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Synaptic Connections Between Spiny Neurons of the Direct and Indirect Pathways in the Neostriatum of the Rat: Evidence from Dopamine Receptor and Neuropeptide Immunostaining

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Abstract

The flow of cortical information through the basal ganglia occurs through the so-called 'direct pathway' and 'indirect pathways'. The object of the present work was to attempt to determine whether spiny neurons in the neostriatum that give rise to the direct pathway (i.e. the striatonigral/entopeduncular pathway) and those giving rise to the indirect pathways (i.e. striatopallidal pathway) are synaptically interconnected. The approach was to carry out double immunocytochemistry at the electron microscopic level using antibodies against peptides or dopamine receptor subtypes that are selectively associated with the neurons that give rise to the direct (substance P or D_1 receptors) and indirect pathways (enkephalin or D_2 receptors). Sections of perfuse-fixed rat neostriatum were immunostained to reveal both substance P immunoreactivity and D_2 receptor immunoreactivity or enkephalin and D_1 receptor immunoreactivity, respectively. Double peroxidase methods were employed using different chromogens that were distinguishable at both the light and electron microscopic levels. In the electron microscope substance P-immunoreactive terminals were seen in synaptic contact with dendritic structures that displayed immunoreactivity for D_2 receptor. Similarly, enkephalin-immunoreactive terminals were seen in synaptic contact with D_1-immunoreactive dendritic structures. Thus, axon collaterals of neurons giving rise to the direct pathway form synaptic contacts with neurons that give rise to the indirect pathway and axon collaterals of neurons giving rise to the indirect pathway form synaptic contact with neurons that give rise to the direct pathway. These results indicate that the two pathways of information flow through the basal ganglia are synaptically linked at the level of the neostriatum.

Introduction

The major cell type of the neostriatum is the so-called medium size densely spiny neuron which may account for up to 90%, or even more, of the total population of striatal neurons (Kemp and Powell, 1971). These neurons are the major projection neurons of the neostriatum, utilize GABA as their major neurotransmitter and are subdivided into two major populations on the basis of their projection region, pattern of axonal collateralization and their neurochemical content (for reviews see Pasik *et al.*, 1979; Chang and Wilson, 1990; Smith and Bolam, 1990a). One sub-population projects to the output nuclei of the basal ganglia, i.e. the substantia nigra pars reticulata (SNr) and the entopeduncular nucleus (EP) (or internal segment of the globus pallidus in primates; GP_i), and expresses, in addition to GABA, the neuropeptides substance P and dynorphin and dopamine D_1 receptors. The second sub-population projects almost exclusively to the globus pallidus (GP) (or external segment of the GP in primates; GP_e) and expresses enkephalin (ENK) and dopamine D_2 receptors (for reviews see Graybiel, 1990; Gerfen, 1992a, b).

Evidence from morphological studies, including intracellular filling of neurons (see Chang and Wilson, 1990) or Golgi-impregnation (see Pasik *et al.*, 1979) have demonstrated that spiny neurons also give rise to extensive local axon collaterals, one of the major synaptic targets of which, are other spiny neurons (Wilson and Groves, 1980; Somogyi *et al.*, 1981). This is supported by the findings that terminals that display immunoreactivity for the neuropeptides expressed by spiny neurons, i.e. ENK or substance P, and possess the morphological features of spiny neuron terminals, make symmetrical synaptic contact with the dendrites, spines and perikarya of spiny neurons (Pickel *et al.*, 1980, 1992; DiFiglia *et al.*, 1982; Somogyi *et al.*, 1982; Bolam *et al.*, 1983; Bouyer *et al.*, 1984; Aronin *et al.*, 1986; Bolam and Izzo, 1988).

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By preferentially projecting to the output nuclei (SNr/EP or GP_i) or to the GP (and thence to the output nuclei either directly or via the subthalamic nucleus), the two subpopulations give rise to the so-called 'direct' or 'indirect' pathways of the information flow through the basal ganglia respectively (Albin *et al.*, 1989; Alexander and Crutcher, 1990; DeLong, 1990; Gerfen, 1992a, b). The two pathways convey different information to the targets of the basal ganglia. Activation of the direct pathway leads to a disinhibition of the neurons in the thalamus, the superior colliculus and the brainstem and is associated with movement, whereas activation of the indirect pathways leads to a greater inhibition of these neurons and is associated with the attenuation of movement. Although, by definition, the direct and indirect pathways are separate and parallel at the level of the neostriatum, this segregation is not maintained at other levels of the basal ganglia. Striatal output neurons that give rise to the direct pathway make convergent synaptic contact with the same neurons in the substantia nigra and the EP or GP_i that receive synaptic input from pallidal or subthalamic neurons, i.e. neurons of the indirect pathway (Smith and Bolam, 1990b, 1991; Bolam and Smith, 1992; von Krosigk *et al.*, 1992; Bolam *et al.*, 1993; Bevan *et al.*, 1994a, b; Smith *et al.*, 1994). However, an important question that arises is whether the synaptic connections between spiny neurons within the neostriatum also represent sites of interaction between the direct and indirect pathways. In other words, are the two populations of spiny neurons that give rise to the direct and indirect pathways synaptically interconnected at the level of the striatum? The objective of the present study was therefore to address this question. The approach was to carry out double immunocytochemistry at both the light and electron microscopic levels taking advantage of the observation that antibodies against dopamine D_1 or D_2 receptors selectively label the dendrites and spines of neurons giving rise to the direct and indirect pathways respectively (for references see Discussion), whereas antibodies against substance P or ENK selectively label the axon terminals of neurons giving rise to the direct and indirect pathways respectively (see Graybiel, 1990; Gerfen, 1992a, b).

Preliminary accounts of the data presented in this paper have been published in abstract form (Yung *et al.*, 1994, 1995b).

Materials and methods

Material from nine rats (female Wistar or male Sprague–Dawley, 200–250 g; Charles River, UK) was used in the present study. The rats were maintained on a 12 h light/12 h dark cycle with free access to food and water. Environmental conditions for housing of the rats, and all procedures that were performed on them, were in accordance with the Animals (Scientific Procedures) Act, 1986.

The rats were deeply anaesthetized with sodium pentobarbitone (Sagital, 60 mg/kg, i.p.) and perfused transcardially with 50–100 ml of saline (0.9% NaCl) followed by 200 ml of the fixative [3% paraformaldehyde and 0.1–0.2% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4] with the aid of a peristaltic pump (flow rate of about 10 ml/min). The brain was quickly removed from the skull and sections of the neostriatum (70 µm) 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 immunoreagents the sections were equilibrated in a cryoprotectant solution (0.05 M PB, pH 7.4, containing 25% sucrose and 10% glycerol), frozen in isopentane cooled in liquid nitrogen and then directly in liquid nitrogen. They were thawed in PBS and then treated with sodium borohydride (0.1% in PBS; BDH chemicals) for 5–10 min at room temperature and washed several times in PBS. Prior to the immunocytochemical staining, the sections were pre-incubated in normal goat serum (NGS, 4% in PBS) for 1 h at room temperature.

Double immunocytochemistry

Two double immunostaining protocols were used to localize dopamine receptor immunoreactivity together with neuropeptide immunoreactivity. In both cases double peroxidase methods were used with different chromogens that produce reactions products that are distinguishable at both the light and the electron microscopic levels. Details of antibody preparations and dilutions are give in Table 1.

Protocol 1

Sections were incubated in monoclonal antibody solution against D_1 receptor or affinity purified polyclonal antibody solution against D_2 receptor overnight at room temperature with constant gentle shaking (Table 1). They were then washed (3 × PBS), incubated in biotinylated secondary antibody solutions (2 h), washed (3 × PBS) and incubated in an avidin–biotin–peroxidase complex (1:100 dilution, ABC, Vector Labs.) for at least 1 h. Immunoreactivity for the receptors was revealed by a peroxidase reaction using tungstate-stabilized tetramethylbenzidine (TMB-tungstate) as the chromogen (Weinberg and van Eyck, 1991; Llewellyn-Smith *et al.*, 1993). Briefly, the sections were washed (2 × PBS; 1 × 0.1 M PB at pH 6.0) and incubated in the TMB-tungstate pre-incubation mixture (0.0023% TMB, 0.046% ammonium paratungstate, 0.0040% ammonium chloride and 0.18% D-glucose in 0.1 M PB, pH 6.0) for 20 min at room temperature. The sections were then transferred to fresh reaction mixture containing 0.1% glucose oxidase (Sigma) and the reaction allowed to continue for 2–5 min. The reaction was stopped by several washes in PB (0.1 M at pH 6.0). The TMB-tungstate reaction product was stabilized for 10 min. in a DAB-cobalt chloride stabilizing mixture (0.1% DAB, 0.0038% ammonium chloride, 0.20% D-glucose, 0.02% cobalt chloride, and 0.1% glucose oxidase in 0.1 M PB at pH 6.0). The sections were then washed several times in PB and then in PBS. Immunoreactivity for substance P or ENK was then revealed using either the ABC method as described above or the peroxidase antiperoxidase method (PAP) with 3,3′-diaminobenzidine (DAB) as the chromogen for the peroxidase reaction. Following incubation overnight in primary antibody solutions against substance P or ENK (see Table 1), the sections were washed (3 × PBS) and then incubated in secondary antibody solutions (biotinylated goat–anti-rat IgG, biotinylated goat–anti-rabbit IgG or goat–anti-mouse) for 2 h. They were then washed (3 × PBS) and incubated in ABC or mouse PAP complex for 1 h. After several washes in PBS and 0.05 M Tris–HCl buffer at pH 7.4 (Tris buffer) the immunoreactive sites were revealed by incubation in 0.025% DAB (Sigma) in Tris buffer containing 0.0048% H_2O_2. The reaction time was normally 5–10 min and was stopped by several washes in Tris buffer and then PBS.

Protocol 2

In the second protocol double immunocytochemistry was carried out essentially as described by Levey and colleagues (1986) using DAB and benzidine dihydrochloride (BDHC) as the chromogens for the peroxidase reactions. Immunoreactivity for ENK or substance P was first revealed by the procedures described above using DAB as the chromogen. After the first peroxidase reaction, the sections were washed and incubated in antibody solutions against the receptors, secondary antibodies and the ABC solution (see above and Table 1). The sections were then washed (2 × PBS; 1 × PB, 0.1 M, pH 6.8 and 1 × PB, 0.01 M, pH 6.8) and reacted with H_2O_2 (0.0048%) in the presence of BDHC (0.01% in 0.01 M PB at pH 6.8) and sodium

TABLE 1. Details of antibody preparations

Antibody directed against	Species of origin	Dilution	Description	Source and/or characterisation
Dopamine D_1 receptor	rat (monoclonal)	1:5000	primary antibody	Levey *et al.* (1993)
Dopamine D_2 receptor	rabbit	1:200	primary antibody	Levey *et al.* (1993)
Substance P	rat (monoclonal)	1:50	primary antibody	Cuello *et al.* (1979)
Met-enkephalin	rabbit	1:2000	primary antibody	Incstar
Leu-enkephalin	mouse (monoclonal)	1:5–10 000	primary antibody	Sera-lab
Mouse IgG	goat	1:50	secondary antibody	ICN
Rabbit IgG	goat	1:100	biotinylated secondary antibody	Vector
Rat IgG	goat	1:100	biotinylated secondary antibody	Vector
Peroxidase–antiperoxidase	mouse	1:100	tertiary reagent	DAKO

nitroprusside (0.025%) for 3–5 min. The reaction was stopped by several washes in PB (0.01 M at pH 6.8). Strict safety precautions were taken with the BDHC. All waste solutions of BDHC were inactivated by oxidation in acidified potassium permanganate solution (Barek *et al.*, 1984; Castergnaro *et al.*, 1985; Barek, 1986; Lunn and Sannsonne, 1990). Briefly, the final concentration of BDHC was reduced to less than 0.9 mg/ml and to each 10 ml of this 5 ml of 0.2 M $KMnO_4$ and 5 ml of 2 M H_2SO_4 were added. The mixture was left for at least 10 h, decolorized by the addition of 5% ascorbic acid, neutralized by the addition of 2M NaOH and washed down the sink with plenty of water. This approach is recommended by the International Agency for Cancer Research and by the Environmental Control and Research Program, NCI-Frederick Cancer Research Facility (Lunn and Sannsonne, 1990).

Controls for double immunocytochemistry

The controls of the experiments consisted of subjecting sections to the whole double immunostaining procedures but with the omission of each of the primary antibodies in turn. This controlled for: (i) cross-reactivities between antibody preparations; (ii) the deposition of one reaction product at the site of another; and (iii) where possibilities of cross-reaction exist, it tested whether the first reaction product obscured the antigen sites.

Processing for light and electron microscopy

On completion of the immunostaining the sections for light microscopy were mounted on gelatine-coated glass slides, dried at room temperature overnight, dehydrated and a coverslip applied using XAM (BDH chemicals) as the mounting medium.

The sections for electron microscopy were treated with osmium tetroxide solution in a buffer (1% in 0.1 M PB at pH 6.8 for those sections reacted with BDHC; at pH 7.4 for sections reacted with other chromogens) for 20–30 min at room temperature. They were then washed in phosphate buffer, dehydrated in a series of increasing concentrations of ethanol and then propylene oxide. After infiltration with resin (Durcupan ACM, Fluka) overnight the sections were mounted on microscope slides, a coverslip applied and the resin cured at 60°C for 48 h. All sections were examined in the light microscope and the areas of interest were re-embedded on blank blocks of resin for further sectioning. Ultrathin sections (silver/grey) were cut using an ultramicrotome (Reichert-Jung), collected on Pioloform-coated copper single slot grids, stained with lead citrate and examined in an electron microscope (Philips 410 or CM10).

Results

Light microscopic observations

Control sections

In the control sections in which one of the primary antibodies had been omitted, only one type of immunoreaction product was present. Thus, in the sections in which either of the antibodies against the receptors was omitted, only the DAB reaction product was visible. Omission of the antibodies against the neuropeptides resulted in the appearance of only the TMB-tungstate or the BDHC reaction products. In each case the distribution of immunostaining was consistent with that observed for single immunostained sections on previous occasions (see below). This indicates that there was no deposition of the second reaction product at the sites of the first reaction and that there was no cross-reactivity between the immunoreagents. Immunostaining using BDHC as a chromogen was only observed with the antibody against the D_1 receptor; due to this low sensitivity we also applied the double immunostaining method with TMB and DAB.

In the control sections of the forebrain, the distribution of immunoreactivity for D_1 or D_2 receptor (TMB-tungstate for D_1 and D_2; BDHC for D_1) was consistent with that described previously using DAB as the chromogen or using immunogold methods (Huang *et al.*, 1992; Levey *et al.*, 1993; Sesack *et al.*, 1994; Hersch *et al.*, 1995; Yung *et al.*, 1995a). In both cases the overall staining of the neostriatum was very dense, as was the staining in other regions of the forebrain including the ventral striatum and the olfactory tubercles. Within the neostriatum many dendrites and spines as well as some perikarya, displayed immunoreactivity for the receptors.

In the control sections in which the receptor antibodies had been omitted, the distribution of immunoreactivity for substance P or ENK (DAB as chromogen) was similar to that described on many occasions in material prepared for electron microscopy from animals that did not receive colchicine treatment (e.g. DiFiglia *et al.*, 1982; Somogyi *et al.*, 1982; Bolam *et al.*, 1983; Bolam and Izzo, 1988; Martone *et al.*, 1992). Both sets of immunostained sections contained many immunoreactive axons and axonal swellings and small numbers of immunostained perikarya. In agreement with previous observations, the immunoreactive perikarya in the substance P-stained sections had the appearance of neostriatal interneurons whereas those in the ENK-stained sections had the appearance of spiny neurons.

Double-stained sections

In the sections double labelled to reveal both dopamine receptor and neuropeptide immunoreactivity, the neostriatum was densely stained by peroxidase reaction products that displayed different colours.

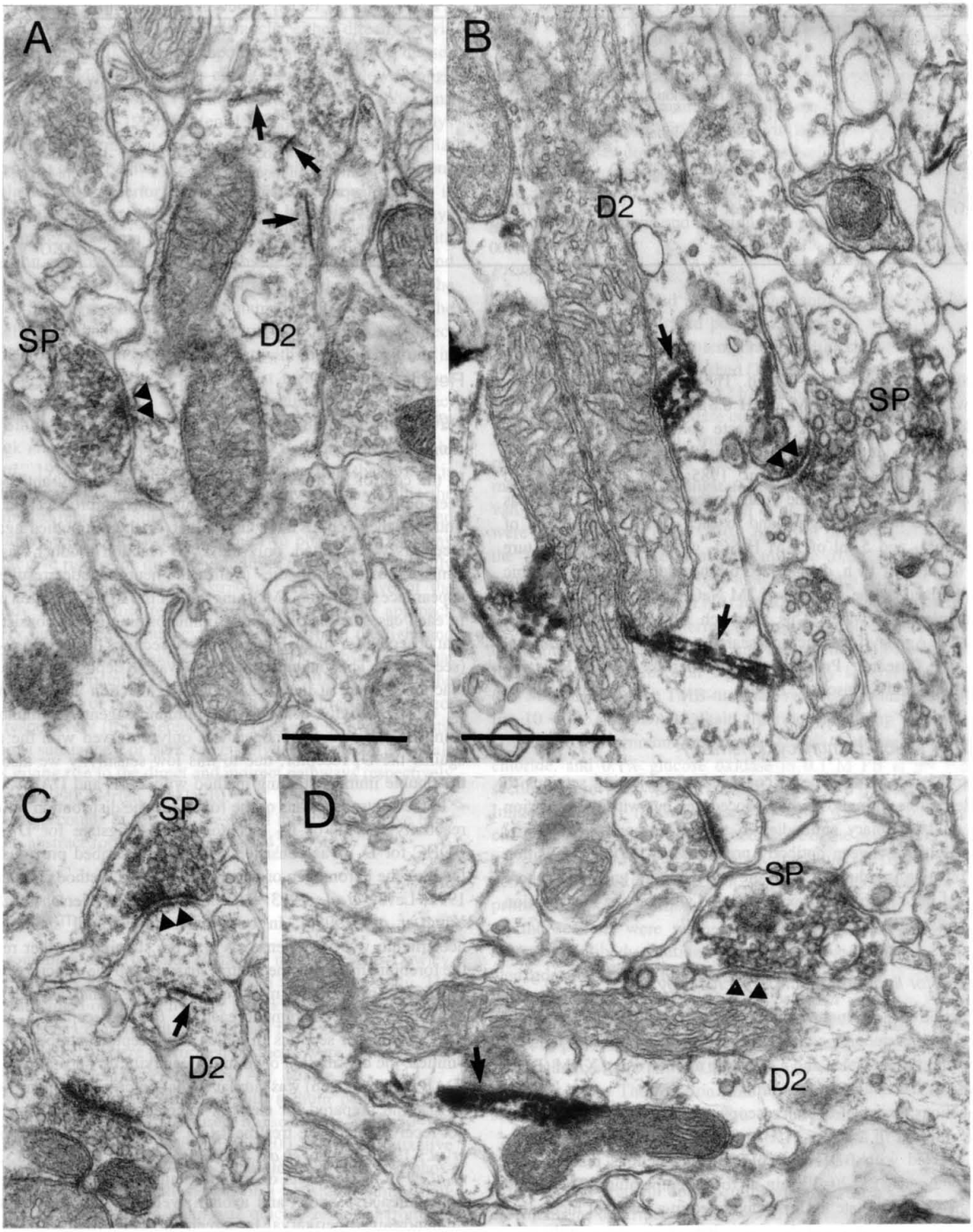

FIG. 1. (A–D) Electron micrographs of the rat neostriatum immunostained to reveal immunoreactivity for D_2 receptor (identified by the TMB-tungstate reaction product, arrows) and immunoreactivity for substance P (identified by DAB reaction product). Substance P-immunoreactive terminals (SP) make symmetrical synaptic contacts (arrowheads) with D_2-immunoreactive dendritic shafts (D_2). Crystals of the D_2 receptor immunoreaction product are indicated by arrows. Scale bars are equivalent to 0.5 μm. The bar in A also applies to C and D.

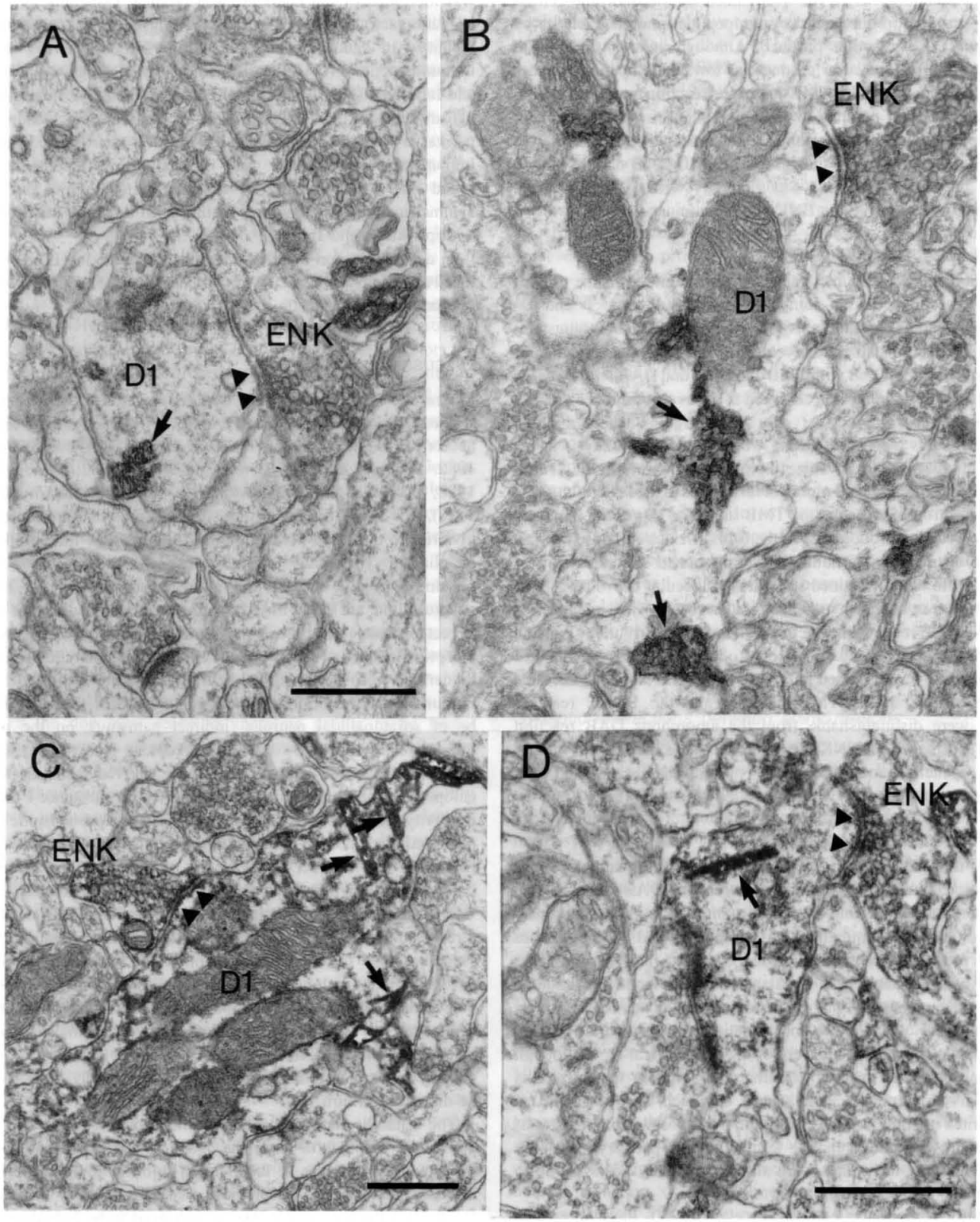

FIG. 2. Electron micrographs of the rat neostriatum immunostained to reveal D_1 dopamine receptor and enkephalin immunoreactivity. (A and B) Enkephalin-immunoreactive axon terminals (ENK) identified by the DAB immunoreaction product make symmetrical synaptic contacts (arrowheads) with D_1-immunoreactive dendritic shafts (D_1) identified by the presence of the BDHC immunoreaction product (some crystals indicated by arrows). (C and D) The enkephalin immunoreactive terminals are also identified by the DAB reaction product and make symmetrical synaptic contacts (arrowheads) with D_1-immunoreactive dendritic shafts but in this case the D_1-immunoreactivity was revealed using TMB-tungstate as the chromogen for the peroxidase reaction (some crystals of reaction product are indicated by arrows). Note that in these two heavily stained dendrites the TMB-tungstate reaction product is also in the form of a floccular precipitate. The dendrite in (D) is also postsynaptic to an immunonegative terminal that probably forms an asymmetrical contact with the dendrite. Scale bars are equivalent to 0.5 μm. The bar in (A) also applies to (B).

Receptor immunoreactivity was recognized by the dark blue TMB-tungstate reaction product and the neuropeptide immunoreactivity by the brown DAB reaction product. Although it was difficult to distinguish the two reaction products at low magnification because of the high density of the TMB-tungstate reaction product, at high magnification it was possible to distinguish the brown neuropeptide-positive punctate structures among the blue receptor-immunoreactive dendrites and perikarya. In the cases of BDHC/DAB double-stained sections, the receptor staining was generally weak (BDHC-D_1) but was distinguishable from the ENK (DAB) immunostaining.

Electron microscopic observations

Two combinations of double staining were examined in the present study, i.e. sections double-stained to reveal substance P and D_2 dopamine receptor immunoreactivities (DAB with TMB-tungstate) and sections double-stained to reveal ENK and D_1 dopamine receptor immunoreactivities (DAB with TMB-tungstate and DAB with BDHC). Although double staining was observed at all levels of the neostriatum at the light microscopic level only those regions where the two reaction products were most strong were selected for analysis at the electron microscopic level. These regions were mainly in the ventral parts of the neostriatum and more dorsal regions at the level of the GP.

In the electron microscope the TMB-tungstate (D_1 or D_2 immunoreactivity) reaction product was in the form of electron-dense crystals that were distributed intermittently within stained structures (Figs 1 and 2C, D). In heavily stained structures a floccular deposit was also present that was attached to subcellular organelles and membranes but, unlike DAB reaction product, had an uneven distribution (Fig. 2C, D). The BDHC (D_1) immunoreaction product was electron dense and crystalline, and was dispersed intermittently within labelled structures (Fig. 2A, B). Both the TMB-tungstate and the BDHC reaction products were distinguishable from the amorphous DAB reaction product (substance P or ENK) that adhered to the membranes of subcellular organelles and the internal plasma membrane, and was distributed homogeneously within labelled structures (Figs 1 and 2).

Double labelling of substance P and dopamine D_2 receptor

The TMB-tungstate (D_2) reaction product was predominantly localised in dendritic elements and perikarya, whereas the DAB (substance P) reaction product was found in axons, axonal boutons and terminals (Fig. 1). Substance P-immunoreactive dendrites and perikarya were not observed in the electron microscope due to their low numbers within the neostriatum. The substance P-immunoreactive boutons were of medium size (~0.5 μm in diameter), contained relatively large round and oval vesicles and sometimes mitochondria (Fig. 1). Most synaptic specializations were of the symmetrical type, although occasional terminals forming asymmetrical synaptic specializations were observed. Dendritic shafts, many of which were identified as the dendrites of spiny neurons, were the main postsynaptic targets of the substance P-immunoreactive terminals. The perikarya of spiny neurons and dendritic spines were also occasionally postsynaptic to the substance P-immunoreactive terminals.

In addition to non-labelled dendritic elements, D_2 immunoreactive dendritic shafts were also found to receive synaptic inputs from substance P-immunoreactive terminals ($n = 31$) (Fig. 1). On each occasion, symmetrical synaptic contacts were formed between the substance P-immunoreactive terminals and the D_2 immunoreactive dendritic shafts.

Double labelling of ENK and dopamine D_1 receptor

Immunoreactivity for D_1 receptor was identified by either the BDHC or TMB-tungstate reaction product and immunoreactivity for ENK was identified by the DAB reaction product. Dopamine D_1 receptor immunoreactivity (BDHC or TMB-tungstate) was predominantly localized in dendritic elements and perikarya, whereas the ENK immunoreactivity (DAB) was found mainly in axons, axonal boutons and terminals (Fig. 2). Presumably due to their low numbers in the neostriatum in this material ENK-immunoreactive dendrites and perikarya were not observed in the electron microscope. The ENK-immunoreactive terminals were of medium size, contained many round and oval vesicles, up to three mitochondria and formed symmetrical synaptic contacts mainly with dendritic shafts but also with perikarya and spines.

In addition to non-labelled dendritic elements, the ENK-immunoreactive terminals made symmetrical synaptic contact with D_1-immunoreactive dendritic shafts ($n = 34$) (Fig. 2).

Discussion

The results of the present study confirm and extend those of previous studies that have demonstrated a synaptic interaction between spiny neurons in the neostriatum (Pickel *et al.*, 1980; Wilson and Groves, 1980; Somogyi *et al.*, 1981; DiFiglia *et al.*, 1982; Somogyi *et al.*, 1982; Bolam *et al.*, 1983; Aronin *et al.*, 1986; Bolam and Izzo, 1988). The major finding is that spiny neurons that are presumed to give rise to the direct pathway of information flow through the basal ganglia are synaptically interconnected with those neurons that are presumed to give rise to the indirect pathways. Thus, neuronal structures that are likely to be of striatonigral/entopeduncular neurons (direct pathway) on the basis of immunoreactivity for D_1 dopamine receptors, receive synaptic input from terminals that are presumed to be derived from striatopallidal neurons that were identified by ENK immunoreactivity. Similarly, neuronal structures that are likely to be of striatopallidal neurons (indirect pathway), on the basis of immunoreactivity for D_2 dopamine receptors, receive synaptic input from terminals that are presumed to be derived from striatonigral/entopeduncular neurons that were identified by substance P-immunoreactivity. Thus, in addition to interacting at the postsynaptic level in the output nuclei of the basal ganglia (see Introduction for references), the direct and indirect pathways of information flow through the basal ganglia also interact at the synaptic level within the neostriatum.

The validity of the conclusions of the present study depends on three issues: first, the reliability of the double staining method; secondly, that D_1 dopamine receptors are expressed exclusively by the striatonigral/entopeduncular neurons and D_2 receptors are expressed exclusively by striatopallidal neurons; and thirdly, that the ENK- and the substance P-immunoreactive terminals are derived solely from the spiny neurons expressing these peptides, i.e. striatopallidal and striatonigral/entopeduncular respectively. Each issue will be discussed in turn.

The reliability and the technical limitations of the use of double peroxidase methods to study the chemical content of both pre- and postsynaptic structures at the electron microscopic level have been discussed extensively on previous occasions (Levey *et al.*, 1986; Bolam and Ingham, 1990). Two points need to be considered. First, it must be ensured that the secondary and tertiary reagents do not cross-react or, if there is a possibility of cross-reactivity, then it must be ensured that the first reaction product prevents cross-reactivity by obscuring antigenic, binding or the peroxidase enzymatic sites. Secondly, it must be ensured that the reaction product formed during the second reaction does not become deposited on the first reaction product. These points were addressed by the controls that were performed in the present study in which the sections were submitted to the whole double staining procedure but the first or second primary

antibody incubations were omitted in turn. The results of these control experiments demonstrated that problems of cross-reactivity, recognition of the chromogen of the second reaction by the peroxidase of the first reaction and non-specific deposition of the one reaction product on another, did not occur in the present study. It should be noted, that pre-embedding double immunostaining methods can only give qualitative data concerning synaptic interactions because of the difficulties of obtaining optimal staining with two antibodies and the problems of differential penetration of the immunoreagents, both of which lead to false-negatives.

The results of *in situ* hybridization studies (Gerfen *et al.*, 1990; Le Moine *et al.*, 1990, 1991; Le Moine and Bloch, 1995) and immunocytochemical studies using the same receptor sub-type specific antibodies as in the present study (Levey *et al.*, 1993; Yung *et al.*, 1994, 1995a, b; Hersch *et al.*, 1995) have demonstrated that striatal medium spiny neurons that express D_1 or D_2 dopamine receptors are largely separate populations of neurons. Furthermore, these studies strongly suggest that D_1 dopamine receptors are associated with substance P- and dynorphin-containing striatonigral (and presumably striatoentopeduncular) neurons, whereas D_2 dopamine receptors are associated with ENK-containing, striatopallidal neurons (see Gerfen, 1992a, b for reviews). It must be noted however, that the conclusion of some electrophysiological, immunocytochemical and *in situ* hybridization studies is that dopamine D_1 and D_2 receptors co-localize to a great extent in spiny neurons (for review see Surmeier *et al.*, 1993; Surmeier and Kitai, 1994). Nevertheless, in our hands (Levey *et al.*, 1993; Yung *et al.*, 1994, 1995a, b; Hersch *et al.*, 1995), immunocytochemical staining with sub-type specific antibodies raised against bacterial fusion proteins that include amino acid sequences of human D_1 and D_2 receptors, labels sub-populations of striatal neurons and differentially labels their terminal fields. Antibodies against sub-classes of the dopamine receptors therefore appear to be useful markers of the neurons giving rise to the direct and indirect pathways. Although other classes of neostriatal neurons express dopamine receptors, their relatively small numbers imply that the majority of receptor-immunoreactive structures encountered in the electron microscope will be derived from spiny neurons. The possibility cannot be excluded however, that the small number of the interneurons that express dopamine receptors account for a large number of dendrites.

The possible sources of the substance P-immunoreactive terminals in the neostriatum include both local neurons and neurons located in regions extrinsic to the neostriatum. All the synaptic contacts observed in the present study formed by substance P-immunoreactive terminals with D_2-immunoreactive dendrites were of the symmetrical type. It is therefore unlikely that they originate in the thalamus, dorsal raphe nucleus or mesopontine tegmentum, i.e. regions containing substance P-positive neurons, as terminals derived from each of these regions form asymmetrical synapses (see Bolam and Bennett, 1995; Clarke *et al.*, 1995). At present it is not possible to distinguish between the terminals of spiny neurons and those of putative substance P-positive interneurons as the latter have not been identified at the electron microscopic level. However, the morphology of the substance P-immunoreactive terminals identified in the present study is similar to that of identified local terminals of spiny neurons within the neostriatum (Wilson and Groves, 1980; Somogyi *et al.*, 1981) or in their projection regions (Chang *et al.*, 1981; Smith and Bolam, 1991; Bolam and Smith, 1992; von Krosigk *et al.*, 1992; Bolam *et al.*, 1993; Bevan *et al.*, 1994a, b). This observation suggests that most of the substance P-immunoreactive terminals seen in contact with the D_2-immunoreactive dendrites are derived from substance P-containing spiny neurons. A similar argument may be put forward for the ENK-positive terminals seen in synaptic contact with the dopamine D_1 receptor dendrites. It is likely that the ENK-positive terminals forming symmetrical synapses that were identified in this and other studies (Pickel *et al.*, 1980, 1992; DiFiglia *et al.*, 1982; Somogyi *et al.*, 1982; Bouyer *et al.*, 1984; Aronin *et al.*, 1986) are derived from spiny ENK-containing neurons because: (i) spiny neurons are the only population of striatal neurons demonstrated to contain ENK; (ii) the morphology of the ENK-positive terminals is similar to that described for the terminals of identified spiny neurons (see above); and (iii) any extrinsic source of ENK-positive terminals is likely to give rise to terminals forming asymmetrical synapses. It should be noted, however, that in primates at least some of the neurons giving rise to the direct (striatonigral) pathway contain ENK (Haber *et al.*, 1994).

In summary, the use of double immunocytochemistry with antibodies against dopamine D_1 and D_2 receptors, together with antibodies against ENK and substance P is a valid approach to address the question of whether spiny neurons giving rise to the direct and indirect pathways are synaptically interconnected via axon collaterals. The approach has advantages over double immunocytochemistry for the neuropeptides as the receptor antibodies preferentially label dendrites and spines, whereas dendritic labelling for the neuropeptides is weak or non-existent in material prepared for electron microscopy. Furthermore, the use of dopamine D_1 and D_2 receptor antibodies as markers of striatonigral/entopeduncular and striatopallidal neurons has advantages over retrograde labelling because the injection of tracers in the GP is likely to also result in the labelling neurons of the direct pathway whose axons travel through the GP and give off minor collaterals (Kawaguchi *et al.*, 1990).

Synaptic interactions between spiny neurons

Previous immunocytochemical studies, alone or in combination with retrograde labelling, have thrown some light on the synaptic relationships between spiny neurons of the different sub-classes. Thus, substance P-immunoreactive terminals have been shown to make synaptic contact with substance P-immunoreactive dendrites (Bolam *et al.*, 1983) and with the spines and dendrites of retrogradely labelled striatonigral neurons (Bolam and Izzo, 1988) indicating that lateral interaction occurs between neurons in the *same* sub-population of striatal neurons. Similarly, neurons giving rise to the indirect pathway (i.e. striatopallidal), identified by ENK immuncytochemistry are synaptically interconnected (Pickel *et al.*, 1980, 1992; DiFiglia *et al.*, 1982; Somogyi *et al.*, 1982). There is also some evidence from previous studies that synaptic interaction occurs between the sub-populations of neurons (Aronin *et al.*, 1986). The present findings demonstrate that neurons giving rise to the direct pathway are synaptically interconnected to those giving rise to the indirect pathway and *vice versa*. Thus, in addition to interacting at the synaptic level in the output nuclei of the basal ganglia (for references see above), the two functionally distinct pathways of information flow through the basal ganglia interact directly via the axon collaterals of the spiny neurons at the level of the neostriatum. It must be remembered that within the complex microcircuitry of the neostriatum spiny neurons are also likely to interact indirectly through many routes (Bolam and Bennett, 1995). One route by which the two pathways may interact is through the cholinergic interneurons. These neurons receive synaptic input from both substance P- and ENK-containing terminals (Bolam *et al.*, 1986; Martone *et al.*, 1992), express substance P and delta opioid receptors (Gerfen, 1991; Kaneko *et al.*, 1993; Le Moine *et al.*, 1994) and in turn, make synaptic contact with spiny

neurons, some of which have been identified as striatonigral neurons (Izzo and Bolam, 1988).

Conclusions

The synaptic connections demonstrated in the present study provide an anatomical substrate for lateral interaction between striatal neurons giving rise to the direct and indirect pathways of information flow through the basal ganglia. As physiological evidence suggests that this interaction is not a fast GABA-mediated event (Jaeger *et al.*, 1994) and spiny neurons do not express substance P receptors (Gerfen, 1991; Kaneko *et al.*, 1993), it remains to be established what neurotransmitter or neuromodulator mediates the interaction, exactly what form the interaction takes and what is its functional role.

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Abbreviations

ABC	avidin–biotin–peroxidase complex
BDHC	benzidine dihydrochloride
DAB	diaminobenzidine
ENK	enkephalin
EP	entopeduncular nucleus
GP	globus pallidus
GP_e	external segment of the globus pallidus
GP_i	internal segment of the globus pallidus
PB	phosphate buffer
PBS	phosphate-buffered saline
SNr	substantia nigra pars reticulata
SP	substance P
TMB	tetramethylbenzidine

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