



IMMUNOCYTOCHEMICAL LOCALIZATION OF D₁ AND D₂ DOPAMINE RECEPTORS IN THE BASAL GANGLIA OF THE RAT: LIGHT AND ELECTRON MICROSCOPY

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Abstract—The modulatory actions of dopamine on the flow of cortical information through the basal ganglia are mediated mainly through two subtypes of receptors, the D₁ and D₂ receptors. In order to examine the precise cellular and subcellular location of these receptors, immunocytochemistry using subtype specific antibodies was performed on sections of rat basal ganglia at both the light and electron microscopic levels. Both peroxidase and pre-embedding immunogold methods were utilized.

Immunoreactivity for both D₁ and D₂ receptors was most abundant in the neostriatum where it was mainly contained within spiny dendrites and in perikarya. Although some of the immunoreactive perikarya had characteristics of interneurons, most were identified as medium-sized spiny neurons. Immunoreactivity for D₁ receptor but not D₂ receptor was associated with the axons of the striatonigral pathway and axons and terminals in the substantia nigra pars reticulata and the entopeduncular nucleus. In contrast, D₂ immunoreactivity but not D₁ immunoreactivity was present in the dopaminergic neurons in the substantia nigra pars compacta and ventral pars reticulata. In the globus pallidus, little immunoreactivity for either D₁ or D₂ receptor was detected.

At the subcellular level, D₁ and D₂ receptor immunoreactivity was found to be mainly associated with the internal surface of cell membranes. In dendrites and spines immunoreactivity was seen in contact with the membranes postsynaptic to terminals forming symmetrical synapses and less commonly, asymmetrical synapses. The morphological features and membrane specializations of the terminals forming symmetrical synapses are similar to those of dopaminergic terminals previously identified by immunocytochemistry for tyrosine hydroxylase. In addition to immunoreactivity associated with synapses, a high proportion of the immunoreactivity was also on membranes at non-synaptic sites.

It is concluded that dopamine receptor immunoreactivity is mainly associated with spiny output neurons of the neostriatum and that there is a selective association of D₁ receptors with the so-called direct pathway of information flow through the basal ganglia, i.e. the striatoentopeduncular and striatonigral pathways. Although there is an association of receptor immunoreactivity with afferent synaptic inputs a high proportion is located at extrasynaptic sites.

The dopaminergic synaptic input from the substantia nigra to the neostriatum has been the focus of extensive analyses because of its degeneration in Parkinson's disease and its role in the control of the normal functioning of the basal ganglia. The activity of the principal cell of the neostriatum, the medium-sized spiny neuron, is regulated by the release of dopamine by the nigrostriatal terminals.^{40,41} Pharmacological and physiological studies³⁷ predicted that the function of dopamine on striatal neurons would be mediated by at least two dopamine receptor subtypes defined as dopamine D₁ and D₂ receptors. To date, by gene cloning, five dopamine receptor subtypes (D₁ to D₅) have been identified. They can be

classified, in structural terms and functional terms into D₁-like (D₁ and D₅) and D₂-like (D₂-D₄) receptors; each type having distinct pharmacological and biochemical properties.^{22,65,71} *In situ* hybridization studies^{8,15,33,46,52,54,55,61,78,79} and ligand binding studies^{5,6,12,17,62} in different species including rats and primates, have demonstrated that the basal ganglia are particularly enriched in dopamine D₁ and D₂ receptor subtypes. Furthermore, recent immunocytochemical studies using antibodies against peptide sequences of cloned D₁ and D₂ receptors, have described the presence of immunoreactivity for the receptors in the basal ganglia of rat and primate.^{2,3,32,51,66}

An issue that is of critical importance to our understanding of the functional organization of the basal ganglia is the precise cellular localization of the receptors in the neostriatum. It has been proposed that there is a separation of dopamine receptors between the so-called direct and indirect neostriatal output pathways. D₁ receptor mRNA is associated

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Abbreviations: ABC, avidin-biotin-peroxidase complex; DAB, 3,3'-diaminobenzidine; 6-OHDA, 6-hydroxydopamine; PAP, peroxidase-antiperoxidase; PB, phosphate buffer; PBS, phosphate-buffered saline; PBS-BSA, PBS supplemented with 0.5% bovine serum albumin and 0.1% gelatin; RT, room temperature.

with neurons that give rise to the direct pathway from neostriatum to substantia nigra pars reticulata or entopeduncular nucleus and also express substance P and dynorphin mRNA, whereas D₂ receptor mRNA is expressed by neurons giving rise to the indirect pathway to the globus pallidus (external segment of the globus pallidus in primates) that also express enkephalin.^{18,19} However, other workers have proposed, using a variety of techniques, that all neostriatal neurons express both D₁ and D₂ receptors^{73,74} or that they co-exist in up to 26% of neostriatal neurons.⁴⁹ Furthermore it has been proposed that up to 60% of striatonigral neurons express mRNA for D₂ receptors.² It has also been suggested that D₁ and D₂ dopamine receptor subtypes are preferentially localized in the sub-compartments of both cat and human neostriatum.^{7,26,34} The first objective of the present experiment was therefore to re-address this issue by comparing the distribution throughout the basal ganglia, of immunoreactivity for D₁ and D₂ receptors using receptor subtype specific antibodies⁵¹ at both light and electron microscopic levels.

A second issue of importance in the understanding of the functional organization of the basal ganglia and the role of dopamine in the basal ganglia is the subcellular localization of dopamine receptors. It is becoming increasingly apparent from immunocytochemical studies of G-protein linked receptors at the electron microscopic level that the subcellular distribution of the receptors is not necessarily associated with afferent synaptic terminals.^{31,32,51,66} Most of these studies used immunoperoxidase methods which, although of importance in the characterization of immunoreactive structures at the cellular level, do not give a precise indication of the subcellular location of the antigenic site because of the diffusion of the reaction product. One approach to address this problem is to use a particulate marker to localize the antigenic sites. The most reliable marker is colloidal gold which can be used by either post-embedding methods or by pre-embedding methods in combination with silver intensification (see Ref. 60). This approach has been successfully applied to the localization of glutamate receptors.^{4,58} The second objective of the present study was to attempt to localize immunoreactivity for D₁ and D₂ receptors at the subcellular level in neostriatum and other regions of the basal ganglia, using pre-embedding immunocytochemistry with colloidal gold conjugated secondary antibodies.

A preliminary report of this study has been published previously in abstract form.⁸⁴

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

Twenty-one rats (Wistar or Sprague-Dawley, 200–250 g; Charles River) were used in the present study for receptor immunocytochemistry. In addition, two 6-hydroxydopamine (6-OHDA) lesioned rats were also used (see below). All the animals were deeply anaesthetized with 3.5% chloral

hydrate in saline (0.9% NaCl) or with sodium pentobarbitone (Sagittal, 60 mg/kg). The animals were then perfused transcardially with 50–100 ml of either calcium-free Tyrode's solution or saline followed by 200 ml of fixative (3% paraformaldehyde with 0.1%–1.0% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4) at a rate of about 10 ml/min. The brain was quickly removed and sections of the basal ganglia (70 µm) including neostriatum, globus pallidus, entopeduncular nucleus, subthalamic nucleus and substantia nigra were cut on a vibrating microtome and collected in phosphate-buffered saline (PBS; 0.01 M, pH 7.4). In order to enhance the penetration of the immunoreagents, the sections were equilibrated in a cryoprotectant solution (PB, 0.05 M, pH 7.4, containing 25% sucrose and 10% glycerol) and freeze-thawed in isopentane (BDH Chemicals) and liquid nitrogen. The sections from those rats perfused with high concentrations of glutaraldehyde (0.5–1.0%) were then treated with sodium borohydride (BDH chemicals, 0.1% in PBS) for 5–10 min. The sections were then washed (3 × PBS) and pre-blocked by incubating in normal serum (4% in PBS) for 1 h at room temperature (RT). In most cases, normal goat serum was used, however, for the monoclonal D₁ receptor antibody (see below), normal rabbit serum was used.

Sections were immunostained by the avidin-biotin-peroxidase (ABC) method or by pre-embedding immunogold method with silver enhancement to reveal immunoreactivity for the D₁ and D₂ dopamine receptors. Various preparations of primary antibody were used in order to give optimal staining, these included polyclonal antisera against both D₁ and D₂ receptors (raised in rabbits), affinity purified polyclonal antibody (rabbit-anti-D₂), and monoclonal antibody (rat-anti-D₁). All the antibodies were raised against fusion proteins derived from amino acid sequences of the C-terminus of the D₁ receptor or intracellular loop 3 of the D₂ receptor. They were biochemically characterized and proved to be highly specific to that particular receptor subtype.⁵¹ The monoclonal antibodies against the D₁ receptor were raised against the same amino acid sequence of the C-terminus. They show specificity for the cloned and native D₁ receptor on Western immunoblots and the immunocytochemical staining was distributed in an identical pattern to the polyclonal antibody. The purified and monoclonal antibodies gave the most intense immunostaining in all regions of the basal ganglia.

Immunocytochemistry: avidin-biotin-peroxidase method

The sections to be immunostained by the ABC method were incubated with primary antibodies (D₁ at 1:2000–1:5000 dilution; D₂ at 1:100–1:2000 dilution; in PBS supplemented with 2% normal serum) at RT for 24 h or at 4°C for 48 h. They were then washed (3 × PBS) and incubated in biotinylated goat-anti-rabbit IgG (1:200; Vector) or biotinylated goat-anti-rat IgG (1:100; Vector) for 1–1.5 h at RT. The sections were then washed (3 × PBS) and incubated in the ABC complex (1:100, Vector) for at least 1 h at RT. After washing (2 × PBS, 1 × Tris-HCl buffer, 0.05 M, pH 7.4), the D₁ and D₂ receptor immunoreactive sites were revealed by incubation in H₂O₂ (0.0048%) in the presence of 3,3'-diaminobenzidine (DAB; Sigma; 0.025%, Tris-HCl buffer, pH 7.4). The reaction was stopped by several washes in Tris-HCl buffer followed by PBS.

Immunocytochemistry: pre-embedding immunogold method

Pre-embedding immunocytochemistry using the silver-intensified immunogold method was carried out essentially as described by Baude *et al.*⁴ The sections which were immunostained by the pre-embedding immunogold method were incubated in primary antibodies as described above. After washing twice in PBS and then twice in PBS supplemented with 0.5% bovine serum albumin and 0.1% gelatin (PBS-BSA), the sections were incubated in goat-anti-rabbit IgG or rabbit-anti-rat IgG conjugated to col-

loidal gold (1.4 nm diameter; Nanogold, Nanoprobes; 1:100–200 dilution in PBS-BSA, with 1–2% normal serum) for at least 2 h at RT. The sections were then washed (3 × PBS-BSA, 3 × PBS) and post-fixed in 1% glutaraldehyde in PBS for 10 min. After two further washes in PBS and two in acetate buffer (0.1 M, pH 7.0), the colloidal gold staining was intensified using a commercially available silver enhancement kit (HQ silver, Nanoprobes). The reaction was carried out for 5–10 min at RT or on ice (4°C) in the dark and stopped by several washes in acetate buffer.

Light and electron microscopy

Those sections for light microscopy were mounted on gelatin-coated microscope slides, dehydrated in a series of dilutions of ethanol and mounted in XAM (BDH Chemicals). Sections for electron microscopy were post-fixed in osmium tetroxide (1% in PB; 0.1 M, pH 7.4) for 20–30 min for DAB-reacted sections or about 10 min for immunogold sections, dehydrated in an ascending series of dilutions of ethanol (with the presence of 1% uranyl acetate in 70% ethanol) followed by propylene oxide (Aldrich) and then embedded in resin overnight, mounted on glass slides and then cured at 60°C for 48 h. All sections were examined in the light microscope. Areas of interest were photographed, cut out from the slide and re-embedded on blank resin blocks. Since the D₁ and D₂ immunoreaction product was very intense in the neostriatum, semi-thin sections (1–2 μm) were cut (Reichert-Jung Ultracut E), collected on gelatin-coated slides, mounted in XAM and were examined in the light microscope. For electron microscopy, serial ultrathin sections were cut (Reichert-Jung Ultracut E), collected on Ploioform-coated slot grids and examined in a Philips 410 or CM10 electron microscope.

6-Hydroxydopamine lesion study

Rats received unilateral stereotaxic injections of 6-hydroxydopamine (6-OHDA) (4 μl of 3 mg/ml in saline containing 0.2 mg/ml ascorbic acid) in the right medial forebrain bundle whilst under pentobarbitone anaesthesia. The injection was administered over a period of 3–5 min and the needle left in place for a further 5–10 min. Twelve days later they were challenged with amphetamine (5 mg/kg i.p.) and the number of times they rotated over a period of 30 min was noted. Sections of the forebrain and the mesencephalon from two of the rats (249 and 242 rotations in 30 min) were immunostained as described above, using a rabbit antiserum against tyrosine hydroxylase⁷⁶ (1:2000 dilution) or they were stained to reveal the dopamine receptor immunoreactivity. These sections were subjected to light microscopic analysis only.

Double immunostaining for dopamine receptors and tyrosine hydroxylase

Some sections of the mesencephalon were double-immunostained to reveal both the receptor immunoreactivity and tyrosine hydroxylase using the double peroxidase method described previously.⁵⁰

Quantitative analysis

Quantitative analysis of the distribution of D₁ and D₂ immunoparticles was performed on immunogold-labelled sections of the neostriatum. Electron micrographs were taken systematically along the interface between the tissue and empty resin. The analysis was carried out on these micrographs at a final magnification of ×45000. A total of 107 micrographs from three animals were used. All the immunoreactive profiles on the micrographs were identified and classified into seven categories: dendrites, spines, axons, axonal boutons (i.e. swelling of axons that contained vesicles), axon terminals forming asymmetrical synapses, axon terminals forming symmetrical synapses, perikarya, plus small immunoreactive profiles that could not be

classified. The relative frequency of each category was calculated.

In order to determine whether immunoreactivity for D₁ and D₂ receptor was selectively associated with membranes, the immunogold particles associated with dendrites and spine was categorized as being associated with membranes or not. Furthermore, the spatial relationship between immunogold particles in spines and afferent synaptic terminals was assessed in single sections. The immunoparticles were categorized as not being associated with a synapse if the spine did not receive synaptic input in that particular single section. The immunoparticles in spines that received afferent synapses were further sub-divided according to their spatial relationship with the synaptic specialization (see legend to Table 2).

The cross-sectional area of immunoreactive perikarya was estimated from drawing-tube drawings. The areas were calculated with the aid of a digitizing pad and MacStereology software.

RESULTS

Light microscopic observations

Neostriatum. Sections of the neostriatum of the rat incubated to reveal immunoreactivity for either D₁ or D₂ receptors were densely stained by the peroxidase reaction product (Figs 1A–C, 2A for D₁; Figs 1D–F, 2B for D₂). The distribution of immunoreactivity for both of the receptors was not homogeneous; although the staining throughout the neostriatum was intense compared with other regions of the brain, the rostral neostriatum displayed areas of differences in the intensity of staining (Fig. 1A, C). The unevenness in the staining for D₂ receptor was greater than that observed for D₁ receptor. The relationship of the uneven staining was not compared with that of the patch/striosome-matrix system in the present study.

Due to the high density of immunoreactive structures it was difficult to distinguish individual structures that displayed immunoreactivity for either D₁ or D₂ receptors at low magnification. At high magnification examination of the vibrating microtome sections and 1–2 μm semi-thin sections revealed that the D₁ and D₂ immunoreaction product was mainly located in dendrites (Fig. 2A, B). Dendritic spines were frequently observed emerging from these immunoreactive dendrites (Fig. 2A, B). The staining of perikarya was less frequently observed and was less intense than the staining of dendrites and dendritic spines (Fig. 2A, B). In most cases, the immunoreactive cell bodies were medium-sized (D₁ mean cross-sectional area ± S.E.M.: 96.3 ± 1.8 μm², diameter 10–15 μm, *n* = 127; D₂ mean cross-sectional area ± S.E.M.: 88.8 ± 1.3 μm², diameter 10–15 μm, *n* = 144) and both types of labelled neuron possessed a large circular nucleus that occupied most of the perikaryon and was surrounded by only a thin rim of cytoplasm. Most of the immunoreactive neurons thus had characteristics of the medium-sized spiny neurons. In addition to spiny neurons, other classes of striatal neuron displayed immunoreactivity for D₁ and D₂ receptors. D₂ receptor immunoreactivity was observed in large or medium-sized neurons that

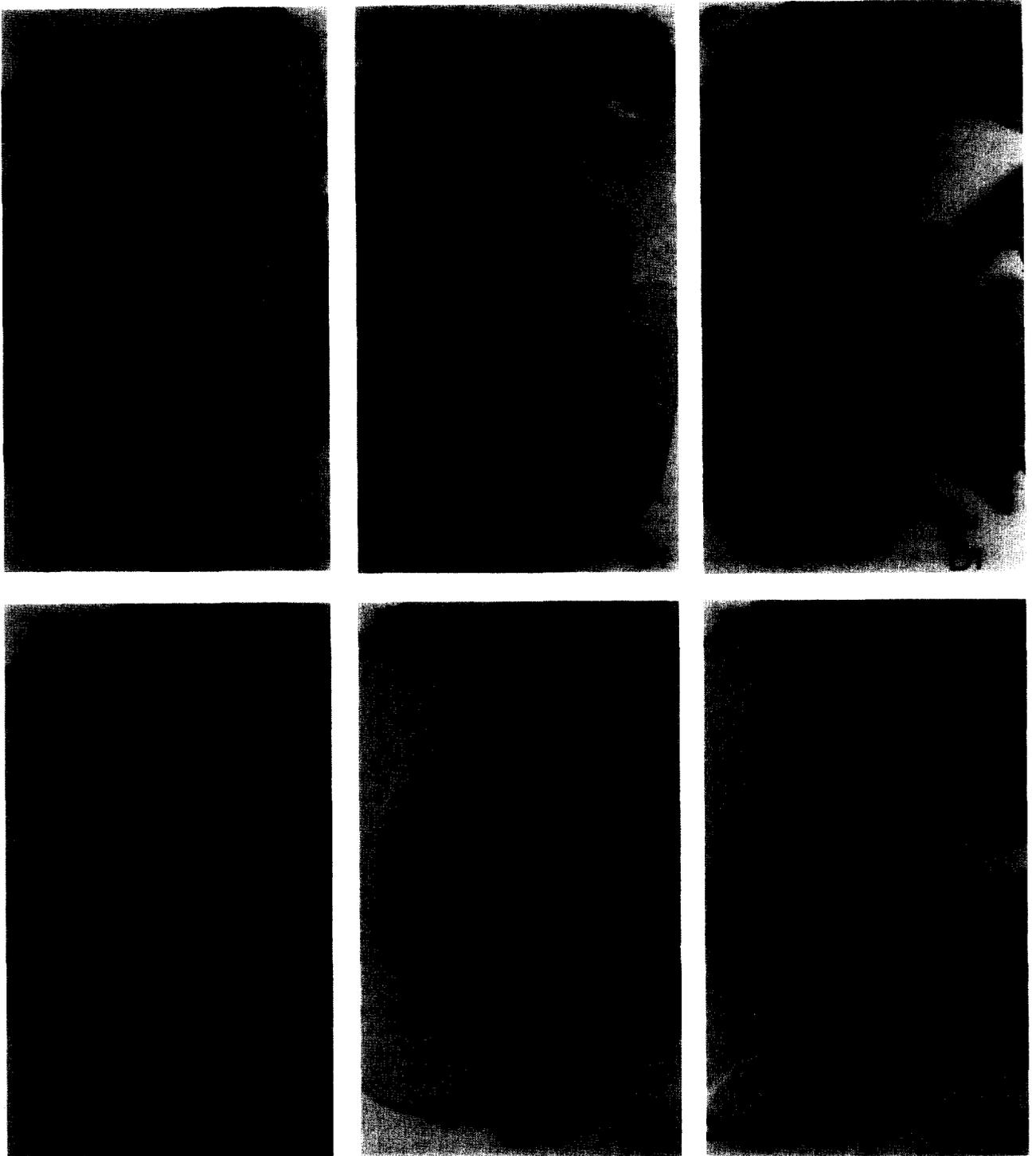


Fig. 1. Light micrographs of the rat brain immunostained to reveal immunoreactivity for D_1 (A–C) and D_2 (D–F) receptors. In A and B the neostriatum (str) displays dense immunoreactivity for D_1 receptor. The nucleus accumbens (Ac) and olfactory tubercle (OT) are also D_1 -immunoreactive (A). The cortex (ctx) shows much less immunoreactivity when compared with the neostriatum. At a more caudal level (C), the neostriatum is densely stained whereas the globus pallidus (GP) contains little immunoreactivity. Only fibres passing through the GP (arrowhead) displayed D_1 immunoreactivity. In D and E the neostriatum (str) displays dense immunoreactivity for D_2 receptor. The nucleus accumbens (Ac) and olfactory tubercle (OT) are D_2 -immunoreactive (D). At a more caudal level (F), the neostriatum is densely stained whereas the globus pallidus (GP) contains little immunoreactivity. No D_2 -immunoreactive fibres passing through the GP were seen. In D–F, the cortex is not D_2 immunoreactive. Scale bar for A–F = 0.95 mm.

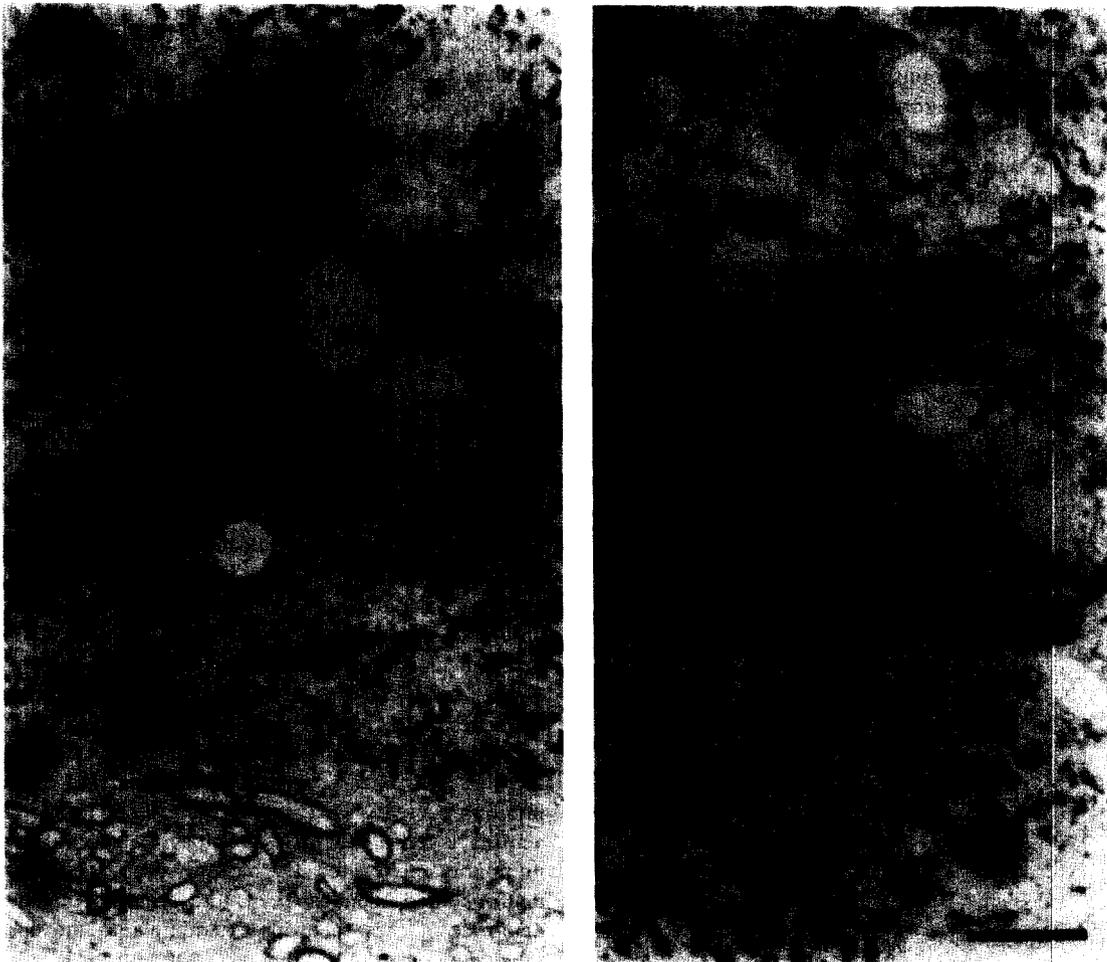


Fig. 2. High magnification light micrographs of semi-thin sections ($1\ \mu\text{m}$) of the neostriatum immunostained by the ABC method. D₁-immunoreactive (A) and D₂-immunoreactive (B) perikarya (P) and dendrites are observed. Most of the immunoreactive punctate structures observed at lower magnification are immunoreactive dendrites. These dendrites are spiny (arrowheads indicate some spines) and densely stained. The perikarya are often less intensely stained. A weakly immunoreactive neuron is also observed (star). In addition, non-reactive neurons are also shown (asterisks) which indicates that not all the neurons in the neostriatum are immunoreactive for the receptors. Scale bar for A and B = $10\ \mu\text{m}$.

possessed nuclear indentations (about 2.8% of immunoreactive neurons). D₁ receptor immunoreactivity was also associated with medium-sized neurons that possessed nuclear indentations (about 4% of immunoreactive neurons).

There was no evidence of staining of glial cells and the bundles of myelinated fibres passing through the neostriatum were immunonegative.

The distribution of the immunoreaction product for D₁ and D₂ receptors stained by the pre-embedding immunogold method was similar to that revealed by the peroxidase method. Thus the neostriatum was densely stained for both receptors and it was difficult to distinguish individual stained neurons and their processes.

In addition to the neostriatum, the more rostral sections revealed that the olfactory tubercle and both the core and shell of the nucleus accumbens were immunoreactive for both D₁ (Fig. 1A) and D₂ recep-

tors (Fig. 1D). As was the case in the dorsal neostriatum, the nucleus accumbens displayed uneven immunoreactivity, particularly for D₂ receptor.

Pallidal complex. The globus pallidus displayed much less immunoreactivity for both D₁ (Fig. 1C) and D₂ receptors (Fig. 1F) than did the neostriatum. The pattern of staining throughout the pallidal complex was the same in both peroxidase- and immunogold-labelled sections.

Immunoreactivity for D₁ was located in what appeared to be, axons and terminals that surrounded pallidal dendrites and perikarya. The intensity of D₁ immunostaining of these structures and the density of stained structures was far less than in the neostriatum. Bundles of fibres passing through the globus pallidus were strongly immunoreactive for D₁ receptors, the intensity of staining was similar to that of positive structures in the neostriatum (Fig. 1C). These bundles of axons were traced to the

entopeduncular nucleus which also displayed strong immunoreactivity for D_1 receptors (Fig. 3A). As was the case for the globus pallidus the staining appeared to be confined to axonal structures including axons of passage and terminals. In addition, the ventral pallidum displayed immunoreactivity for D_1 receptors with a moderate intensity of staining. In each of these

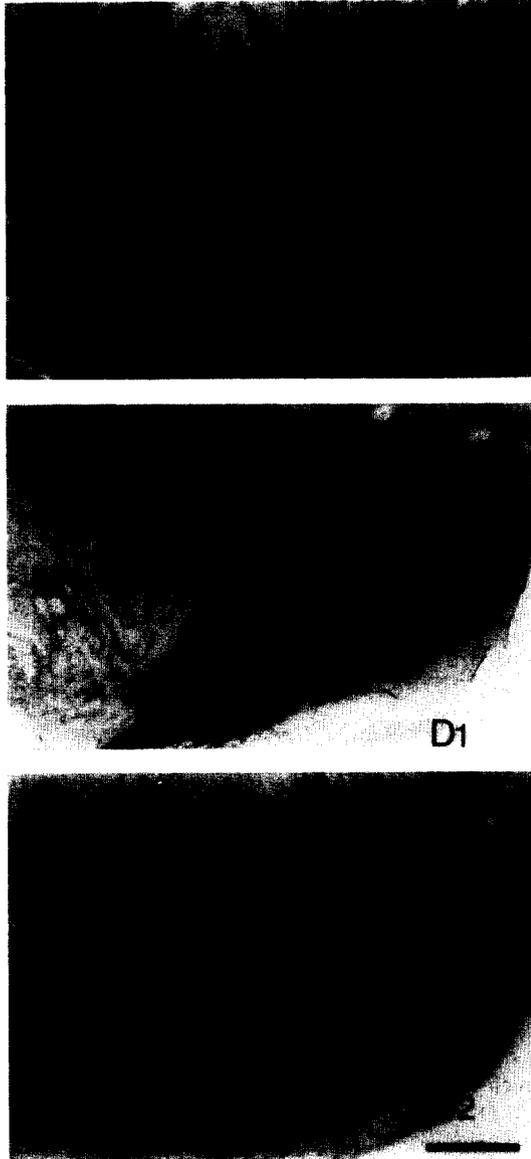


Fig. 3. Light micrographs of rat entopeduncular nucleus (EP; A) and the substantia nigra (SN; B and C) immunostained by the ABC method to reveal immunoreactivity for D_1 (A and B) and D_2 receptors (C). (A) D_1 immunoreactivity is mainly in the neuropil of the EP and probably represents axons and terminals. There is no obvious neuronal perikaryal or dendritic staining. D_1 immunoreactivity in the neostriatum (str) is still observed at this level. (B) D_1 immunoreactivity in the substantia nigra pars reticulata (SNr) is present in the neuropil. (C) D_2 immunoreactivity is in the perikarya of neurons in the substantia nigra pars compacta (SNc) and their dendrites that extend into the pars reticulata. The ventral tegmental area (VTA) is also D_2 -immunoreactive. Scale bar for A-C = 0.5 mm.

three structures, the globus pallidus, the ventral pallidum and the entopeduncular nucleus, neuronal perikarya and dendrites did not display immunoreactivity for D_1 receptors.

In contrast to the staining produced with the antibodies to D_1 receptors, the entopeduncular nucleus, the ventral pallidum and the bundles of axons of the striatofugal pathway were negative for D_2 receptor immunoreactivity (Figs 1E, F). In the globus pallidus, immunoreactivity for D_2 receptor was either very low or undetectable (Fig. 1F). At high magnification it was evident that the neuropil of the globus pallidus contained a population of D_2 -immunoreactive small punctate structures that appeared to be more discrete than structures stained for D_1 receptors. Occasionally, D_2 -immunoreactive spiny dendrites of apparently complete spiny neurons were found within the globus pallidus.

Substantia nigra. The substantia nigra displayed intense immunoreactivity for both D_1 and D_2 receptors but the distribution of the two were markedly different. The substantia nigra pars reticulata was dense in D_1 immunoreactivity which was associated with axonal structures but not neuronal perikarya or dendrites (Fig. 3B). In sections that were double stained for D_1 receptor immunoreactivity and for tyrosine hydroxylase, it was evident that the dense immunoreactivity for D_1 receptors extended into the ventral layer of the pars compacta. In contrast, immunoreactivity for D_2 receptor was present in neurons and dendrites of the pars compacta, dendrites of these neurons extending into the pars reticulata (Fig. 3C) and in a group of neurons located in the ventral part of the pars reticulata. The immunoreactive perikarya were large (mean cross-sectional area \pm S.E.M.: $161.6 \pm 10.5 \mu\text{m}^2$, diameter $16\text{--}26 \mu\text{m}$, $n = 27$), and oval in shape. Some of their dendrites extended in the plane of the pars compacta and others extended into the pars reticulata. Spines were occasionally observed emerging from the immunoreactive dendrites but they were at a much lower density than that found on dendrites in the neostriatum. The morphology and distribution of the D_2 -immunoreactive neurons was similar to that of tyrosine hydroxylase-immunoreactive neurons in the pars compacta (data not shown). Immunoreactivity for D_2 was also found in neuronal perikarya and dendrites in the ventral tegmental area (Fig. 3C).

Subthalamic nucleus. No immunoreactivity for either the D_1 and D_2 receptor was found by light microscopic observation (data not shown).

6-Hydroxydopamine lesioned animals. Sections of the neostriatum from animals that received unilateral 6-OHDA lesions, showed a marked depletion of tyrosine hydroxylase immunoreaction product on the lesioned side. There was no apparent difference in the density of both D_1 and D_2 receptor immunoreactivity between the lesioned and non-lesioned sides.

In the sections of the mesencephalon of the lesioned animals, there were no, or very few, tyrosine

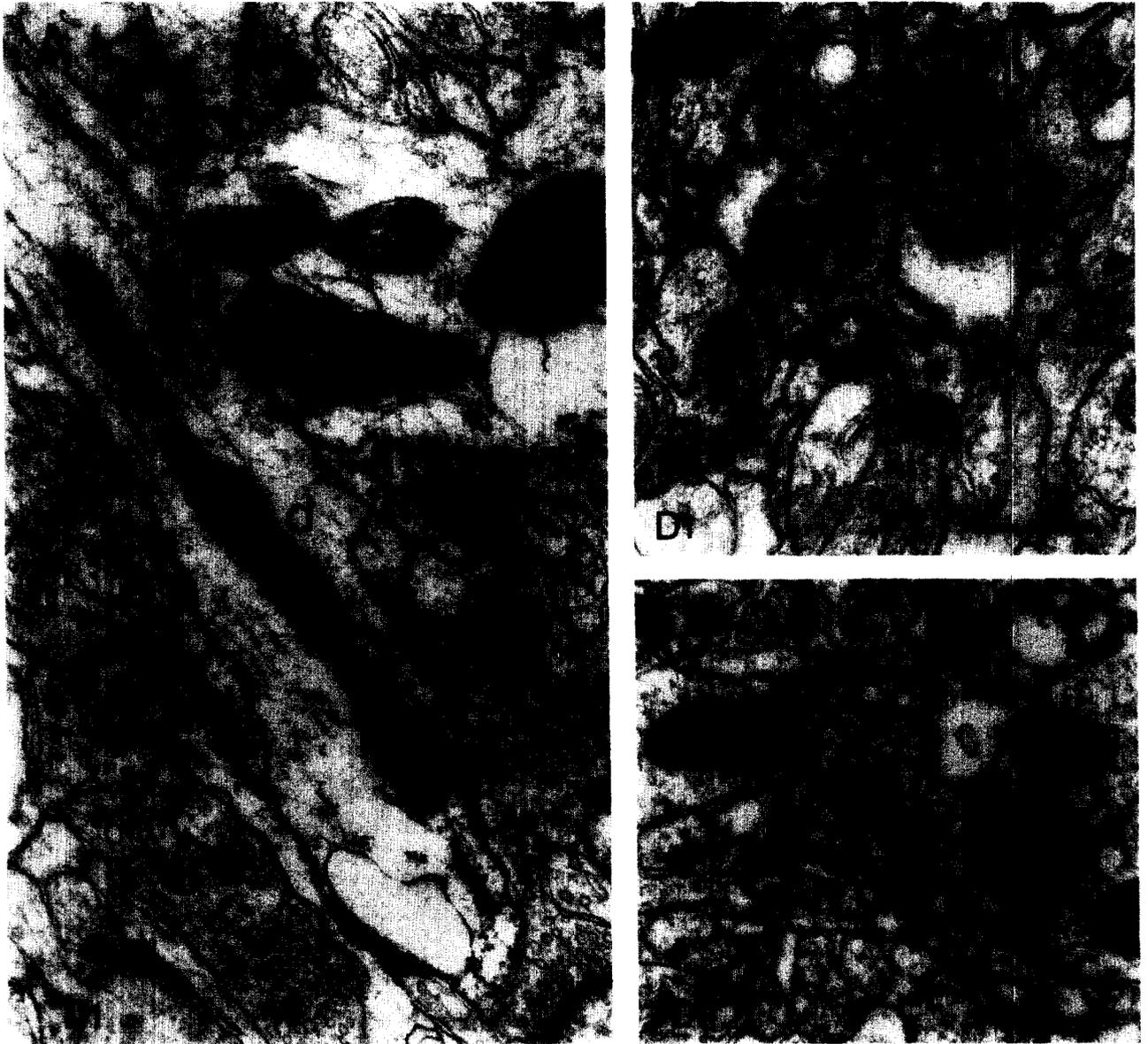


Fig. 4. Electron micrographs of the neostriatum immunostained by the ABC method to reveal immunoreactivity for D₁ receptor. In A a D₁-immunoreactive dendrite (d) contains patches of immunoreaction product (open arrows). B shows an immunoreactive dendrite (d) that gives rise to a non-reactive spine (*). C shows a D₁-immunoreactive spine (s) associated with afferent synaptic inputs, one forming an asymmetrical synapse (arrowhead) which has the characteristics of a cortical terminal, and the other forming symmetrical synaptic contact (two arrowheads). Scale bars for A and B = 0.5 μ m; for C = 0.5 μ m.

hydroxylase-immunoreactive neurons found in the substantia nigra pars compacta on the lesioned side but there were many on the non-lesioned side. The D₂ immunoreactivity in the compacta neurons followed the same pattern as the tyrosine hydroxylase immunoreactivity, i.e. the number of D₂-immunoreactive neurons was markedly reduced on the lesioned side. In the substantia nigra pars reticulata, however, there was no difference between the lesioned and non-lesioned sides in the intensity of D₁-immunostaining.

Electron microscopic observations

Neostriatum. In the electron microscope the peroxidase reaction product was identified as an electron dense precipitate associated with the membranes of subcellular organelles and the internal plasma membrane (see Fig. 4 for D₁-immunostained structures and Fig. 5 for D₂-immunostained structures). Both D₁ and D₂ immunostained structures were frequently encountered in the electron microscopic sections. The majority of immunostained structures were small

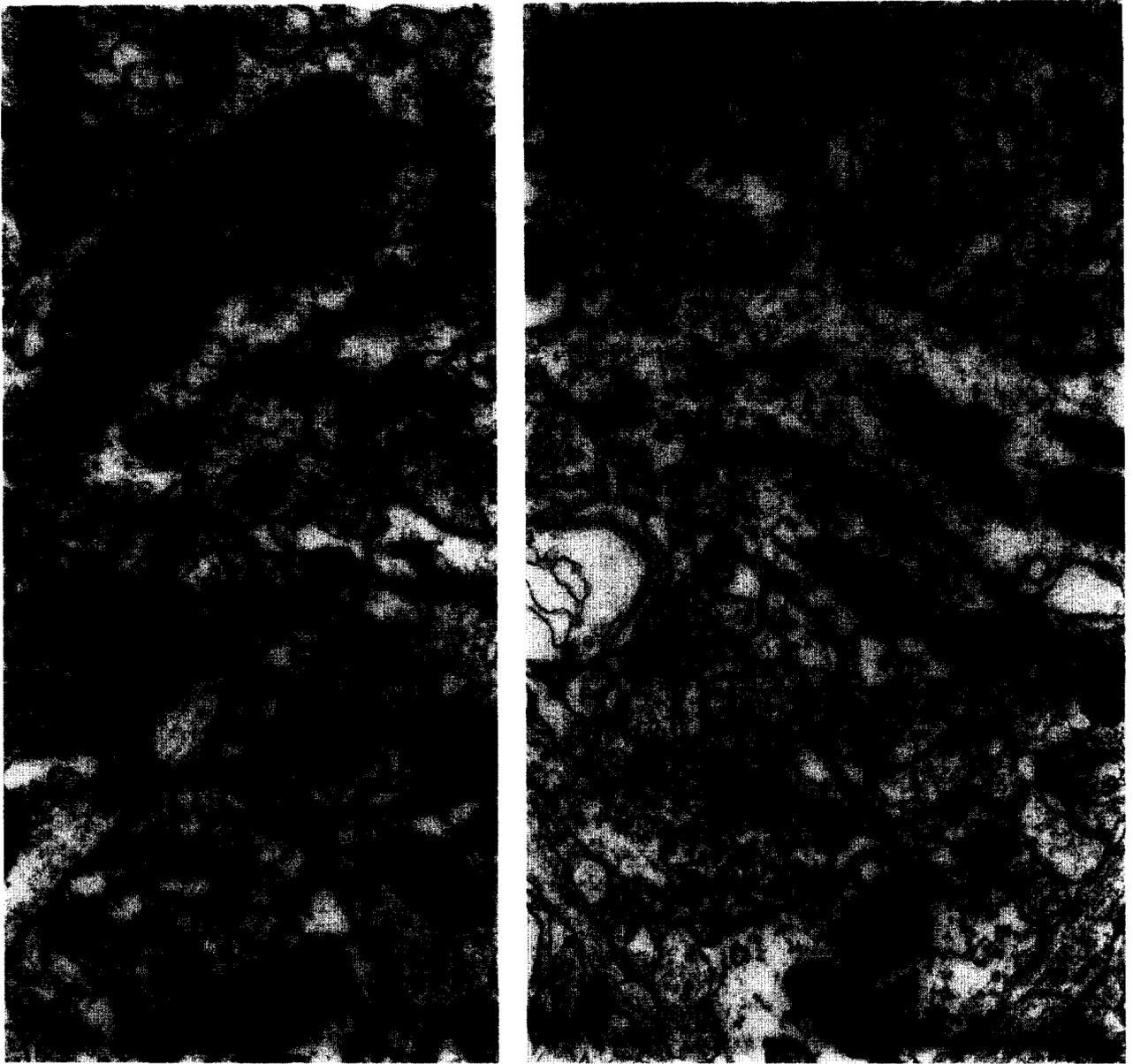


Fig. 5. Electron micrographs of the neostriatum immunostained by the ABC method to reveal immunoreactivity for D_2 receptor. In A dendrites (d) and a bouton (b) display D_2 -immunoreactivity. The immunoreactivity is distributed unevenly as a patch (open arrow) in one of the dendrites. In B a D_2 -immunoreactive dendrite (d) gives rise a non-reactive spine (*). A D_2 -immunoreactive spine (s) is receiving an asymmetrical synaptic input from a D_2 -immunoreactive terminal (b). Other immunoreactive dendrites (d) with patches of immunoreaction product (open arrows) are present. Scale bar for A and B = $0.5 \mu\text{m}$.

Fig. 6. Electron micrographs of the neostriatum immunostained by the pre-embedding immunogold method with silver enhancement to reveal the immunoreactivity for D_1 receptor. A shows a low magnification electron micrograph of the perikaryon (p) of a neuron that is D_1 immunoreactive. The immunogold particles (some indicated by curved arrows) are primarily associated with cell membrane and are unevenly distributed along its surface. The nucleus is large and unindented and surrounded by a thin rim of cytoplasm. These are characteristics of medium-sized spiny neurons. B and C show electron micrographs of dendrites (d) that display immunoreactivity for the D_1 receptor. The immunogold particles are mostly associated with the internal surface of the cell membrane of the dendrites and often occur in clumps. In C a bouton forms synaptic contact (two arrowheads) with a D_1 immunoreactive dendrite: the granules of reaction product are associated with a synapse. A bouton (b) in B also contains a granule of reaction product. D shows two D_1 -immunoreactive spines (s) both of which receive asymmetrical synaptic input at the head (indicated by an arrowhead in the upper one). One of the spines also receives input (two arrowheads) from a bouton that forms a symmetrical synapse that is associated with immunoreactivity for D_1 receptor. Scale bars for A, $1 \mu\text{m}$; for B-D = $0.5 \mu\text{m}$.

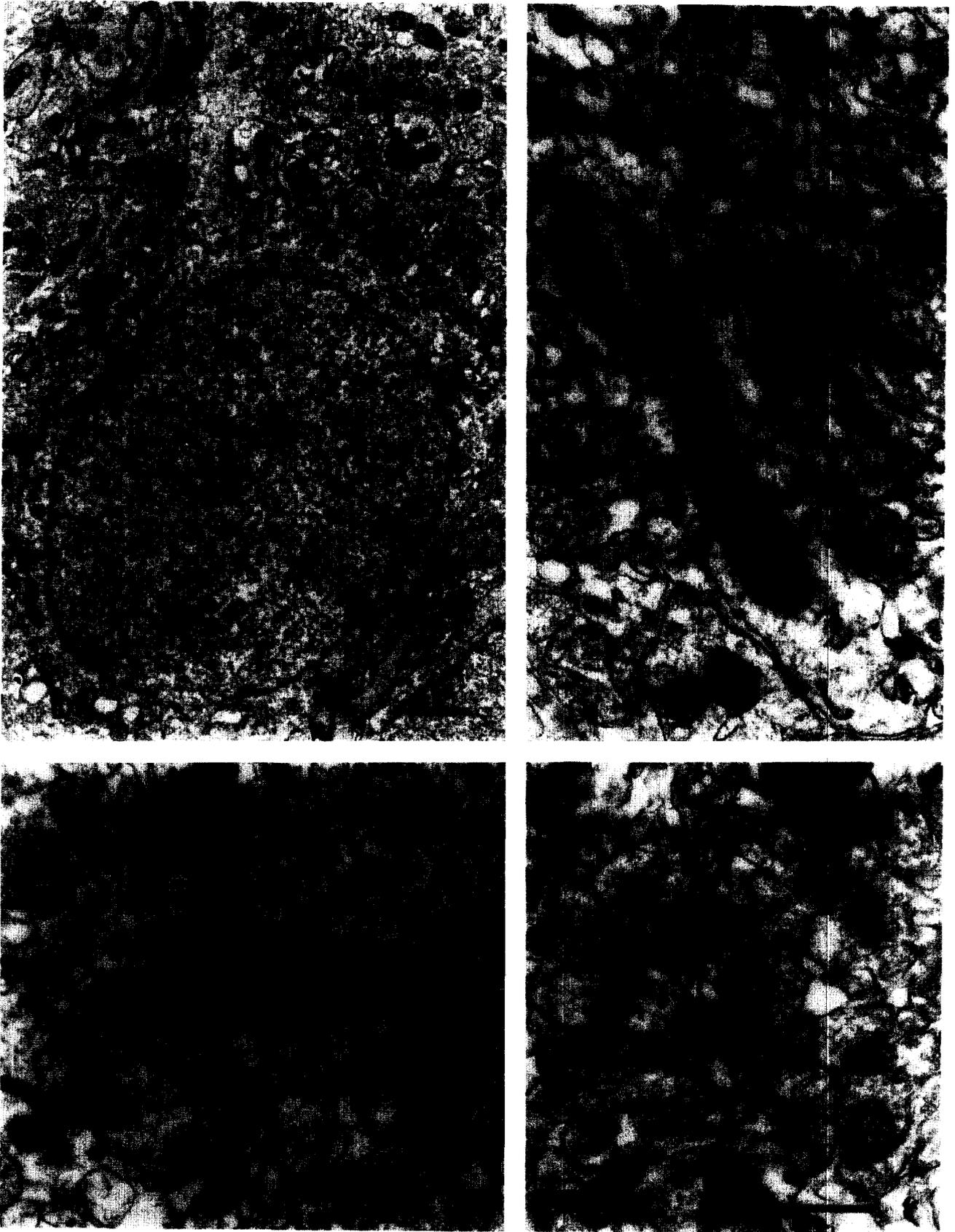


Fig. 6

diameter dendrites (Figs 4A, B, 5) and dendritic spines (Figs 4C, 5B), although immunoreactive large diameter dendrites, perikarya and axonal structures (see below) were also observed.

The majority of neuronal perikarya that displayed immunoreactivity for either D_1 or D_2 receptor were of medium-size, had a thin rim of cytoplasm with only few subcellular organelles and a relatively large, circular, unindented nucleus (see Fig. 6A which is a D_1 -immunoreactive neuron labelled by the immunogold method). These morphological characteristics are those of the most common type of striatal neuron, the medium-sized densely spiny neuron. In addition, a small number of D_2 -immunoreactive perikarya were observed that possessed indented nuclei (not illustrated), a characteristic of interneurons in the neostriatum.

At the subcellular level the peroxidase reaction product usually filled an immunoreactive profile, however, it was often the case that in dendrites and perikarya it was unevenly distributed. Patches of peroxidase reaction product were localized within dendrites (Fig. 4A for D_1 ; Fig. 5A, B for D_2) and perikarya whereas other parts were free of the immunoreaction product. Individual immunoreactive dendrites were often seen to give rise to spines that possessed no reaction product (Fig. 4B for D_1 -immunoreactive structures; Fig. 5B for D_2 -immunoreactive structures). Similarly, in single sections, immunoreactive spines were seen emerging from dendrites that did not apparently possess immunoreaction product. Occasionally the patches of peroxidase reaction product in dendrites or the immunoreactive spines were associated with afferent synapses (Figs 4C, 5B; see also sections stained by the pre-

embedding immunogold method Figs 6C, D, 7, 8A, B, 9). However, analyses made on serial sections revealed that many of the patches of reaction product were not associated with afferent synapses.

In order to localize more precisely the sites of D_1 and D_2 immunoreactivity at the subcellular level the pre-embedding immunogold method with silver intensification was employed. The distribution and types of structures stained by this method were essentially the same as those observed in the peroxidase stained sections. Thus the granules of silver-intensified immunogold localizing either D_1 (Figs 6, 7) or D_2 immunoreactivity (Figs 8, 9) were found mainly in dendrites, spines and occasionally in axonal structures (see below). Both D_1 - (Fig. 6A) and D_2 -immunoreactive perikarya, with the characteristics of the spiny neurons, were observed. The granules of silver-intensified immunogold for both D_1 (Figs 6, 7) and D_2 (Figs 8, 9) receptors were primarily associated with the plasmalemma of the stained structures although intracellular immunoparticles were observed (also see *Quantitative analysis* section). However, intracellular immunoparticles may represent the periphery of particles that do, in fact, originate on membranes (Fig. 9C, D). As with the peroxidase immunostained sections the distribution of the silver-intensified immunogold particles was uneven (Figs 6–9), clusters of particles were located intermittently along the length of the cell membranes. On some occasions the immunoreactivity for either receptor was immediately adjacent to the membrane postsynaptic to terminals forming asymmetrical synapses (Figs 6D, 8B). Rarely immunogold particles were observed on the postsynaptic membrane of this type of synapse (Fig. 9A, B). Silver-intensified

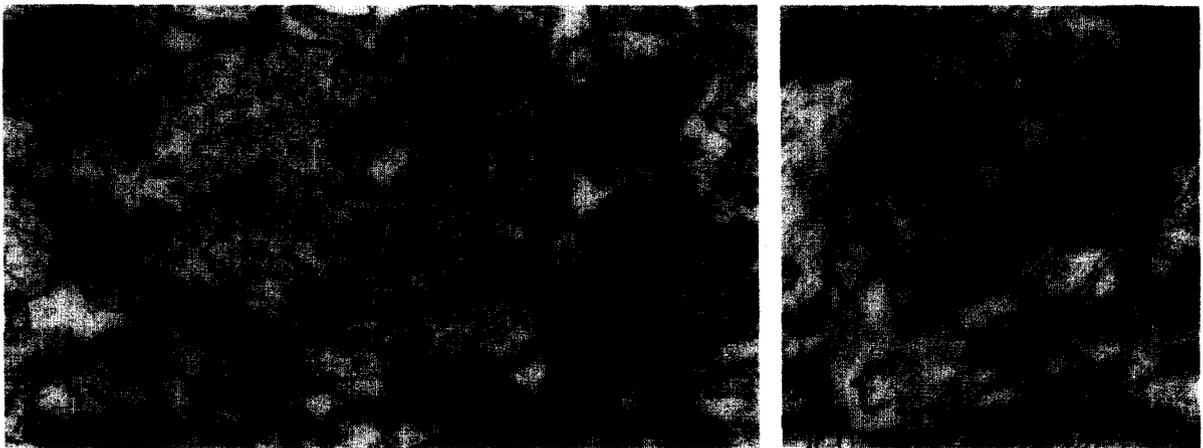


Fig. 7. Electron micrographs of the neostriatum illustrating an association of immunoreactivity for D_1 receptor with the membrane postsynaptic to terminals forming symmetrical synaptic specializations. (A) A dendrite (d) has many immunoparticles associated with the cell membrane. It receives synaptic input from a bouton (b) that forms symmetrical membrane specializations (arrowheads). The specialized membrane has silver-intensified immunogold particles adhering to it indicating the presence of D_1 receptor immunoreactivity. (B) A dendritic spine (s) receives synaptic input from two terminals, the one on the left forms an asymmetrical synapse (arrowhead). The terminal on the right (b) is in symmetrical synaptic contact with the spine (two arrowheads) and the postsynaptic membrane has an immunogold particle associated with it. Scale bar for A and B = 0.5 μm .

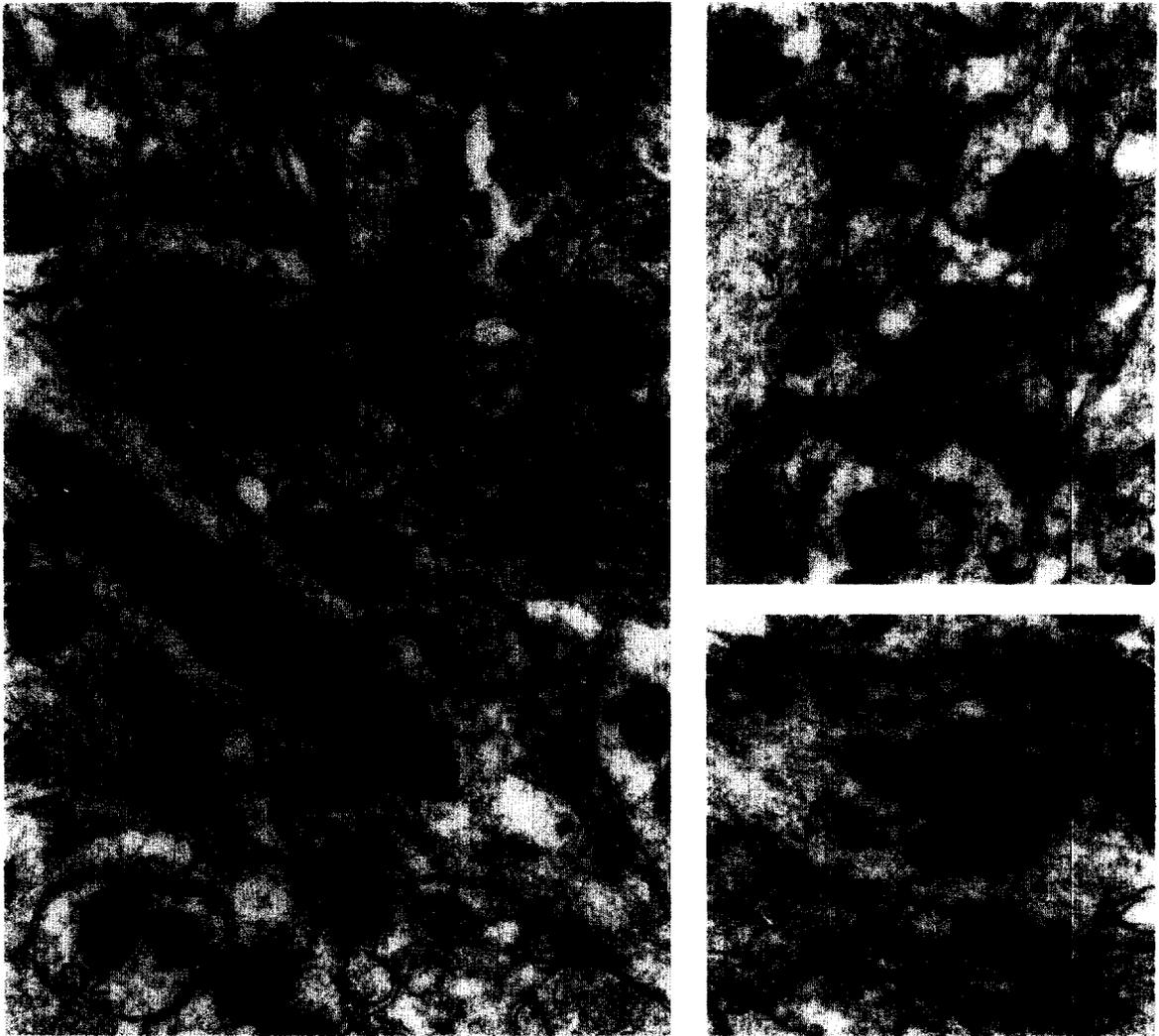


Fig. 8. Electron micrographs of the neostriatum immunostained by the pre-embedding immunogold method with silver enhancement to reveal immunoreactivity for D₂ receptor. Immunoreactivity for D₂ receptor is present in dendrites (d), spines (s) and occasional boutons (b). The immunogold particles are primarily associated with membranes. The immunoreactive spines are often associated with terminals forming asymmetrical synapses (arrowheads), in cases adjacent to the synaptic specialization. Scale bar for A-C = 0.5 μ m.

immunogold particles were more commonly seen on the membranes postsynaptic to terminals forming symmetrical synapses with dendrites (Figs 6C, 7A, 8C) or spines (Figs 6D, 7B, 9C-F). However, in single sections as well as in serial sections, it was evident that this was not necessarily the case, immunoparticles were often associated with parts of the plasmalemma that did not receive afferent synaptic input (Figs 6, 8).

In addition to perikarya and dendritic structures, D₁ and D₂ immunoreactivity was observed in axonal structures. Both D₁ and D₂ immunoreactivity was found in pre-terminal as well as terminal boutons (Fig. 6B for D₁; Fig. 5A, B for D₂). Some of the D₁- and D₂-immunoreactive boutons formed symmetrical synapses with non-reactive structures (not shown) and occasional D₂-immunoreactive boutons were

seen forming asymmetrical synapses with immunoreactive dendritic spines (one example is shown in Fig. 5B immunostained by the peroxidase method).

Globus pallidus. Consistent with the light microscopic observations, when examined in the electron microscope the globus pallidus contained only little immunoreactivity for D₁ or D₂ receptors. D₁-immunoreactive small unmyelinated axons and occasional myelinated axons were observed (not shown). Occasionally terminal boutons that formed symmetrical synaptic contacts with non-reactive dendrites were observed (not shown).

Entopeduncular nucleus. As observed at the light microscopic level, electron microscopic analysis of the entopeduncular nucleus revealed D₁ but not D₂ immunoreactivity. The immunoreactivity for D₁ receptor, whether localized by the peroxidase

(Fig. 10A) or the immunogold method, was present in large numbers of small diameter non-myelinated axons and to a lesser extent, myelinated axons (Fig. 10A). Immunoreactive boutons were also ob-

served, some of which were in symmetrical synaptic contact with non-reactive dendrites (Fig. 10B).

Substantia nigra pars compacta. The peroxidase immunoreaction product for D₂ receptor was local-

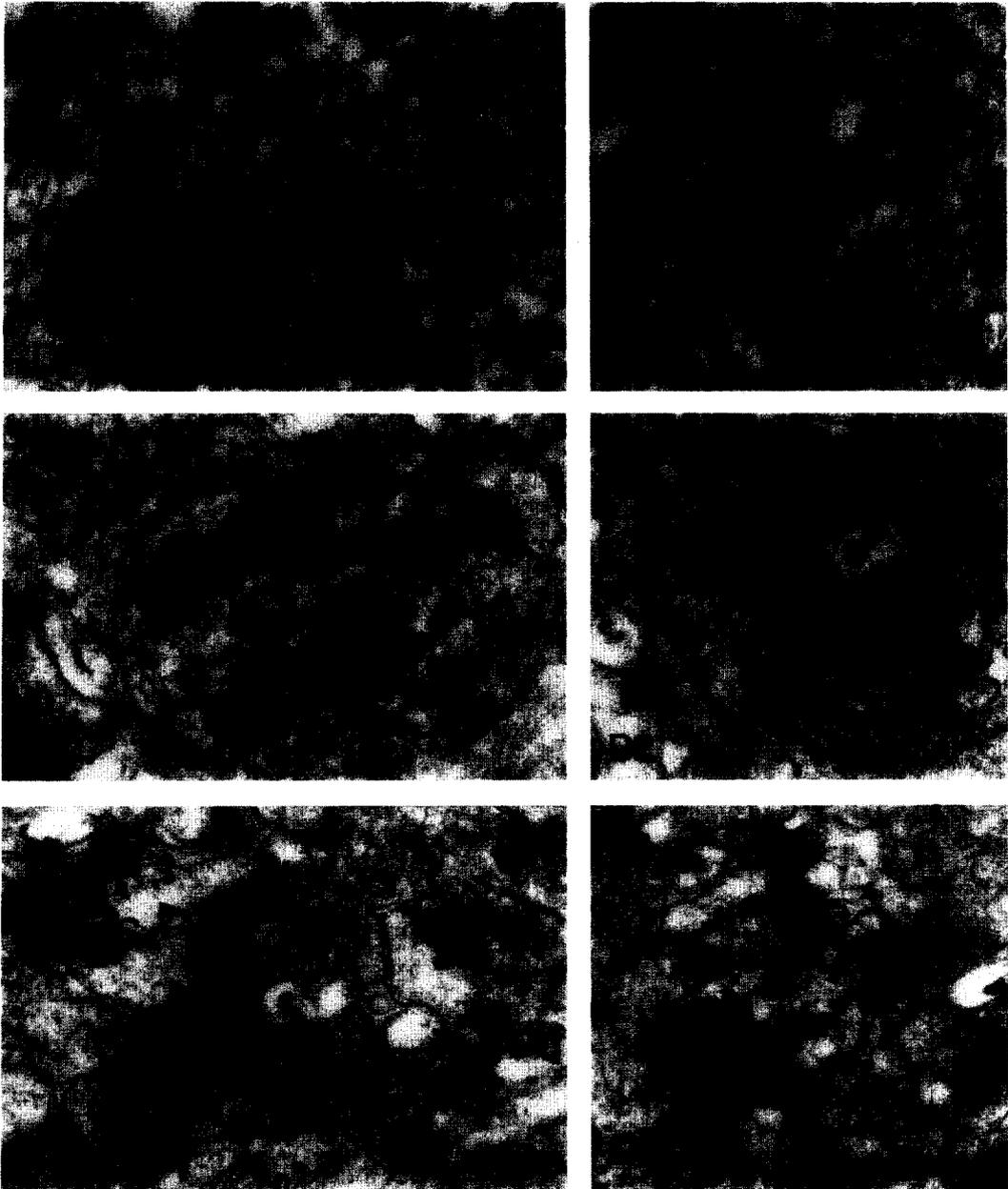


Fig. 9. Electron micrographs of the neostriatum illustrating an association of immunoreactivity for D₂ receptor with postsynaptic membranes. (A, B) Dendritic spines (s) receiving afferent synaptic input from terminals forming asymmetrical synaptic specializations (arrowhead). In both cases the postsynaptic membranes have immunoparticles adhering to them indicating the presence of immunoreactivity for D₂ dopamine receptor. (C, D) Serial sections of a spine (s) that receives synaptic input from a terminal forming asymmetrical synaptic specializations. A second bouton (b) closely apposes the spine but a synaptic specialization was not observed. Immunogold particles are associated with the membrane apposed to the bouton indicating the presence of immunoreactivity for D₂ receptor. Note that in D the silver-intensified immunogold particles are at their periphery and no longer touching the membrane. (E, F) Dendritic spines (s) that each receive synaptic input from two boutons. One of the boutons (b) forms symmetrical membrane specializations (two arrowheads) and is associated with immunoparticles. The other forms an asymmetrical synapse (arrowhead). All micrographs are at the same magnification, bar = 0.5 μ m.

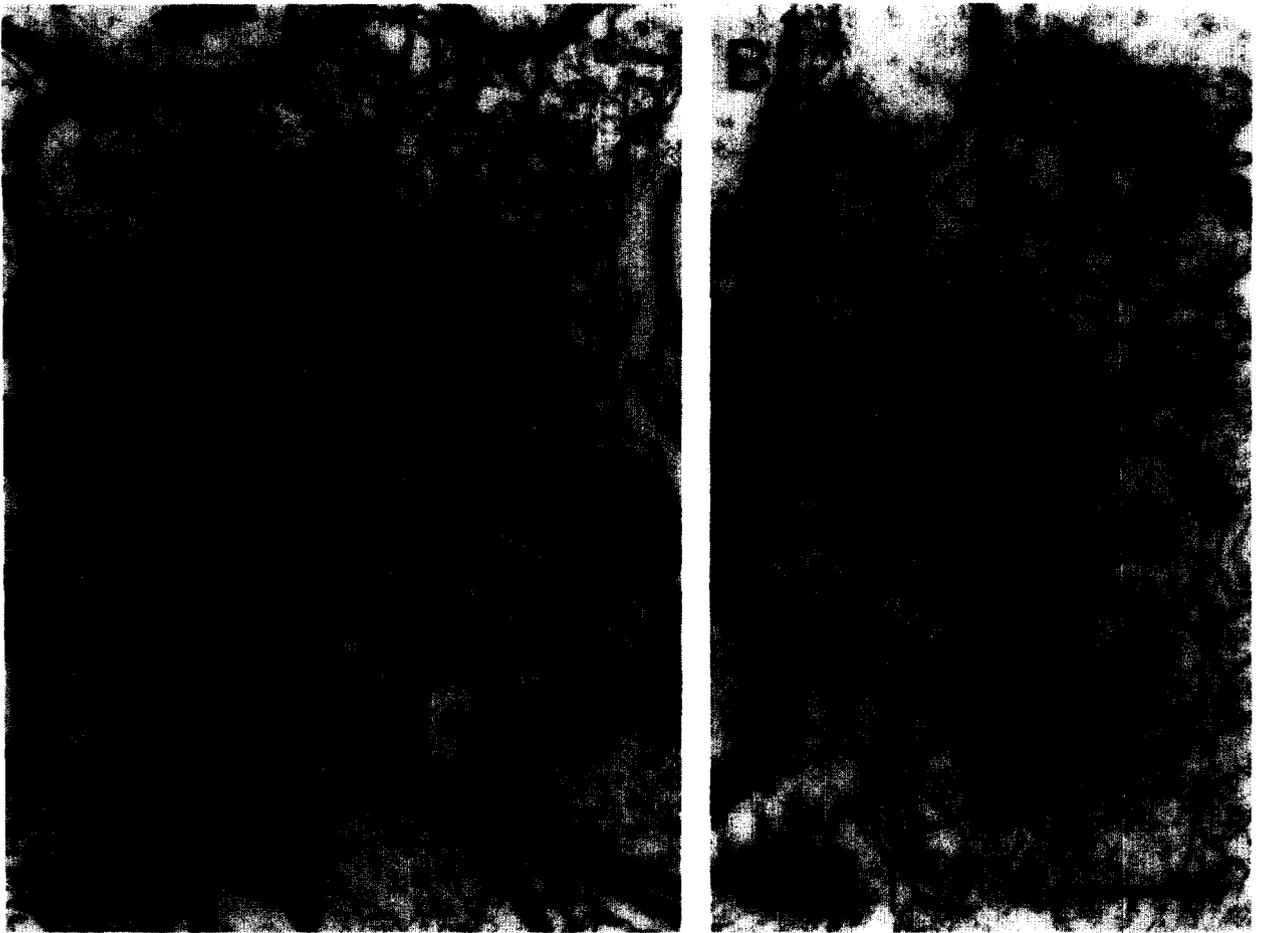


Fig. 10. Electron micrographs of the entopeduncular nucleus immunostained by the ABC method to reveal immunoreactivity for D₁ receptor. A shows many axons (some indicated by asterisks) in the EP that are D₁ immunoreactive. They include both unmyelinated and myelinated axons. The immunoreaction product usually fills the structures and does not show the clumping that was characteristic in the neostriatum. In B a D₁-immunoreactive bouton (b) forms symmetrical synaptic contact (arrowheads) with a non-reactive dendrite (star). Scale bar for A and B = 0.5 μ m.

ized in perikarya and dendrites. These immunoreactive neurons were large and the nucleus possessed indentations and the large volume of cytoplasm contained many organelles (Figs. 11A, B). Proximal and distal dendrites (Fig. 11C) of these compacta neurons were also immunoreactive for D₂ and they were filled with the peroxidase reaction product. Rarely axonal elements that were immunoreactive for D₂ receptors were observed. As with neurons in the neostriatum the subcellular distribution of the immunoreaction product showed some unevenness although to a much lesser extent than in striatal neurons. Patches of immunoreaction product were found in the cytoplasm of the cell and sometimes reaction product was associated with endoplasmic reticulum (Fig. 11B). Reaction product was sometimes associated with afferent synapses (as in Fig. 11C, mostly asymmetrical), but as occurred in neostriatum, it was not necessarily the case.

The distribution of silver-intensified immunogold for D₂ receptors in the pars compacta was similar to

that of the peroxidase immunoreaction product in that it was found in perikarya and dendrites. Many of the granules were found within the cytoplasm of the perikarya and were sometimes associated with the endoplasmic reticulum although most were associated with the plasmalemma of the immunoreactive perikarya and dendrites. The silver-intensified immunogold particles as well as the peroxidase reaction product did not show a selective association with afferent synapses.

Substantia nigra pars reticulata. The distribution of D₁ immunoreactivity in the substantia nigra pars reticulata at the electron microscopic level was similar to that observed in the entopeduncular nucleus. The peroxidase immunoreaction product (Fig. 12A) and silver intensified immunogold particles were mainly found in small diameter axonal structures. These D₁-immunoreactive axons were present in clusters and were also scattered in the neuropil. They included both myelinated and unmyelinated axons (Fig. 12A). D₁-immunoreactive boutons were also found, and in

some cases, these were seen to form symmetrical synaptic contacts with non-reactive dendrites (Fig. 12B).

Quantitative analysis. The relative frequency of D₁- and D₂-immunoreactive profiles in the neostriatum are shown in Table 1. Most of the D₁- and D₂-

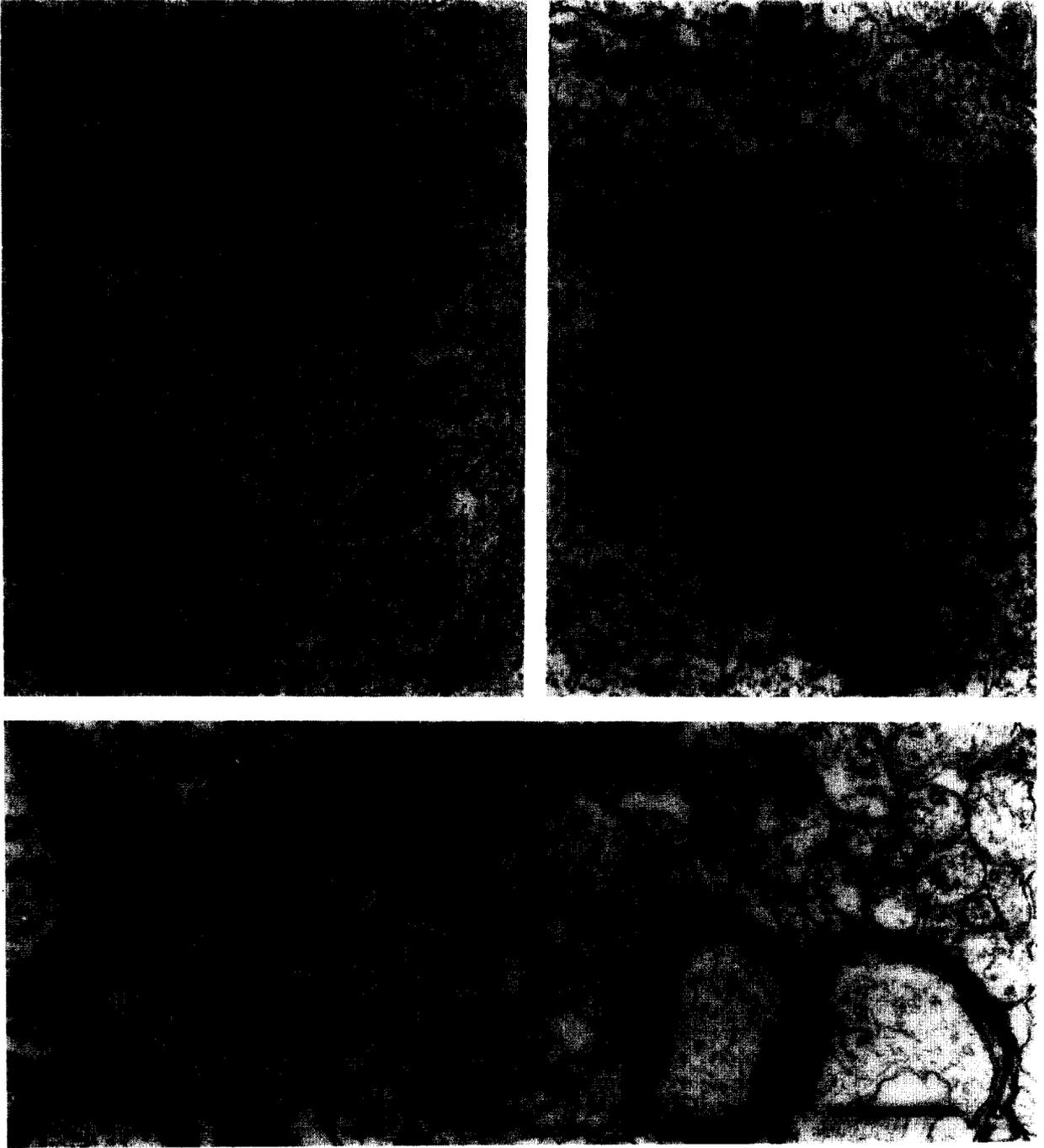


Fig. 11. Electron micrographs of the substantia nigra pars compacta (SNc) immunostained by the ABC method to reveal immunoreactivity for D₂ receptor. A and B show a D₂-immunoreactive neuron (p) and some dendritic profiles (d) in the SNc immunostained by the peroxidase method. In A, the nucleus of the neuron shows slight indentations and is surrounded by large area of cytoplasm that contains many organelles, characteristic of dopaminergic neurons in the pars compacta. The D₂-immunoreactivity is mainly in the cytoplasm. B shows the region of the cytoplasm which is bounded by dotted lines at a higher magnification. The D₂-immunoreactivity is distributed within the cytoplasm and some is associated with endoplasmic reticulum. C shows an electron micrograph of a D₂-immunoreactive dendrite (d) in the SNc. The immunoreactive dendrite is in asymmetrical synaptic contact (arrowhead) with a non-reactive bouton.

Scale bars for A = 2 μm; for B = 1 μm; for C = 0.5 μm.

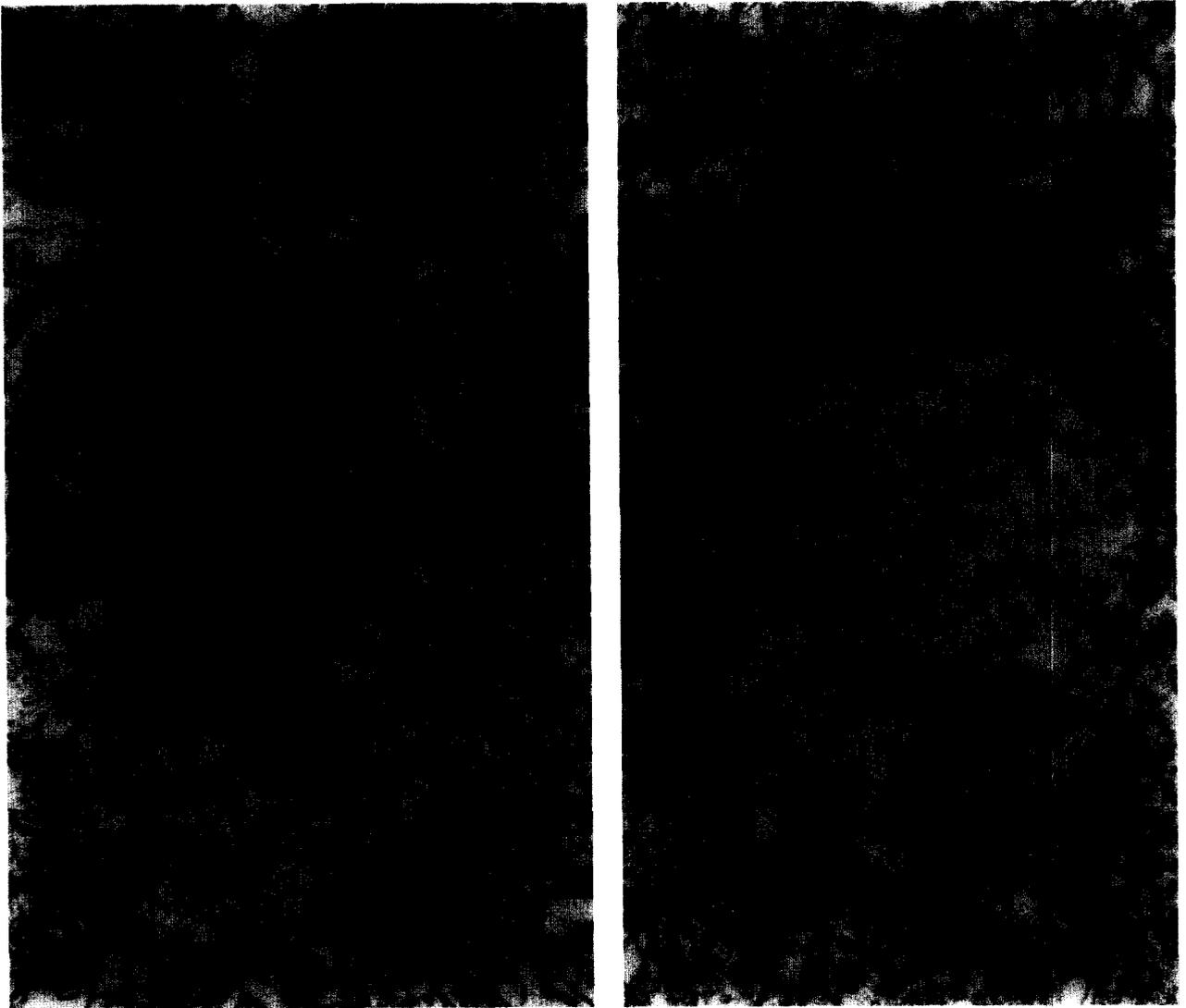


Fig. 12. Electron micrographs of the substantia nigra pars reticulata (SNr) immunostained by the ABC method to reveal immunoreactivity for D₁ receptor. A shows many axons (some indicated by asterisks) that are D₁-immunoreactive in the SNr. These axons are both unmyelinated and myelinated. The small axons are usually filled with peroxidase immunoreaction product. In B a D₁-immunoreactive bouton (b) in the SNr forms symmetrical synaptic contact (arrowheads) with a non-reactive dendrite (star). Scale bar for A and B = 0.5 μ m.

immunoreactive profiles in the neostriatum (approximately 75%) were dendrites and dendritic spines (Table 1). In dendrites, a large proportion of D₁ and D₂ immunogold particles were associated with the cell membranes (D₁, 75.6%; D₂, 80.2%), whereas a

smaller proportion were distributed within the cytoplasm (D₁, 24.4%, D₂ 19.8%). In dendritic spines, almost all the D₁ and D₂ immunogold particles (approximately 95%, Table 2) were associated with the cell membranes. Approximately half of the D₁

Table 1. Distribution of D₁- and D₂-immunoreactive profiles in the neostriatum

	Dendrites	Spines	Axons	Non-synaptic boutons		Synaptic boutons		Perikarya	Unclassified
				Non-synaptic boutons	Synaptic boutons	Asymmetrical	Symmetrical		
D ₁ <i>n</i> = 625	54.4%	23.0%	3.8%	7.2%	2.7%	0.8%	0.5%	7.5%	
D ₂ <i>n</i> = 358	53.4%	24.3%	2.2%	10.3%	2.2%	1.4%	0.3%	5.9%	

The distribution of D₁- and D₂-immunoreactive profiles in the neostriatum was carried out on immunogold-labelled sections. All the immunoreactive profiles on the electron micrographs, taken from three animals, were classified into eight categories. *n* indicates the number of immunoreactive profiles identified in each case.

Table 2. Distribution of D₁- and D₂-immunoparticles associated with dendritic spines

	On membranes					Within the spine
	Associated with symmetrical synapses	Distant from symmetrical synapses	Associated with asymmetrical synapses	Distant from asymmetrical synapses	Not associated with synapses	
D ₁ <i>n</i> = 184	3.8%	0.5%	12.0%	26.1%	52.7%	4.9%
D ₂ <i>n</i> = 127	7.1%	3.1%	11.0%	27.6%	44.9%	6.3%

Analyses of the distribution of D₁- and D₂-immunoparticles associated with dendritic spines was carried on the same sets of electron micrographs from which the data in Table 1 were obtained. The immunogold particles were defined as "not associated with a synapse" if the spine did not receive an afferent terminal in that particular single section. In those spines that did receive an afferent synaptic input the immunoparticles were defined as "associated with a synapse" if the particle was either touching the membrane specialization or was immediately adjacent to it. Immunoparticles were defined as "distant from the synapse" when present on the membrane at the opposite side of the spine. *n* indicates the numbers of immunogold particles quantified in each case.

and D₂ immunogold particles were in spines that did not have any afferent synaptic input in the single ultrathin section (Table 2). For those immunogold particles in spines that did receive afferent synapses, about one-third of them were localized on the cell membrane facing asymmetrical synapses, i.e. on the opposite side of the spine (Table 2). Approximately one-tenth of the immunogold particles were localized either on the postsynaptic membrane or immediately adjacent to the specialization of asymmetrical synapses (Table 2). The remainder were either on the postsynaptic membrane or immediately adjacent to the specialization of symmetrical synapses (Table 2).

A relatively small proportion of D₁ or D₂ immunoreactivity was associated with axonal components (<17%, Table 1). D₁- and D₂-immunoreactive perikarya were rarely found (<1%, Table 1).

DISCUSSION

The results of the present study confirm and extend previous light and electron microscopic observations of the distribution of immunoreactivity for D₁ and D₂ dopamine receptors in the basal ganglia of the rat.^{2,3,10,32,51,53} They confirm the presence of D₁ and D₂ receptors in medium-sized spiny neurons of the neostriatum and in neuronal structures in other regions of the basal ganglia, the distribution being consistent with previous ligand binding^{5,62} and *in situ* hybridization^{33,78,79} studies. Our electron microscopic findings confirm that the distribution of immunoreactivity for both of the receptors within dendrites and spines is uneven. They also demonstrate that immunoreactivity is primarily located in membranes, and although present on the postsynaptic membrane at some synaptic specializations, a large proportion of the immunoreactivity is not necessarily associated with afferent synaptic terminals.

Characterization of neuronal elements expressing dopamine receptor immunoreactivity

The immunostaining of the neostriatum for both the D₁ and D₂ receptor was the most dense observed

in any region of the brain. Due to this high density of labelled structures, the low power light microscopic analysis did not allow us to unequivocally identify the neuronal elements displaying immunoreactivity. The high power light microscopic analysis and the electron microscopic analysis, however, revealed that most of the staining was associated with the neuropil and not with neuronal perikarya. Stained perikarya were present, but their numbers were low and they were often obscured by the dense staining of the neuropil. Most of the stained elements were dendrites and dendritic spines (about 75%) although occasionally stained axons and terminal boutons (<17%) were also observed. The majority of the labelled neuronal perikarya were of medium-size and possessed a round unindented nucleus which was surrounded by a thin rim of cytoplasm, i.e. they possessed features that are characteristic of the most common neuron of the neostriatum, the medium-sized densely spiny neuron.⁶⁷ This observation, together with the fact that most of the neuropil staining was associated with spines and with dendrites that often gave rise to spines, leads to the conclusion that most of the staining for D₁ and D₂ receptors is associated with medium spiny projection neurons (see also below). This is in agreement with previous immunocytochemical studies and with *in situ* hybridization studies (for references see the Introduction).

Occasionally D₁- and D₂-immunoreactive neurons which had morphological features different from those of spiny neurons were also observed. These included cells that were larger than the spiny neurons, had larger volumes of cytoplasm and possessed indented nuclei. This latter feature is characteristic of striatal interneurons and indeed double immunocytochemical studies have shown that each of the major classes of interneurons, i.e. cholinergic, GABAergic and somatostatin/neuropeptide Y-containing, receive afferent synaptic input from dopaminergic terminals.^{11,13,42,43,44,77} Furthermore, 80% of cholinergic neurons express D₂ mRNA^{48,79} and a small proportion also express D₁ mRNA.⁴⁶ Thus at least the

cholinergic interneuron has been shown to receive input from dopaminergic terminals, to possess the mRNA for the receptors and to express the receptor protein. The exact identity of the other types of immunoreactive neurons remains to be established.

The entopeduncular nucleus and the substantia nigra pars reticulata, as well as the ventral aspects of the pars compacta, were rich in D₁ receptor immunoreactivity that was confined to axons and to axon terminals. Furthermore, within the globus pallidus and at more caudal locations bundles of fibres were strongly immunoreactive. These bundles are presumed to be the fibres of the striatoentopeduncular and striatonigral pathways as their location corresponds to that of striatofugal axons labelled with the anterograde tracer, neurobiotin (unpublished observations). In contrast to these structures, the neuropil of the globus pallidus contained only a sparse distribution of D₁-immunoreactive axons and terminals. Our findings thus demonstrate that spiny neurons within the neostriatum, the axons of the striatonigral pathway and their terminals in the substantia nigra and the entopeduncular nucleus are rich in D₁ dopamine receptor immunoreactivity whereas the axons of the striatopallidal pathway either do not possess or are poor in D₁ receptor immunoreactivity. These observations are consistent with the proposal derived from combining tracing and *in situ* hybridization studies,¹⁹ double *in situ* hybridization studies,^{46,47} combined tracing and binding studies^{28,29} and functional analyses^{19,20,21,63,64} which indicate that dopamine D₁ receptors are selectively associated with substance P-containing striatonigral and striatoentopeduncular neurons, the so-called direct pathway, and not with enkephalin-containing striatopallidal neurons. Our data however, do not preclude the possibility that at least low levels of D₁ receptors are expressed by striatopallidal neurons.

Although we cannot be sure of the origin of the axon and terminal staining for D₁ receptors in the globus pallidus, these may represent the minor axon arborizations that have been shown to arise from intracellularly filled spiny striatonigral neurons that send their main axon to both the substantia nigra and the entopeduncular neuron.³⁶ It is interesting to note that although the globus pallidus is generally considered to be free of substance P,^{1,27} high magnification examination of resin-embedded sections that have been stained to reveal substance P immunoreactivity contain a sparse population of axons stained to the same intensity as those in the substantia nigra and entopeduncular nucleus (unpublished observations). Although quantitative analyses were not performed, the density of substance P-immunoreactive structures is in the same order of magnitude as the D₁-immunoreactive profiles. It is possible that both may be derived from the minor axonal collateral arborizations of striatonigral/striatoentopeduncular neurons and are not a component of the main striatopallidal pathway.

In contrast to the D₁ receptor, immunoreactivity for D₂ receptor was not detectable in the neuropil of the entopeduncular nucleus or the substantia nigra pars reticulata and was absent from the fibres of the striatonigral pathway. This absence of D₂-immunoreactivity in locations associated with the so-called "direct pathway" is consistent with the observations suggesting that D₂ receptors are not associated with substance P-immunoreactive striatonigral/striatoentopeduncular neurons.^{19,28,29,46,47,63,64} However, no or very weak D₂ receptor staining was detected in the neuropil of the globus pallidus. Since the globus pallidus represents the target of the striatal neurons that give rise to the "indirect" pathway and have been proposed to contain both enkephalin and D₂ receptors,^{19,21,47} one would expect the neuropil of the globus pallidus to be as dense in D₂ receptor immunoreactivity as it is for enkephalin immunoreactivity^{1,27} or the substantia nigra/entopeduncular nucleus is for D₁ receptor immunoreactivity. As this was not the case and D₂ receptor immunoreactivity could not be detected in *any* of the terminal arborizations of striatal output neurons, the present findings neither support nor refute the hypothesis that D₂ receptors are selectively associated with the neurons that give rise to the "indirect" striatopallidal pathway. It should be noted, however, that in primate, immunostaining for D₂ receptor, albeit light, has been detected in the external segment of the globus pallidus.⁵¹

It remains unclear why D₂ receptor immunoreactivity was not associated with the axons and terminals of striatal projection neurons when D₁ immunoreactivity was strongly associated with axons and terminals. There are several possible explanations for these observations. Firstly, D₂ receptor protein may not be transported along axons of striatal projection neurons or only transported to a minor degree. This is consistent with the low levels of D₂ binding sites that are detected in the globus pallidus and substantia nigra reticulata.^{5,12,17} The differential distribution of receptors within neurons is not without precedent, subtypes of muscarinic receptors show selective localization within perikarya and dendrites.³¹ Secondly, the receptor protein may have been transported, but at levels below the limit of detection by the immunocytochemical protocol that we used. If this is the case it implies that the *degree* of transport is different between D₁ and D₂ receptors since our protocol can readily visualize D₁ receptors associated with the axons and terminals of striatonigral and in the striatoentopeduncular neurons. Thirdly, the fixation protocol may have differentially affected the receptor in axons and perikarya/dendrites or reduced their already low levels, to undetectable levels. Fourthly, the receptor may have been altered in some way during transportation such that it is no longer recognized by the antibodies or is no longer accessible to the antibodies.

In contrast to the neuropil of substantia nigra pars

reticulata, D₂ receptor immunoreactivity was present in neurons of the substantia nigra pars compacta, the ventral tegmental area and a group of neurons in the ventral pars reticulata. The anatomical location, the morphology, the fact that staining of these cells is no longer present in sections ipsilateral to a 6-OHDA lesion of the medial forebrain bundle, indicate that they represent the dopaminergic nigrostriatal cell group, A9 and the mesolimbic group, A10. This conclusion is consistent with other immunocytochemical studies of D₂ receptor immunoreactivity,^{3,10,51,66} radioligand binding studies,^{5,12,17} *in situ* hybridization studies^{8,15,54,55,56,78,79} and functional analyses⁴⁵ implicating the association of D₂ receptors with dopaminergic neurons.

Despite the absence or low level of staining for D₂ receptor in the neuropil of the globus pallidus we observed a rare population of neurons that were stained for D₂ receptor. The morphological characteristics were different from typical pallidal neurons in that they were of medium size, multipolar and gave rise to dendrites radiating in all directions that possessed spines. The nature of these immunoreactive neurons remains unclear. They may represent the class of small-medium size spiny neurons that have been identified in an intracellular staining study of the rat globus pallidus,³⁹ although the possibility that they are misplaced striatal neurons cannot be excluded.

Subcellular localization of D₁ and D₂ receptor immunoreactivity

One of the original objectives of the present study was to address the issue of the *subcellular* localization of D₁ and D₂ receptors. In particular it was considered important to determine whether immunoreactivity for the receptor showed any association with afferent synaptic terminals. The results from the peroxidase-stained tissue were consistent with those of previous studies^{32,51,66} in that there was uneven staining of dendrites. In addition, immunopositive spines arising from immunonegative dendrites and immunonegative spines were seen arising from immunopositive dendrites. As discussed in the Introduction, although peroxidase reactions are ideal for the localization of antigens at the *cellular* level, the fact that the reaction products can diffuse within membrane limited structures means that they are not ideally suited to the precise localization of antigens at the *subcellular* level. Thus in the present experiment the DAB immunoreaction product in spines generally filled the whole structure and the patches of immunoreaction product in dendrites were associated with the plasmalemma as well as the membranes of subcellular organelles. Despite the rather diffuse location of the reaction product it was possible to gain some indication of the relationship of receptor immunoreactivity and afferent synapses. Thus by analysis of a full series of electron microscopic sections through a "patch" of immunoreaction product in a dendrite

or an immunoreactive spine, it was possible to identify any afferent synaptic terminals that were present. This analysis demonstrated that immunoreactivity for both the D₁ or the D₂ receptors was often associated with afferent synaptic boutons forming symmetrical or asymmetrical synapses. Indeed, preliminary double labelling studies from this laboratory^{85,86} and the labelling studies of others⁶⁶ have indicated that some of the afferent terminals associated with dopamine receptor immunoreactivity on the postsynaptic membrane are dopaminergic terminals as identified by TH immunocytochemistry. However, it was often the case that a patch of immunoreactivity in a dendrite was not associated with an afferent terminal. Immunoreactive spines, in which the reaction product usually filled the entire structure, often received afferent synaptic terminals. This occurred more frequently at synapses forming asymmetric specializations, i.e. the type of synapse usually associated with the cortical input than with terminals forming symmetrical synapses, i.e. the type usually associated with dopaminergic terminals.^{9,14,16,30,38,43,59,67,68,69,82} It must be remembered, however, that asymmetrical synapses are far larger and more easily recognized than the small synaptic specializations that are formed by dopaminergic terminals, particularly when the contact is made with a spine.

The use of the immunogold method confirmed and extended these observations. This material demonstrated that the immunoreactive sites for both the D₁ and D₂ receptors were predominantly located on membranes of dendrites and spines. In the case of dendrites, 80% of immunoparticles were associated with the plasmalemma and in keeping with the predicted topology of the dopamine receptors, this association was with the internal surface of the membrane. This figure is likely to be an underestimate of the number of immunoparticles associated with membranes as the serial section analysis revealed that a silver-intensified immunogold particle may, at its periphery in a serial section, be at a distance from the membrane (see Fig. 9). In agreement with the observations in the peroxidase-stained material the immunoparticles were often seen in association with afferent synaptic terminals, i.e. were touching the postsynaptic membrane or were immediately adjacent to the specialization. Although in the quantitative analysis of dendritic spines about 50% of immunoparticles were found in spines that did not receive an afferent synapse in the single section, this is likely to be an underestimate of the true degree of association with afferent terminals as virtually all spines receive afferent synaptic input. In a single ultrathin section it is possible that the immunoparticles are situated at the periphery of a synapse that does not appear in the section. When closely associated with synapses, immunoparticles touching the membrane specialization occurred more commonly at synapses with symmetrical membrane

specializations than at synapses with asymmetrical specializations. The morphology of those terminals forming symmetrical specializations, the size of the membrane specializations and their location on dendritic spines are features consistent with those of dopaminergic terminals identified by immunocytochemistry for tyrosine hydroxylase.^{9,16,43,59,68} Although it was very rare to observe immunoparticles on the postsynaptic membrane of terminals forming asymmetrical synaptic specializations they were commonly observed immediately adjacent to them. Terminals forming this type of specialization, as mentioned above, are likely to be derived from the cortex.^{14,30,38,68,69,82} It is unclear at present whether this represents a selective association of dopamine receptors with membranes postsynaptic to cortical terminals, a receptor that is randomly situated in the cell membrane, or whether it is related to dopaminergic inputs to spines. It is thus evident that dopamine receptor immunoreactivity is associated with the postsynaptic membrane at afferent synapses that have the features of dopaminergic synapses, with non-synaptic sites and possibly with the postsynaptic membrane of non-dopaminergic synapses. It is noteworthy that other ultrastructural studies of different classes of receptors using immunocytochemical techniques have identified relatively high concentrations of receptor immunoreactivity at extrasynaptic as well as synaptic sites.^{4,31,57,58,70,75}

The detection of large amounts of dopamine receptor immunoreactivity at non-synaptic sites raises questions as to why they are located extrasynaptically and what are their functional roles. There are several possibilities that may account for their presence at extrasynaptic sites. Firstly, one must assume that at least some of the receptor immunoreactivity detected at non-synaptic sites along the membrane of dendrites represents receptors that are being transported from their sites of synthesis to their site of action at synapses. Secondly, it is possible that the receptor antigens are more accessible to the immunoreagents at extrasynaptic sites, i.e. the synaptic specialization in some way restricts the access of the immunoreagents. What appears to be a relative enrichment at non-synaptic sites may simply be a failure to stain the receptors at the synaptic site. Thirdly, it is possible that the receptors are in some way altered at the synaptic sites such that they are less efficiently recognized by the antibodies.

It should be noted that although the quantitative data presented in this paper are representative of the observations that we made, they may not be representative of the true distribution of the receptors as immunolabelling by pre-embedding methods are not ideal for the quantitative localization of antigens. A more accurate quantitative analysis of the location of immunoreactivity for the receptors awaits the application of post-embedding methods which overcome the problems of penetration of the immunoreagents and access to the antigenic sites.⁵⁹

When considering the roles of dopamine receptors located at non-synaptic sites it is important to know whether they are functional receptors. In the present study this could not be determined, however if they are linked to their second messenger systems then stimulation of the receptors is likely to elicit responses in the dendrites or spines. This response, as with other inputs to striatal neurons, will presumably be dependent on the state of activity of the membrane^{80,81} and the nature, activity and location of afferent synaptic terminals.¹⁶ These receptors may thus represent part of the physiological system of dopaminergic transmission in the neostriatum in which dopamine released by nigrostriatal terminals will have its immediate and major effect on receptors located at the synapses and then have a delayed and presumably lesser effect on receptors located at a distance from the synapse. The latter effect will be dependent on the rates of diffusion, uptake and metabolism of the released dopamine.³⁵ The circumstances under which the receptors at the two locations are stimulated may relate to the mode of firing of the dopaminergic neurons. Two patterns of firing are characteristic of dopaminergic neurons in the midbrain: single spikes and bursts.^{24,25} The bursting mode of firing elicits the release of dopamine in the neostriatum and nucleus accumbens that is disproportionately higher than occurs with regularly spaced spikes^{23,72} and indeed it has been suggested that the concentration of dopamine that is reached at the edge of the synapses may be high enough to stimulate receptors that are outside of the synaptic specialization.³⁵ Thus at a low frequency of firing with regularly spaced spikes dopaminergic transmission will occur at the synaptic specializations of the nigrostriatal terminals and during burst firing transmission may occur by stimulation of receptors at *both* synaptic and a non-synaptic sites. It is clearly important to establish whether the receptors located at non-synaptic sites are indeed functional.

An additional point that arises from the observed distributions of dopamine receptor immunoreactivity relates to the pharmacology of the neostriatum. If we again assume that all of the dopamine receptor immunoreactivity that we identified represents functional receptors then the overall response, i.e. the effect on firing of the neurons or indeed the behavioural response of the animal, to an exogenously administered drug that acts on dopamine receptors is likely to be different to the response that occurs following the release of dopamine at the synaptic site. The response to stimulation of the nigrostriatal or mesolimbic pathways or the response to indirectly acting dopamine receptor stimulants (e.g. amphetamine) is likely to be different from that of a directly acting dopamine receptor agonist.⁸³ The exogenous agonist will presumably stimulate the receptors and elicit responses at the extrasynaptic sites to the same, or to a greater degree, than the receptors located within the synapse, whereas endogenously released

dopamine will have its preferential, and greatest effect, on receptors located at synapses. It is clear that the presence of immunoreactivity for dopamine receptors at extrasynaptic sites raises issues about the mode of transmission in the dopaminergic system. The critical issue, as pointed out above, that remains to be established is whether the receptors located at the non-synaptic sites are functional receptors and are exposed to dopamine under physiological conditions.

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