THE DISTRIBUTION OF SYNAPSES ON A PHYSIOLOGICALLY IDENTIFIED MOTOR NEURON IN THE CENTRAL NERVOUS SYSTEM OF THE LEECH

An Electron Microscope Study after the Injection of the Fluorescent Dye Procion Yellow

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ABSTRACT

The fine structure of a physiologically identified motor neuron in the segmental ganglion of the leech central nervous system and the morphology of synapses on it were studied after injection of the fluorescent dye Procion yellow as a marker. The injected cell and its processes within the neuropil were located in thick or thin sections with fluorescence optics after initial fixation with glutaraldehyde and brief treatment with osmium tetroxide. The same or adjacent thin sections could then be examined in the electron microscope. Comparison with uninjected cells showed that the general features of the injected cell are retained although some organelles are distorted. The main features of the geometry of this neuron are the same from animal to animal: a single large process runs from the soma through the neuropil to bifurcate and enter the contralateral roots. Within the neuropil the main process gives off long branches (up to 150 μ), but these are greatly outnumbered by short branches and spines, one or a few microns in length, which were not appreciated in previous light microscope studies after injection of Procion yellow. Serial thin sections of selected areas along the main process within the neuropil showed that there are synapses on most of the shorter branches and spines; occasional synaptic contacts were also made on the main process itself and on longer branches. At least two morphologically distinct types of synapse could be recognized. A minimum estimate of the total number of synapses on the motor cell is 300, based on their occurrence in reconstructed segments.

INTRODUCTION

The segmental ganglion of the leech *Hirudo medicinalis* is one of several invertebrate preparations that have been studied with the aim of determining neural mechanisms underlying simple patterns of behavior (6, 12, 13, 17). Such preparations have the advantage of a relatively small number of neurons (about 350 in the leech ganglion) many of which can be identified from ganglion to ganglion and functionally characterized. In the leech segmental ganglion, for instance, sensory and motor cells have been identified, their receptive fields and the muscles they innervate defined, and

the connections between some of them determined as well as the mechanism of these connections (2, 11, 19, 13). A valuable complement to these electrophysiological studies would be knowledge about the morphology of synapses on identified cells. For example, one would like to know whether terminals of particular presynaptic neurons end on specific regions of the postsynaptic cell, how terminals with different synaptic mechanisms are distributed on the postsynaptic processes, and whether such terminals differ in structure. This information would further serve as a basis for evaluating whether morphological changes accompany long-lasting changes in function determined by physiological experiments (for example reference 8).

Although the fine structure of the leech ganglion has been investigated by Coggeshall and Fawcett (4), and by Gray and Guillery (5), it has not been possible to pursue these questions by relating the electron microscope findings to cells identified with intracellular electrodes. The reason for this is that the processes of the ganglion cells ramify in a dense central mass of fibers, the neuropil, where the synaptic connections are made. The structural complexity of the neuropil makes it difficult to determine the origin of particular profiles seen in the electron microscope. Furthermore, one cannot always be sure which cell bodies correspond to physiologically identified neurons because clues of size and position that are obvious in the intact ganglion are lost when the tissue is sectioned.

The purpose of the present experiments was to study electron microscopically the cell body, processes, and synaptic contacts on an identified neuron by means of a marking technique that would generally preserve its fine structure. We have done this by adapting the electrophoretic injection of the fluorescent dye Procion yellow (18) to the requirements of electron microscope fixation and staining.

For this study we chose a motor cell (the "large longitudinal motor neuron") that innervates the longitudinal muscles of a segment because it can be readily identified and much of its functional organization is already known. For example, this motor cell receives monosynaptic inputs from three modalities of sensory cell in the same and adjacent ganglia and, together with its electrically coupled contralateral homologue, executes a monosynaptic shortening reflex in response to mechanical stimulation of the skin (19, 13). One class of sensory cell makes chemical synaptic connections on the motor cell, another class makes electrical contacts, while a third class makes synapses that transmit by both chemical and electrical means (13).

A brief account of this study has appeared elsewhere (10).

MATERIALS AND METHODS

The techniques of dissection and identification of cells in leech segmental ganglia have previously been described in detail (11, 19, 13). Isolated ganglia were pinned in a shallow bath of modified leech Ringer's solution containing 8 mM/L Ca^{++}, or L-15 culture medium, Grand Island Biological Co., Grand Island, N. Y. (Gibco) adjusted to a tonicity of 260 mosmols. The motor cell was recognized by its size and position in the ganglion, and its identity confirmed by recording the action potential with a dye-filled electrode. The action potential of the large longitudinal motoneuron is distinguished by its shape and small amplitude (19).

Electrodes were filled with 6% Procion yellow M4RAN by weight (18, 13) and selected for resist-

FIGURE 1 Comparison of injected and uninjected motor cell bodies. Fig. 1 A, thick section (3 μ) of injected motor cell seen in the fluorescence microscope. Fluorescence is unevenly distributed; there is a cytoplasmic ring of relatively low intensity giving the cell a characteristic target appearance. X 1170. Fig. 1 B, low power electron micrograph of a nearby thin section of same cell showing corresponding ring of cytoplasmic organelles. Cellular elements surrounding the nerve cell are processes from the packet glial cell. X 1560. Fig. 1 C, uninjected cell from same preparation (presumptive contralateral homologue of injected motor neuron in Figs. 1 A and 1 B) showing similar arrangement of organelles. N, nucleus. X 1510. Figs. 1 D and 1 E, higher power view of injected motor cell and uninjected cell, respectively. The injected cell shows typical vacuolated appearance of the cytoplasm apparently due to swollen endoplasmic reticulum (ER) and disrupted mitochondria (MI). Other structures in the injected cell such as dark bodies (D), granular vesicles (V), the nuclear envelope (NE), and Golgi apparatus (G) are readily recognizable. Note also the denser and more homogeneous matrix of both nucleus (N) and cytoplasm of the injected cell. X 13,300.
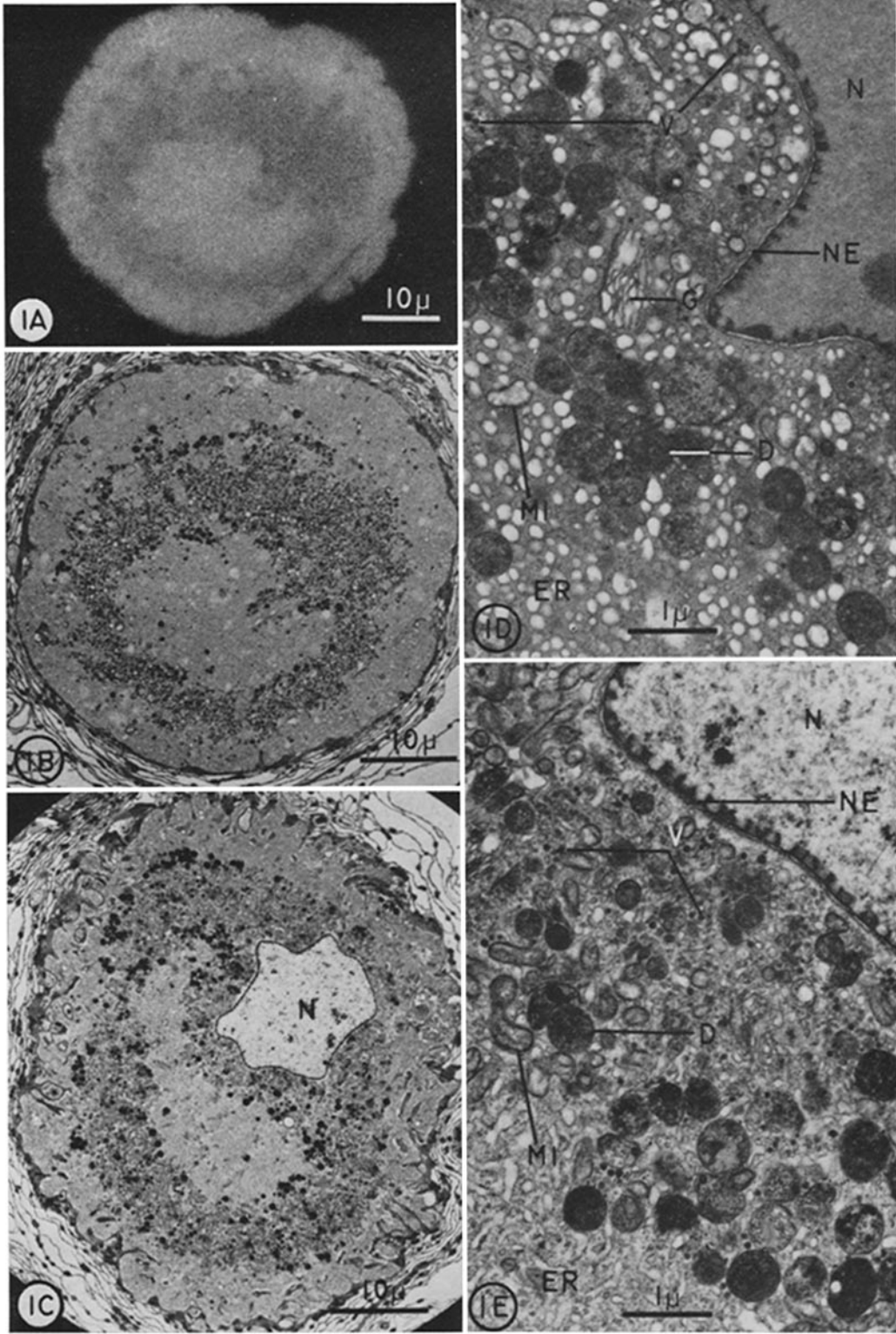
ances between 200–400 M ohms. Those with lower resistances often caused excessive injury to the cells (see below), while those with higher resistances did not inject dye fast enough. Dye was injected by passing 500 msec pulses of hyperpolarizing current at 1/sec for 20–60 min; the current ranged from 1 × 10^{-8} to 5 × 10^{-8} A. Under these conditions the cell became appreciably yellow within 2–3 min, and was deeply yellow at the end of the injection period. The dye was allowed an additional diffusion time of 30 min to 5 hr; in general, we used times in the range of 1–2 hr since the processes that we were concerned with are within a few hundred microns of the cell body.

Ganglia were then fixed for 30 min with 0.8% glutaraldehyde and 1.0% paraformaldehyde in phosphate buffer at pH 7.4. The preparation was postfixed in 1.0% osmium tetroxide in phosphate buffer for 90 sec–4 min with agitation, and stained for 2 hr at 4°C with 0.5% uranyl acetate buffered with tris maleate. After dehydration with a graded series of ethanol solutions, the preparation was embedded in Epon 812 or Maraglas (Polysciences, Inc., Rydal, Pa.) and cured for 45–90 min in a vacuum oven at 90°C.

Ganglia were sectioned transversely through the axis of the roots (see Fig. 4 A); 3-μ sections were cut on glass knives and examined with a conventional fluorescence microscope using a BG 12 exciter filter and a 530 nm barrier filter. The injected cell was thus encountered first at the level of its cell body, and its processes were followed in cross-sections through the neuropil; at any point alternate thin sections could be examined in the electron microscope.

Exposures to osmium tetroxide greater than several minutes led to pronounced quenching of the fluorescence in sections, while exposures shorter than about 90 sec resulted in inadequate contrast of the tissue when viewed in the electron microscope. The small size of the leech ganglion (about 0.3 mm thick) is probably an important factor in achieving adequate contrast with brief exposure times, because after 90 sec structural details were sometimes well seen at the periphery of the neuropil but marginally defined at its center. In general, 3 min resulted in both adequate fluorescence and staining throughout the ganglion. Shorter times (90 sec) were used when maximum fluorescence was desired (e.g. observing fluorescence in thin sections; see below). Even with very short exposures fluorescent cells were not visible in whole mounts because OsO_4 blackens the tissue. An unexpected advantage of OsO_4 treatment, however, was a marked reduction in the usual background fluorescence present in sections with aldehyde fixation alone.

In order to locate selected details for study with the electron microscope we found it useful in some cases to reembed 3-μ sections and cut them into thin sec-

tions. Thick sections were transferred by a wire loop to slides that had previously been immersed in 1.0% glycerol in absolute alcohol and been allowed to dry. The sections could be examined and photographed with fluorescence optics, and subsequently floated off the slide by placing a drop of water on them. Sections were transferred to a second drop of water in an aluminum weighing dish, and the drop was evaporated by gentle heating. They were then mounted on a small cube of cured Maraglas by placing a drop of epoxy cement (Ross Chemical & Mfg. Co., Detroit, Mich.) on the section and then the cube of Maraglas on top of this. After curing the epoxy cement for 4 hr at 60°C, the aluminum was peeled away leaving the 3-μ section glued to the surface of a block which could be trimmed in the usual way. Usually 20–30 thin sections were recovered from the original thick section.
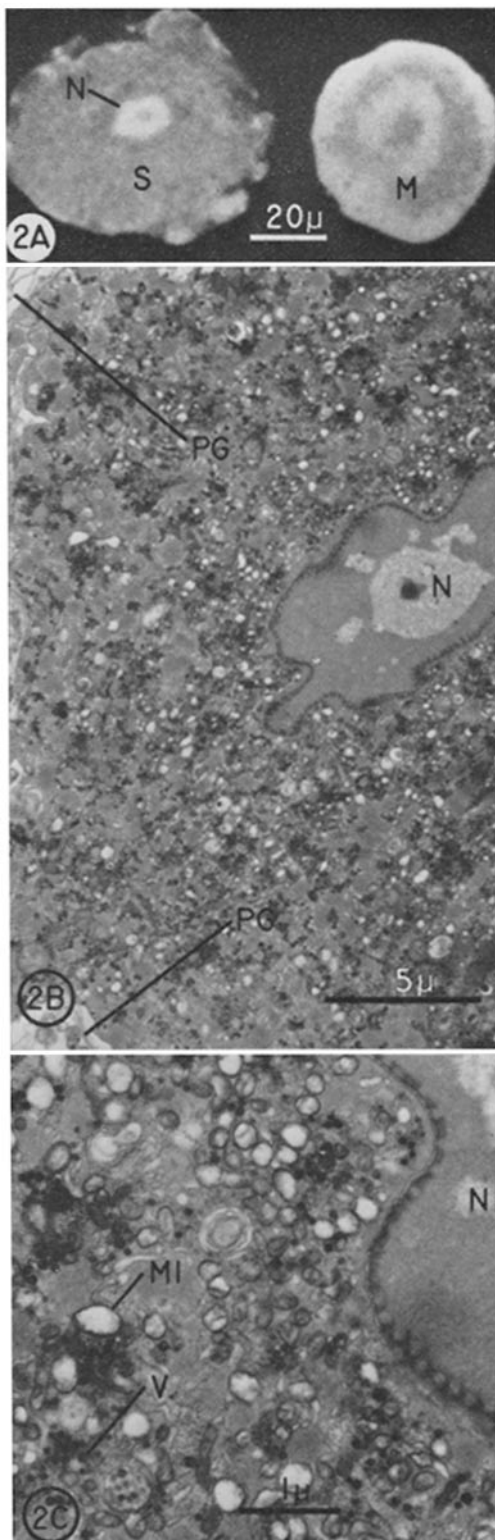
Processes of the injected cell were also localized on occasion by mounting thin sections on glycerol-treated slides. A thin section was photographed with the fluorescence microscope, subsequently floated off, placed on a 75-mesh parlodion-coated grid, and stained with either uranyl acetate and lead citrate, or lead citrate alone. This enabled us to examine the same section with fluorescence and electron optics.

For some purposes serial thin sections (about 1000 A thick) were taken. Owing primarily to loss behind grid bars the effective recovery rate of injected cell profiles in these experiments was about 50–60%; however, no more than five profiles were ever obscured in succession.

RESULTS

Fine Structure of the Cell Body of the Motor Neuron; Comparison of Injected and Uninjected Cells

The cell body of an injected motor neuron contains dye throughout its cytoplasm and nucleus when viewed with the fluorescence microscope. The distribution of dye however is not uniform: the peripheral cytoplasm is highly fluorescent, while central to this there is a less intense ring (Figs. 1 A, 2 A). In the electron microscope the same cell shows zones of its cytoplasmic constituents which fit the distribution of fluorescence (Fig. 1 B). The ring of relatively low fluorescence is composed of mitochondria, dark bodies 0.2–0.7 μ in diameter that probably represent deposits of lipofuscin pigment, endoplasmic reticulum, and granular vesicles 500–800 A in diameter (Figs. 1 D, 1 E), while the remaining cytoplasm is generally lacking these structures, being composed largely of granular endoplasmic reticulum. This arrangement of or-

ganelles found in the motor neuron has already been described in one of two main morphological cell types in the segmental ganglia of the leech by Coggeshall and Fawcett (4).

The effect of dye injection on the fine structure of the motor neuron was evaluated by comparing it with a cell having the same cytoplasmic characteristics and in the same position on the opposite side of the ganglion (Fig. 1 C). This cell was presumably the homologous motor neuron, its identity being only tentative in the absence of a marker. The injected cell was distinguished by characteristic small vacuoles in its cytoplasm, apparently caused by distortions of endoplasmic reticulum and mitochondria (Fig. 1 D). In addition, the ground substance of the cytoplasm (and of the nucleus) is more homogeneous and darker than that of the control cell. Other structures such as the nuclear envelope, dark bodies, and granular vesicles remain largely unchanged by the injection procedure. The arrangement of the processes of the surrounding packet glial cell was also similar for injected and uninjected cells (Fig. 1 B).

In another series of experiments we injected the lateral sensory cell that responds to noxious stimuli (11). Its appearance was characteristically different from the motor cell, the fluorescence being more evenly distributed throughout the cytoplasm (Fig. 2 A). As expected, in the electron microscope organelles were not arranged in distinct zones but rather were scattered throughout the cytoplasm (Figs. 2 B, 2 C). In addition, this cell contains numerous granular vesicles, thus corresponding to the second major category of leech neurons recognized by Coggeshall and Fawcett (4). We did not determine to what extent the differences we have

Figure 2   Injected sensory cell. Fig. 2 A, a motor cell (M) and sensory cell (S) have been injected in the same preparation and photographed in a thick section (3 μ) with fluorescence optics. Motor cell has typical target appearance while the sensory cell cytoplasm is diffusely fluorescent. N, nucleus. × 475. Fig. 2 B, low power electron micrograph of an injected sensory cell. Ring of organelles that characterizes the motor cell is absent; rather organelles are scattered throughout the cytoplasm. N, nucleus, PG, packet glial cell. × 4050. Fig. 2 C, higher power view of injected sensory cell shown in Fig. 2 B. As in the injected motor cell the cytoplasm has a vacuolated appearance due to disruption of some mitochondria (MI) and swelling of the endoplasmic reticulum. Numerous granular vesicles (V) are present. N, nucleus. × 13,000.

described for this particular motor and sensory cell can be generalized to other motor and sensory cells. Structural changes of the endoplasmic reticulum and mitochondria similar to those already described were also seen in the injected sensory cell (Fig. 2 C).

Occasionally the periphery of the cell body and the main process of an injected cell had a fragmented appearance when viewed in thick sections; this distortion seemed associated with difficulty in penetrating the cell and was presumably due to excessive damage. Such preparations were discarded.

## Fine Structure of the Main Process and its Branches within the Neuropil

In general, alterations induced by the injection procedure, such as the vacuolated appearance of the cell and the distortion of mitochondria, are present throughout the main process within the neuropil; in addition there are dense patches of material scattered throughout the axoplasm (Fig. 3). These features are helpful in identifying the injected profile in the electron microscope, although this is not true of smaller branches (see for example Fig. 6). As shown in Fig. 3 the changes are less marked with increasing distance from the cell body. The main process contains numerous mitochondria which are rounder than those in the cell body, microtubules, occasional granular vesicles, and one or more dense bundles of filaments. These characteristics have been described in axons of ganglion cells in general (5, 4), and are seen in other uninjected axon profiles in our preparations (see for example Fig. 9 C). Thus, as in the cell body, while the injection of dye causes visible changes in the fine structure of the main process

within the neuropil, its characteristic constituents can still be recognized.

With fluorescence optics, branches of various sizes can be seen arising from the main process. Most obvious are about 20 large branches which run for distances of 50–100 μ or more (Fig. 4 A). Many shorter branches are also seen as well as spines only one or a few microns in length. These are difficult to detect with the light microscope (Figs. 4 B, 4 C). Fig. 4 C shows several spines in the same thin section viewed in the fluorescence and electron microscopes (see also Fig. 3); processes with diameters as small as 0.5 μ can be correlated in this way.

To determine the distribution and configuration of the short branches we examined five regions, each 10 μ in length, of one injected motor cell by making reconstructions of these areas from serial thin sections (Figs. 4 A, 5). In region I, near the cell body, only two branches were seen arising from the main process; both were large and could be followed for 15–25 μ before they were lost; no spines or short branches were seen in this area. In another preparation a 25 μ stretch was examined in serial section from this same region and again only rare large branches and a very occasional spine were seen. In contrast, in regions II–V short branches and spines are numerous, occurring with a frequency of greater than 1/μ. Thus, while in light microscope reconstructions the 20 or so long branches are the most obvious feature, these are greatly outnumbered by several hundred shorter branches and spines arising from the major process which are evident with electron optics. The distribution of short processes is not uniform, as the distribution of large branches appears to be: they are rarely seen in region I, but increase in number as the midline is approached (regions II–III) and

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Figure 3 Injected motor process at selected points in the neuropil. Fig. 3 A, main process of injected motor cell (*MP*) in its initial course in the neuropil (region I in Fig. 4 A). Two large branches (*BR*) can be followed out of field. Main process and branches are surrounded by an investment of neuroglial processes (*NG*). Note the disruption of mitochondria (*MI*) and clumping of cytoplasmic material; these features made it possible to recognize injected profiles in the neuropil with only occasional reference to the fluorescence microscope. Subsequent parts of this figure (3 B–3 E) are cross-sections from the same preparation. Ventral is top, anterior to the right. × 6900 in Figs. 3 A–3 E. Fig. 3 B, main process as it nears midline (region II, Fig. 4 A). Changes seen in Fig. 3 A are present but less marked. Note presence of spines (*SP*). Fig. 3 C, main process in midline (region III, Fig. 4 A) showing similar cytoplasmic changes. Fig. 3 D, main process in contralateral neuropil (region IV, Fig. 4 A). Fig. 3 E, posterior branch of main process beyond bifurcation (region V, Fig. 4 A). Cytoplasmic changes still present, but less obvious.

5μ
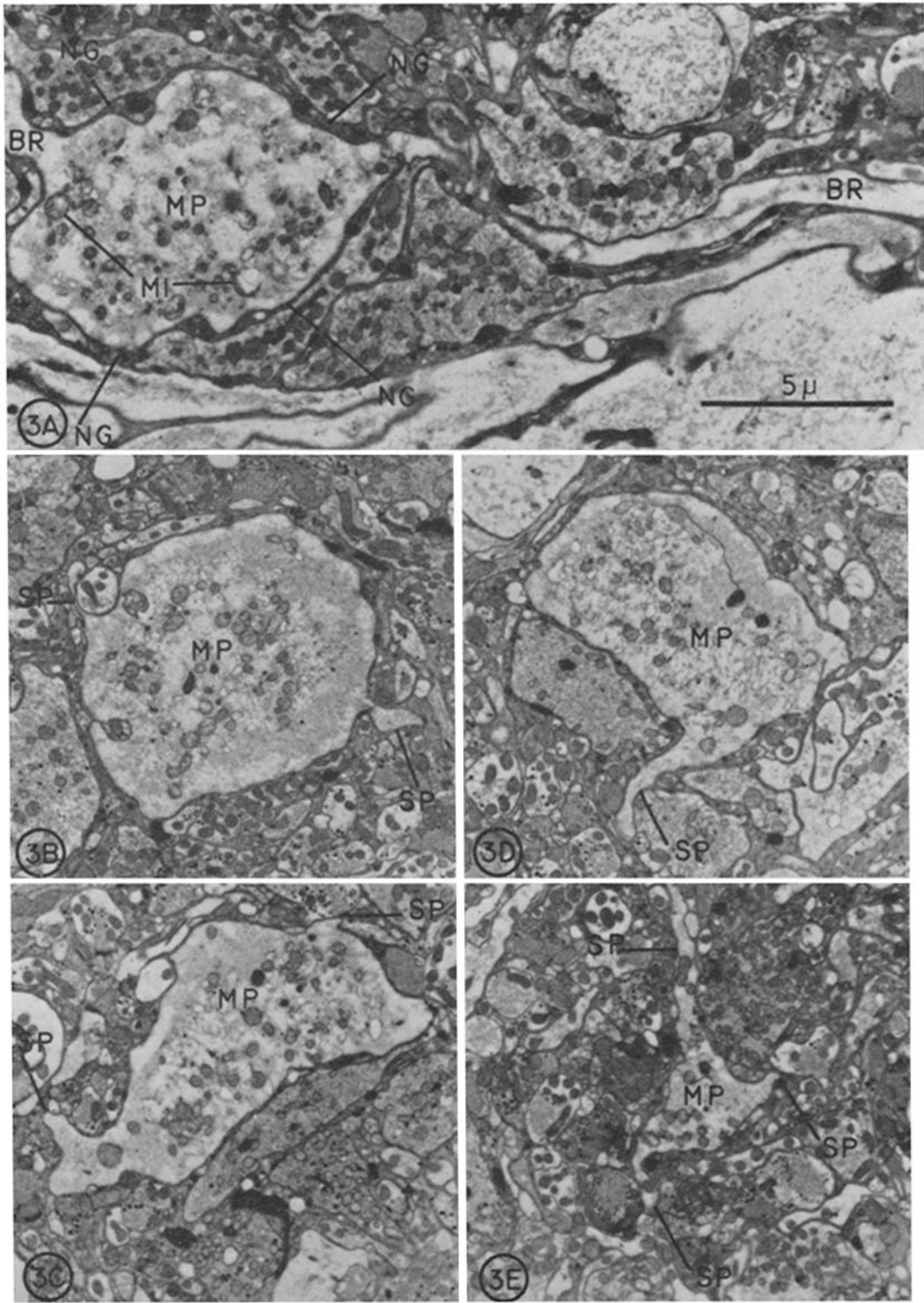
are prominent in the contralateral neuropil (regions IV–V).

In order to examine longer branches at greater distances from the main process without resorting to serial sections, we used the technique of reembedding thick sections containing a branch that could be seen in the fluorescence microscope (see Materials and Methods). Fig. 6 is a montage of several thin sections taken from the original thick section (fluorescence micrograph in Fig. 6) and is representative of several branches studied in this way. Only occasional spines similar to those of the main process were seen arising from such branches (for example see Fig. 8 C).

Synapses on the Motor Cell

The structure of synapses in the leech neuropil (Figs. 7 A, 7 B) is basically similar to that in vertebrate nervous systems (4). Presynaptic profiles contain numerous vesicles which are clustered near the presynaptic membrane, the synaptic cleft is wider than the nonsynaptic extracellular space, the pre- and postsynaptic membranes are increased in density, and there is a band of dense material attached to the postsynaptic membrane. Additional features of synapses in the leech neuropil are that two or more postsynaptic processes which sometimes contain vesicles usually share a single presynaptic specialization (Fig. 7 B), and that the postsynaptic profiles slightly indent the presynaptic terminal, which has a well defined tuft of dense material on its membrane just opposite the space separating adjacent postsynaptic processes.

Presynaptic profiles from unidentified cells made synaptic contacts on both the main process and branches of injected motor neurons. We studied the morphology and distribution of synapses on one

motor cell in detail by looking at the serial sections of selected 10 μ stretches described above (Figs. 4 A, 5). Random sections from areas between the reconstructed segments were also examined, as well as serial sections of some of these regions in injected motor cells from other ganglia.

Synaptic contacts were rarely seen in region I (Fig. 5) either on the main process or on the large branches that emerge from it. These branches are generally surrounded by a glial investment, and are only occasionally in direct contact with other neural processes (see for example Fig. 6). It is possible, however, that long branches have more synapses on them in their distal portions, which we did not examine.

In contrast, numerous typical presynaptic profiles make contact on short branches and spines of the motor cell in regions II–V, in addition to occasional synapses on the main process itself and on larger branches (Fig. 8). These could be divided into two general categories on the basis of the appearance of the presynaptic profile. The most frequent type of presynaptic element (Figs. 8 A–8 C) is a small (often less than 1 μ) profile densely packed with agranular vesicles about 300–500 A in diameter, an occasional granular vesicle 500–800 A in diameter, and scattered mitochondria. This type of synapse was distributed about equally in regions II–V.

The second type of presynaptic profile in contact with the motor cell is larger (up to several microns in diameter). Synaptic vesicles are similar to those in the first type but are found only in the region of membrane specialization, giving the terminal a characteristic empty appearance (Figs. 8 D–8 E). In some cases this type of ending made multiple en passant contacts with processes of the motor cell; an example of this is shown in Fig.

FIGURE 4 A   Light microscope reconstruction of an injected motor cell (see Nicholls and Purves, Fig. 1). Main process traverses neuropil, bifurcates, and enters roots (R) contralateral to cell body. Large branches arise from the main process and run for distances up to about 150 μ. Five segments (I–V) bracketed by dotted lines indicate approximate location of 10 μ regions examined in serial thin sections; reconstructions from electron micrographs of these regions are shown in Fig. 5. C, posterior connective. $\times$ 130.

FIGURE 4 B   Section (7.5 μ thick) cut in same plane as in Fig. 4 A, showing injected main process of motor cell from midline (ML) to its bifurcation (large arrows). Numerous short branches and spines (small arrows) several microns or less in length arise from main process; these are not shown in Fig. 4 A. Aldehyde fixation only. $\times$ 270.

FIGURE 4 C   Electron micrograph of a cross-section of an injected motor cell main process with several spines (A, B, C, D). Inset is the same section photographed in the fluorescence microscope before transfer to grid for examination with the electron microscope (see Materials and Methods). $\times$ 8800. Inset, $\times$ 1600.
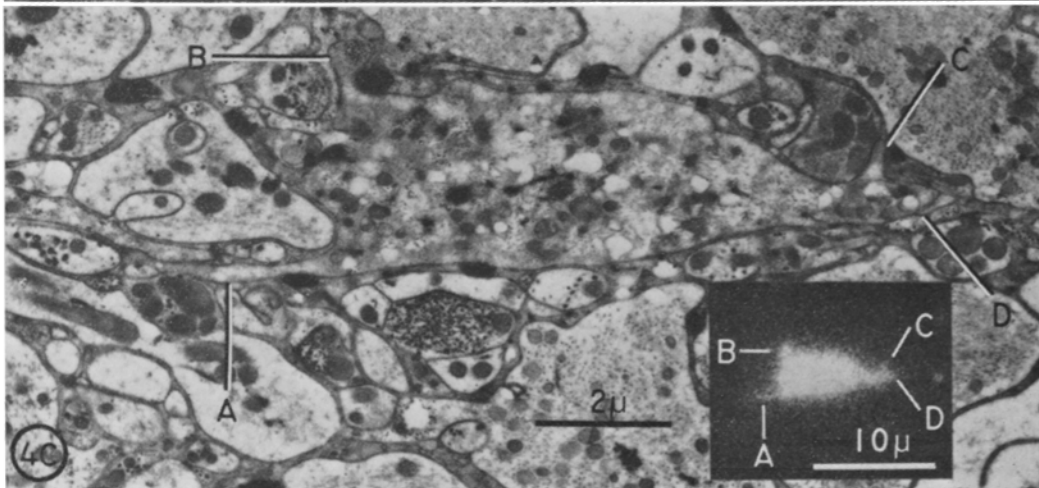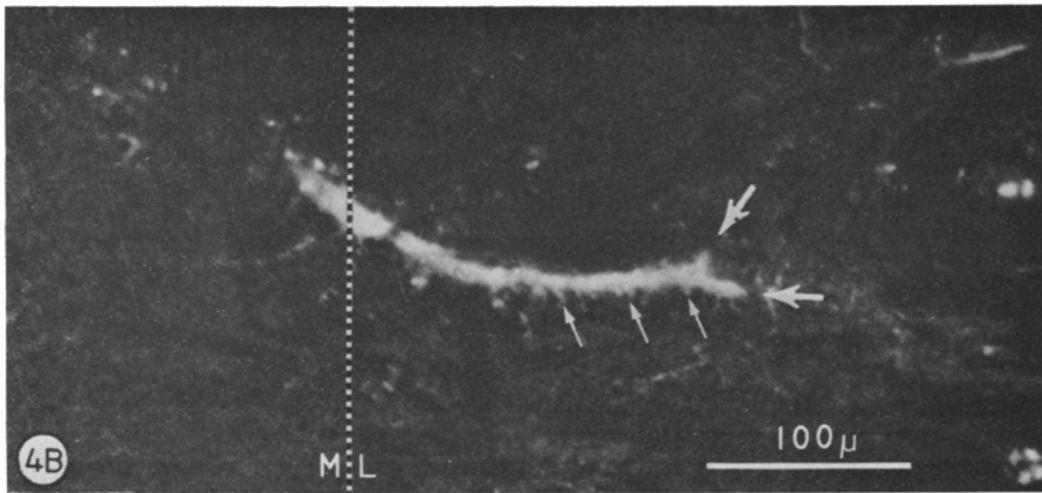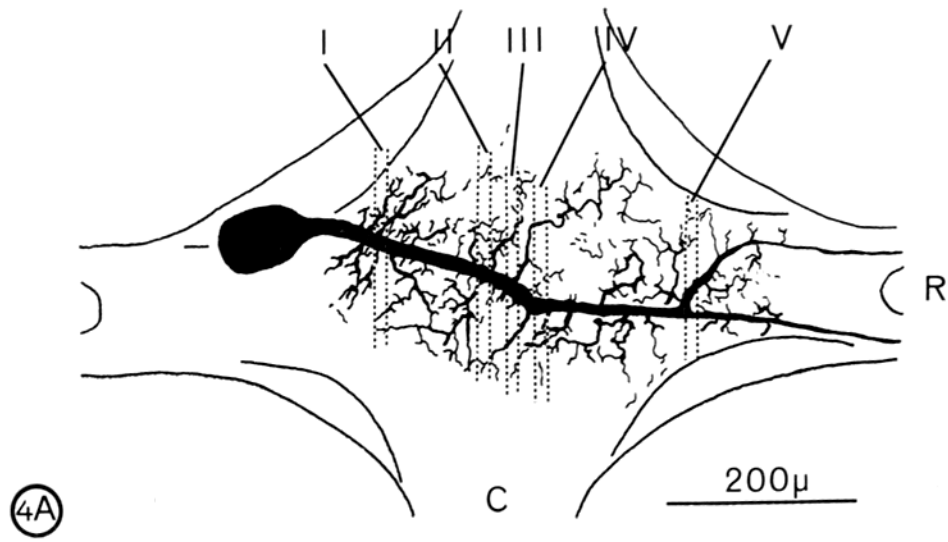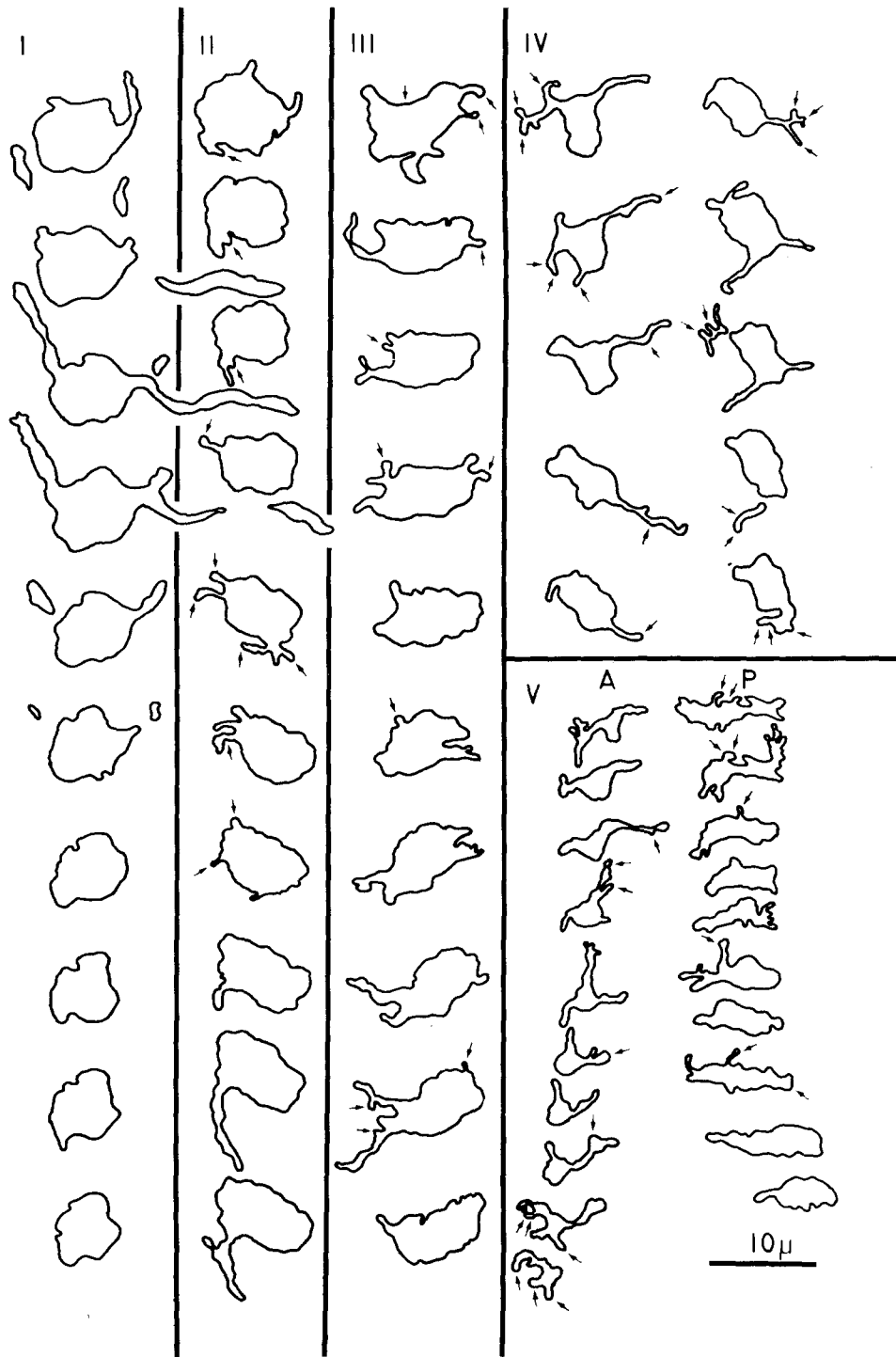
FIGURE 5 Reconstruction of main process of an injected motor cell and some of its branches from serial thin sections through selected 10 μ regions in the neuropil. The approximate locations of these regions are bracketed in Fig. 4 A and indicated by corresponding Roman numerals. Each profile is a composite drawing of 1 μ intervals; sequence is progressing away from the injected cell body as one moves down the page. Arrow indicates site of a synaptic contact on the motor cell. Short branches and spines are rare in region I and synapses are absent; both are prevalent in other four regions. Note that in region V there are two profiles for each 1 μ interval representing the anterior (A) and posterior (P) branches of the main process which has bifurcated. Ventral is top, anterior to the right. × 1530.
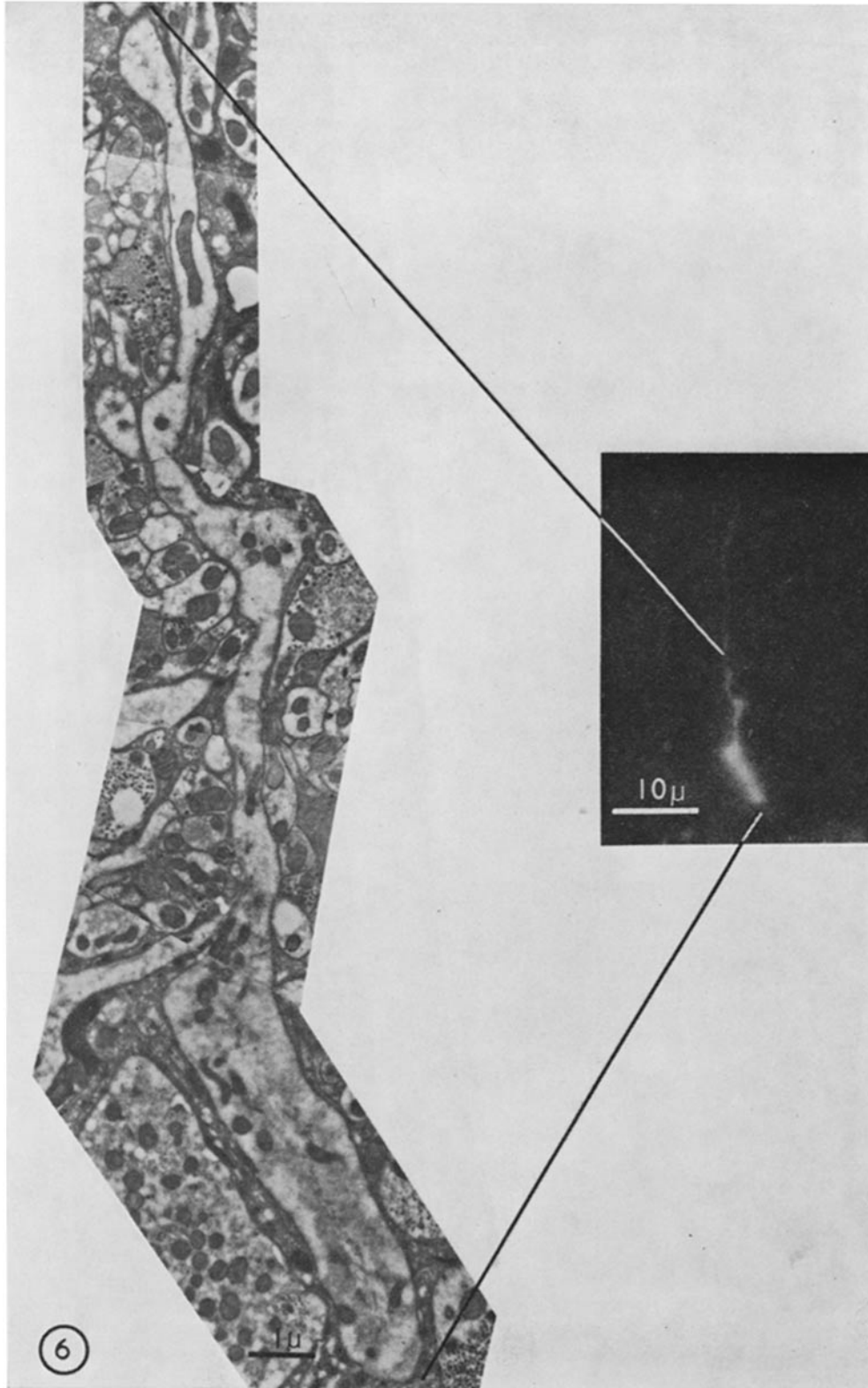
FIGURE 6   Light micrograph, portion of a larger branch running from about 25 μ to about 70 μ from the main process seen in 3 μ section with fluorescence optics. $\times$ 1000. Electron micrograph, after reembedment and thin sectioning of the original thick section the same branch was found in the electron microscope and a montage constructed by combining micrographs of several thin sections; lines indicate the extent of the branch included in the montage. No synapses were seen on this process in serial thin sections through the original thick section. $\times$ 9600.
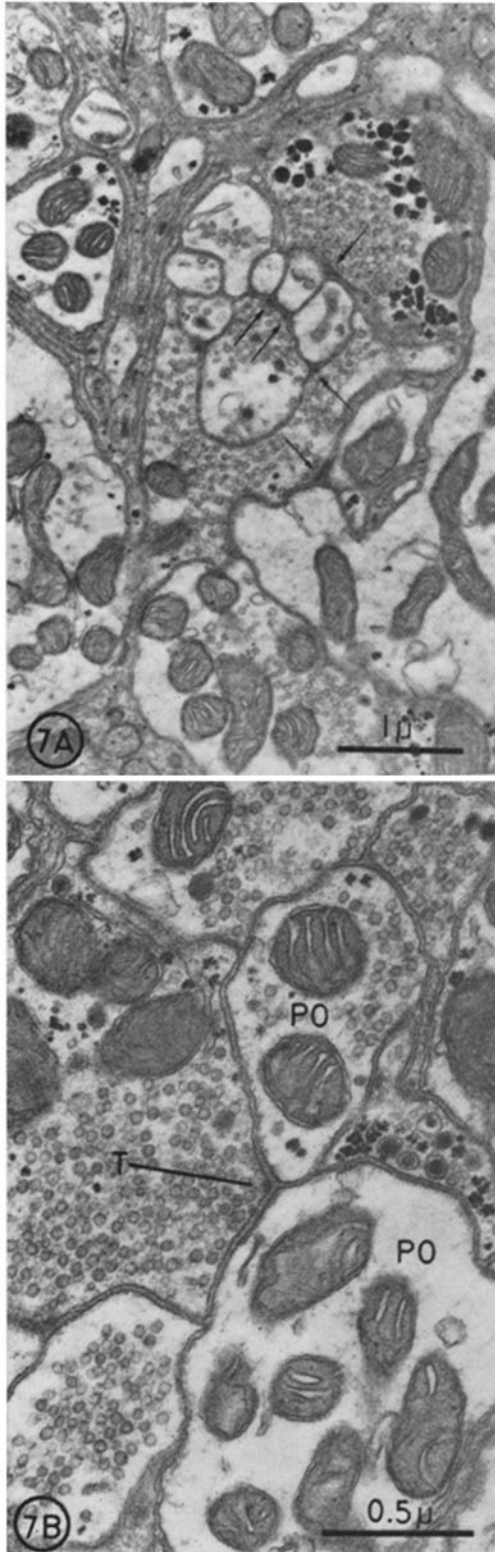
9 A. A total of 93 synapses was seen on the cell examined in greatest detail—63 were of the first type, 27 fell into the second category, and three could not be classed definitely in either group (e.g. Fig. 9 B). In contrast to the small terminals densely packed with vesicles, the large clear profiles were not evenly distributed, all 27 being encountered in or near the midline (regions II–IV). Both types of terminal also made synapses on other unidentified processes.

In most cases it was not possible to determine the origin of the postsynaptic profile sharing a presynaptic element with the motor cell. Occasionally, though, the plane of section included the origin, as in Fig. 9 C where the conjoint profile is a spine from a nearby unidentified process.

Our results permit a minimum estimate of the total number of synapses made on the motor cell studied in greatest detail. In the regions examined in serial section other than region I, synapses occurred on short branches and spines with a frequency of 1–2 μ; since the distance from region II to the opposite roots where the neuropil ends is about 300 μ there are at least 300–600 synaptic contacts. This figure underestimates the total because the effective recovery of sections was only 60% at best, and because many branches whose continuity was in doubt were not followed; also the distal portions of longer branches were not examined. Hence the actual number of synapses making contact with the motor cell may be as high as several thousand.

The contacts we have described fit anatomical criteria for chemical synapses in vertebrates. It is known that the motor cell is also electrically coupled to other cells by rectifying and nonrectify-

Figure 7 Synapses between unidentified cells. Fig 7 A, low power view of portion of neuropil showing several vesicle-filled profiles making specialized contacts (arrows) with two or more adjacent postsynaptic processes. Fig. 7 B, at higher power the specialized regions of contact are similar to synaptic contacts in vertebrates: the two opposed plasma membranes are increased in density, there is a slight widening of the extracellular space, and there is a band of dense material on the membrane of the presumed postsynaptic processes (PO). There is some dense material on the presynaptic membrane also, but it is mainly confined to a tuft (T) directly opposite the space separating conjoint postsynaptic processes. Vesicles in the presynaptic process are focused on the specialized region; one of the postsynaptic processes also contains vesicles. Preparation fixed for 30 min in OsO_4. Fig. 7 A, $\times$ 14,700. Fig. 7 B, $\times$ 39,000.
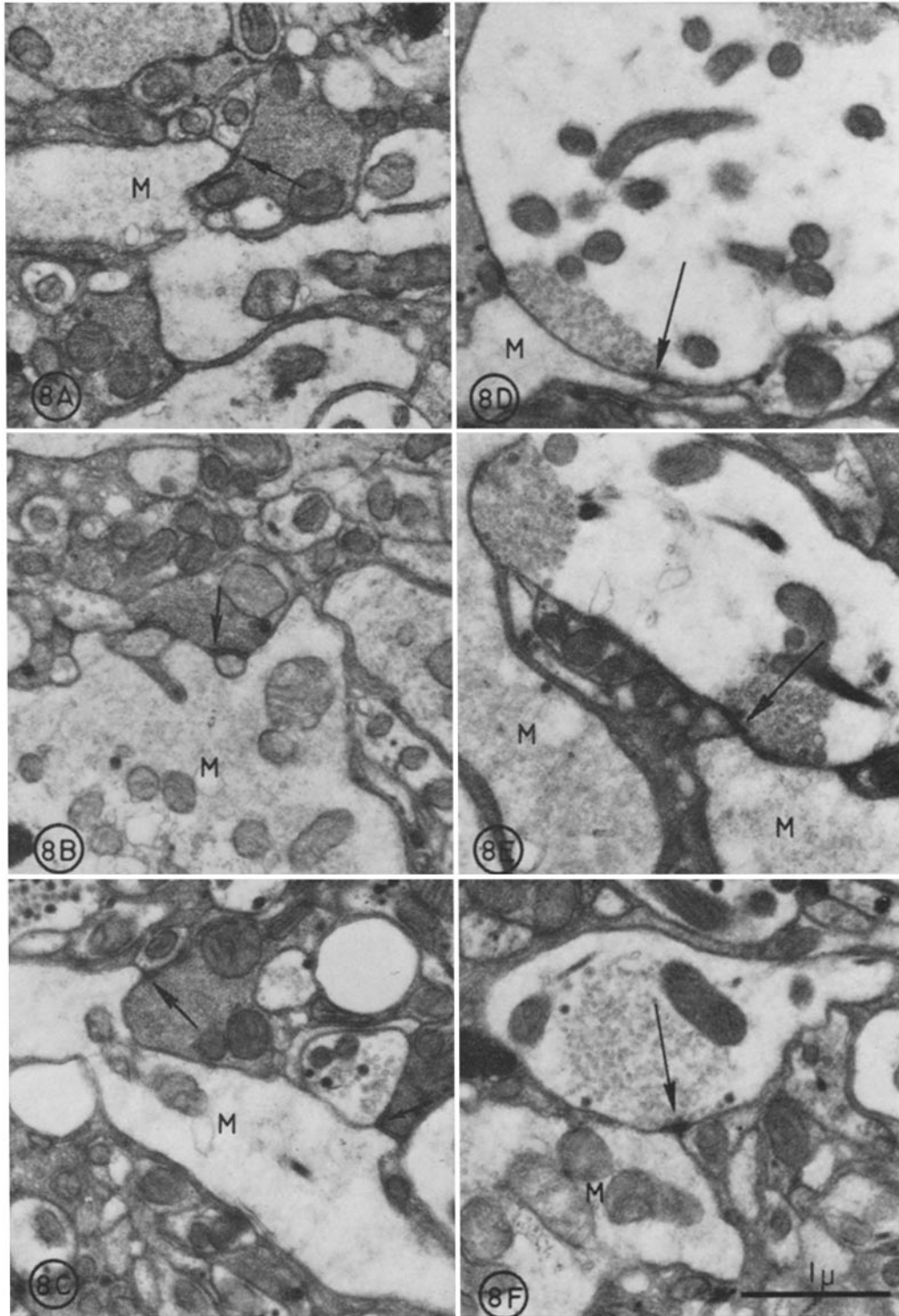
FIGURE 8   Presynaptic profiles in contact with processes of injected motor cell. Figs. 8 A–8 C, examples of smaller type of presynaptic profile densely packed with agranular vesicles on motor cell processes (M). In Fig. 8 A synapse (arrow) is on short branch about 2 μ from main process; in Fig. 8 B synapse is on spine of main process, while in Fig. 8 C synapses are on a larger branch approximately 17 μ from the main process. Figs. 8 D–8 F, examples of larger type of presynaptic profile in which vesicles are generally clustered at the synaptic specialization. In Fig. 8 D the synapse is on a short branch about 4 μ from the main process, while in Figs. 8 E and 8 F the synapses are on short, broad spines of the main process. Figs. 8 A–8 F, $\times$ 22,600.

ing electrical junctions (13, 19). Gap junctions thought to be the morphological correlate of electrical transmission in vertebrates (3, 16) and invertebrates (14) were not seen in this study.

DISCUSSION

Since the initial experiments of Stretton and Kravitz (18), the fluorescent dye Procion yellow has been widely used to determine the structure of
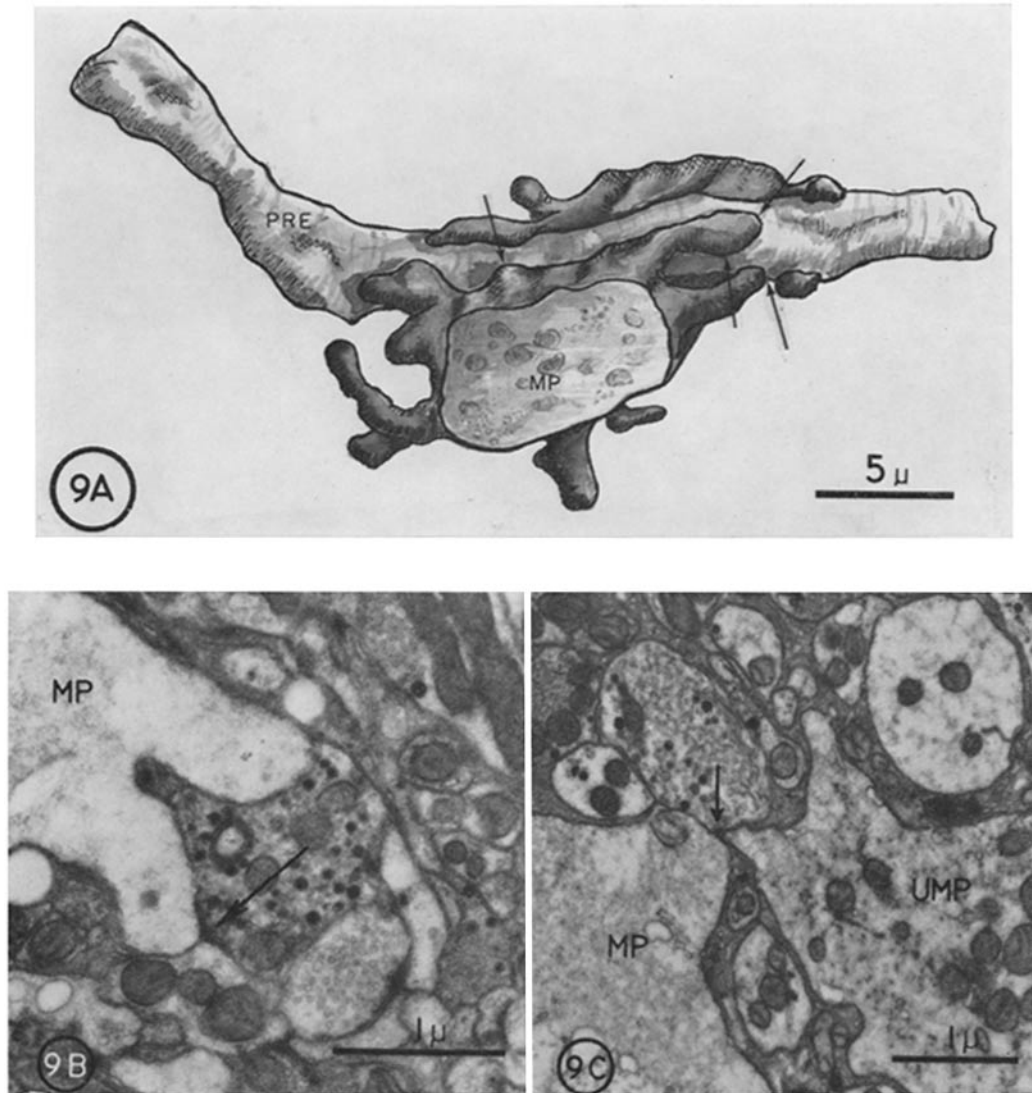

FIGURE 9 A   *En passant* synapses on the motor cell. Reconstruction from serial thin sections of main process of motor cell (*MP*) over a 4.5 μ segment near the midline (region III, Fig. 5) showing its relationship to a presynaptic element (*PRE*) which makes four of the larger type of synaptic contacts (Figs. 8 D–8 F) on it (approximate locations shown by arrows). Ventral is upward, anterior to the right. × 3558.

FIGURE 9 B   Unclassified synapse on a motor cell. Example of a synapse (arrow) on a spine of the main motor process (*MP*) containing numerous dense core vesicles. This profile does not fit either category of synapse described. × 22,600.

FIGURE 9 C   Origin of a conjoint postsynaptic profile. Example of a synapse (arrow) on the main motor cell process (*MP*) where the adjacent postsynaptic element is a spine from a nearby unidentified main process (*UMP*). × 16,400.

both vertebrate and invertebrate nerve cells at the level of the light microscope (for example references 7, 9, 13, 17, 19); it has also been used to study electrical coupling between neurons (15) and to make quantitative estimates of neuronal surface area (1). A particular advantage of the technique over such classical methods as silver stains is that one can physiologically identify the cell which is to be marked by recording from it through the dye electrode. At the level of the light microscope, however, the nature and distribution of synapses on neurons made visible by Procion yellow injection cannot be determined, and thus one's understanding of how the injected cell is structurally related to other nerve cells is limited.

The method of dye injection and fixation we describe allows one to study the same injected profile in both light and electron microscopes. Although the fine structure of the injected cell is distorted in some respects, its general features are preserved; most importantly, synapses on the motor cell appear normal and their morphological type and distribution can be studied.

A central feature of the organization of synapses on the motor cell is the prevalence of contacts on short branches and spines of the main process as it traverses the neuropil, and their relative scarcity on the main process itself as well as the proximal parts of larger branches. Although synapses on the motor cell were only occasionally encountered in the initial course of the main process (region 1, Fig. 5), they occurred frequently as the process approached the midline and throughout the contralateral neuropil. This initial description could be carried further by double injections of the motor cell and other cells known to be presynaptic to it on the basis of physiological experiments.

It is known that the large longitudinal motor neuron makes electrical synapses with other cells (19, 13). In the course of these studies we did not see the usual morphological correlates of electrical transmission (see above). Nor did we find processes of the motor cell making chemical synapses on processes of other neurons. We cannot rule out, however, that the motor cell makes chemically transmitting contacts with other postsynaptic cells, because a relatively small portion of the main process and only a few of its branches were studied in serial sections. It is unlikely that injection would obscure such contacts because granular vesicles remain intact even in injected cell bodies, and because in other experiments we found intact

terminals of an injected sensory cell containing large numbers of agranular vesicles. So far, electrophysiological experiments have also failed to demonstrate chemically transmitting contacts from the motor cell to other neurons.

The main drawbacks inherent in this method of studying the structure of a complex neuropil are: (a) it requires the use of small pieces of tissue, since a successful result depends on the ability of osmium tetroxide to fix the specimen adequately before quenching the fluorescence. With leech ganglia (about 0.3 × 0.8 mm), fixation was routinely satisfactory once we had determined the appropriate exposure times. (b) While the main process within the neuropil could be easily recognized in the electron microscope on the basis of the morphological changes described, branches could be identified as belonging to the injected cell only if they were followed in continuity, or if they were selected from thick sections that had been mounted and sectioned for the electron microscope. Sectioning thick sections was a useful method for studying isolated branches, but it was time consuming, as was examination of the same thin section in both light and electron microscopes. Correlating isolated small profiles by either method was difficult because of distortions caused by resectioning and the heating of the plastic by the electron beam.

In spite of these limitations we believe this approach will be generally useful in examining the specific arrangement of synaptic contacts on identified cells in the leech neuropil and in other preparations where intracellular recording is feasible.

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