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Structure and localization of synaptic complexes in the cardiac ganglion of a portunid crab

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Summary

The cardiac ganglion of *Portunus sanguinolentus* exhibits spontaneous rhythmic activity when isolated. The ganglion contains five large and four small intrinsic neurons and is innervated by three pairs of fibres originating in the thoracic ganglia.

We have identified the processes of the large neurons in electron micrographs by injecting these cells with two electron-dense markers, horseradish peroxidase (HRP) and Procion Rubine (PR). In addition we have studied the processes of the four smaller neurons by light microscopy serial reconstructions and by electron microscopy of selected regions. Both markers were found only in neuronal processes and not in glial cells nor in the extracellular space, except close to the soma of the injected cell.

We found contacts between the small secondary (collateral) processes of the large cells but not between their somata or their primary processes (axons and dendrites). Two specialized structures present at the contacts between the collateral processes were small membrane close appositions, possibly the site of electrotonic junctions, and chemical synapses. Contacts between processes marked by HRP and those marked by PR were common, as were contacts between processes marked by either HRP or PR and those of the other intrinsic neurons. Adjacent processes stained by PR could contain PR deposits of different densities, but it is unclear whether this finding was due to intercellular diffusion of the dye or to its diffusing at different rates into branches of the same process. Identified processes of all the intrinsic neurons contained the same type of vesicles, which were different from those found in processes of the extrinsic fibres. Chemical synapses were present at contacts between processes of the extrinsic and intrinsic neurons, as well as at contacts between processes of the intrinsic neurons.

The axons of three small cells made a series of contacts at which extensive arrays of membrane close appositions, but not chemical synapses, were found. These three axons also formed contacts, either directly or through their collateral branches, with processes of the large cells, at which both membrane close appositions and chemical synapses were present. The axon of the fourth small cell could not be followed in our series.

Introduction

The nervous system which controls the heart beat of decapod Crustacea has been the object of considerable physiological research because it appears to be a relatively simple model for the analysis of the mechanisms resulting in rhythmic neuronal activity (Hagiwara, 1961; Hartline, 1979; Tazaki & Cooke, 1979a, b, 1983). A ganglionic trunk contains the somata of five large and four small neurons as well as an extensive neuropil (Alexandrowicz, 1932). When the ganglionic trunk is isolated from the surrounding tissues, the system continues to be spontaneously active with each cell generating bursts of impulses separated by silent periods. The bursts are tightly coordinated, and in some crab species the action potentials of the five large cells are synchronous (Tazaki, 1972; Tazaki & Cooke, 1979a; Berlind, 1982).

The main factor assuring a coordinated spike discharge is thought to be a widespread system of electrotonic junctions. It is known that the large cells are strongly electrically coupled to each other and to the small cells (Hagiwara, 1961; Tazaki & Cooke, 1979a), and there is indirect evidence that the small cells are also coupled to each other (Tazaki & Cooke, 1979a). Although the physiological evidence for these electrical junctions is clear, their localization and structure are a matter of debate. Irisawa & Hama (1965) and Ohsawa (1972) have found axo-axonic contacts in the ganglia of *Squilla* and *Panulirus*, respectively, and have suggested that these may be the seat of the electrotonic junctions. However, the presence of axo-axonic contacts has not been confirmed by Aizu (1975) who has investigated the

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ganglion of *Peneaus*, nor by Hawkins & Howse (1978) who have studied the ganglion of *Callinectes*.

Tazaki & Cooke (1983) have injected Lucifer Yellow (Stewart, 1978) in the large neurons of the isolated ganglion of the crab *Portunus sanguinolentus*, one of the species showing synchronous spike discharge of the large cells. When injected into any one of the five large cells, Lucifer Yellow diffused to the other four, but did not pass, apparently, into the small cells. Dye coupling, as revealed by Lucifer Yellow, did not involve conspicuous axo-axonic contacts, but appeared to depend on a diffuse system of junctions distributed throughout the entire ganglionic trunk. Assuming that the same junctions also provide the structural substrate for dye and electrical coupling (Stewart, 1978, 1981), it is conceivable that these entities could have been overlooked in previous electron microscopical studies because of their small size.

We have studied the distribution of horseradish peroxidase (Adams, 1977) and Procion Rubine (Christensen, 1973; Stead, 1973) in the ganglion of *Portunus sanguinolentus*. Intracellular injection of these substances in the large neurons resulted in the deposit of recognizable electron-dense precipitates in the somata and processes of the injected cells. Using these two markers we have been able to document the presence of numerous contacts between the terminal arborizations of the secondary processes of the large cells, called short collaterals by Alexandrowicz (1932), but not between their somata or their larger, primary processes, including their axons. By contrast, extensive contacts between the axons of (at least) three of the four small cells and between collaterals of the large and small cells were present. Small, isolated membrane close appositions, possibly the sites of the electrotonic junctions, were present at the contacts between the collateral processes of the large cells and at those between collaterals of the large and small cells, while extensive arrays of similar membrane appositions characterized the contacts between the axons of the small cells. Assuming that the close appositions identified in our sections are indeed sites of electrotonic junctions, these data would suggest that the junctions between the small cells may act as high-pass filters, while those found between the large cells act as low-pass filters. We also describe the structure of the chemical synapses found in this simple nervous system.

Material and methods

Fixation procedure and selective marking of the large neurons

Twenty ganglia were used in this study. Ten, used as controls, were fixed immediately after completion of the dissection in a 2% buffered solution of glutaraldehyde and then postfixed in a 0.5% solution of OsO_4 (King, 1976b).

In six of the remaining ten specimens Procion Rubine (PR), an electron-dense dye, was injected by hyperpolarizing current pulses into the soma of one of the five large neurons present in the ganglion (see Figs 1a, 1b, 2). The sample of dye used was PR MX-B (molecular weight 716.5), kindly supplied by ICI America, Inc. For the injection, the tips of the micropipettes were filled with a 2% solution of PR in 0.25 M KCl; the remainder of the pipette was filled with 3 M KCl. The pulses had an amplitude of between 5 and 10 nA, a duration of about 1 s and were delivered at the frequency of 0.5 per second for about 1 to 2 h until the soma of the injected cell was bright red. After injection the specimens were left to incubate for several hours in saline at room temperature and then fixed. During the incubation the dye could be seen to diffuse slowly from the soma of the injected cell into the initial part of its axon and dendrites (see legend to Fig. 1). The incubation was stopped when diffusion of the dye, as observed at the dissection microscope, was complete.

In the four other specimens, one large cell was injected with PR and another one with horseradish peroxidase (HRP; Sigma type VI). HRP was dissolved in 0.25 M KCl and the solution brought to pH 10 with borate buffer. Pipettes for injecting HRP were filled in the same manner as those used for PR. The same parameters used for injecting PR were also used for HRP except that pulse polarity was depolarizing. After the injections of PR and HRP were completed, the preparations were left overnight in saline at 15° C and then fixed. After a few rinses in 0.1 M phosphate buffer (pH 7.2) they were incubated with diamino-benzidine (Isopac, Sigma) and then made to react with a dilute solution of hydrogen peroxide (Adams, 1977). During injection of PR and HRP the spontaneous rhythmic activity of the cells was continuously monitored to check the state of the preparations and to be sure of the intracellular position of the micropipettes.

None of the ganglia used were stained *en bloc* with uranyl acetate.

Sectioning procedures

Control and experimental specimens alike were dehydrated in an ethanol series, cleared in propylene oxide and embedded in Epon. In all cases the ganglionic trunk was sectioned perpendicularly to its long axis. Six of the ten control specimens were cut in sections, 0.75 µm thick, which were then stained with hot 5% paraphenylenediamine (Mirolli & Talbott, 1972) for light microscopy. From two of these specimens, complete series of the entire trunk were obtained. The two series were used to reconstruct the course of the axons of the four small cells (Fig. 1c; see legend) which, because of their size, could not be injected with intracellular markers. Partial series were cut from four other specimens; the series were limited to the anterior end in two of the specimens, and to the posterior end in the other two. A preliminary study of this material showed that the main structural features of each region of the trunk were repeated, with only minor variations, in all the specimens examined. Most of the neuropil was concentrated close to the two ends where the initial segments of the axons of the large cells were also

found. These results suggested that a reasonably accurate survey of the ultrastructural details of the neuropil could be obtained by cutting groups of thick and thin sections at intervals of ~ 5 μm in these two regions of the ganglionic trunks. This procedure was followed for the four remaining control specimens and for those in which dyes were intracellularly injected. The interval between the groups of thick sections was sufficient to follow large neural processes (5 μm or more in diameter) so that many of the structures visible in electron micrographs of the corresponding thin sections could be traced back to identified neurons.

Results

Anatomy of the cardiac ganglion

The ganglion of *Portunus* contains, as is the rule in the brachyuran Crustacea, five large and four small neurons (Alexandrowicz, 1932). The somata of these cells are found in the enlarged ends of a short ganglionic trunk; those of three of the large neurons are located in the anterior end and those of the other six in the posterior end. Alexandrowicz (1932) distinguished the large processes of these neurons into axons and dendrites, depending on their length and their course (Fig. 1a–c). It is not known whether the dendrites have conduction properties that are different from those of the axons (Tazaki & Cooke, 1983), but since it has been suggested that they may have mechanoreceptive peripheral endings (Hagiwara, 1961), it seemed advisable to maintain the distinction introduced by Alexandrowicz. Dendrites and axons of the intrinsic neurons give off secondary processes or short collaterals (Alexandrowicz, 1932) (Fig. 1a–c). Similar short collaterals are also given off by the three pairs of fibres which provide the extrinsic innervation of the ganglion (not shown in Fig. 1). The short collaterals branch, close to their points of origin, into bushy arborizations (Alexandrowicz, 1932; Tazaki & Cooke, 1983). Arborizations of the intrinsic neurons overlap with each other and with those of the extrinsic fibres to form the neuropil (Alexandrowicz, 1932).

Distribution of Procion Rubine and horseradish peroxidase

The somata of cells injected with either PR or HRP could be identified in whole mounts of the ganglionic trunk (Fig. 2). HRP also stained the axons and other processes of the injected cells, including their short collaterals. In agreement with the results of Alexandrowicz (1932) and of Tazaki & Cooke (1983), most of the collaterals appeared to be born from the initial segment of the axons and dendrites. PR was only visible at the light microscopic level in the soma and in the initial segments of the large processes of the injected cells (Fig. 2). Neurons marked by PR and HRP could also be identified in sections stained with uranyl acetate and lead citrate (Figs 3–6) as well as in unstained sections (Fig. 7).

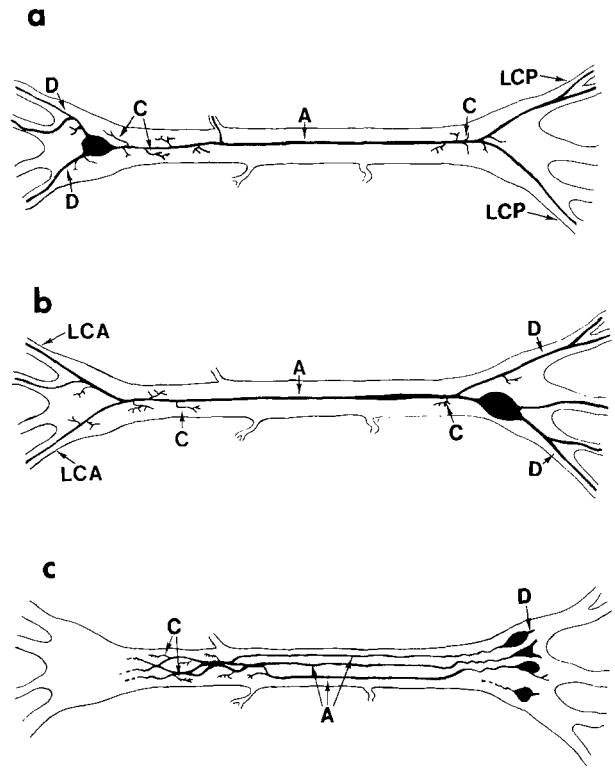


Fig. 1. Shape of the intrinsic neurons of the cardiac ganglion of *Portunus sanguinolentus*. (a, b) Examples of the shape of the anterior (a) and the posterior (b) large cells. The two figures, based on drawings of *in toto* preparations of cells injected with HRP, demonstrate the course in the trunk of the axons (A) and dendrites (D) of these cells as well as the origin and position of their secondary processes (C), called short collaterals by Alexandrowicz (1932). As shown in the drawings the axons of the anterior cells run backwards to the posterior end where they bifurcate and exit the trunk through the posterior roots of the lateral connectives (LCP) while the axons of the posterior large cells have a symmetrical course, running forward in the trunk and exiting through the anterior roots of the lateral connectives (LCA). (c) Schema showing the course in the trunk of the axons of three of the small posterior cells as reconstructed through the study of a light microscopic series. These axons could be followed up to the anterior end of the trunk where they formed numerous close contacts and gave off collateral branches. The axon of the fourth small cell could not be followed in our series. This figure is not drawn to scale and has only illustrative value. See Fig. 17 for a more accurate representation of the contacts made by the three small axons.

The cells injected with HRP contained a dense, floccular precipitate which was not uniformly distributed but appeared condensed in relatively large aggregates over the inner face of the plasma membrane and on the outer mitochondrial membrane (Fig. 6). The preservation of the structure of the

processes stained by HRP was uniformly poor (Figs 7–10) irrespective of how far they were from the soma; only mitochondria among the cellular organelles could be recognized. However, in the soma itself the organelles were less affected (Fig. 6), as if the structural damage provoked by the HRP reaction was somehow dependent on the size of the cellular compartments in which the damage took place. With the exception of traces found close to the point of injection, HRP precipitate was only found in the injected cell.

The cells injected with PR appeared darker than the surrounding structures because of the presence of a fine, electron-dense precipitate (Figs 3, 7–9). Signs of shrinkage and of disrupted fine structure, although present, were much less severe than in the cells injected with HRP. In the soma (Fig. 3) and in the processes found close to the soma itself (Fig. 4), PR appeared to bind selectively to the membranes of mitochondria, of lysosomes and of the endoplasmic reticulum (Figs 3, 4, 9). In the axons and the initial segments of the short collaterals, reticular cisternae were shrunk and microtubules and other cytoskeletal components could not be recognized (Fig. 3). However, clumps of small particles of moderate electron density, about 20 nm in diameter, were present (Figs 4, 11). The origin of these structures is unknown, but it seems likely that they may represent condensed bundles of cytoskeletal elements bound by PR. Mitochondria and synaptic vesicles were preserved, even in processes in which signs of damaged ultrastructure were pronounced (Figs 4, 7, 9). PR, apparently, did not diffuse well through the membranes of mitochondria and of synaptic vesicles, causing these organelles to appear, in a number of sections, as pale profiles contrasting with the dense background provided by the precipitate of the dye (Fig. 9). In processes found further from the soma this precipitate was still recognizable, and the preservation of the fine structure was satisfactory although some mitochondria and some of the larger vesicles were swollen (Figs 11, 18, 20). We have not observed evidence of phagocytosis of PR-injected cells by glia, such as has been seen in the nervous system of some vertebrates after injection of Procion Brown, another electron-dense dye (Christensen & Ebner, 1978).

PR was not present in the extracellular space, at least not in quantities appreciable in our electron micrographs, nor in glial cells except in the region close to the point of injection (Fig. 5). Even here, although some glial cells were stained and the extracellular space appeared contaminated, PR was well localized, and there was no evidence of passage of the dye from the glia or the extracellular space into the neighbouring neural processes or vice versa.

Somata, axons and large collaterals of the cells not injected with PR did not contain recognizable traces

Figs 2–6. Characteristics of neurons stained with HRP and PR. All electron micrographs were taken from sections stained with uranyl acetate and lead citrate.

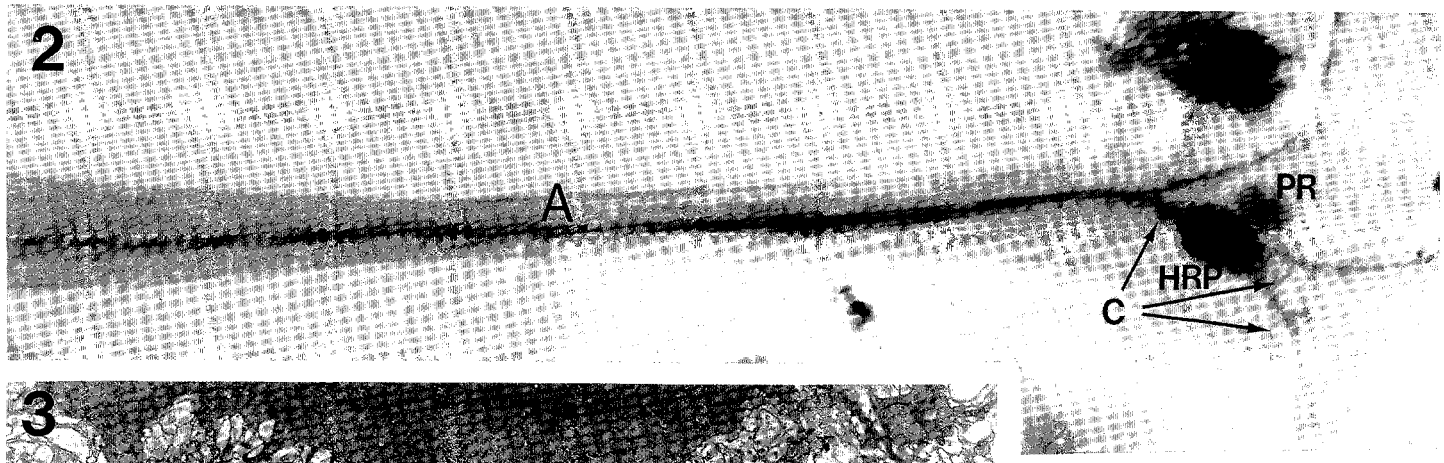
Fig. 2. Low power light micrograph of the posterior end and the central part of a ganglionic trunk in which one of the two large posterior neurons was injected with PR and the other with HRP. Only the soma of the neuron injected with PR can be distinguished in the photograph, although the initial part of the dendrites and about 0.5 mm of the axon of this neuron were also marked by a faint reddish tinge. By contrast, in addition to the soma, the axon (A), dendrites and initial part of some of the short collaterals (C) of the neuron injected with HRP are also visible. Other collaterals are given off by the axons of the large posterior neurons in the anterior end (not shown). The anterior cells have a similar structure, with their axons giving off collaterals in both the anterior and the posterior ends of the trunk (not shown). × 72.

Fig. 3. Electron micrograph of a section cut through the peripheral cytoplasm of the soma of an anterior neuron injected with PR. The cytoplasm appears dark because of a fine, uniformly distributed PR precipitate and also because the small reticular cisternae, found in the somatic cytoplasm, are shrunk. PR appears to bind selectively to the membrane of lysosome-like bodies (L) and to other small unidentified membranous profiles, possibly elements of the endoplasmic reticulum. Note the somatic processes (SP) extending into the surrounding glia. × 5000.

Fig. 4. Axon of an anterior cell injected with PR. The section was cut close to the soma of the injected cell where the axon has a small cross-sectional area. The axon appears shrunk and only mitochondria (M) can be recognized. × 11 200.

Fig. 5. Low power micrograph of the neuropil of the anterior end of the trunk of a specimen in which a neuron was injected with PR. Glial cells are also marked by PR, presumably due to leakage of the dye from the microelectrodes during penetration of the sheath. GS, Glial cell soma; GL, profiles of some of the processes of stained glial cells. The extracellular space also appears contaminated by PR (arrow); however, none of the neuronal processes whose profiles are seen in the micrograph contain detectable amounts of PR. The two bodies showing dense PR deposits (SP) are somatic processes of the injected cell. Note the three small neural processes (asterisks) in close contact. × 5000.

Fig. 6. Cortical region of the soma of a cell injected with HRP. Mitochondria (M) as well as cisternae of endoplasmic reticulum (ER) can be recognized in the soma, but not in the somatic processes extending into the surrounding neuropil (asterisks). The large arrow points to a possible synapse between a process of an unidentified neuron and one of the HRP-stained cell. × 17 000.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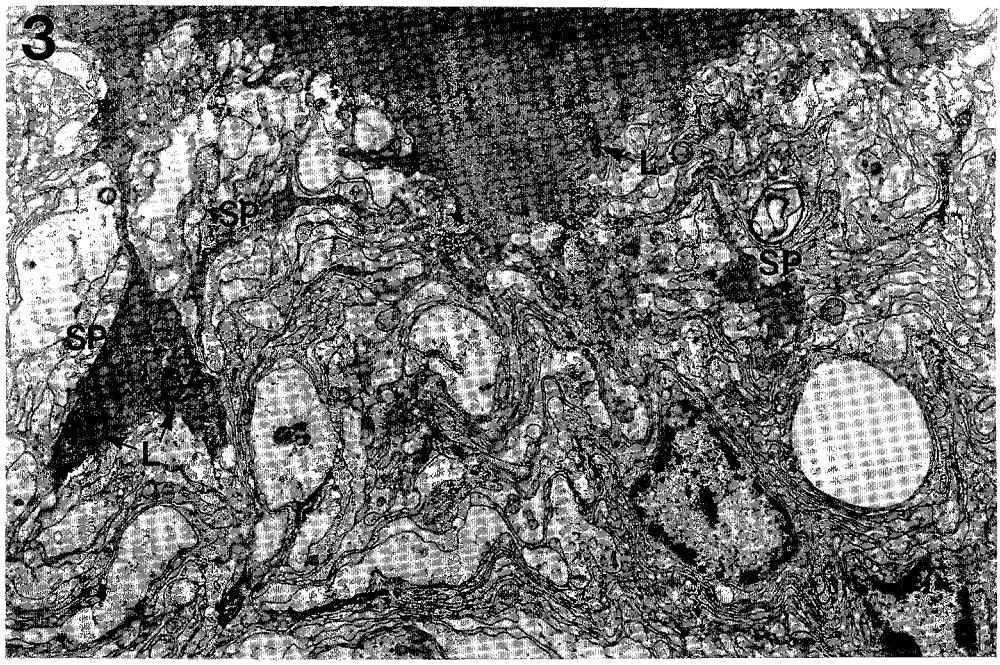
A
PR
HRP
C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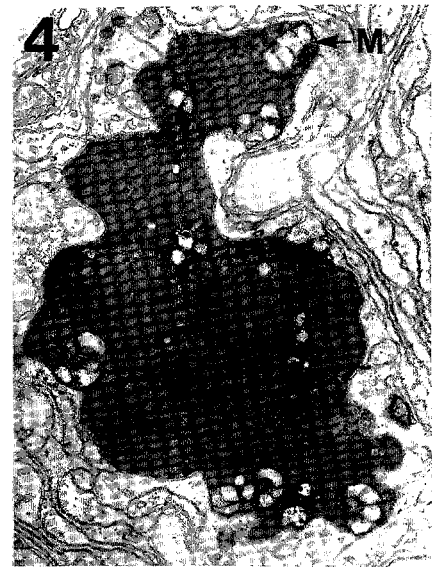
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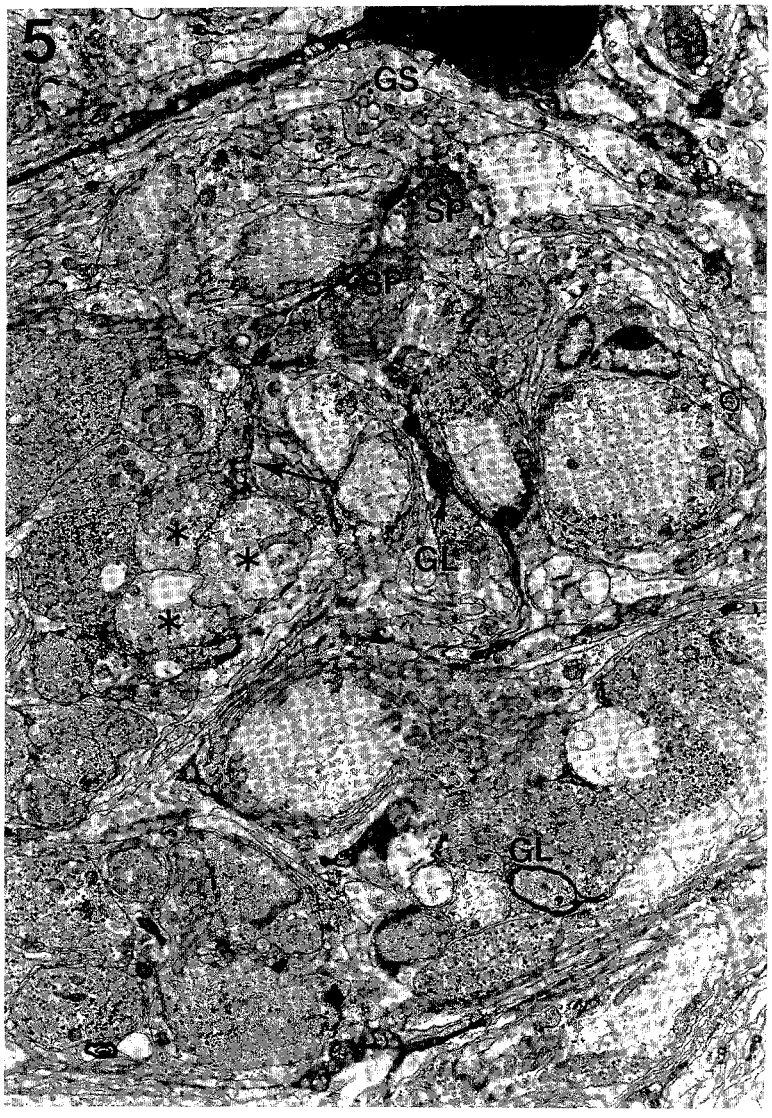
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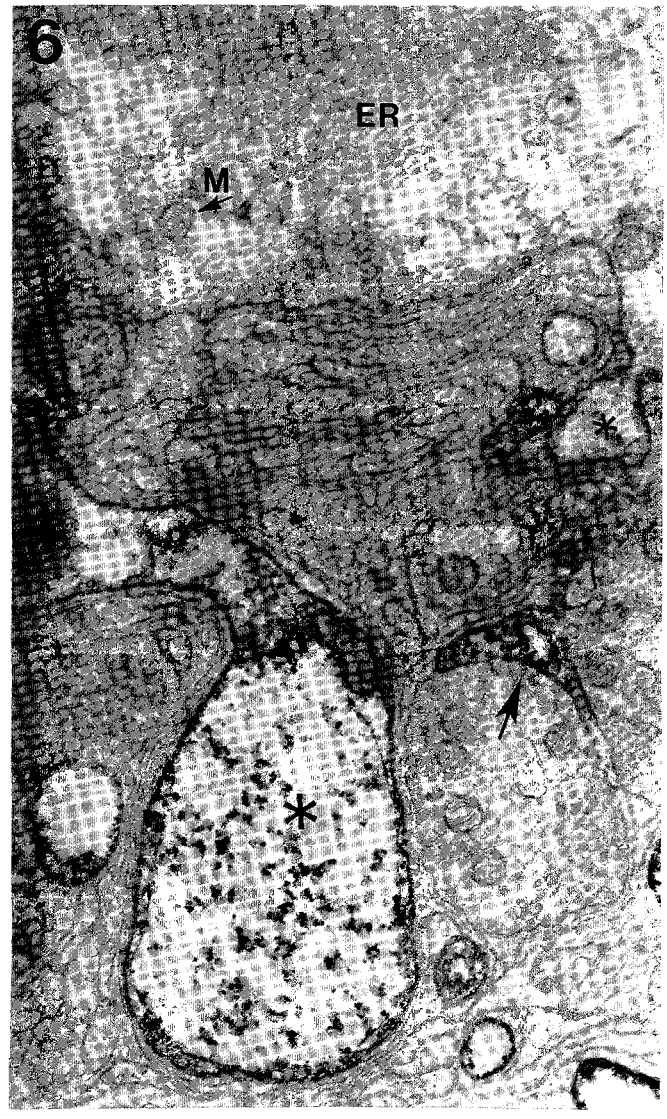
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ER
M

of the dye. However in the neuropil, we have consistently found clusters of processes containing PR deposits of different densities (Figs 11, 18). This finding was particularly clear in regions of the neuropil found far from the soma of the injected cell, and thus at the edge of the diffusion range of PR (Figs 11, 18). It could also be observed among processes found closer to the soma, but in this region it was less clearly visible due to the poor preservation of the fine structure (data not shown). Given the molecular weight of PR MXB (716 Da) it would be reasonable to expect that this dye should diffuse from the injected large cells to the others, as does Lucifer Yellow (Slack, 1984; Tazaki & Cooke, 1983), and these results would seem to support this hypothesis. However, no structural specializations suggesting the presence of intercellular channels between adjacent processes containing PR deposits of different density could be recognized. It is possible, therefore, that these findings may simply reflect different rates of diffusion of PR into the terminal branches of the same collateral of the injected cell.

Processes of the large cells

The surface of the somata of these neurons was highly infolded with glial cells indenting the soma surface. In addition, short somatic processes extended into the surrounding glial sheath (Figs 3, 6). Most of these processes appeared to contact only glial cells, but in a few cases we have observed evidence of possible synapses (Fig. 6) suggesting that these small somatic processes could be analogous to the dendrites of polarized neurons. Because of the presence of these processes and the numerous glial infoldings, the surface of the somata of these neurons should be significantly larger than that of a regular spheroid of the same volume (Mirolli & Talbott, 1972).

The cytoplasm of the axons of the large cells contained regularly spaced microtubules and filaments as well as small mitochondria (Fig. 18). Small clusters of round, dark-core vesicles, measuring about 120 nm in diameter, were also found scattered in the peripheral part of axons (data not shown). The initial part of the large processes called dendrites by Alexandrowicz (1932) had a similar structure. We have not studied the peripheral portion of these processes.

All the secondary processes of the large cells which we have been able to identify, either by tracing them in contiguous sections (Fig. 19) or, more directly, because they were stained by PR (Fig. 20), contained a mixture of round, clear vesicles and larger, dark-core vesicles. Most of the dark-core vesicles were found in the middle of large and medium-sized processes (about 1 μm or more wide), while the smaller, electron-lucent vesicles occupied a more peripheral position. These processes branched into finer, finger-like processes which were devoid of vesicles (see Fig. 28).

No direct membrane contacts with other neural elements were made by the axons and the initial part of the dendrites of the large cells, which were covered by a multilayered glial mantle (Figs 3, 4, 6). A thick mantle also covered the initial part of their collaterals, but as these processes extended away from their sites of origin and divided into smaller branches their glial sheaths became less conspicuous (Figs 16, 18) and were pierced by the processes of other neurons. The

Figs 7–11. Figs 7 to 10 show neuropil of the anterior end of the trunk in a specimen in which one of the three anterior cells was injected with HRP and another with PR. Contacts between processes of the two cells can be recognized in unstained sections (Fig. 7) as well as in sections stained with uranyl acetate and lead citrate (Figs 8–10). Processes stained with PR contain a fine electron-dense precipitate, uniformly distributed. Mitochondria and synaptic vesicles are preserved, although mitochondria appear swollen. Processes of neurons injected with HRP contain a coarser, floccular precipitate; moreover, apart from distorted mitochondria, no other organelles can be recognized. Fig. 7, × 33 500.

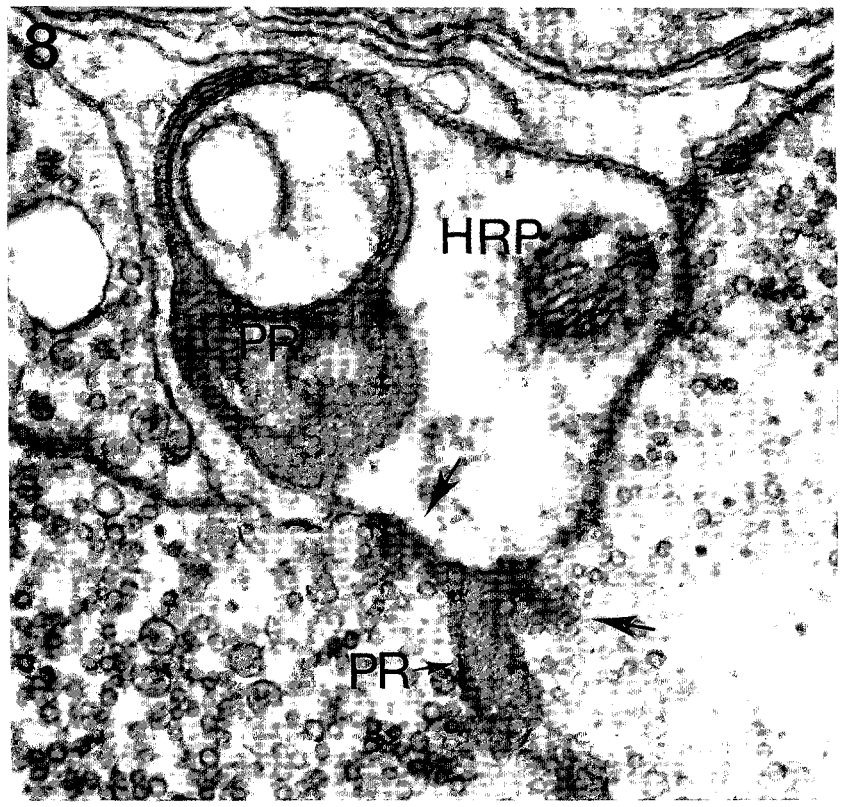
Fig. 8. Synapses (arrows) between a process containing round, clear and dark-core vesicles and process marked by HRP and PR. Size and shape of the vesicles found in this process identify it as of type A (see text). Synapses are characterized by vesicles clustered around densities in the presynaptic element, by a thickened membrane in the postsynaptic element and by a widening of the extracellular space. × 43 000.

Fig. 9. PR does not diffuse in the synaptic vesicles (at least in large quantities) so that processes marked by this dye appear as if they were negatively stained. × 25 000.

Fig. 10. A cluster of PR- and HRP-stained neural endings in contact with each other and with unmarked processes. Arrows point to synaptic contacts. × 25 000.

Fig. 11. Cluster of PR stained processes (PR) in the neuropil of a specimen in which one anterior cell had been injected with the dye. This section was cut at about 0.5 mm from the soma of the injected cell at the edge of the limit of diffusion of PR. The density of the PR precipitate found in adjacent processes is not uniform, possibly because of uneven diffusion of PR in the terminal branches of the same collateral (see Discussion). All the processes stained by PR can be classified, on the basis of the vesicles which they contain, as type A. They synapse (arrows) with other processes, B and C, containing different types of vesicles (see text). × 25 000.


7
PR
HRP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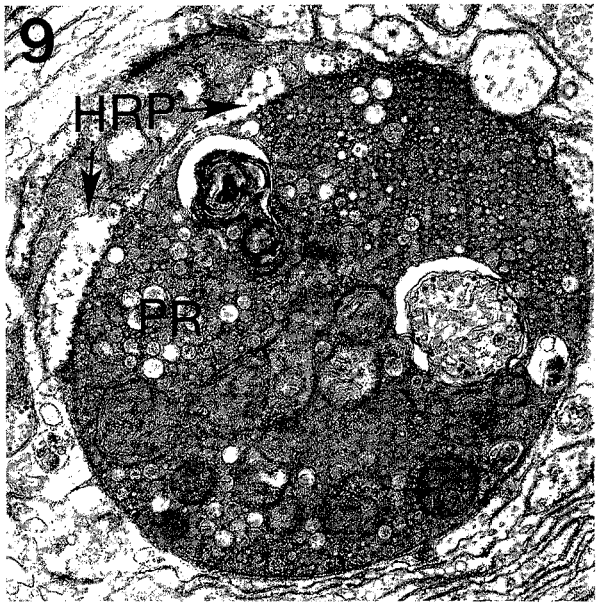
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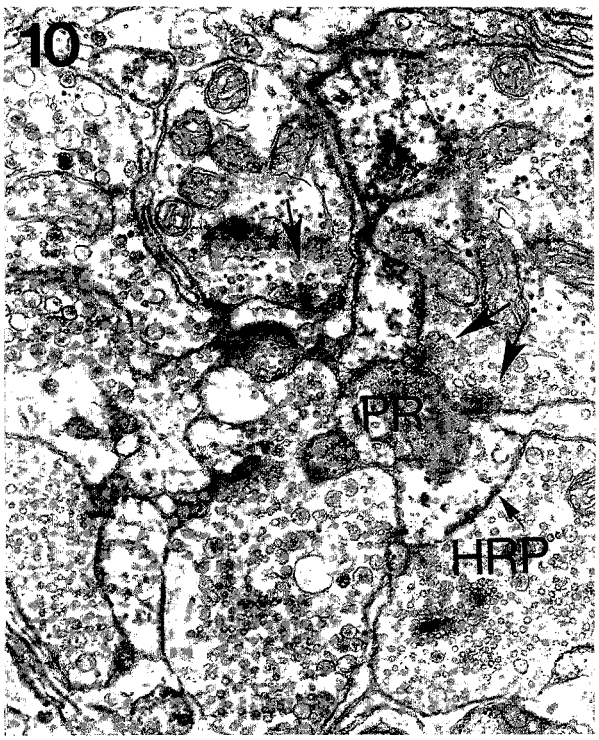
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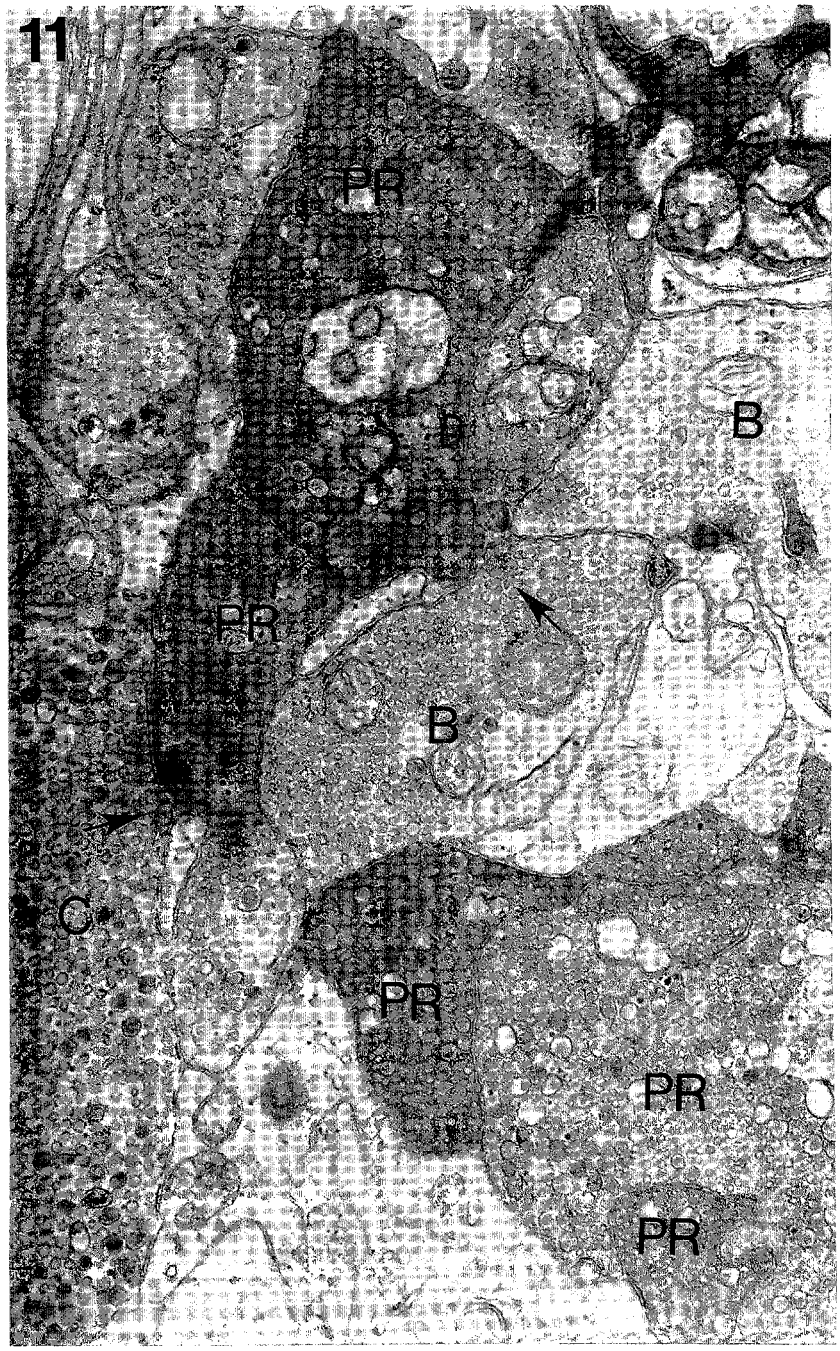
11
PR
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B
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PR
PR

fine endings of the arborizations of the collaterals were only partially covered by glia and were contacted, over most of their surface, by endings of other large cells, of the small cells and of the extrinsic fibres (Figs 18, 19).

Processes of the small cells

The somata and the major processes of the four small cells were also enveloped by a multilayered glial sheath (Fig. 12). However, the somata of the small cells, unlike those of the large cells, had a smooth surface with no processes extending into the glia or into the neighbouring neuropil (Fig. 12). As shown by Alexandrowicz (1932) several dendrites originated from the somata of the small cells, but we have not been able to identify them in the neuropil.

Our reconstruction of the course taken by the axons of the small cells is entirely based on the study of two complete and four partial light microscopy series, as explained in the Material and methods section. We have been able to identify and follow in the trunk only the axons of three of the four cells. The axon of the fourth cell was very small, had a tortuous course and could not be followed in our series. The soma of this cell appeared to have a cytoplasm richer in smooth reticular cisternae and mitochondria than the other three (data not shown). These data suggest that the fourth cell could be morphologically and, by implication, functionally different from the other three. However, we cannot exclude that the axon of the fourth cell may also run in the trunk up to the anterior end, as suggested by Alexandrowicz (1932).

In the posterior two-thirds of the trunk the three small axons which we could follow ran separately from each other, each one being covered by its individual glial sheath (Fig. 13). However, in the anterior third of the trunk they came close together and formed a series of contacts characterized, at the light microscopic level, by a lack of glial wrapping around the individual axons and by a sharp boundary separating their cytoplasm (Figs 14, 15). Contacts involving all the three axons were present, though rare (Fig. 14). More frequently only two of the three were in contact in any given segment of the trunk (Fig. 15); in the next segment the two separated with one joining the third axon to form a similar contact and so on. This sequence was repeated four to five times and resulted in the formation of a chain-like structure (Fig. 17). Each contact was between 20 and 30 μm long, and subsequent regions of contact were separated by approximately the same length. In cross-sections the contacts between the small axons involved between one-third and one-quarter of their perimeter (Figs 14, 15, 19).

In the region where the contacts were found, the shape and cross-section of the small axons varied significantly from one segment to the next (Figs 13–15), reflecting the tortuous course of these fibres. The cytoskeletal elements were organized in bundles oriented at various angles from each other (Figs 18, 19). Moreover, in this region the glial sheath of the small axons was incomplete and their surface was, in part, covered by processes of other neurons (Fig. 19). Finally, in this region the small axons gave rise to numerous secondary processes, containing small, clear vesicles and larger, dark-core vesicles, which extended into the surrounding neuropil where they contacted endings of other neurons (Figs 15, 18, 27).

We have been able to trace the small axons only up to the anterior end of the trunk. Here they divided into smaller branches which could not be followed in our light microscopic series. In electron micrographs of this region we have consistently found clusters of between three and seven small and medium-sized neural processes (1 to 3 μm in diameter), containing either only a few clear, round vesicles or no vesicles at all, which were in contact with each other (Fig. 5). Position and structure of these processes as well as the contacts that they made suggested that some of them could be terminal branches of the small cells.

Structure of the neural endings in the neuropil

Our study of the neuropil was based on several hundred medium and high power micrographs,

Figs 12–16. Light micrographs from sections cut in different parts of the same ganglionic trunk illustrating the structure of the small cells and the contacts made by their axons. × 1000.

Fig. 12. The soma of one of the four small cells. Note the smooth surface of the soma and the thick glial sheath surrounding it. Arrows point to the initial segment of the axon and to the origin of a short collateral.

Fig. 13. Section cut in the central part of the trunk. The axons of eight out of the nine intrinsic neurons are identified. The axons of the three anterior cells are labelled 1, 2 and 3, those of the two large posterior cells are labelled 4 and 5, and those of the three small posterior cells which can be followed in our serial sections are labelled 6, 7 and 8. The unlabelled profiles are either the axons of the extrinsic modulator fibres or unidentified branches of the axons of the intrinsic neurons.

Figs 14, 15. Contacts made by the axons of the small cells in the anterior region of the trunk. The arrow in Fig. 14 points to a collateral of a large axon contacting one of the small axons, and the arrow in 15 to the origin of a collateral of a small axon.

Fig. 16. Section cut in the anterior end of the trunk showing two short collaterals given off by the axon of anterior cell 2. Note the branching of the collaterals close to their origin.


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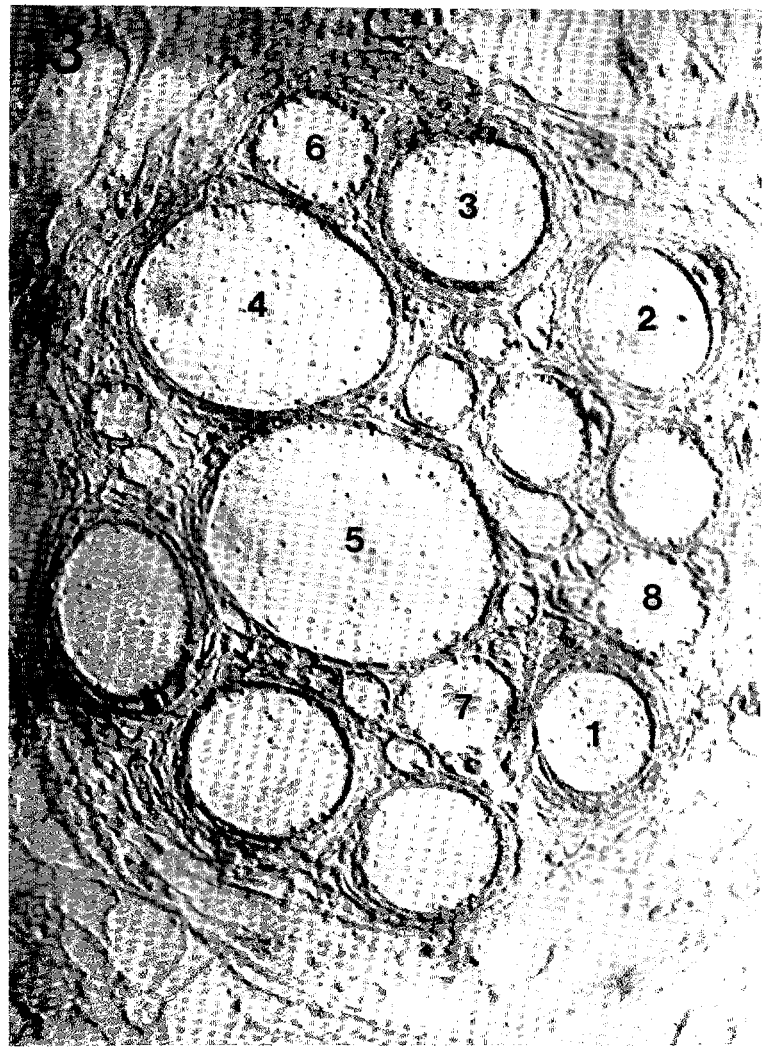
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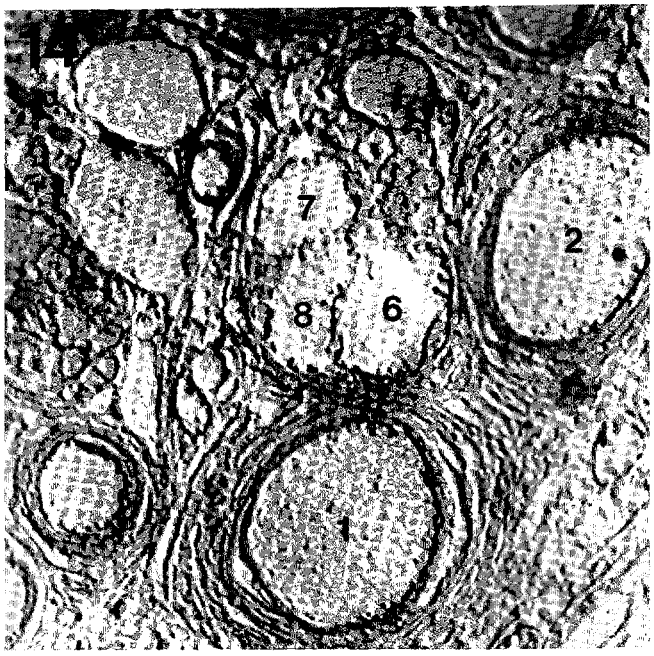
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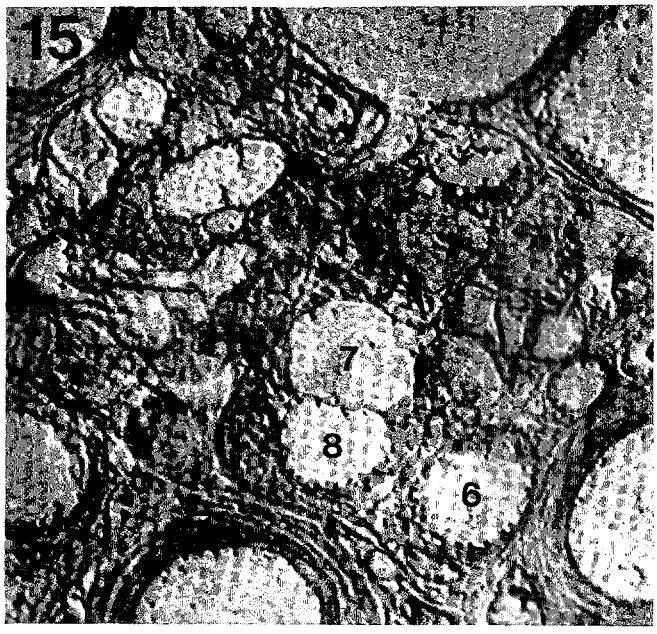
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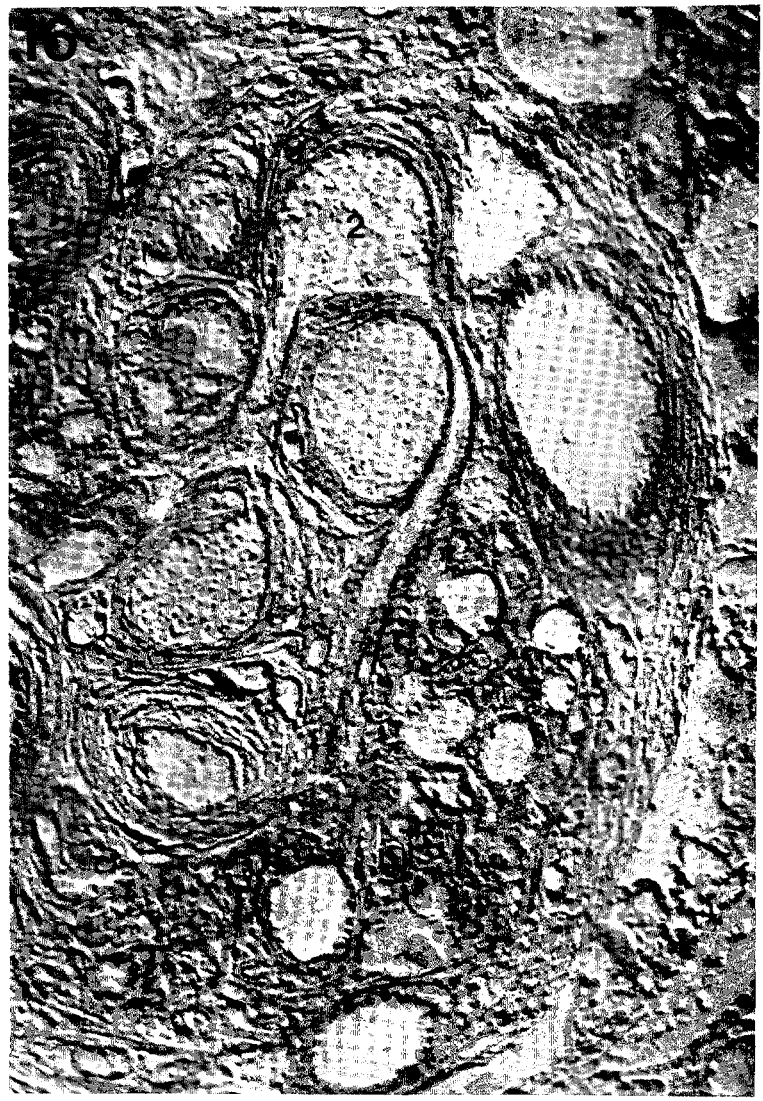
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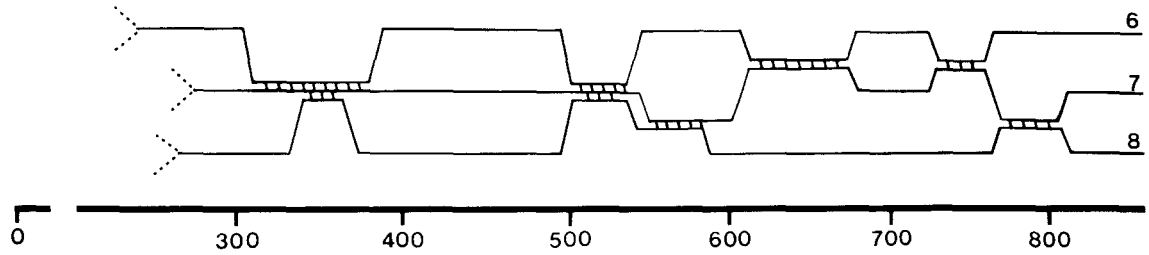


Fig. 17. Schematic drawing of the chain of contacts made by the three small axons as reconstructed from a complete series cut for light microscopy. The horizontal scale shows the distance (μm) of the contacts found in this series from a reference section in the anterior end of the trunk. Results consistent with those summarized in the figure were also obtained in the other complete serial reconstruction of the ganglion and in the two reconstructions of the anterior half of the trunk.

covering about 10 000 μm^2. Most of the neuropil was found in the anterior part of the ganglionic trunk, but some (approximately 30% of the total) was found in the middle part of the trunk and in the posterior end. In all these regions the neuropil had the same structure, being formed by the intertwining of three types of neural endings, each characterized by a distinct population of vesicles which we have classified as types A, B and C.

Endings of type A were by far the most numerous, accounting for about 65% of all those present. They were characterized by a mixture of clear and dark-core, round vesicles similar to those present in all the identified offshoots of the large cells, including those stained by PR (Figs 7, 9, 11, 29). The clear vesicles, measuring on average about 45 nm in diameter, were about ten times as numerous as the dark-cored ones, which were larger, having an average diameter of 110 nm. Collaterals of the small cells also contained vesicles of this type with, possibly, a minor difference in the relative proportion of clear and dark-cored vesicles (Fig. 27).

Type B endings, about 20% of the total, contained clear pleomorphic vesicles, often with elliptical profiles, whose small axis measured, on average, 35 nm, plus a few round, dark-core vesicles 85 nm in diameter (Fig. 11). They could be traced directly to two large fibres which did not originate from the intrinsic neurons and which could, on this basis, be identified as two of the six fibres providing the extrinsic innervation of the ganglion (data not shown).

Type C endings, about 5% of the total, had round, clear vesicles and dark-cored vesicles which were larger and more variable in size and shape than those found in type A endings. The average diameter of the clear vesicles was 55 nm and that of the dark vesicles 110 nm. Type C endings which, as already emphasized, were relatively rare, could not be traced back to any identified axon, and it would seem a safe conclusion that they should also originate from the extrinsic fibres since the processes of all the intrinsic neurons which we have been able to identify contained type A vesicles.

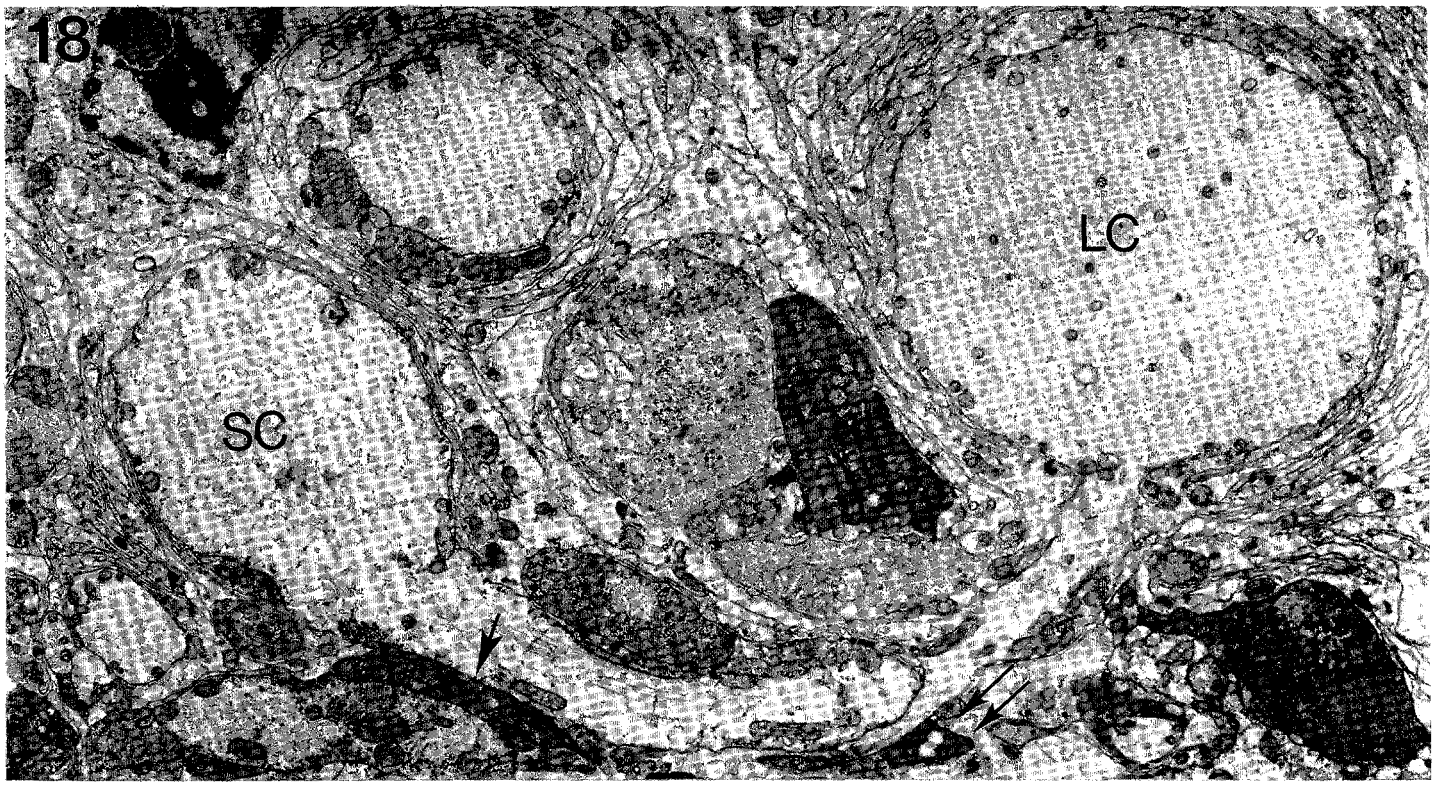
Figs 18–21. Contacts between the intrinsic neurons of the ganglion.

Fig. 18. Contact between the collateral of a small cell axon (SC) and the collateral of the axon of an anterior cell (LC). Apart from their size, the axons of the small cells can be distinguished from those of the large cells because their cytoskeletal elements are more sparse and more irregularly arranged in the anterior region of the trunk. In addition, the small axons have only a few mitochondria in their central portion. This section was cut from a specimen in which one anterior cell had been injected with PR, and a number of processes in the neuropil are marked by the dye. One of these processes contacts the small cell collateral (arrow) and another (double arrows) contacts the collateral of the large cell. Note the variable density of adjacent PR-labelled processes; note also that different parts of the same process (asterisk) may contain PR deposits of different densities. × 6 500.

Fig. 19. Contacts between two small axons (SC) and between them and the collateral of a large cell (LC). Small mitochondria and vesicular cisternae are lined up against the outer membranes at the region of contact. Membrane close appositions present at these contacts are shown at higher magnification in Figs 22 and 26. Other, unidentified processes (arrows) are also in contact with the two small axons. The vesicles contained in these processes are of type A (see text) as are those contained in the process marked LC. × 11 600.

Fig. 20. Higher magnification of the contact between the large cell (LC) and the small cell (SC) collaterals shown in Fig. 18. Note the absence of glia at the region of contact. Arrows point to possible membrane close appositions between the two collaterals and between the collateral of the large cell and a PR-stained process. × 32 000.

Fig. 21. Synapse between a type A process and two small axons (SC). × 70 000.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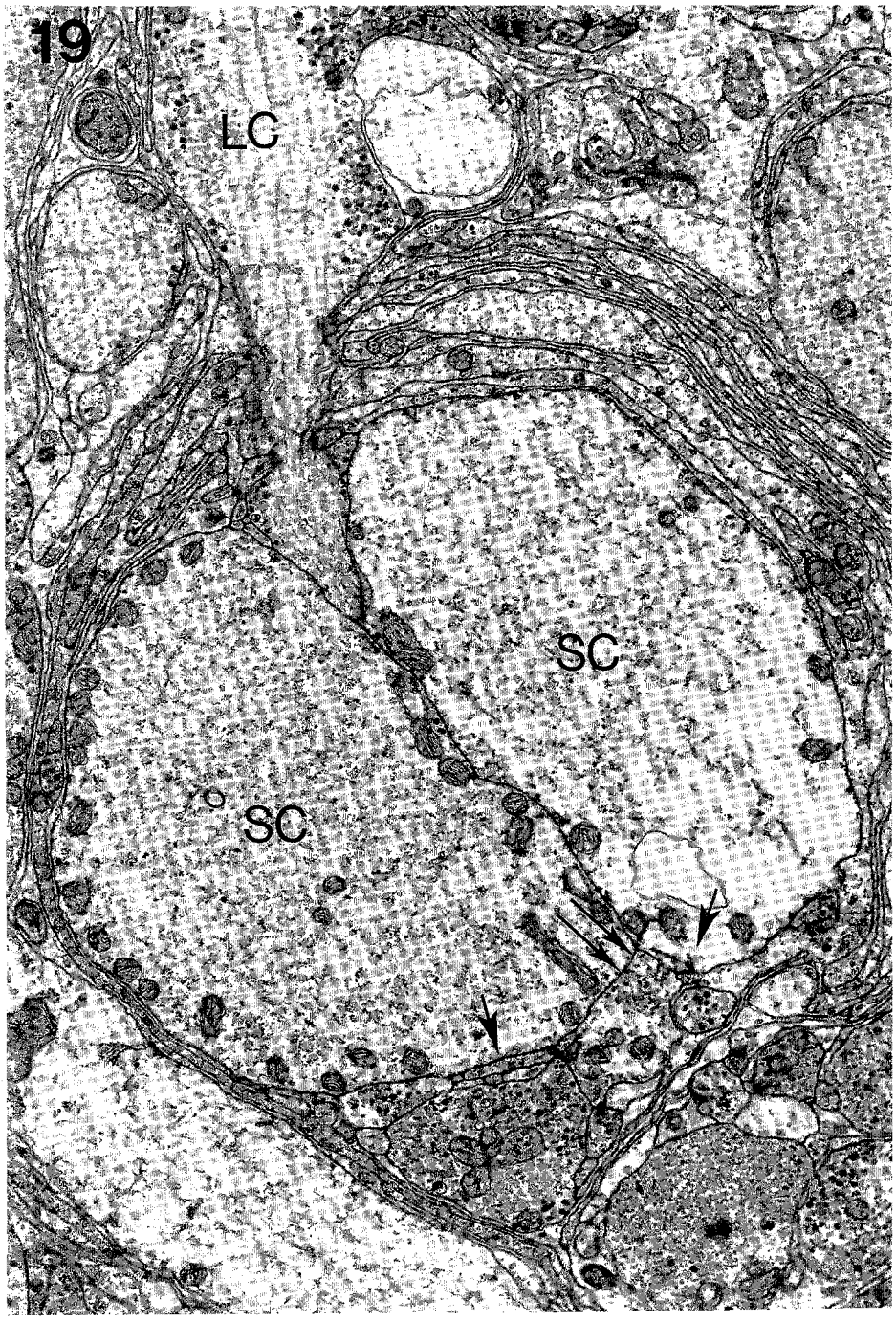
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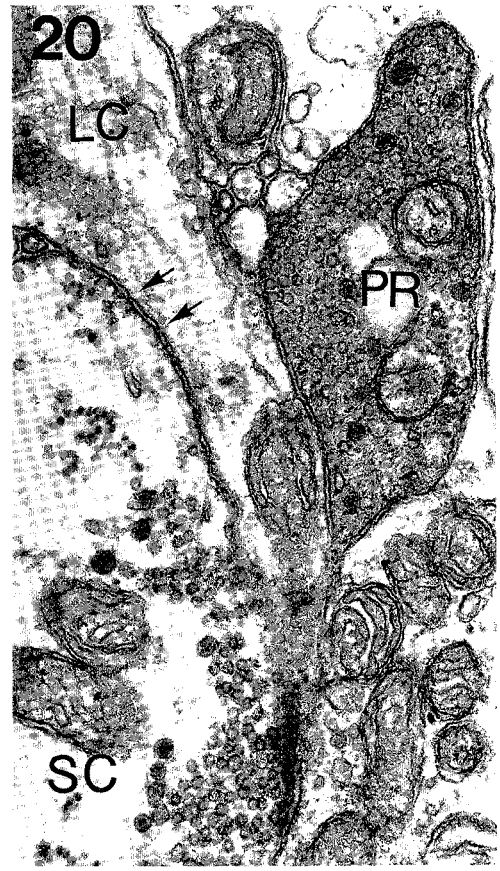
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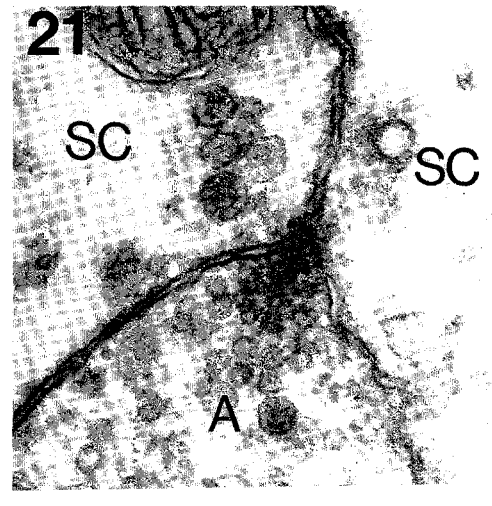
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A

Most of the processes contacting the small axons and their collaterals were of type A and could, on this basis, be identified as offshoots of either the large cells or of other small cells. Direct identification of processes of the large cells contacting small axons has been possible (Figs 18, 19, 27). Endings of type B were also in contact with the small axons.

Two types of junctions were present at the contacts between adjacent neural processes: small membrane close appositions, similar to the punctate appositions identified by Kensler *et al.* (1979) as possible sites of electrical synapses, and larger junctions, similar to those identified in other crustacean neuropils as chemically mediated synapses (King, 1976a, b).

Membrane appositions

At these junctions the outer leaflets of the membranes of adjacent neural processes appeared to touch each other (Figs 22, 23). Membrane appositions have been found at the contacts between the three small axons (Fig. 22), at those between the small axons and identified processes of the large cells (Fig. 26) and also at the contacts between fine type A endings in the neuropil (Fig. 29). In all cases observed the appositions were small (between 20 and 50 nm in length) and for this reason they could be identified only in grey and silver-grey sections, since in thicker sections they could not be distinguished from artifacts resulting from the overlapping of membranes in the plane of the sections (Figs 23, 24). Their localization and structure suggest that these junctional specializations could mark the site of electrical synapses.

Extensive arrays of membrane appositions, spaced at intervals of about 0.5 μm, have been found at all the contacts between the small axons. The resolution of our micrographs was not sufficient to distinguish a space between the apposing membranes (Fig. 23), although without the use of an extracellular marker, the presence of a narrow gap could not be excluded. Between the regions of apparent fusion the apposing membranes ran parallel, delimiting an extracellular space of regular width (about 200 nm wide) which was partially filled by a fine-grained, electron-dense material. Small subsurface cisternae and mitochondria (Figs 22, 26) were found close to the apposing membranes. Similar, although less extensive, arrays of membrane appositions were also present at the contacts made by identified processes of the large cells with the small axons.

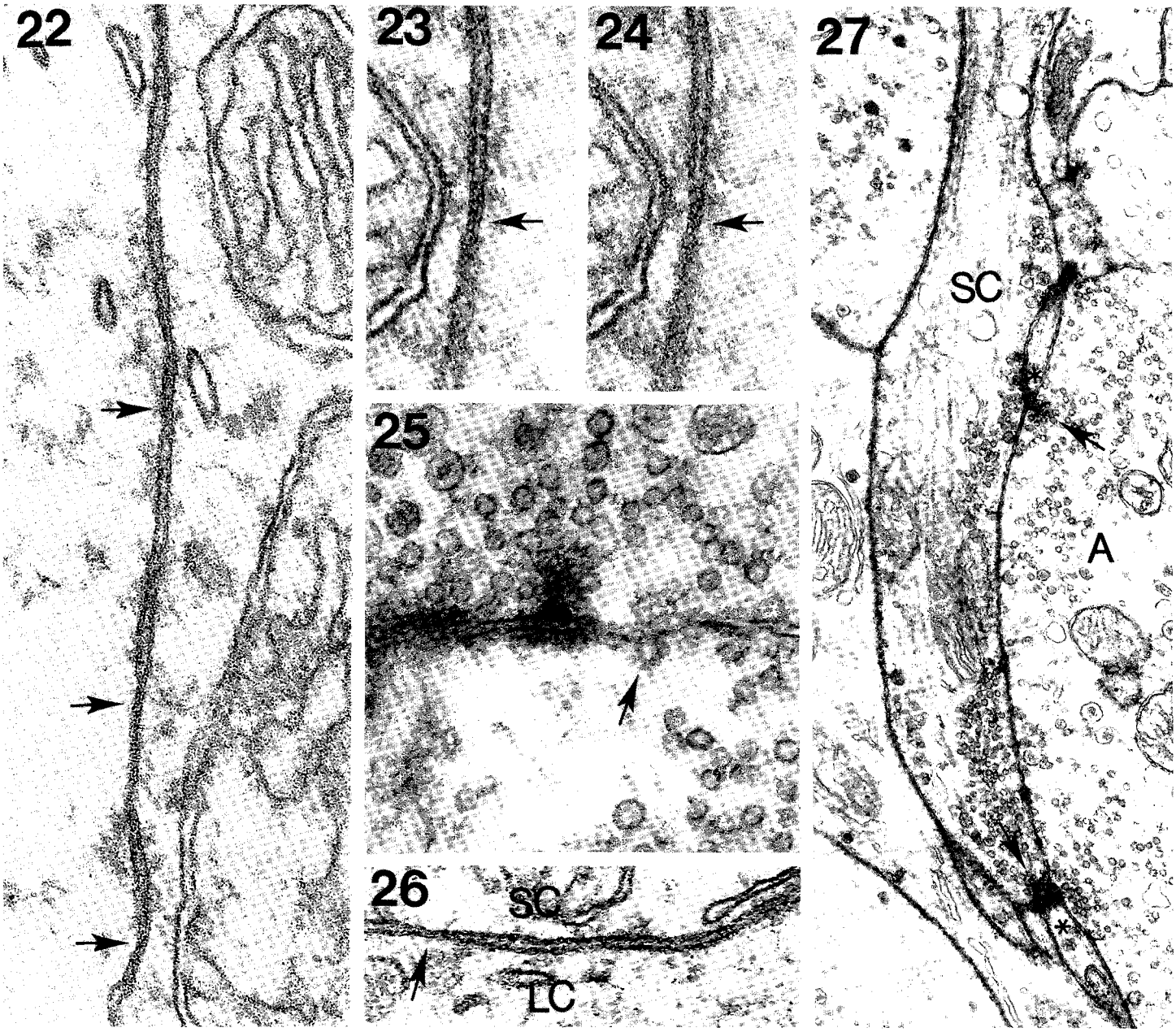
Only isolated membrane appositions were found scattered at the contacts between type A endings in the neuropil (Fig. 29). Because of the irregular and wavy outline of these (as well as other) processes, membrane appositions were difficult to recognize and therefore we cannot give a reliable estimate of their frequency. They were widespread, however, as they have been recognized in sections cut from all the regions of the neuropil of control material. We have been unable to identify them in the specimens injected with PR (or with PR and HRP), perhaps because of the difficulty in routinely obtaining ultra-thin (grey) sections from this material.

Chemical synapses

Chemical synapses were characterized by the following structural specializations (Figs 8, 25): (a) presence in the presynaptic element of clusters of clear vesicles around variously shaped densities; (b) thickening of the postsynaptic membrane facing the density; (c) widening of the extracellular space; (d) accumulation in this space of a fine granular material. Coated and uncoated vesicles attached to presynaptic membranes (Figs 25, 29) could also be identified.

In the several hundred micrographs that we have examined, we have found only 41 examples of synapses in which both the pre- and postsynaptic elements could be classified as being of type A, B or C on the basis of the vesicles that they contained (see section on structure of the neural endings in the neuropil). In the remainder of the synapses studied (over 200) the postsynaptic elements were small, finger-like processes, devoid of vesicles which, therefore, could not be classified except in a few favourable sections (Fig. 28). Of the 41 cases in which both elements could be classified, 25 were between type A endings (Figs 8, 25), 11 between B and A endings, B being presynaptic (Figs 11, 28), and 5

Figs 22–29. Structural details of membrane close appositions and synapses. Figs 22–24 show close appositions (arrows) between contacting small axons. Figs 23 and 24 were taken at a 30° tilt to show the apparent touching of the outer leaflets of the apposing membranes. Fig. 25 shows a synapse between two unidentified type A endings. Arrow points to a coated vesicle. Fig. 26 shows a membrane close apposition (arrow) between a small cell axon (SC) and the collateral of a large cell (LC). Fig. 27 shows synapses (arrowheads) made by a collateral of a small axon (SC), containing type A vesicles, and by an unidentified type A ending (A) with small processes devoid of vesicles (asterisks). Fig. 28 shows a synapse (asterisk) between a type B process and a small finger-like branchelet of a type A process. This micrograph also demonstrates the intricate shape of the neural endings making up the neuropil. Fig. 29 shows close appositions (asterisks) between two unidentified type A processes. Arrows point to synaptic vesicles attached to the plasma membrane. Fig. 22, × 100 000; Figs 23 and 24, × 170 000; Fig. 25, × 80 000; Fig. 26, × 90 000; Fig. 27, × 25 000; Fig. 28, × 18 000; Fig. 29, × 92 000.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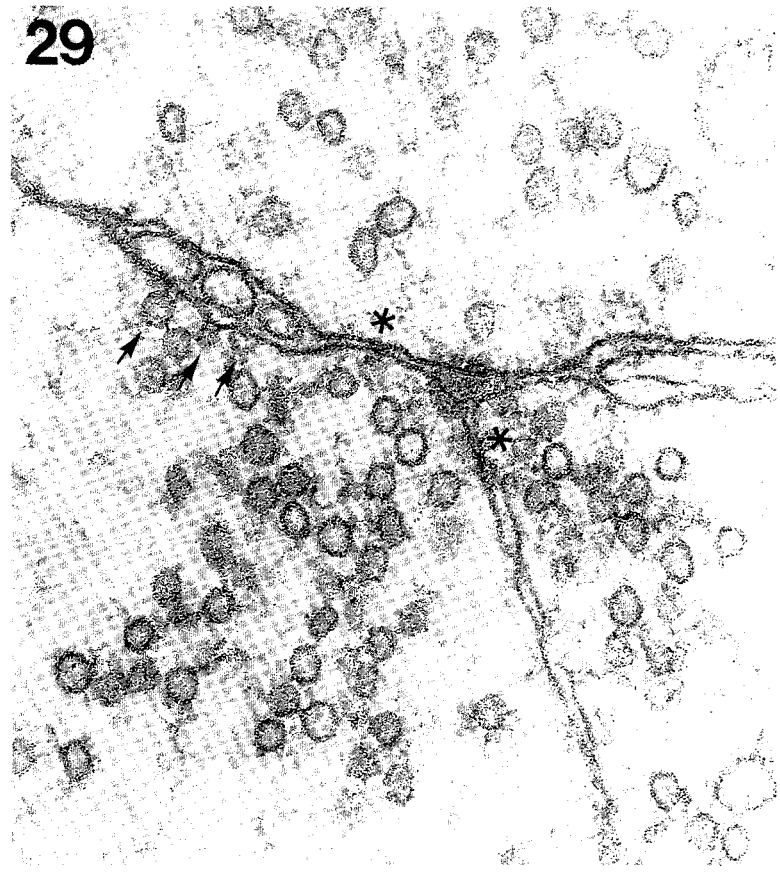
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between C and A endings, C being presynaptic (Fig. 11). A few possible cases of synapses at which A endings were presynaptic and B or C endings were postsynaptic were also found, but in none of these cases was the identification of the postsynaptic elements sure (data not shown). Synapses between two type B endings or two type C endings were not observed, nor were synapses between types B and C although, given the scarcity of type C endings, the possible occurrence of these junctions was difficult to exclude.

Some of the synapses between identified type A endings were between processes of the small and large cells, with either cell being pre- or postsynaptic (Figs 21, 27). In the neuropil of specimens double-injected with PR and HRP, contacts between processes containing the two markers were commonly found. Structural specializations indicative of synapses, such as widening of the extracellular space and thickening of the membranes, were present (Figs 7, 8), although clusters of vesicles around densities could not be recognized over the intense background provided by the dye. We have not identified synaptic specializations at the contacts made by contiguous PR-stained processes.

Discussion

Electrical junctions between large cells

Our results suggest that the anatomical substrate for electrical coupling between the large neurons of the cardiac ganglion of *Portunus sanguinolentus* should be a diffuse system of small junctions found at the contacts between the endings of their short collaterals. We have not found, despite an intensive light and electron microscopic survey, contacts between the somata or the large processes of these cells. By contrast, contacts between the fine processes of their collaterals could be routinely demonstrated. It is clear, therefore, that the site of the electrotonic junctions between these cells must be found at the level of the fine arborizations of their processes.

Contacts between type A endings, most if not all of which are offshoots of the large cells, are characterized by the presence of small membrane close appositions. We think that these appositions should be the sites of the electrotonic junctions, although further studies using *en bloc* staining and freeze-fracture are required to reveal the possible presence of gap junctions at these structures. Aside from these appositions we have not been able to identify other structures which could be reasonably interpreted as the sites of intercellular channels. According to Heitler *et al.* (1985) small membrane appositions are the sites of intercellular channels in the crayfish abdominal ganglia. Because the coupling between the cells of *Portunus* is large (Tazaki & Cooke, 1979a), it could be argued that the small size of the appositions makes them unlikely candidates for these electrical synapses; however, this argument has no validity. As shown by Hall *et al.* (1983), small, scattered clusters of intercellular channels can account for large electrical coupling, provided that they are present in sufficient numbers.

The position as well as the distribution of the aggregates of the channels is of functional significance, because it determines the filtering characteristics of the junctions. In *Portunus*, intercellular spread of hyperpolarizing currents or subthreshold depolarizing currents between the large cells is more effective the lower the frequency (Tazaki & Cooke, 1979a), suggesting that the electrotonic junctions between these cells act as low-pass filters as is the case in the lobster ganglion (Watanabe, 1958; Hagiwara *et al.*, 1959; Watanabe & Bullock, 1960). If the coupling between the large cells occurs primarily through a diffuse network of small junctions sited on the fine terminal arborizations of their collaterals, as suggested by our data, their low-pass filtering characteristics could be readily explained (Bennett, 1966), since these processes, being located at some distance from the zone of spike initiation, interpose a load capacitance between the site of intercellular current spread and that of the excitable membrane. It is interesting to note that the junctions between the giant neurons of *Navanax*, which are made up by numerous but small patches of intercellular channels (Hall *et al.*, 1983) between fine neuronal processes, also act as a low-pass filter (Levitan *et al.* 1970).

Electrical junctions between the small cells

In the cardiac ganglion of the crab the action potentials of the large cells are normally initiated by a powerful excitatory synaptic drive from the small cells (Tazaki & Cooke, 1979a, b, 1983). Although the firing of the small cells is not synchronous, the action potential of each small cell results in synchronous excitatory synaptic potentials in each of the five large cells (Tazaki & Cooke, 1979a). These results suggest that these axons should be linked by electrotonic junctions which, in this case, should act as high-pass filters allowing the spread from one axon to the others of fast-rising depolarizing currents, thus ensuring that the collaterals of all three axons will be approximately equally depolarized whenever any one of them is invaded by an action potential. The extensive arrays of close appositions found at the contacts between the three small axons are in parallel with the excitable membrane of the axons and, therefore, should act as high-pass filters and so satisfy the main requirement imposed by the physiological data. Accordingly, the contacts between the

small axons would be the crucial element providing synchronization of the output of the ganglion.

The presence of extensive contacts between the axons of pacemaker cells in the crustacean cardiac ganglia appear to be a general, if not an absolute rule. Our results confirm those of Irisawa & Hama (1965) and Ohsawa (1972), already briefly summarized in the Introduction. Ohsawa did not identify the three contacting axons in the ganglion of *Panulirus*, but the structural similarities between the axo-axonic contacts illustrated by this author, as well as the size and the number of the fibres involved, suggest that in this species the contacting axons are also those of small cells. The 14 cells making up the more primitive ganglion of *Squilla* are all of equal size and therefore a direct comparison with the situation in *Portunus* is not possible. However, the three contacting axons in the ganglion of *Squilla* were identified by Irisawa & Hama (1965) as being those of pacemaker cells. Axo-axonic contacts were not found in the ganglia of *Peneaus* (Aizu, 1975) and of *Callinectes* (Hawkins & Howse, 1978) but it is possible that they may have been overlooked in these studies, since these structures, although quite obvious, are found only in a restricted region of the ganglionic trunk in *Portunus*.

Chemically mediated synapses

As we have already stated, type B endings are offshoots of the extrinsic fibres, as are, most probably, those of type C. The relative abundance of these endings and the characteristics of the vesicles which they contain give additional clues to their function. Studies using Methylene Blue (Alexandrowicz, 1932) demonstrated that all the three pairs of extrinsic fibres distribute terminals throughout the ganglion, one pair being consistently larger than the others. Shimahara (1969a, b) found that in *Panulirus* the excitation of one pair, the inhibitors, resulted in postsynaptic potentials recordable from the somata of large neurons, while the excitation of the other two resulted only in a weak enhancement of the ganglion bursting activity, without any detectable somatic excitatory postsynaptic potentials. Similar results have been obtained by Watanabe *et al.* (1968, 1969) in *Squilla*. If these observations can be extended to the crab, we suggest that the type B endings described here represent the terminals of the inhibitory fibres. Consistent with this identification is the similarity in size and form of the small, clear, pleomorphic vesicles which these endings contain with the vesicles of identified inhibitory synapses in Crustacea (Atwood & Pomeranz, 1977; Hirosawa *et al.* 1981; Tao-Cheng *et al.*, 1981). As shown by Nakajima & Reese (1983), the size of the vesicles (although not necessarily their morphology) is, at least in crustacean preparations, a good indicator of the excitatory or inhibitory nature of the synapses. Type C endings might be attributable to the extrinsic excitatory fibres, the scarcity of these types of endings being well correlated with their weak effect (Shimahara, 1969b; Watanabe *et al.*, 1969).

We cannot exclude the possibility that some of the type A endings may originate from one of the two pairs of the extrinsic fibres whose endings have not been identified (presumably the excitors). However, as already emphasized our results suggest that the majority of type A endings should be offshoots of the intrinsic neurons. Therefore, most of the synapses between endings of this type should occur either between collaterals of large and small cells or between collaterals of different large cells. In agreement with these conclusions, electrophysiological data have localized chemically mediated excitatory synapses between small and large cells to the anterior region of the ganglion mid-trunk (Tazaki & Cooke, 1979b, 1983). There is no direct physiological evidence for chemically mediated reciprocal synapses between the large cells. It should be noted, however, that there are aspects of the electrophysiological behaviour of the large cells, such as the sustained plateau underlying their action potential bursts (Tazaki & Cooke, 1979a; 1983), which cannot be explained on the basis of their known properties. They could be accounted for most simply by assuming the presence of reciprocal excitatory synapses between collaterals of the large cells, such as those suggested by our morphological data.

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