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The ultrastructure and somatic efferent synapses of small granule-containing cells in the superior cervical ganglion

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INTRODUCTION

Recent electron microscopic studies of sympathetic ganglia have revealed small, granule-containing cells in addition to the typical large sympathetic neurons of the ganglion (Siegrist, de Ribaupierre, Dolivo & Rouiller, 1966; Grillo, 1966; Williams, 1967*a*, *b*; Elfvin, 1968; Matthews & Raisman, 1968). These cells are of particular interest in that they are quite possibly sympathetic interneurons. Grillo (1966) and Siegrist *et al.* (1966) independently found that cells of this type in the superior cervical ganglion of the rat have features in common with both chromaffin cells and sympathetic neurons. Williams (1967*a*, *b*) described these small cells in the same ganglion as giving off processes which form efferent synapses with structures which he considered to be dendrites. He put forward the hypothesis that the small cells were 'connector neurons interposed in effector circuits between preganglionic neurons and postganglionic dendrites'. Elfvin (1968) has described what are probably cells of the same type in the inferior mesenteric ganglion of the rabbit, and noted that in electron-density the granules resemble those of the noradrenaline-containing type of chromaffin cell in the adrenal medulla.

Earlier studies with the light microscope did not clearly distinguish a cell type corresponding with the small granule-containing cell (e.g. Cajal, 1911; Ranson & Billingsley, 1918; De Castro, 1932). Small groups of cells giving the chromaffin reaction are known to occur in certain sympathetic ganglia of various species (see Coupland, 1965*a*); in the superior cervical ganglion of the adult rat, however, these cells are not readily revealed by standard techniques for the demonstration of chromaffin elements (Lempinen, 1964; Coupland, 1965*a*; Eränkö & Härkönen, 1965). But since the development of the highly sensitive method of Falck & Hillarp (Falck, Hillarp, Thieme & Torp, 1962) for the demonstration of biogenic amines there have been numerous accounts of a type of small cell, scantily distributed in sympathetic ganglia, which may be induced to fluoresce intensely by this procedure (e.g. Norberg & Hamberger, 1964; Norberg, Ritzén & Ungerstedt, 1966; Norberg & Sjöqvist, 1966; Eränkö & Härkönen, 1965; Csillik, Kálmán & Knyihár, 1967; Olson, 1967). In their size, grouping and distribution these small intensely fluorescent cells appear to correspond with the small granule-containing cells which are the subject of this paper.

Although the differentiation of cell types in the ganglion has been relatively recent, the quest for a sympathetic interneuron is of some antiquity. Dogiel (1896) implied

that there might be synaptic interaction between neurons in sympathetic ganglia. Johnson (1918, 1921) performed experiments devised to test Dogiel's hypothesis but found no evidence to support it. Pharmacological studies subsequently showed that, though the preganglionic fibres were thought to act by liberating acetylcholine, adrenaline could modify transmission through a sympathetic ganglion (Marrazzi, 1939), and that an adrenaline-like substance was liberated during stimulation (Bülbring, 1944). The electrophysiological studies of Eccles & Libet (1961) provided evidence for the occurrence of an inhibitory process (the P wave) in a sympathetic ganglion, in the rabbit, and this was attributed to the action of adrenaline diffusing from intraganglionic chromaffin cells. Dunant & Dolivo (1967) have demonstrated a similar P wave in the superior cervical ganglion of the rat. Recent intracellular recordings in frog sympathetic ganglia (Tosaka, Chichibu & Libet, 1968) have shown inhibitory postsynaptic potentials which Tosaka *et al.* suggest may be due to the action of cells such as the small cells described by Williams (1967*a*, *b*).

The possibility is, therefore, that the small granule-containing cells may be a type of sympathetic interneuron and may prove to mediate intraganglionic inhibitory processes. The present paper describes the general character and distribution of the small cells in the superior cervical ganglion of the rat. It is confirmed that they give rise to efferent synapses within the ganglion. Many of these efferent synapses arise directly from the cell soma, without the intervention of any process which might be regarded as an axon (cf. Hama, 1966; Sétáló & Székely, 1967), and to this arrangement is applied the term 'somatic efferent synapse'.

MATERIAL AND METHODS

Young adult albino rats (Wistar strain) of about 3 months of age were used in this study. Fixation in the earlier part of the series was by intracardiac perfusion with a buffered ice-cold mixture of 4 % formaldehyde and 1 % glutaraldehyde. In some animals a phosphate buffer was used, and in others cacodylate. The rats were anaesthetized briefly with ether and cooled in ice before the perfusion was begun. After perfusion for 30 min the tissues were left undisturbed for a further 30 min, then the superior cervical ganglia were gently dissected out and postfixed in 2 % osmium tetroxide for 1 h. In order to test whether the stress of ether anaesthesia and the preliminary cooling might cause sufficiently extensive liberation and depletion of catecholamines to affect the morphology and distribution of granular vesicles, one rat was fixed by perfusion under chloral hydrate anaesthesia administered by intraperitoneal injection, and was not cooled until after the perfusion with chilled fixative was begun; the adrenal medulla was prepared for electron microscopy in addition to the superior cervical ganglia.

In the later part of the series the ganglia were removed under chloral hydrate anaesthesia and were fixed by immersion in buffered ice-cold fixative. The fixatives used were 1 % osmium tetroxide buffered with veronal acetate, or 4 % glutaraldehyde with cacodylate or phosphate buffer, or the same aldehyde mixture as was used for the perfusions. The aldehyde-fixed ganglia were postfixed in 2 % osmium tetroxide for 1 h.

After fixation was complete, all the ganglia were dehydrated in alcohol and em-

bedded in Araldite. Groups of small cells were located with the light microscope in thick (1–2 μm) sections stained by a mixture of 1 % methylene blue and 1 % Azur II, and ultrathin sections from the selected area were stained with lead citrate and uranyl acetate for examination with the electron microscope.

One perfused ganglion was sectioned at 2 μm throughout and a 1 in 2 series was mounted and examined in the light microscope for small cells.

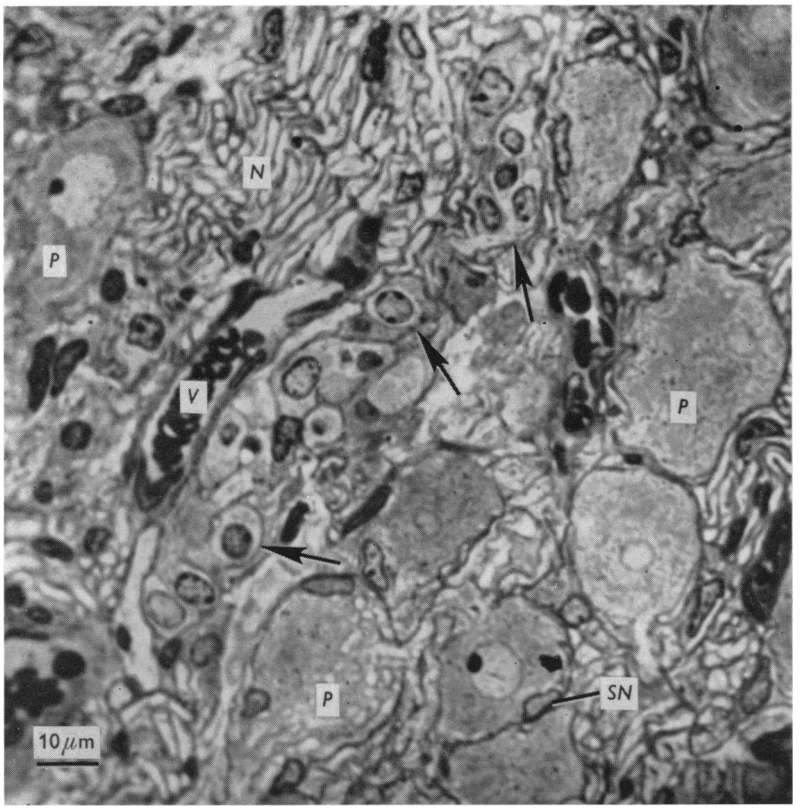

Fig. 1. Light micrograph. Intraganglionic cord of small cells (arrows), with adjacent blood vessel (*V*), principal sympathetic neurons (large cells e.g., *P*), nuclei of satellite cells (e.g., *SN*) and bundles of nerve fibres. (*N*). Aldehyde immersion.

RESULTS

1. *Light microscopy*

In sections 1–2 μm thick, stained by methylene blue and Azur II, the small cells may be distinguished from the typical sympathetic neurons of the ganglion and from the numerous satellite cells by their size, shape, arrangement and staining affinity. Final confirmation of their identity may then be made with the electron microscope, which reveals their characteristic granules. In the present study the small cells have been found, either by light or by electron microscopy, in 23 superior cervical ganglia from 21 rats. In 18 ganglia their identity has been confirmed with the electron microscope.

Some typical small cells are shown in Fig. 1. They are of about 6–12 μm in diameter, and in shape they are rounded or polyhedral, sometimes elongated, with an approximately central nucleus which has a darkly staining rim and does not show a conspicuous nucleolus. The cytoplasm lacks Nissl bodies and appears pale and clear. The cells are arranged compactly in small clusters, or sometimes in cords or strands; where they form a compact cluster the boundaries of adjacent cells are closely apposed. These features distinguish them clearly from the typical large sympathetic neurons, which are 15–40 μm in diameter, lie singly rather than in clusters, contain masses of Nissl substance and have a lightly staining 'vesicular' nucleus with one or more conspicuous nucleoli. In the following description these typical sympathetic neurons will be referred to as 'principal cells' or 'principal neurons' for the sake of simplicity. The arrangement of the small cells in clusters and their relatively abundant pale cytoplasm and rounded nucleus distinguish them also from satellite cells, which tend to have an elongated or irregular dark nucleus with a narrow rim of cytoplasm. The term 'satellite cell' is here used to include all those cells which provide cytoplasmic sheaths for the nerve cells and nerve fibres within the ganglion, and incidentally also for the clusters of small cells, as will be described later.

The number of small cells appearing in a 2 μm thick section of a cluster may vary from 2 to over 12; occasional larger groups are formed by the aggregation of several clusters. It is usual for the clusters or groups to be associated with one or several thin-walled capillary vessels, and on occasion the small cells may form a continuous cuff surrounding a vessel. With experience it becomes possible to identify with some confidence single small cells, perhaps as the first sign of an approaching cluster in serial sections. The impression has been obtained that it is not common in the superior cervical ganglion of the rat for small cells to occur singly, except in the neighbourhood of clusters or groups.

In order to obtain some idea of the number and distribution of small cells, a single superior cervical ganglion was sectioned serially and examined at intervals of 4 μm. In this ganglion 30 clusters (including 6 larger groups) of small cells were identified, with an additional 4 clusters (including 2 larger groups, of which one was particularly large) at the base of the branches along the external carotid artery (cf. Eränkö & Härkönen, 1965; Norberg *et al.* 1966; Olson, 1967). One of the large groups within the ganglion was placed at the headward end, near to a mass of efferent axons leaving the ganglion, and the others lay for the most part along or around the central core of the ganglion, principally in its cranial two-thirds. In this part of the ganglion there was a group or cluster of small cells within at the most 500 μm of every principal cell. The small cells were distinctly less numerous in the caudal one-third of the ganglion, and were not found in the zone of entry of the afferent fibres to the ganglion.

2. *Electron microscopy*

Comparison of small cells with principal cells

The appearance of a typical sympathetic neuron is illustrated in Fig. 2, and at higher magnification in Figs. 8 and 9. The ultrastructure of these cells in the rat has been described by several workers, e.g. Forsmann, 1964; Taxi, 1965; De Lemos &

Pick, 1966. Fig. 2 illustrates the completeness of the satellite sheath which surrounds the neurons. It shows also that their cytoplasm contains many compact arrays of granular endoplasmic reticulum and scattered lysosome-like bodies, and that the nucleus has finely granular chromatin without dense peripheral aggregations.

In Fig. 3, for comparison, is shown part of a large, compact cluster of the small granule-containing cells. At various points they lie with their cell membranes in apposition, but elsewhere they are ensheathed by satellite cell cytoplasm. Their cytoplasm is made conspicuous by the presence of numerous granular vesicles (dense-cored vesicles), which tend to lie peripherally. In the principal neurons it is rare to find more than an occasional granular vesicle; and although, when found, such a vesicle closely resembles those seen in the small cells, it lies typically near to the Golgi apparatus rather than in the peripheral rim of cytoplasm. The cytoplasm of the small cells differs in other respects also from that of the principal neurons. It shows relatively few cisternae of granular endoplasmic reticulum, and these tend to lie singly (Fig. 4); regular arrays of cisternae, corresponding to Nissl bodies, are rare. There is, however, an abundance of ribosomes which are not membrane-bound (Fig. 4). The mitochondria, which are numerous, are typically round or oval, seldom elongated, and frequently show a striking arrangement of straight, parallel cristae with regular spacing (Figs. 4, 16), which is not seen in the mitochondria of the principal neurons. One or several Golgi zones may be seen, lying in the deeper part of the cytoplasm, often disposed in a circle or arc near one pole of a somewhat eccentric nucleus. Lysosome-like dense bodies, which are numerous in the principal cells, are rare in the small cells. Multivesicular bodies and cilia are occasionally seen. The nucleus of the small cell is conspicuously different from the nucleus of the principal cell. Although in both cell types the nuclear profile tends to be rather regular, lacking deep indentations, in the small cell the nuclear chromatin is considerably more electron-dense, especially after fixation with aldehydes, and has many dark aggregations lying against the inner layer of the nuclear membrane (e.g. Figs. 4, 10). Nucleoli may sometimes be distinguished, more readily after osmium fixation, but are more or less obscured by adjacent dark aggregations (Figs 3, 10).

Dense-cored vesicles

The dense-cored vesicles of the small cells are seen best after perfusion fixation with aldehydes (Fig. 4). They are of about 65–120 nm in diameter in our material. Although they are not confined solely to the peripheral rim of cytoplasm they are much more numerous here than in the deeper part of the cell (Figs. 3, 4). The dense core or granule of the vesicle is most often centrally placed and of about 30–75 nm

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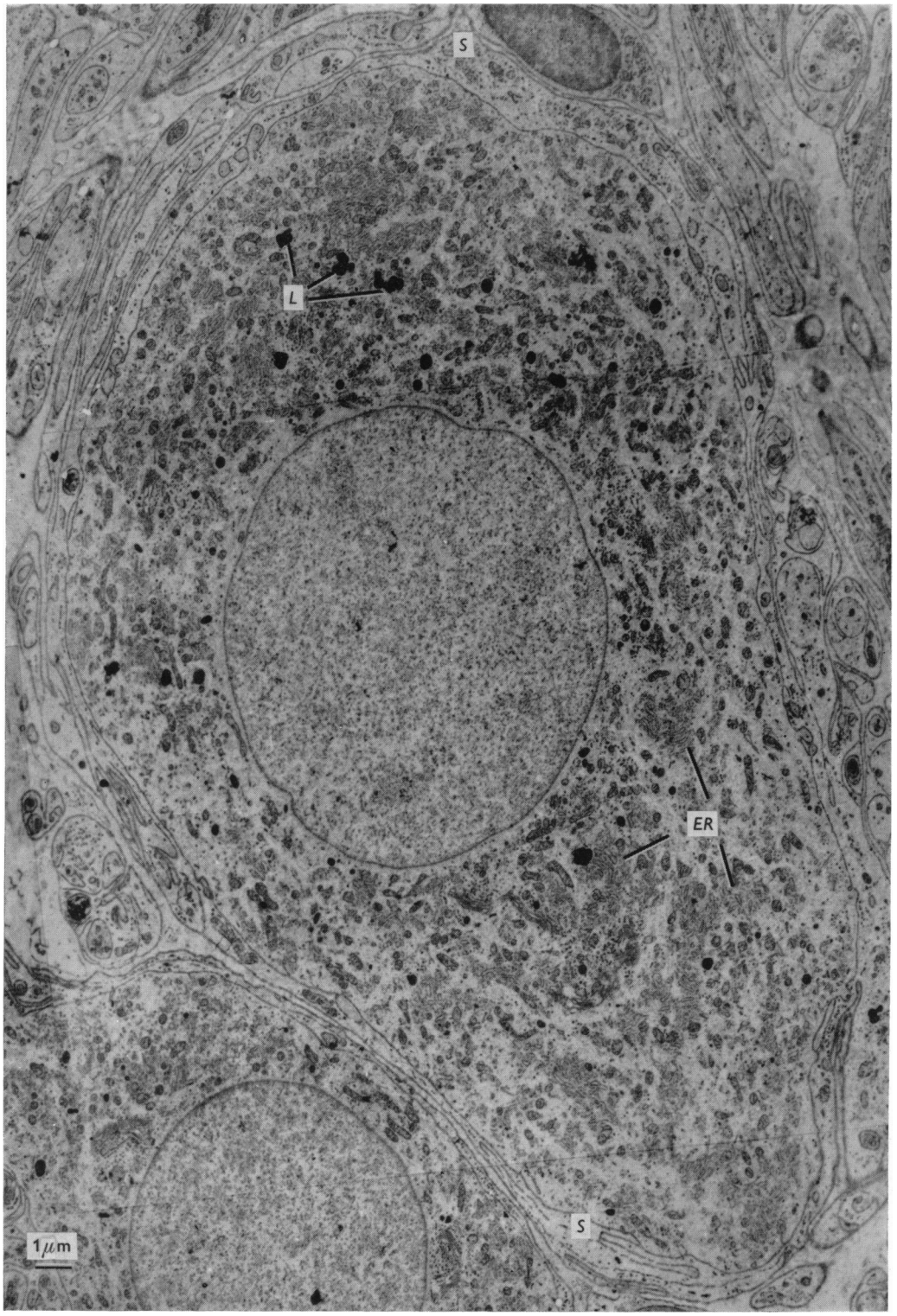
Fig. 2. Electron micrograph. Montage showing a principal sympathetic neuron, enclosed by an entire sheath of satellite cytoplasm (*S*). *ER*, Endoplasmic reticulum; *L*, Lysosome-like bodies. Osmium immersion.

Fig. 3. Electron micrograph. Montage showing part of a large cluster of small granule-containing cells. In some places their cell membranes are apposed; elsewhere they are ensheathed by satellite cytoplasm (e.g. *S*). A small vessel (*V*), with endothelial cell nucleus (*EN*), lies near the centre of the field. Close to this vessel there are occasional gaps in the satellite sheaths of small cells and their processes (e.g. arrows). An afferent synapse is seen at *A*, and probable efferent synapses at *E*. Nuclei of a satellite cell (*SN*) and a pericyte (*PN*) are also present. Osmium immersion.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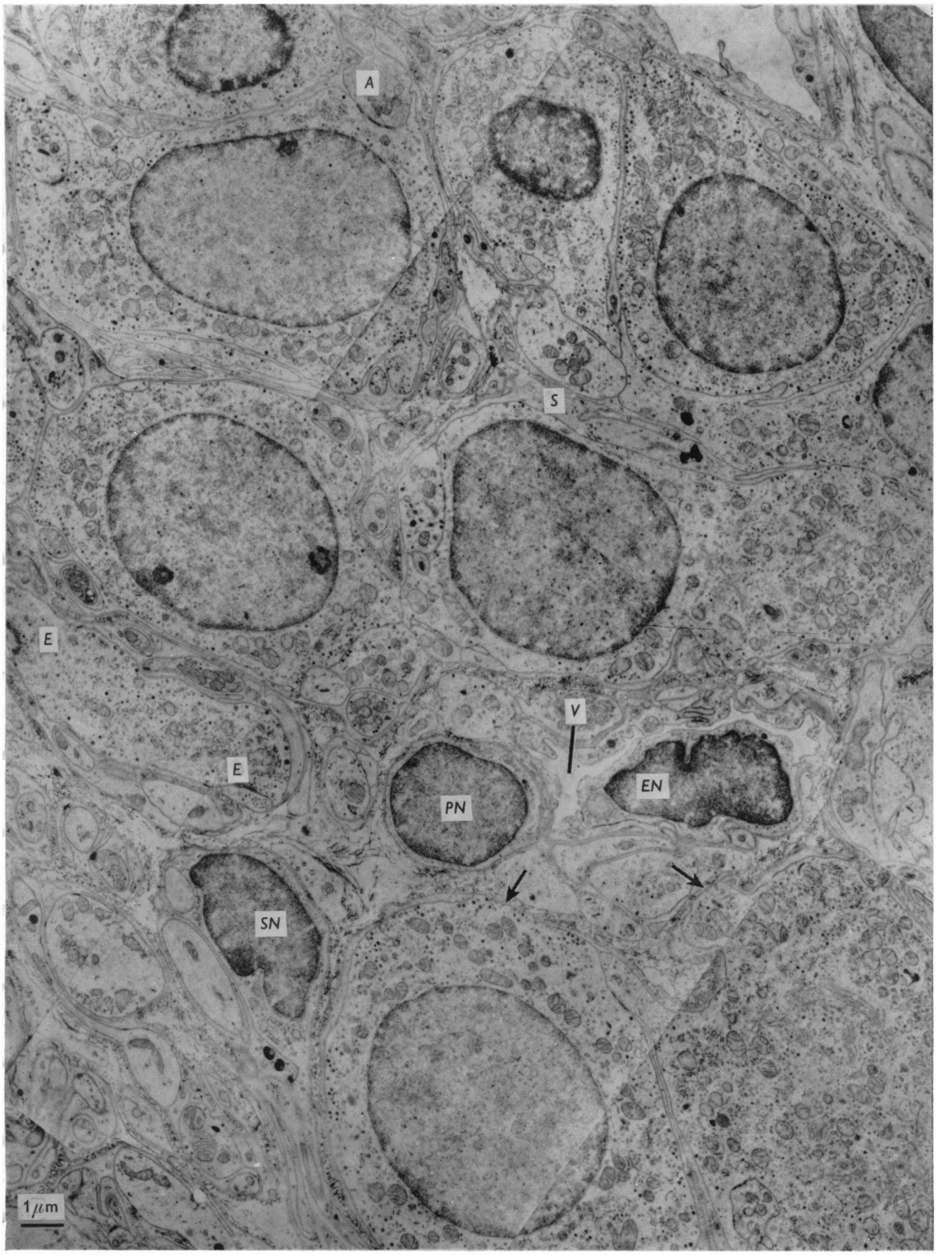
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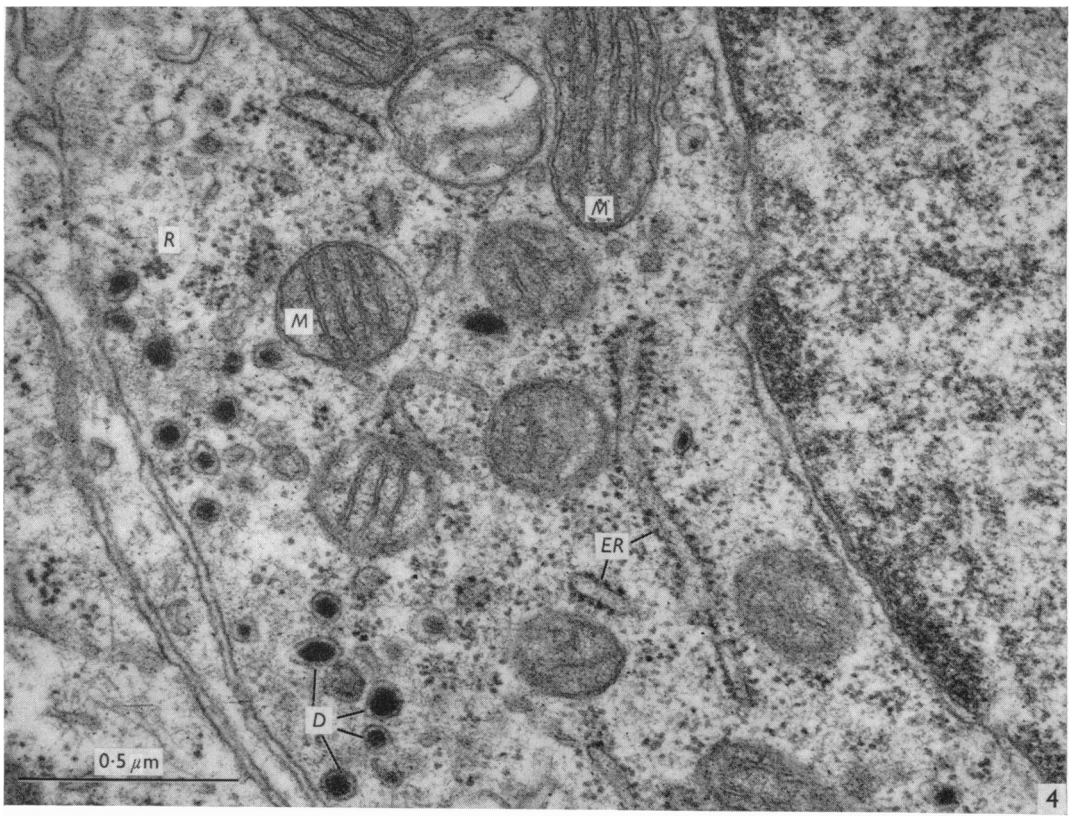
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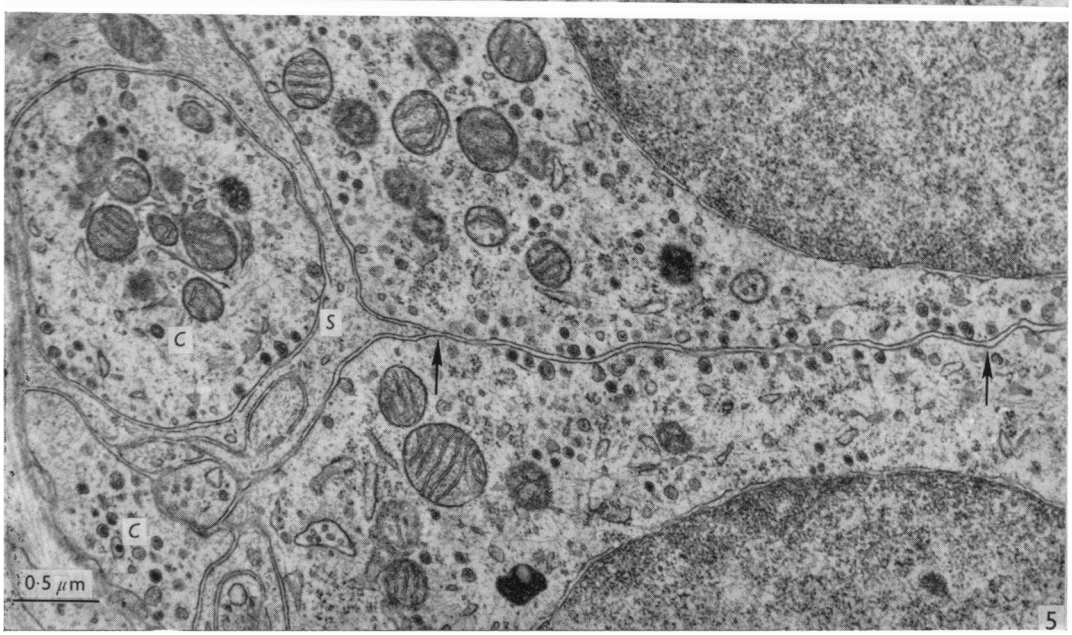
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in diameter. It is usually highly electron-dense, and in this it resembles the granule of the noradrenaline-containing type of chromaffin cell of the adrenal medulla (Coupland, 1965*b*); but the electron-density is variable, even for adjacent granules within the same cell, and some are much less dense than others (e.g. Fig. 4). It is not known whether this variability is due to fixation, to plane of section or to differences in composition or in physiological state of the granules. The surrounding halo is relatively electron-lucent and is most often about 15–20 nm wide. There is a distinct triple-layered limiting membrane. These granular vesicles differ from those of the noradrenaline-containing cell of the adrenal medulla in being smaller and in lying chiefly within the peripheral cytoplasm; in the adrenal medullary cells the granular vesicles are 50–350 nm in diameter and tend to be fairly densely packed throughout most of the cytoplasm (Coupland, 1965*b*).

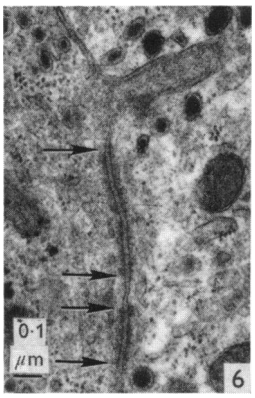
In one superior cervical ganglion of the rat which was fixed by perfusion under chloral anaesthesia without preliminary cooling, in an attempt to avoid some of the stress associated with ether anaesthesia and with hypothermia, the small cells have been found to have the appearance already described, although in some ways the quality of the fixation is slightly impaired. The size, form and distribution of the granular vesicles lie within the usual range. The appearance of the adrenal medullary chromaffin cells from the same animal agrees with the morphology described by Coupland (1965*b*) and by Elfvin (1965*a*), and they do not appear to be grossly degranulated.

Arrangement and satellite sheaths of small cells

The cluster of small cells shown in Fig. 3 illustrates the compact arrangement of the cells. Adjacent cells may lie with regions of their cell membranes closely apposed (Fig. 5), linked in some places by attachment plaques involving an increased intermembrane gap with symmetrical aggregations of electron-dense material to the membranes, and additional dense material between them (Fig. 6). In other places the membranes of adjacent cells may show small, mutually interlacing finger-like projections (Fig. 7). These are often, though not exclusively, placed at the angles of cells. The presence of these interlacing projections is not found to be associated with expansions of the extracellular space, and in this they are unlike the otherwise comparable projections of the chromaffin cells of the adrenal medulla (cf. Elfvin, 1965*a*; Coupland, 1965*b*). Regions of close apposition of cell membranes may, however, not be extensive or numerous, and are sometimes not seen at all in a section of a cell cluster (e.g. Fig. 10). Where the membranes of the small cells are not in mutual apposition, they are ensheathed by satellite cell cytoplasm, and adjacent satellite sheaths may be separated by channels of the intercellular stroma of the ganglion, containing collagen fibres (Fig. 10), an arrangement which is typical for the

Fig. 4. Cytoplasm and edge of nucleus of small cell, showing dense-cored vesicles (*D*), mainly in the peripheral cytoplasm, and mitochondria with regular parallel cristae (e.g., *M*). Contrast the few ribosomes associated with cisternae of granular endoplasmic reticulum (*ER*) with the many free ribosomal arrays (*R*). Aldehyde perfusion.

Fig. 5. Adjacent small cells with extensive apposition of surface membranes (arrows). Satellite cytoplasm (*S*) common to both cells also ensheathes other profiles, including two processes of small-cell cytoplasm (*C*). Osmium immersion.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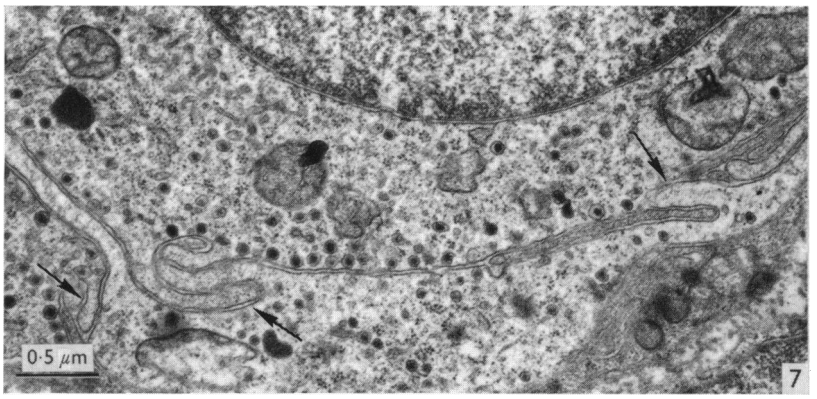
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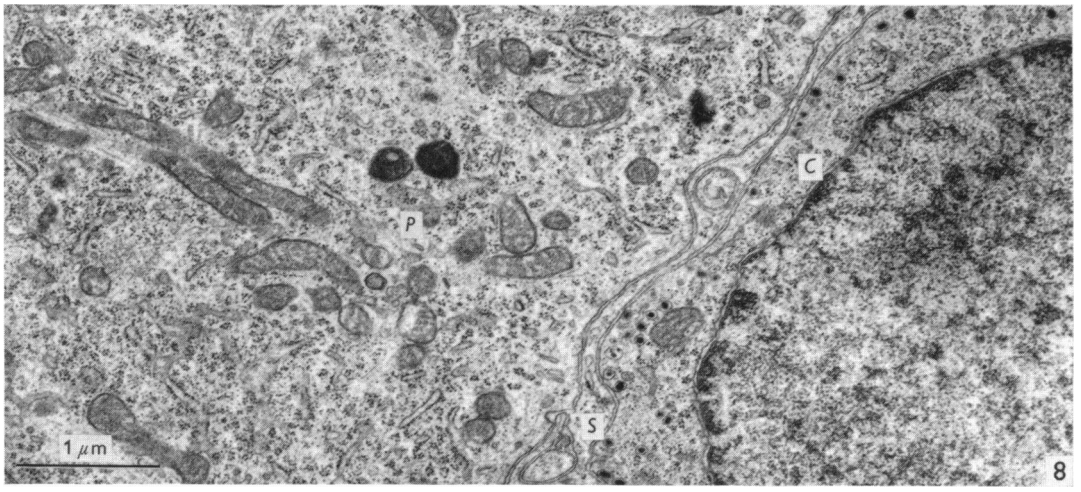
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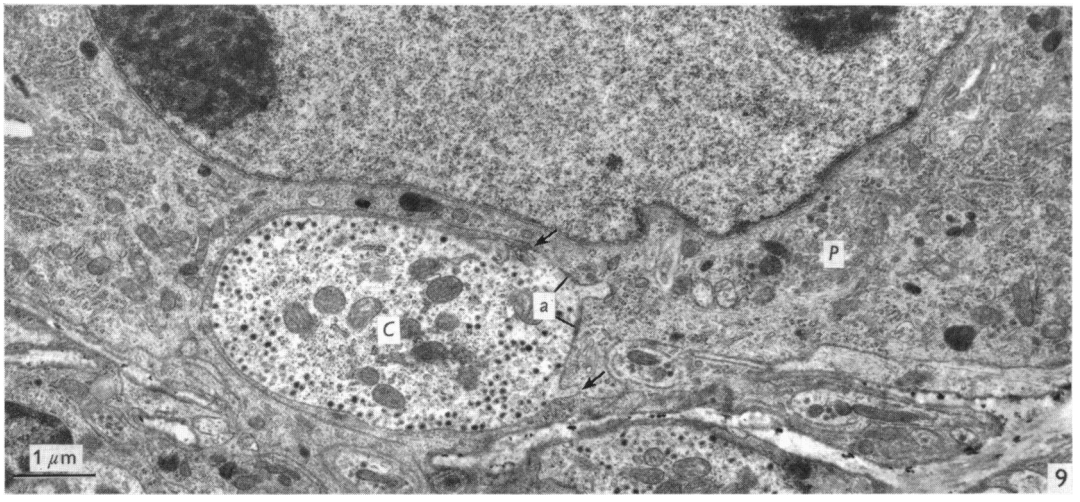
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principal sympathetic neurons. Alternatively, the same satellite cytoplasm may be shared by two or more small cells, as in Fig. 5. Occasionally a small cell, or a substantial process of one, is seen sharing the same sheath cell as one of the principal neurons (Fig. 8); and a small-cell process has been seen to lie closely apposed to the membrane of a principal neuron, linked with it by attachment plaques and projecting a short process into its cytoplasm (Fig. 9). (No region which could be regarded as a synaptic specialization was seen in this case.) It is most unusual for two principal cells to lie with their cell membranes apposed in this way.

The satellite sheath of the small cell is not everywhere complete, but is here and there deficient over a small area (cf. Elfvin, 1968). In these areas the plasma membrane of the small cell or of one of its processes is separated only by basement membrane from the stroma of the ganglion (see Fig. 11). Such an exposed area of small cell surface membrane is sometimes seen to be in direct communication across the basement membrane with a pericapillary tissue space (Fig. 12), and the capillary vessel may here be very thin-walled, with fenestrations (Fig. 12). Fig. 13 shows a fenestrated vessel lying close to parts of several small cells.

Processes of the small cells

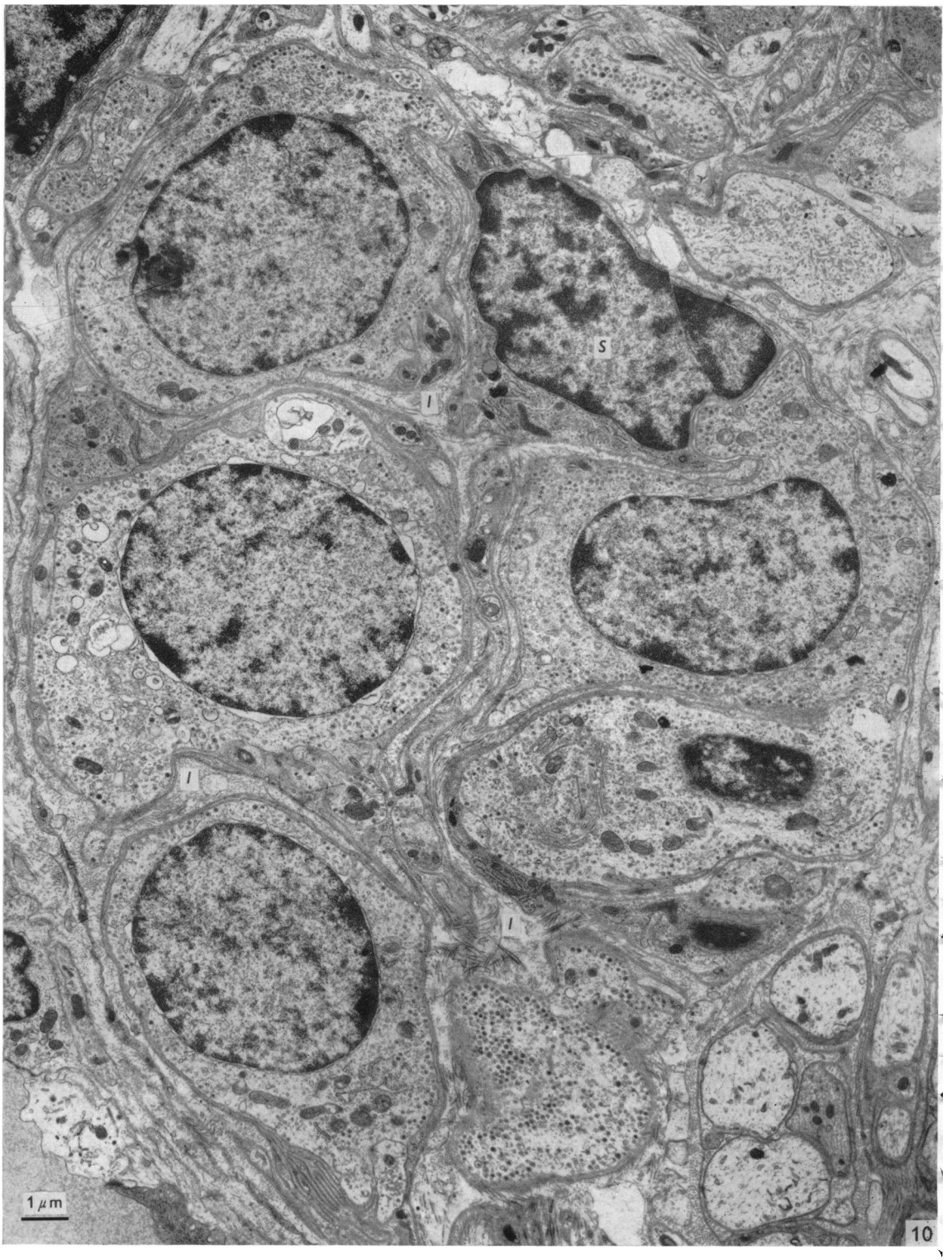
Because the small cells may have the status of interneurons the question of their processes and of whether an axon can be identified is of particular interest. Apart from the small interlocking projections already described, processes of varying form and dimensions are seen to arise from the small cells, or are encountered close to a cluster of small cells. It is not certain for what distance they may extend from the cell body; in our electron microscopic preparations lengths of up to 10 μm have been observed, but Norberg *et al.* (1966) in fluorescence studies with the light microscope have seen processes extending up to 40 μm from the small strongly fluorescent cells of the superior cervical ganglion in the rat. In the present study two main types of processes have been seen. The commoner type is rather broad, blunt and more or less irregular, often arising as an apparent extension of one pole of the soma (e.g. Fig. 14). In such a process there may be an appearance as of crowding of dense-cored vesicles throughout the cytoplasm, but this could be due to tangential sectioning close to the limiting membrane of the process. A few tubules resembling neurotubules may be seen lying along the long axis of the process. One such process, 1·3 μm wide, of which a non-tapering portion 7 μm long has been observed, gives rise to two efferent synapses in this part of its course. Less commonly a type of process is seen which is slender and is provided with a core of filaments (Fig. 15). Sometimes, as in Fig. 15, the process comes to lie alongside another small cell, and here (though not in the case

Fig. 6. Attachment plaques (between arrows) linking apposed membranes of small cells. Note the symmetry of the attachment zones and the local absense of dense-cored vesicles. Aldehyde perfusion.

Fig. 7. Interlocking finger-like projections (arrows) of adjacent small cells. The dense-cored vesicles do not extend into these projections. Aldehyde immersion.

Fig. 8. Small cell (*C*) and principal sympathetic neuron (*P*) related to same ensheathing process of satellite cytoplasm (*S*). Aldehyde perfusion.

Fig. 9. Principal sympathetic neuron (*P*) with closely related process of small cell (*C*). Between arrows their surface membranes are in apposition and are linked by attachment plaques (*a*). Aldehyde perfusion.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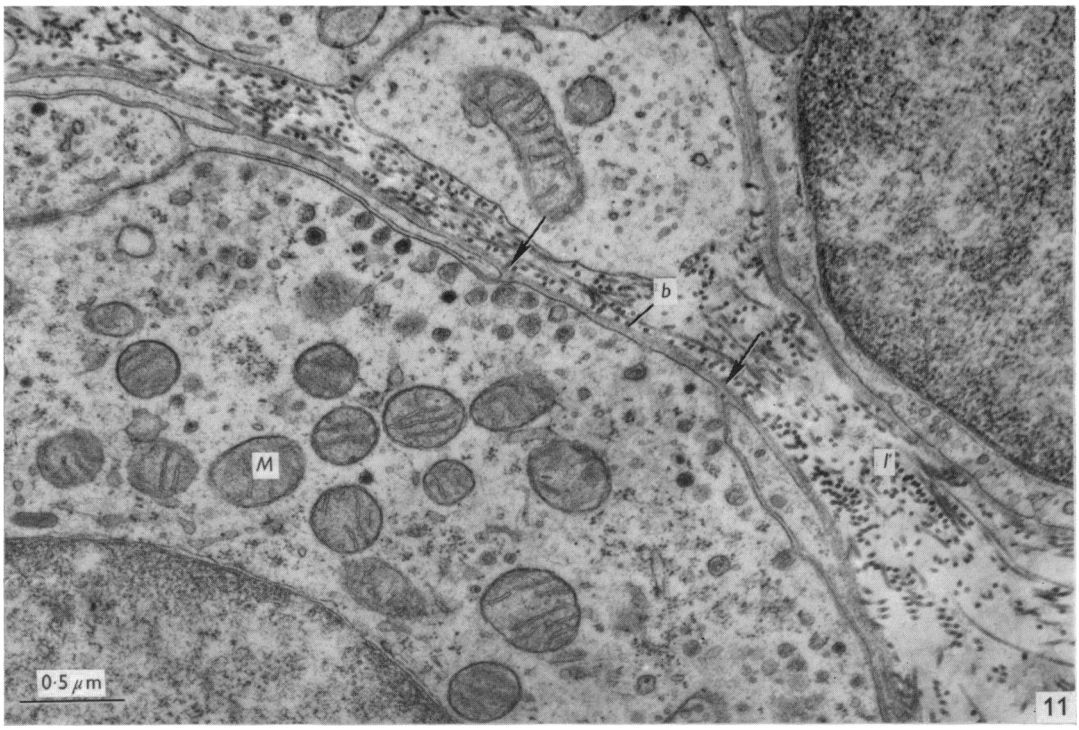
illustrated) may be linked with it by an attachment plaque. The mitochondria of the processes may be of the form typical for the somata of the small cells but may be elongated; and sometimes a much larger, rounded mitochondrion with regular parallel cristae is seen, occupying much of the profile of a process (Fig. 16). Such a profile has been seen to give rise to an efferent synapse.

Afferent synapses

Processes containing many vesicles of synaptic type are found lying in close apposition with the small cells, with vesicles clustered towards regions of mutual membrane specialization (Figs. 17–20). These are interpreted as afferent synapses. The vesicles may be entirely of the small clear type, or may include a few dense-cored vesicles of 60–80 nm in diameter with a core of moderate electron-density. The appearance of the terminals is essentially the same, whether the initial fixation is by aldehydes or by osmium. The afferent terminals often occur in pairs, placed close together upon the same small cell (Figs. 17, 18), and an instance has been seen of three terminal profiles upon adjacent regions of the same small cell, with other afferent terminals upon small cells in close proximity. The afferents are sometimes seen to originate as expanded profiles from a slender stem (Fig. 19), which has on occasion been seen to arise as a lateral branch from a substantial unmyelinated nerve fibre (Fig. 18). The contacts are placed more commonly on the cell body than upon a process, often lie at one pole of an elongated cell, and may deeply indent the cell body (e.g. see Fig. 27). In this they are unlike the afferent synapses upon the principal neurons of the ganglion, which are predominantly axo-dendritic in the rat (Taxi, 1965). In the afferent synapses upon the small cells, multiple small, inconspicuous zones of membrane specialization are found, each with a little dense material aggregated to the membrane without conspicuous asymmetry or notable increase of membrane gap, and with an associated cluster of small clear vesicles in the presumed afferent terminal. These specializations occupy only a small proportion of the region of apposition of the membranes, and from one to five may be seen in a single section of a profile. This type of synapse has features in common with Gray's Type II axosomatic synapses in the cerebal cortex (Gray, 1959). In the cytoplasm of the small cells underlying the area of contact, dense-cored vesicles are typically few or absent; but on occasion they may be clustered close to the membrane at the same point, an appearance which raises the question whether the synapse might not sometimes be reciprocal. We suspect, however, that the quality of fixation may have some influence on this point, as we have observed an unusually regular alignment of dense-cored vesicles in a single row in contact with the plasma membrane and with membrane-bounded structures such as mitochondria or multivesicular bodies in a preparation in which other criteria, such as dilatation of cisternae of endoplasmic reticulum and wide separation of the two layers of the nuclear membrane in the intervals between pores, suggested that the preservation was not ideal. The identification of efferent synapses from the small cells has therefore been treated with some caution.

Fig. 10. Montage showing a cluster of small granule-containing cells with few areas of apposed membranes. Collagen-containing channels of intercellular stroma (*I*) in most places separate the narrow satellite sheaths of adjacent cells. The nucleus of a satellite cell (*S*) is included in the cluster. Aldehyde immersion.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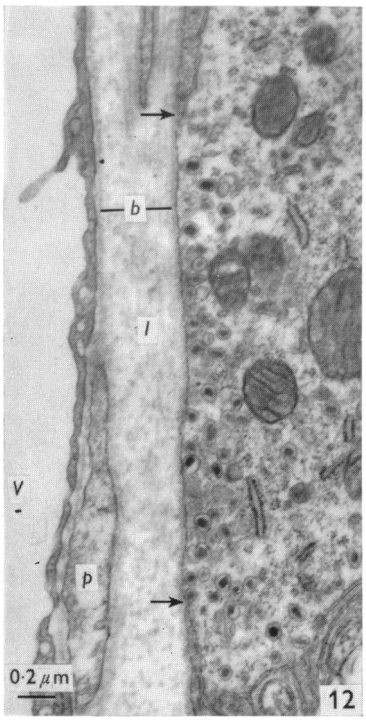
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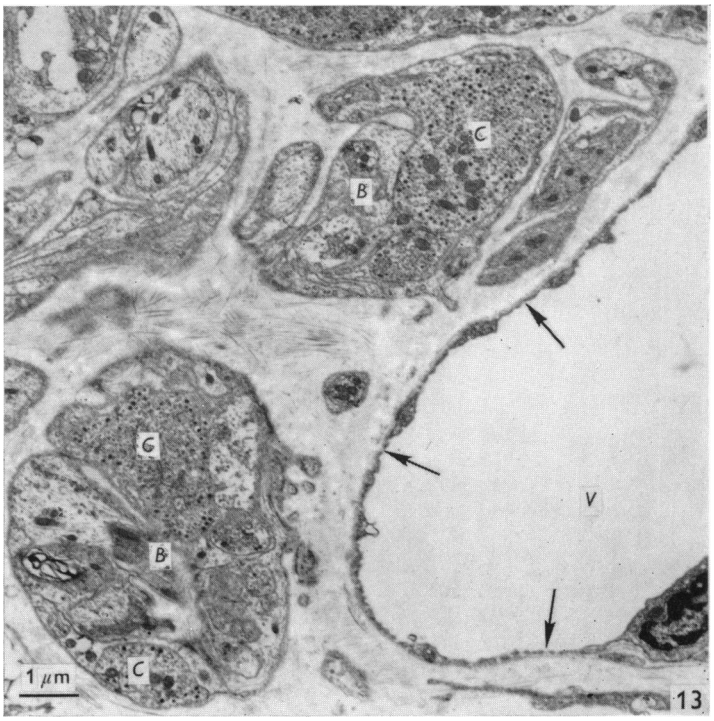
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Efferent synapses

The presence of numerous dense-cored vesicles in close relation to the plasma membrane, coupled with the frequent occurrence of attachment plaques, has complicated the identification of the efferent synapses. However at certain points where the small cell comes into contact with another process a particular combination of features is seen which, taken together, appear to satisfy all the accepted criteria for identification of a synapse, with outward polarity from the small cell. These features include a cluster of vesicles on the presynaptic side (often associated with dense projections from the presynaptic membrane), a marked postsynaptic thickening and usually an increased interval between pre- and postsynaptic membranes. Membrane specializations having this configuration are regarded as efferent synapses. That they may be distinguished from attachment plaques is illustrated in Fig. 21. This shows part of a small cell (*C*) which is linked with a darker profile (*Pr*) by a complex membrane specialization which has two distinct zones. One (to the right) has the symmetry of an attachment plaque and is without a vesicle cluster; and the other (to the left) shows an asymmetry of the dense material aggregated to the membranes, and has a cluster of vesicles (which include dense-cored vesicles) lying close to the membrane and associated with dense projections from it, in the cytoplasm of the small cell. The latter arrangement is regarded as an outgoing synapse.

Efferent synapses thus defined have been found to arise both from the processes of the small cells and from their cell bodies. Figures 24 and 25 show what seems to be an unequivocal efferent synapse and is an example of a somatic efferent synapse. A process is here seen indenting the cell body of a small cell by a spine-like projection which has membrane specializations on each side. These show asymmetrical aggregations of electron-dense material to the membrane, greater on the postsynaptic side, an increase of inter-membrane gap with an accumulation of denser material between the membranes, and associated vesicle clusters in the cytoplasm of the small cell. The vesicle clusters include dense-cored vesicles one of which is lying in close contact with the membrane. Smaller clear vesicles are seen but are not conspicuous in these vesicle clusters. The dense-cored vesicles are more closely crowded here than in adjacent regions of the cell periphery, and are lying in more than one row. The postsynaptic structure in this case is a short spine arising from a larger process which contains both neurotubules and ribosomes and, therefore, may well be a dendrite.

Other examples of efferent synapses from the small cells are shown in Figs. 22 and 23. The area of membrane specialization occupies only a part of the region of ap-

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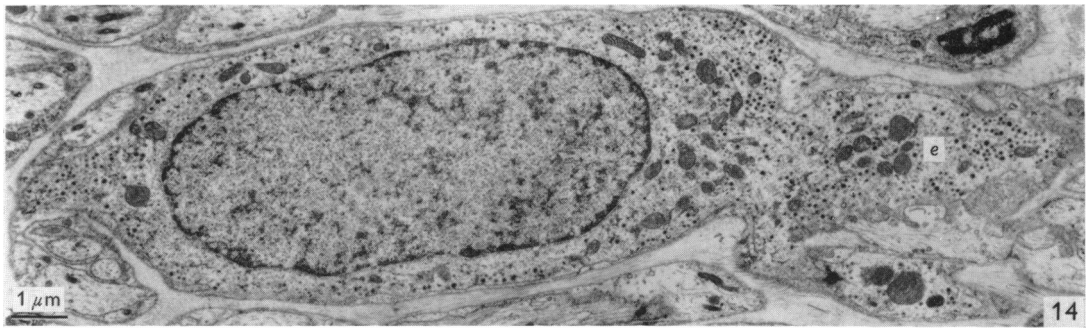
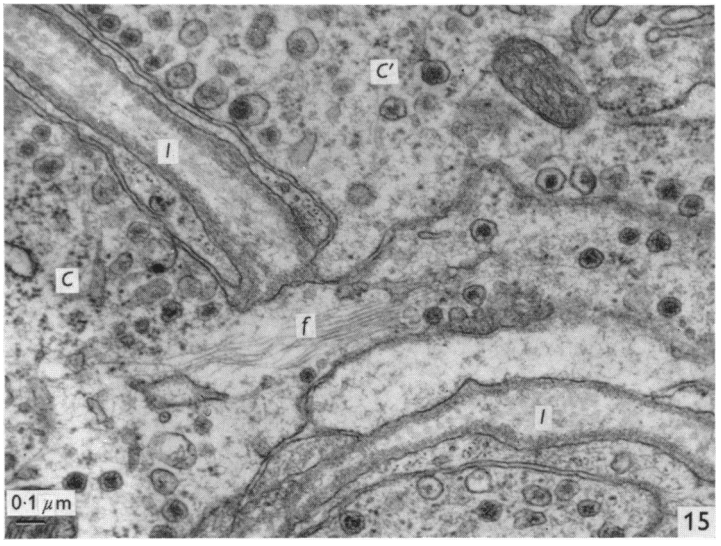
Fig. 11. (Detail from Fig. 3). Interval (between arrows) in satellite sheath of small cell, where the surface of the small cell is exposed across a basement membrane (*b*) to the collagen-containing intercellular stroma (*I*). Note the numerous mitochondria (*M*) in the small cell. Osmium immersion.

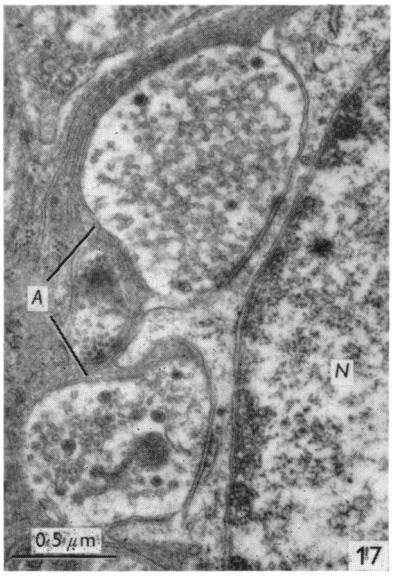
Fig. 12. 'Exposed' area of small cell surface (between arrows) separated by a basement membrane and a narrow channel of intercellular stroma (*I*) from the basement membrane of a fenestrated capillary vessel (*V*). A pericyte process (*p*) is present. *b*, basement membranes. Aldehyde perfusion.

Fig. 13. Bundles (*B*) containing parts of several small cells (e.g., *C*) lying close to a vessel (*V*) with many fenestrations, some of which are indicated by arrows. Aldehyde perfusion.


e
1 μm
14


C'
I
C
f
I
0·1 μm
15


M'
M
C
0·5 μm
16


A
N
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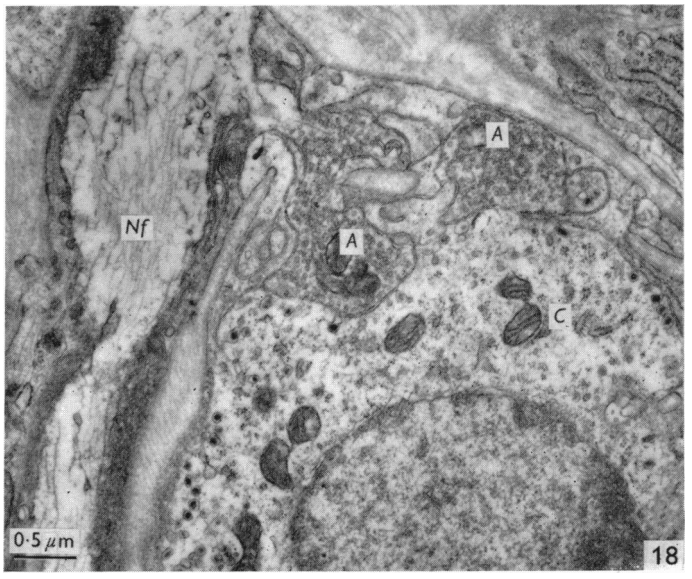
17


A
Nf
A
C
0·5 μm
18

position of the pre- and postsynaptic membranes. Most commonly there is a single area of membrane specialization, but there may be two (as in Fig. 25), which may be inclined at an angle to each other or may lie in the same plane. The synaptic area is commonly concave towards the postsynaptic process, as in the examples shown, but may be flattened or curved in the opposite direction. The characteristic dense-cored vesicles of the cells are in all cases clustered towards the region of membrane specialization in more than one row and are sometimes seen to lie in contact with the membrane. Within these clusters, as in other parts of the cell, the cores of the vesicles may show considerable variation in electron-density. The postsynaptic cytoplasm occasionally shows a well-defined row of sub-junctional bodies (cf. Taxi, 1965), as in Fig. 23, which illustrates a synapse arising from a process of a small cell. The latter contains an example of a rather large mitochondrion with particularly regular parallel cristae.

These efferent synapses of the small cells are very different in character from their afferent synapses. Instead of multiple small and inconspicuous zones of membrane specialization they show one or two more extensive zones, which have conspicuous aggregations of associated dense material, more pronounced on the postsynaptic side, and there is frequently an increase of inter-membrane gap, which is not typical for the afferent synapses. In particular, however, the vesicles associated with the efferent synapses differ from those in the afferent terminals in being predominantly dense-cored and in including only a minority of small clear vesicles (e.g. compare Figs. 17–20 with Figs. 21–25). Unlike the afferent synapses, the efferent synapses are less well displayed after osmium fixation than after aldehyde fixation, because osmium is less effective in preserving the dense-cored vesicles, although it gives better preservation of basement membranes and collagen.

In the course of the present study, 60 efferent synapses of the small cells have been identified, in or near small cell clusters. Regions more remote from known small cell clusters have also been studied; here typical small cell processes are uncommon, and synapses resembling the efferent synapses of the small cells as described above are rarely seen. Synaptic terminal profiles containing a few dense-cored vesicles of about 80 nm diameter in addition to large clusters of small clear vesicles are regularly found, but there is insufficient evidence as yet to connect these with processes of the small

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Fig. 14. Montage. Elongated small cell with an irregular extension (*e*) at one pole which contains cytoplasmic microtubules, mitochondria, ribosomes and dense-cored vesicles. Aldehyde perfusion.

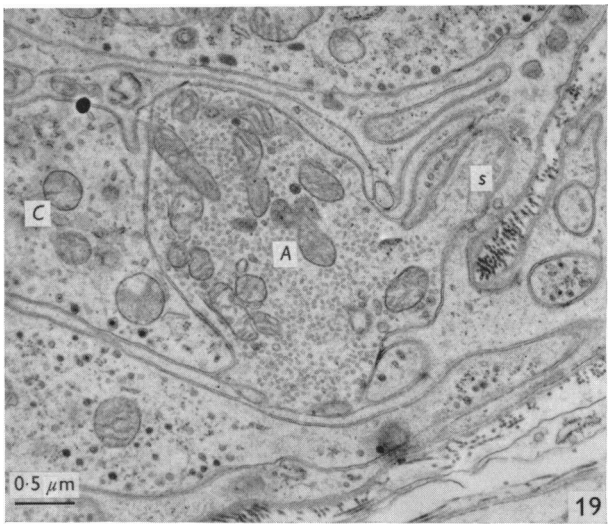
Fig. 15. Slender process with core of filaments (*f*) arising from one small cell (*C*) and becoming apposed to another (*C'*). Note intercellular channels (*I*) bounded by basement membrane. Aldehyde immersion.

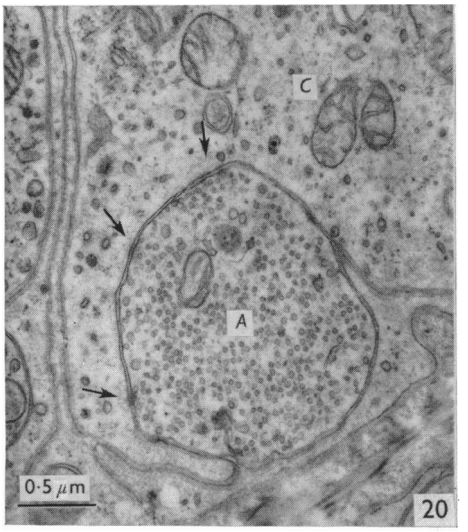
Fig. 16. Process (*C*) of small cell containing a very large rounded mitochondrion (*M*) with regular parallel cristae. Mitochondria (*M'*) of small cell soma (above) for comparison. Osmium immersion.

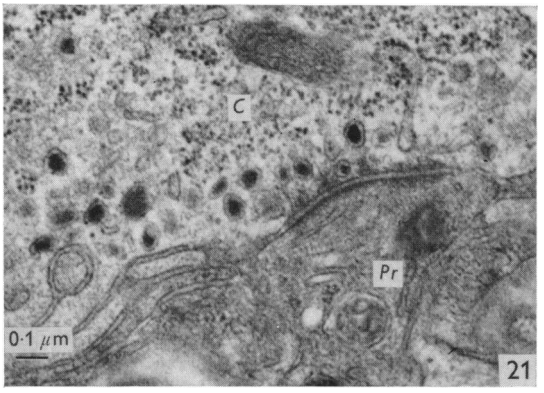
Fig. 17. Pair of afferent terminal profiles (A) synapsing upon and indenting the soma of a small cell, the nucleus of which (*N*) appears on the right. Note dense-cored vesicles intermingled with clear vesicles in the terminal profiles. Aldehyde immersion.

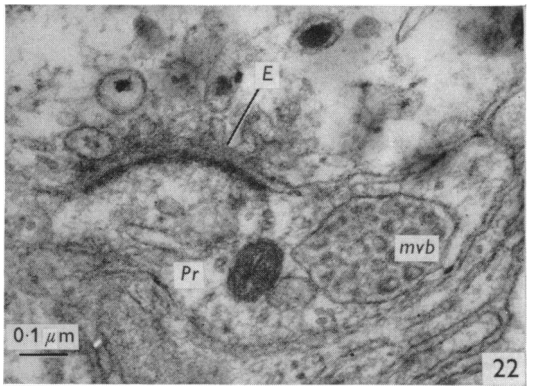
Fig. 18. Unmyelinated nerve fibre (*Nf*) giving rise to a slender lateral branch which is continuous with one of a pair of afferent terminal profiles (*A*) upon the soma of a small cell (*C*). Aldehyde perfusion.

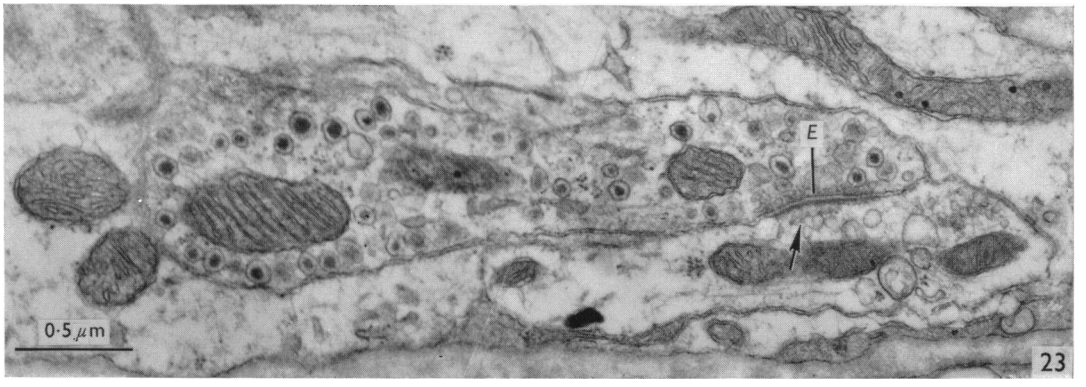
cells, and it has been shown above that the afferents to the small cells also may contain a few dense-cored vesicles. In the large mass of small cells situated at the base of the branches along the external carotid artery (cf. Eränkö & Härkönen, 1965)











efferent synapses from the small cells, both from processes and of somatic type, have been recognized, in addition to many afferent synapses. Of the 60 intraganglionic efferent synapses which have so far been observed, 21 arise from profiles containing a nucleus and are situated on a part of the cell profile circumferential to the nucleus, i.e. not on a cell process. These have therefore been identified as somatic efferent synapses. The remaining 39 are situated upon profiles lacking a nucleus, of which eleven are apparently processes as judged from their size and configuration, while the others are large enough to be tangential sections of the cell body. Thus, at least one-third of the efferent synapses of the small cells which have been identified in this study are of the somatic efferent type. Of these, an interesting example is illustrated in Figs. 26–28. Figure 26 shows a small cell which receives an afferent process at one pole of the soma and gives an efferent synapse at the opposite pole. The synapses are shown at higher magnification in Figs. 27 and 28. Each of the contacting processes is embedded in the small cell, which here exhibits neither a dendrite nor an axon. Only the cell body of the small cell intervenes between the afferent synaptic terminal and the process with which the cell in turn synapses. In another example, 6 efferent synapses have been seen to arise at widely separated points from a single nucleated profile of a small cell. The postsynaptic profiles upon this cell are small, round or ovoid, containing a few irregular clear vesicles but otherwise almost featureless.

Although one synapse has been found in which the postsynaptic element is spine-like and arises from a broad process which is continuous with the cell body of a principal neuron, the postsynaptic structure in the efferent synapses of the small cells has not in every case been identified with certainty. It is not clear whether the small cell may synapse with more than one type of postsynaptic element. The spine of Fig. 25 arises from a process which is likely to be a dendrite; but the postsynaptic profile does not often show such characteristic features. It is usually a small, ovoid or irregular process, which in about 12 % of cases has contained a multivesicular body (e.g. see Fig. 23). Often it exhibits an almost uniformly granular, rather dark cytoplasm such as is found in the dendritic and somatic spines of the principal neurons, but it may contain vesicles resembling those seen in postganglionic axons within the ganglion.

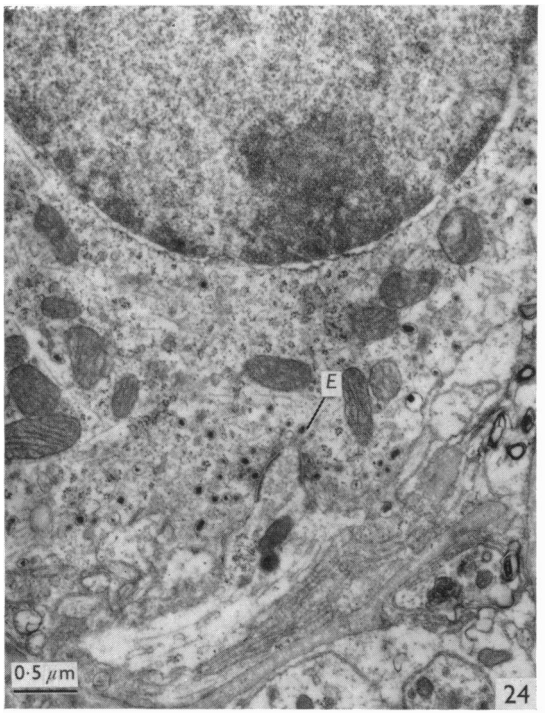
Fig. 19. Afferent terminal profile (*A*) in continuity with a slender stem (*s*) and synapsing upon a process (*C*) of a small cell. The area of contact shows several inconspicuous regions of membrane specialization. Osmium immersion.

Fig. 20. Afferent terminal profile (*A*) upon small cell soma (*S*) showing three inconspicuous regions of membrane specialization (arrows), with slight clustering of vesicles. Osmium immersion.

Fig. 21 Part of a small cell (*C*) linked with a darker profile (*Pr*) by a complex membrane specialization, the right-hand part of which resembles an attachment plaque, while the left-hand part has the features of a synapse with outward polarity from the small cell. Dense-cored vesicles are included in the vesicle cluster. Aldehyde perfusion.

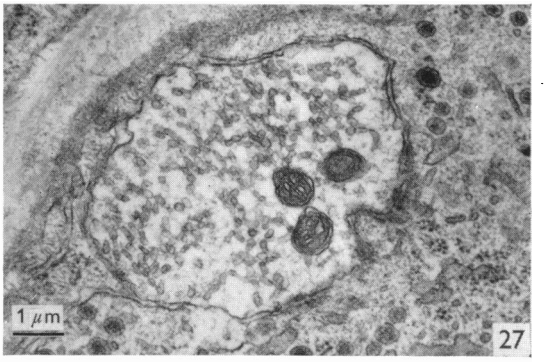
Fig. 22. Efferent synapse (*E*) from a small cell on to a profile (*Pr*) containing a multivesicular body (*mvb*). Aldehyde perfusion.

Fig. 23. Montage. Efferent synapse (*E*) arising from what is probably a process of a small cell. A row of subjunctional bodies (arrowed) is seen in the postsynaptic cytoplasm. Aldehyde perfusion.


E
0·5 μm
24


R
n
0·1 μm
25


A
E
Pr
0·2 μm
26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28

DISCUSSION

These results confirm the observation of Williams (1967*a*, *b*) that the small, granule-containing cells of the superior cervical ganglion of the rat give rise to efferent synapses within the ganglion. These cells are, therefore, clearly in a position to act as interneurons in the conventional sense. There is in addition the possibility that they may also act by liberating active substances, across the exposed areas of membrane we have described, directly into the connective tissue spaces of the ganglion to take effect by diffusion. It appears likely that they will prove to mediate intraganglionic inhibitory processes (Eccles & Libet, 1961; Dunant & Dolivo, 1967; Tosaka *et al.* 1968). The extent to which they show affinity with chromaffin cells is discussed later.

In the present study it has been shown that many of the outgoing synapses from the small cells are of somatic efferent type (cf. Hama, 1966; Sétáló & Székely, 1967), arising directly from the cell body. Since the afferent synapses to these cells also tend to lie upon the cell soma, an interesting situation is disclosed in which afferent and efferent synapses may be placed at opposite poles of the cell, without dendrite or axon, so that only the cell body intervenes. The afferent synapses are often multiple, having two or even three expanded terminals contacting the small cell close together, but they have not been seen to reach a small cell at widely separated points. On the other hand, efferent synapses have been seen spread far apart over a large profile containing the nucleus, and this introduces the possibility that in some cases the efferent and afferent synapses may not be at opposite poles of the cell but may be closer together, perhaps even on occasion lying side by side, as was found by Sétáló & Székely in neurons of the optic tectum of the frog.

The vesicle clusters associated with the efferent synapses are of particular interest. In all cases the characteristic dense-cored vesicles of the cell are included in the vesicle cluster at the cell membrane in the region of the specialization, and they may often be seen in contact with it. We have not found any evidence of the membrane of the dense-cored vesicles becoming disrupted at this point. The clustering of dense-cored vesicles at the synapse is distinguished by the number and close crowding of the vesicles in more than one row from the tendency of these same vesicles to lie in a single row in contact with non-specialized regions of the plasma membrane. The latter tendency may be enhanced when the quality of fixation is impaired (as judged by other criteria), and in such cases may occur also in relation to other membrane-bounded structures within the cytoplasm, such as mitochondria and multivesicular bodies.

These vesicle clusters appear to be composed predominantly of dense-cored vesicles, and to include relatively few small clear vesicles. It is not possible to exclude

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Figs. 24, 25. Somatic efferent synapse (*E*) from a small cell on to a spine-like projection arising from a process containing neurotubules (*n*) and ribosomes (*R*). Fig. 25 shows detail of synaptic region. (Further description in text.) Aldehyde perfusion.

Fig. 26–28. Small cell receiving an afferent synapse at one pole of the soma and giving off a somatic efferent synapse (*E*) at the other. Both the afferent terminal profile (*A*) (shown in detail in Fig. 27) and the postsynaptic profile (*Pr*) (detail in Fig. 28) deeply indent the body of the cell and are partly covered by vesicle-free extensions of its cytoplasm. Aldehyde immersion.

the presence of the latter, for they are much less conspicuous than the dense-cored vesicles and might more readily be obscured within the cluster. The efferent synapse of the small cells is clearly, however, of an unusual type in respect of its vesicle population. It is quite unlike the various terminal specializations of the post-ganglionic sympathetic neurons, for example those in the pineal organ (e.g. Wolfe 1965), where our own methods of preparation reveal satisfactorily the characteristic small (40–60 nm) vesicles with a central or eccentric osmiophilic dot (unpublished observations); it is also unlike the post-ganglionic sympathetic endings in the vas deferens, although here these tend to form discrete endings (Richardson, 1962). Nonetheless it appears undoubtedly to be a synapse, to judge from the asymmetrical membrane specialization, the increased inter-membrane gap, the clustering of vesicles on one side only and the occasional presence of a subsynaptic apparatus.

Although it is possible to characterize at least some of the efferent synapses of the small cells it is not known what may be the form and extent of such axons as they may possess, nor, indeed, whether they have true axons. Processes giving rise to efferent synapses have been seen in the present study but have not been traced further than 7 μm in the ultrathin sections. It appears likely from fluorescence studies that the cells give off processes of moderate length, since Norberg *et al.* (1966) in the rat have seen processes of small strongly fluorescent cells in this ganglion of up to 40 μm in length, and Csillik *et al.* (1967) have made a similar observation in the cat; but these might extend their sphere of activity either on the efferent or on the afferent side. The fluorescence studies also demonstrate a fine fluorescent pericellular network surrounding some at least of the typical large sympathetic neurons of the ganglion (Hamberger, Norberg & Ungerstedt, 1965). The identification of these networks is obscure, and there is as yet insufficient evidence to indicate whether or not they may be correlated with processes of the small granule-containing cells; but it is of interest that Hamberger & Norberg (1965) found that they persist after deafferentation of the ganglion, and that they are therefore presumably of intraganglionic origin.

The extent of the contribution which the small cells might make as sympathetic interneurons must depend in some degree upon their number and distribution within the ganglion. The present study has indicated that there may be approximately 30 clusters or larger groups of these cells in the superior cervical ganglion of the rat. In the ganglion examined, these were distributed chiefly along the cranial two-thirds; here they lay within about 500 μm of every principal neuron, and were in many cases much closer. The radius of the dendritic territory of the principal sympathetic neuron in the rat, as seen in Golgi preparations, is of the order of at least 150 μm (unpublished observations). Thus, even if the processes of the small cells do not extend far beyond the limits of the group or cluster, the cells would appear to be so placed as to be capable of coming into contact with a large proportion of the principal neurons or their dendrites.

The superior cervical ganglion is, however, a complex structure in which there may be more than one pathway of transmission; and the small cells need not necessarily be involved in every pathway. There are various reasons for a possible heterogeneity in the superior cervical ganglion. Like other sympathetic ganglia, it contains both cholinergic and adrenergic neurons; in addition, it is of multisegmental origin, representing a fusion of several segmental elements of the sympathetic system in

the cervical region, and it has diverse functions, relating not only (for example) to sweat glands, skin hairs and vasomotor control but also to the special functions of pupillary dilation and the innervation of the pineal organ.

So far the ultrastructure of the small granule-containing cells has been described chiefly in the superior cervical ganglion of the rat (Siegrist *et al.* 1966; Grillo, 1966; Williams, 1967*a*, *b*; Matthews & Raisman, 1968), but there is a recent account of cells which are probably of the same type in the inferior mesenteric ganglion of the rabbit (Elfvin, 1968). The cells described by Elfvin are larger (10–25 μm in diameter, instead of 6–12 μm), and the vesicles are both larger (150–200 nm) and more numerous, sometimes being distributed throughout the cytoplasm (although they may be predominantly peripheral). In these features the cells are more like adrenal medullary chromaffin cells, but they have processes of a length which would be unusual in a chromaffin cell. The question of particular interest is whether they will be found to bear efferent synapses.

There is suggestive evidence from fluorescence studies that comparable cells may also occur in other species. Csillik *et al.* (1967) have found small, intensely fluorescent cells scattered in the superior cervical ganglion of the cat; and Olson (1967) has made a similar observation in the same ganglion of the mouse. Olson recognized a large group of these cells 'surrounding the root of a nerve trunk leaving the ganglion about the middle of the lateral border' which probably corresponds with the 'well vascularized organ composed of these cells... accidentally observed near the ganglion' in the rat (Eränkö & Härkönen, 1965) and with the particularly large group which we have observed at the base of the branches along the external carotid artery. Ultrastructural studies would, however, be required to show whether all these cells are precisely comparable.

It is important to consider so far as is possible the degree of affinity of the small granule-containing cells with the chromaffin cells of the adrenal medulla, with which they clearly have many features in common. The electron density of the granules of the dense-cored vesicles in the small cells of the rat superior cervical ganglion is comparable with that of the granules in the noradrenaline-containing cells in the adrenal medulla (Coupland, 1965*b*, Elfvin 1965*a*), but the granules are smaller (about 30–75 nm in diameter, entire vesicle about 65–120 nm in diameter, as compared with a granule diameter of 50–350 nm in the adrenal medulla). The granular vesicles of the small cells are also less densely packed in the cells, lying in the processes and in the peripheral rim of the perinuclear cytoplasm rather than throughout the cytoplasm. In the experiment in the present study, in which an attempt was made to minimize the stress involved in the method of preparation, the same order of difference was seen in size and in distribution between the granules of the small cells in the superior cervical ganglion and those of the noradrenaline-containing type of chromaffin cell in the adrenal medulla.

The grouping of the small cells and the relationships between adjacent cells are in many cases rather similar to those in the adrenal medulla. Like chromaffin cells, the small cells lie often in clusters and may have their cell membranes in close apposition, linked here and there by attachment plaques and bearing small interlocking finger-like projections (cf. Coupland, 1965*b*; Elfvin, 1965*a*). But the small cells are more often intimately related to satellite cells, in various ways, than are the chromaffin

cells of the adrenal medulla, where extensive areas of the cell surfaces are separated only by basement membrane from the perivascular spaces of the fenestrated capillaries, and where satellite cell covering, if present, is more or less restricted to the regions of the afferent nerve terminals (Elfvin, 1965*a*; Coupland, 1965*c*). Clusters of small cells are always surrounded by satellite sheaths. Mutual apposition of membranes between the small cells of a cluster may be virtually absent and the cells may, instead, have independent satellite sheaths, separated wholly or in part by extensions of the intercellular stroma containing collagen fibres. Some of the small cells may have considerable independence and may lie singly, scattered among principal neurons and bundles of nerve fibres (e.g. Fig. 14). In this they closely resemble the principal neurons of the ganglion.

It is noteworthy, however, that here and there the sheath of satellite cytoplasm is deficient over a small area, leaving part of the membrane of a small cell exposed across a basement membrane to the connective tissue stroma of the ganglion or to the perivascular space of a fenestrated capillary (cf. Elfvin, 1965*b*, for an account of capillary fenestrae in the adrenal medulla). The presence of these occasional gaps in the satellite wrappings of the small cells is a feature which is hardly ever seen in relation to the principal cells of the ganglion, and it may be highly significant. This arrangement would allow the small cells to exchange biologically active material with the tissue fluids and perhaps, in the manner of chromaffin cells, with the bloodstream, through the fenestrated capillary vessels which are sometimes seen in relation to small cell clusters. But the possession by the small cells of relatively long processes and above all of efferent synapses set them apart from typical chromaffin cells. Taken all together, the various features of the small cells place them in a category intermediate between the typical chromaffin cell and the typical sympathetic neuron. Fig. 29 illustrates diagrammatically the principal features of these three cell types. It is possible that all three types represent divergent lines of development from the same stem cell, the primitive sympathetic neuron (Coupland, 1965*a*; Coupland & Weakley, 1968). It is also possible that the small cells themselves may not form a completely homogeneous group but may prove to exhibit gradations of character between the two extremes of the range. It is interesting that the adrenal medullary chromaffin cell has a peripheral arrangement of granules at a certain stage of development (Coupland & Weakley, 1968), and that the developing sympathetic neuron also has similar granules in the cell body, which are later seen only in the processes (Wechsler & Schmekel, 1967). In the small cell the retention of granules in the cell body might perhaps be partly related to the presence of efferent synapses on or near to the cell soma; and the relative scarcity of membrane-bound ribosomes and the condensed state of the nuclear chromatin might prove to be correlated with the possibility that these cells do not possess a long axon.

Since the small granule-containing cells of the rat superior cervical ganglion in so many ways resemble noradrenaline-containing chromaffin cells, it is interesting that they are not a prominent feature of material prepared by the standard light microscopic techniques for chromaffin tissue (cf. Lempinen, 1964; Coupland, 1965*a*). Eränkö & Härkönen (1965) explored this point specifically. It may be that the smaller size and relatively smaller number of the granules explains this discrepancy, or it may be that the material is not noradrenaline but some other monoamine such as sero-

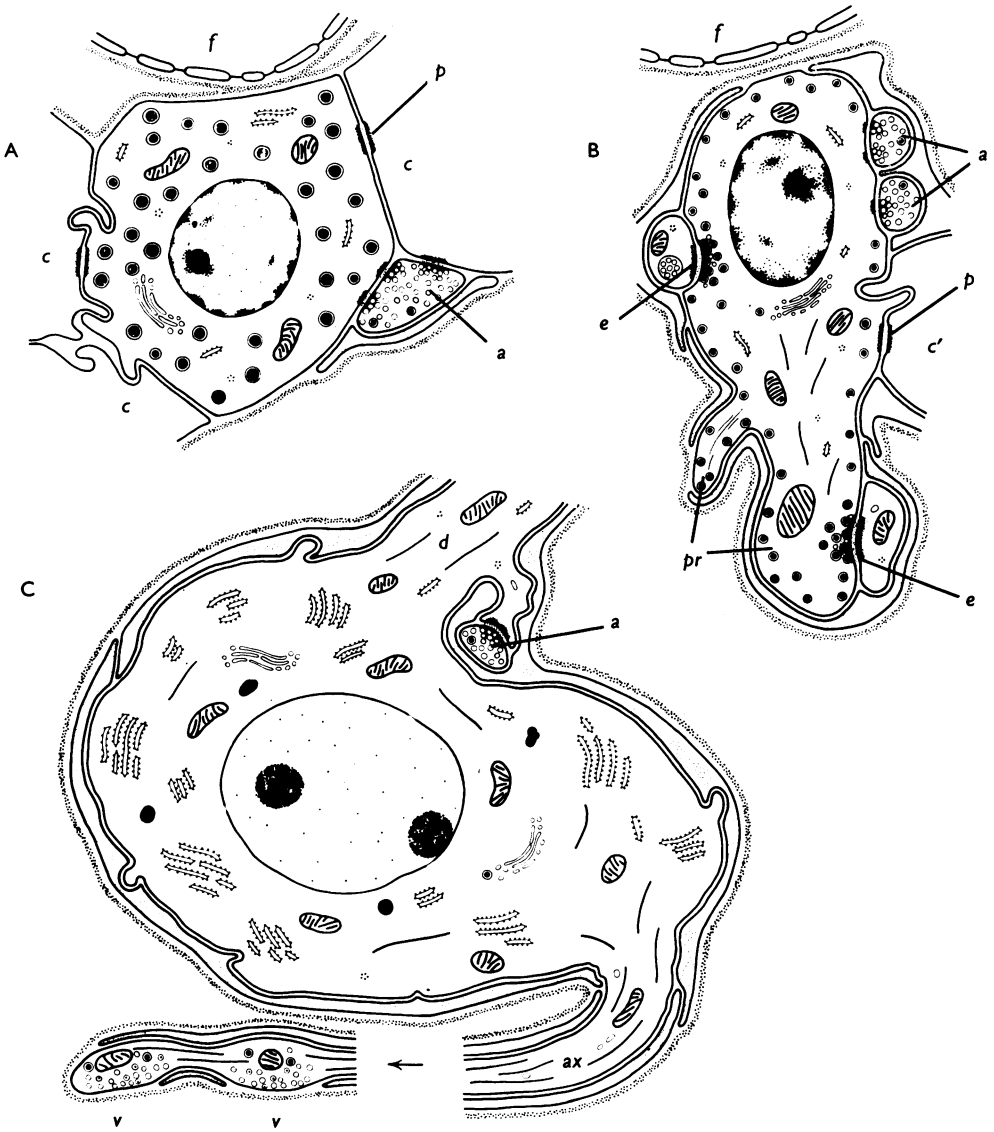

Fig. 29. Diagram comparing some features of an adrenal medullary chromaffin cell (A), a small granule-containing cell (B) and a principal sympathetic neuron (C). In the series A to C the covering of satellite cytoplasm (fine stippling) becomes progressively more extensive, while the distribution of intracytoplasmic dense-cored vesicles is progressively more restricted. Cell A, which secretes into the bloodstream, is freely related across basement membrane (coarse stippling) to fenestrated capillaries (*f*); and cell C, which directs the release of transmitter substance to its axon terminals, has a complete covering of satellite cytoplasm. Cell B, with efferent synapses (*e*) and cytoplasmic prolongations (*pr*) in addition to areas of cell surface free of satellite covering, appears to represent an intermediate cell type, exhibiting some of the features both of the neuron and of the chromaffin cell.

a, afferent synapses; *p*, attachment plaques; *c*, chromaffin cells adjacent to cell A; *c'*, small granule-containing cell adjacent to cell B; *d*, base of dendrite, *ax*, origin of axon, *v*, terminal varicosities of axon of cell C.

tonin, as suggested by Eränkö & Härkönen (1965) on the basis of the colour of the fluorescence. Norberg *et al.* (1966), however, conclude that it is noradrenaline, and point out that the apparent colour of fluorescence may vary according to the concentration of a substance; the observations of Mayor & Kapeller (1967) on constricted adrenergic nerves would lend support to this. Corrodi & Jonsson (1967) have given an account of certain problems involved in the discrimination of biologically active monoamines by the fluorescence method.

The structures with which the small cells are in synaptic contact have not in all cases been firmly identified. The afferent terminals might be derived, for example, either from preganglionic axons or from collaterals, if they occur, of the postganglionic axons (cf. Bessou, Laporte & Planel, 1959). In the afferent terminals the small vesicles are of the clear type and are not seen to contain any osmiophilic dot; this finding, therefore, sets them apart from the extraganglionic axon terminals of the principal neurons and makes it less likely that they are derived from axon collaterals of these cells. The more likely possibility is therefore that they arise from preganglionic axons. In electrophysiological studies, Dunant & Dolivo (1967) have found evidence which suggests that the intraganglionic inhibitory process in the rat superior cervical ganglion is under the influence of cholinergic nerves; this they attribute to preganglionic afferent fibres (of the C group) synapsing upon cells of the type we are considering.

In respect of the postsynaptic structure, we have evidence which indicates that in some cases at least it may be a spine arising from a dendritic process of a principal sympathetic neuron. Our observations, therefore, support the view that the small granule-containing cells are interneurons innervated by preganglionic fibres and forming synapses upon the principal sympathetic neurons.

SUMMARY

1. The small, granule-containing cells in the superior cervical ganglion of the rat have been located by light microscopy in Araldite-embedded material and studied with the electron microscope. The cells occur in small clusters which lie singly or in groups, and 30 clusters or groups have been found in a single ganglion.

2. These small cells are about 6–12 μm in diameter and contain numerous dense-cored vesicles (granular vesicles) of diameter about 65–120 nm, which lie mainly in the peripheral part of the cytoplasm. The mitochondria are rounded and tend to show a regular pattern of straight, parallel cristae. Cisternae of granular endoplasmic reticulum are scanty, but there are numerous free ribosomes. The nucleus is rounded and contains peripheral aggregations of darkly granular material.

3. The cells give rise to processes which contain dense-cored vesicles and characteristic mitochondria, and may show longitudinally-oriented tubules or filaments.

4. Adjacent small cells often lie with regions of their cell membranes in close apposition and may here be linked by attachment plaques. Elsewhere the cells or cell clusters are ensheathed by the cytoplasm of satellite cells. The satellite sheath shows here and there small deficiencies, and in these areas the small cell surface membrane is separated only by a basement membrane from the connective tissue stroma of the ganglion. Such an area of 'exposed' membrane may abut on the perivascular space of a fenestrated capillary vessel.

5. Processes containing many small clear vesicles and occasional dense-cored vesicles (of diameter 60–80 nm) establish specialized contacts with the small cells, which are regarded as afferent synapses. In addition the small cells or their processes show areas of specialized contact with other profiles, which are interpreted as being efferent synapses. Both the afferent synapses and the efferent synapses may be placed upon the cell body of the the small cell. To the latter arrangement is applied the term 'somatic efferent synapse'. In some cases, at least, the postsynaptic structure has been identified as the dendrite of a principal sympathetic neuron.

6. It is concluded that these small granule-containing cells are capable of acting as interneurons within the ganglion, and that, morphologically, they show many features which place them in a category intermediate between chromaffin cells and principal sympathetic neurons.

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