Neuroscience Vol. 20, No. 2, pp. 577–587, 1987
Printed in Great Britain

0306-4522/87 $3.00 + 0.00
Pergamon Journals Ltd

CHARACTERIZATION OF SUBSTANCE P- AND [MET]ENKEPHALIN-IMMUNOREACTIVE NEURONS IN THE CAUDATE NUCLEUS OF CAT AND FERRET BY A SINGLE SECTION GOLGI PROCEDURE

P. N. IZZO, A. M. GRAYBIEL* and J. P. BOLAM
MRC Anatomical Neuropharmacology Unit, Department of Pharmacology, South Parks Road, Oxford OX1 3QT, U.K. and *Whitaker College and Department of Psychology and Brain Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A.

Abstract—Modifications of the single-section Golgi-impregnation procedure of Gabbott and Somogyi[23] are described. The modifications allow easier and more rapid preparation of the sections for Golgi-impregnation and easier handling of large numbers of serial sections. The technique consists of placing a section that has been treated with osmium tetroxide and potassium dichromate on a microscope slide and "sandwiching" it with a second microscope slide. The two slides are held together at one end by tape and the assembly is dipped into a solution of silver nitrate. Golgi-impregnation of neurons occurs within a few hours and is generally complete within 12 h.

The technique has been applied to sections through the caudate nucleus of the cat and ferret in order to define the morphological characteristics of striatal substance P- and methionine enkephalin-immunoreactive neurons. Sections were first incubated to reveal the immunoreactive structures and then subjected to the Golgi method. Golgi-impregnated neurons that were immunoreactive for either substance P or methionine enkephalin had medium-size perikarya from which several dendrites emerged. The dendrites branched close to the perikaryon; secondary and higher order dendrites were densely laden with spines, as many as 15 spines per 10 μm of dendrite. It is concluded that both striatal substance P-containing and methionine enkephalin-containing neurons are of the medium-size densely spiny type. Medium-size densely spiny neurons may be homogeneous with respect to their somatodendritic morphology but heterogeneous with respect to their chemical characteristics and axonal morphology.

The application of Golgi-impregnation to electron microscopy, particularly in combination with anterograde and retrograde tract tracing methods, has led to advances in the elucidation of the position of morphologically characterized neurons within the neural circuits of the central nervous system.[46] To gain a better understanding of the role of a particular neuron in the circuits, it is important to know the chemical characteristics of the neuron and its afferent synapses. A major innovation in this field was the combination of immunocytochemistry or histochemistry for a neurotransmitter or related substance with Golgi-impregnation which allowed chemical characteristics of a Golgi-impregnated neuron as well as its afferent synapses to be established.[22,45] This was not possible with classical Golgi techniques because immunoreagents have only limited penetration into tissue in the order of 50 μm maximum whereas the Golgi procedures require thick slices of tissue that are at least 1 mm thick. Freund and Somogyi[22] overcame this problem by first carrying out immunocytochemistry or histochemistry on sections of 80–100 μm thickness and then stacking them together to form a "block" of about 1 mm thick which was embedded in agar and treated as a standard Golgi block. The procedure has been successfully applied to sections incubated to reveal intracellularly injected or retrogradely transported horseradish peroxidase,[22] sections stained by immunocytochemical methods[4,6,21,45] and sections stained histochemically to reveal acetylcholinesterase (AChE).[3]

The original procedure of Freund and Somogyi[22] was modified by Gabbott and Somogyi[23] to allow Golgi-impregnation of a single section of about 80–100 μm without having to stack them together into a block. Golgi-impregnation is effected by "sandwiching" single sections between microscope cover slips and then exposing the "assembly" to the reagents for Golgi-impregnation. This modification has all the advantages of the method of Freund and Somogyi[23] but in addition is easier to perform; it minimizes the handling of the sections so that much less damage occurs and it allows the degree of cell staining to be assessed in the light microscope during the impregnation.

In carrying out a study of the relationship of the histochemical compartments of the striatum to the types and form of Golgi-impregnated neurons, we made modifications to the single-section Golgi technique of Gabbott and Somogyi[23] in order to make the

Address correspondence to: Dr J. P. Bolam, MRC Anatomical Neuropharmacology Unit, Department of Pharmacology, South Parks Road, Oxford OX1 3QT, U.K.

Abbreviations: AChE, acetylcholinesterase; GABA, gamma-aminobutyric acid; Met, methionine; PAP peroxidase–antiperoxidase; PBS, phosphate-buffered saline.

technique more reliable and more easily and rapidly performed. We have combined the single-section procedure with immunocytochemistry in sections of cat and ferret forebrain and describe in this paper the modifications of the procedure and the morphological characteristics of Golgi-impregnated substance P- and [Met]enkephalin-immunoreactive neurons in the caudate nucleus.

EXPERIMENTAL PROCEDURES

Tissue from 3 cats (4–16 months) and 2 ferrets (about 6 months) was used in this study. The animals were deeply anaesthetized and perfused through the heart with 200–500 ml 0.9% NaCl at room temperature followed by approximately 2 l of fixative (10–15°C) over a period of 5–10 min. The fixative consisted of either 4% paraformaldehyde and 0.05% glutaraldehyde, or 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M Na phosphate buffer (pH 7.4) containing 0.9% NaCl. After perfusion, the brains were removed from the skull, cut into blocks which included the caudate nucleus and putamen and stored in 0.1 M phosphate buffer (pH 7.4) at 4°C for about 48 h. They were then washed in buffer and sectioned at 80 μm on a Vibratome. The sections were then washed in phosphate-buffered saline (0.01 M phosphate, PBS) at pH 7.4 and processed for immunocytochemistry with antisera directed against substance P and [Met]enkephalin. One of the cats received bilateral injections of colchicine (500 μg in 10 μl saline) into the lateral ventricles 24 h before perfusion. In all the animals every third section was cut at 50 μm and stained for AChE by a modified version of that described by Geneser-Jensen and Blackstad.[25]

Immunocytochemical procedure

Immunocytochemistry was carried out by the peroxidase–antiperoxidase (PAP) technique.[50] For substance P, sections were washed in 20% (1 h) then 1% (0.5 h) normal rabbit serum in PBS. They were then incubated as follows: 70 h in monoclonal antibodies against substance P[14] (1:1000 dilution in 1% normal rabbit serum) at 4°C; 3 h in rabbit anti-rat IgG (Miles) (1:50 dilution in 1% normal rabbit serum in PBS) at room temperature and 3 h in rat PAP (Sternberger, Meyer Inc.) (1:100 dilution in PBS) at room temperature. Each incubation was followed by three washes in PBS over a period of 30 min. The same protocol was used for the rabbit anti-[Met]enkephalin antiserum (1:600 dilution, antiserum number R170 donated by R. P. Elde[37]) except that the normal serum was from goat, the secondary antibody was goat anti-rabbit IgG (1:50 dilutions; Miles) and the PAP was prepared in rabbit (1:100 dilution; Miles). Some sections were incubated by a protocol which includes methanol and hydrogen peroxide pretreatments and the use of a higher molarity buffer; details of this protocol are given elsewhere.[27] The antigens were then localized by incubation (5–15 min) in 3,3′-diaminobenzidine (50 mg/100 ml in 0.05 M Tris HCl buffer pH 7.6, containing 0.01% H_2O_2). The sections were then washed in Tris buffer, and postfixed (30 min) in 1% osmium tetroxide in 0.1 M phosphate buffer. Care was taken at this stage to ensure that the sections were flat.

Golgi-impregnation procedure for single sections

After osmium postfixation the sections were washed in buffer and free-floated in a solution of $K_2Cr_2O_7$ (3.5%, aqueous) for 1–3 h. Each section was then laid flat on the centre of a microscope slide using a fine artist's brush. Excess $K_2Cr_2O_7$ was removed from the slide with filter paper and another microscope slide was placed on top of the section so as to "sandwich" it between the two slides. Care was taken to avoid crushing the section or shearing it by allowing the slides to move with respect to one another. The two slides were held in position by gently taping them together at one end with electrical insulation tape. The slide assemblies were then placed in an upright position (taped side up) in 1–2% $AgNO_3$ solution in a small beaker.

The essential differences between the method that we used and that described by Gabbot and Somogyi[23] are as follows: (1) microscope slides are used instead of cover slips to "sandwich" the sections; (2) the slides are not glued together but adhere to each other by surface tension and are lightly held together at one end with tape; (3) one end (non-taped end) of the slide assembly is immersed in the silver nitrate solution in an upright position, rather than having the assembly totally immersed.

The duration of $AgNO_3$ treatment was determined by monitoring the course of impregnation with a light microscope. The time varied from 6 to 24 h. When satisfactory impregnation occurred the process was terminated and the sections gold-toned. If the impregnation was not satisfactory the sections were washed well in distilled H_2O and returned to the $K_2Cr_2O_7$ step.

Section removal

Sections were retrieved from the slide assemblies by removing the tape, gently separating the slides with the aid of a razor blade and removing the section with a fine artist's brush or razor blade. They were then either mounted in dry glycerol between coverslips for subsequent gold-toning and processing for light and electron microscopy or washed in distilled H_2O for re-impregnation. Gold-toning was carried out by a modified version of the method of Fairén *et al.*[19] (for details see Ref.[22]). The sections were dehydrated and embedded on microscope slides in epoxy resin (Durcupan ACM, Fluka).

Factors influencing impregnation

The most important factor governing the Golgi-impregnation of neurons was the manner of diffusion of the silver nitrate solution into the tissue. We believe that it is necessary for the silver nitrate to penetrate the sections from their edges, i.e. in the plane parallel to the plane of section. If the silver solution flows over the section, the surface becomes covered in crystals (presumably silver chromate); under these conditions, impregnation rarely occurs. When the silver solution does not flow over the section and diffuses slowly in from the edges, then good impregnation usually follows. If physical barriers to the diffusion of the silver solution are present, e.g. air bubbles, cerebral ventricles or large masses of myelinated fibres, then the impregnation is usually much poorer or does not occur at all. In studying the caudate nucleus we therefore (1) removed the septum to obviate the barrier of the lateral ventricle, (2) cut off the corpus callosum, (3) oriented the section so that the caudate would be the first area to be exposed to the silver solution, and (4) positioned the section so that when they were first dipped into the silver solution they were quickly surrounded by it without the formation of bubbles. This final precautionary step permits the diffusion of the silver from all edges of the sections. If air bubbles form around the section they can sometimes be removed by carefully inserting a razor blade between the slides at the untaped end and carefully forcing them apart until the fluid retreats past the air bubble; the slides are then slowly lowered together again. If $AgNO_3$ solution runs over the surface of the section, crystals form and the section should be removed, washed in distilled H_2O and returned to the $K_2Cr_2O_7$. Silver chromate crystals sometimes form in the ridges left by the Vibratome blade; to have the smoothest surface possible, sections should be cut with high amplitude of vibration and slow rate of blade advance. Finally, the quality of impregnation is dependent upon the state of the tissue; well-fixed and "robust" sections were invariably impregnated to a better degree than poorly fixed sections or those exposed to

penetration enhancement techniques (freeze–thawing or detergent treatment).

Light and electron microscopy

After curing the resin for 48 h at 60°C all sections were examined in the light microscope. The immunostaining and Golgi-impregnation were assessed and neurons that were both Golgi-impregnated and immunostained were identified. Selected neurons in the cat material were drawn with the aid of a microscope drawing tube (Leitz) and measurements of the size of perikarya and dendrites were made from these drawings using a Bioquant image analysis system. No account was taken for shrinkage of the tissue.

To confirm the presence of immunoreactivity in the perikarya of Golgi-impregnated neurons, some neurons were re-embedded in blocks of resin suitable for re-sectioning. From these, 1 μm sections for light microscopy and ultrathin (silver/grey) sections for electron microscopy were cut. The latter sections were collected on Formvar-coated single-slot grids counterstained with lead citrate and examined in a Philips EM201 electron microscope. To improve contrast for electron microscopy, the tissue was stained *en bloc* with 1% uranyl acetate (in 70% ethanol) during dehydration.

RESULTS

The degree and quality of Golgi-impregnation was similar to that seen with conventional Golgi techniques. The impregnated neurons appeared sometimes in groups but were often isolated (Figs 1B, and 3A). Perikarya, dendrites, dendritic spines and axons were all impregnated (Figs 1 and 3). The types of neurons that were impregnated were similar to those described on previous occasions in rat, cat and monkey (see Refs 3, 10, 28, 40 and 52). Although the majority of impregnated neurons were the medium-size densely spiny type (see Figs 1 and 3), other types including large neurons and medium-size aspiny neurons were also observed. No obvious differences were seen between the caudate nucleus and putamen, or between the cat and ferret.

Immunostaining

Substance P. At the light microscopic level substance P-immunoreactivity in the neostriatum was remarkably uneven. In the caudate and particularly in its rostral part, high concentrations of immunoreaction product were present in patches of irregular shape (see Fig. 1B). These regions represent the striosomal compartments of the caudate nucleus and correspond to AChE-poor regions.[27] Detail of the structure of substance P-positive/AChE-poor patches and their relationship to Golgi-impregnated neurons will be the subject of a separate communication.

Most of the immunoreaction product was associated with neuronal perikarya and sometimes proximal dendrites (Fig. 1B). The immunoreactive neurons were of medium-size and the majority were similar to the type 1 substance P-positive neuron described in the rat.[5] The neuronal staining occurred predominantly in the striosomes (as defined by the dense neuropil staining; see Fig 1B), but there were also many immunoreactive perikarya with no apparent association with the striosomes. Immunoreactive axon or fibre-like structures were also present; these were predominantly in the superficial layers of the tissue and appeared evenly distributed throughout the sections.

Immunoreactive neurons, in which indentations of the nucleus were visible in the light microscope, were rarely seen. These neurons probably correspond to the type 2 substance P-immunoreactive neurons observed in the rat.[5] The apparent rarity of these cells probably reflects their low density in the striatum and the difficulty in identifying indented nuclei in the light microscope.

Methionine enkephalin. [Met]enkephalin immunoreaction product was present within medium-size perikarya, their proximal dendrites and many apparently isolated dendrites (Fig. 3A, B). In addition, in some areas terminal staining occurred in the most superficial layers of the sections. Perikaryal staining was most prominent in the colchicine-treated cat. The distribution of the immunoreactive neurons was different for colchicine and non-colchicine animals as has been reported previously;[27] in the latter animals the immunoreactive-neurons tended to avoid the striosomes (i.e. AChE-poor zones) while in the colchicine-treated cat they were distributed more evenly throughout the caudate nucleus and putamen.

Specificity and controls. The specificity of the antibodies used in the present experiments has been characterized previously[14,37] (see also Refs 5, 27 and 47). In sections that were incubated by the immunocytochemical procedure but with omission of the primary antibodies, no specific immunostaining occurred. In addition, some sections from each animal were incubated with other antisera raised in rabbits (directed against glutamate decarboxylase, tyrosine hydroxylase, somatostatin) and monoclonal antibodies (directed against choline acetyltransferase). The patterns of staining obtained with these antibodies were markedly different from those obtained with the anti-substance P or anti-enkephalin antibodies. The pattern of staining described above is therefore specific to the substance P and [Met]enkephalin antibodies we used and not a result of other steps in the procedure.

Golgi-impregnation combined with immunostaining

A total of 33 Golgi-impregnated neurons that were also immunoreactive for substance P were identified (25 in cat, 8 in ferret). In the [Met]enkephalin-immunoreacted material 55 Golgi-impregnated immunoreactive neurons were identified (41 in cat and 14 in ferret). The immunoreaction end product appeared as a brown homogenous staining of the perikarya and was easily distinguishable from the grey/black secondary Golgi deposit. Immunoreaction product was identified in Golgi-impregnated neurons at the light microscopic level when (1) the gold-toning of the perikarya was pale (Fig. 1B), (2) Golgi-impregnated dendrites arose from non-impregnated

Fig. 1. (A) Drawing of a Golgi-impregnated, gold-toned neuron that is also immunoreactive for substance P. Micrographs of this neuron are shown in subsequent figures. The unshaded dendrite on the left was not Golgi-impregnated but expressed immunoreactivity; see the dendrite labelled d in (B) and (C). This neuron was found within a substance P-immunoreactive "patch", the boundaries of which are shown by the solid black lines above and below the neuron. The dendritic spines were drawn free-hand and are therefore an approximation of the true density. The neuron shows the characteristics of medium-size densely spiny neurons, i.e. medium-size perikaryon, secondary and higher order dendrites densely laden with spines. (B) Partial light microscopic photomontage of the same neuron (N) as in (A). Note the high density of spines on the second and higher order dendrites and the non-impregnated but immunoreactive dendrite (d) emerging from the left of the perikaryon. Note the darker appearance of the neuropil below and lower right of the perikaryon; this is due to the high density of substance P-immunoreactive structures forming the "patch". Three immunoreactive perikarya are indicated by stars. A non-impregnated, non-immunoreactive neuron (n) and a capillary (c) are labelled for correlation with electron micrograph (C). (C) Low power electron micrograph of the perikaryon of the Golgi-impregnated, immunostained neuron (N) and its immunostained proximal dendrite (d). Bars: (A) 50 μm; (B) 25 μm; (C), 10 μm.

Fig. 2. (A) Medium power electron micrograph of the same neuron (N) shown in Fig. 1. Note the round nucleus with smooth unindented nuclear envelope and the relatively small area of cytoplasm, features that are typical of medium-size densely spiny neurons. The cytoplasm is darker than the surrounding neuropil because of the Golgi-deposit and the immunoreaction product. The proximal dendrite (d) is only immunostained. The boxed areas and (B) and (C) are shown at higher power. (B and C) Higher power electron micrographs of the regions shown by boxes in (A). In both micrographs the electron dense granules of the secondary Golgi-deposit are indicated by arrowheads. The immunoreaction product (arrows) appears as an amorphous electron dense material, often associated with organelle membranes and often in clumps. An immunoreactive profile in the neuropil is also shown in (B). Bars: (A) 2 μm; (B and C) 0.5 μm.

Fig. 3. (A) Low power light micrograph of a section through the caudate nucleus of the cat, processed to demonstrate [Met]enkephalin-immunoreactivity and then Golgi-impregnated. The field contains many immunoreactive perikarya, some of which are indicated by open arrows, and several unstained neurons indicated by stars. The neurons labelled N_1 and N_2 are both immunoreactive and Golgi-impregnated. The perikaryon of N_1 was only partially impregnated by the Golgi deposit (small arrow in (B)) which enabled easy identification of the immunoreaction product. Two of the dendrites (arrowheads) of this neuron are in the plane of the micrograph and are densely laden with spines. The perikaryon of neuron N_2 was not impregnated but one of its dendrites is impregnated (small arrows) and is densely laden with spines. (B) High power light micrograph of Golgi-impregnated, [Met]enkephalin-immunoreactive neuron N_1 in (A). The arrowheads indicate part of the perikaryon that is partially impregnated. [Met]enkephalin-immunoreactive neurons are indicated by open arrows and non-immunoreactive neurons by stars. (C) Drawings of the two neurons in (A) and (B). The unshaded parts of the cells are those regions that were not Golgi-impregnated but displayed [Met]enkephalin-immunoreactivity. The dendrites indicated by arrows or arrow heads are the same as those that appear in light micrograph (A). Bars (A) 25 μm; (B) 12.5 μm; (C) 50 μm.

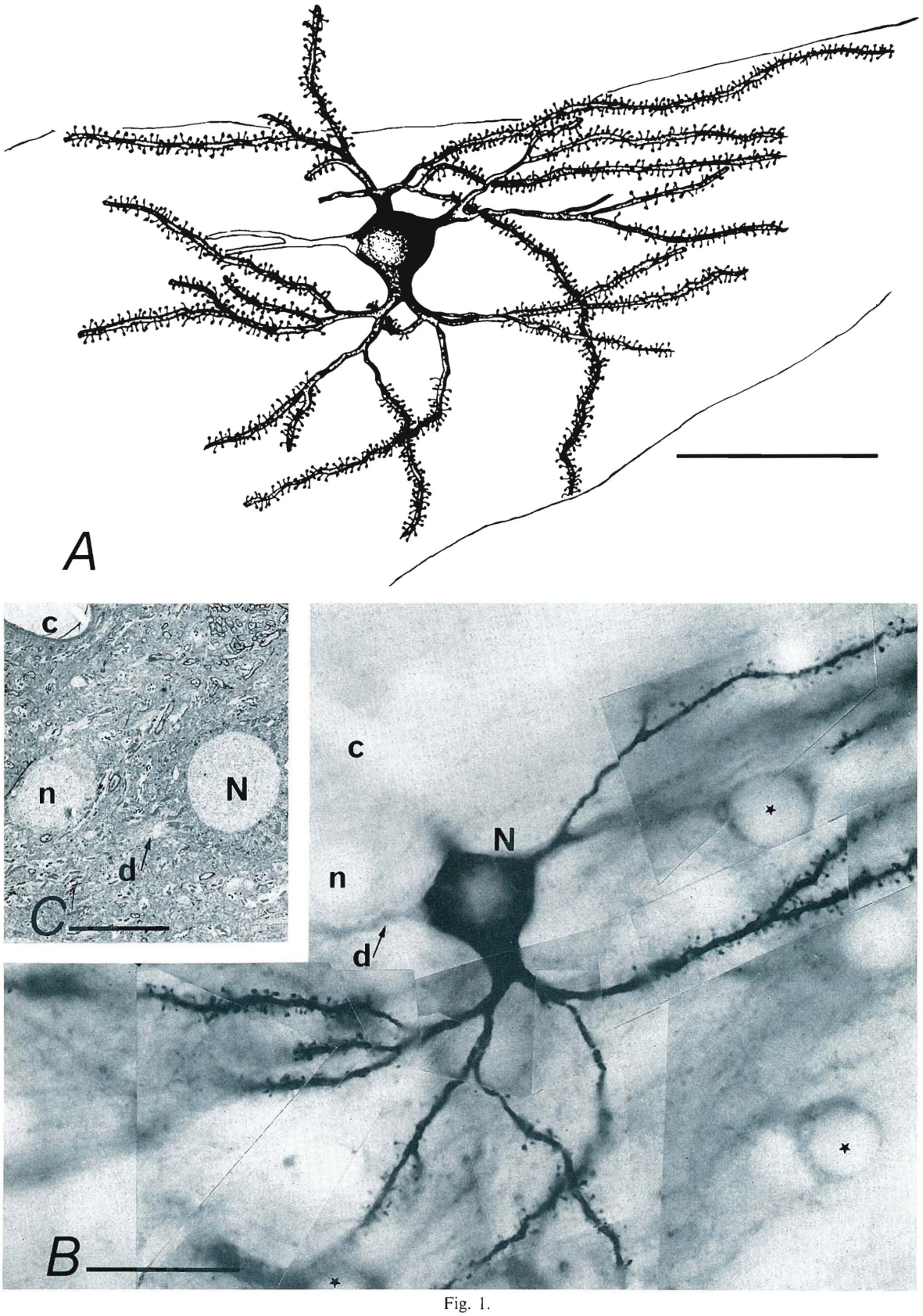

Fig. 1.

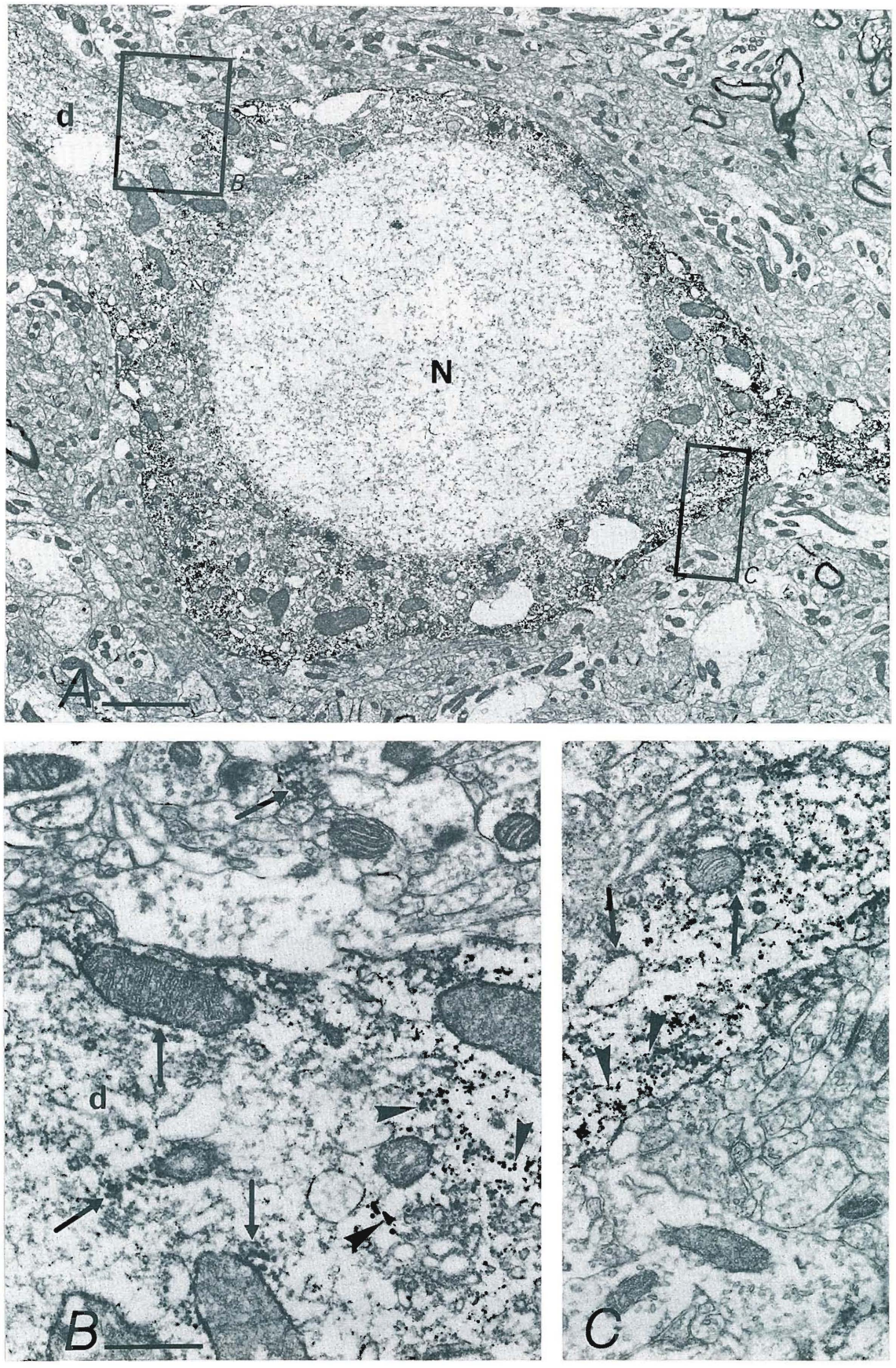

Fig. 2.

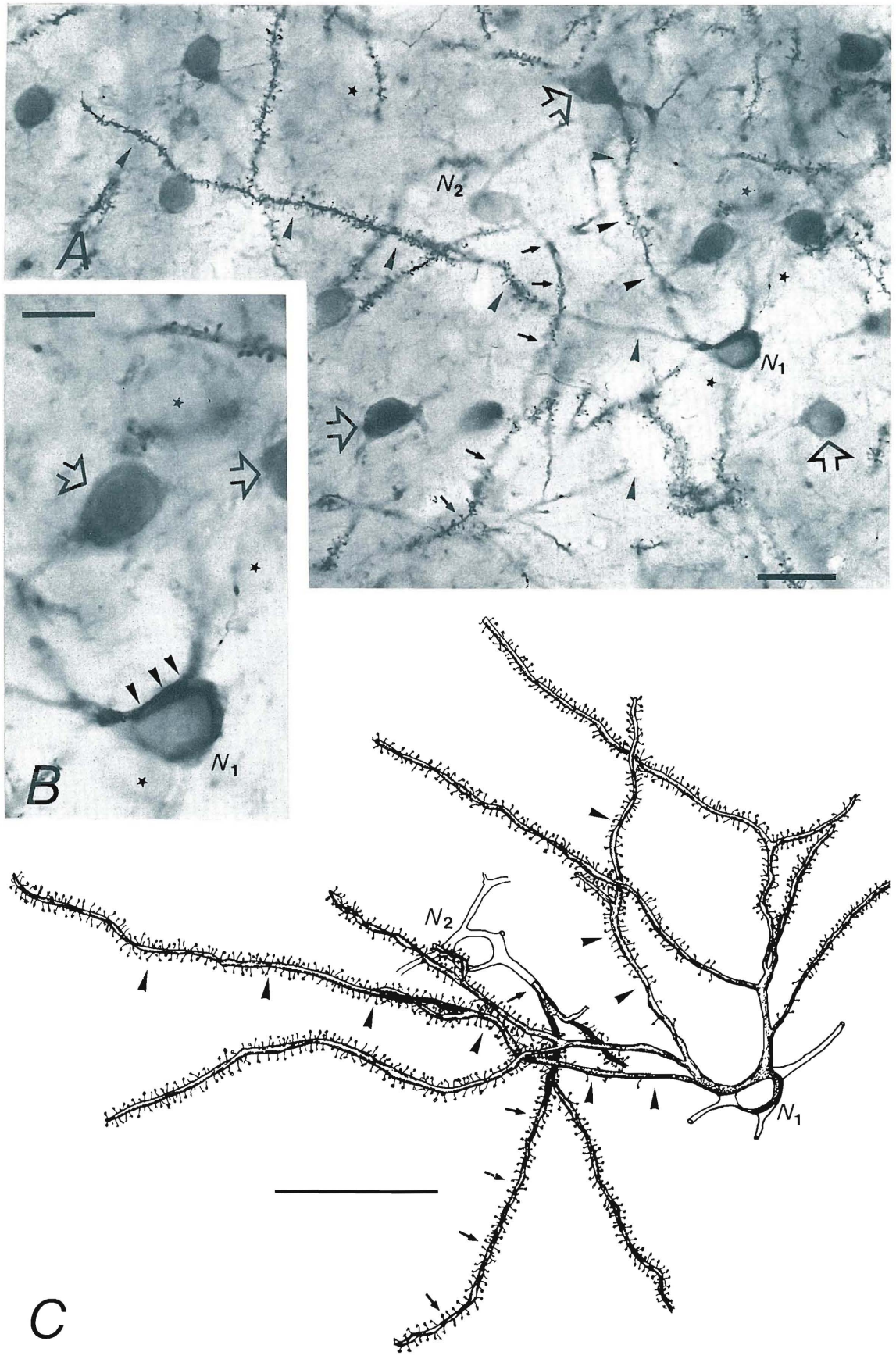

Fig. 3.

or partially impregnated perikarya (Fig. 3A), or (3) non-impregnated processes of the neuron displayed immunoreactivity (Fig. 1B). The immunostaining of some of the neurons was confirmed by taking semi-thin (1 μm) sections of the perikarya (not illustrated) and/or by electron microscopy (Fig. 2). In semi-thin sections the brown immunoreaction product was much easier to identify because the secondary Golgi-deposit was usually confined predominantly to the cell membrane. In the electron microscope the immunoreaction product appeared as an electron dense deposit associated with the external membranes of subcellular organelles and the internal side of the cell membrane (Fig. 2). The secondary Golgi-deposit was distinguishable from the peroxidase reaction product by its greater electron density and its granular form (Fig. 2B, C).

Golgi-impregnated substance P-positive neurons

All neurons that were both Golgi-impregnated and substance P-immunoreactive were medium-sized neurons and displayed characteristics of the class of neuron that is described as medium-sized and densely spiny[40] (Figs 1 and 2). The main features of these neurons include: (a) the medium-size, round or oval perikaryon (mean of 14.5 by 18.2 μm; cross-sectional are $206.3 \pm 12.3\ \mu m^2$), (b) 3–5 primary dendrites (mean diameter at first branch point; 3.6 μm) that branch within 20 μm of the mid-point of the cell body, and (c) the presence of spines on secondary and higher order dendrites, with densities as high as 15 spines/10 μm.

Ultrastructural examination of the substance P-immunoreactive, Golgi-impregnated neurons in the electron microscope showed that the perikarya of these neurons all had round unindented nuclei surrounded by a scant rim of cytoplasm which was poor in organelles (Fig. 2).

None of the Golgi-impregnated, substance P-positive neurons had their axons impregnated by the Golgi deposit to any significant degree. In some of the neurons the axon initial segment was impregnated; this emerged from the perikaryon or a thick primary dendrite.

Golgi-impregnated [Met]enkephalin-positive neurons

As with the substance P-immunostained neurons, all neurons that expressed immunoreactivity for [Met]enkephalin were of the medium-size densely spiny type (Fig. 3). Thus they possessed medium-size perikarya (mean of 13.8 by 18.4 μm; cross-sectional area $196.5 \pm 8.8\ \mu m^2$) and three to five primary dendrites (mean diameter 3.8 μm at first branch point) that branch within 22.5 μm of the mid-point of the perikaryon. Spines were only present on secondary and higher order dendrites with a density of up to 16 spines/10 μm of dendrite. When impregnated, the axon initial segment was seen to emerge from the perikaryon or a proximal dendrite.

Golgi-impregnated substance P- and [Met]enkephalin-immunoreactive neurons were indistinguishable upon visual comparison. Comparison of 10 substance P- and 11 [Met]enkephalin-positive neurons from cat caudate nucleus, revealed no statistically significant differences between cross-sectional area and dimensions of cell bodies, numbers and diameters of primary and higher order dendrites or the distance from the mid-point of the perikaryon to the densely spiny part of the dendrites.

In the sections immunostained with antibodies against choline acetyltransferase, somatostatin or glutamate decarboxylase, Golgi-impregnated medium-size densely spiny neurons were never immunostained.

DISCUSSION

The relative advantages and disadvantages of the use of single sections for Golgi impregnation have been discussed extensively on previous occasions.[22,23] The only comment required here is that we believe the minor modifications of the technique developed by Gabbott and Somogyi[23] make the preparation of the section/slide assembly much easier, more rapid and more reliable. The procedure, as modified, allows the manipulation of large numbers of serial sections. Finally, single section methods allow the impregnation of very large sections; in the present experiments sections as large as 7×10 mm were successfully impregnated.

Our results demonstrate that the predominant type of striatal neuron that expresses substance P-like immunoreactivity and probably the only type of striatal neuron that expresses [Met]enkephalin-immunoreactivity are of similar somatodendritic morphology and are categorized as medium-size densely spiny neurons. These observations support the conclusions of previous ultrastructural studies of substance P-immunoreactive[5] and [Met]enkephalin-immunoreactive neurons[18,43] in which it was demonstrated that both types of neuron have the ultrastructural characteristic of medium-size densely spiny neurons. Similarly, medium-size densely spiny neurons have been shown to be the major type of striatal projection neuron[44,48] and neurochemical or immunochemical studies have shown the presence of both substance P[7,8,24,31–35,38,41,49] and enkephalin[12,13,15,17,49] or related opiate peptides[51,53] in striatal output pathways.

The finding that striatal neurons expressing substance P or [Met]enkephalin immunoreactivity are of a similar somatodendritic morphology raises the question of whether substance P and [Met]enkephalin co-exist in the same neurons. One might expect that neurons of a similar somatodendritic morphology would have similar chemical characteristics. Indeed Penny *et al.*[42] have recently demonstrated that a population of medium-sized striatal neurons express both neuropeptides, however the larger proportion of

the neurons expressed only one of the peptides. There is further evidence to support the view that substance P and [Met]enkephalin are mainly present in separate populations of spiny neurons. First, immunocytochemical studies of substance P- and [Met]enkephalin-immunoreactive neurons in the caudate nucleus of the cat have shown that the neurons expressing these peptides have different distributions. Substance P-immunoreactive neurons are primarily located in striosomes, i.e. in zones that are AChE-poor, whereas [Met]enkephalin-immunoreactive neurons are primarily located outside these zones (at least in the absence of colchicine).[27] Second, the three major regions that receive fibre projections from the striatum have sharply different distributions of these neuropeptides. The entopeduncular nucleus (internal segment of the globus pallidus) and the substantia nigra are rich in substance P-containing terminals and have restricted distributions of [Met]enkephalin immunoreactive neuropil, whereas the external segment of the globus pallidus is rich in [Met]enkephalin-containing terminals and has only limited amounts of substance P-positive neuropil.[2,11,16,26,29,30,36] Since the substance P in the substantia nigra and the [Met]enkephalin in the pallidum are thought to be derived predominantly from striatal afferents terminating in these regions and since there is evidence in primates that the striatonigral and the striatopallidal projections arise mainly from different populations of striatal neurons,[20,39] it seems likely that substance P and enkephalin are expressed mainly in separate populations of spiny efferent neurons.

In view of these observations and our finding that striatal neurons expressing substance P-like immunoreactivity have a somatodendritic morphology virtually indistinguishable from that of striatal neurons expressing [Met]enkephalin-like immunoreactivity makes it possible that neurons of a similar morphological type have different neuropeptide contents. This conclusion supports the view that separate control can be exerted over the morphology and neurotransmitter content of a given neuronal population.

The chemical differences between populations of medium-size densely spiny neurons may be reflected in differences in axonal morphology even though the somatodendritic morphology of these neurons is similar. There is evidence that they may be subdivided on the basis of the patterns of arborization of their axons. In an analysis of striatal neurons labelled by the intracellular injection of horseradish peroxidase, Chang *et al.*[9] identified two groups of medium-size densely spiny neurons with different axonal arbors. Both types had extensive local axon collaterals in the vicinity of their dendritic trees but there were differences in their patterns of innervation of the globus pallidus. One type had extensive axonal arbors in the globus pallidus arising from more than one branch of the main axon. The second type gave rise to only sparse terminal fields in the globus pallidus, and these arose from a few fine collaterals of the main axon. For both types there was evidence that the neurons also innervated other (more caudal) structures. The axons of our Golgi-impregnated immunoreactive neurons were never impregnated beyond their initial segments, so we cannot directly compare our sample of Golgi-impregnated immunostained neurons to the two types described by Chang *et al.*[9] Nevertheless, it seems plausible that the substance P-containing medium-size densely spiny neurons are of the class that only gives rise to a sparse innervation of the external pallidum and that the other medium-size densely spiny type of Chang *et al.*[9] represents the [Met]enkephalin-containing medium-size densely spiny neuron giving rise to the dense plexus of [Met]enkephalin-containing (and presumably GABA-containing[1]) terminals in the pallidum.

Acknowledgements—We are grateful to G. Ungpakorn, L. Hayes and H. F. Hall for their excellent technical assistance, to Dr A. C. Cuello for the substance P antibodies, to Dr R. P. Elde for the [Met]enkephalin antibodies and to Dr P. L. Gabbott for his advice and discussion about the "single section Golgi" technique. This work was supported by grants from the Medical Research Council and the E. P. Abraham Cephalosporin Trust (JPB and PNI) and grants from the National Science Foundation (BNS83-19547) and the Seaver Institute (AMG).

REFERENCES

1. Aronin N., DiFiglia M., Graveland G. A., Schwartz W. J. and Wu J.-Y. (1984) Localization of immunoreactive enkephalins in GABA synthesizing neurones in rat neostriatum. *Brain Res.* **300,** 376–380.
2. Beckstead R. M. and Kersey K. S. (1985) Immunohistochemical demonstration of differential substance P-met-enkephalin-, and glutamic-acid-decarboxylase-containing cell body and axon distributions in the corpus striatum of the cat. *J. comp. Neurol.* **232,** 481–498.
3. Bolam J. P., Ingham C. A. and Smith A. D. (1984) The section Golgi impregnation procedure 3. Combination of Golgi-impregnation with enzyme histochemistry and electron microscopy to characterize acetylcholinesterase-containing neurons in the rat neostriatum. *Neuroscience* **12,** 687–709.
4. Bolam J. P., Powell J. F., Wu J.-Y. and Smith A. D. (1985) Glutamate decarboxylase-immunoreactive structures in the rat neostriatum. A correlated light and electron microscopic study. *J. comp. Neurol.* **237,** 1–20.
5. Bolam J. P., Somogyi P., Takagi H., Fodor I. and Smith A. D. (1983) Localization of substance P-like immunoreactivity in neurons and nerve terminals in the neostriatum of the rat: a correlated light and electron microscopic study. *J. Neurocytol.* **12,** 325–344.
6. Bolam J. P., Wainer B. H. and Smith A. D. (1984) Characterization of cholinergic neurons in the rat neostriatum. A combination of choline acetyltransferase immunocytochemistry, Golgi-impregnation and electron microscopy. *Neuroscience* **12,** 711–718.

7. Brownstein M. J., Mroz E. A., Kizer J. S., Palkovits M. and Leeman S. E. (1976) Regional distribution of substance P in the brain of the rat. *Brain Res.* **116,** 299–305.
8. Brownstein M. J., Mroz E. A., Tappaz M. L. and Leeman S. E. (1977) On the origin of substance P and glutamic acid decarboxylase (GAD) in the substantia nigra. Brain Res. **135,** 315–323.
9. Chang H. T., Wilson C. J. and Kitai S. T. (1981) Single neostriatal efferent axons in the globus pallidus; a light and electron microscopic study. *Science* **213,** 915–918.
10. Chang H. T., Wilson C. J. and Kitai S. T. (1982) A Golgi study of rat neostriatal neurons: light microscopic analysis. *J. comp. Neurol.* **208,** 107–126.
11. Chesselet M.-F. and Graybiel A. M. (1983) Met-enkephalin-like and dynorphin-like immunoreactivities of the basal ganglia of the cat. *Life Sci.* **33,** 37–40.
12. Correa F. M. A., Innis R. B., Hester L. D. and Snyder S. H. (1981) Diffuse enkephalin innervation from caudate to globus pallidus. *Neurosci. Lett.* **25,** 63–68.
13. Cuello A. C., Del Fiacco M., Paxinos G., Somogyi P. and Priestly J. V. (1981) Neuropeptides in striato-nigral pathways. *J. Neural Trans.* **51,** 83–96.
14. Cuello A. C., Galfre G. and Milstein C. (1979) Detection of substance P in the central nervous system by a monoclonal antibody. *Proc. natn. Acad. Sci. U.S.A.* **76,** 3532–3536.
15. Cuello A. C. and Paxinos G. (1978) Evidence for a long leu-enkephalin striopallidal pathway in rat brain. *Nature* **271,** 178–180.
16. Cuello A. C. and Kanazawa A. (1978) The distribution of substance P immunoreactive fibres in the rat central nervous system. *J. comp. Neurol.* **178,** 129–157.
17. Del Fiacco M., Paxinos G. and Cuello A. C. (1982) Neostriatal enkephalin-immunoreactive neurons project to the globus pallidus. *Brain Res.* **231,** 1–17.
18. DiFiglia M., Aronin N. and Martin J. B. (1982) Light and electron microscopic localization of immunoreactive leu-enkephalin in the monkey basal ganglia. *J. Neurosci.* **2,** 303–320.
19. Fairén A., Peters A. and Saldanha J. (1977) A new procedure for examining Golgi impregnated neurons by light and electron microscopy. *J. Neurocytol.* **6,** 311–337.
20. Feger J. and Crossman A. R. (1984) Identification of different subpopulations of neostriatal neurons projecting to the globus pallidus or substantia nigra in the monkey: a retrograde fluorescent double-labeling study. *Neurosci. Lett.* **49,** 7–12.
21. Freund T. F., Martin K. A. C., Smith A. D. and Somogyi P. (1983) Glutamate decarboxylase-immunoreactive terminals of Golgi-impregnated axoaxonic cells and of presumed basket cells in synaptic contact with pyramidal neurons of the cat's visual cortex. *J. comp. Neurol.* **221,** 263–278.
22. Freund T. S. and Somogyi P. (1983) The section–Golgi impregnation procedure. I. Description of the method and its combination with histochemistry after intracellular iontophoresis or retrograde transport of HRP. *Neuroscience* **9,** 463–474.
23. Gabbott P. L. and Somogyi J. (1984) The 'single' section Golgi-impregnation procedure: methodological description. *J. Neurosci. Meth.* **11,** 221–230.
24. Gale K., Hong J.-S. and Guidotti A. (1977) Presence of substance P and GABA in separate striatonigral neurons. *Brain Res.* **136,** 371–375.
25. Geneser–Jensen F. A. and Blackstad T. W. (1971) Distribution of acetylcholinesterase in the hippocampal region of the guinea pig. I. Entorhinal area, parasubiculum and presubiculum. *Z. Zellforsch. mikrosk. Anat.* **114,** 460–481.
26. Graybiel A. M. (1984) Neurochemically specified subsystems in the basal ganglia. In *Functions of the Basal Ganglia.* Ciba Foundation Symposium 107 (eds Evered D. and O'Connor M), pp. 114–149. Pitman, London.
27. Graybiel A. M. and Chesselet M.-F. (1984) Compartmental distribution of striatal cell bodies expressing [Met]enkephalin-like immunoreactivity. *Proc. natn. Acad. Sci. U.S.A.* **81,** 7980–7984.
28. Graybiel A. M. and Ragsdale C. W. (1983) Biochemical anatomy of the striatum. In *Chemical Neuroanatomy* (ed. Emson P. C.), pp. 427–504. Raven Press, New York.
29. Haber S. N. and Nauta W. J. H. (1983) Ramifications of the globus pallidus in the rat as indicated by patterns of immunohistochemistry. *Neuroscience* **9,** 245–260.
30. Haber S. N. and Watson S. J. (1983) The comparison between enkephalin-like and dynorphin-like immunoreactivity in both monkey and human globus pallidus and substantia nigra. *Life Sci.* **33,** 33–36.
31. Hong J. S., Yang H.-Y., Racagni G. and Costa E. (1977) Projections of substance P containing neurons from neostriatum to substantia nigra. *Brain Res.* **122,** 541–544.
32. Jessel T. M., Emson P. C., Paxinos G. and Cuello A. C. (1978) Topographic projections of substance P and GABA pathways in the striato- and pallido-nigral system; a biochemical and immunohistochemical study. *Brain Res.* **152,** 487–498.
33. Kanazawa I., Emson P. C. and Cuello A. C. (1977) Evidence for the existence of substance P-containing fibres in striatonigral and pallido-nigral pathways in rat brain. *Brain Res.* **119,** 447–453.
34. Kanazawa I., Mogaki S., Muramoto O. and Kuzuhara S. (1980) On the origin of substance P-containing fibres in the entopeduncular nucleus and the substantia nigra of the rat. *Brain Res.* **184,** 481–485.
35. Kohno J., Shiosaka S., Shinoda K., Inagaki S. and Tohyama M. (1984) Two distinct strio-nigral substance P pathways in the rat: an experimental immunohistochemical study. *Brain Res.* **308,** 309–317.
36. Ljungdahl Å., Hökfelt T. and Nilsson G. (1978) Distribution of substance P-like immunoreactivity in the central nervous system of the rat—I. Cell bodies and nerve terminals. *Neuroscience* **3,** 861–943.
37. Micevych P. and Elde R. (1980) Relationship between enkephalinergic neurons and the vasopressin-oxytocin neuroendocrine system of the cat: an immunohistochemical study. *J. comp. Neurol.* **190,** 135–146.
38. Mroz E. A., Brownstein M. J. and Leeman S. E. (1977) Evidence for substance P in the striato-nigral tract. *Brain Res.* **125,** 305–311.
39. Parent A., Bouchard C. and Smith Y. (1984) The striatopallidal and striatonigral projections: two distinct fiber systems in primates. *Brain Res.* **303,** 385–390.
40. Pasik P., Pasik T. and DiFiglia M. (1979) The internal organisation of the neostriatum in mammals. In *The Neostriatum* (eds Divac I. and Oberg R. G. E.), pp. 5–36. Pergamon Press, Oxford.

41. Paxinos G., Emson P. C. and Cuello A. C. (1978) Substance P projections to the entopeduncular neucleus, the medial preoptic area and the lateral septum. *Neurosci. Lett.* **7,** 133–136.
42. Penny G. R., Afsharpoor S. and Kitai S. T. (1986) The glutamate decarboxylase- leucine enkephalin-, methionine enkephalin- and substance P-immunoreactive neurons in the neostriatum of the rat and cat: evidence for partial population overlap. *Neuroscience* **17,** 1011–1045.
43. Pickel V. M., Sumal K. K., Beckley S. C., Miller R. J. and Reis D. J. (1980) Immunocytochemical localization of enkephalin in the neostriatum of rat brain: a light and electron microscopic study. *J. comp. Neurol.* **189,** 721–740.
44. Somogyi P., Bolam J. P. and Smith A. D. (1981) Monosynaptic cortical input and local axon collaterals of identified striatonigral neurons. A light and electron microscopic study using the Golgi-peroxidase transport-degeneration procedure. *J. comp. Neurol.* **195,** 567–584.
45. Somogyi P., Freund T. F., Wu J.-Y and Smith A. D. (1983) The section Golgi impregnation procedure. 2. Immunocytochemical demonstration of glutamate decarboxylase in Golgi-impregnated neurons and in their afferent synaptic boutons in the visual cortex of the cat. *Neuroscience* **9,** 475–490.
46. Somogyi P., Hodgson A. J. and Smith A. D. (1979) An approach to tracing neuron networks in the cerebral cortex and basal ganglia. Combination of Golgi staining, retrograde transport of horseradish peroxidase and anterograde degeneration of synaptic boutons in the same material. *Neuroscience* **4,** 1805–1852.
47. Somogyi P., Priestly J. V., Cuello A. C., Smith A. D. and Bolam J. P. (1982) Synaptic connections of substance P immunoreactive nerve terminals in the substantia nigra of the rat: a correlated light and electron microscopic study. *Cell Tissue Res.* **223,** 469–486.
48. Somogyi P. and Smith A. D. (1979) 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. *Brain Res.* **178,** 3–15.
49. Staines W. A., Nagy J. I., Vincent S. R. and Fibiger H. C. (1980) Neurotransmitters contained in the efferents of the striatum. *Brain Res.* **194,** 391–402.
50. Sternberger L. A. (1979) *Immunocytochemistry*, 2nd edition, Wiley, New York.
51. Vincent S., Hökfelt T., Christensson I. and Terenius L. (1982) Immunohistochemical evidence for a dynorphin immunoreactive striato-nigral pathway. *Eur. J. Pharmac.* **85,** 251–252.
52. Woolf N. J. and Butcher L. L. (1981) Cholinergic neurons in the caudate-putamen complex proper are intrinsically organised: a combined Evans Blue and Acetycholinesterase analysis. *Brain Res. Bull.* **7,** 487–507.
53. Zamir N., Palkovits M., Weber E., Mezey E. and Brownstein M. J. (1984) A dynorphinergic pathway of leu-enkephalin production in rat substantia nigra. *Nature* **307,** 643–645.

(*Accepted* 16 *June* 1986)