SYNAPTIC LOCALIZATION OF IONOTROPIC GLUTAMATE RECEPTORS IN THE RAT SUBSTANTIA NIGRA

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Abstract—Glutamatergic neurotransmission in the substantia nigra pars compacta and pars reticulata is mediated through N-methyl-d-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxaline propionic acid/kainate (AMPA) type receptors as well as other glutamate receptors and is critical for basal ganglia functioning. A major glutamatergic input to the substantia nigra originates in the subthalamic nucleus, and the long-lasting stimulation of the dopaminergic cells of the substantia nigra pars compacta by the subthalamic neurons has been implicated in the pathophysiology of Parkinson’s disease. The objectives of the present study were to determine the subcellular and subsynaptic localization of subunits of the N-methyl-d-aspartate and AMPA receptors in the substantia nigra, and also to determine whether co-localization of N-methyl-d-aspartate and AMPA receptor subunits occur at individual synapses. To achieve this, pre-embedding and post-embedding immunocytochemistry was applied to sections of substantia nigra using antibodies that recognize the NR1 and NR2A/B subunits of the N-methyl-d-aspartate receptor, and GluR2/3 subunits of the AMPA receptor.

In both regions of the substantia nigra, immunolabelling for each of the subunits was observed in numerous perikarya and proximal dendrites. At the subcellular level, silver-intensified immunogold particles localizing N-methyl-d-aspartate and AMPA receptor subunits were most commonly present within dendrites where they were associated with a variety of intracellular organelles and with the internal surface of the plasma membrane. Post-embedding immunogold labelling revealed immunoparticles labelling for NR1, NR2A/B and GluR2/3 to be enriched at asymmetric synaptic specializations, although a large proportion of asymmetric synapses were immunonegative. Double immunolabelling revealed, in addition to single-labelled synapses, the co-localization of subunits of the N-methyl-d-aspartate receptor and subunits of the AMPA receptor at individual asymmetric synapses. Similarly, double immunolabelling also revealed the co-localization of the NR1 and NR2A/B subunits of the N-methyl-d-aspartate receptor at individual asymmetric synapses. Labelling for NR1 and GluR2/3 was, on average, relatively evenly distributed across the width of the synapse with a gradual reduction towards the periphery when analysed in single sections.

In summary, the present results demonstrate that AMPA and N-methyl-d-aspartate receptors are selectively localized at a subpopulation of asymmetric synapses in the substantia nigra pars compacta and reticulata and that the two receptor types, at least partially co-localize at individual synapses. It is concluded that glutamatergic transmission in the substantia nigra pars compacta and pars reticulata occurs primarily at asymmetric synapses and, at least in part, is mediated by both N-methyl-d-aspartate and AMPA receptors. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: basal ganglia, synapses, immunolabelling, AMPA receptors, NMDA receptors.

The basal ganglia comprise a group of interconnected subcortical nuclei which are involved in a variety of functions including motor control, memory and cognition.5,21,29,44,45,47,111 One division of the basal ganglia, the substantia nigra (SN), consists of two major components, the substantia nigra pars reticulata (SNr) and the pars compacta (SNC). The SNr contains one of the populations of basal ganglia output neurons39,40 and the SNC contains the dopaminergic nigrostriatal neurons which are involved in the modulation of the flow of cortical information through the basal ganglia.16,40,96 The dopaminergic neurons degenerate in Parkinson’s disease.13,35,36,46 by processes that are not fully understood, however, glutamate receptor-mediated excitotoxicity has been suggested to be a contributory factor.13,22,29 The SNC and SNr receive glutamatergic afferents arising in a variety of regions including the cortex, the pedunculopontine nucleus and the subthalamic nucleus (STN).12,53,57,89 The effects of glutamate in the SN are mediated by the three principal types of glutamate receptors, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxaline propionic acid/kainate (AMPA) and metabotropic receptors.49,69,92 Fast synaptic transmission is proposed to be mediated by both AMPA and NMDA receptors and physiological evidence suggests that the stimulation of AMPA receptors results in fast excitatory postsynaptic potentials whereas stimulation of NMDA receptors is considered as a mechanism to modulate this response.23,43,61,97 As part of one of the indirect pathways (striato-pallido-subthalamonigral pathway)49,96 the subthalamonoigral projection has been proposed to be over-active in Parkinson’s disease.4,5,29,48,63 Long-lasting stimulation of glutamate receptors by the STN neurons may thus play a role in the degeneration of dopaminergic neurons in Parkinson’s disease.15,29

Molecular biology techniques have identified two families...
of genes that code for NMDA receptor subunits, termed NMDA receptor 1 (NR1) and NMDA receptor 2 (NR2). One gene codes for NR1 and eight splice variants are known, whereas four distinct genes code for the NR2 subunits (NR2A–D). It has been proposed that functional NMDA receptors consist of hetero-oligomeric structures comprising at least one NR1 and one NR2 subunit, although NR1 subunits have been found to form functional homomeric receptors when expressed in vitro.

### EXPERIMENTAL PROCEDURES

#### Animals and tissue preparation

Wistar rats (Charles River, Margate, Kent; 200–500 g) maintained on a 12-h light/dark cycle with free access to food and water were used in this study. Environmental conditions for housing of the rats, and all procedures performed on them, were in accordance to the Animals (Scientific Procedures) Act 1986 and in accordance with the European Communities Council Directive (86/609/EEC). All efforts were made to minimize the number of animals used and to keep any suffering to a minimum.

The animals were deeply anaesthetized with sodium pentobarbitone (Sagatal, Rhone Merieux, Tallaght, Dublin, Ireland; 200 mg/kg, i.p.) and then perfused transcardially with 100 ml of phosphate-buffered saline (PBS; 0.01 M phosphate, pH 7.4) or saline (0.9% NaCl) for 1–2 min 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 a period of 20 min.

Following fixation, the brain was quickly removed from the cranium, and 70-μm thick sections were cut on a vibratome and collected in PBS. To enhance immunoreagent penetration into the tissue the sections were equilibrated in a cryoprotectant solution (0.05 M PB, pH 7.4, containing 25% sucrose and 10% glycerol) and freeze-thawed by freezing in isopentane (BDH Chemicals, Letterworth, Leicestershire, UK) that had been cooled in liquid nitrogen, dipping into liquid nitrogen and finally thawing in PBS. The sections were then washed several times in PBS.

#### Immunohistochemistry

Immunoreactivity for the NR1 subunit of the NMDA receptor was detected using two monoclonal antibodies raised in mouse [NR1 (60012A); Pharmingen, Becton and Dickinson, Oxford, UK, and NR1 (MAB3631); Chemicon International, Harrow, UK]. Both NR1, antibodies were raised against a recombinant fusion protein containing the amino acids 660–811 corresponding to a putative extracellular loop between transmembrane regions III and IV of the NR1 subunit.

The antibody was raised against a synthetic peptide (LNSCNRVRVKKMPSESIDV) corresponding to the C-terminal of rat NR2A receptor subunit, and recognizes both NR2A and NR2B subunits and shows slight cross-reactivity with NR2C and NR2D. It has been characterized previously and used extensively in immunohistochemical studies. The GluR2/3 AMPA receptor subunits were detected using a polyclonal antibody raised in rabbit [NR2A/B (AB1548); Chemicon International]. The antibody was raised against a synthetic peptide (GLUR2/3) and a polyclonal antibody raised in rabbit [GluR2/3 (AB 15161) Chemicon International]. These antibodies were raised against synthetic peptides derived from the intracellular C-terminus of the GluR2 subunit. Both anti-GluR2/3 antibodies recognize GluR2 and GluR3 subunits of the AMPA receptor. The antibodies have been characterized and extensively used for immunohistochemical studies.

#### Pre-embedding immunoperoxidase method

The sections were incubated for 15–20 h at room temperature with constant gentle shaking in primary antibody solutions (NR1, NR2A/B or GluR2/3; see Table 1) diluted in PBS (with or without 1% normal goat serum). They were then washed (3 × PBS) and incubated in biotinylated goat anti-rabbit or goat anti-mouse IgGs (1:100, Vector Laboratories, Peterborough, UK) for a minimum of 1 h at room temperature. The sections were then washed (3 × PBS) and incubated in avidin–biotin–peroxidase complex (ABC; 1:100, Vector Laboratories, Peterborough, UK) for a minimum of 1 h at room temperature. The sections were incubated in 0.0048% 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, Poole, Dorset, UK; 0.05% in TB). The reaction was stopped by several washes in TB.

#### Pre-embedding immunogold method

The pre-embedding immunogold method was carried out as previously described. Briefly, sections were incubated in primary antibody solutions as described above. After washing (2 × PBS, 2 × PBS supplemented with 0.5% bovine serum albumin and 0.1% gelatine (PBS–BSA)), they were incubated in goat anti-rabbit or goat anti-mouse IgGs conjugated to colloidal gold (1.0 nm diameter; Nanoprobes, Stony Brook, NY, USA; 1:100 in PBS–BSA) for 2 h at

### Table 1. Details of antibody preparations

<table>
<thead>
<tr>
<th>Receptor subunits</th>
<th>Species of origin</th>
<th>Source</th>
<th>Pre-embedding</th>
<th>Post-embedding</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1</td>
<td>mouse (monoclonal)</td>
<td>PharMingen (MAB363)</td>
<td>0.9 μg/ml</td>
<td>45 or 90 μg/ml</td>
</tr>
<tr>
<td>NR1</td>
<td>mouse (monoclonal)</td>
<td>Chemicon (AB1548)</td>
<td>2.5 μg/ml</td>
<td>6.67 μg/ml</td>
</tr>
<tr>
<td>NR2A/B</td>
<td>rabbit</td>
<td>Chemicon (AB1548)</td>
<td>0.2 μg/ml</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td>GluR2/3</td>
<td>mouse (monoclonal)</td>
<td>Refs 72 and 77</td>
<td>0.5 μg/ml</td>
<td>1 or 2 μg/ml</td>
</tr>
<tr>
<td>GluR2/3</td>
<td>rabbit</td>
<td>Chemicon (AB1506)</td>
<td>0.5 μg/ml</td>
<td>1 or 2 μg/ml</td>
</tr>
</tbody>
</table>

This table lists the species of origin, source, and immunoreactivity of the antibodies used in the study.
room temperature. The sections were then washed (2 × PBS–BSA; 2 × PB) and post-fixed in 1% glutaraldehyde in PBS for 10 min. After washing (2 × PBS, 2 × sodium acetate buffer; 0.1 M, pH 7.0), the colloidal gold labelling was intensified using a silver enhancement kit (HQ silver, Nanoprobes) for 3–5 min at room temperature in the dark. The sections were washed in sodium acetate buffer and then PB.

**Preparation for electron microscopy**

Immunoperoxidase- and immunogold-treated sections were post-fixed in osmium tetroxide (1% in PB) for 25 min for the DAB-reacted sections or 10 min for the immunogold-reacted sections at room temperature. After washing (3 × PB), they were dehydrated in an ascending series of dilutions of ethanol. Uranyl acetate (1%) was included in the 70% ethanol solution. They were then treated with propylene oxide (2 × 10 min) and equilibrated in resin overnight (Durcupan, ACM, Fluka, Gillingham, Dorset, UK), mounted on glass slides and cured at 60°C for 48 h. The sections were first examined in the light microscope. Areas of interest were photographed and some regions from the immunogold-treated sections were cut out from the slide and glued to blank cylinders of resin. Ultrathin sections were cut on a Reichert Ultracut E and collected on single slot copper grids coated with pioloform (Agar Scientific). The sections were stained with lead citrate as described above.

**Controls of specificity of the immunolabelling**

The specificity of the pre-embedding and post-embedding techniques was demonstrated by the absence of labelling for the respective antigens when the primary antibodies were omitted. Since double labelling was performed, it was necessary to control for the specificity of the secondary antibodies. Sections were incubated with either a primary antibody raised in mouse or rabbit, at the appropriate dilutions, and then with a secondary, gold-conjugated antibody directed against immunoglobulins of rabbit or mouse respectively. In parallel, positive controls for each antibody were included. In each case the inappropriate gold-conjugated secondary antibody failed to produce specific labelling, although the positive controls revealed specific labelling of asymmetric synapses and membranes.

**Quantitative analysis**

Quantitative analyses were carried out on both the SNC and SNr. Regions of overlap between SNC and SNr were excluded from the analyses.

**Post-embedding immunogold method**

After perfusion and fixation as described above, the substantia nigra was prepared for embedding in Lowicryl resin using the freeze substitution method as described previously. In brief, vibratome sections (500 µm) of mesencephalon were cut and washed extensively in PB. The substantia nigra was dissected out to give blocks of approximately 5 × 3 mm. These were equilibrated in ascending concentrations of sucrose solution (0.5 M, 1 M and 1.5 M in PB) and then were slammed on a cooled copper block with a Leica MM80E cryofixation apparatus. The blocks of substantia nigra were transferred to a Leica CS Auto at −80°C, where freeze substitution followed. In both cases slices were immersed in 0.5% uranyl acetate (TAAB Laboratories, Reading, Berkshire, UK) in methanol at −80°C for 16 h, methanol for 36 h and then the temperature was increased at 10°C/h to −50°C and all the following steps were conducted at this temperature. The blocks were then infiltrated with a 1:1 mixture of methanol and Lowicryl HM20 (Chemische Werke Lowi GmbH) for 2 h, a 1:2 mixture for 2 h, undiluted Lowicryl for 2 h and then fresh Lowicryl overnight. The slices were then placed in embedding capsules in fresh Lowicryl and polymerized under UV light for a minimum of 48 h. Ultrathin sections were cut from the SN blocks and collected on single-slot nickel grids coated with pioloform in preparation for the subsequent post-embedding immunohistochecmistry.

The sections were immunolabelled by the post-embedding method essentially as described previously. Briefly, they were washed in de-ionicized water and in Tris-buffered saline (TBS) (0.9% NaCl in 50 mM TB, pH 7.6). They were then incubated for 30 min in 2% human serum albumin (HSA; Sigma-Aldrich) in TBS containing 0.1% Triton X-100 (TBST) at room temperature. This was followed by a 15–20 h incubation in primary antibody solutions at room temperature. Primary antibodies were diluted in TBST containing 2% HSA (see Table 1 for details). After several washes in TBS, the sections were incubated in the appropriate secondary antibodies conjugated to different sized colloidal gold particles, 10 nm gold-conjugated goat anti-mouse IgG (British BioCell, 1:60) and 15 nm gold-conjugated goat anti-rabbit IgG (British BioCell, 1:80); for NR1 and GluR2/3 particles across the width of asymmetric synaptic specializations was analysed. The distance of each particle from the nearest edge of the synapse was measured and normalized, to take into account different sizes of synapses. The data were expressed as the proportion of immunoparticles in five bins along the half-width of the synapse.

**Double immunolabelling using the post-embedding procedure**

Double immunolabelling was carried out on the same ultrathin section using mixtures of primary antibodies. The following combinations were used: anti-NMDA and anti-AMPA antibodies (either one or both monocolonal NRI antibodies and the polyclonal GluR2/3 antibody; or the polyclonal NR2A/B antibody and the monocolonal GluR2/3 antibody; see Table 1). The same post-embedding procedure was followed as described above, except that the sections were incubated on drops of a cocktail of primary antibodies diluted to the same concentrations used in the single labelling experiments. After several washes in TBS, the sections were incubated in a cocktail of 10 nm gold-conjugated goat anti-mouse IgG (British BioCell, 1:60) and 15 nm gold-conjugated goat anti-rabbit IgG (British BioCell, 1:80) in 2% HSA in TBST supplemented with polyethylene glycol (5 mg/ml). They were then post-fixed in glutaraldehyde and contrasted with uranyl acetate and lead citrate as described above.

**Pre-embedding immunogold.** Analysis of the subcellular distribution of NR1 and GluR2/3 immunoreactivity was carried out at the electron microscope level on immunogold sections of SNC and SNr from three animals. In order to quantify the distribution of immunogold labelling for NR1 and GluR2/3, a continuous strip of tissue from each animal was analysed at a magnification of 21,000. Every immunogold particle was noted according to the location within a neuronal element.

**Post-embedding immunogold.** Quantitative analysis of the distribution of immunogold particles and distribution of labelled structures was performed on sections or electron micrographs of Lowicryl embedded SNC and SNr from three animals, for the NR1 and GluR2/3 subunits and two animals for NR2A/B. Sections immunlabelled for NR1, NR2A/B and GluR2/3 were systematically scanned and immunopositive synapses were identified and photographed. A synapse was considered immunopositive when two or more immunoparticles were present within the specialization. The proportion of asymmetric synapses immunopositive for the receptor subunits was determined in systematic scans in the electron microscope. In the double labelling experiments (NR1 and GluR2/3 or NR2A/B and GluR2/3), the sections were systematically scanned in the electron microscope and each labelled synapse was characterized as NR1- or NR2A/B-positive, GluR2/3-positive or double labelled.

The distribution of NR1 and GluR2/3 particles across the width of asymmetric synaptic specializations was analysed. The distance of each particle from the nearest edge of the synapse was measured and normalized, to take into account different sizes of synapses. The data were expressed as the proportion of immunoparticles in five bins along the half-width of the synapse.
RESULTS

Light microscopic observations

Immunoreactivity for the NR1 and NR2A/B subunits of the NMDA receptor and the GluR2/3 subunits of the AMPA receptor was revealed in the SNc and SNr by the immunoperoxidase and the pre-embedding immunogold (except NR2A/B) methods. Since immunolabelling was essentially as has been described by others, only a brief account will be given here. In both the SNc and SNr immunolabelling for NR1, NR2A/B and GluR2/3 was observed in numerous perikarya and proximal dendrites (Fig. 1). In the SNc the immunopositive neurons were of medium size and many exhibited elongated perikarya along the plane of the compacta, typical of SNc neurons (Fig. 1A, C, E). In the SNr cells immunopositive for NR1, NR2A/B and GluR2/3 possessed medium size round or oval perikarya and showed the typical distribution pattern of reticulata neurons (Fig. 1B, D, F).

Electron microscopic observations

Localization of the N-methyl-D-aspartate receptor in the substantia nigra. The subcellular distribution of subunits of the NMDA receptor was determined using the pre-embedding immunogold method for NR1 and the post-embedding immunogold method for both NR1 and NR2A/B. In the pre-embedding immunogold-labelled sections immunoreactive sites were identified by the slightly irregularly-shaped, electron dense immunometal particles (Fig. 2). The most commonly labelled profiles for the NR1 subunit were dendritic shafts and perikarya in both the SNc and SNr (Fig. 2A, B). The immunoparticles were associated with both the plasma membrane and intracellular sites. The membrane-associated particles, defined as those particles touching the plasma membrane, in the SNc (21.5%; SNr 24.5%) were located on the internal surface of the membrane at extrasynaptic sites and sometimes closely associated with asymmetric synapses (Fig. 2A, B). The synapse-associated immunolabelling (SNc, 0.6%; SNr, 0.8%) was observed in the case of NR1 and NR2A/B.
Fig. 2. Pre-embedding immunogold detection of the NR1 subunit of the NMDA receptor (A, B) and the GluR2/3 subunits of the AMPA receptor (C–E) in the substantia nigra pars compacta (SNc; A, C, D) and substantia nigra pars reticulata (SNr; B, E). (A, B) Immunolabelling for NR1 in dendrites (d) in the SNc (A) and SNr (B). Immunogold particles are localized within the cytoplasm (some indicated by arrowheads) and on the plasma membrane at the periphery of an asymmetric synapse (large arrow in A) formed by the bouton labelled b. (C) Immunolabelling for GluR2/3 in the perikaryon of a neuron in the SNc. Immunolabelling is associated with the external surface of lamellae of the endoplasmic reticulum (some immunoparticles indicated by small arrows). (D) Immunolabelling for GluR2/3 in a dendrite in the SNc. Immunogold particles are localized within the cytoplasm (arrowhead) and on the plasma membrane (small arrows). One of the membrane-associated particles is located at the periphery of an asymmetric synapse (large arrow) formed by a large bouton (b). (E) Immunolabelling for GluR2/3 in a dendrite in the SNr which is apposed by many boutons (some indicated by b). The immunogold labelling is localized within the cytoplasm (arrowheads) and associated with the plasma membrane (small arrows). Immunogold particles are sometimes closely associated with asymmetric synapses (large arrow) and sometimes possibly with boutons forming symmetrical synapses (curved arrow). (Both the antibodies were obtained from Chemicon; see Table 1). Scale bar = 0.5 μm.
Table 3. Proportion of asymmetric synapses labelled for NR1 and NR2A/B or GluR2/3

<table>
<thead>
<tr>
<th></th>
<th>NR1</th>
<th>NR2A/B</th>
<th>GluR2/3</th>
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</thead>
<tbody>
<tr>
<td>SNc</td>
<td>6%</td>
<td>17%</td>
<td>15%</td>
</tr>
<tr>
<td>unlabelled</td>
<td>94%</td>
<td>83%</td>
<td>85%</td>
</tr>
<tr>
<td>SNP</td>
<td>4%</td>
<td>17%</td>
<td>8%</td>
</tr>
<tr>
<td>unlabelled</td>
<td>96%</td>
<td>83%</td>
<td>92%</td>
</tr>
</tbody>
</table>

Proportion of plasma membrane labelling associated with asymmetric synapses as revealed by the pre-embedding and post-embedding immunogold techniques. For the pre-embedding immunogold method, SN tissue was systematically scanned and the number of immunogold particles counted in SNc (NR1, 479 particles; GluR2/3, 893 particles) and SNP, (NR1, 331 particles; GluR2/3, 787 particles). For the post-embedding method, the number of immunogold particles were counted from electron micrographs of immunopositive synapses. In order to avoid bias, the synapses that were the focus of the micrographs were excluded from the analysis. The total number of particles counted using the post-embedding immunogold technique the SNc were: NR1, 2681; GluR2/3, 4156; and in the SNP were: NR1, 1879; GluR2/3, 2049. Data points were obtained from at least two animals.

Localization of AMPA receptors in the substantia nigra. In pre-embedding immunogold-labelled sections of substantia nigra, the distribution of GluR2/3 immunolabelling was found to be similar to that observed with the NR1 antibodies and was essentially as previously described in peroxidase-stained sections in the squirrel monkey. As predicted by the light microscopic analysis, labelling for GluR2/3 was detected in perikarya and dendrites in both the SNc and SNP. The distribution of the immunoparticles was similar to that revealed with the NR1 antibodies and immunolabelling observed for ionotropic glutamate receptors in other regions of the basal ganglia. About one third of the particles were associated with the plasma membrane (SNc and SNP, 31%) and about two thirds associated with intracellular sites (SNc, 64.8%; SNP, 62.2%) including the endoplasmic reticulum (Fig. 2C) and the cytoplasmic side of the nuclear membrane. The membrane-associated immunoparticles mostly occurred at extrasynaptic sites although a small proportion were located at the periphery of asymmetric synaptic specializations (Figs 3D–I).

Figures in parentheses represent the numbers of synapses observed.
Fig. 3. Post-embedding immunogold detection of the NR1 and NR2A/B subunits of the NMDA receptor and the GluR2/3 subunit of the AMPA receptor in the SNc and SNr. (A, B) Immunolabelling for NR1 in the SNc (A) and SNr (B) revealed with 10 nm gold particles. In both cases the immunogold labelling (arrows) is localized at asymmetrical synapses formed between boutons (b) and dendritic shafts (d). (C, D) Immunolabelling for NR2A/B in the SNc (C) and SNr (D) revealed with 15 nm gold particles. As with the NR1 labelling the immunogold labelling for NR2A/B (some indicated by large arrows) is localized at asymmetrical synapses formed between boutons (b) and dendritic shafts (d). (E, F) Immunolabelling for GluR2/3 subunits of the AMPA receptor in the SNc (E) and SNr (F) revealed with 15 nm gold particles. In both regions the AMPA receptor labelling (some indicated by large arrows) is primarily associated with asymmetric synapses formed between boutons (b) and dendritic shafts (d). In E, the upper synaptic specialization is clearly associated with subjunctinal dense bodies and the lower has two active zones. (Each antibody was obtained from Chemicon; see Table 1). Scale bar = 0.5 μm.
Fig. 4. Co-localization of subunits of the NMDA receptor with the GluR2/3 subunits of the AMPA receptor and co-localization of different subunits of the NMDA receptor at synapses in the substantia nigra. (A, B) Double labelling for the NR1 subunit of the NMDA receptor (10 nm gold particles; some indicated by small arrows) and the GluR2/3 subunits of the AMPA receptor (15 nm gold particles; some indicated by large arrows) at individual asymmetric synapses formed between dendrites (d) and boutons (b) in the SNc (A) and SNr (B). (C, D) Double labelling for the NR2A/B subunits of the NMDA receptor (15 nm gold particles; some indicated by large arrows) and the GluR2/3 subunits of the AMPA receptor (10 nm gold particles; some indicated by small arrows) at individual asymmetric synapses formed between dendrites (d) and boutons (b) in the SNc (C) and SNr (D). (E, F) Double labelling for the NR1 subunit (10 nm gold particles; some indicated by small arrows) and NR2A/B subunits (15 nm gold particles; some indicated by large arrows) at individual asymmetric synapses formed between dendrites (d) and boutons (b) in the SNc (E) and SNr (F). Note that the large bouton in E gives rise to two active zones. Many of the asymmetric synapses (particularly those in C–F) are associated with prominent subjunctional dense bodies. In addition to the synaptic labelling, single or clusters of immunogold particles were observed at intracellular sites. These were sometimes associated with mitochondria (A, D, E). (A, B: NR1 antibody from PharMingen and GluR2/3 from Chemicon; C, D: monoclonal GluR2/3 antibody, NR2A/B antibody from Chemicon; E, F: NR1 antibody from PharMingen and NR2A/B antibody from Chemicon; see Table 1). Scale bar = 0.5 μm.
GluR2/3 (Table 3). As suggested for the NR1 and NR2A/B labelling, these values are likely to be underestimates of the true proportions.

Analysis of the distribution of immunoparticles encoding for the NR1 subunit of the NMDA receptor and GluR2/3 subunits of the AMPA receptor along asymmetrical synapses in the SNc (A) and SNr (B) labelled by the post-embedding immunogold method. The distribution was even over the width of the synapses with a fall off towards the edge, particularly in the SNc. The analysis was carried out on single sections. In the SNc, 266 synapses (1427 gold particles) were examined for GluR2/3 and 83 synapses (242 gold particles) for NR1; in the SNr, 117 synapses (559 gold particles) were examined for GluR2/3 and 58 synapses (156 gold particles) for NR1. The gold particles were assigned to five bins over the half width of the synapses. Only synapses labelled with two or more immunoparticles were included in the analysis.

Table 4. Double labelling for AMPA and/or NMDA receptor subunits in the substantia nigra

<table>
<thead>
<tr>
<th>Region</th>
<th>Combination</th>
<th>NR1</th>
<th>GluR2/3</th>
<th>Double</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNc</td>
<td>NR1</td>
<td>5.9 (7)</td>
<td>70.3 (83)</td>
<td>23.7 (28)</td>
</tr>
<tr>
<td>SNr</td>
<td>NR1</td>
<td>9.0 (6)</td>
<td>62.7 (42)</td>
<td>28.3 (19)</td>
</tr>
<tr>
<td>SNc</td>
<td>NR2A/B</td>
<td>33.7 (34)</td>
<td>31.7 (32)</td>
<td>34.7 (35)</td>
</tr>
<tr>
<td>SNr</td>
<td>NR2A/B</td>
<td>39.7 (31)</td>
<td>15.4 (12)</td>
<td>44.9 (35)</td>
</tr>
<tr>
<td>SNc</td>
<td>NR1</td>
<td>15.3 (11)</td>
<td>31.9 (23)</td>
<td>52.8 (38)</td>
</tr>
<tr>
<td>SNr</td>
<td>NR1</td>
<td>20.3 (16)</td>
<td>27.9 (22)</td>
<td>51.9 (41)</td>
</tr>
</tbody>
</table>

Figures represent the percent of immunopositive synapses single or double labelled for AMPA and/or NMDA receptor subunits in the three combinations of antibodies used for the double labelling experiments. Figures in parentheses represent the number of synapses observed. Data were obtained from at least two animals.

Double immunolabelling was carried out using 10 or 15 nm immunogold particles coupled to the secondary antibodies for monoclonal primary antibodies and polyclonal antibodies respectively (Table 1).

Double labelling with either or both NR1 (monoclonal) antibodies and the GluR2/3 (polyclonal) antibody revealed that the NMDA receptor subunit and the AMPA receptor subunits co-localize at individual asymmetric synapses in both the SNc and SNr. Approximately one quarter of immunopositive asymmetric synapses were labelled for both NR1 and GluR2/3 in SNc and SNr (Table 4). A high proportion of the synapses immunopositive for NR1 was also immunolabelled for GluR2/3 (80% of NR1-positive synapses in the SNc and 76% in SNr). A smaller proportion of synapses immunopositive for GluR2/3 were also positive for NR1, (SNc, 25%; SNr, 31%). Double labelling for the NR2A/B subunits and the GluR2/3 subunits also revealed the co-localization of NMDA and AMPA receptor subunits at individual asymmetric synapses. In this case, more than one third of all labelled synapses were immunopositive for both NR2A/B and GluR2/3 (Table 4).

Double immunolabelling for the NR1 and NR2A/B subunits of the N-methyl-D-aspartate receptor. The post-embedding immunogold technique was also utilized to reveal the relative localization of the NR1 and NR2 subunits of the NMDA receptor (Fig. 4E, F). Co-localization of both of the NMDA subunits was identified at individual asymmetric synapses in both the SNc and SNr. More than one half of immunopositive asymmetric synapses were double labelled for both NR1 and NR2A/B subunits (Table 4). Of the NR1-positive synapses, more than 70% were double labelled for the two NMDA subunits and of the NR2A/B-positive synapses, more than 60% were positive for both.

Immunolabelling with a cocktail of N-methyl-D-aspartate and AMPA receptor antibodies. In order to determine whether the small proportion of asymmetric synapses labelled in the post-embedding experiments was due to the low sensitivity of the procedure, a cocktail of all the NR1, NR2A/B and GluR2/3 subunit antibodies at concentrations routinely used
in this study was applied to sections of the SN (Table 1). Using this approach, preliminary findings indicate that 16% and 13% of asymmetric synapses were immunopositive for the cocktail of antibodies in SNc and SNr respectively (Fig. 6A, B), figures that are in similar orders of magnitude to the labelling produced by each antibody singly. Thus, they are slightly greater than the proportion of asymmetric synapses labelled with either NR1 or GluR2/3 alone, but slightly smaller than the proportion labelled with NR2A/B alone (Table 3).

DISCUSSION

The results of the present study confirm and extend previous studies on the cellular localization of subunits of the NMDA and AMPA receptors in the SN and, for the first time, provide a detailed analysis of their subcellular localization in relation to synaptic specializations and their co-localization at individual synapses. First, in confirmation of previous findings, they demonstrate the widespread distribution of NMDA and AMPA receptor subunits among neurons of both the SNc and SNr. Secondly, they demonstrate by the pre- and post-embedding immunogold methods, that immunolabelling for the receptors is selectively associated with the plasma membrane and that a high proportion of this is localized on the membrane postsynaptic to boutons forming asymmetric synapses where it is relatively evenly distributed across the width of the synapse. Thirdly, they demonstrate by post-embedding double immunogold labelling, that different subunits of the NMDA receptor co-localize at individual asymmetric synapses in the SNc and SNr. Finally, the results demonstrate that subunits of both the NMDA receptor and the AMPA receptor co-localize at individual asymmetric synapses in both the SNc and SNr. Taken together these findings suggest that, as in other regions of the basal ganglia and in other regions of the brain, glutamate transmission in both the SNc and SNr occurs primarily at synaptic specializations and, at some synapses at least, is mediated by both AMPA and NMDA receptors.

Cellular localization of AMPA and N-methyl-D-aspartate receptors

The light microscopic observations, in which we demonstrated a high proportion of neurons in the SNc and SNr immunolabelled with antibodies that recognize the NR1 subunit of the NMDA receptor and antibodies that recognize the GluR2/3 subunits of the AMPA receptor, are essentially in agreement with previous immunocytochemical studies and in situ hybridization studies in rats and primates. Thus, each study described extensive labelling of neurons in both the SNc and SNr. Paquet and co-workers found that almost all of the dopaminergic neurons in the SNc of squirrel monkeys expressed both AMPA and NMDA receptor subunits. Our data extend these findings as the post-embedding immunogold technique demonstrated directly the co-localization of AMPA and NMDA receptor subunits in neurons in the SNc and SNr. Extensive labelling for the NR2A/B subunits of the NMDA receptor was found in both the SNc and SNr which is in contradiction to the findings of studies in the squirrel monkey but not the findings of Tse and Yung in the SNr of rat. This discrepancy may relate to species differences or variations in immunocytochemical techniques. It is interesting to note however, that in situ hybridization studies have demonstrated that the signals for NR2C and D in the SN are stronger than those for NR2A and B. This, together with the fact that the NR2A/B antibody that we used shows slight cross-reactivity with NR2C and NR2D, may account for the discrepancies between our findings and those of others. Nevertheless, the important point is that both the dopaminergic neurons of the SNc and the output neurons of the basal ganglia in the SNr express both NMDA and AMPA receptor subunits and that they co-localize in at least some nigral neurons.

Subcellular localization of subunits of AMPA and N-methyl-D-aspartate receptors

The immunogold methods revealed immunolabelling for subunits of the NMDA and AMPA receptors at both intracellular sites and on the plasma membrane of neurons of the SNc and SNr. The pre-embedding technique revealed that 2–4.8%...
of membrane-associated gold particles labelling for NR1 or GluR2/3 in both regions of the SN were localized at the periphery of asymmetrical synapses. This apparently low proportion reflects the low proportion of the plasma membrane of dopaminergic and reticulata neurons that is occupied by asymmetric synapses and the fact that immunogold particles in the pre-embedding procedure have restricted access to receptors localized within the synaptic specialization. In contrast to this, analysis of the sections labelled by the post-embedding method for NR2A/B and GluR2/3, revealed a prominent association of labelling for each antibody with the membrane specializations of asymmetric synapses in both the SNc and SNr. Although it is not possible to localize the labelling to the pre- or postsynaptic membrane with certainty when using the post-embedding immunogold method (see Results), the results of the pre-embedding labelling indicate that immunolabelling is predominantly associated with the postsynaptic neuron and membrane. The quantitative analysis confirmed these findings.

A large proportion of the immunogold particles labelling for NR1, NR2A/B and GluR2/3 was observed at intracellular sites. This is consistent with previous anatomical studies showing the presence of glutamate receptor subunits associated with endoplasmic reticulum, mitochondria, Golgi apparatus and the external side of the nuclear membrane. These probability reflect receptor subunits in the process of synthesis and/or breakdown. Intracellular labelling may also include non-specific labelling.

The current, widely accepted model for ionotropic glutamate receptor subunit topology indicates that the N-terminal is extracellular, the C-terminal is intracellular and in addition to the three transmembrane domains (I, III, IV) there is a domain (II) between domains I and III forming a cytoplasmic-facing “hairpin loop”. The C-terminal antibodies used in the present study (NR2A/B and GluR2/3 antibodies; see Table 1) did indeed show immunolabelling on the intracellular side of the plasma membrane in the pre-embedding immunogold experiments. The monoclonal NR1 antibodies (Table 1) are directed against a sequence of amino acids which are considered to be located in the extracellular loop between transmembrane domains III and IV and consequently immunolabelling with these antibodies would be expected to be located primarily on the extracellular surface of the plasma membrane. However, NR1 labelling with these antibodies showed a similar distribution pattern to the immunolabelling found with the polyclonal glutamate receptor subunit antibodies; the majority of membrane-associated NR1 labelling was found on the intracellular side of the plasma membrane. In the striatum NR1 immunolabelling using the same monoclonal NR1 antibodies as used in the present study, was also found to be associated with the internal, rather than external, surface of the plasma membrane. This discrepancy is puzzling but may be a technical artefact relating to the penetration of the immunogold particles. Whatever the situation, when taken together, the NR1 immunolabelling generated by both the polyclonal and monoclonal NR1 antibodies indicate that immunolabelling is predominantly associated with the postsynaptic neuron and membrane.

Membrane-associated immunolabelling in the post-embedding experiments accounted for a relatively small proportion of the total number of immunoparticles, but 5.3–28.4% of these were localized at asymmetrical synapses. Since asymmetric synapses represent only a small proportion of the synapses afferent to both dopaminergic neurons in the SNc and basal ganglia output neurons in the SNr, these figures indicate a selective enrichment of labelling at asymmetric synapses. Although extrasynaptic labelling occurred, it was not in the form of accumulations of several particles that occurred at asymmetric synapses but rather, single particles scattered along the membrane suggesting that the concentration or density of the receptor subunits is lower than that at synapses. These results allow us to conclude that glutamatergic neurotransmission occurs primarily at asymmetric synapses and provide an anatomical basis for the role of NMDA and AMPA receptors in the regulation of the activity of neurons of the SNc and SNr.

The NMDA and AMPA receptor-positive synapses in the SNc and SNr accounted for a relatively small proportion of the total number of asymmetric synapses. The low proportions are likely to be underestimates of the true proportions of synapses that express the receptors for technical reasons. First, synapses labelled with a single immunoparticle may in fact be receptor-positive. Secondly, it cannot be excluded that some apparently immunonegative synapses contain receptors in neighbouring sections but were not present in the section that was analysed. Thirdly, the number or density of receptors may be below the level of detection with the post-embedding procedure. However, when we used a mixture of all the primary antibodies to maximize the labelling of synapses, we found that a similar proportion were labelled as when the antibodies were used singly. It is thus likely that a proportion of the boutons forming receptor-negative, asymmetric synapses are, in fact, non-glutamatergic. Indeed, serotonergic terminals from the raphe and cholinergic terminals probably derived from the pedunculopontine nucleus, give rise to boutons in the SN that form asymmetric synapses. It should be noted however, that at least some of the cholinergic terminals in the entopeduncular nucleus that are derived from the pedunculopontine nucleus, are also enriched in glutamate immunoreactivity and this may also be the case in the SN. Preliminary analyses using double post-embedding immunogold labelling of glutamate receptor subunits and glutamate itself in SN, indicate that almost every bouton forming NR1- or GluR2/3-positive asymmetric synapses is also glutamate immunopositive, however, a high proportion of boutons forming asymmetric synapses were glutamate negative and formed NR1- and GluR2/3-negative synapses. These findings, together with the data in the present study, provide confirmation that NMDA and AMPA receptors are expressed at glutamatergic, asymmetric synapses in the SN, and support the presence of non-glutamatergic afferents that form asymmetric synapses in the SN.

It should be noted that the quantitative estimates that we have made reflect the labelling present in the sections that we examined. Due to variations in the “robustness” of immunolabelling with different antibody preparations, the values may reflect more the “quality” of immunolabelling with a particular antibody and may not be a true representation of the distribution of the receptor subunits in the brain. Furthermore, since it is unlikely that we labelled every antigenic site, the values must, therefore, be considered as minimum values for the true distributions of AMPA and NMDA subunits in the SN.

The analysis of the distribution of immunogold particles across the width of the synapses revealed that, on average, the
immunolabelling for both the NR1 subunit of the NMDA receptor and GluR2/3 subunits of the AMPA receptor is evenly distributed across the synaptic specialization with a gradual fall-off at the periphery. The distribution of NR1 and GluR2/3 labelling is essentially the same as found previously in other regions of the basal ganglia (neostriatum and globus pallidus, entopeduncular nucleus and subthalamic nucleus). This pattern of distribution for the GluR2/3 subunits has also been observed in other areas of the brain including the hippocampus and cerebellum and suggests that both NMDA receptors and AMPA receptors containing GluR2 and/or 3 subunits in the same postsynaptic density in the SN will have equal access to synthaptically released glutamate. This is not the situation, however, in the hippocampus, where NMDA receptor labelling is found to be concentrated at the centre of the synaptic specialization indicating that the mechanisms controlling the selective trafficking of receptors to synapses and within the synaptic specialization vary in different regions and from synapse to synapse. The analysis carried out in this study was on single sections and hence the data are an average for the whole population; analysis of serial sections may reveal differences in individual or subpopulations of synapses.

Co-localization of N-methyl-d-aspartate receptor subunits at individual synapses

Double labelling for the NR1 and NR2A/B subunits of the NMDA receptor using the post-embedding immunogold technique revealed co-localization at more than half of all immunopositive asymmetric synapses in SNc and SNr. This finding is consistent with suggestions that functional NMDA receptor complexes require the presence of at least one NR1 and NR2 subunit (see Introduction). On the basis of this, one would predict that all NR2A/B-positive synapses would also be immunopositive for NR1; the failure to detect 100% co-localization presumably relates to the technical limitations of the procedure as discussed above. The failure to detect immunolabelling for NR2A/B at all NR1-positive synapses may also relate to technical limitations but it could also be that they express different NR2 subunits (NR2C, NR2D) that were not analysed in the present study, but are highly expressed in the SN.

Co-localization of N-methyl-d-aspartate and AMPA receptor subunits at individual synapses

The double immunolabelling demonstrated that NMDA and AMPA receptors co-localize at individual asymmetric synapses in the SNc and SNr. These findings are similar to those found in other regions of the basal ganglia and in other regions of the brain. Immunolabelling for the NR1 and the GluR2/3 subunits was co-localized at one-quarter of the immunopositive synapses, and the labelling for NR2A/B and GluR2/3 at more than one-third of immunopositive synapses. This difference is likely to be related to the robustness of immunolabelling for the NR2A/B subunits compared to that for the NR1 subunit which is reflected in the higher proportion of asymmetric synapses, labelled with the NR2A/B antibody than with the NR1 antibodies (see Table 3). Interestingly, in sections double labelled for NR1 and GluR2/3, 70—80% NR1-positive synapses were also GluR2/3 positive. However, only up to one-third of synapses positive for GluR2/3 were also positive for NR1. This suggests that synapses expressing NMDA receptors also express AMPA receptors but that there is a subpopulation of synapses that express only AMPA receptors.

Functional considerations

It is clear from many physiological studies that glutamate excitation of both dopamine neurons of the SNc and non-dopamine neurons of the SNr is mediated by both NMDA and AMPA receptors. Burst firing of dopamine neurons, which is related to increased release of dopamine in the striatum and to behavioural responses, is an NMDA receptor-dependent phenomenon. Furthermore, stimulation of the STN or prefrontal cortex induces burst firing in dopamine neurons that is dependent on NMDA but not AMPA receptors. In contrast, the excitatory responses of dopamine neurons following stimulation of the pedunculopontine nucleus is not associated with burst firing, is not blocked by NMDA antagonists but is dependent on AMPA receptors. These observations, taken together with the finding that only a proportion of neurons that respond with burst firing following STN or prefrontal cortex stimulation are associated with synapses that only expressed AMPA receptors. It is interesting to note that terminals in the STN and entopeduncular nucleus, and presumably also the SN, that are derived from the pedunculopontine nucleus, stimulation of which produces only AMPA-dependent responses. It is also possible that theafferent fibres that were stimulated when SNc neurons failed to respond to STN or prefrontal cortex stimulation with burst firing were associated with synapses that only expressed AMPA receptors. It is interesting to note that terminals in the STN and entopeduncular nucleus, and presumably also the SN, that are derived from the pedunculopontine nucleus, also contain acetylcholine; co-release of glutamate and acetylcholine may produce the richness and subtlety of response that is brought about at other glutamatergic synapses by the presence of multiple types of glutamate receptors. One way to clarify these issues is to combine anterograde labelling with receptor immunolabelling to identify the origin of synaptic terminals forming receptor-positive synapses.

Since we conclude that the most synapses that express NMDA receptors also express AMPA receptors, then the failure of AMPA-receptor antagonists and the success of NMDA-receptor antagonists in modulating burst firing following subthalamic nucleus or prefrontal cortex stimulation, implies that some mechanism other than, or in addition to, AMPA receptor-mediated depolarization, is required to sufficiently depolarize the neurons to overcome the magnesium block of the NMDA receptors.

Neurons in the SNr are similarly responsive to cortical stimulation. As in the SNc, there is also evidence of differences in the compliment of excitatory amino acid receptors at different synapses. Thus, high-pressure neurological syndrome in rats is dependent on NMDA receptor activity in the SNr and convulsive seizures in models of epilepsy are selectively sensitive to blockade of different populations of glutamate receptors. The elucidation of the precise compliment of excitatory amino
acid receptors at different synapses awaits the application of combined anterograde labelling and receptor labelling techniques.

CONCLUSIONS

The findings of the present study demonstrate that excitatory amino acid receptors are localized at asymmetric synapses in both the SNc and SNr, and that AMPA and NMDA receptors co-localize within at least some synapses. It is thus likely that glutamatergic transmission occurs primarily at synaptic specializations in the SN although non-synaptic transmission by glutamate diffusing from the synapse cannot be excluded. Any involvement of glutamate-mediated excitotoxicity in the cell death that occurs in Parkinson’s disease presumably relates to the receptors localized at synaptic sites and perhaps also at extrasynaptic sites.

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