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PROJECTION OF NEOSTRIATAL SPINY NEURONS TO THE SUBSTANTIA NIGRA. APPLICATION OF A COMBINED GOLGI-STAINING AND HORSERADISH PEROXIDASE TRANSPORT PROCEDURE AT BOTH LIGHT AND ELECTRON MICROSCOPIC LEVELS

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SUMMARY

One type of striatonigral neuron in the rat has been characterized. Golgi impregnation of striatal neurons that had been retrogradely labeled by horseradish peroxidase has shown that the medium-sized, densely spiny neurons project to the substantia nigra.

Some of the synapses on three of these identified striatonigral neurons have been studied in the electron microscope following replacement of the Golgi deposit by means of the 'gold-toning' method. Synapsing axonal boutons were found on the following sites: soma and axon initial segment (symmetrical, with flattened or pleomorphic vesicles); primary and secondary dendritic shafts (symmetrical with pleomorphic vesicles); dendritic spines (asymmetrical, with spheroidal vesicles).

These findings show that new information concerning neuronal connectivity can be obtained by combining three classical procedures in the same material: first, the Golgi method, that characterizes the type of neuron on the basis of its dendritic morphology; second, a retrograde tracing method, that identifies the projection area of the neuron; and, third, ultrastructural analysis of the nature of afferent terminals on the neuron.

INTRODUCTION

Application of the Golgi method has established that, in a variety of species, the neostriatum contains several morphologically distinct types of neuron (for references

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see Di Figlia et al.[11]). Since differences in dendritic and axonal morphology are related to differences in input and output respectively, it would greatly help our understanding of the neostriatum if the interconnections of these morphologically identified neurons and their relationships to striatal afferents were known.

However, there are many unresolved problems which have so far hindered progress in this field. Opinions are divided, for example, even about the exact number of distinct cell types, which is not entirely due to species' differences (see, for example, Fox et al.[14,15] and Di Figlia et al.[11] for the monkey, Kölliker[28] and Kemp and Powell[21] for the cat). There is even less agreement about which neuron or neurons project from the neostriatum. For example, while all studies agree that the medium-sized neuron with densely spiny dendrites is a major component of the neostriatum, it was considered to be a local interneuron in the monkey and cat[14,21] whereas in other studies in the same two species this neuron type was shown to have an axon of considerable length[11,26]. The latter authors suggested that this type of cell might be an efferent neuron, but they could not demonstrate whether or not the axon leaves the striatum[26]. This discrepancy is partly explained by the nature of Golgi preparations, for it is rarely possible to follow axons over large distances because they either leave the section or, if myelinated, are not impregnated at all. Nevertheless, in a Golgi study[30] the axon of a striatal spiny neuron was traced as far as the globus pallidus in the mouse, suggesting an efferent role for this neuron. It is noteworthy that this type of neuron has also been demonstrated by using the intracellular horseradish peroxidase (HRP) filling technique and the morphological characteristics described in Golgi studies, such as size of the cell, densely spiny secondary and tertiary dendrites, extensive local axon collaterals and a long main axon, could all be confirmed by this independent procedure[26,27].

Striatal efferent cells have been labeled by retrograde transport of HRP[7,17], and *Herpex simplex* virus[4] from the substantia nigra. These studies show that the cells labeled are predominantly of medium size (13–20 μm) but, since the HRP reaction end-product is mainly localized to the soma and proximal dendrites, this method alone does not make it possible to establish which of the several morphologically distinct neurons of similar size project from the striatum to the substantia nigra.

Recently, we have succeeded in combining the Golgi staining procedure, which provides fine detail of the dendritic and local axonal morphology of single neurons, with the axonal transport of HRP, which helps to identify the target areas of projecting neurons when used as a retrograde marker. Several combinations of the two procedures can provide new information on the connectivity of neuron networks, especially when used in combination with anterograde degeneration at the electron microscope level[37]. One of these combinations is the retrograde HRP labeling and subsequent Golgi staining of the same neuron so that both the projection area and the detailed dendritic morphology are revealed at the same time. The present study describes our initial efforts to apply this combined approach to the striatum.

METHODS

Nine male Wistar albino rats (160 g) were anesthetized with chloral hydrate and

received unilateral injections of HRP (Sigma type VI, 20% in distilled water) into the substantia nigra at stereotaxic co-ordinates A, 2.0 mm; L, 2.1 mm; V, 7.3 mm after König and Klippel[29]. Two further animals were injected at the same anteroposterior level but with an oblique approach at an angle of 45°. HRP (30–80 nl) was delivered during 15–30 min through glass micropipettes (50–100 μm tip diameter) using a controlled gas pressure system. Twenty to twenty-nine hours later the animals were anesthetized with chloral hydrate and perfused through the heart with a fixative containing 2.5% glutaraldehyde, 0.5% paraformaldehyde and 0.1 M, pH 7.4, sodium phosphate buffer. Following perfusion, the brain was sectioned in the stereotaxic apparatus and sections were immersed in the fixative for 2–5 h and washed subsequently in buffer. Thereafter specimens were processed for HRP histochemistry (tolidine as substrate) followed by Golgi staining consisting of a consecutive potassium dichromate and silver nitrate treatment basically as described earlier[36]. Some of the specimens were 'gold-toned' (ref. 12) using a slightly modified procedure to that described originally. The Golgi sections were immersed in 0.07% $NaAuCl_4 \cdot 2H_2O$ for 30–60 min at 0 °C; this was followed by washing, treatment with oxalic acid (0.1%) and removal of the original Golgi deposit by sodium thiosulphate[12]. As a result, the original Golgi deposit is replaced by a fine, granular, less dense precipitate which makes it possible to study intracellular structures; it has been suggested that this secondary precipitate is metallic gold[12]. The sections were mounted in Durcupan for light microscopy and areas of interest were subsequently re-embedded for electron microscopy[36]. The specimens were stained en bloc with uranyl acetate and thin sections with lead citrate. Electron microscopic section series were mounted on single slot, Formvar coated grids. The whole procedure takes 4–5 days and the detailed method will be described separately[37].

Terminology. We shall refer to the colored or electron-dense material produced by the action of HRP as 'HRP reaction end-product'. The term 'Golgi deposit (or stain)' will refer to the substance(s) formed during the original Golgi impregnation and the substance(s) formed as a result of 'gold-toning' will be called 'secondary gold precipitate'.

RESULTS

Labeling of striatal neurons following injection of horseradish peroxidase into the substantia nigra

After carrying out the procedure for HRP histochemistry and Golgi staining, retrogradely labeled neurons could be identified in sections from various parts of the striatum, depending upon the part of the substantia nigra where HRP was injected (Figs. 2 and 3). The HRP labeled cells could be distinguished from Golgi-impregnated cells on the basis of the color and distribution of the reaction end-product. Lightly labeled cells contain numerous dark brown granules containing HRP reaction end-product but the cytoplasm of the neuron is also filled to a varying degree with a reddish-brown reaction end-product (Figs. 2 and 3).

When the neuron is more heavily labeled with HRP, or the sections are incubated

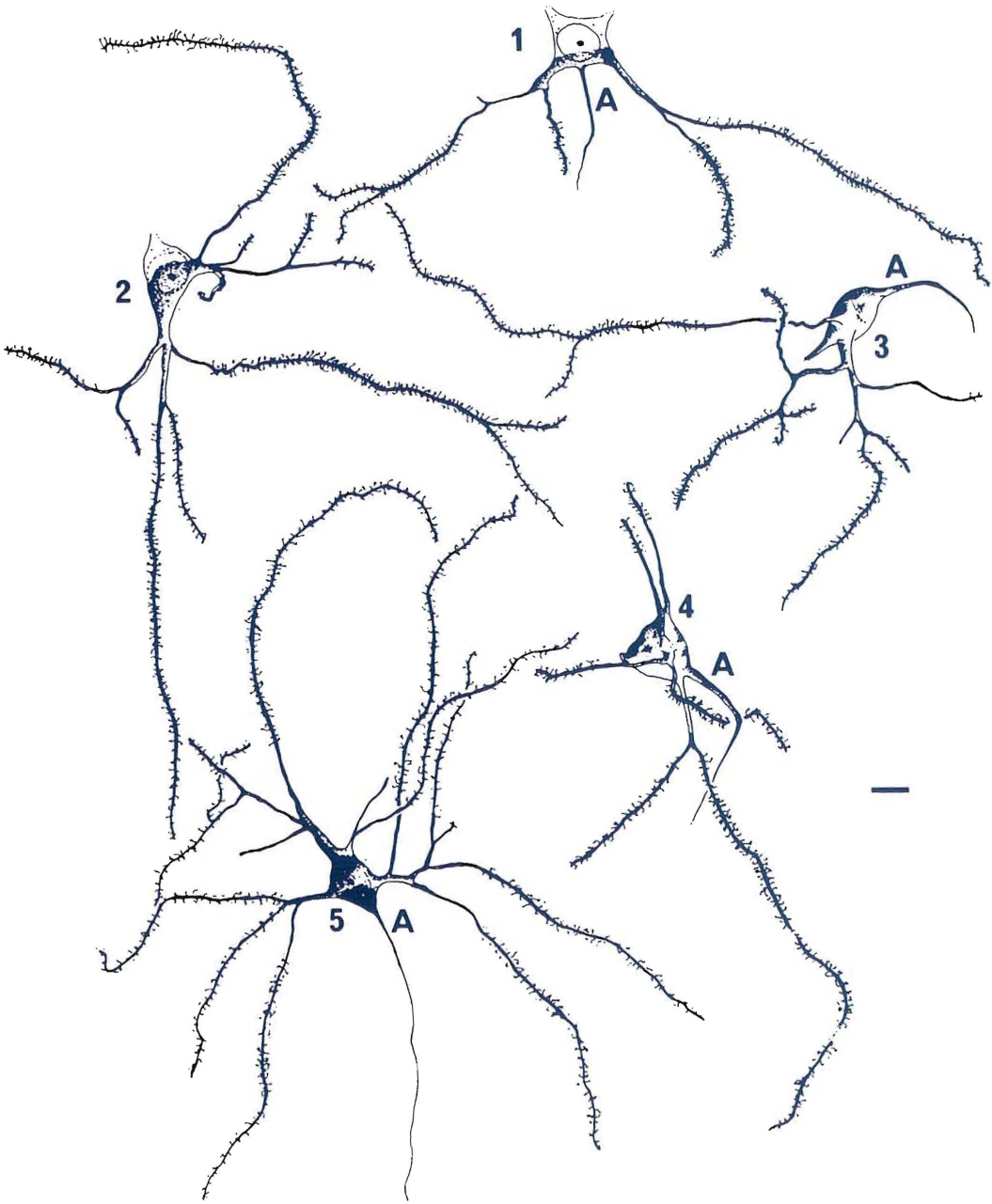


Fig. 1. Drawings of Golgi-stained striatonigral neurons identified by retrograde labeling after injection of horseradish peroxidase into the substantia nigra. Neurons nos. 1 and 2 are partially impregnated. Nos. 1 and 5 are shown on photographs (Figs. 5a, 4a respectively). A, axon, Scale = 10 μm.

longer, the reaction end-product fills the soma and proximal dendrites homogenously, exactly delineating the neurons. However, it is not possible to tell to which neuronal type the HRP labeled cells correspond since most parts of the dendrites and the local axons are not filled by the HRP reaction end-product.

Retrograde transport of horseradish peroxidase in Golgi-impregnated neurons

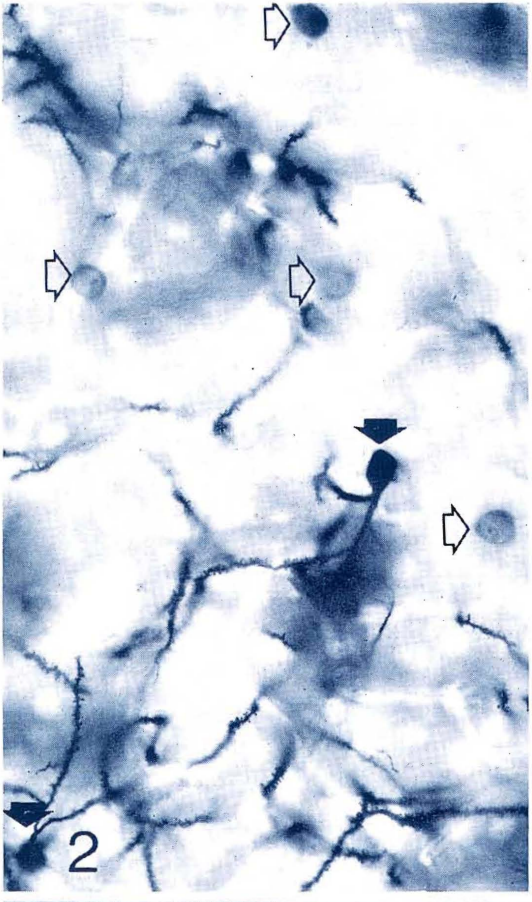
In general, the neurons impregnated by the Golgi stain in sections of rat striatum were very similar to the types of neuron described in other species (see Introduction). In particular, we noted the common occurrence of medium-sized neurons whose dendrites were densely covered with spines (see also ref. 8). A different type of medium-sized neuron, with long smooth, or very sparsely spinous dendrites, was impregnated most frequently in the rostral striatum, especially in the supracommissural region. Since the aim or this study was to identify Golgi-impregnated neurons that contained retrogradely transported HRP, we will not further describe our findings on the different types of Golgi-impregnated neurons.

Identification of the HRP reaction product in Golgi-stained neurons.

Following 'gold-toning' of the Golgi-stained sections, the original reddish-black Golgi deposit is replaced by a lighter gray to black precipitate, the amount of which can be regulated during 'toning'. HRP labeled neurons are more conspicuous in this preparation (Fig. 3). When a moderate amount of secondary 'gold' precipitate is deposited in the somas of stained neurons they are sufficiently transparent to allow a search for HRP reaction end-product. At the same time the finest detail of dendrites can be seen so that spines and other characteristics can easily be recognized under the light microscope (Fig. 5a). In many cases the primary Golgi stain fills only part of the soma and the dendrites originating from this part of the neuron, and this pattern is exactly followed by the secondary 'gold' precipitate (Fig. 5a, b). Since parts of the soma may be completely free from Golgi deposit, it is easily possible to recognize HRP reaction product should the neuron be labeled; the remaining part of the neuron, with Golgi-stained dendrites, can be used for characterization of the cell type.

We have so far identified 8 Golgi-stained neurons that were also labeled by retrograde transport of HRP from the substantia nigra; four of these were completely, and four partially, impregnated by the Golgi stain. Each of these neurons could be identified, on the basis of its densely spiny dendrites (Fig. 1), as the medium-sized spiny neuron described in Golgi studies on other species (see above).

Several methods were used to see whether a Golgi-stained cell was also labeled by HRP. In the simplest case, when the neuron is partially Golgi-impregnated (see neurons 1 and 2 in Fig. 1), the HRP reaction end-product can easily be recognized in the parts of the soma free of Golgi deposit in the thick Golgi section (Fig. 5a). When the HRP reaction end-product in a Golgi-impregnated 'gold-toned' cell is not readily visible in the Golgi section, 1 μm plastic sections are taken in order to reveal the HRP reaction end-product granules, which show up as dark brown particles within the cytoplasm (Figs. 4b, 5c). Finally, it is possible to recognize these reaction end-product granules under the electron microscope even in Golgi-impregnated cells which were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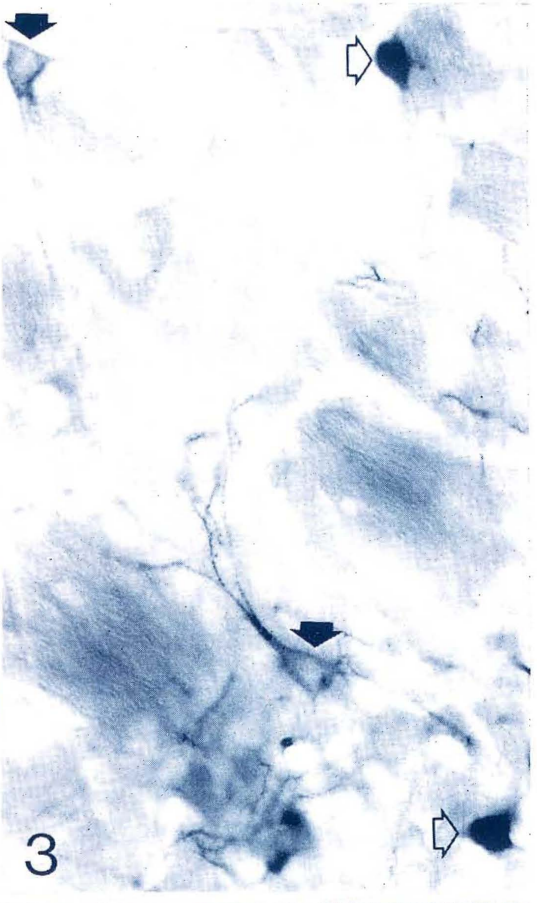
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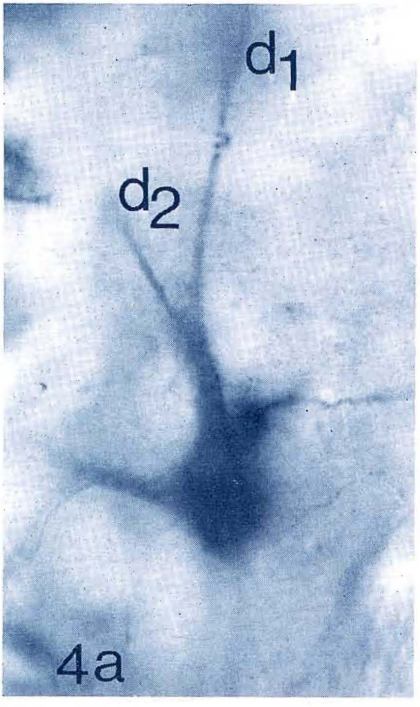
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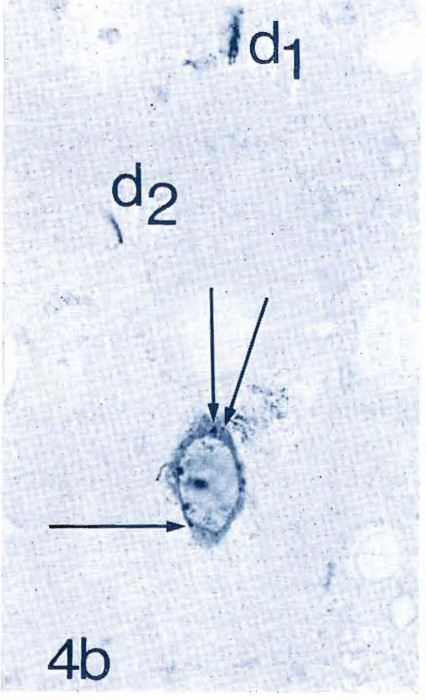
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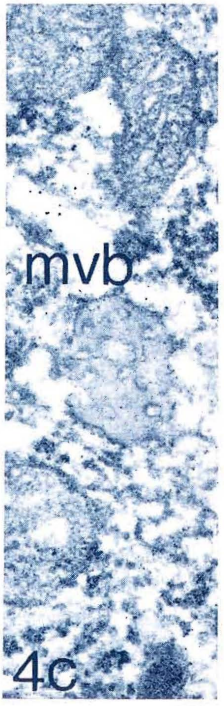
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not 'gold-toned'. Although the HRP reaction end-product in our procedure is not osmiophilic and so does not have the high electron density of the product formed from diaminobenzidine, it appears as homogenous low electron density material in membrane-limited granules and multivesicular bodies (Figs. 4c and 9). Neither the original Golgi deposit nor the secondary 'gold' precipitate penetrates into these membrane-limited bodies.

Identification of synapses on the striatonigral medium-sized spiny cells

When Golgi-impregnated or 'gold-toned' neurons are re-embedded for electron microscopy it becomes possible to characterize the boutons synapsing on any part of the cell[5,12,36]. By continuous cross-correlation, the light microscopically identified structures can be followed and the number and types of boutons received can be assessed. Three Golgi-stained, and subsequently 'gold-toned', striatonigral neurons were studied in this way using serial sections and all the main parts of the neuron were sampled for boutons received; the total number of each type of bouton observed is given below. However, we will illustrate (Figs. 5–10) the cross-correlations on only one of these neurons.

Axo-somatic synapses. Eighteen boutons synapsing on the cell bodies of striatonigral neurons were analyzed: they all made symmetrical membrane contacts and contained either flattened or pleomorphic vesicles (Figs. 9, 10). The boutons containing flattened vesicles could be distinguished from those containing pleomorphic vesicles and so possibly have a different origin. Boutons with pleomorphic vesicles were encountered on the axon initial segments of identified efferent neurons (Fig. 8).

Axo-dendritic synapses. The primary dendritic shaft of striatonigral spiny neurons is free of spines, as are the most proximal parts of secondary dendrites (Figs. 1, 5a). These parts of the neuron also receive symmetrical synapses (9 were identified) but they are not numerous. Soon after the first branching the dendrites become densely covered with spines of varying length and shape. The spines of neurons projecting to the substantia nigra receive boutons which make asymmetrical contacts (25 were observed) and contain spheroidal vesicles (Figs. 5d, 6). However, as has already been shown for axon-spine synapses in general in the striatum[1,3,22,34], these boutons are heterogenous; thus, the size and packing density of the vesicles varies greatly. Boutons are not restricted to the spines on this part of the dendrite, for the shaft also receives synapses, but these (6 were observed) are of the symmetrical type (Fig. 7).

Fig. 2. Light micrograph of part of Golgi-stained striatum which was also processed for HRP histochemistry. Peroxidase labeled cells (open arrows). Golgi-stained cells (solid arrows). × 360.

Fig. 3. Similar preparation as in Fig. 2 but after 'gold-toning'. Note heavily HRP labeled (open arrows) and lightly 'gold-toned' Golgi-stained (solid arrows) neurons. × 360.

Fig. 4. Neuron heavily labeled with horseradish peroxidase (a) in 100 μm Golgi section and (b) in 1 μm plastic section not counterstained. Lightly 'gold-toned' dendrites (d_1, d_2) emerge from the soma. Long arrows point to HRP-containing granules; one of them, a multivesicular body (mvb), is shown in (c) on an electron micrograph to contain reaction end-product. a,b, × 1100; c, × 50,000.

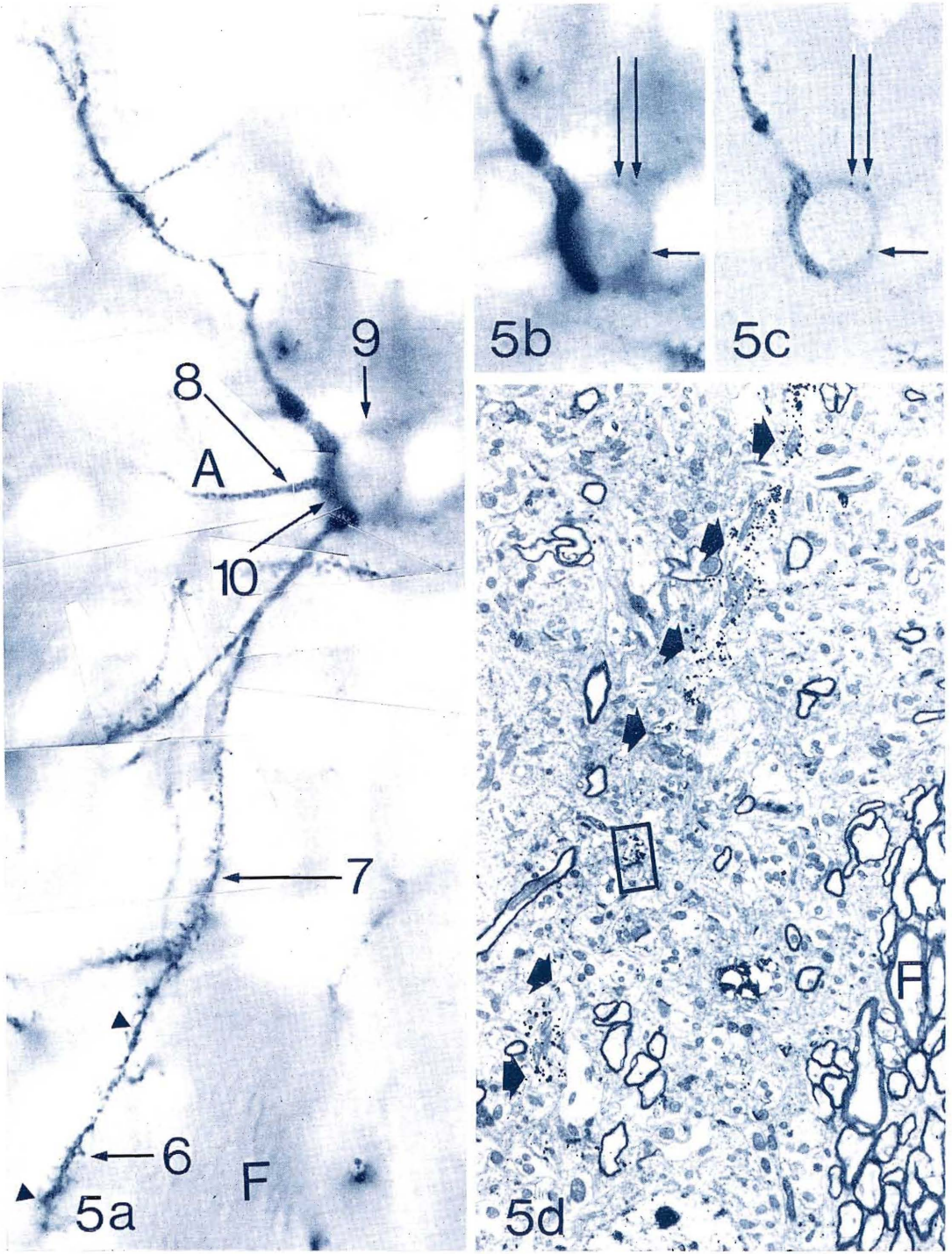


Fig. 5. a: photomontage of a strongly 'gold-toned' striatonigral neuron labeled with horseradish peroxidase. Note densely spiny dendrites and partially impregnated soma. The soma is shown (b) in Golgi section after 'gold-toning', and (c) in 1 μm plastic section. Long arrows in b and c point to HRP reaction end-product granules. In a, the numbers indicate the parts of neuron where electron micrographs (Figs. 6–10) were taken. Part of the dendrite between the triangles in a is shown on a low power electron micrograph in d. The area indicated by arrow no. 6 in a and enclosed by a rectangle in d is shown in high magnification in Fig. 6. A, axon; F, fibre bundle. Magnifications: a, $\times$ 1070; b and c, $\times$ 1250; d, $\times$ 6000.

DISCUSSION

In so far as we have analyzed the distribution of retrogradely labeled neurons in the striatum following injection of HRP in the substantia nigra, our results agree with earlier studies on the cat[17] and rat[7]. Likewise, our Golgi studies on the rat reveal a variety of neuron types, just as in earlier and more extensive studies on other species[11,14,15,21,28,30,35]. These studies all described the medium-sized spiny neuron and our results now show that this type of neuron projects to the substantia nigra. This conclusion can be drawn with certainty because the Golgi stain impregnated the whole dendritic tree of some of the neurons that were retrogradely labeled by HRP.

Afferents to the striatum

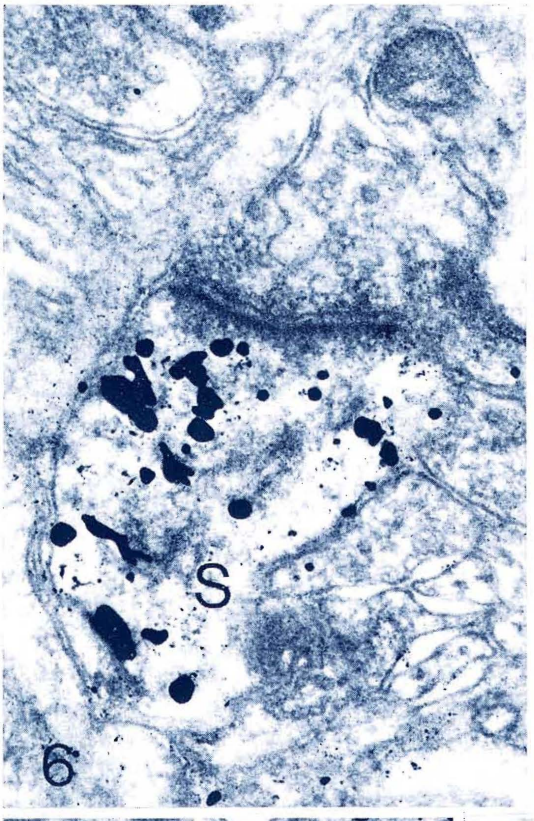
By examining some of the identified striatonigral neurons in the electron microscope, we were able to describe some of the types of boutons that form synaptic contacts on them.

A great variety of boutons has been described in previous studies on the striatum and using anterograde degeneration[9,23], monoamine cytochemistry[1,18] and autoradiography[2,32] the origin of some of these terminals could be identified. However, such studies were limited because there was no means of identifying the origin of the elements that were postsynaptic to the boutons, i.e., whether they belonged to interneurons and/or to efferent neurons. Consequently, it has not been possible to assess the role of such boutons in the circuitry of the striatum. The present work provides a morphological approach to this problem. A combination of Golgi impregnation with the identification of boutons undergoing anterograde degeneration has already been successfully applied in studies on the cerebral cortex[36,38] and it will now be possible to combine this procedure with the labeling of projecting neurons by the retrograde transport of HRP. The application of such a three-pronged approach will allow us to identify the input of striatonigral and other striatal efferent neurons.

An alternative approach to the study of the input of morphologically identified striatal neurons has been described by Kocsis et al.[26,27] who used intracellular recordings from neurons in the cat caudate nucleus and then marked the cells with intracellularly applied HRP. They suggested that striatal spiny neurons receive monosynaptic input from the cerebral cortex, the intralaminar thalamus and substantia nigra. This is in good agreement with the results of Kemp and Powell[24], who showed, using long survival times, that following lesions of the cerebral cortex and/or the thalamus the number of impregnated spines significantly decreased on the medium-sized spiny cells in the cat's caudate nucleus. This was interpreted as a result of loss of monosynaptic input from the structures. In both studies the spiny neurons were the same type as in the present study.

Possible functional implications

In addition to the possibilities raised above concerning the afferents to the spiny neurons, there are certain other implications of our finding that these striatal neurons project to the substantia nigra.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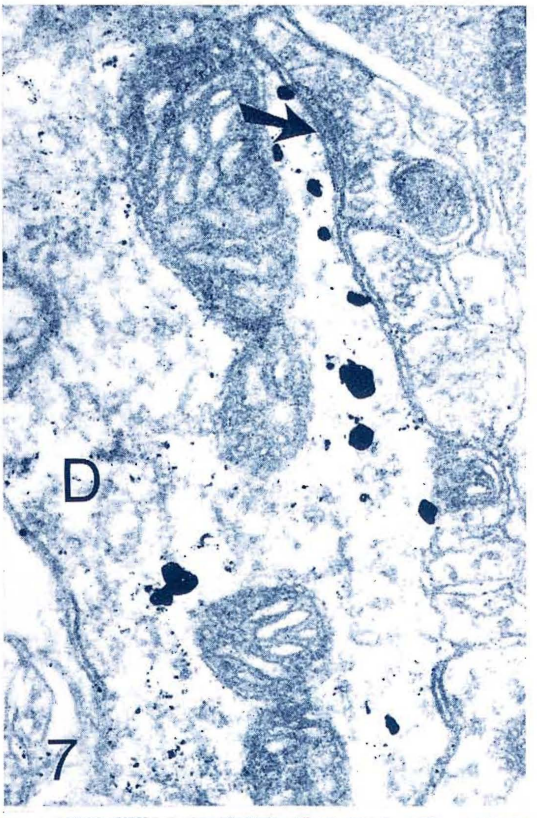
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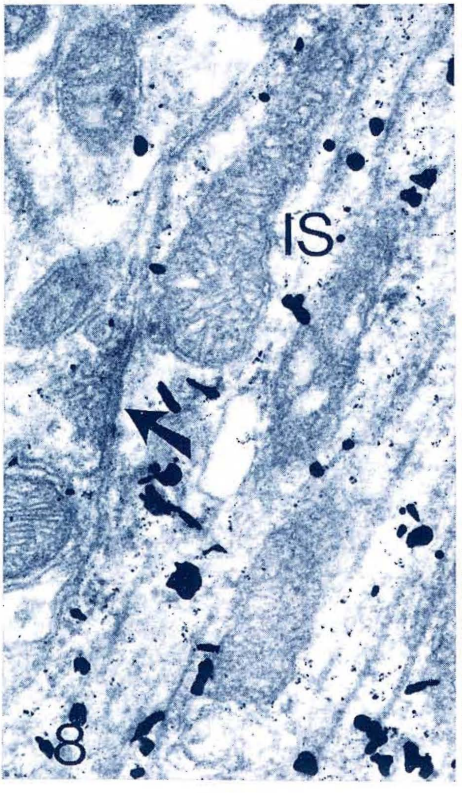
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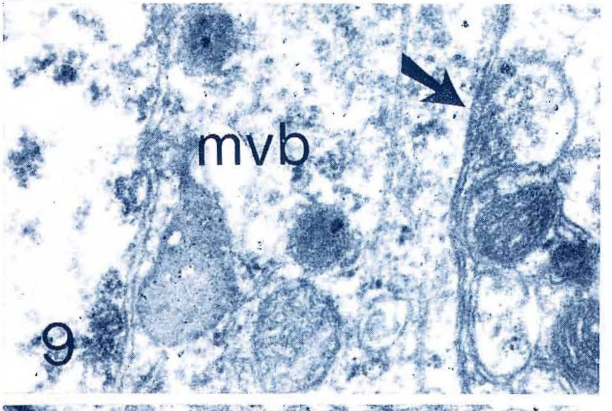
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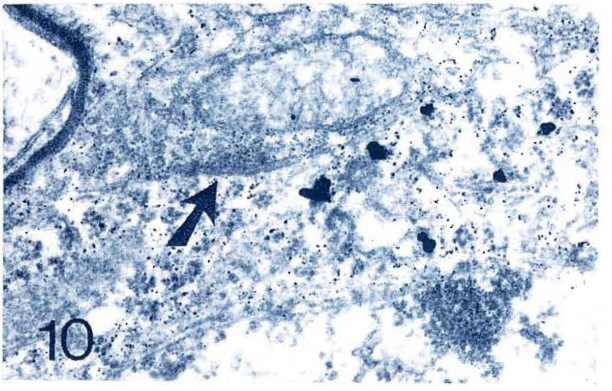
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Striatal spiny neurons have been shown to have an extensive local axon collateral system in the cat and monkey[11,14,26] and we found this in the rat too (unpublished observations). In many cases, however, the axon is not stained beyond the initial segment, presumably because it becomes myelinated; therefore it is not yet clear whether the spiny neuron population is homogenous with regard to axon morphology. However, when intracellular HRP filling was used to reveal the morphology of these neurons, a method which stains myelinated axons beyond their initial segment, axon collaterals were readily visualized[26,27]. If striatonigral spiny neurons also have local axon collaterals, this has important implications for the circuitry of the striatum.

The substantia nigra receives both a GABA-containing[6,13,16,25] and a substance P-containing[6,16,19,20] projection from the striatum in the rat. Thus either of these transmitters may be used in the striatum at the synapses of local axon collaterals. However, substance P-containing neurons have been shown to be situated mainly in the rostral part of the rat's caudoputamen[6,10,19,31] while spiny neurons in Golgi preparations can be found over the whole rostrocaudal extent of the striatum which suggests that they are unlikely to be responsible for substance P-like immunoreactivity in the substantia nigra. On the other hand, it has been suggested that GABA may be a transmitter in the striatum[33], and the substantia nigra receives a GABA-containing projection from the whole rostrocaudal extent of the caudoputamen[19]. It is tempting to speculate, therefore, that the striatal spiny neurons represent the GABA-containing projection to the substantia nigra and that local axon collaterals of these neurons might be involved in GABAergic interactions in the striatum.

All 8 neurons which were, in our study, both Golgi-stained and labeled retrogradely by HRP injected in the substantia nigra belong to the densely spiny type, but this does not exclude that other types of neurons may also project to substantia nigra. It should be possible, using the present method, to establish which other types of striatal neurons project to substantia nigra and, even more important, to identify the input of striatonigral neurons using anterograde degeneration or autoradiography.

Figs. 6–10. Electron micrographs illustrating synapses on different parts of one of the identified striatonigral spiny neurons (drawing no. 1 in Fig. 1; illustrated in Fig. 5). Note the granular secondary 'gold' precipitate formed as a result of 'gold-toning' of the Golgi section.

Fig. 6. Bouton containing spheroidal vesicles forms an asymmetric contact on a spine (S) of the dendrite shown at arrow 6 in Fig. 5a and d. × 67,000.

Fig. 7. Symmetrical synapse on a distal dendritic shaft (D), of the neuron shown in Fig. 5a (arrow 7). × 67,000.

Fig. 8. Bouton containing pleomorphic and large dense-cored vesicles forms a symmetrical synapse on the axon initial segment (IS), shown by arrow 8, of the neuron in Fig. 5a. × 52,500.

Fig. 9. Symmetrical axosomatic synapse formed by a bouton containing pleomorphic vesicles on an unimpregnated part of the soma, indicated by arrow 9 in Fig. 5a. mvb, multivesicular body containing HRP reaction end-product. × 52,500.

Fig. 10. Symmetrical axo-somatic synapse formed by a bouton containing flattened synaptic vesicles on the impregnated part of the soma indicated by arrow 10 in Fig. 5a. × 44,500.

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