D). Terminals forming symmetrical synapses that had the morphological characteristics of those derived from the globus pallidus (n = 28; Fig. 6D–F, 7E–G; Smith et al., 1990; Bevan et al., 1997) and terminals forming receptor-immunonegative asymmetrical synapses (n = 9) were observed in synaptic contact with the dendrites or soma of STN neurons that also received glutamate receptor subunit-immunopositive inputs. As was the case in the EP, the quantitative analysis of the distribution of immunoparticles for each of the glutamate receptor subunits revealed that most of the immunolabelling that was associated with synapses was confined to the synaptic specializations. Two percent (GluR1), 5% (GluR2/3), and 3% (NR1) of immunoparticles near synaptic sites fell apparently outside of the postsynaptic specialization, within a distance of 25 nm, which is equivalent to 10% of the width of the postsynaptic membrane specialization (Fig. 8), a value that again could be due to sterical distortion. On average for the whole population of synapses analysed, the immunolabelling was evenly distributed along the synaptic specialization, and the distributions of the different subunits along the synapse were not significantly different (Kruskal-Wallis, $P = .518$; Fig. 8).

Co-localization of immunolabelling for glutamate receptor subunits at synapses

The analysis of serial sections on separate grids that were immunolabelled with different antibodies revealed that individual synapses in both the EP and the STN expressed immunoreactivity for multiple AMPA receptor subunits and for both AMPA and NMDA receptor subunits (Figs. 1A–H, 3A,C, 4A,B, 7A,C). In the EP, 69% of those synapses that were identified as GluR1 immunopositive and subsequently identified in the serial section were also immunopositive for the GluR2/3 subunit (n = 20). Similarly, 61% of GluR4-immunopositive synapses and 44% of NR1-immunopositive synapses that were identified in the serial section were also immunopositive for the GluR2/3 subunit (n = 20 and 12, respectively). In the STN similar

Fig. 7. Serial sections of the subthalamic nucleus that were immunolabelled to reveal NR1-, glutamate- (GLU), GluR2/3-, or GABA-immunoreactive sites by using the postembedding immunogold method. **A–D:** Sections through a bouton (b_1) that forms an asymmetrical synapse (arrow) with a dendrite (d). The synapse is immunopositive for both the NR1 subunit (A) and the GluR2/3 subunit (C). The terminal is associated with a high index of glutamate immunoreactivity (B) (3.53) and is GABA-immunonegative (D; index = 1.28). An adjacent bouton (b_2) forms symmetrical synaptic contact with a dendrite (d); the synapse is receptor-immunonegative (open arrowhead). The bouton has undetectable levels of glutamate immunoreactivity (B), but is GABA-immunopositive (index = 15.19). This bouton has the characteristics of a terminal derived from the globus pallidus. **E–G:** Sections of three boutons (b_1, b_2, and b_3) that form synapses with a dendrite (d). Terminal bouton, b_1, forms an asymmetrical synapse (arrow) with a dendrite, is immunopositive for the NR1 subunit (E), and has undetectable levels of glutamate immunoreactivity (F) but is GABA-immunopositive (G; index = 12.89). The bouton b_2 makes asymmetrical synaptic contact with a dendrite, is receptor-immunonegative (solid arrowhead), and is associated with a high index of glutamate immunoreactivity (F; index = 13.74) and is GABA-immunonegative (G; index = 2.17). The bouton b_3 forms a symmetrical synapse that is receptor-immunonegative (open arrowhead) and has a low level of glutamate immunoreactivity (F; index = 1.98), but is GABA-immunopositive (G; index = 13.91). This bouton has the characteristics of a terminal derived from the globus pallidus. Scale bar = 0.5 μm (for A–G).

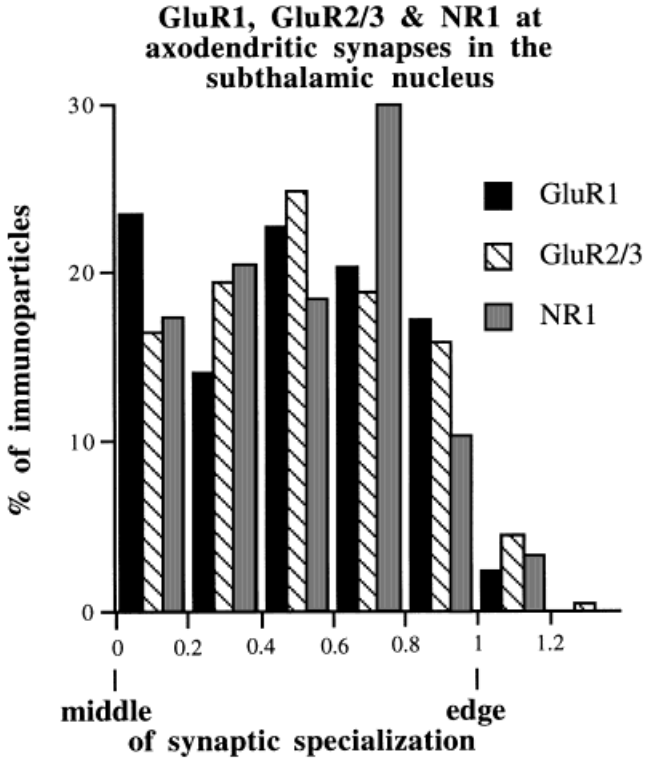

Fig. 8. The average distribution of immunoparticles for GluR1, GluR2/3, and NR1 subunits along the synaptic membrane at axodendritic synapses in the subthalamic nucleus labelled by the postembedding immunogold method. A similar distribution was obtained for all subunits (GluR1, 30 synapses [mean length ± SEM, 338 ± 3 nm], 128 immunometal particles; GluR2/3, 39 synapses [mean length ± SEM, 372 ± 3 nm], 298 immunometal particles and NR1, 44 synapses [mean length ± SEM, 391 ± 2 nm], 185 immunometal particles). On average, immunoparticles for each subunit are evenly distributed along the synaptic membrane, and the distributions for each subunit are not significantly different from one another (Kruskal-Wallis test). Only synapses labelled with two or more immunoparticles were included in the analysis.

values were obtained. Thus, 73% of GluR1-immunopositive synapses and 32% of NR1-immunopositive synapses were found also to be immunopositive for the GluR2/3 subunit (n = 11 and 10, respectively).

Glutamate and GABA immunoreactivity in the entopeduncular nucleus and subthalamic nucleus

The patterns of glutamate and GABA immunolabelling in the EP and STN were similar to those observed previously by the postembedding method in epoxy resin–embedded tissue (Bolam and Smith, 1992; Bevan and Bolam, 1995; Bevan et al., 1995; Clarke et al., 1996, 1997). Thus, in the EP and the STN immunogold particles were concentrated over synaptic terminals and preterminal boutons (Figs. 2–4, 6). Glutamate immunolabelling was mainly associated with the vesicles and mitochondria of terminals forming asymmetrical synapses, many of which were identified, in serial sections, as GABA-immunonegative. Immunolabelling for GABA was mainly associated with the vesicles and mitochondria of terminals forming symmetrical synapses. The quantitative analysis revealed that the levels of glutamate immunolabelling in boutons forming asymmetrical synaptic contacts were significantly higher than the levels associated with boutons forming symmetrical synaptic contacts (Figs. 2B,E, 3B, 6B,E, 7B). Boutons forming symmetrical synaptic contacts, on the other hand, exhibited levels of GABA immunoreactivity greater than the levels associated with any other structure, including cell bodies, dendritic structures, and the majority of terminals forming asymmetrical synaptic contacts (Figs. 2C,F, 6C,F, 7D,G). Additionally, as has been previously observed (Bevan and Bolam, 1995; Bevan et al., 1995; Clarke et al., 1997), a small proportion of boutons forming asymmetrical synaptic contacts in the STN were GABA-immunopositive (Fig. 7G) and were usually associated with a relatively low level of glutamate immunoreactivity (Fig. 7F).

Glutamate and GABA immunoreactivity at AMPA and NMDA receptor subunit–immunopositive synapses

The multiple immunolabelling on serially adjacent sections revealed that the terminals presynaptic to synapses that were immunopositive for glutamate receptor subunits in both the EP and the STN possessed significantly higher levels of glutamate immunoreactivity than the appropriate reference terminals and were GABA-immunonegative (Figs. 9–12). Thus, boutons that formed synapses that were positive for the NR1 subunit of the NMDA receptor or the GluR2/3 subunit of the AMPA receptor, or were positive for both of them, had high levels of glutamate immunoreactivity overlying them. The same boutons were GABA-immunonegative. However, it is clear from the distributions of the index of immunoreactivity for the amino acids (Figs. 9–12) that the levels of immunolabelling in the boutons forming receptor-positive synapses were heterogeneous with respect to glutamate and GABA. Thus, in the EP, some boutons that

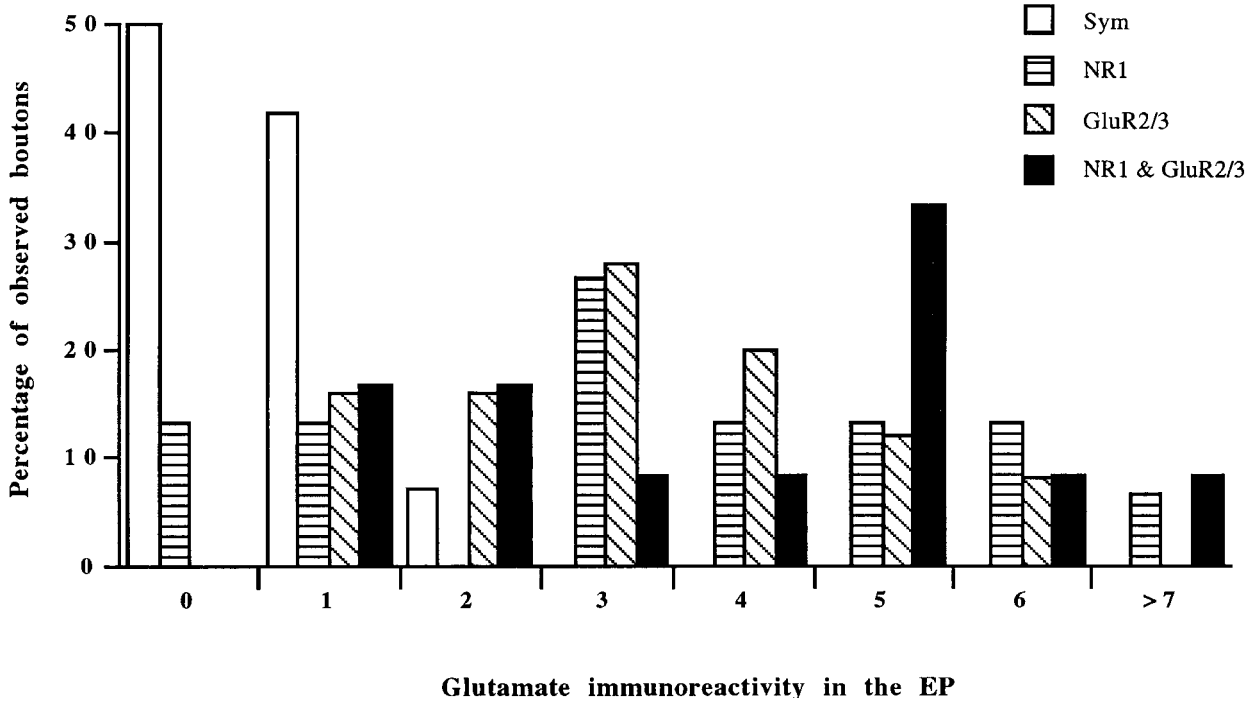

Fig. 9. Frequency distribution of the levels of glutamate immunoreactivity associated with presynaptic terminals of glutamate receptor subunit–positive synapses and of symmetrical synapses in the entopeduncular nucleus. Glutamate immunoreactivity is represented as the ratio of the density of immunogold particles overlying the synaptic terminals to that overlying terminals forming symmetrical synapses in the same section. The receptor-immunopositive terminals had significantly greater levels of glutamate immunoreactivity than terminals forming symmetrical synapses (Mann-Whitney U-test; $P < .0001$). Sym, terminals forming symmetrical synapses (mean glutamate immunoreactivity ± SEM = 0.99 ± 0.08; range: 0–2.37, n = 43); NR1, terminals forming NR1-positive synapses (mean ± SEM = 3.78 ± 0.57; range: 0.37–7.51, n = 15); GluR2/3; terminals forming GluR2/3-positive synapses (mean ± SEM = 3.74 ± 0.29; range: 1.06–6.33; n = 25); NR1 & GluR2/3, terminals forming synapses that were positive for both antibodies (mean ± SEM = 4.48 ± 0.67; range: 1.04–9.15; n = 12).

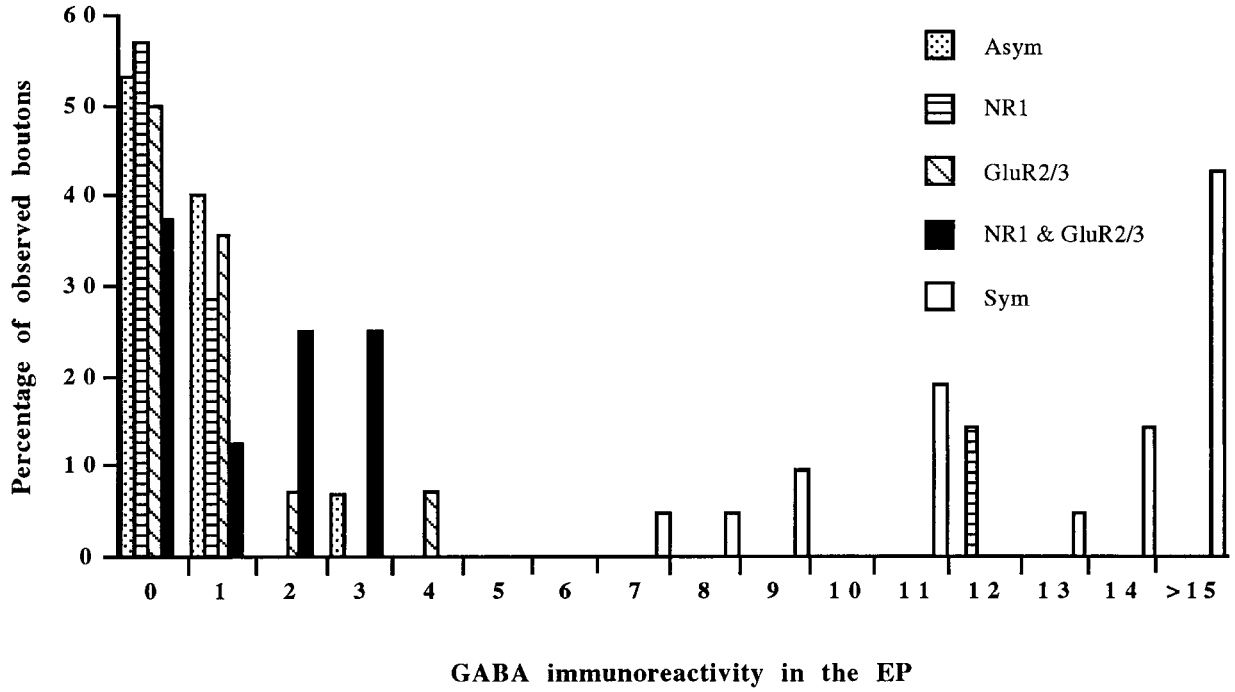

Fig. 10. Frequency distribution of the levels of GABA immunoreactivity associated with the terminals presynaptic to glutamate receptor subunit-immunopositive and -immunonegative synapses in the entopeduncular nucleus. GABA immunoreactivity is represented as the ratio of the density of immunogold particles overlying the synaptic terminals to that overlying terminals forming asymmetrical synapses in the same section. The terminals presynaptic to both receptor-immunopositive and -immunonegative asymmetrical synapses have significantly lower levels of GABA immunoreactivity compared to that associated with terminals forming symmetrical synapses (Mann-Whitney U-test; $P < .0001$). Sym, terminals forming symmetrical synapses and immunonegative for the glutamate receptor subunits (mean GABA immunoreactivity ± SEM = 14.82 ± 1.16; range: 7.14–28.93; n = 21); NR1, terminals forming NR1-positive synapses (mean ± SEM = 2.41 ± 1.61; range: 0.21–12.00; n = 7); GluR2/3, terminals forming GluR2/3-positive synapses (mean ± SEM = 1.30 ± 0.30; range: 0.11–4.39; n = 14); NR1 & GluR2/3, terminals forming synapses that were positive for both antibodies (mean ± SEM = 1.97 ± 0.51; range: 0.33–3.88; n = 8); Asym, terminals forming asymmetrical synapses that did not display immunolabelling for any of the glutamate receptor subunits (mean ± SEM = 1.00 ± 0.24; range: 0–3.55; n = 15).

formed synapses that were immunopositive for NR1 and/or GluR2/3 had levels of glutamate immunoreactivity (mean ± SEM = 1.11 ± 0.19; n = 7) comparable to the levels in terminals forming symmetrical synapses (i.e. GABAergic terminals). The levels of GABA immunoreactivity in these terminals (mean ± SEM = 1.67 ± 0.58; n = 5), was however, lower than in known GABAergic terminals (i.e., still less than five). Additionally, in the STN some boutons that were presynaptic to glutamate receptor subunit-immunopositive synapses had levels of glutamate (mean ± SEM = 0.72 ± 0.25; n = 7) comparable to terminals forming symmetrical synapses; however, in contrast to those in the EP, these terminals also had high levels of GABA (mean ± SEM = 11.09 ± 2.65; n = 7) comparable to known GABAergic terminals (n = 4 NR1-positive, n = 1 GluR2/3-positive, and n = 2 NR1 and GluR2/3-positive).

DISCUSSION

AMPA and NMDA receptor subunit distribution in the entopeduncular nucleus and subthalamic nucleus

The data in the present study demonstrate that immunolabelling for AMPA and NMDA receptor subunits was localized primarily on membranes and concentrated at the active zones of asymmetrical synapses. This observation is consistent with that seen in other post-embedding immunolabelling studies of ionotropic glutamate receptors both in the basal ganglia and in other regions of the brain (Nusser et al., 1994; Baude et al., 1995; Kharazia et al., 1996; Matsubara et al., 1996; Popratiloff et al., 1996; Bernard et al., 1997a; Landsend et al., 1997; Nusser and Somogyi, 1997; Rubio and Wenthold, 1997; Somogyi et al., 1998). Since it is recognised that postembedding immunolabelling, although allowing better access of the antibodies to the synapses, is less sensitive than pre-embedding methods (Baude et al., 1995; Lujan et al., 1996; Bernard et al., 1997a), our findings suggest that the highest density of AMPA and NMDA receptor subunits in the EP and STN is within asymmetrical synaptic specializations. Furthermore the quantitative analysis of the distribution of the immunolabelling associated with synapses demonstrated that the number of immunogold particles fell to virtually zero within 25 nm of the edge of the synaptic specialization (Figs. 5, 8). Presumably because of technical limitations, only a small proportion of synapses were labelled; we therefore cannot make quantitative statements concerning the absolute numbers or genuine proportions of receptor-positive synapses. Nevertheless, the results indicate that there is a wide range of receptor densities within postsynaptic membrane specializations at different synapses.

The profile of ionotropic receptor subunit immunolabelling in the EP (GluR1, GluR2/3, and GluR4 subunits of the

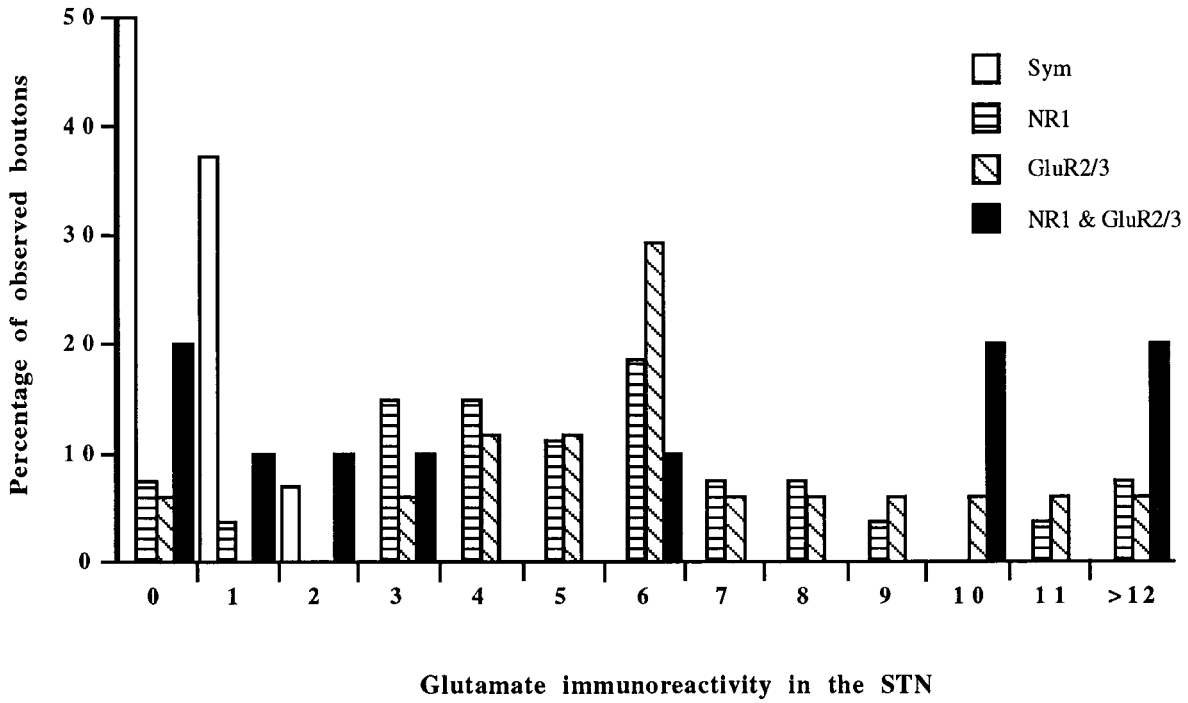

Fig. 11. Frequency distribution of the levels of glutamate immunoreactivity associated with terminals presynaptic to glutamate receptor subunit-immunopositive and -immunonegative synapses in the subthalamic nucleus. Glutamate immunoreactivity is represented as the ratio of the density of immunogold particles overlying the synaptic terminals to that overlying terminals forming symmetrical synapses in the same section. The receptor-immunopositive terminals had significantly greater levels of glutamate immunoreactivity than terminals forming symmetrical synaptic contacts (Mann-Whitney U-test; $P < .0001$). Sym, terminals forming symmetrical synapses and immunonegative for the glutamate receptor subunits (mean glutamate immunoreactivity ± SEM = 1.00 ± 0.11; range: 0–2.88; n = 43); NR1, terminals forming NR1-positive synapses (mean ± SEM = 6.17 ± 0.75; range: 0–16.90; n = 27); GluR2/3, terminals forming GluR2/3-positive synapses (mean ± SEM = 6.76 ± 0.69; range: 0.86–12.15; n = 17); NR1 & GluR2/3, terminals forming synapses that were positive for both antibodies (mean ± SEM = 6.11 ± 1.64; range: 0–13.40; n = 10).

AMPA receptor and the NR1 subunit of the NMDA receptor) and the STN (GluR1 and GluR2/3 subunits and the NR1 subunit) is largely consistent with the results of both in situ hybridization studies (Sato et al., 1993; Standaert et al., 1994) and pre-embedding immunocytochemical studies (Petralia and Wenthold, 1992; Martin et al., 1993; Petralia et al., 1994; Bernard et al., 1996, 1997b; Paquet and Smith, 1996). However, there are some discrepancies and inconsistencies (see Table 1) that presumably relate to the specificity of the reagents, species differences, and technical factors. It should be noted, however, that we have directly demonstrated that the highest density of immunolabelling for the receptor subunits is located at synaptic membrane specializations. In addition we have demonstrated that the distribution of subunits along the length of the synaptic membrane is, on average for the population, even for all glutamate receptor subunits studied in both nuclei. This distribution of the AMPA receptor subunits is similar to that in the striatum and hippocampus (Bernard et al., 1997a; Somogyi et al., 1998). The distribution of NR1 however, is different from that found in the hippocampus where the NR1, NR2A and NR2B subunits have been shown, on average, to be concentrated at the centre of the synapse (Somogyi et al., 1998; R. Lujan personal communication). One possible reason for these differences might be that the terminals in the hippocampus that were studied were homogeneous in origin, whereas those in the EP and STN were heterogeneous; our sampling could therefore have obscured an uneven distribution associated with terminals of a single origin.

The findings of the present study also demonstrate that individual neurons in the EP and STN express multiple subunits of the AMPA glutamate receptor and express subunits of both the AMPA receptor subunit and the NMDA receptor and, furthermore, that the co-localization occurs within individual asymmetrical synaptic specializations. Thus, in the EP, the co-localization of the GluR1, GluR4, or NR1 subunits with the GluR2/3 subunit was observed within single synapses on perikarya, dendrites, and spines. In the STN, the GluR1 or the NR1 subunits and the GluR2/3 subunit were similarly co-localized within individual synapses. These findings suggest, therefore, that glutamatergic transmission is mediated by a combination of AMPA and NMDA receptors in at least a subpopulation of synapses in the EP and STN. It remains to be established whether this is a general principle at all asymmetrical synapses in the EP and STN. The co-localization of AMPA and NMDA receptor subunits has been observed at synapses in the cortex (Kharazia et al., 1996), hippocampus (R. Lujan, personal communication), and other regions of the basal ganglia (Bernard and Bolam, 1997).

Functional AMPA receptor channels are believed to exist as either homomeric or heteromeric structures, and func-

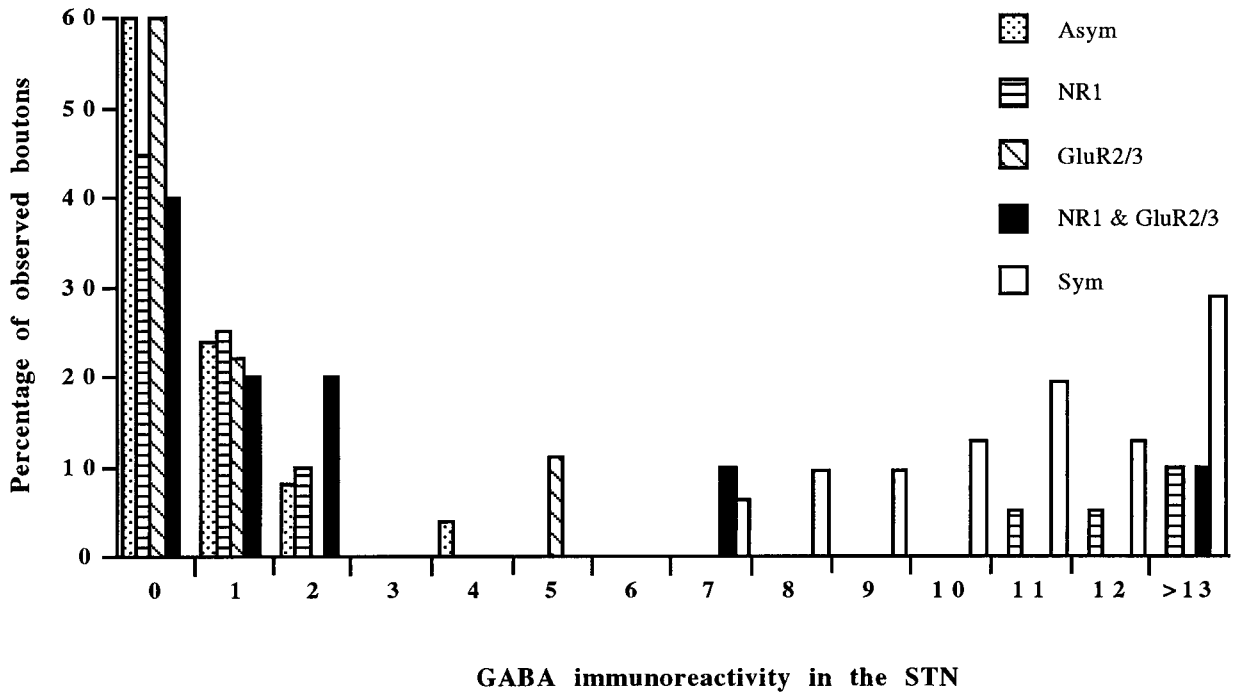

Fig. 12. Frequency distribution of the levels of GABA immunoreactivity associated with the terminals presynaptic to glutamate receptor subunit-immunopositive and -immunonegative synapses in the subthalamic nucleus. GABA immunoreactivity is represented as the ratio of the density of immunogold particles overlying the synaptic terminals to that overlying terminals forming asymmetrical synapses in the same section. The terminals presynaptic to both receptor-immunopositive and -immunonegative asymmetrical synaptic specializations have significantly lower levels of GABA immunoreactivity compared to that associated with terminals forming symmetrical synaptic contacts (Mann-Whitney U-test; $P < .0001$). Sym, terminals forming symmetrical synapses and immunonegative for the glutamate receptor subunits (mean GABA immunoreactivity ± SEM = 12.00 ± 0.55; range: 7.77–19.66; n = 31); NR1, terminals forming NR1-positive synapses (mean ± SEM = 4.04 ± 1.42; range: 0.07–24.16; n = 20); GluR2/3; terminals forming GluR2/3-positive synapses (mean ± SEM = 1.21 ± 0.62; range: 0–5.99; n = 9); NR1 & GluR2/3, terminals forming synapses that were positive for both antibodies (mean ± SEM = 2.92 ± 1.33; range: 0–13.34; n = 10); Asym, terminals forming asymmetrical synapses that did not display immunolabelling for any of the glutamate receptor subunits (mean ± SEM = 1.00 ± 0.19; range: 0–4.77; n = 25).

tional NMDA receptors are believed to consist of the NR1 subunit and at least one subtype of the NR2 subunit (Monyer et al., 1992; Nakanishi, 1992; Hollmann and Heinemann, 1994; Bettler and Mulle, 1995; Mori and Mishina, 1995; Sucher et al., 1996). The present study revealed the presence of specific AMPA and NMDA receptor subunits within the same synapses but cannot resolve the subunit composition of the individual channels within these synapses. The physiological analysis of Gotz and co-workers (1997) demonstrated that STN neurons have the highest calcium permeability mediated by AMPA receptors of all basal ganglia neurons studied, suggesting that STN neurons express calcium-permeable AMPA receptors on their somatic surface. These calcium-permeable AMPA receptors have been shown to lack the GluR2 (also known as the GluRB) subunit (Hollmann and Heinemann, 1994; Jonas and Burnashev, 1995; Gu et al., 1996; Lu et al., 1996); however, because the antibody used in the present study detected both the GluR2 and GluR3 subunits, we were unable to distinguish between the two subunits. Whether these channels also exist on the dendrites of STN neurons or if there is differential targeting of receptor subunits, as demonstrated recently in the dorsal cochlear nucleus (Rubio and Wenthold, 1997), remains to be established.

Glutamate and GABA immunoreactivity at AMPA- and NMDA-immunopositive synapses in the entopeduncular nucleus and subthalamic nucleus

The main finding of the analysis of the amino acid content in synaptic terminals is that the majority of terminals forming AMPA and/or NMDA receptor subunit-positive synapses have high levels of glutamate immunoreactivity and are GABA immunonegative. This finding provides direct evidence that glutamate is the neurotransmitter at these synapses because two of the criteria that a substance is a neurotransmitter are fulfilled, i.e., that the substance is selectively enriched in the presynaptic terminal and the receptors are expressed in the postsynaptic membrane.

In the EP, terminals derived from the STN (Bevan et al., 1994b) and the mesopontine tegmentum (Clarke et al., 1996, 1997) have been shown to contain large numbers of vesicles, have elevated levels of glutamate immunoreactivity, and form asymmetrical synaptic specializations with dendrites, spines, and perikarya that are occasionally associated with postjunctional dense bodies. In the present study the terminals presynaptic to the AMPA and NMDA

glutamate receptor subunit-immunopositive synapses had similar morphological characteristics to those of STN and MTg terminals and also contained elevated levels of glutamate immunoreactivity. This finding suggests, therefore, that the excitatory effects of STN neurons and MTg neurons at individual synapses in the EP are mediated by a combination of AMPA- and NMDA-dependent mechanisms. Similarly, in the STN, terminals derived from the mesopontine tegmentum (Bevan and Bolam, 1995; Clarke et al., 1997), the cortex and the thalamus (Bevan et al., 1995) have morphological and neurochemical similarities to those presynaptic to the glutamate receptor subunit–immunopositive synapses in this study. Our results therefore suggest that the actions of excitatory afferents from the MTg, cortex, and thalamus on STN neurons are mediated by AMPA- and NMDA-dependent mechanisms. The profile of receptor subunits at synapses formed by terminals of known origin remains to be established directly by a combination of anterograde labelling and receptor immunolabelling.

An additional observation of this study was that the levels of the amino acids in terminals at glutamate receptor–positive synapses were heterogeneous. Thus, in both the EP and STN, terminals with low levels of glutamate immunoreactivity (similar to those in GABAergic terminals) had glutamate receptor subunits in their postsynaptic membrane specializations. This distribution of glutamate immunolabelling is similar to that observed in previous analyses of the EP and STN (Bolam et al., 1993; Bevan and Bolam, 1995; Bevan et al., 1995; Clarke et al., 1996, 1997). The findings suggest that there are marked differences in the levels of glutamate in terminals at synapses, defined as glutamatergic, on the basis of the presence of glutamate receptor subunits in the EP and STN. If this is indeed the case, then the criterion used in previous experiments to establish whether glutamate is the neurotransmitter utilized in the neuronal pathways, i.e., that the presynaptic terminal is significantly enriched in glutamate compared to the levels in GABAergic terminals (Somogyi et al., 1986; Ottersen, 1989; Van den Pol, 1991; Llewellyn-Smith et al., 1992; Phend et al., 1992; Rinvik and Ottersen, 1993; Kharazia and Weinberg, 1994; Valtschanoff et al., 1994; Bevan and Bolam, 1995; Bevan et al., 1995; Ericson et al., 1995; Blomqvist et al., 1996; Clarke et al., 1996, 1997), may result in some populations that use glutamate as a neurotransmitter being overlooked. It is quite possible, however, that in individual synaptic boutons glutamate may be heterogeneously distributed, and unequivocal identification of a terminal with low or undetectable levels of glutamate may require serial section analysis.

The terminals forming asymmetrical synapses with low levels of glutamate immunoreactivity and elevated levels of GABA have been previously identified as originating from the MTg (Bevan and Bolam, 1995). However, the precise functional significance of GABA-immunopositive terminals with glutamate receptor subunits in their postsynaptic specialization remains to be established. Interestingly, the reverse situation has been found in the cerebellum, where the α_6 subunit of the $GABA_A$ receptor was found at both inhibitory and excitatory glutamatergic synapses on granule cells (Nusser et al., 1996).

CONCLUSIONS

The findings of the present study demonstrate that immunolabelling for the AMPA and NMDA receptor subunits is localized predominantly within asymmetric synapses in the EP and STN. Furthermore we demonstrate the colocalization of AMPA and NMDA receptors in individual synapses and their exact spatial location in relation to the excitatory glutamatergic inputs of the EP and STN. The elucidation of the specific glutamate receptor subunit composition at synapses of known origin in the EP and STN may lead to the development of new therapies in the treatment of Parkinson's disease (Klockgether and Turski, 1989, 1990; Brotchie et al., 1991; Klockgether et al., 1991).

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