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Localization of substance P-like immunoreactivity in neurons and nerve terminals in the neostriatum of the rat: a correlated light and electron microscopic study

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Summary

An antiserum, to substance P has been used to study the neostriatum of rats which has received intracerebral injections of colchicine. Both cell bodies and nerve fibres were found to display immunoreactivity. Some of the fibres were swollen and could be traced back to their parent cell body.

Examination in the electron microscope of structures that had first been identified in the light microscope showed that there are two different types of substance P-immunoreactive cell body. The first kind (type I) of immunoreactive cell body was of medium size and had a smooth surfaced nucleus. It displayed the ultrastructural features typical of medium-size spiny neurons. Identified axons of type I neurons gave rise to immunoreactive axon collaterals within the neostriatum: boutons along these collaterals were found to form symmetrical synaptic contacts. The second kind (type II) of immunoreactive cell body was also of medium-size and had a round or oval shape, but the nucleus was deeply indented and was surrounded by a thin rim of cytoplasm. Synaptic input to this neuron was sparse and consisted of small boutons that made symmetrical contacts with the perikaryon and proximal dendrites.

Many immunoreactive dot-like structures could be seen in the light microscope: upon examination in the electron microscope these were found to be boutons. All fifty-six synaptic boutons that were studied made symmetrical synaptic contacts. These boutons were indistinguishable from the boutons of axon collaterals of identified type I immunoreactive neurons. The most common postsynaptic structures were dendrites, including some dendritic spines, although synapses between immunoreactive boutons and several perikarya, and an axon initial segment were observed. The morphological features of the immunoreactive boutons in the

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neostriatum were very similar to one type of substance P-immunoreactive bouton in the substantia nigra and to a bouton type in the substantia nigra which is labelled following the anterograde transport of horseradish peroxidase from the striatum.

It is suggested that there are two kinds of substance P-containing neurons in the striatum and that one of these is likely to belong to the medium-spiny class. The latter type of neuron is probably the source of the striatonigral substance P-containing projection and of the immunoreactive boutons within the striatum. The finding of substance P-immunoreactive synaptic boutons within the neostriatum provides a morphological basis for the view that substance P might serve as a neurotransmitter in the neostriatum.

Introduction

Although substance P-like immunoreactivity is present in the neostriatum, its concentration in this part of the basal ganglia is much lower than in the substantia nigra and it has been considered to be present in the neostriatum mainly within neuronal cell bodies of a striatonigral pathway (see review by Nicoll *et al.*, 1980). However, immunohistochemical studies have shown a moderately rich network of fibres containing substance P-like material in the neostriatum of the rat (Cuello & Kanazawa, 1978; Ljungdahl *et al.*, 1978a, b) and this raised the question of whether such fibres are merely axons of passage or whether there is local release of substance P within the neostriatum. This was found to be the case by Michelot *et al.* (1979) who detected substance P in the perfusates from push–pull cannulae implanted in the caudate nucleus of the cat: the release occurred spontaneously but could be increased markedly by perfusion with 50 mM K^+. *In vitro* studies also showed that the release of substance P could be evoked from slices (Starr, 1978) or from minced striatal tissue of the rat, in a calcium-dependent manner (Pettibone *et al.*, 1980).

It is possible, therefore, that locally released substance P could serve as a neurotransmitter or modulator within the neostriatum. There is some evidence for possible actions of this peptide in the striatum: thus, it potentiates the K^+-evoked release of [^{3}H]dopamine and [^{3}H]5-hydroxytryptamine from striatal slices (Starr, 1978) and has some effect on the electrical activity of striatal neurons (Le Gal La Salle & Ben-Ari, 1977). By use of the ultracentrifuge it has been shown that 61% of the substance P in the rat neostriatum is recovered in the crude mitochondrial fraction and that 72% of this is present in a fraction containing synaptosomes (Pettibone *et al.*, 1980). However, the nature of the structures storing the peptide is not known.

We have used an immunocytochemical procedure at both light and electron microscopic levels in an attempt to answer several questions. 1. What morphological class or classes of neuron contain substance P-immunoreactivity in the neostriatum? 2. Is this peptide contained within axonal boutons that form synapses and so might represent sites from which it could be released within the neostriatum? 3. If the latter is the case, what is the origin of the boutons and what are the postsynaptic structures?

Methods

All experiments were performed on female albino Wistar rats (160–180 g) that had received injections of colchicine (BDH) under chloral hydrate anaesthesia either into the lateral ventricle (20 μl containing 70 μg) or directly into the neostriatum (5 μg in 1 μl) 24 h before death. The animals were perfused through the heart whilst under chloral hydrate anaesthesia (350 mg/kg i.p) with 20 ml of Tyrode's solution followed by 200 ml of fixative consisting of 4% paraformaldehyde, 0.05% glutaraldehyde and 30 ml saturated picric acid, all in 0.1 M sodium phosphate buffer, pH 7.4 (Somogyi & Takagi, 1982). The brains were then removed from the skull and post-fixed for 30 min to 3 h at 4°C. Blocks of neostriatum were dissected out, washed in the sodium phosphate buffer, infiltrated with sucrose (10% w/v, then 20%) and frozen in liquid N_2 twice. The blocks were then cut to give sections of 60–80 μm using an Oxford Vibratome and the sections were washed overnight in phosphate-buffered saline (PBS) at 4° C.

Immunocytochemistry for substance P was carried out using Sternberger's peroxidase–antiperoxidase technique according to the procedure of Somogyi & Takagi (1982). Very briefly, the sections were incubated in rabbit anti-substance P antiserum (1/1500 dilution; antiserum described by Sakanaka *et al.*, 1981) overnight at 4° C with shaking; 3 × 30 min washes in PBS; 2–4 h in goat anti-rabbit antiserum (1/40 dilution; supplied by Miles); 3 × 30 min washes in PBS; 1.5 h or overnight in rabbit peroxidase–antiperoxidase (1/100 dilution; supplied by Capel); 3 × 30 min washes. The antigen was localized by incubation with 3,3′-diaminobenzidine (50 mg/100 ml 0.05 M Tris HCl pH 7.4 + 0.01% H_2O_2) for 5–10 min. The sections were then washed for 5 min in the Tris buffer and then in the phosphate buffer, treated with osmium tetroxide, dehydrated and mounted on microscope slides in resin (Durcupan ACM, Fluka).

The sections were examined in the light microscope and areas of interest were photographed and re-embedded for examination in the electron microscope. Serial ultrathin sections were collected on single slot formvar-coated grids and examined in a Philips 201C electron microscope at 60 kV using an objective aperture of 20 μm. To improve contrast for electron microscopy, the sections were stained *en bloc* with 1% uranyl acetate in the 70% ethanol; but lead stain was not used. Correlated light and electron microscopy was carried out on the striata of three rats.

To test the specificity of the immunocytochemical procedure the following control experiments were performed: 1. Omission of the anti-substance P serum; 2. replacement of the antiserum with normal rabbit serum; 3. adsorption of the anti-substance P antiserum with substance P; 4. adsorption of the anti-substance P serum to leu-enkephalin, somatostatin, neurotensin, met-enkephalin. Adsorption experiments were carried out by incubation of 400 μg of the peptides per ml of the diluted serum, overnight at 4° C followed by centrifugation at 100 000 g for 1 h to remove any precipitated material.

Results

SPECIFICITY OF THE ANTISERUM

All immunostaining was abolished: 1. upon omission of the substance P antiserum; 2. when the antiserum was replaced by normal serum; 3. when the antiserum was adsorbed with substance P. However, no reduction in immunostaining was observed when the substance P antiserum was adsorbed with any of the other peptides (see Methods).

Although there is good evidence that the substance P-like immunoreactivity in the neostriatum is largely due to substance P itself (Ben-Ari *et al.*, 1979; Pettibone *et al.*,

1980; Sperk & Singer, 1982), we shall call the material we study 'substance P-immunoreactivity'.

LIGHT MICROSCOPIC APPEARANCE OF SUBSTANCE P-IMMUNOREACTIVE STRUCTURES

In the light microscope both neuronal perikarya and fibres were labelled by the immunocytochemical procedure using the antiserum to substance P. However, cell bodies were only occasionally labelled, sometimes only one, or no, cells per section could be seen. In animals that had received intraventricular colchicine the immunoreactive neurons were generally found close to the ventricle wall or in the ventral part of the striatum. Immunoreactive cell bodies were of medium-size (10–15 μm) and were round or oval in shape (Figs. 1A, 2B, 4B).

Fibre and terminal labelling occurred mainly in areas within the influence of the injected colchicine and consisted of numerous isolated bouton-like structures and occasional lengths of fibre with several varicosities or swellings. The fibres with swellings were sometimes seen in continuity with the parent cell body (Figs. 1A, 2B).

As the immunostaining was so dependent upon the effect of colchicine we cannot make any comment about the regional distribution of substance P-immunoreactive neurons and fibres. It is noteworthy that in the same animals from which striatal tissue was examined, other brain regions, both close to the striatum e.g. bed nucleus of the stria terminalis and distant from the striatum, e.g. substantia nigra or interpeduncular nucleus, showed strong, even staining with the substance P antiserum.

ELECTRON MICROSCOPY

Appearance of the peroxidase reaction endproduct

As described previously for the localization of substance P in the substantia nigra and enkephalin in the neostriatum (Somogyi *et al.*, 1982a,b), the peroxidase reaction endproduct was associated with the surfaces of most cell organelles including

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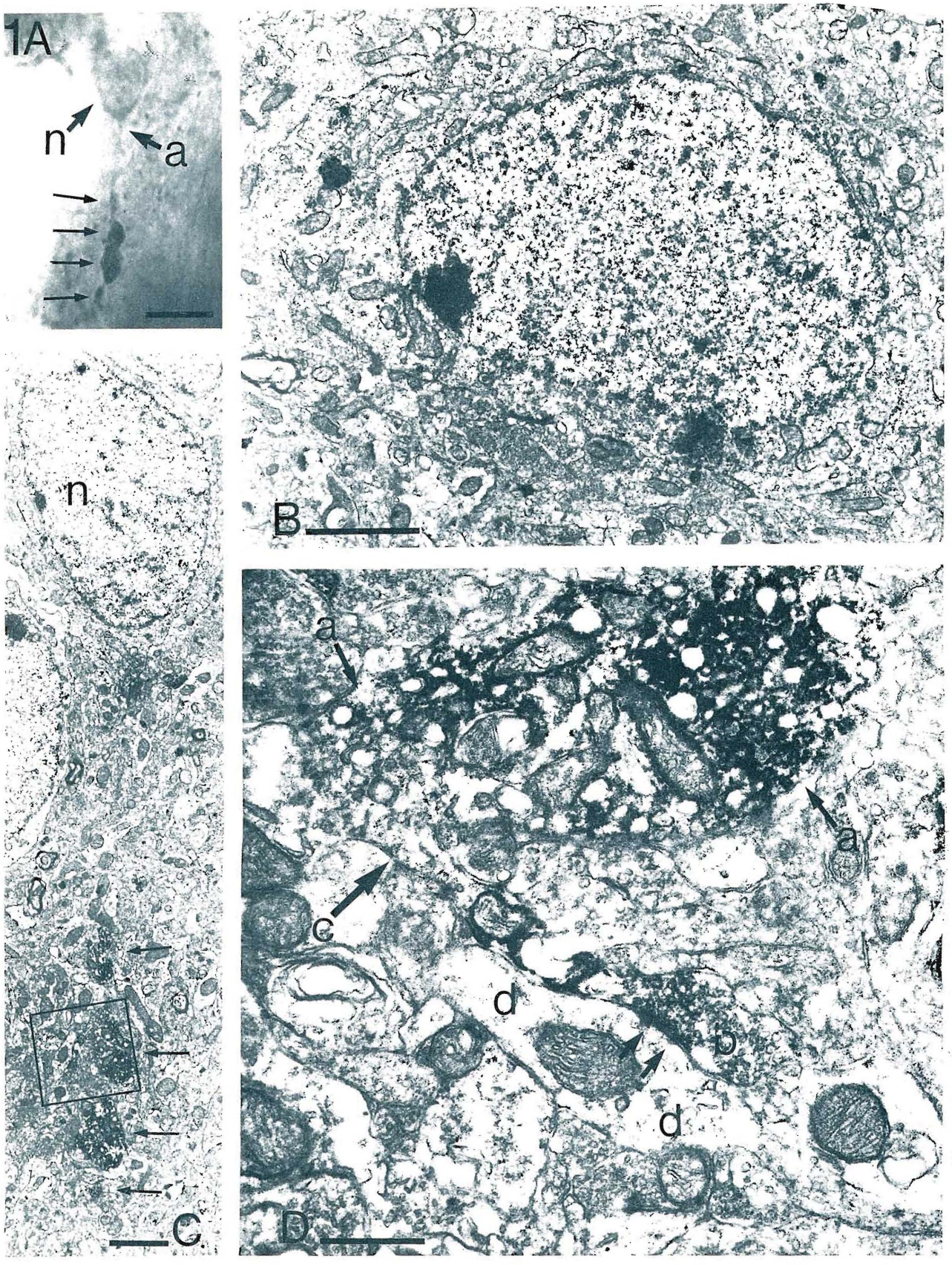
Fig. 1. (A) Light micrograph of a type I substance P-immunoreactive neuron. The perikaryon is indicated by n and the origin of its axon by a. Four swellings of the axon, which also appear in C, are indicated by arrows. The swellings are due to colchicine treatment. The weak staining of the perikaryon is due to its location deep within the thick section. (B) Electron micrograph of the perikaryon of the same immunoreactive neuron as in A. (C) Low-power electron micrograph of the perikaryon (n) and swellings of the axon (arrows) of the neuron in A and B. A collateral arose (boxed area) from one of the swellings and is shown at a higher magnification in D. (D) High-power electron micrograph of the boxed area in C but rotated through 90° in a clockwise direction. The swollen (a) gives rise to a collateral (c) which then develops a vesicle-containing bouton (b). Within the axon there are a number of structures which might represent intra-axonal organelles which have become swollen due to colchicine treatment. The bouton makes symmetrical synaptic contact (arrows) with a dendrite (d). A serial section of the same bouton is shown at a higher magnification in Fig. 5F. Scale bars: A, 10 μm; B, 20 μm; C, 2 μm; D, 0.5 μm.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microtubules (Figs. 2A, 2D), mitochondria, endoplasmic reticulum, the cell membrane, and the outer surface of electron-lucent vesicles (Figs. 1B, D; 2A, C, D; 3C, D; 4A, C, E; 5). Reaction product was also found in the core of some large granulated vesicles (Figs. 2D, 5C).

Substance P immunoreactive neurons

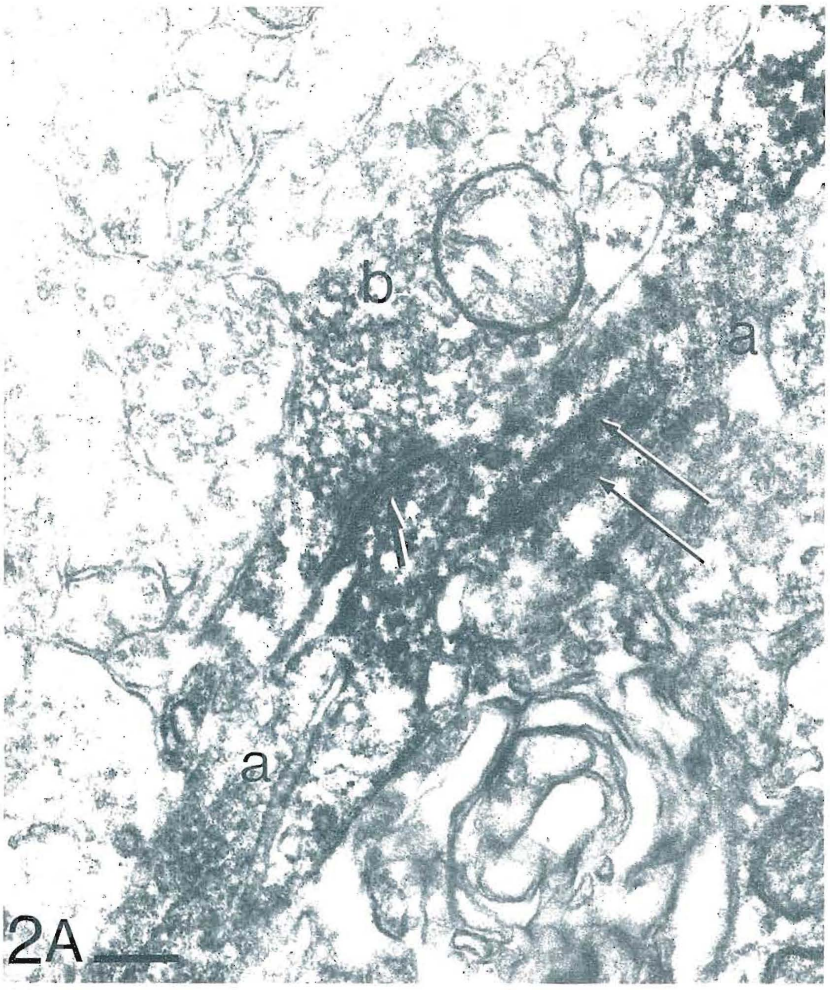
In all, 12 immunoreactive neurons were identified in the light microscope and then re-embedded for examination in the electron microscope. On the basis of ultrastructural features, the substance P reactive neurons may be divided into two distinct morphological classes.

Type I substance P-immunoreactive neurons (4 were studied) were of medium size, had round or oval cell bodies and a centrally located round or oval nucleus (Figs. 1, 2). The nuclear envelope was smooth and was not indented (Fig. 2B). The amount of cytoplasm and the number of organelles was low.

In some neurons of type I the initial part of the axon exhibited strong substance P-immunoreactivity (see Figs. 1A and 2B). These axons had large swellings which, when examined in the electron microscope (Figs. 1D; 2A, D), were found to contain numerous mitochondria, electron-lucent vesicles and occasional immunoreactive dense core vesicles. These swellings were presumed to have been formed as a result of the treatment with colchicine. Serial sections of the axons revealed the presence of collaterals (Figs. 1C, D; 2C) that gave rise to boutons containing large round and oval electron-lucent vesicles (Figs. 1D, 2C, 5F). One of these boutons (Fig. 1D, 5F) was seen to form a symmetrical synaptic contact with a non-immunoreactive dendritic shaft.

Examination of serial sections of the dendrites of type I neurons revealed the presence of dendritic spines (Figs. 3A, B). Afferent synapses were observed on all the parts of the neurons that were examined. Perikarya received only a few afferent synapses, consisting of boutons containing pleomorphic vesicles and making symmetrical

Fig. 2. (A) Electron micrograph of an axonal swelling (a) of the type I substance P-immunoreactive neuron shown in B. The swollen axon contains a large amount of reaction product that is associated with all organelles, including microtubules (thin arrows). The axonal swelling is postsynaptic to a bouton (b) that is also immunoreactive for substance P. A large amount of reaction product is associated with the electron dense undercoating of the postsynaptic membrane (thick short arrows). (B) Partial light microscopic photomontage of the type I substance P-immunoreactive neuron. The perikaryon is labelled n and the axon a. The axonal swelling (due to colchicine treatment) is indicated by an open arrow and is the same as that shown in A. (C) An immunoreactive bouton (b) containing large round and oval vesicles that arose from a collateral of the same axon shown in A and B. (D) An axonal swelling of another type I substance P-immunoreactive neuron. Reaction product is associated with all cell organelles including microtubule fascicles (long thin arrow) and it is also present in large dense-cored vesicles. The swelling receives a symmetrical synaptic input from a bouton containing pleomorphic vesicles. Scale bars: A, 0.2 μm; B, 10 μm; C and D, 0.2 μm.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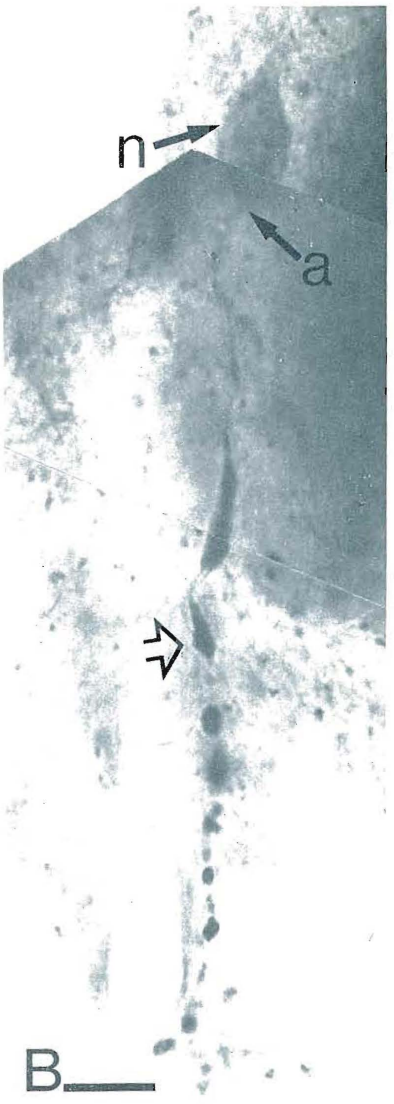
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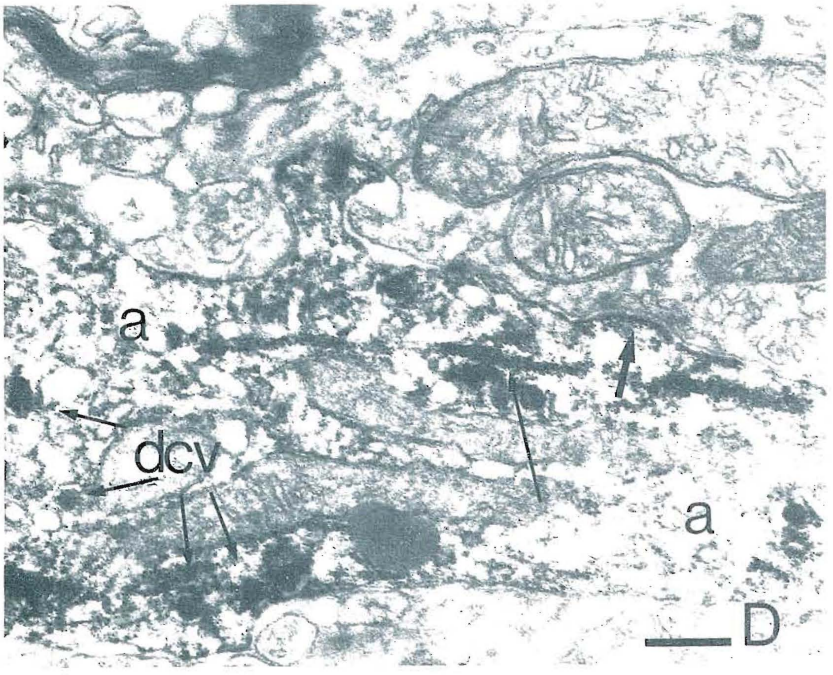
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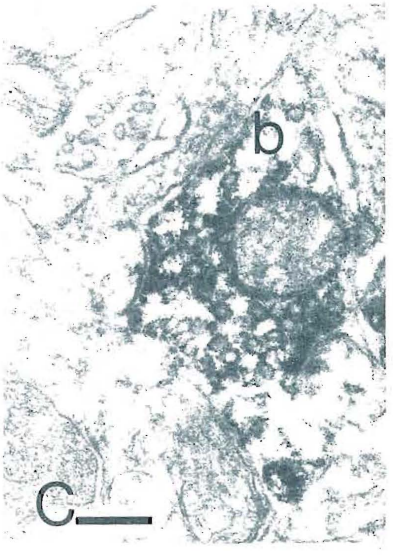
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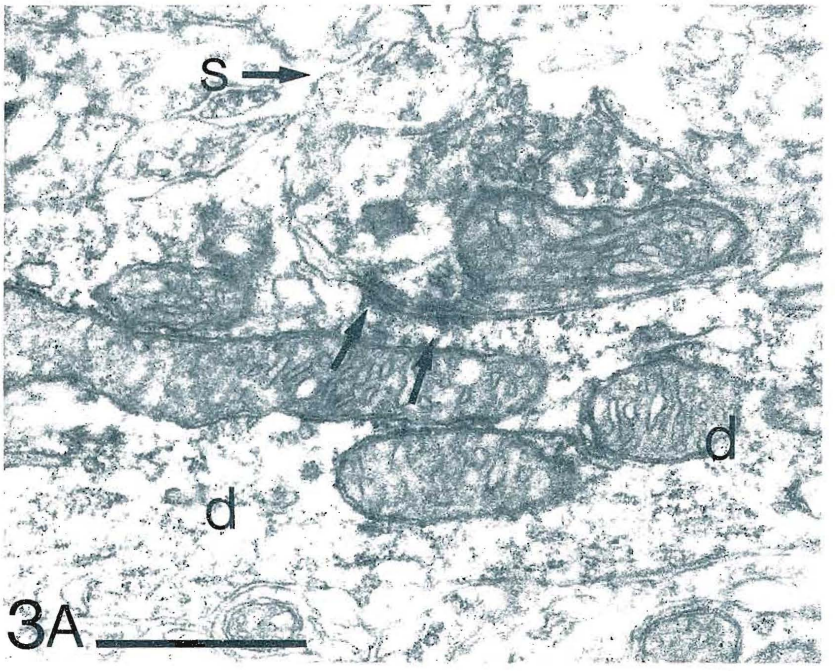
membrane contacts (Fig. 3C). Boutons containing large round and oval vesicles (distinct from the pleomorphic type) making symmetrical membrane contacts were also found on the perikarya of these neurons (Fig. 3D). The dendrites received boutons that contained pleomorphic (Fig. 3A) or large round and oval vesicles and which made symmetrical synaptic contacts. Dendritic spines received asymmetrical synapses from boutons containing round vesicles (Fig. 3B).

The proximal axons of type I neurons also received afferent synapses (Figs. 2A, D). These synapses were symmetrical and the presynaptic boutons contained pleomorphic vesicles (Fig. 2D). One immunoreactive axonal varicosity received an input from a

Fig. 3 (A) A dendrite of the type I substance P-immunoreactive neuron that gave rise to the axon in Fig. 2D. The dendritic shaft (d) is in contact (probably symmetrical synaptic contact) with a bouton containing pleomorphic vesicles (arrows). The same dendrite gives rise to a spine (s). (B) A dendritic spine (s), which in serial sections was shown to be continuous with the dendrite (d), of the same type I immunoreactive neuron. The spine is in asymmetrical synaptic contact with a bouton (arrow). (C) Symmetrical synaptic contact (arrow) between a bouton containing pleomorphic vesicles and the perikaryon of the immunoreactive neuron shown in A and B. (D) Symmetrical synaptic contact (arrow) between a bouton containing large pleomorphic vesicles and the type I immunoreactive neuron shown in Fig. 2. The scale bar in A is 0.5 μm and is the same for all four micrographs.

Fig. 4. (A) Low-power electron micrograph of substance P-immunoreactive neuron of type II. The small arrows indicate areas of a high concentration of reaction endproduct. The large arrows indicate deep nuclear invaginations. Note the small amount of cytoplasm and the scarcity of organelles. A glial cell which also appears in the light micrograph of 4B is labelled g. (B) Light micrograph of the substance P-immunoreactive neuron (n) and the glial cell (g) shown in 4A. (C) High-power electron micrograph of the boxed area in A. The immunoreactive perikaryon is in symmetrical synaptic contact (arrow) with a small bouton containing pleomorphic vesicles. (D) A bouton containing many pleomorphic vesicles is in symmetrical synaptic contact (arrow) with the proximal dendrite (d) of another type II substance P immunoreactive neuron. (E) A small bouton containing pleomorphic vesicles is in synaptic contact with the perikaryon of a type I substance P-immunoreactive neuron. Scale bars: A, 2 μm; B, 10 μm; C, D and E are the same magnification, the bar in D = 0.5 μm.

Fig. 5. Structures postsynaptic to substance P-immunoreactive boutons in the neostriatum. (A) Synaptic contact (arrow) between an immunoreactive bouton (b) and a dendrite (d) which gives rise to a spine (s). The spine is in synaptic contact with a non-reactive bouton (small arrow). (B) An immunoreactive bouton (b) which makes symmetrical synaptic contact at two sites (arrows) with a dendrite (d). (C) An immunoreactive bouton (b) forming a symmetrical synapse (small arrow) with a dendrite or spine. The bouton contains an immunoreactive dense cored vesicle (thin arrow). (D) A lightly immunoreactive bouton (b) makes symmetrical synaptic contact (arrow) with the perikaryon (p) on a non-reactive striatal neuron. (E) A dendritic spine (s) receives symmetrical synaptic input from an immunoreactive bouton (b) and asymmetrical input from a non-reactive bouton. Note the relatively large size of the vesicles in the immunoreactive bouton. (F) Serial section of the synaptic bouton that arose from the axon collateral of the type I neuron in Fig. 1. The bouton (b) makes symmetrical synaptic contact (arrow) with a dendrite (d). Note the similar morphology of this bouton and the other immunoreactive boutons. The magnification in A–F is the same. Scale bar: 0.5 μm.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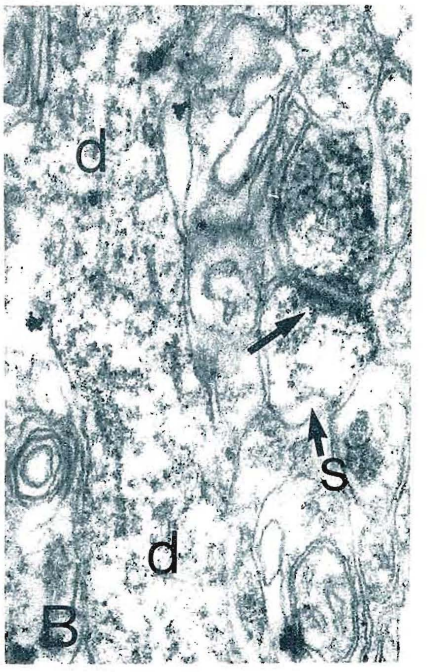
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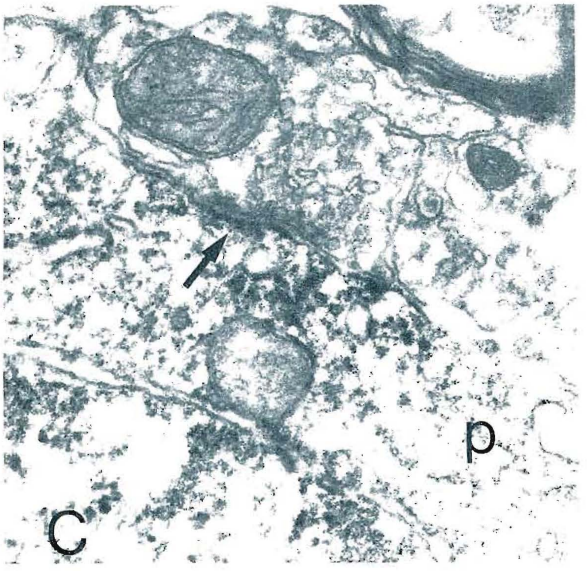
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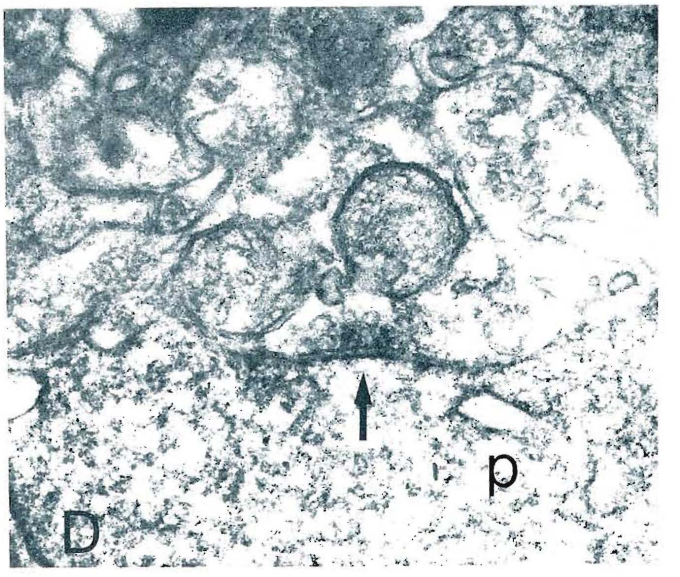
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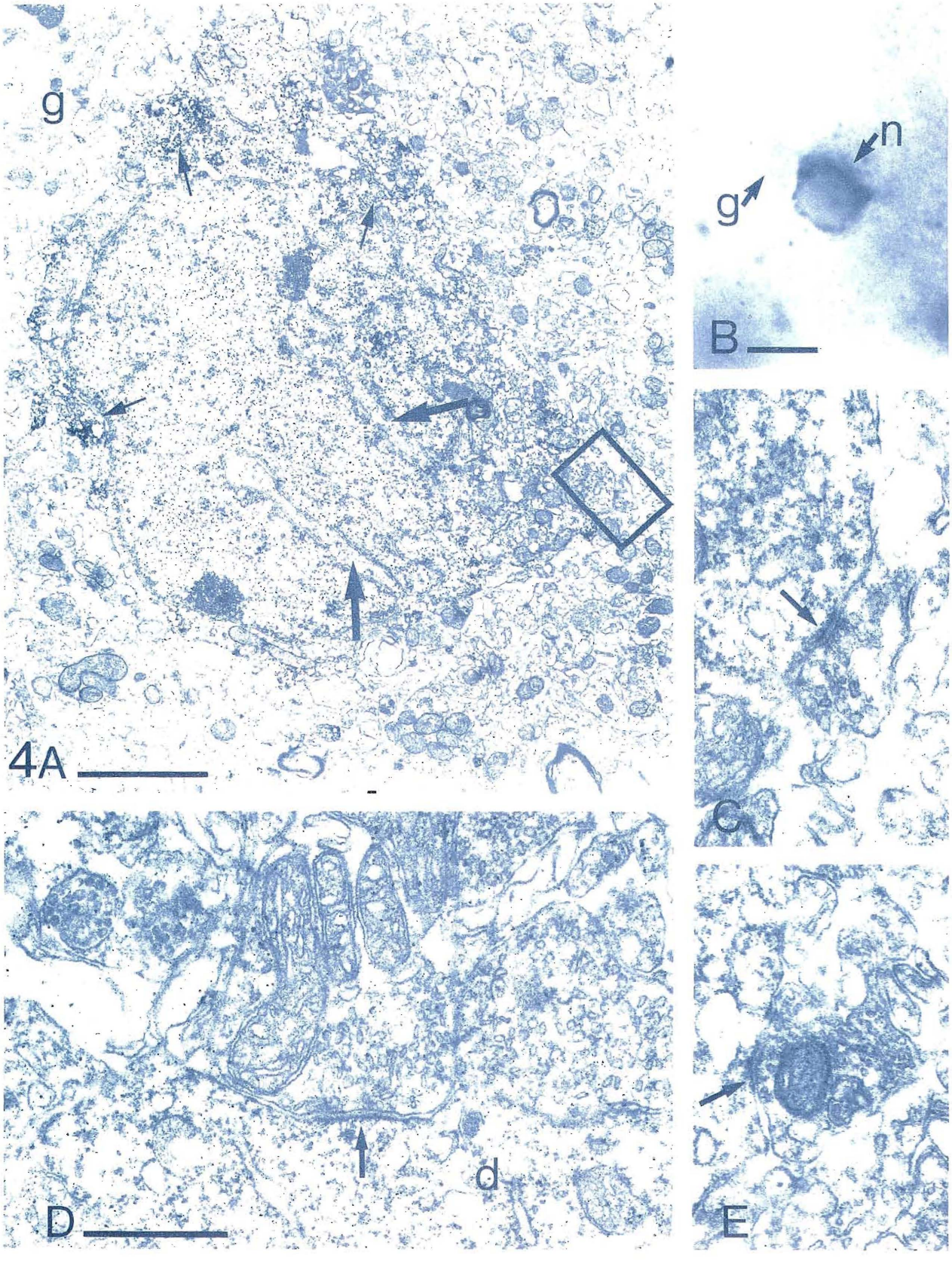
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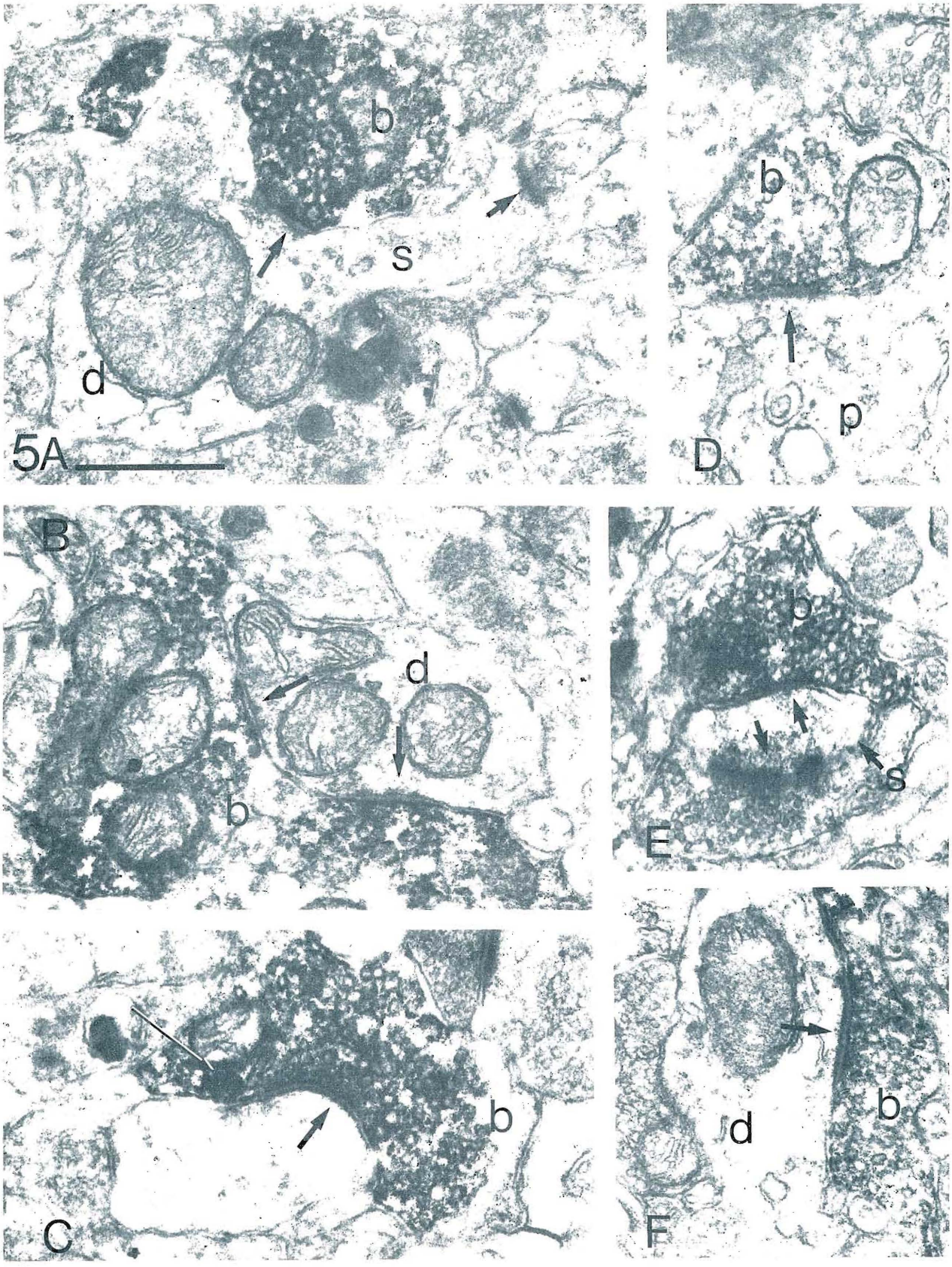
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bouton that was also immunoreactive for substance P (Fig. 2A). The bouton was very similar in morphology to the boutons originating from the collaterals of the immunoreactive axons. (Compare Fig. 2A with 2C, 1D and 5F.)

Type II substance P-immunoreactive neurons (8 were studied) were also of medium size and had round or oval perikarya (Fig. 4B). However, in contrast to neurons of type I, the centrally located nucleus characteristically had several deep invaginations (Fig. 4A). The nuclear invaginations often gave the nucleus a polylobulated appearance. There was only a thin rim of cytoplasm and it contained very few organelles; less cytoplasm and organelles than in type I neurons. (Compare Figs. 1B and 4A.) The synaptic input to the perikaryon was very sparse, consisting of only occasional small boutons, containing pleomorphic vesicles, which made symmetrical membrane contacts (Figs. 4C, 4E). This type of bouton was similar to the boutons containing pleomorphic vesicles that made contact with the type I substance P-immunoreactive neurons. (Compare Figs 4C and E with 3C.) Proximal dendrites were similarly sparse in organelles; the synaptic input also consisted of boutons making symmetrical contact and containing pleomorphic vesicles (Fig. 4D).

SUBSTANCE P-IMMUNOREACTIVE BOUTONS

Many immunoreactive varicosities were seen in the electron microscope, but not all of them were studied in serial sections. Fifty-six substance P-immunoreactive synaptic boutons were examined in detail. These boutons were fairly large (0.5 μm to 1 μm in cross-section), contained many electron-lucent large round and oval vesicles and all of them formed symmetrical membrane contacts (Figs. 5A–F). They occasionally contained dense core vesicles which were sometimes immunoreactive (Fig. 5C), although both reactive and non-reactive dense core vesicles have been observed in the same bouton. The boutons formed by the axon collaterals of type I immunoreactive neurons (Figs. 1D, 2C, 5F) were of this type.

Forty of the identified immunoreactive boutons made synaptic contact with dendritic shafts (Figs. 1D, 5A, B). Four of the postsynaptic dendrites were observed to possess spines and two other postsynaptic dendrites were the proximal dendrites of neurons that were ultrastructurally similar to medium-size densely spiny neurons. Seven of the boutons formed contacts with dendritic spines (Fig. 5E) and the postsynaptic elements of three boutons could not be distinguished but were either spines or small dendrites (Fig. 5C). Five boutons made contact with perikarya and four of these were ultrastructurally similar to medium-size densely spiny neurons (Fig. 5D). The other neuron had an indented nucleus and so did not belong to the medium-size spiny class. Only one immunoreactive bouton was seen to make an axo-axonic contact and that was with the initial segment of an immunoreactive axon; see above (Fig. 2A).

Discussion

The results allow us to suggest answers to the questions set out in the Introduction, although sometimes we have to do this by comparison between the present findings

and those obtained by other methods, rather than by direct analysis. Unequivocal answers to most of the questions would require combination in the same material of several different techniques, such as Golgi-staining, autoradiography, retrograde transport and immunocytochemistry; such combination methods are being developed (see Smith *et al.*, 1981), but for the time being we shall have to be content with the use of correlated light and electron microscopic immunocytochemistry. As pointed out previously (Somogyi & Takagi, 1982), this correlation method makes it possible to study in the electron microscope structures which are so rarely stained that it would be almost impossible to find them in electron microscopic sections taken at random, and also to trace structures like axons in the light microscope to their parent cell prior to examination in the electron microscope. We will now discuss the application of this approach to the study of cells and processes of substance P-immunoreactive structures in the neostriatum.

COMPARISON OF SUBSTANCE P-IMMUNOREACTIVE NEURONS WITH OTHER CHARACTERIZED NEURONS

Kanazawa *et al.* (1977b), Cuello & Kanazawa (1978) and Ljungdahl *et al.* (1978a) have described substance P-immunoreactive cell bodies in the rat striatum in light microscopic studies. The latter authors described small and medium-sized immunoreactive cells in rostral regions and occasional large multipolar cells somewhat more caudally. Thus, these studies already hinted at the possible presence of more than one type of substance P-immunoreactive cell body, something the present work has confirmed. Since we are able to study the immunoreactive cells in the electron microscope we can compare them with the types of striatal neuron that have been morphologically characterized and also examined ultrastructurally.

Type I substance P-immunoreactive neurons and their identified processes

Many of the features of this type of immunoreactive neuron are similar to those of the neuron most frequently impregnated with the Golgi method, the medium-size densely spiny neuron (Kemp & Powell, 1971; Pasik *et al.*, 1979). At the light microscope level type I neurons were of medium size and had a thin rim of cytoplasm. In the electron microscope these characteristics were confirmed and the ultrastructural features of the cell were found to be similar to those of medium-size spiny neurons that have been studied in the electron microscope after Golgi staining (Somogyi & Smith, 1979; Somogyi *et al.*, 1979, 1981a; DiFiglia *et al.*, 1980; Dimova *et al.*, 1980; Frotscher *et al.*, 1981; Bolam *et al.*, 1981b; Smith *et al.*, 1981) or after filling with horseradish peroxidase (Wilson & Groves, 1980; Bishop *et al.*, 1982). The features in common are a smooth, unindented nucleus, a relatively small amount of perinuclear cytoplasm, and a characteristic type of axosomatic synaptic input. Examination of the processes of the type I substance P-immunoreactive neuron showed other similarities to medium-size spiny neurons. The observation in serial sections of dendrites arising from the cell bodies revealed the presence of spines. The axodendritic and axospinous synaptic input was similar to that of characterized medium-size spiny neurons, as was the type of

synapse on the axon initial segment (Somogyi & Smith 1979; DiFiglia *et al.*, 1980). Furthermore, the boutons formed by identified axon collaterals of these neurons are similar to those of the identified local axon collaterals of medium-size spiny neurons (Wilson & Groves, 1980, Figs. 11–15; Somogyi *et al.*, 1981a, Figs. 4D, 8A, B; Smith *et al.*, 1981, Fig. 3; Bishop *et al.*, 1982, Fig. 6A, B).

Thus, although the evidence is indirect, it seems reasonable to suggest that type I substance P-immunoreactive neurons belong to the class of striatal neuron defined by Golgi staining as the medium-size densely spiny type. Some implications of this suggestion will be discussed below.

Type II substance P-immunoreactive neurons

The comparison of this type of neuron with morphologically characterized neurons in the striatum is more difficult. In contrast to the type I neuron, this immunoreactive neuron had an idented nucleus. However, such a feature is found in several types of neuron in the striatum and so cannot be used for positive identification. We can nevertheless distinguish neurons of type II from several types of identified neuron in the striatum. 1. Type II neurons are not of the medium-size densely spiny type (see above). 2. They are distinct from the second type of identified striatonigral neuron (striatonigral type 2 of Bolam *et al.*, 1981b) since the latter neurons are larger, have a large amount of cytoplasm and a heavy axosomatic synaptic input. 3. There are marked differences between type II neurons and the class of smooth dendrite neuron that accumulates locally administered [^{3}H]GABA (Clarke *et al.*, 1982; Bolam *et al.*, 1983). The presumed GABAergic neurons do not have such prominent nuclear indentations, they have a larger amount of cytoplasm, and they receive asymmetrical as well as symmetrical synapses on their perikaryon. 4. The giant neuron that is present in Golgi material (Pasik *et al.*, 1979) is clearly much larger. 5. The neuron that is immunoreactive for somatostatin (Takagi *et al.*, unpublished observations) is similar in that it has an indented nucleus but can be distinguished on the basis of its axosomatic input: the somatostatin-immunoreactive neuron receives asymmetrical as well as symmetrical synapses on its perikaryon and the symmetrical synapses are formed by large boutons, in contrast to the small boutons in contact with the perikaryon of type II substance P-immunoreactive neurons. 6. Finally, the neuron that is immunoreactive for enkephalin (Pickel *et al.*, 1980; DiFiglia *et al.*, 1982) does not have an indented nucleus and may be of the medium-size spiny type.

We suggest that the type II substance P-immunoreactive neuron may belong to the medium-size aspiny class, which has been shown to be heterogeneous (Pasik *et al.*, 1979; Dimova *et al.*, 1980; Danner & Pfister, 1981; Bishop *et al.*, 1982), although further work is clearly necessary.

SUBSTANCE P-IMMUNOREACTIVE BOUTONS

Earlier studies at the light microscopic level (Cuello & Kanazawa, 1978; Ljungdahl *et al.*, 1978a, b) have shown the presence in the neostriatum of a moderately dense network of fine substance P-immunoreactive fibres, but it required ultrastructural studies to see

whether any of these structures were actually nerve terminals. Our finding that substance P-immunoreactivity occurs within vesicle-containing varicosities that form synaptic contacts provides such evidence. It is noteworthy that all 56 immunoreactive varicosities studied in detail, using serial sections where necessary, formed typical synaptic contacts. A similar finding was made for substance P-immunoreactive boutons in the substantia nigra (Somogyi *et al.*, 1982a) and for enkephalin-immunoreactive boutons in the striatum (Somogyi *et al.*, 1982b). Our findings thus provide a morphological basis for the view that substance P can act as a synaptic transmitter in the neostriatum.

The immunoreactive synaptic boutons observed at random in the striatum were all of one morphological type and were similar to the boutons of local axon collaterals from identified type I substance P-immunoreactive neurons (see above) and these in turn, despite the colchicine-treatment, were very similar to the boutons of local axon collaterals of medium-size spiny neurons. It is possible, therefore, that all the substance P-immunoreactive boutons observed may have been from axon collaterals of a type of medium-size spiny neuron.

Some of the postsynaptic targets of the substance P-immunoreactive neurons had the ultrastructural characteristics of medium-size spiny neurons. This is consistent with other studies which have indicated that the recurrent axon collaterals of medium-size spiny neurons may form synapses on other medium-size spiny neurons (Park *et al.*, 1980; Wilson & Groves, 1980; Somogyi *et al.*, 1981a; Smith *et al.*, 1981; Bishop *et al.*, 1982). It is clear, however, that there are other postsynaptic targets of the substance P-immunoreactive boutons. Thus, one of the perikarya receiving such a bouton had an indented nucleus and thus was not a medium-size spiny neuron. It is noteworthy that the postsynaptic targets differ in one fundamental respect from those of enkephalin-immunoreactive boutons: the latter occur all around the cell body of a neuron that is very similar to the second type (Bolam *et al.*, 1981b) of striatonigral neuron (DiFiglia *et al.*, 1982; Somogyi *et al.*, 1982b); substance P-immunoreactive boutons were never observed to form such contacts.

POSSIBLE IMPLICATIONS FOR NEURONAL CIRCUITS

Biochemical and immunohistochemical studies after the placement of lesions have led to the view that substance P is one of the major transmitters of striatal efferent pathways to the substantia nigra, globus pallidus and entopeduncular nucleus (see review by Nicoll *et al.*, 1980; Staines *et al.*, 1980; Kanazawa *et al.*, 1980; Sperk & Singer, 1982). The identification of two types of substance P-immunoreactive neurons in the striatum raises the question whether one or both cell types project from the striatum and whether they have different projection areas. Since we were able to study in the electron microscope synaptic boutons derived from axon collaterals of identified type I neurons, we can compare their ultrastructural features with those of substance P-immunoreactive boutons in the substantia nigra. Two types of such immunoreactive bouton have been found in the substantia nigra (Somogyi *et al.*, 1982a): one type (type 1 of the latter

study; Fig. 1C) contained large electron-lucent vesicles, occasional large granulated vesicles and formed symmetrical synapses. These are the features of the boutons of the local axon collaterals of our type I substance P neurons in the striatum and of boutons in the nigra that can be labelled by the anterograde transport of horseradish peroxidase (HRP) from the striatum (Somogyi *et al.*, 1981b; Figs 5, 9). Thus, we suggest that the type I neurons (assumed to be medium-size spiny neurons: see above) might be the source of the substance P projection to the substantia nigra. This suggestion is compatible with direct evidence from combined HRP and Golgi studies which have shown that the medium-size spiny neurons project to the nigra (Somogyi & Smith, 1979; Somogyi *et al.*, 1979) and that some of these identified projection neurons have local axon collaterals in the striatum whose boutons have similar features to those of our type I substance P-immunoreactive axon collaterals (Somogyi *et al.*, 1981a; Smith *et al.*, 1981).

The question may now be asked: what are the morphological features of the terminals of the type II substance P-immunoreactive neuron, since no bouton could be traced along an axon to one of these neurons? There are two possible answers to this question. First, it is possible that the striatal substance P-immunoreactive terminals are in fact heterogeneous but that we were unable to distinguish more than one type, or only detected one type. Second, it is possible that type II neurons are also projection neurons but that they do not have any local axon collaterals in the striatum. Neurons with the morphological features of type II neurons have not been identified so far in ultrastructural studies on striatal neurons that have been retrogradely labelled from the substantia nigra (Bak *et al.*, 1978; Somogyi & Smith, 1979; Somogyi *et al.*, 1979, 1981a; Bolam *et al.*, 1981b; Henderson, 1981). So far, there do not appear to have been any ultrastructural studies on striatal neurons that have been retrogradely labelled from the globus pallidus or entopeduncular nucleus and so it remains possible that our type II neurons project to one of these regions, although it must be pointed out that medium-size spiny neurons have also been shown to project to the globus pallidus (Leontovich, 1954; Chang *et al.*, 1981; Bishop *et al.*, 1982).

It can be concluded that only our observations on the type I substance P-immunoreactive neuron can be related at present to neuronal circuits. It seems possible that this type of neuron may belong to the class of medium-size spiny neurons that project to the substantia nigra, and that its local axon collaterals in the striatum can influence at least two types of neurons, including other medium-size spiny neurons. Since a high proportion of medium-size spiny neurons are projection neurons (Bolam *et al.*, 1981a) it is probable that projecting substance P-containing neurons could influence other projecting neurons. It is noteworthy that we observed a substance P-immunoreactive bouton forming a synapse on an axon initial segment of a substance P-immunoreactive neuron, so the influence of local substance P-containing terminals may not be confined to dendrites and perikarya.

POSSIBLE FUNCTIONAL IMPLICATIONS

Very little is known about the possible actions of substance P in the striatum. There has been a brief report about responses of neurons in the rat striatum to substance P applied

by microiontophoresis (Le Gal La Salle & Ben-Ari, 1977). These authors found that the spontaneous rate of firing of half of the neurons studied was slightly increased, but that the effect was slow in onset. At higher currents there was initially a brief depression of the firing rate. As mentioned in the Introduction, substance P can influence the rate of release of dopamine and 5-hydroxytryptamine from slices of striatum (Starr, 1978). Our finding of synaptic boutons immunoreactive for substance P suggests that neurons in the striatum might well be sensitive to this peptide, but we did not find the type of synaptic relationship that could account for the effects of the peptide on the release of transmitters from the terminals of afferent fibres.

Probably the most significant of our findings from a functional point of view is that of synaptic boutons that are likely to contain substance P and are therefore probably the site from which it is released. Since the release of substance P has been demonstrated from the striatum (see the Introduction), further work is now necessary to identify the possible actions of substance P in this part of the brain. The degeneration of striatonigral and striatopallidal substance P-containing pathways is thought to occur in Huntington's disease (Kanazawa *et al.*, 1977a; Gale *et al.*, 1978; Emson *et al.*, 1980) and a large decrease in the striatal concentration of substance P has been reported in the disease, most notably in the putamen (Buck *et al.*, 1981). Our findings of substance P-immunoreactive synaptic boutons along local axon collaterals of presumptive striatonigral neurons raises the question whether the loss of substance P in the striatum has any local consequences for the functioning of the striatum in Huntington's disease, as well as being a reflection of the loss of substance P-containing neuronal cell bodies which project to other regions.

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