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# Localization of Dopamine D1 and D2 Receptors in the Rat Neostriatum: Synaptic Interaction With Glutamate- and GABA-Containing Axonal Terminals

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*KEY WORDS* dopamine D1 and D2 receptor; gamma-aminobutyric acid; glutamate; basal ganglia; immunocytochemistry; immunogold staining; electron microscopy

*ABSTRACT* In order to determine the synaptic interactions between the glutamate- and GABA-containing axonal terminals and the two subpopulations of medium spiny neurons in the rat neostriatum, double immunocytochemistry was performed. Sections of perfuse-fixed rats were used. Immunoreactivity for dopamine D1 and D2 receptors was used as a marker for the two subpopulations of spiny neurons that give rise to the direct and indirect pathways, respectively. Receptor immunoreactivity was first revealed by preembedding immunostaining. Postembedding colloidal gold labeling was then performed to reveal immunoreactivity for glutamate or GABA. The results were analyzed at the electron microscopic level. Both the D1-immunoreactive, presumed striatonigral/entopeduncular neurons, and the D2-immunoreactive, presumed striatopallidal neurons, were found to receive qualitatively similar synaptic inputs from glutamate-immunoreactive terminals and GABA-immunoreactive terminals. The present results indicate that the different classes of spiny neurons are thus likely to be under a similar regime of excitatory and inhibitory control. **Synapse 38:413–420, 2000.** 

## INTRODUCTION

Medium-sized spiny neurons of the neostriatum are the main recipients of the afferent synaptic inputs to the basal ganglia (see Gerfen and Wilson, 1996; Smith and Bolam, 1990), the major ones of which are derived from the cortex (Frotscher et al., 1981; Kemp and Powell, 1971a,b; Somogyi et al., 1981), the thalamus (Chung et al., 1977; Dubé et al., 1988; Kemp and Powell, 1971a,b; Sadikot et al., 1992; Xu et al., 1991) and the substantia nigra pars compacta (Freund et al., 1984). Spiny neurons also receive inputs that originate in other divisions of the basal ganglia and from local striatal neurons, including interneurons and other spiny neurons (Bennett and Bolam, 1994; Bolam and Bennett, 1995; Bolam and Izzo, 1988; Smith and Bolam, 1990; Somogyi et al., 1981; Wilson and Groves, 1980; Yung et al., 1996). The afferents of spiny neurons differ in their neurochemical content; thus glutamate is utilized by terminals that are derived from the cortex and thalamus and GABA by terminals that are derived from at least some striatal interneurons and spiny neurons (see reviews: Bolam and Bennett, 1995; Gerfen, 1992; Kawaguchi, 1997; Kawaguchi et al., 1995).

Spiny neurons are the major projection neurons of the neostriatum. They are subdivided into two subpopulations that give rise to the major pathways of information that flow through the basal ganglia, i.e., the direct striatonigral/striatoentopeduncular pathway and those that give rise to the indirect striatopallidal pathway (Albin et al., 1989; Alexander and Crutcher, 1990; DeLong, 1990; Gerfen, 1992; Gerfen and Wilson, 1996; Graybiel, 1990; Smith et al., 1998). Although the somato-dendritic morphology of the two subpopulations is very similar, they can be distinguished on the basis of neurochemistry. The striatonigral/striatoentopeduncular neurons contain substance P and dynorphin and express the D1 dopamine receptor, whereas striatopallidal neurons contain enkephalin and express

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the D2 dopamine receptor (Graybiel, 1990; Gerfen, 1992; Gerfen et al., 1990; Le Moine and Bloch, 1995; Le Moine et al., 1991; Levey et al., 1993; Hersch et al., 1995; Yung et al., 1995, 1996). It should be noted, however, that physiological analyses and molecular biological analyses suggest that both populations of neurons express both receptor types (Surmeier and Kitai, 1994). A recent report suggests that although functional D2 receptors may not be totally segregated to striatopallidal neurons, they are expressed in a higher proportion of striatopallidal neurons than striatonigral neurons (Waszczak et al., 1998). Immunoreactivity for D1 and D2 receptors is thus a useful neurochemical marker for the subpopulations of spiny neurons (Hersch et al., 1995; Yung et al., 1995, 1996).

Activity in the two pathways is altered in abnormal conditions such as in Parkinson disease; it is thus important to know the nature of the synaptic input to the spiny neurons to the two pathways that ultimately controls their output. The objective of the present study was to determine whether spiny neurons giving rise to the direct and indirect pathways receive qualitatively similar synaptic input from glutamate- and GABA-containing immunopositive terminals. The two subpopulations of spiny neurons were first labeled by revealing immunoreactivity for D1 and D2 receptors (Hersch et al., 1995; Yung et al., 1995, 1996); postembedding immunogold labeling was then employed in order to reveal immunoreactivity for glutamate or GABA in axonal terminals of the neostriatum.

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

## MATERIALS AND METHODS

### Animals and tissue preparation

Material from two rats (female, Wistar, 200–250 g, Charles River) were used. The housing of the animals and all procedures performed on them were in accordance with the Animals (Scientific Procedures) Act, 1986, UK. The animals were deeply anesthetized with sodium pentobarbitone (Sagatal, 60 mg/kg, i.p.). They were then perfused transcardially with 50–100 ml of saline (0.9% NaCl) followed by 200 ml of fixative consisting of 3% paraformaldehyde and 1% glutaraldehyde in PB (0.1 M, pH 7.4). Sections of the neostriatum (70 μm) were cut on a vibrating microtome and collected in 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 in liquid nitrogen and thawed. They were 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 preembedding immunocytochemical staining, the sections were incubated with normal goat serum (4% in PBS) for about 1 h at room temperature.

*Abbreviations:*

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| ABC | avidin-biotin-peroxidase complex |
| DAB | diaminobenzidine |
| EP | entopeduncular nucleus |
| GABA | gamma-aminobutyric acid |
| 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 |
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline with Triton |

### Immunocytochemistry

Sections were incubated in affinity-purified polyclonal antibody or monoclonal antibody solutions against D1 receptor (1:1,000–2,000 dilutions in PBS; Levey et al., 1993; Hersch et al., 1995) or affinity-purified polyclonal antibody against D2 receptor (1:100–200 dilutions in PBS; Levey et al., 1993) overnight at room temperature with constant gentle shaking. They were then washed (3× PBS), incubated in biotinylated secondary antibody solutions (goat-antirabbit IgG or goat-antirat IgG, 1:100–200 dilutions, Vector Laboratories, Burlingame, CA) for 2 h, washed (3× PBS), and then incubated in an avidin-biotin-peroxidase complex (1:100 dilution, ABC, Vector) for at least 1 h. Immunoreactivity for the receptors was revealed by a peroxidase reaction using DAB as chromogen. The sections were washed (2× PBS; 1× 0.05 M Tris-HCl buffer) and incubated in 0.025% DAB in Tris-HCl buffer (pH 7.4) containing 0.0048% $H_2O_2$ for ~5–10 min. The reaction was stopped by several washes in Tris buffer and then PBS.

### Processing for electron microscopy

The sections were treated with osmium tetroxide (1% in 0.1 M PB, pH 7.4) for ~30 min at room temperature. They were washed in PB (0.1 M) and dehydrated in a series of increasing concentrations of ethanol and then propylene oxide. After infiltration with resin (Durcupan ACM, Fluka, Buchs, Switzerland) overnight, the sections were mounted on microscope slides, coverslips 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 cut out from the slide and glued to blank blocks of resin. Ultrathin sections (silver/gray) were cut using an ultramicrotome (Reichert-Jung) and collected on Pioloform-coated, gold, single-slot grids. These sections were then subjected to postembedding immunogold labeling.

### Postembedding immunogold labeling

Postembedding immunogold labeling followed the procedures of Phend et al. (1992) with slight modifica-

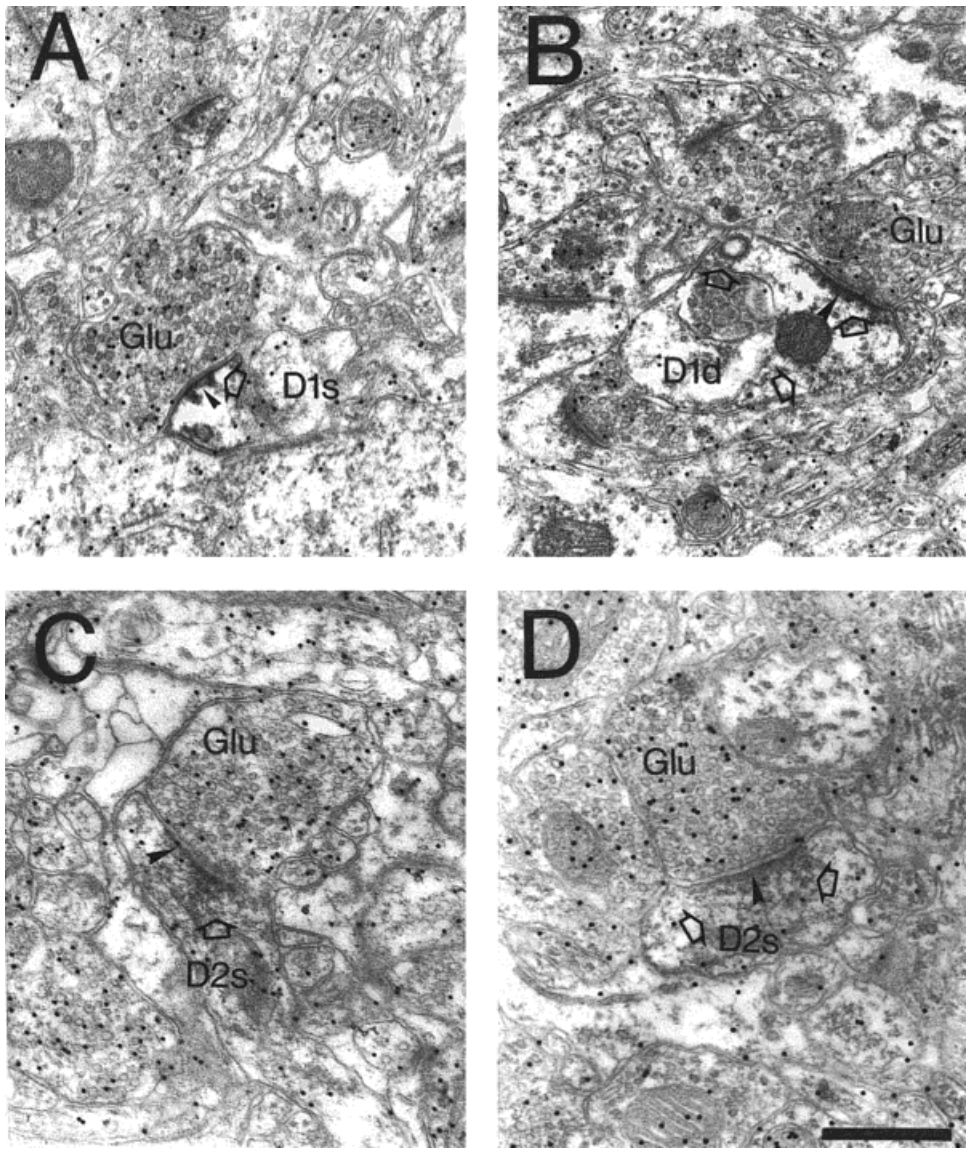


Fig. 1. Electron micrographs of rat neostriatum immunostained to reveal immunoreactivity for D1 or D2 receptor (identified by DAB reaction product, some clumps of which are indicated by open arrows) and immunoreactivity for glutamate (identified by the relative enrichment of immunogold particles). Glutamate-immunoreactive boutons (Glu) form asymmetrical synaptic contacts (arrowheads) with D1-immunoreactive spines (D1s; **A**), dendritic shafts (D1d; **B**) and D2-immunoreactive spines (D2s; **C,D**). Scale bar = 0.5 μm.

tions. Adjacent serial ultrathin sections, which were collected on separate gold grids, were incubated for 14–18 h in polyclonal antibody solutions against GABA (1:2,000–5,000 dilutions; Hodgson et al., 1985; Somogyi and Hodgson, 1985; Somogyi et al., 1985) or glutamate (1:2,000 dilution; Arnel Products, New York, NY) in Tris-buffered saline with Triton X-100 (TBST, 0.05M, pH 7.6, 0.01% Triton X-100) at room temperature overnight in a large glass petri dish. The sections were then washed (2× TBST, 1× TBS, pH 8.2), and incubated in secondary antibody solution conjugated with colloidal gold (goat-antirabbit IgG, 10 nm in diameter, 1:25 dilution in TBS, pH 8.2; British Biocell, Cardiff, UK) at room temperature for 1–1.5 h. They were then washed (2× TBS, 6× distilled $H_2O$), treated with 1% uranyl acetate for at least 1.5 h, washed again (4× distilled $H_2O$), stained with lead citrate, and examined in an electron microscope (Philips 410 or CM10).

### Quantitative analysis of the distribution of immunogold particles

A quantitative analysis of the distribution of immunogold particles in different structures was performed because of the inherent variability in the immunogold labeling technique and, in the case of glutamate, because of its ubiquitous distribution in the nervous system as a metabolic intermediate, a precursor of GABA, and as a neurotransmitter. The number of immunogold particles overlying presumed GABA-immunoreactive or glutamate-immunoreactive terminals that formed symmetrical or asymmetrical synaptic contacts with D1- or D2-immunoreactive structures were counted on electron micrographs. The area of each immunoreactive terminal was measured with the aid of a digitizing pad and MacStereology software. The density of the immunogold particles overlying each terminal was cal-

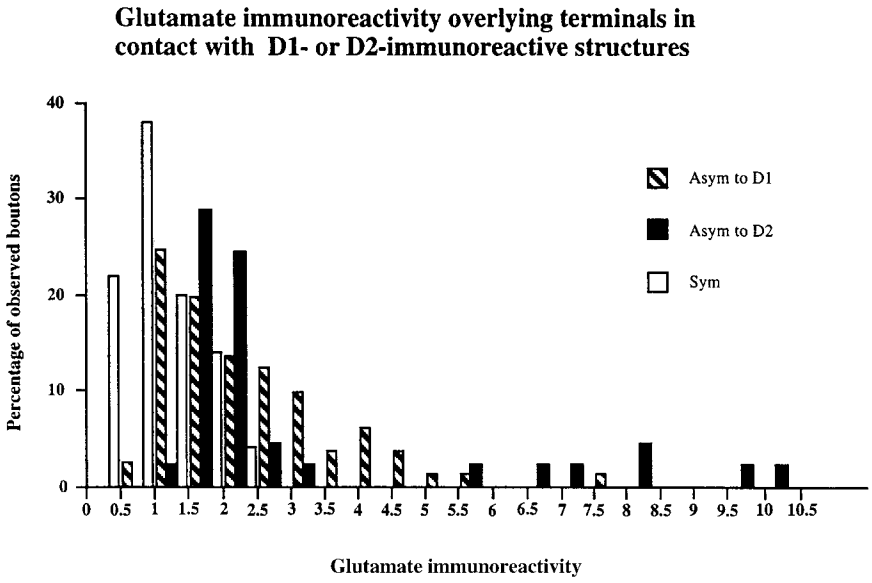


Fig. 2. Frequency distribution of the levels of glutamate immunolabeling associated with terminals in synaptic contact with D1- or D2-immunoreactive structures. Glutamate immunoreactivity is expressed as the ratio of the density of immunogold particles overlying terminals forming asymmetric contacts with D1-immunoreactive (Asym to D1) or D2-immunoreactive (Asym to D2) dendritic structures to that overlying terminals forming symmetrical synapses (sym) in the same section. The levels of glutamate immunoreactivity associated with terminals forming asymmetrical synapses with both D1- and D2-immunoreactive structures (n = 81 and 45, respectively) are significantly higher than the levels associated with terminals forming symmetrical synapses ($P < 0.001$, Mann Whitney U-test).

culated. Background values of the immunogold labeling were obtained by measuring the density of immunogold particles in terminals forming asymmetrical (in the case of GABA immunolabeling) or symmetrical (in the case of glutamate immunolabeling) membrane specializations in the same ultrathin sections. Nonspecific adhesion of gold particles to the resin was taken into account by subtracting the density of immunogold particles overlying the resin in the lumen of capillaries in the same grid. In order to take into account variability between animals, between experimental runs and between different grids, the densities of immunogold particles overlying putative glutamatergic and GABAergic terminals was normalized. The densities of immunogold particles overlying the presumed glutamate-immunoreactive terminals was normalized by expressing it as a ratio of the density overlying terminals forming symmetrical synaptic contacts. The densities of immunogold particles overlying the presumed GABA-immunoreactive terminals was expressed as a ratio of that overlying terminals forming asymmetrical synaptic contacts. The ratios for glutamate or GABA immunoreactivity were then statistically compared to the values obtained for terminals forming symmetrical or asymmetrical synapses, respectively, by the Mann-Whitney U-test. Issues relating to the quantification of immunogold labeling for GABA and glutamate have been discussed extensively previously (Bevan et al., 1995; Somogyi et al., 1986).

## RESULTS

### Light microscopic observations

In confirmation of previous findings (Levey et al., 1993; Hersch et al., 1995; Yung et al., 1995, 1996), dense D1 or D2 immunoreactivity was observed in the neuropil of the neostriatum. At high magnification, immunoreactive perikarya with a large unstained nucleus and a thin rim of cytoplasm, i.e., features of the medium size spiny neuron, could be identified among dense dendritic and spine-like labeling (data not shown).

### Electron microscopic observations

#### Double-labeling for dopamine receptors and glutamate

In double-labeled sections, immunoreactivity for D1 or D2 receptor was identified by the electron-dense, floccular DAB reaction product, whereas immunoreactivity for glutamate was identified by the presence of immunogold particles. Enrichment of immunogold particles was found in axonal terminals that formed asymmetrical synapses with dendritic elements (Fig. 1A–D). Many of these dendritic elements were either D1- (Fig. 1A,B) or D2-immunopositive (Fig. 1C,D). The majority of the glutamate-containing terminals in contact with receptor-immunoreactive structures formed synapses with spines. In each case, synaptic contacts were formed primarily at the heads of spines (Fig. 1A,C,D). A smaller proportion of glutamate-containing terminals made synaptic contacts with receptor-immunoreactive dendritic shafts (Fig. 1B).

Glutamate immunolabeling associated with terminals that formed asymmetrical synaptic contacts with D1-immunoreactive structures (n = 81) was significantly greater ($P < 0.001$) than that associated with terminals that formed symmetrical synapses in the same ultrathin sections. Most of the glutamate-enriched terminals were found to contain glutamate immunoreactivity of at least 1.5-fold higher than the terminals that formed symmetrical synapses (Fig. 2). Similarly, significantly higher levels of glutamate immunoreactivity were associated with terminals forming asymmetrical synaptic contacts with D2-immunoreactive profiles (n = 46) than terminals forming

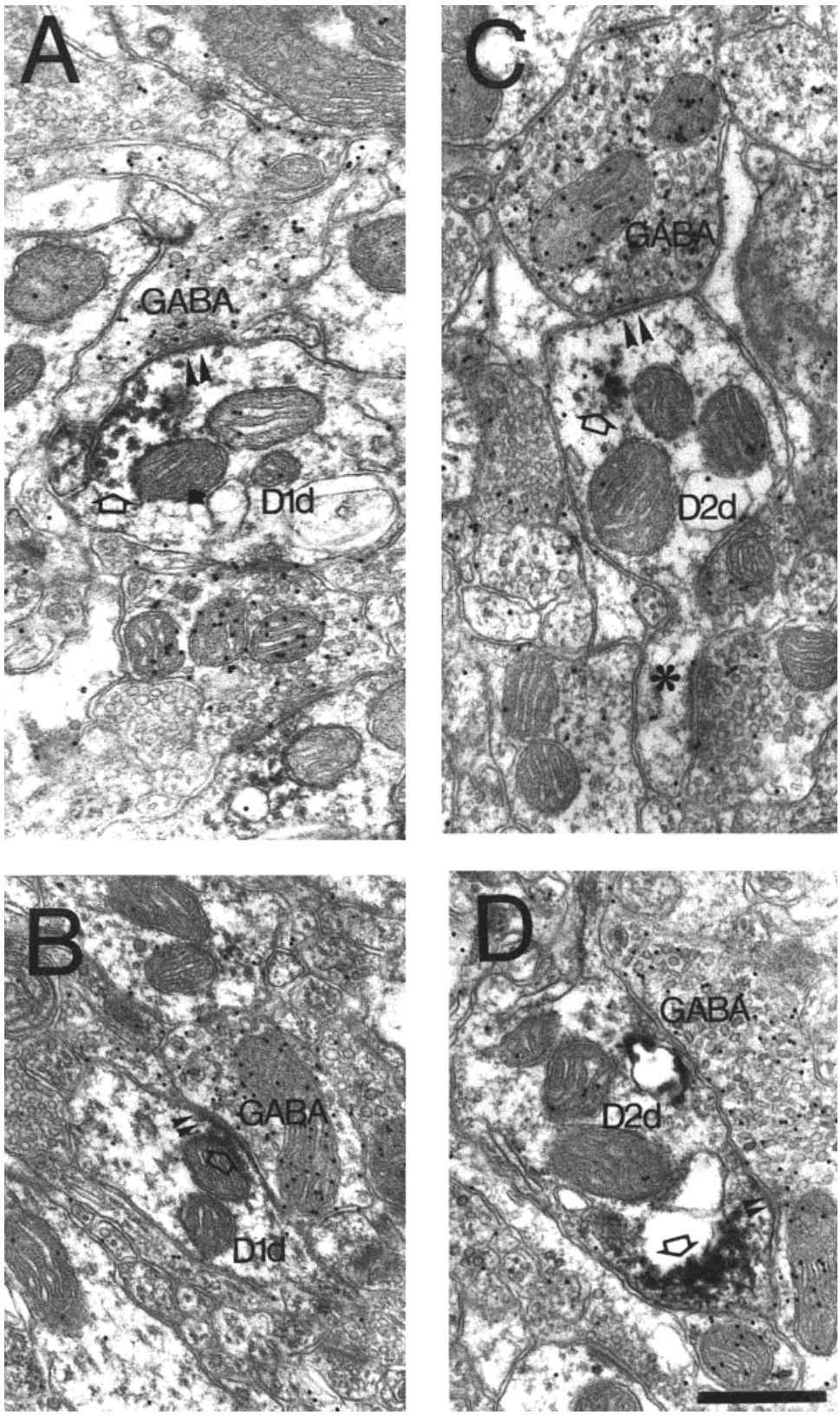


Fig. 3. Electron micrographs of rat neostriatum immunostained to reveal immunoreactivity for D1 or D2 receptor (identified by DAB reaction product, some clumps of which are indicated by open arrows) and immunoreactivity for GABA (identified by the enrichment of immunogold particles). GABA-immunoreactive boutons (GABA) make symmetrical synaptic contacts (two arrowheads) with D1-immunoreactive dendrites (D1d; **A,B**) and D2-immunoreactive dendrites (D2d; **C,D**). Some D1-immunoreactive dendrites that receive synaptic input from GABA-immunoreactive terminals were seen to give rise to nonreactive spines (one is indicated by a star in **C**). The dendrites are likely belong to spiny neurons. Scale bar = 0.5 μm.

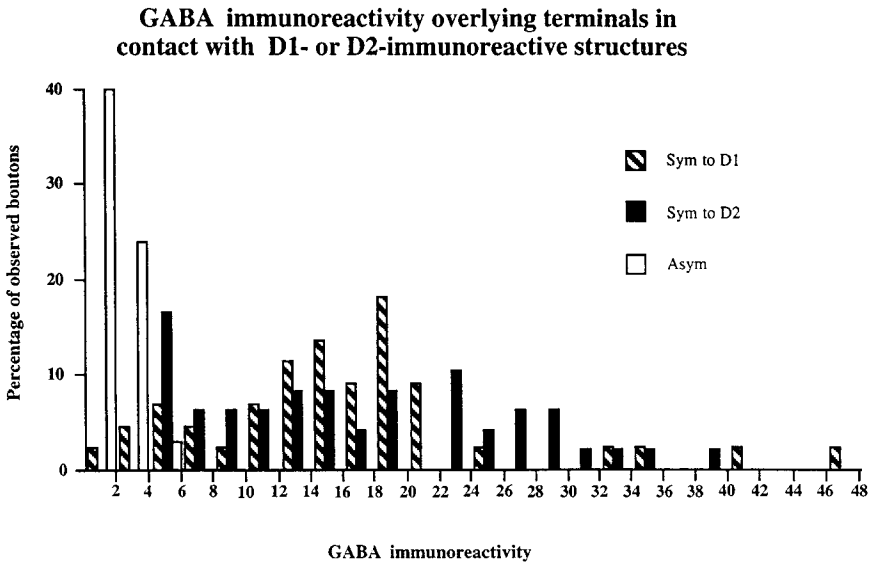


Fig. 4. Frequency distribution of the levels of GABA immunolabeling associated with terminals in synaptic contact with D1- or D2-immunoreactive structures. GABA immunoreactivity is expressed as the ratio of the density of immunogold particles overlying terminals forming symmetrical contacts with D1-immunoreactive (Sym to D1) or D2-immunoreactive (Sym to D2) dendritic structures to that overlying terminals forming asymmetrical synapses (Asym) in the same sections. The levels of GABA immunoreactivity associated with terminals in synaptic contact with D1-immunoreactive structures (n = 44) and D2-immunoreactive structures (n = 49) are significantly higher than those associated with terminals forming asymmetrical synapses ($P < 0.001$, Mann Whitney U-test).

symmetrical synapses in the same sections ($P < 0.001$; Fig. 2).

Many of the glutamate-enriched terminals were also identified in adjacent GABA-labeled sections and in each case were found to be GABA-negative (data not shown).

### Double-labeling for dopamine receptors and GABA

Accumulation of gold particles in the GABA-immunolabeled sections was predominantly found in axonal structures and terminals that formed symmetrical synapses with dendritic elements (Fig. 3). Many of the dendritic elements were either D1- (Fig. 3A,B) or D2- (Fig. 3C,D) immunoreactive.

The levels of GABA immunoreactivity associated with terminals that formed symmetrical synaptic contacts with D1-immunoreactive profiles (n = 44) were significantly higher than the levels associated with terminals forming asymmetrical synaptic contacts in the same sections ($P < 0.001$). Most of the GABA-enriched terminals had levels of GABA immunoreactivity at least 4 times higher than the terminals that formed asymmetrical synapses (Fig. 4). Similarly, GABA immunoreactivity associated with terminals forming symmetrical synaptic contacts with D2-immunoreactive profiles (n = 48) was also significantly higher than the levels associated with terminals forming asymmetrical synaptic contacts ($P < 0.001$).

On the basis of morphological criteria, the GABA-enriched terminals forming synaptic contacts with D1- and D2-immunoreactive dendrites could be subdivided. Some of the GABA-immunoreactive terminals were relatively large in size (more than 1.0 $\mu m^2$), packed with a large number of synaptic vesicles, with one or two large mitochondria and contained a relatively high number of immunogold particles (Fig. 3C,D). Others were much smaller in size (less than 0.5 $\mu m^2$), with fewer synaptic vesicles, with no mitochondria, and contained a relatively low density of immunogold particles (Fig. 3A).

## DISCUSSION

The present results demonstrate that both the D1-immunoreactive structures which are presumed to represent neurons of the direct pathway and the D2-immunoreactive structures which are presumed to represent neurons of the indirect pathway receive qualitatively similar synaptic inputs from glutamate- and GABA-immunoreactive terminals in the neostriatum of the rat. These data thus suggest that, in qualitative terms, the medium spiny neurons that give rise to the direct or indirect pathways in the basal ganglia are similarly endowed with glutamatergic and GABAergic afferents.

### Glutamate-containing afferents to the spiny neurons

The synaptic contacts between the spiny neurons and the glutamate-containing afferents are found mainly on the head of dendritic spines and, to a lesser extent, on dendritic shafts. Tracing studies have revealed that the glutamatergic boutons that terminate on the head of spines of the spiny neurons in the neostriatum mainly originate in the cortex (Bouyer et al., 1984; Dubé et al., 1988; Frotscher et al., 1981; Hassler et al., 1978; Hersch et al., 1995; Ingham et al., 1998; Kemp and Powell, 1971a,b; Smith et al., 1994; Somogyi et al., 1981; Xu et al., 1991). The present results show that both the D1-immunoreactive and D2-immunoreactive neurons receive similar inputs from glutamatergic terminals which presumably drive from the cortex. The findings support the observation of Hersch et al. (1995), who demonstrated by antero-

grade labeling that corticostriatal afferents make synaptic contacts with both D1-immunoreactive spines and D2-immunoreactive spines.

Both D1-immunoreactive and D2-immunoreactive dendritic shafts were also found to receive glutamatergic inputs in the present observations. The possible origins of these glutamatergic projections include the centromedian/parafascicular nucleus of the thalamus (Dubé et al., 1988; Kemp and Powell, 1971b; Smith et al., 1994; Xu et al., 1991), and other thalamic nuclei such as the centrolateral, paracentral, ventrolateral, and ventromedial (Xu et al., 1991), although a high proportion of these afferent contact spines, glutamate-enriched terminals in contact with dendritic shafts, may also be derived from the contralateral cortex (Hersch et al., 1995). It is interesting to note that the thalamostriatal afferents that originate in the centromedian nucleus in primates have been shown to preferentially innervate neurons that project to the internal segment of the globus pallidus, i.e., those neurons giving rise to the indirect pathway (Sidibé and Smith, 1996).

### GABA-containing innervation of spiny neurons

The present results demonstrate that both the D1- and D2-immunoreactive neurons in the neostriatum receive similar inputs from GABA-containing boutons. In agreement with previous observations (see reviews: Bolam and Bennett, 1995; Smith and Bolam, 1990), symmetrical membrane specializations are formed in all the cases of synaptic contacts. Possible origins for the GABA-containing synaptic inputs to the spiny neurons include pallidal neurons (Beckstead, 1983; Kita et al., 1991; Staines et al., 1981; Walker et al., 1989), although most pallidostriatal terminals are in contact with interneurons (Bevan et al., 1998), GABAergic interneurons (Wilson and Groves, 1980; see reviews: Bennett and Bolam, 1994; Bolam and Bennett, 1995; Kawaguchi, 1997; Kawaguchi et al., 1995), and spiny neurons themselves that give rise to the direct striatonigral or the indirect striatopallidal pathways (Somogyi et al., 1981; Yung et al., 1996). The different morphological characteristics of the GABA-positive boutons are consistent with this suggestion. One type of GABA-immunoreactive bouton (large in size, packed with a high number of synaptic vesicles, with one or two large mitochondria and containing a high number of immunogold particles; Fig. 3C,D) are similar to the morphology of the boutons formed by the pallidostriatal axons or possibly the parvalbumin-positive GABA interneurons (see reviews: Bolam and Bennett, 1995; Smith et al., 1998). The second type of GABA-immunoreactive bouton (smaller in size, with fewer synaptic vesicles, with no mitochondria observed in the terminal, and containing a low number of immunogold particles; Fig. 3A) are similar in morphology to substance P- (Bolam et al., 1986; Bolam and Izzo, 1988) or enkephalin- (Aronin et al., 1986; Somogyi et al., 1982) immunoreactive boutons in the neostriatum, which are presumed to be derived from spiny neurons (see also Yung et al., 1996). This type of GABA-positive terminal may thus be formed by the local collaterals of spiny neurons.

In conclusion, the present results provide evidence that the D1-immunoreactive (presumed spiny neurons of the direct pathway) and the D2-immunoreactive (presumed spiny neurons of the indirect pathway) receive qualitatively similar synaptic inputs from glutamate-immunoreactive terminals and GABA-immunoreactive terminals.

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