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Synaptic connections of enkephalin-immunoreactive nerve terminals in the neostriatum: a correlated light and electron microscopic study

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Summary

Two different antisera to leucine-enkephalin were used to study the localization of enkephalin-like immunoreactive material in the neostriatum and globus pallidus of the rat, by means of the unlabelled antibody–enzyme method. Thin immunoreactive varicose fibres are scattered throughout the neostriatum. In the ventral striatum, fibres come together and follow a relatively straight course for several micrometers, forming tube-like structures which can be traced to cell bodies; these cell bodies are completely surrounded by immunoreactive fibres. Occasional immunoreactive varicose fibres are also found close to another type of neuron throughout the whole neostriatum.

Examination by electron microscopy of immunoreactive structures that had been identified first in the light microscope, showed that each of the nearly 200 varicosities examined was a vesicle-containing bouton that formed a synaptic contact. Rarely were asymmetrical synaptic contacts found between immunoreactive boutons and dendritic spines. All other synapses formed by enkephalin-immunoreactive boutons were symmetrical. Two types of postsynaptic neuron were identified; the first type was a medium-sized neuron with the ultrastructural features of a typical striatal spiny neuron. The second type had a larger perikaryon surrounded by numerous immunoreactive varicosities that were found to be boutons forming symmetrical synapses. The long dendrites of this second type of neuron likewise received a dense input of immunoreactive boutons forming symmetrical synapses; such ensheathed dendrites were found to be the

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tube-like structures seen in the light microscope. The ultrastructural features of these neurons, notably a highly indented nucleus, were those of a rare type of striatonigral neuron. In the globus pallidus, all the enkaphalin-immunoreactive boutons studied formed symmetrical synapses with ensheathed dendrites and perikarya that were similar to the latter type of postsynaptic neuron in the neostriatum. Axo-axonic synapses involving immunoreactive boutons were not seen in our material.

The results are consistent with the view that enkephalin-like substances may be synaptic transmitters in the neostriatum and that they may have different actions according to the nature of the postsynaptic target. The finding that one type of neostriatal neuron, and a very similar neuron in the globus pallidus, receives multiple enkephalin-immunoreactive boutons all over its perikaryon and along its dendrites indicates a potentially important role of enkephalin in the convergence of information within the neostriatum and pallidum on to output neurons.

Introduction

The discovery of endogenous opioid peptides in the brain (Hughes *et al.*, 1975) and of their presence in the basal ganglia of rats (Hong *et al.*, 1977; Yang *et al.*, 1977) and man (Cuello, 1978; Gramsch *et al.*, 1979; Emson *et al.*, 1980) has thrown new light on the earlier pharmacological studies which had shown that opiates can influence the chemistry and functioning of the basal ganglia (see reviews by Eidelberg, 1976; Kuschinsky, 1976; Pert, 1978). There is now considerable evidence that the opioid peptides (enkephalins) might be neurotransmitters in the basal ganglia since they occur in nerve fibres (Elde *et al.*, 1976) and nerve cells (Hökfelt *et al.*, 1977); they can be released *in vivo* (Cesselin *et al.*, 1981) and *in vitro* by a calcium-dependent process (see review by Iversen *et al.*, 1980); and they can influence the activity of neurons in the basal ganglia (see review by North, 1979).

Neuroanatomical studies (Cuello & Paxinos, 1978; Correa *et al.*, 1981; Del Fiacco *et al.*, 1982) have shown that in the rat most, if not all, of the enkephalin-containing fibres in the globus pallidus come from neurons in the neostriatum. However, there are also many enkephalin-containing fibres, as well as cells, in the neostriatum of the rat (Elde *et al.*, 1976) and monkey (Haber & Elde, 1982) and the neostriatum of the monkey is even richer in opiate receptors than the globus pallidus (Kuhar *et al.*, 1973). Furthermore, the release of enkephalins can be demonstrated from striatal tissue *in vitro* (Henderson *et al.*, 1978) and from the caudate nucleus *in vivo* (Cesselin *et al.*, 1981). Thus, as well as being a transmitter in a striatopallidal pathway, enkephalin may be a transmitter within the neostriatum. In electron microscopic studies, Pickel *et al.* (1980) have shown enkephalin-like immunoreactivity in synaptic boutons in the rat neostriatum. We have now studied the localization of enkephalin immunoreactivity in the rat neostriatum by correlated light and electron microscopy, using the unlabelled antibody–enzyme method (Sternberger *et al.*, 1970), in order to provide more information about the types of immunoreactive boutons and their postsynaptic targets.

Methods

These studies are based on two series of experiments using different antisera. As both gave identical results, they will be described together. In the first part of this study male Wistar rats, and in the second part CFY strain albino rats, were used. The rats were anaesthetized with Equithesin (composition given in Tagerud & Cuello, 1979) or with chloral hydrate (350 mg/kg i.p.). The animals were perfused with a mixture of paraformaldehyde and glutaraldehyde as fixative (Somogyi *et al.*, 1982) and in later experiments with a picric acid (0.2%)–paraformaldehyde (4%)–glutaraldehyde (0.05%) fixative (Somogyi & Takagi, 1982). Small blocks of the striatum and surrounding areas were washed in 0.1 M sodium phosphate buffer pH 7.4, followed by the same buffer containing 10% sucrose and then the same buffer containing 20% sucrose, each time until the blocks sank. They were then frozen in liquid nitrogen and thawed in 0.1 M phosphate buffer pH 7.4. Sections (70 μm) were cut on a Vibratome (Oxford Instruments) and washed overnight at 4° C in 0.1 M sodium phosphate buffer at pH 7.4.

Sections were incubated for immunocytochemistry as follows: 30 min in 20% goat serum (Cappel); 30 min wash; overnight at 4° C in rabbit anti-leu-enkephalin serum (see below); 3 × 40 min washes; 2 h in goat anti-rabbit IgG fraction diluted 1:40; 3 × 40 min washes; 2 h in rabbit peroxidase–antiperoxidase complex (Cappel) diluted 1:100; 3 × 40 min washes. All dilutions were made with phosphate-buffered saline (PBS) which was used for the washes as well. Goat serum (1%) was added to the rabbit sera.

In the first series of experiments an antiserum (A) to leu-enkephalin that had been prepared and tested by Miller *et al.* (1978) was used at a dilution of 1:300. In the second series of experiments, another antiserum (B) against leu-enkephalin was used (Senba *et al.*, 1982) at a dilution of 1:500.

Specificity of the immunocytochemical reaction was tested with the following control incubations. 1. Replacement of the specific serum by normal rabbit serum; 2. Omission of the specific serum; 3. adsorption of the anti-leu-enkephalin serum to leu-enkephalin (Miles-Yeda Ltd); 4. adsorption of anti-leu-enkephalin serum B to substance P, neurotensin, somatostatin and met-enkephalin (all from Sigma). Adsorption was carried out overnight at 4° C at the final dilution, with each peptide (0.4 mg/ml), followed by centrifugation at 100 000 g for 1 h. The supernatant was used for the test.

Following incubation, the peroxidase activity was detected using 3,3′-diaminobenzidine 4HCl (Sigma) as described elsewhere (Somogyi *et al.*, 1982). Sections were treated with 1% OsO_4 in 0.1 M sodium phosphate buffer pH 7.4 for 1 h at room temperature, dehydrated in ethanol and mounted on slides in Durcupan (Fluka) resin. To enhance the contrast, 1% uranyl acetate was included in the 70% ethanol for 40 min.

Immunocytochemically stained structures were selected in the light microscope for subsequent electron microscopic study of their synaptic connections using the correlation method described elsewhere (Somogyi & Takagi, 1982; Somogyi *et al.*, 1982). Serial ultrathin sections were studied without lead staining at 60 kV, using 20–30 μm objectives in a Phillips 201C electron microscope.

Results

Specificity of the antisera and the method

Antiserum A produced against leu-enkephalin was shown to crossreact less than 1% with met-enkephalin, and much less with β-endorphin in radioimmunoassay (Miller *et al.*, 1978). Antiserum B showed less than 1% crossreactivity with met-enkephalin and

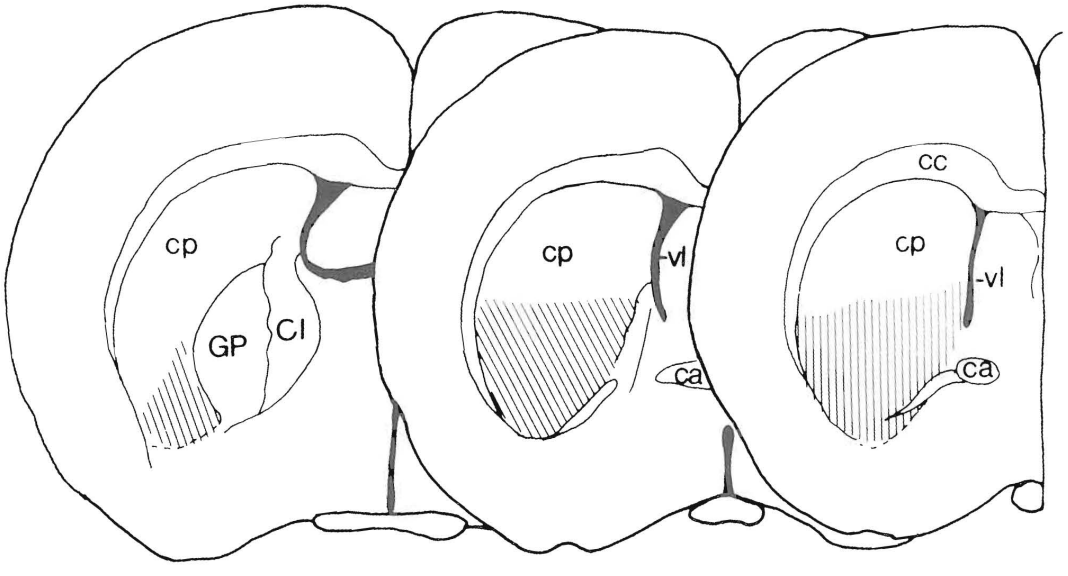


Fig. 1. Drawings of coronal sections of the rat forebrain indicating the parts (shaded) of the ventral caudatoputamen (cp) where neurons and dendrites of type-2, postsynaptic to enkephalin-immunoreactive boutons, were found. ca, commissura anterior; cc, corpus callosum; CI, capsula interna; GP, globus pallidus; vl, ventriculus lateralis.

no crossreactivity with β-endorphin (Senba *et al.*, 1982). Adsorption of both antisera A and B to leu-enkephalin completely abolished immunostaining. Adsorption of antisera B to high concentration of met-enkephalin also greatly attenuated immunostaining. Immunostaining was not affected by adsorption to the other peptides. It is uncertain to what extent crossreactivity between various opioid peptides contributes to immunocytochemical staining in fixed tissue, therefore the terms enkephalin immunoreactivity or immunostaining will be used in the text. This qualification is also necessary because a heptapeptide containing the met-enkephalin sequence has been found in the striatum (Stern *et al.*, 1979).

In controls, including those in which normal rabbit serum was used or the specific serum was omitted, peroxidase reaction endproduct was only found in cell bodies, where it was in characteristic granulated bodies. Endogenous peroxidase activity was never observed in axons or terminals.

Distribution of enkephalin immunostaining

Within the neostriatum–globus pallidus complex very strong immunoreactivity was found in the globus pallidus (Fig. 2A, B). This strong staining ends very sharply at the border with the neostriatum, but at the most ventral aspect of the globus pallidus this

boundary is less abrupt (Fig. 2A, B). The intensity of staining also changes gradually at the border between the neostriatum and the so-called ventral pallidum. Fibre bundles cross from the neostriatum into the globus pallidus, and some immunoreactive fibres (not illustrated) can be traced across the neostriatum/globus pallidus border. The fibre bundles cut in frontal sections in the ventral striatum contain many more immunoreactive fibres (Fig. 5A, B); thus there is a gradient in the staining of fibre bundles from dorsal to ventral striatum. Immunostaining is differently distributed in the ventral and dorsal parts of the neostriatum (Fig. 1), but the difference can be seen only at high magnification. In the present study no attempt was made to relate the sample sites to the patches of enkephalin-positive islands of the caudoputamen described in the cat (Graybiel *et al.*, 1981) as these were not conspicuous in our rat material. Immunoreactive perikarya and dendrites were found in the neostriatum, in agreement with Pickel *et al.* (1980), but we shall confine this report to a description of immunoreactive fibres and the postsynaptic targets of their boutons.

Light microscopic appearance of enkephalin-immunoreactive fibres and the structures closely associated with them

In both the dorsal and ventral caudoputamen, varicose fibres can be observed running without any particular orientation (Fig. 2C). The fibres are very thin and rarely branch. Most of the varicosities are scattered in the neuropil (Fig. 2C) among cell bodies, the outlines of which can be seen clearly in the thick sections mounted in resin (Figs. 3A, 7A). However, in the ventral caudoputamen (Fig. 1) some of the varicose fibres gather together and follow a relatively straight, common course (Figs. 7A, B, 9A). They form a thick sheath around structures presumed to be dendrites (subsequently confirmed by electron microscopy; see below). Where these dendrites branch, the enkephalin immunoreactive fibre and varicose sheath follows the branches. The immunoreactive *'tubes'* formed this way may run for several hundred micrometers. In cross-sections such a structure appears as a ring of immunoreactivity (Figs. 7B, 9A).

The unstained cell bodies show up so clearly, that their size, the emerging dendrites, nucleus and nucleolus all can be seen in the light microscope (Figs. 3A, 4A, 5A–E, 7A). Two types of neuronal perikarya were found to be associated with immunoreactive varicose fibres.

'Type-1' neurons are medium sized with a small amount of cytoplasm around the nucleus (Figs. 3A, 4A). Three to four dendrites taper gradually from the cell soma. Only a few immunoreactive varicosities are situated close to the cell bodies and proximal dendrites; usually one or two were observed. Some of the fibres may provide several varicosities to the same perikaryon (Figs. 3A, 4A).

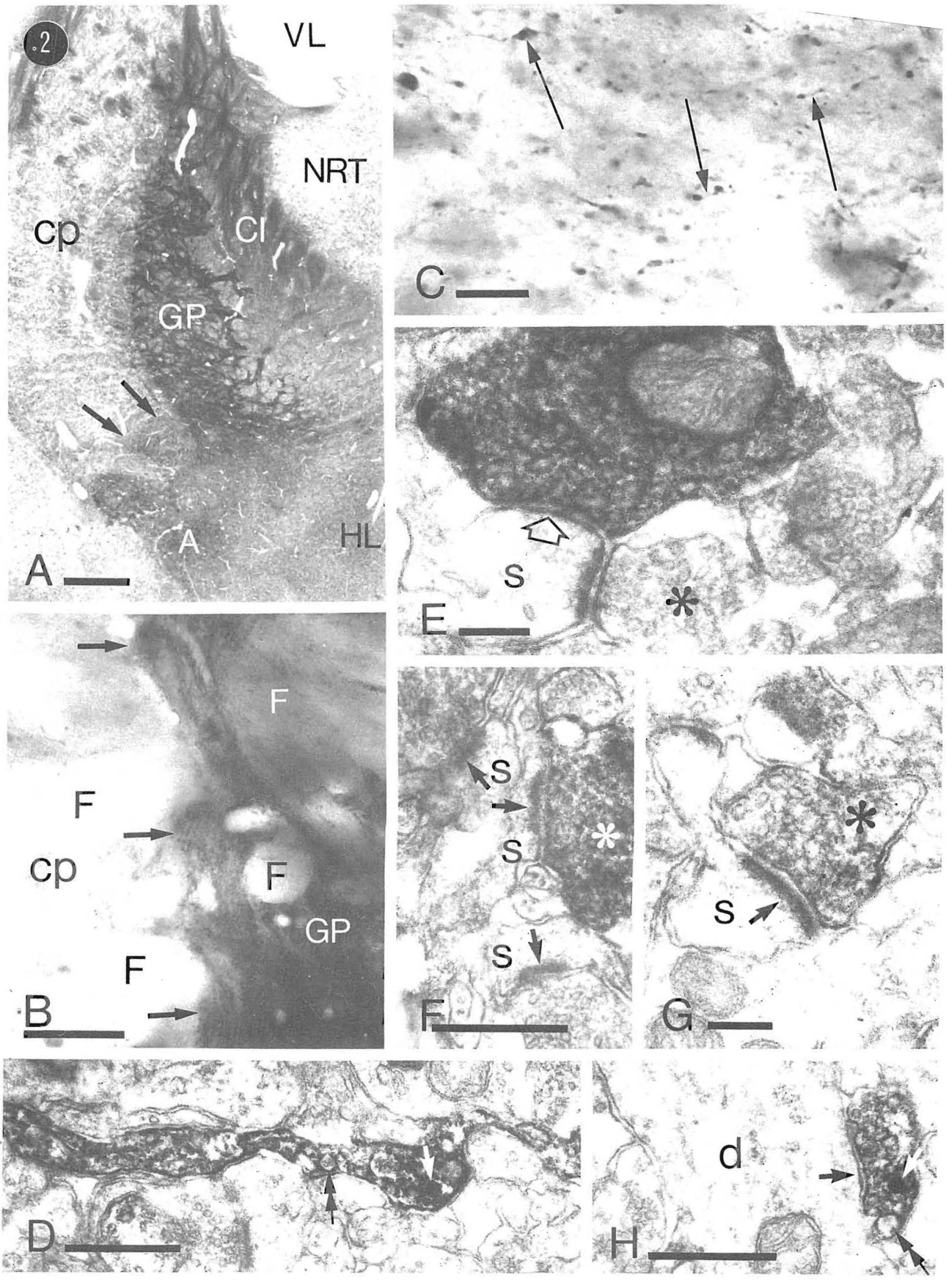
'Type-2' neurons associated with immunoreactive structures are larger and usually ovoid or spindle-shaped (Figs. 5A–E, 7A). Rarely are they embedded in fibre bundles (Fig. 5A–E). Two to three thick dendrites leave the perikaryon and sometimes begin

branching immediately. So far these neurons have only been found in the ventral striatum (Fig. 1). They are completely surrounded by immunoreactive varicose fibres, which also follow the dendrites. The dendrites seen in isolation (Figs. 7B, 9A) and surrounded by immunoreactive varicosities undoubtedly belong to these neurons. The cell bodies have only been seen more ventrally than the isolated dendrites, which are scattered over the area indicated in Fig. 1.

Fig. 2. (A–C) Light micrographs of 70 μm thick osmium-treated sections reacted for leu-enkephalin and mounted in epoxy resin. Strong immunoreactivity is observed in the globus pallidus (GP) in (A) and (B). Arrows in (B) indicate where the strong immunoreactivity ends abruptly at the lateral border of the globus pallidus with the caudatoputamen (cp). Arrows in A indicate the 'ventral striatum' where the immunoreactivity is stronger. Fibre bundles (F) in the neostriatum and globus pallidus contain little or no immunoreactivity. The neuropil in the neostriatum contains many immunoreactive varicose fibres (arrows in C). (D–H) Electron micrographs from the neostriatum. (D) Small diameter immunoreactive unmyelinated axon containing both stained (white arrow) and unstained (double arrow) large granulated vesicles. (E) Spine (s) receiving a symmetrical synapse (arrow) from an immunoreactive bouton and an asymmetrical synapse from an unstained bouton (asterisk). (F) Spines in the caudal neostriatum receiving asymmetrical synapses; one of the boutons is strongly immunoreactive. (G) Another stained bouton (asterisk) making an asymmetrical synapse (arrow) with a spine (s) in the rostral neostriatum. (H) Small immunoreactive bouton, containing an immunoreactive large granulated vesicle (white arrow) and a non-reactive vesicle (double arrow), making a symmetrical synapse (arrow) with a type-1 dendrite (d). Abbreviations: see caption to Fig. 1; A, amygdala; HL, hypothalamus lateralis; NRT, nucleus reticularis thalami; VL, ventriculus lateralis. Scale bars: (A) 0.5 mm; (B) 50 μm; (C) 10 μm; (D, F, H) 0.5 μm; (E, G) 0.2 μm.

Fig. 3. (A) Light micrograph of a medium sized type-1 neuron (N_1) emitting a dendrite (d) and in close apposition to an immunoreactive fibre consisting of five varicosities (b_{1-5}). (B) The same neuron (N_1) and the five varicosities (b_{1-5}) at the electron microscopic level; the framed area is shown in (E). (C) Four sections away from the section shown in (B), three boutons (b_{1-3}) of the fibre can be seen to make symmetrical synaptic contact (arrows) with the neuron. (D) The synapse (arrow) of one bouton (b_1) is shown at high magnification. (E, F) Serial sections of one bouton (b_5) which establishes a synapse (arrow) with a dendrite (d) which was not seen to originate from neuron N_1. Scale bars: (A) 10 μm; (B) 1 μm; (C, E, F) 0.5 μm; (D) 0.2 μm.

Fig. 4. (A) Light micrograph of the same neuron (N_1) as in Fig. 3. Two varicosities ($b_{1,2}$) of the associated immunoreactive fibre are shown at a different focal depth. (B) Electron micrograph of the neuron in contact with the two boutons ($b_{1,2}$) at the shoulder of an emerging dendrite (d). (C) The two boutons ($b_{1,2}$) are seen at higher magnification; one of them (b_2) is in synaptic contact (open arrow) with the perikaryon. Immunoreactive (arrow) and nonreactive (double arrow) large granulated vesicles are indicated. (D) One bouton (b_2) is seen on the next serial section making symmetrical synaptic contact (open arrow). The synaptic vesicles of bouton b_2 can be compared with those in a non-immunoreactive bouton making an asymmetrical synaptic contact (thick arrow) with a spine and another bouton (asterisk) containing small flattened vesicles. Note prominent presynaptic dense projections (white arrows). Scale bars: (A) 5 μm; (B) 1 μm; (C) 0.5 μm; (D) 0.2 μm.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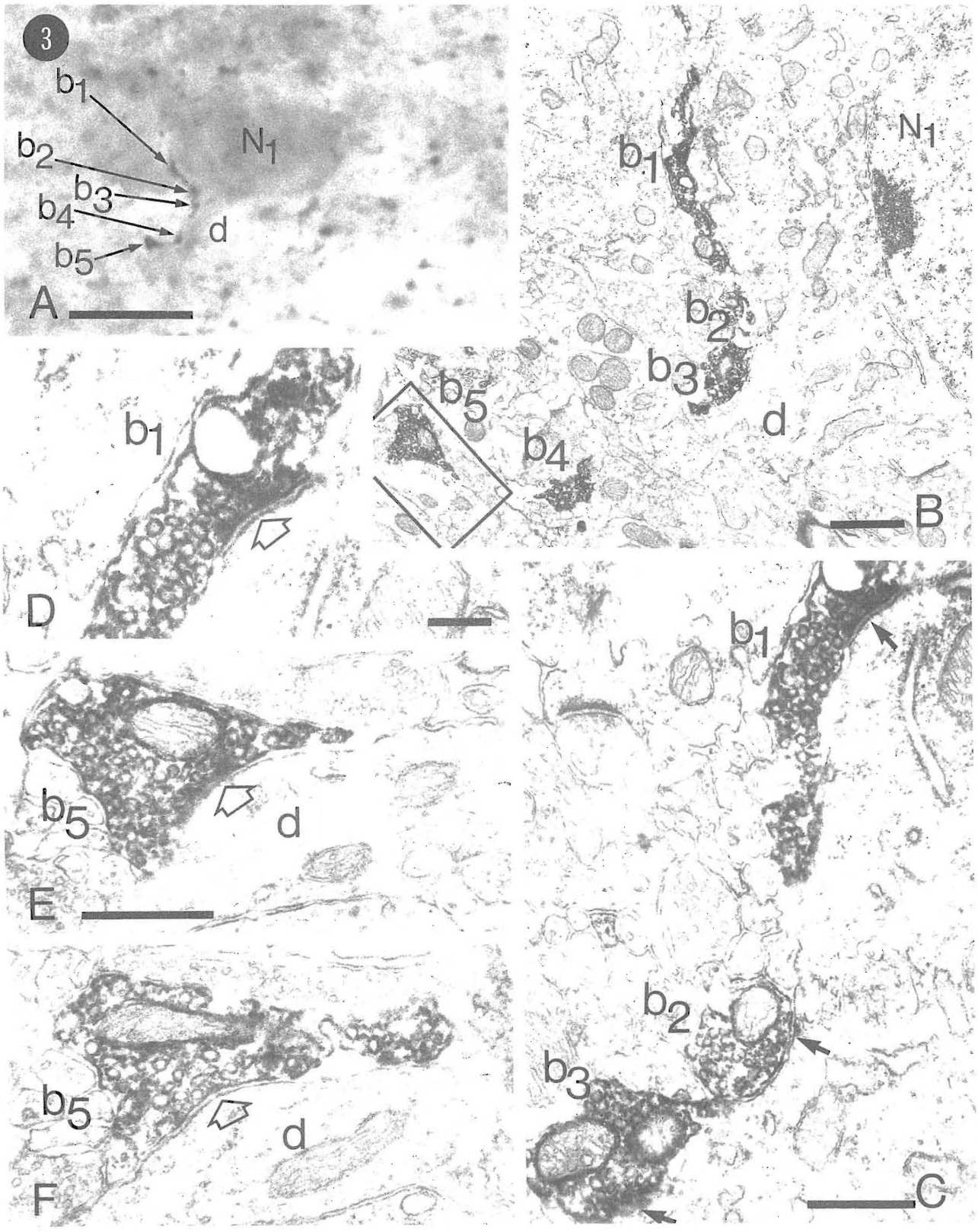
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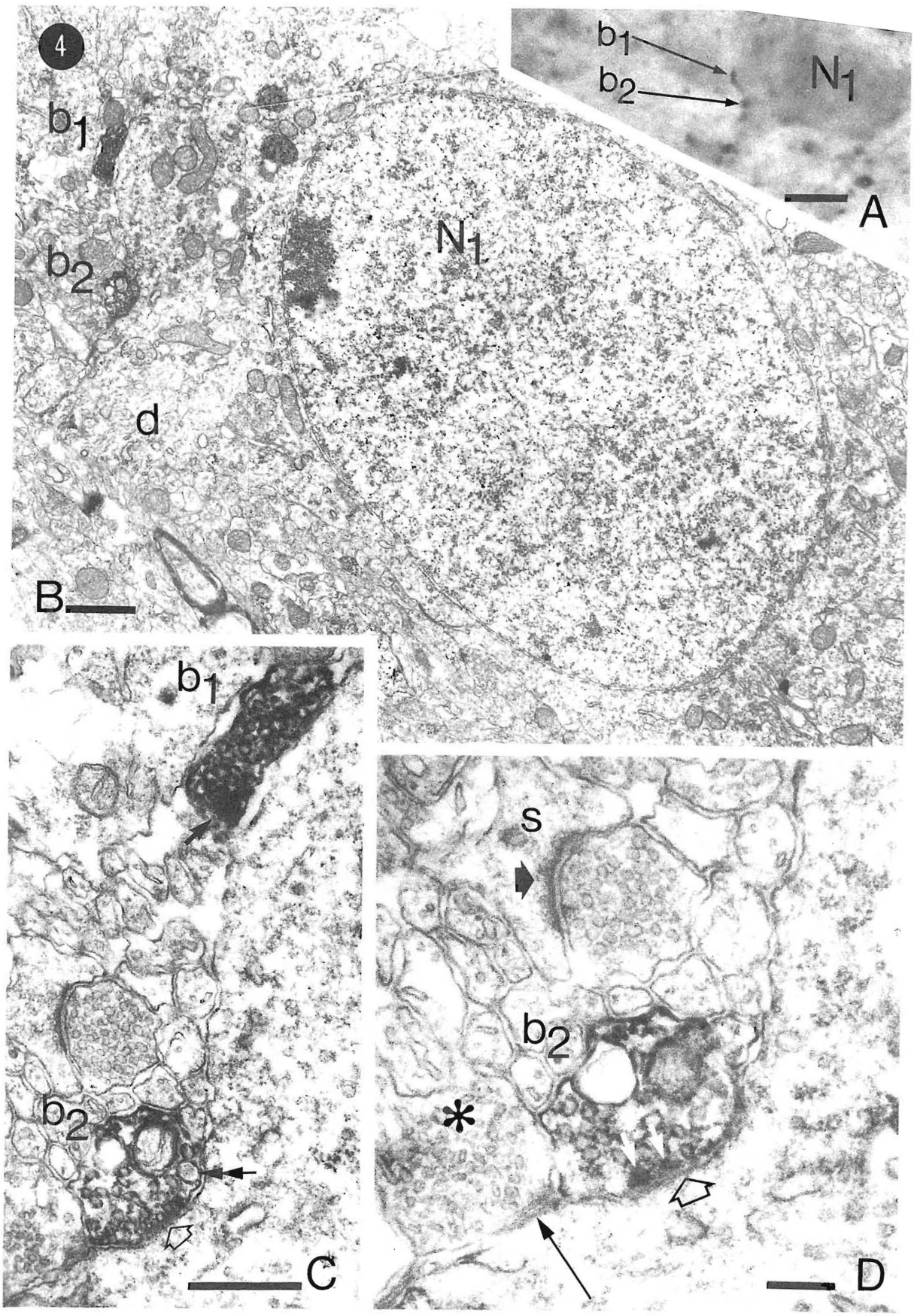
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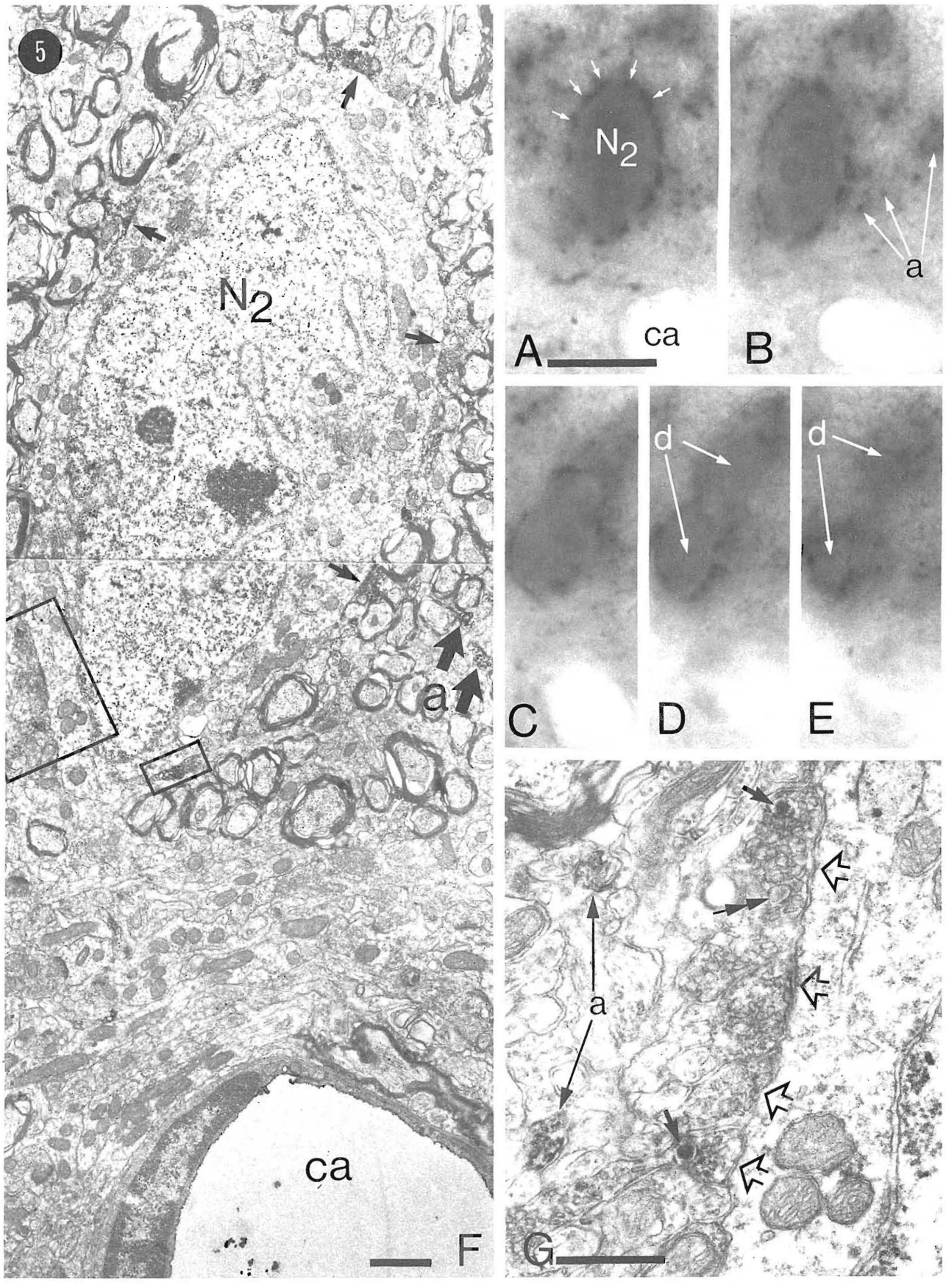
A third type of neuron recognizable in the light microscope, the typical striatal giant cell (Leontovitch, 1954; Pasik *et al.*, 1979), was not seen in association with immunoreactive varicosities.

The way our sections were processed made it possible to select light microscopically identified immunoreactive structures for electron microscopy. The following structures were studied: 1. immunoreactive fibres and varicosities in the neuropil from both the dorsal and ventral striatum; 2. immunoreactive fibres and varicosities following the presumed dendrites of type-2 neurons; 3. type-1 neurons with associated immunoreactive varicosities from both the dorsal and ventral striatum; 4. type-2

Fig. 5. (A–E) Light micrographs taken at different focal depths of a type-2 neuron (N_2) completely surrounded by immunoreactive varicosities (some marked by short white arrows) in the rostral neostriatum. Long white arrows in (B) mark immunoreactive axons (a) running perpendicular to the plane of the section in a fibre bundle in which the neuron is embedded. At one pole of the neuron two thick dendrites (d) also surrounded by immunoreactive structures are emerging. (F) Low-power electron micrograph showing the same neuron (N_2) surrounded by immunoreactive synaptic boutons (small arrows). Note the immunoreactive axons (a) in the fibre bundle (thick arrows). A capillary (ca), also seen in (A), serves as an identifying feature. The electron-dense lining on the inner surface of the endothelial cell is due to uranyl acetate staining. The large framed area is shown in (G) and the small framed area in Fig. 6G. (G) Weakly immunoreactive preterminal boutons completely ensheath the perikaryon (arrows). Some large granulated vesicles are immunoreactive (arrows); others are not (double arrow). Small diameter immunoreactive unmyelinated axons (a) can be seen nearby. Scale bars: (A–E) 10 μm; (F) 1 μm; (G) 0.5 μm.

Fig. 6. (A) The same type-2 neuron as in Fig. 5. Immunoreactive boutons in the framed areas are shown at a higher magnification in B, C and E, where it can be seen that they make symmetrical synapses (thick arrows). Open arrows mark small unmyelinated immunoreactive axons running along the surface of the neuron. The filled arrow marks a synapse established by an immunoreactive bouton that is shown in a serial section in (D); besides the symmetrical synaptic contact (thick arrow), this bouton also shows a puncta adherentia (asterisk) with the perikaryon. (E, F) Serial sections of a bouton within one of the framed areas in (A). Note prominent presynaptic dense projections (white arrows, C, F), immunoreactive (small arrow, F) and nonreactive (double arrow, F) large granulated vesicles and the pleomorphic clear vesicles in the boutons. (G) High-power micrograph of the area in the small frame shown in Fig. 5F; immunoreactive bouton forms a symmetrical contact (thick arrow). Scale bars: (A) 0.5 μm; (B–G) 0.2 μm.

Fig. 7. (A) Light micrograph of a type-2 neuron (N_2) in the neostriatum. Both the perikaryon and dendrites (d) are surrounded by immunoreactive fibres. (B) Light micrograph of a large branching type-2 dendrite (d) surrounded by immunoreactive fibres and varicosities. One branch (open arrow) runs between the outlines of a neuron (N), a fibre bundle (f) and an astroglial cell (Gl) as also shown in the electron micrograph in Fig. 8A. A segment of the dendrite between the arrows (C) is shown in electron micrographs of serial sections in (C) and (D). Double arrow points to a similar dendrite in cross-section which is surrounded by immunoreactive boutons. (C and D) Serial sections of the portion of the dendrite between arrows C in (B); the dendrite is completely ensheathed in boutons, most of which are immunoreactive (asterisk) and which make symmetrical synapses (arrows) and sometimes puncta adherentia (curved arrows) with the dendrite. Scale bars: (A) 50 μm; (B) 10 μm; (C, D) 0.5 μm.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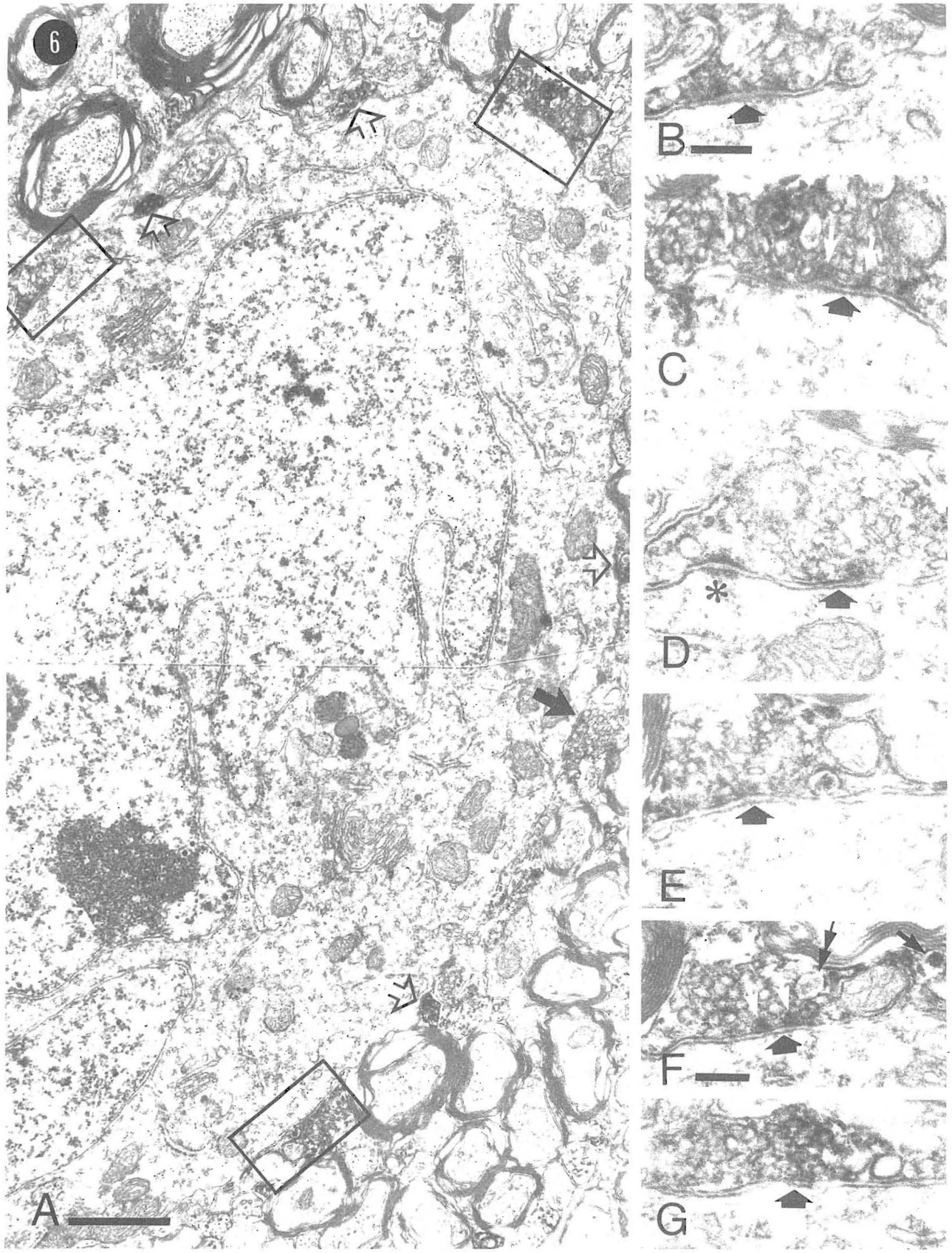
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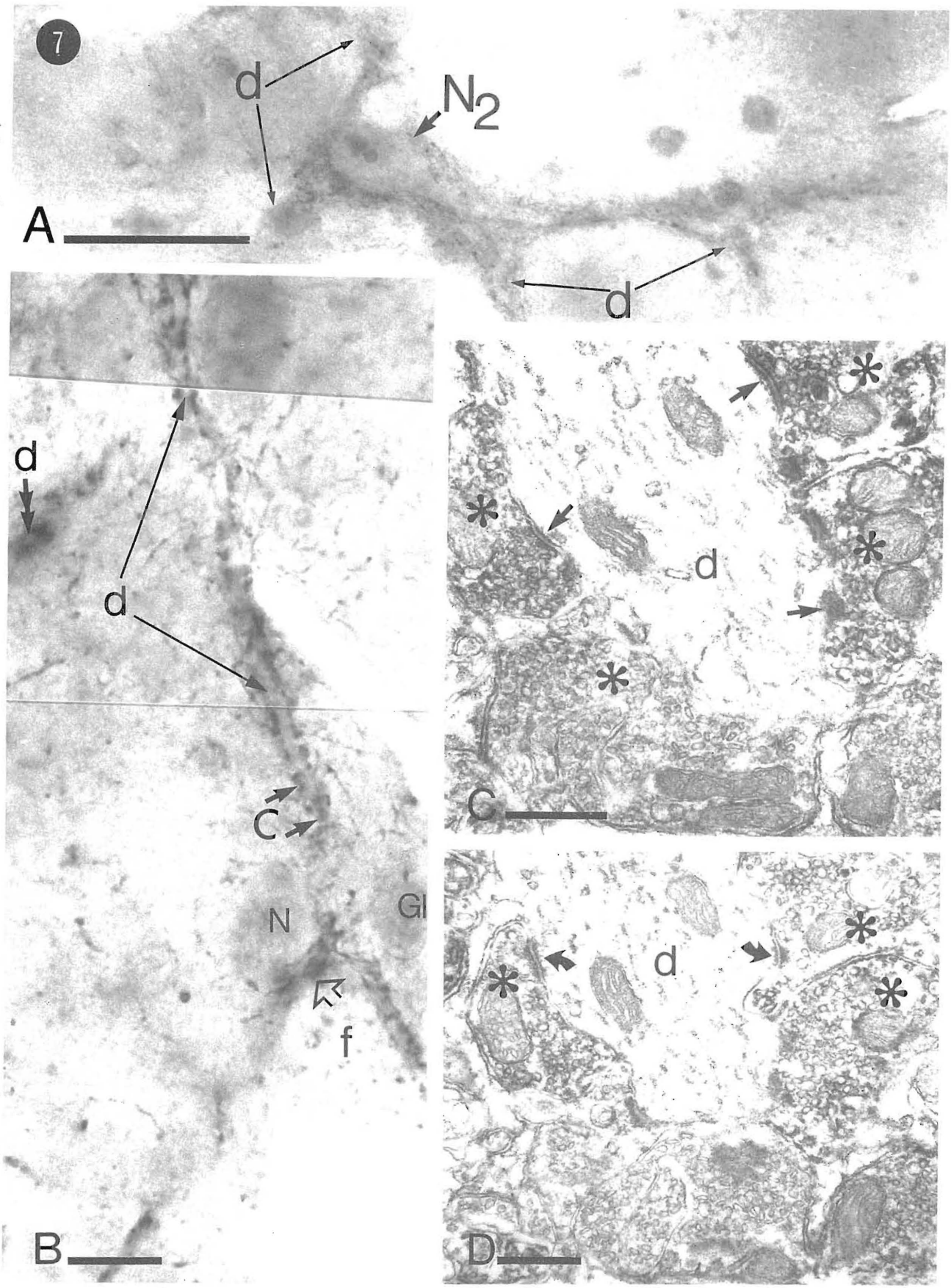
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neurons in the ventral striatum; 5. two areas of the globus pallidus were also studied for comparison of bouton and synapse types. Besides the identified structures, a large number of other immunoreactive boutons were studied when they came into the plane of the serial sections.

Electron microscopic characteristics of enkephalin-immunoreactive axons and nerve terminals

All axons in the neuropil and almost all immunoreactive axons in the fibre bundles were very thin and unmyelinated (Figs. 2D, 5F, G).

The peroxidase reaction endproduct within any stained structure was associated with the surface of all cell organelles; e.g. with the outer surface of microtubules, mitochondria, smooth endoplasmic reticulum and small electronlucent vesicles, as well as with the inner surface of the plasma membrane.

Large granulated vesicles in immunoreactive axons and nerve terminals contained reaction endproduct both around their membrance as well as over their granulated core (Figs. 2D, H, 4C, 5G, 6C, E, F). In an individual terminal there were only a few large granular vesicles, and some immunoreactive terminals that were completely sectioned serially were found not to have any. In many boutons some granulated vesicles were immunoreactive while others were completely devoid of reaction endproduct (Figs. 2D, 4C, 5G, 6F).

Two different types of enkephalin-immunoreactive boutons

Almost two hundred varicosities identified in the light microscope were sectioned serially and studied in the electron microscope. All of the varicosities that contained clear vesicles made synaptic contact. At least two types of synaptic contact could be distinguished.

Type A synaptic contacts made by enkephalin immunoreactive boutons were asymmetrical (or Gray's type I, Fig. 2F, G). They were found in both the dorsal and ventral part of the caudoputamen but they were extremely rare, only four having been found in the present study. The postsynaptic structure was in all cases a dendritic spine. The immunoreactive boutons contained small clear synaptic vesicles (Fig. 2F, G).

Type B synaptic contacts made by enkephalin immunoreactive boutons were

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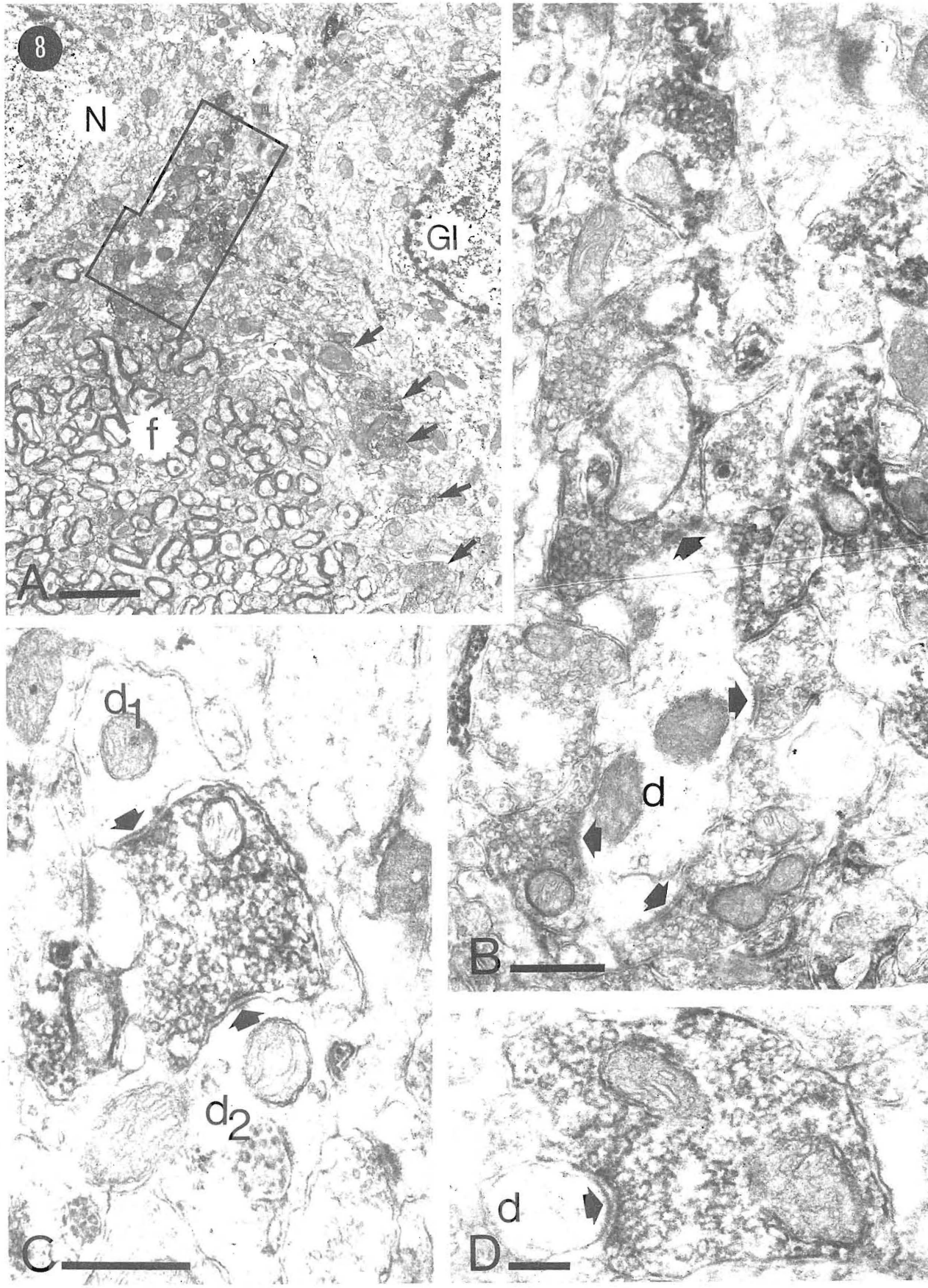
Fig. 8. (A) Low-power electron micrograph of the branch of a type-2 dendrite indicated by the open arrow in Fig. 7B. A neuron (N), a fibre bundle (f) and an astroglial cell (Gl) serve as identification marks for the correlation of the light and electron micrographs. Arrows indicate a row of immunoreactive boutons which followed the other branch of the dendrite (d) but which is not in the plane of the section at this level. Framed area is shown in (B). (B) The dendrite (d) is surrounded by tightly packed immunoreactive axons and boutons, some of them making symmetrical synapses (thick arrows). (C) An imunoreactive bouton makes symmetrical synapses (arrows) with two type-1 dendrites ($d_{1,2}$) which do not receive any other synapses in this section. (D) A distal type-1 dendrite (d) receives a symmetrical synapse from an immunoreactive bouton. Scale bars: (A) 2 μm; (B, C) 0.5 μm; (D) 0.2 μm.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symmetrical (Gray's type II) with a small postsynaptic density, but sometimes they had striking presynaptic dense projections (Figs. 3D, F, 4D, 6C, F) enhanced by the accumulation of horseradish peroxidase (HRP) reaction endproduct. The boutons contain pleomorphic clear vesicles that were larger than those in most nerve terminals in the neostriatum; for example, they were larger than those in boutons forming asymmetrical axo-spinous contacts (Figs. 2H, 3–6, 7C, D, 8, 9C, D). Type B boutons also contained both immunoreactive and non-reactive large granulated vesicles. Some of the boutons were small and of the *en passant* type (Fig. 2H, and b in Figs. 3, 4) others were large bulbous swellings of the thin axons (Figs. 5–8). Some fibres were tested by serial sectioning of several varicosities along their course to establish whether they each made the same type of synapse. Up to four boutons originating from the same fibre were studied this way and they were all found to make type B symmetrical contacts (Figs. 3, 4). Boutons forming type B contacts were often found to make a *punctum adherens* with the postsynaptic structure in addition to the synaptic contact (Figs. 6D, 7D).

The postsynaptic structures of enkephalin immunoreactive boutons

Axo-somatic synapses were established with two distinct types of postsynaptic neuron. Type-1 neurons were of medium size and had a round or ovoid nucleus with homogeneously distributed chromatin. They had a thin rim of cytoplasm, except at the base of the three to four primary dendrites which tapered gradually from the perikaryon (Figs. 3, 4). Except for microtubules and mitochondria, the dendrites were poor in cell organelles. Synaptic contacts were rare on both the proximal dendrites and on the perikaryon. All these synapses were symmetrical and two distinct types of boutons provided them. One type contained small flattened vesicles (Fig. 4D) the other large pleomorphic vesicles. Most of these latter boutons were immunoreactive type-B boutons (Figs. 3, 4). One immunoreactive fibre made only a few, usually just one, synaptic contact with an individual perikaryon; the maximum number of synapses received by a type-1 neuron from a single fibre was three (Figs. 3, 4). Type-1 neurons received few synapses from immunoreactive boutons; the maximum number on an individual perikaryon was seven.

Type-2 neuronal perikarya that were postsynaptic to enkephalin immunoreactive boutons were distinct from type-1 neurons both in their fine structure and in their synaptic input (Figs. 5, 6). The light microscopic identification of these neurons prior to electron microscopy was essential because they were sparse and found only in the

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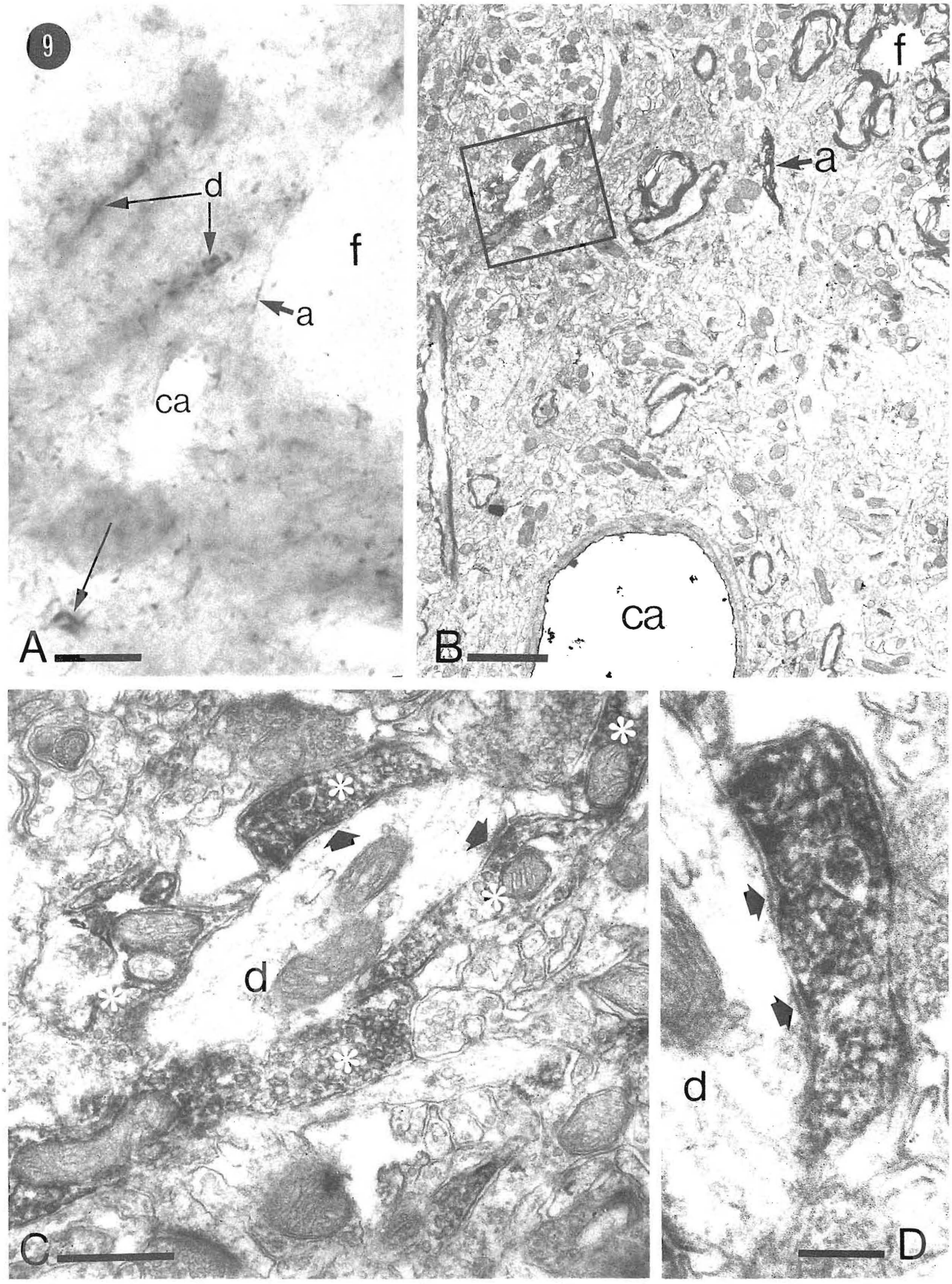
Fig. 9. (A) Small distal type-2 dendrites (long arrows) are seen surrounded by immunoreactive varicosities. One dendrite near a capillary (ca), a fibre bundle (f) and an immunoreactive axon (a) can also be seen in the electron micrograph in (B). The framed area in (B) is shown at higher magnification in (C). (C) Immunoreactive boutons (asterisks) are completely surrounding the small dendrite (d). Symmetrical synaptic contacts are indicated by thick arrows. (D) One of the synaptic boutons shown at higher magnification. Scale bars: (A) 10 μm; (B) 2 μm; (C) 0.5 μm; (D) 0.2 μm.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ventral striatum. The perikarya were elongate, often spindle-shaped, with very thick dendrites emerging from the two poles of the cell (Figs. 5A–E, 7A). The shape of the nucleus was irregular with deep invaginations penetrating it from all sides. The most conspicuous feature of type-2 neurons was their synaptic input. Both the perikarya and proximal dendrites were ensheathed by boutons making symmetrical synaptic contacts (Figs. 5G, 6B–G). Almost without exception these boutons were enkephalin immunoreactive (Figs. 5F, 6A). They were type-B boutons with large, clear, pleomorphic vesicles containing both immunoreactive and non-reactive large granulated vesicles (Figs. 5G, 6B–G). Not only the boutons, but also the immunoreactive axons were found to surround the neuron in a nest-like formation.

Axo-dendritic synapses were more common than axo-somatic ones and were established by type-B boutons only. Two types of dendrite were found postsynaptic to enkephalin-immunoreactive boutons. Type 1 dendrites were thin (Fig. 8D) or medium sized (Figs. 2H, 3E, F, 8C) containing microtubules and a few mitochondria but few other organelles. Rarely spines could be connected to them in serial sections, and the irregularities (Fig. 8C) on their surface also suggested that they were spiny. They received few synapses, apart from the enkephalin-immunoreactive ones. There was no preferential association between an immunoreactive fibre and any particular dendrite.

Type-2 dendrites postsynaptic to enkephalin-immunoreactive boutons were shown by direct examination to be the tube-like structures seen in the light microscope (Figs. 7C, D, 8A, B, 9B–D). The great majority of the boutons were immunoreactive (Figs. 7A, B, 8B, 9C). Occasional boutons making asymmetrical synaptic contacts were found but these were devoid of reaction endproduct (Fig. 10A). These dendrites received similar input on both their thick proximal (Fig. 7A, B) and their distal (Figs. 8A, B, 9) portions. The thick part of the type-2 dendrites was identical with the proximal dendrites of type-2 neurons.

Axo-spinous contacts involving immunoreactive boutons were rare, although most synapses in the neostriatum are of this type. Axo-spinous synapses were established by both type A (Figs. 2F, G) and type B (Fig. 2E) boutons. In cases when type B boutons made symmetrical synaptic contact with a spine, the same spine also received asymmetrical contact from a bouton free of reaction endproduct (Fig. 2E).

Axo-axonic synaptic contacts with preterminal axons, boutons or with any profile containing synaptic vesicles were not seen, although we carefully examined a large number of immunoreactive boutons in serial sections. In many cases (Figs. 3E, F, 4D,

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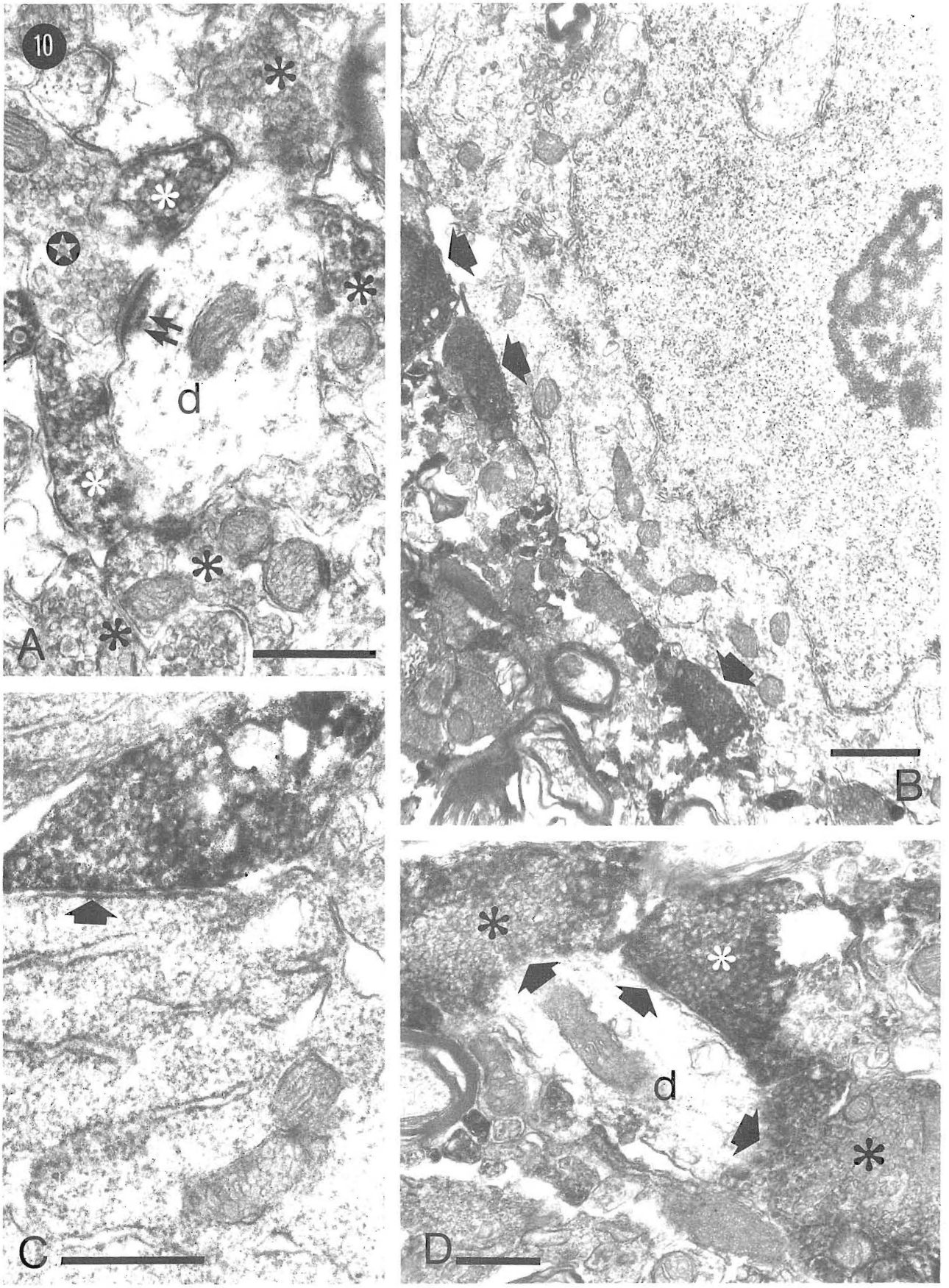
Fig. 10. (A) Type-2 dendrite in the neostriatum surrounded by immunoreactive boutons (asterisks) receives an asymmetrical synapse with postjunctional dense bodies (arrows) from a nonreactive bouton (star). (B) The perikaryon of a large neuron in the globus pallidus is surrounded by boutons, most of which show immunoreactivity (arrows). (C) Symmetrical axo-somatic synapse established by an immunoreactive bouton in the globus pallidus. (D) Immuno-reactive boutons (asterisks) making symmetrical synapses (arrows) surround a dendrite (d) in the globus pallidus. Scale bars: (A, C, D) 0.5 μm; (B) 1 μm.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9C, D) immunoreactive boutons lay in close membrane apposition with other boutons and vesicle-containing profiles, being separated only by the extracellular space (Figs. 2E, 3E, 4D, 8D) but no morphological sign of any membrane specialization could be observed.

Some characteristics of enkephalin immunoreactive boutons in the globus pallidus

Since two types of enkephalin-immunoreactive boutons were observed in the neostriatum, and the neostriatum is known to send enkephalin fibres to the globus pallidus (see the Introduction), two areas were studied in the globus pallidus to establish the types of immunoreactive boutons there. The proportion of enkephalin immunoreactive boutons in the globus pallidus is much higher than in the neostriatum. All boutons which have been studied made symmetrical synaptic contacts (Figs. 10C, D) and were very similar to type B boutons described in the neostriatum. The postsynaptic elements were dendrites (Fig. 10D) or large perikarya (Figs. 10B, C) and both postsynaptic structures received a large number of synapses from immunoreactive terminals. The dendrites, both proximal and distal, were often ensheathed by immunoreactive terminals (Fig. 10D) and so resembled the type-2 cells in the neostriatum.

Discussion

Origin of enkephalin immunoreactive boutons in the neostriatum (see Fig. 11)

In contrast to a previous study (Pickel *et al.*, 1980), enkephalin-immunoreactive boutons were found to make symmetrical, or Gray type II, as well as asymmetrical, or Gray type I (Gray, 1959), synaptic contacts. This difference is not due to the specificity of the antibodies as one of the antisera used in this study is identical to that used by Pickel *et al.* (1980). The difference is rather a question of interpretation since most of the synaptic contacts formed by immunoreactive boutons in the study of Pickel *et al.*, which they considered to be asymmetrical, would be regarded as symmetrical (Gray's type II) in the present investigation (except the synapse in their Fig. 9A). In the present study, the type of synaptic contact was identified from that section of a series cut from a bouton in which the plane of the section was perpendicular to the synaptic cleft. In this way, the type-A and type-B synaptic bouton populations could clearly be separated (see, for example, Fig. 2E–H). A similar situation exists in the substantia nigra where substance P immunoreactive boutons can form either symmetrical or asymmetrical synaptic contacts (Somogyi *et al.*, 1982).

The finding of two types of enkephalin-immunoreactive boutons raises the question of their origin. The evidence obtained here that one fibre makes the same type of contact at all of its boutons which could be studied, suggests that type A and type B boutons originate from different populations of neurons. As all type A boutons contacted spines, it could be argued that it is the postsynaptic element which determines the extent of the postsynaptic specialization. However, since type B boutons also occasionally contacted

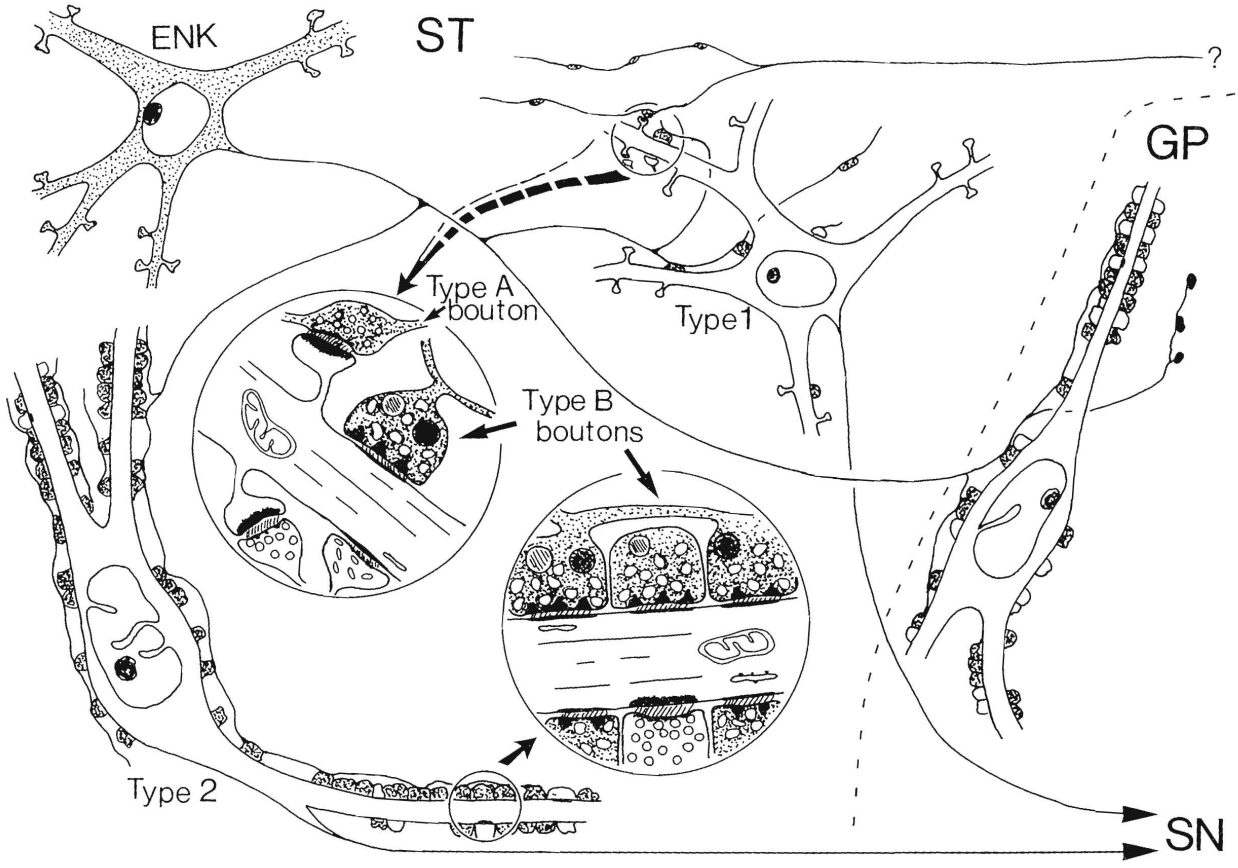

Fig. 11. A diagrammatic representation of the synaptic connections of enkephalin-immunoreactive boutons (dotted) in the neostriatum (ST). Type-A boutons form asymmetrical synapses with spines, probably from medium sized spiny neurons. The origin of type-A boutons is unknown, but they may come from neurons extrinsic to the neostriatum. The enkephalin-immunoreactive cells (ENK) of the neostriatum have been shown to be of the medium sized spiny type (Pickel *et al.*, 1980) and many of them project to the globus pallidus (GP). Type-B boutons, which make symmetrical synapses, probably originate from the medium sized spiny neurons. They contain large clear pleomorphic vesicles and both immunoreactive and nonreactive large granulated vesicles. Type-B boutons establish synaptic contacts with the perikarya and dendrites of type-1 and type-2 neurons. Neurons of these two morphological categories have been shown to project to the substantia nigra (SN). The great majority of synapses received by type-2 neurons are enkephalin-immunoreactive, but they also receive a few asymmetric synapses from nonreactive boutons.

spines and spines have been shown to receive symmetrical synapses in the neostriatum (Wilson & Groves, 1980; Somogyi *et al.*, 1981) this suggestion can be ruled out. So, where are the neuronal perikarya giving rise to type A and type B boutons?

It has been demonstrated that the neostriatum contains many enkephalin-immunoreactive perikarya (Hökfelt *et al.*, 1977; Sar *et al.*, 1978; Wamsley *et al.*, 1980; Pickel *et al.*, 1980; Finley *et al.*, 1981; Del Fiacco *et al.*, 1982) and that most of them project to the globus pallidus (Cuello & Paxinos, 1978; Del Fiacco *et al.*, 1982). It was not the aim of the present study to investigate these perikarya since their fine structural characteristics have been described in detail (Pickel *et al.*, 1980). The same authors suggested that the enkephalin-boutons in the neostriatum are derived from the local axon collaterals of striato-pallidal neurons. However, since no immunoreactive bouton or axon has been traced back to neurons intrinsic to the neostriatum, the possibility exists that some or all of the immunoreactive boutons originate from extrinsic nuclei which are known to project to the neostriatum and which contain enkephalin-immunoreactive perikarya. Thus it is known that the following regions contain enkephalin-immunoreactive perikarya (first reference) and project to the caudoputamen (second reference): parafascicular nucleus of the thalamus (Wamsley *et al.*, 1980; Veening *et al.*, 1980); the substantia nigra (Hökfelt *et al.*, 1977; Nauta *et al.*, 1974); the amygdala (Finley *et al.*, 1981; Kelley *et al.*, 1982); the raphe dorsalis (Glazer *et al.*, 1981; see Azmitia, 1978); and the neocortex (Finley *et al.*, 1981; Oka, 1980).

On the other hand, electron microscopic degeneration studies (Kemp & Powell, 1971; Chung *et al.*, 1977; Hassler *et al.*, 1978; Somogyi *et al.*, 1981; Frotscher *et al.*, 1981) and autoradiographic studies (Hattori *et al.*, 1979) indicate that most of the extrinsic afferents to the neostriatum terminate with asymmetric synapses on spines. Thus it can be speculated that type-A terminals originate from one of the brain areas containing enkephalin cells which project to the neostriatum.

Type-B boutons make symmetrical contacts and it has been demonstrated that only boutons making symmetrical contacts survive in the isolated neostriatum (type IX of Hassler *et al.*, 1977; Hassler, 1979) suggesting that some, at least, were local in origin. Further evidence of the striatal origin of type-B boutons comes from their fine structural features. The small postsynaptic membrane density, the prominent presynaptic dense projections and the large clear pleomorphic vesicles make type-B immunoreactive boutons very similar to the boutons of local axon collaterals originating from projecting striatal spiny neurons (Somogyi *et al.*, 1981), to the pallidal boutons of efferent striatal spiny neurons (Chang *et al.*, 1981), and to the local boutons of intracellularly labelled striatal spiny neurons (Wilson & Groves, 1980). Thus type-B boutons may very well originate from the local axon collaterals of efferent, striatal medium sized spiny neurons. The fine structural characters of the enkephalin-immunoreactive perikarya (Pickel *et al.*, 1980) were indeed similar to those of identified medium spiny neurons (Somogyi & Smith, 1979; Dimova *et al.*, 1980; Di Figlia *et al.*, 1980; Wilson & Groves, 1980; Frotscher *et al.*, 1981; Somogyi *et al.*, 1981).

The neurons that are postsynaptic to immunoreactive boutons (see Fig. 11)

The most important finding of the present study is that there are two distinct types of neurons receiving input from enkephalin immunoreactive terminals in the striatum. The first type of perikaryon and the type-1 dendrites are very similar to those of the identified medium sized spiny neuron described in electron microscopic studies (Somogyi & Smith, 1979; Dimova *et al.*, 1980; Wilson & Groves, 1980; Di Figlia *et al.*, 1980). It seems that these neurons receive rare synapses from type-A boutons on their spines and some synapses from type-B boutons on their perikarya, dendritic shafts and very rarely on their spines. Rarely, immunoreactive fibres made multiple synapses with an individual type-1 neuron but in general, the input of type-1 neurons from enkephalin fibres seems to be sparse and distributed throughout the neuron.

A high proportion of neurons in the striatum are projection neurons (Bolam *et al.*, 1981a) and we suggest that many of the postsynaptic type-1 neurons might be efferent cells, as it has been shown that medium sized, spiny neurons project to the substantia nigra (Somogyi & Smith, 1979) and to the globus pallidus (Leontovich, 1954; Preston *et al.*, 1980; Chang *et al.*, 1981). The striatonigral spiny neurons receive two types of synapse on their perikarya (Somogyi & Smith, 1979) just as do type-1 neurons in the present study, and the enkephalin-immunoreactive type-B boutons are very similar to the boutons with pleomorphic vesicles that occur on identified striatonigral neurons. This type of bouton probably originates from the local axon collaterals of enkephalin-immunoreactive spiny cells in the striatum as discussed above. Indeed, several lines of evidence suggest that medium spiny neurons are interconnected via their local axon collaterals (Park *et al.*, 1980; Somogyi *et al.*, 1981; Wilson & Groves, 1980).

Type-2 neurons receive a different type of input from immunoreactive boutons. There is little doubt that both type-2 dendrites and perikarya represent the same cell type and it is apparent that the major input of this neuron type is from enkephalin-immunoreactive type-B boutons, probably of striatal origin. There are few places known in the C.N.S. in which a neuron receives such an homogeneous input with regard both to the type of synaptic bouton and its putative transmitter. The multiple synapses made by enkephalin fibres running along the dendrites and perikarya of type-2 neurons make their potential interaction very powerful. Furthermore, this interaction indicates a high degree of convergence of enkephalin-immunoreactive fibres upon type-2 neurons.

This type of striatal neuron was discovered only recently in the ventral striatum in combined Golgi-electron microscopic-HRP studies (Bolam *et al.*, 1981b; Smith *et al.*, 1981) and it was shown that the dendrites run for long distances in the dorso-ventral direction. In the present study type-2 dendrites that received enkephalin-immunoreactive input were found to have a similar distribution to that described by Bolam *et al.* (1981b), but some perikarya were found more dorsally, in the ventral one-third of the rostral caudoputamen.

It has been pointed out (Bolam *et al.*, 1981b) that the types of afferent synapses and

their arrangement on these striatal neurons is strikingly similar to neurons of the globus pallidus and substantia nigra zona reticulata, the two main efferent targets of the neostriatum. The present study shows that this similarity applies in a chemical sense to the neurons of the globus pallidus, since they also receive a heavy enkephalin input from similar types of boutons to those received by type-2 neurons. As the ventral striatum is in close proximity to the ventral pallidum all along its ventral aspect, it is likely that the scattered type-2 neurons in the neostriatum are homologous with pallidal neurons, with one possible difference. Type-2 neurons are in a position to receive input from striatal afferents which do not terminate in the ventral pallidum. Furthermore, they may receive input from a different set of enkephalin-immunoreactive neurons than cells of the ventral pallidum. The occasional asymmetrical synaptic contacts along the dendrites of type-2 neurons suggest that they also receive input other than from the collaterals of striatal immunoreactive neurons.

The significance of the heavy enkephalin input to type-2 neurons is increased by the finding of Bolam *et al.* (1981b) that many of these neurons (called striatonigral type 2 in that study) project to the substantia nigra. Thus while the nigra has not been shown to receive enkephalin input from the striatum (Hong *et al.*, 1977), both types of neurons projecting to the nigra are under the influence of enkephalin-immunoreactive boutons and one of them receives most of its input from this type of terminal. If the assumption, that most type-B boutons originate from local axon collaterals of striatopallidal neurons is correct, then it is the enkephalin striatopallidal system which will also influence the striatonigral output via terminals on striatonigral neurons in the striatum. It was suggested (Bolam *et al.*, 1981b) that there was a feedback between the two types of striatonigral neurons mediated by the local axon collaterals of striatonigral type 1 neurons (i.e. the medium sized spiny neurons). This suggestion has to be modified now because it is more likely that there is an interplay between the striatopallidal and striatonigral neurons. Just what this interplay means in the functional sense remains to be established.

It is noteworthy that although in the present study serial sectioning was used, we were unable to detect any axo-axonic synapses. This is in contrast to a previous report where the existence of such contacts was proposed: 'axon terminals . . . form specialized junctions with unlabelled axons or axon terminals' (Pickel *et al.*, 1980, p. 727). Although enkephalin-immunoreactive nerve terminals have been found in direct apposition to non-immunoreactive vesicle-containing structures (including boutons) both in the present study and in that of Pickel *et al.* (1980), this is no different from the apposition of immunoreactive boutons to any other part of a neuron or glial cell. Thus, on morphological grounds alone, there is no criterion which allows us to postulate presynaptic actions of endogenous opiates on the terminals of other neurons which would account for pharmacological observations, such as the action of opiates on the release of dopamine (Chesselet *et al.*, 1981).

Possible functional implications

Actions of opiates in the striatum are complex. After systemic administration the effects could, of course, be due to actions either directly on elements in the neostriatum or via neurons which send projections to the neostriatum; a good example of the latter is the effect of opiates on dopaminergic neurons in the substantia nigra (McMillen, 1980; Jurna, 1981). However direct administration of opiates into the striatum can also lead to different effects, e.g. a low dose of morphine injected into the neostriatum causes analgesia while a ten-fold higher dose causes hyperalgesia (Jacquet & Lajtha, 1973). Our finding that enkephalin-immunoreactive boutons and synapses in the neostriatum are of two distinct types might conceivably be relevant to these, and related, pharmacological observations since the postsynaptic targets presumably mediate different effects of the opiates.

Finally, all the immunoreactive boutons selected from the light microscope for electron microscopic examination were found, without exception, to make classical symmetrical or asymmetrical synaptic contacts. This is at variance with previous work (Pickel *et al.*, 1980) in which it was argued that some of the enkephalin-immunoreactive boutons in the neostriatum appear to lack synaptic specialization and was suggested that such boutons would exert their influence diffusely by releasing a modulator. While diffuse interactions between neurons without synaptic contacts are possible, our results on enkephalin-immunoreactive boutons in the neostriatum of the rat suggest that most, if not all such boutons have the opportunity to exert their effect via synaptic contacts. This indicates that the enkephalin-immunoreactive material may have a function as a synaptic transmitter. A similar conclusion was reached for substance P-immunoreactive boutons in the substantia nigra (Somogyi *et al.*, 1982) since there too, all identified boutons were found to make synaptic contacts.

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