PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The Functional Organization of Motor Neurons in an Insect Ganglion

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THE FUNCTIONAL ORGANIZATION OF MOTOR NEURONS IN AN INSECT GANGLION

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[Plates 27 to 31]

The distribution of motor nerve cell bodies in the metathoracic ganglion of the cockroach *Periplaneta americana* was mapped and displayed in three dimensions. A dense ring of ribonucleic acid (*RNA*) appears in the perinuclear cytoplasm of a nerve cell body whose axon has been cut in a peripheral nerve trunk. Using this *RNA* ring as the primary marker, 5 cell maps of ganglia from different animals were constructed to indicate which motor nerve cell body sends its axon out a particular peripheral nerve trunk. We count about 3000 neurons in the ganglion, and of these about 230 are above 20 μm in diameter. About 100 of these larger cells are generally arranged in bilaterally symmetrical pairs. These cell pairs have been assigned numbers and can be identified from one animal to another. Nerve cell bodies associated with nerves 3 through 6 send their axons out the ipsilateral nerve trunks. Cells associated with nerve 2 send their axons out the contralateral nerve trunk. This study may provide a basis for understanding the structural and metabolic organization responsible for the particular behavioural capacities of certain populations of neurons.

Introduction

There are a number of problems in neurobiology whose solution requires that we understand the critical factors which determine the functional characteristics of neuron populations. These problems have to do with such phenomena as the ability to learn, to evoke specific sensations, or to organize a locomotor pattern. The specific properties of different neuronal groups may depend upon two types of variables. One is structural and involves the geometry of excitatory and inhibitory connexions. The other variable may be chemical in nature and have to do with specific metabolic capabilities of individual neurons within the population. There is increasing evidence for the chemical differentiation of neurons (Levi-Montalcini 1966), and the possibility that these chemical differences may be in part responsible for the specific properties of integrative systems should be seriously considered.

In many instances the approach to such questions has been to examine a system that consists of a relatively small number of neurons and yields a specific well-defined bit of behaviour. The aim is to identify the structural and functional properties of the neurons in the particular system responsible for the behavioural act. Examples of such simple systems that have been fruitful in recent years are the crustacean cardiac ganglion (Hagiwara & Bullock 1957), the octopus brain (Young 1964), the crustacean ventral nerve cord (Wiersma & Mill 1965) and the neural elements governing insect locomotion (Wilson 1961, 1966). Understanding the implications of such structural–functional relationships in groups of neurons may depend upon discovering preparations in which the specific

neurons can be identified and the three-dimensional distribution of cell bodies and connexions defined. The precise identification of individual neurons involved in particular behavioural acts allows the focus of chemical and electrophysiological analyses on single units rather than on large masses of tissue. This should make more apparent the differences responsible for the specific behavioural potential of various neural systems.

The thoracic ganglia of the cockroach *Periplaneta americana* are relatively small pieces of nervous tissue that give rise to a variety of complex behaviour patterns. They are involved with the control of locomotion (Pringle 1940; Wilson 1965), endogenous activity (Milburn Weiant & Roeder 1960), nerve regeneration (Bodenstein 1957) and learning (Eisenstein & Cohen 1965). Their involvement in such diverse behaviour prompted us to investigate the organization of the cell bodies within one of these ganglia, the 3rd or metathoracic ganglion. An attempt is made in this paper to identify and map in three dimensions the major motor nerve cell bodies of this ganglion. The mapping procedure makes use primarily of ribonucleic acid (*RNA*) changes in the cell bodies of motor neurons whose axons have been injured (Cohen & Jacklet 1965). This technique permits the identification of the peripheral nerve trunk containing the axon of a particular neuron soma and also the specific limb muscle innervated by a given neuron. The purpose of this work is twofold. (1) It provides a basis for identifying the individual nerve cells in a small volume of central nervous tissue involved in many types of interesting behaviour. This may permit a start in relating the structural and chemical parameters of a group of neurons to the observed behavioural attributes of the system. (2) It is hoped that this study will serve as a model for examining the structural and chemical properties of nerve cells in other simple integrative systems.

Materials and methods

The experimental animal used in this study is the cockroach *Periplaneta americana*. Newly moulted adult males were selected from the colony by using the criterion of a light tan colour as evidence that the animal had undergone a moult within the last 24 h. These animals were then operated upon within 2 to 10 days after moulting to the adult stage. Ninety-two of these adult males were subjected to experimental operations. Ten late-stage nymphs between the 10th and 12th instar were also used as experimental animals. Ten normal unoperated adult males and one late-stage nymphal male were used as part of additional control procedures.

Cutting an axon causes a change in the *RNA* distribution of the attached nerve cell body. This change in the *RNA* of the neuron soma was used as the primary indicator for determining which peripheral nerve trunk contains the axon of a given central cell body. The procedure used was that described by Cohen & Jacklet (1965) and only the essence of the technique is given here. The metathoracic ganglion and associated peripheral nerve trunks were exposed as shown in figure 6, plate 28, by laying back a flap of ventral cuticle. The appropriate nerve trunks were cut about 1 mm distal to the ganglion and the cuticular flap was then sealed back in place with warm wax. Two to four days after cutting the nerve trunks, the ganglion was fixed in Zenker's solution for 2 h, sectioned at 10 μm, and stained for ribonucleic acid according to the pyronine-malachite green technique of Baker & Williams (1965). In some cases the operated animals were left up to 60 days before fixing the ganglion. Within this longer period after cutting the nerves one can

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

detect the shift of the nucleus to an eccentric position in the regenerating neurons (see figures 3 and 4, plate 27).

The stained serial sections of the experimental ganglia were then examined with bright-field and phase microscopy to determine which central cell bodies exhibited a dense perinuclear ring of *RNA*. The matched cell of the unoperated side of the ganglion serves as a control as seen in figures 1 and 2, plate 27. This perinuclear *RNA* ring has been shown by Cohen & Jacklet (1965) to be a response of the cell body to injury of its axon. It is apparent within 48 h after injury and has disappeared 10 days after cutting the axon. The formation of the *RNA* ring was used as the main marker to determine which central neuron soma was attached to a given peripheral axon. At approximately 4 weeks after nerve section, when regeneration of the axon is well under way (Bodenstein 1957; Guthrie 1962), the nucleus of the regenerating neuron shifts to an eccentric position in the soma, usually to a point where the axon emerges from the cell body. This is seen by comparing figures 3 and 4, plate 27. This change in nuclear position was sometimes used as evidence, in addition to the perinuclear ring response, for linking a given cell to a particular axon.

Using the markers indicated above, cell maps were constructed from the 10 μm transverse serial sections with the technique described by Pusey (1939). This involves the projection of each section on to tracing paper and drawing the outline of the ganglion and cell bodies. The tracings are then alined with respect to a grid oriented in a plane that is parallel to the dorsal and ventral surfaces of the ganglion. The location and dimension of each cell body are transferred to the grid to give cell maps as seen in figures 5 and 9, plates 28 and 30. In one case the data from such a map was transferred to a perspective grid to give the three-dimensional representation of cell-body distribution seen in figure 10. Only the cell bodies 20 μm in diameter and above and lying in the ventral and lateral portions of the ganglion were drawn on the maps. The peripheral nerves are designated by number according to the scheme followed by Pringle (1939) and Pipa & Cook (1959). The cell bodies that could be consistently associated with nerves 2 through 6 were given numbers. The groups of dorsal cells that did not show any consistent response to peripheral nerve trunk injury are not included in the maps and will be treated in another paper.

Results

The symmetry of cell distribution

Cell distribution maps, drawn to include the cells defined above, were constructed from metathoracic ganglia of four adults and one late-stage nymph. Examination of any individual map shows a striking bilateral symmetry of cell body distribution. Approximately 50 cells on one side can be consistently identified and matched with cells on the opposite side of the ganglion. These cell pairs have been assigned numbers, starting with the most anterior cells as no. 1. Numbers have been assigned primarily to motor cell bodies whose peripheral axons have been identified. Certain large cells in this area whose axons have not been identified can be seen in figures 5 and 9. Numbers have been reserved for these pending identification of their function and this accounts for some of the numerical gaps that are present. This was done to preserve the anterior-to-posterior numbering procedure when these cells are eventually identified. The bilateral symmetry occasionally breaks down for certain cells located near the mid-line as seen for cell 11A in figures 5 and 9

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The consistency of cell location

The fixity of cell location within this ganglion is demonstrated by comparing the distribution of cells in ganglia from different individuals. This can be seen by examining the location of similarly numbered cells in ganglia from animals Y3 and N2 as shown in figures 5 and 9 respectively. Y3 is a late stage nymph and N2 is a newly moulted adult. One can identify most of the specifically numbered cells from one animal to another. This consistent location of cells from one animal to another has been borne out in the five ganglion maps constructed.

Preparation N2 has been mapped in two dimensions in figure 9. A perspective representation of the cells in this preparation has been constructed in figure 10 to display the major motor-cell body locations in three dimensions. This accurately portrays the cells shown in the flat projection of figure 9 along three axes and gives a picture of their distribution within the ganglionic mass. Individual cells can be identified by using the transparent labelled overlay of figure 10 and comparing this with the numbered cells of figure 9. An additional aid to picturing the vertical distribution of cells within the ganglion is provided by the representative transverse sections through the middle and posterior portion of the ganglion as seen in figures 7 and 8. The level of these transverse sections is indicated by the appropriately labelled parallel lines in figures 5 and 6.

Relationship between cell bodies and peripheral nerve trunks

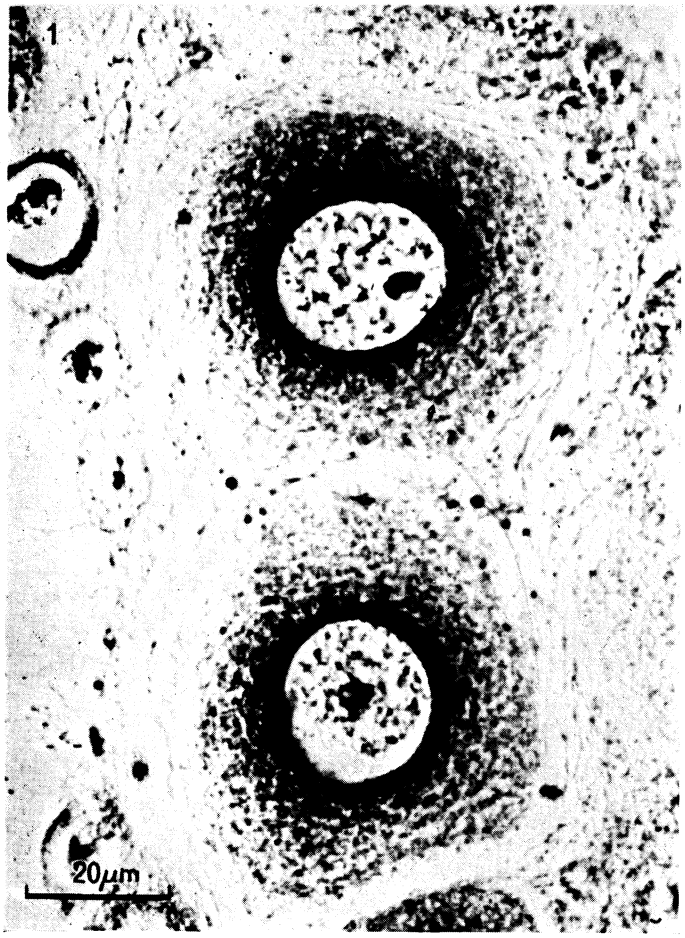
In order to determine which central nerve-cell bodies send their axons out any particular peripheral nerve trunk, the nerve trunks on one side were cut singly or in various combinations while those of the opposite side were left intact as a control. The presence or absence of a perinuclear *RNA* ring was always determined by comparing differences in stain density and distribution in the cell whose axon had been cut, to the appearance of the contralateral matched cell whose axon was intact. A total of 102 animals were examined in this manner and the results tabulated in table 1.

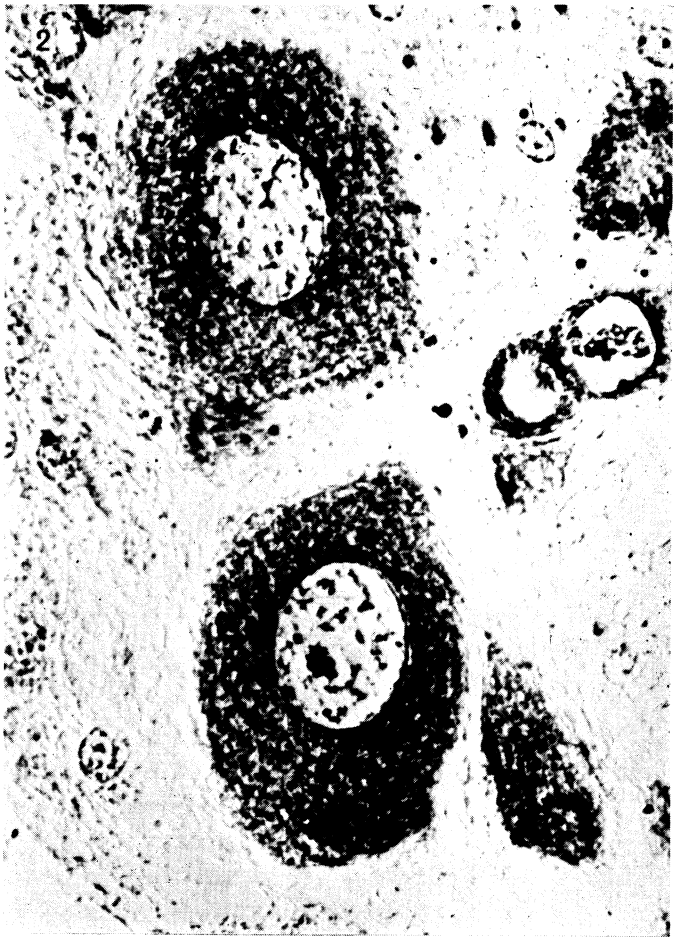
The number and location of cells showing *RNA* perinuclear rings were reasonably constant from animal to animal following damage to a specific nerve trunk. The average number of cells responding to section of individual nerve trunks or various combinations is shown in the last column of table 1.

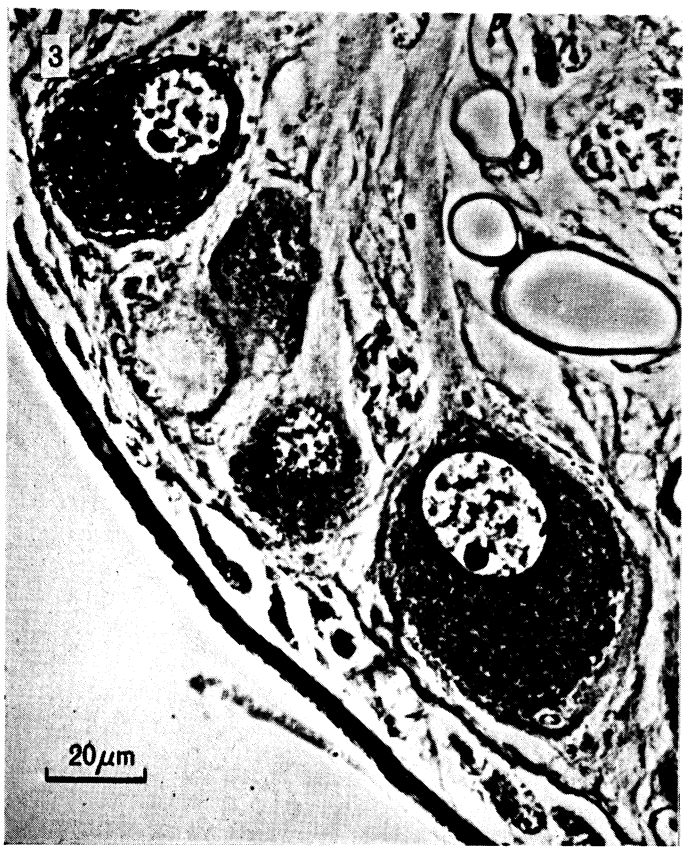
FIGURES 1 AND 2. Cell bodies of neurons in the metathoracic ganglion stained with pyronine-malachite green and viewed with bright-field microscopy. Figure 1 shows cells whose axons were cut 2 days previous to fixing the ganglion. Figure 2 shows the bilaterally matched cells from the opposite side of the same ganglion. The axons of the cells in figure 2 were left intact as a control. Note the dense perinuclear ring of pyronine stain in the injured cells of figure 1 and the relative lack of this ring in the matched control cells of figure 2.

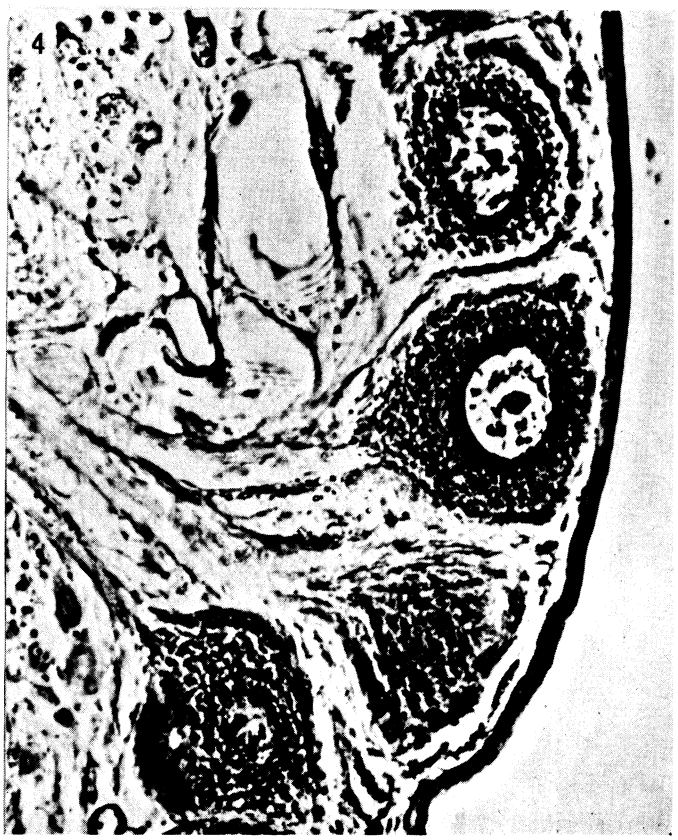
FIGURES 3 AND 4. Cells of the metathoracic ganglion stained with pyronine-malachite green and viewed with phase-contrast microscopy. The axons of the cells in figure 3 were cut 28 days previous to fixing the ganglion. The corresponding control cells on the opposite side of the same ganglion had their axons left intact. Note the eccentric location of the nucleus in the regenerating cells of figure 3 as compared to the central location of the control cells in figure 4.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES









FIGURES 1 to 4. For legends see facing page.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

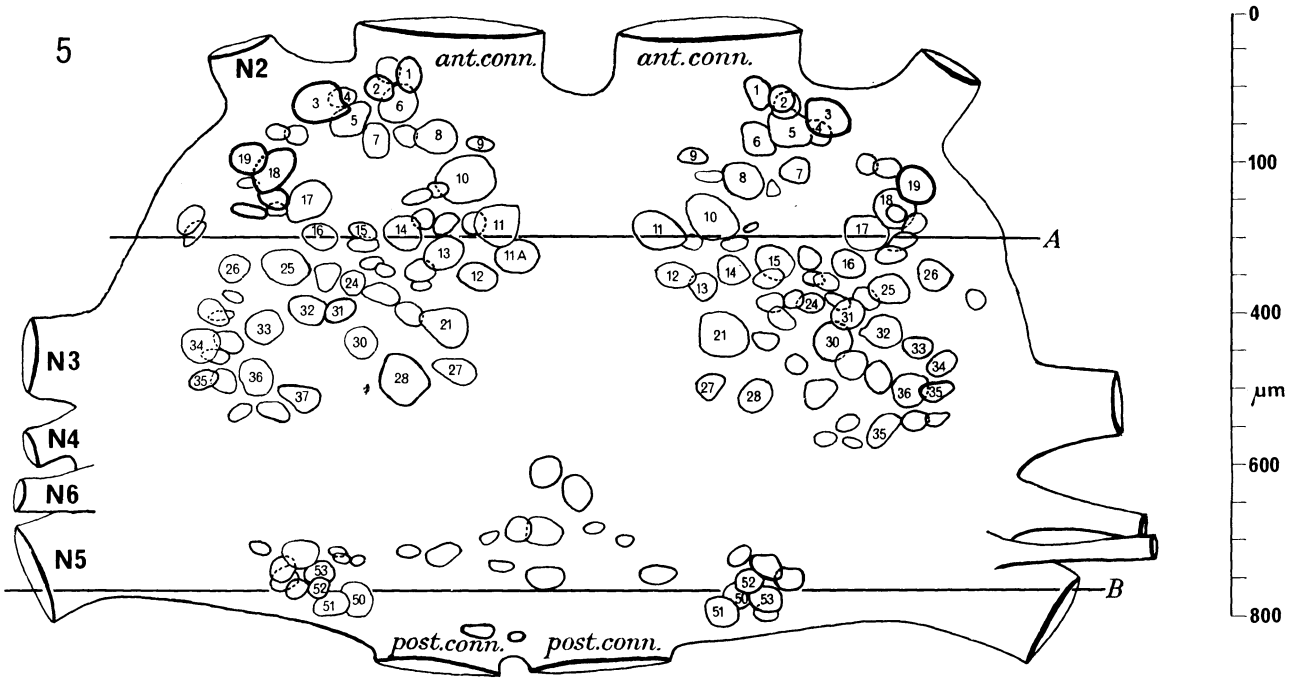


FIGURE 5. Cell map of the metathoracic ganglion from a late-stage nymph. The map was constructed from 10 μm transverse serial sections and shows the ganglion in dorsal view. Cells that are specific landmarks or that can be consistently associated with particular nerve trunks are numbered on one side, starting at the anterior end of the ganglion. The corresponding cell of the opposite side is given the same number. Line *A* corresponds to the plane of the section shown in figure 7. Line *B* indicates the plane of the section seen in figure 8. N2 to N6, nerve trunks; *ant. conn.*, anterior connectives; *post. conn.*, posterior connectives.

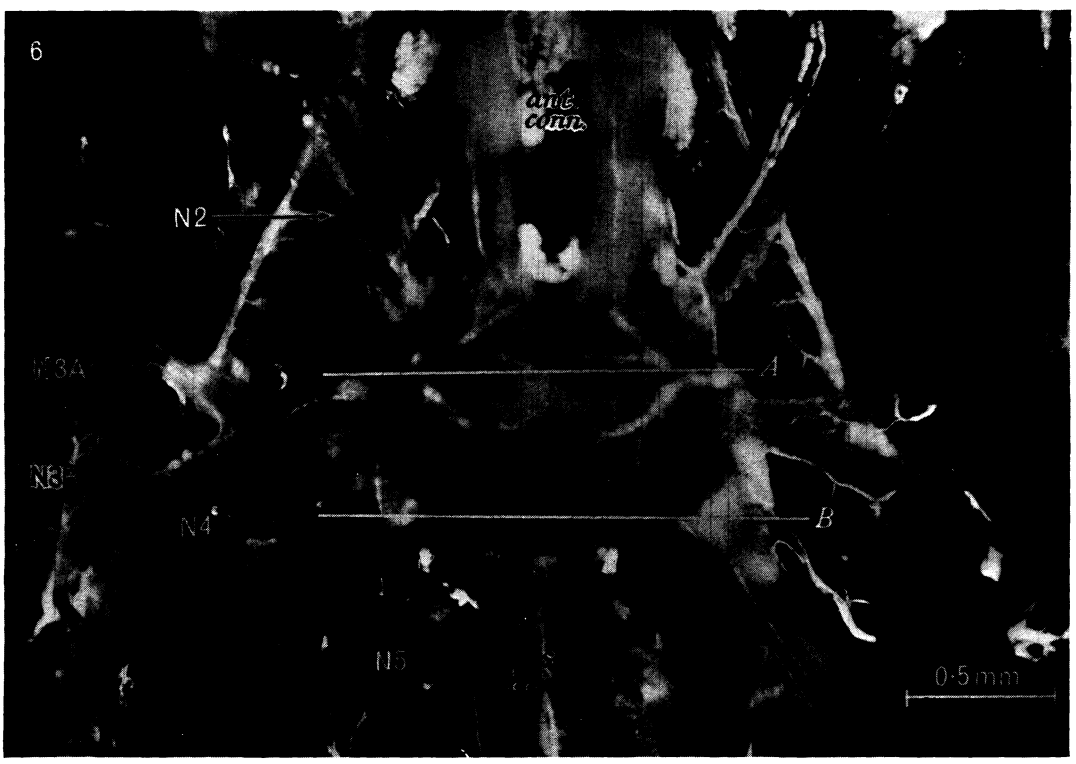
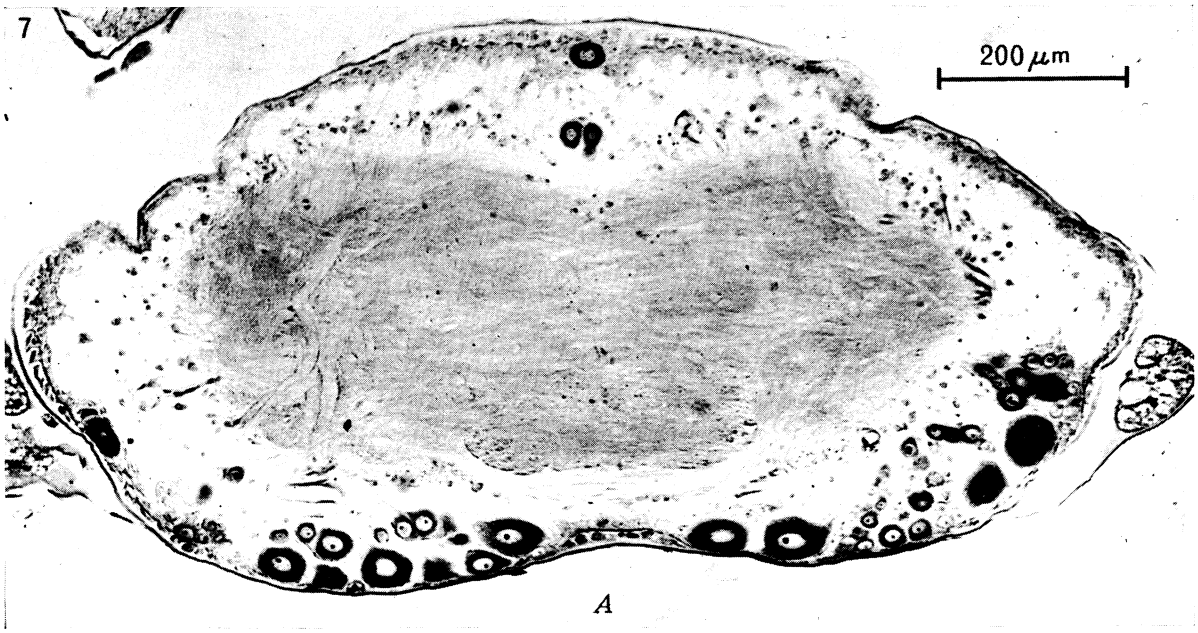


FIGURE 6. Ventral view of an unfixed metathoracic ganglion *in situ*. The large superficial tracheal trunks have been removed to show the peripheral nerve trunks. Labels are the same as in figure 5. The parallel white lines indicate the plane of the sections shown in figures 7 and 8.



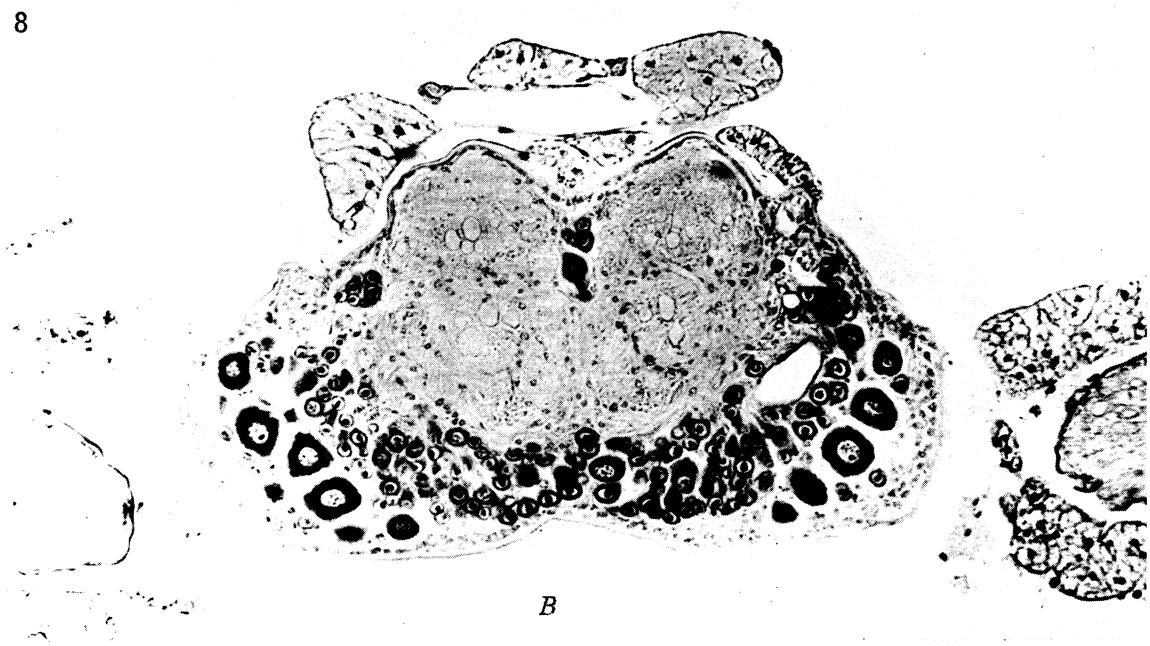


FIGURE 7. Transverse section through a metathoracic ganglion in the plane indicated by line *A* in figures 5 and 6. Dorsal is toward the top of the page; stain is pyronine-malachite green. The matched pair of cells on either side of the mid-line are cells 11 as indicated in the maps of figures 5 and 9. Cell 10 is seen just to the right of cell 11 on the right side of the photograph.

FIGURE 8. Transverse section through a posterior portion of the same ganglion as in figure 7. The large cells stepped on either side of the section are cells of the '50' group indicated in the maps of figures 5, 9 and 10. The plane of section is indicated by line *B* in figures 5 and 6. Magnification same as figure 7.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

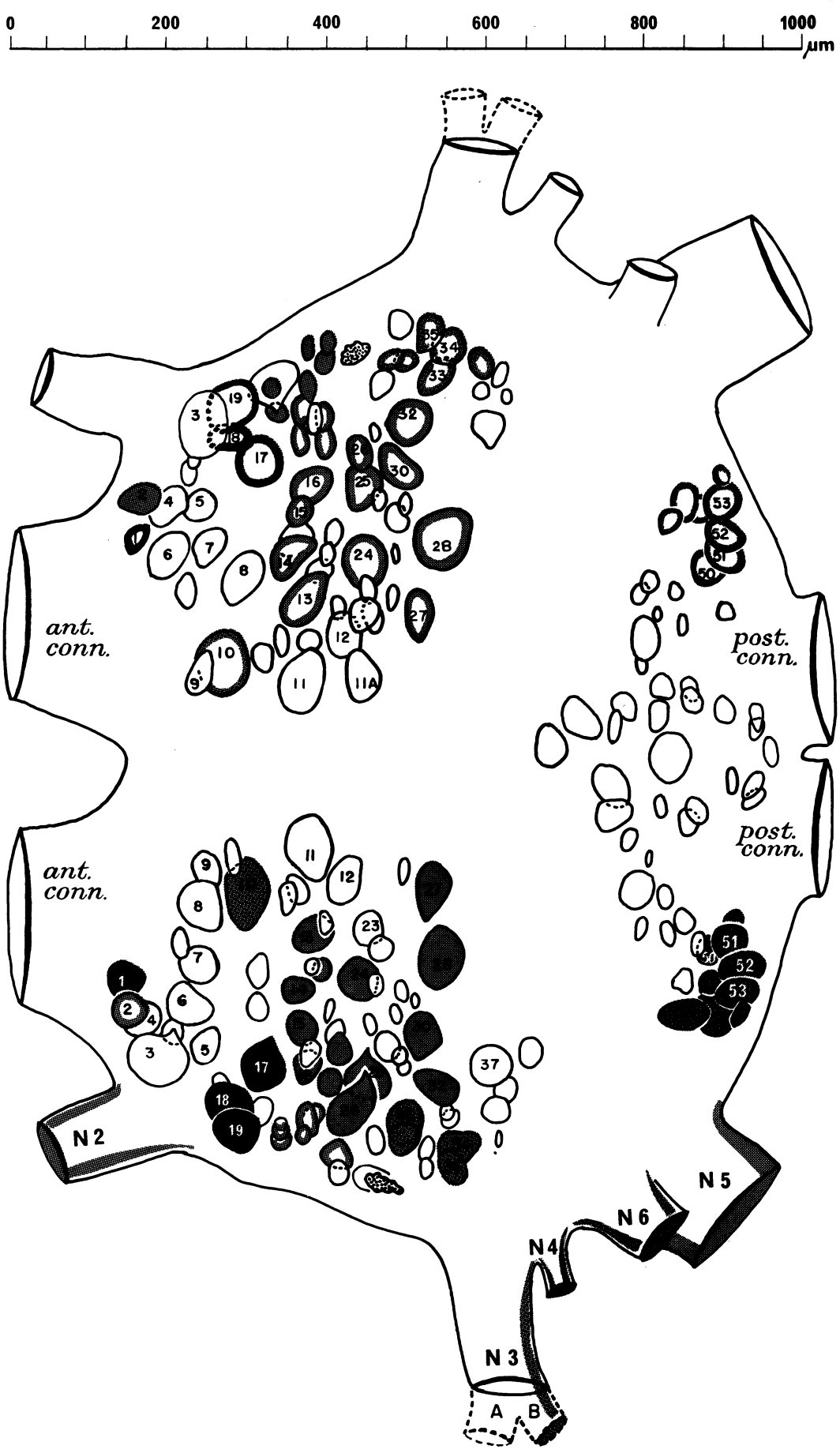


FIGURE 9. For legend see facing page.

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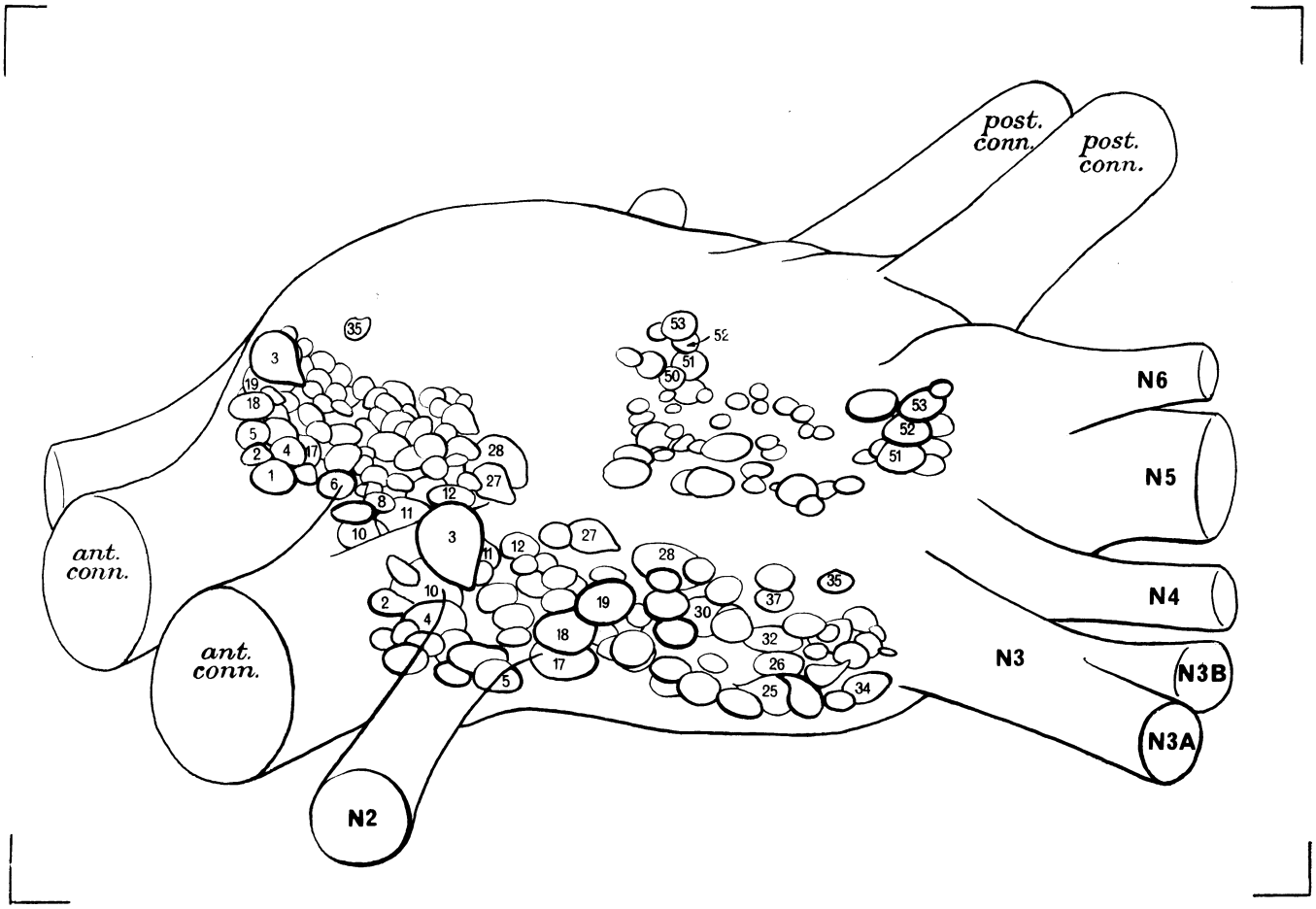

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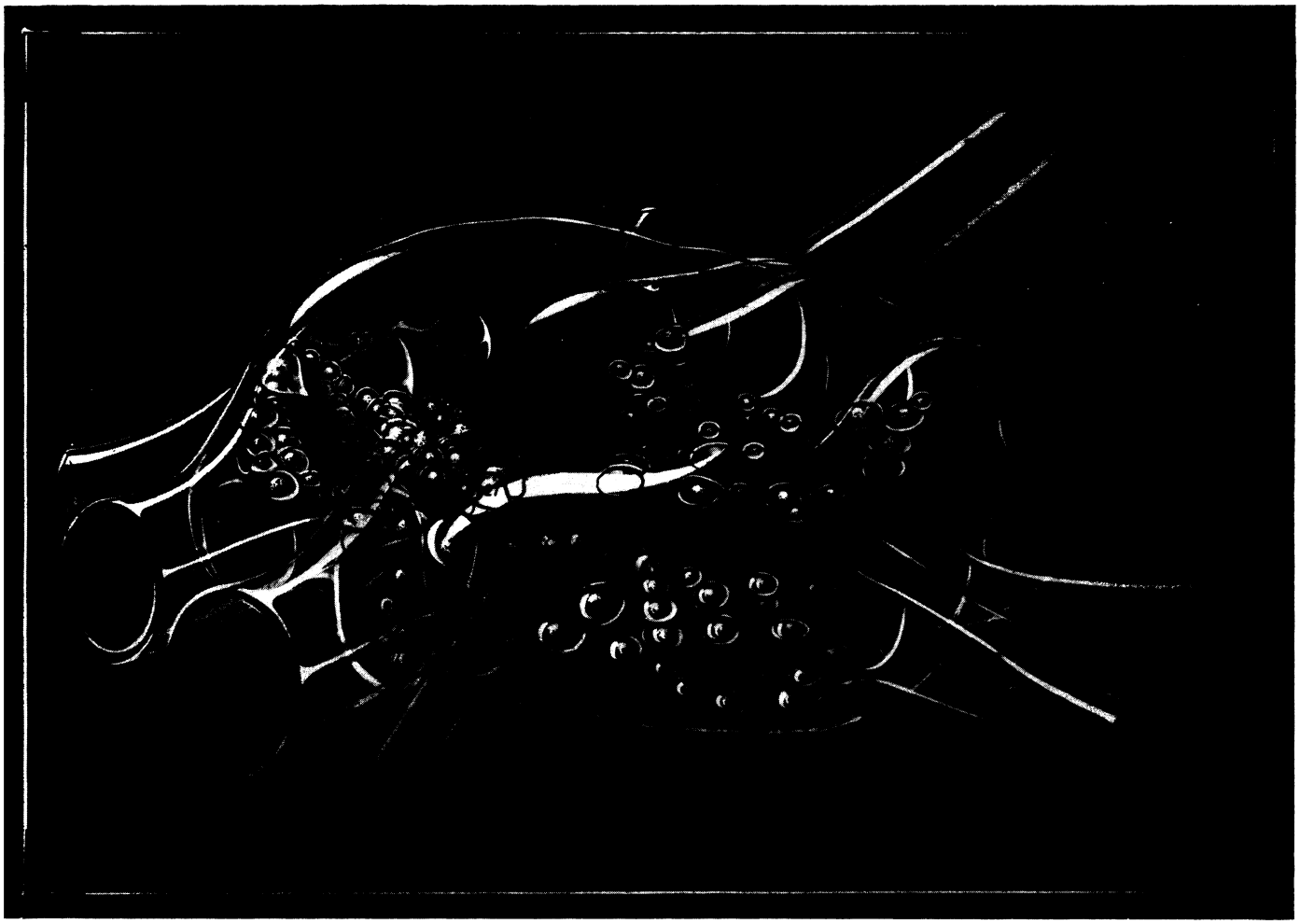

FIGURE 10. A three-dimensional representation of the same ganglion that is mapped in figure 9. The overlay is labelled to indicate the nerve trunks and some of the key cell bodies shown in figure 9. The scale is the same as in figure 9.

FIGURE 9. A cell map of the metathoracic ganglion of a young adult male. Constructed from 10 μm serial sections. The solid coloured cells send their axons out from nerve trunks of the corresponding colour. Note that all the cells except those associated with nerve 2 send their axons out from the ipsilateral nerve trunks. Nerve 2 cells send their axons out from the contralateral trunk. The open coloured cells send their axons out from the the unlabelled nerve trunks of the side on which they are located, except again for the cells contributing axons to nerve 2. Labelling same as in figure 5.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

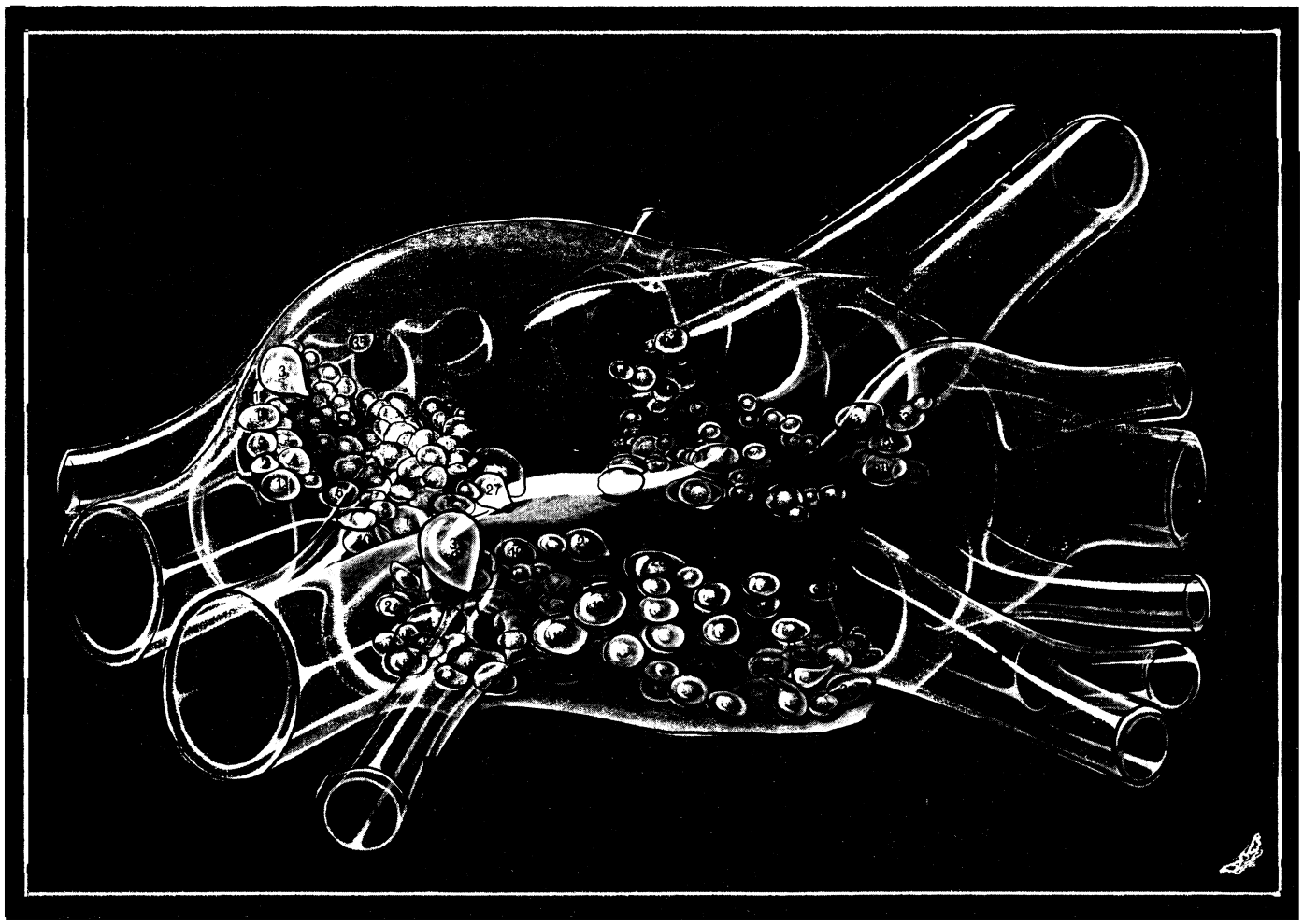

FIGURE 10. A three-dimensional representation of the same ganglion that is mapped in figure 9. The overlay is labelled to indicate the nerve trunks and some of the key cell bodies shown in figure 9. The scale is the same as in figure 9.

FIGURE 9. A cell map of the metathoracic ganglion of a young adult male. Constructed from 10 μm serial sections. The solid coloured cells send their axons out from nerve trunks of the corresponding colour. Note that all the cells except those associated with nerve 2 send their axons out from the ipsilateral nerve trunks. Nerve 2 cells send their axons out from the contralateral trunk. The open coloured cells send their axons out from the the unlabelled nerve trunks of the side on which they are located, except again for the cells contributing axons to nerve 2. Labelling same as in figure 5.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The cells that could be consistently identified with a given nerve trunk from one preparation to another are indicated in the coloured map of figure 9. This map of cell outline is constructed from serial sections of preparation N2. The colour coding matches the nerve-cell bodies to the nerve trunk in which their axons leave the ganglion and is based on the averaged data indicated in table 1. Nerves 3 through 6 contain the motor axons that innervate the leg musculature (Pipa & Cook 1959). As shown in figure 9, the cell bodies

TABLE 1.

nerves cut	no. of animals	average no. of cells per animal that show rings
N 2	5	8
N 3A	3	10
N 3B	4	8
N 4	3	4
N 5	13	16
N 4, N 6	4	12
N 2, N 3, N 4, N 5, N 6	11	54
N 2, N 3, N 4, N 5, N 6, *ant. conn.*	30 (9)*	54
same	4 (1)	54
N 3B, N 5	18	24
ant. conn.	7	?
	102	

* Numbers in parentheses indicate nymphs within the group.

associated with these nerves send their axons out from the ipsilateral nerve trunks. The cell bodies of nerve 2, whose axons go to body musculature (Pipa & Cook 1959), send their axons out from the contralateral nerve trunk. The bulk of the cells sending axons out from any particular nerve trunk are generally grouped together within the ganglion. However, there are instances such as for nerves 2, 3 and 4 where a single cell body is set apart from the main group contributing axons to the particular nerve trunk. The most conspicuous of these is the large medially placed cell 10 associated with nerve 3B. It is possible that these separate groupings of cell bodies which send their axons out from the same nerve trunk may correlate with functional differences such as fast and slow activity.

In the instances where the anterior connectives of the ganglion were cut, no clear *RNA* ring responses could be consistently observed in the cell bodies. This indicates either that the cell bodies of the axons in the connectives lie in other ganglia or that they are too small to be resolved by this technique. After examining the data of Zawarzin (1924), Bullock & Horridge (1965) arrive at the conclusion that the majority of fibres in the thoracic connectives of the dragonfly larva *Aeschna* are interneurons. This implies that the cell bodies attached to the axons in the connectives may be distributed throughout the ventral nerve cord in other ganglia and could explain why we do not consistently find cell bodies showing the injury response after cutting the anterior connectives of the meta-thoracic ganglion.

Cell numbers

A comparison of the number of cells in the cockroach ganglion to the data available for other arthropod ganglia may be instructive. Dresden & Nijenhuis (1958) have counted what they believe to be motor axons in nerve trunks 3 through 6 on one side of the meso-thoracic ganglion in *Periplaneta americana*. They arrive at approximately 102 fibres between

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

2 and 20 μm in diameter. We count approximately 103 nerve-cell bodies above 20 μm in diameter on one side only in the metathoracic ganglion. Assuming that the larger axons come from the larger cells, the good correlation between our cell body count and the motor axon count of Dresden & Nijenhuis substantiates the idea that the large cells of the ganglion are probably motor neurons. Of the 103 cells above 20 μm on one side of the metathoracic ganglion, we have numbered and can consistently locate on our maps approximately 54 cells. These are generally composed of the larger cell bodies. Dresden & Nijenhuis indicate 50 motor axons of the mesothoracic nerve trunks in the 10 to 20 μm diameter range. The numerical correspondence of the two sets of data strongly suggests that the 54 numbered cells of our map give rise to the large motor axons of the nerve trunks.

In one ganglion stained for *RNA*, photographs were taken of each 10 μm serial section and an attempt made to count all the nerve cells in the ganglion. For the large cells above 20 μm this was relatively simple and one arrives at a total of 230 cells for the entire ganglion. For the cells below 20 μm, counts were made in the photographs of every other section to avoid counting the same cell twice. We find a total of 3192 cells in this category. Addition of the two groups yields a total number of 3422 cells in the entire ganglion. We feel we could distinguish the smaller nerve cells down to 15 μm in diameter from glial cells by their form and affinity for the pyronine-malachite green stain. It is interesting to note that the cell counts made by Wiersma (1957) for the thoracic ganglia of the crayfish range approximately between 2000 and 4000 cells and thus compare quite favourably with our count of the insect ganglion.

Discussion

Cytological changes induced by axon injury can be used for mapping the central neurons of insects as well as vertebrates. These cytological changes in both groups of organisms are associated with the processes of axonal repair but may differ in their temporal and quantitative aspects. The primary difference is that the major response in the vertebrates consists of a dispersal of cytoplasmic *RNA* aggregates (Nissl 1892, 1894; Brattgård, Edström & Hydén 1957) while in the insect the initial response is an aggregation of *RNA* in the perinuclear cytoplasm (Cohen & Jacklet 1965; Cohen 1967).

The bilaterally symmetrical distribution of cell bodies within the ganglion and their consistent location from one animal to another has several important implications. Bodian (1947) has pointed out the great variability of *RNA* metabolism in adjacent central neurons of mammals. We have noted differences in *RNA* staining properties of the cockroach central neurons and this has been seen previously by other workers (Hess 1958; Wigglesworth 1960). It is our impression that the stain density of pyronine-malachite green is remarkably similar for both members of a bilateral pair of central neurons in the cockroach. By using one member of a bilateral cell pair as an experimental object and the opposite member as a control, it is possible to eliminate genetic variability between different animals and, perhaps even more important, the variability in *RNA*-protein metabolism between one cell and another in the same ganglionic mass. This allows one to note reasonably small deviations between the experimental and control cells by directly comparing the appearance of members of a bilateral pair. By being able to identify certain cell bodies from one animal to another, it is possible to determine that the *RNA*

differences seen between specific cells of an individual appear consistently in different animals. This indication that the nerve cells do consistently differ in certain metabolic properties may allow one to correlate some of the observed histochemical differences with the specific functional properties of these neurons. It also serves to emphasize that neurons within the same bit of central nervous tissue can differ in their metabolic capacity and that the entire explanation of functional organization may not lie in circuitry alone but could also depend upon specific metabolic abilities of individual neurons.

The cellular bilateral symmetry of this ganglion also permits one to substantiate a major assumption of this mapping technique, namely, that the *RNA* injury response is confined to the cell bodies whose axons have been injured. One must rule out the chance that the change in cytoplasmic *RNA* is due to a general protein response to injury of the organism as indicated by the work of Berry, Krishnakumaran & Schniederman (1964). This possibility is eliminated by comparing the appearance of the injured cell to its bilateral mate. Differences in members of matched pairs of cells are consistently seen as shown in figures 1 and 2, plate 27. This indicates that the responses are confined primarily to the injured side and are not a generalized systemic reaction evoked by surgery.

Another possibility is that the effect might be transynaptic and evoked by eliminating the afferent input to the central cell bodies when cutting the peripheral nerve trunk. Afferent input, however, influences the musculature of both the contralateral and ipsilateral limbs (Pringle 1940; Wilson 1965). If disturbance of the afferent input were the responsible factor for the observed *RNA* responses, then one would expect to see rings generally scattered on both sides of the ganglion when the nerve trunks are cut. The fact that the responses to injury of leg nerves 3 to 6 are confined to the injured side only indicates that the transynaptic factor is not involved and we are seeing a direct response of the cell body to injury of its own axon.

Still another factor indicating that we are dealing with motor neurons is the close agreement between the number of mapped motor neurons and the number of medium and large motor axons described in the peripheral nerve trunks by Dresden & Nijenhius (1958).

The identification of central motor neurons in the metathoracic ganglion is presently being extended to include the connexion of these neurons to individual leg muscles. This should enable the identification of specific motor neurons engaged in particular locomotor patterns, either learned or unlearned. It represents an initial step in defining the individual nerve cells involved in specific behavioural acts. There remains to be developed a method for identifying the specific sensory and interneurons involved and their synaptic connexions. The beautiful studies of Zawarzin (1924) on dragonfly larvae demonstrate the power of the methylene-blue technique for showing nerve processes as well as cell bodies in central nervous tissue. However, this technique is capricious and one is never quite certain how many cells are not stained. The *RNA* procedure described here should allow one to determine which particular motor cells are important in any given bit of behaviour. By then concentrating on these few cells with methylene blue and silver stains, one might be able to locate some of the tracts involved and at least the most probable areas of synaptic contact. It is also hoped to use the nerve-terminal degeneration techniques that have proved so powerful in the vertebrates (Gray & Guillery 1966) to localize further the critical

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

synaptic areas between sensory fibres, interneurons and motor neurons. This may then permit the detailed specification of critical synapses by concentrating an electron-microscopic analysis on a very localized bit of neuropile.

One would hope by this means to characterize the major nerve cell bodies and synaptic elements involved in specific acts of behaviour. This should provide insight into the structural and metabolic organization responsible for the specific behavioural capabilities of certain populations of neurons.

We wish to thank Professor J. W. S. Pringle, F.R.S., for generously providing the facilities of his Department, where this work was initiated while M.J.C. was on sabbatical leave from the University of Oregon. The counsel of Dr J. R. Baker on cytological techniques was invaluable. The excellent technical assistance of Mr David Young is most appreciated. We are indebted to Mr Peter Parks for his fine rendering of figure 10.

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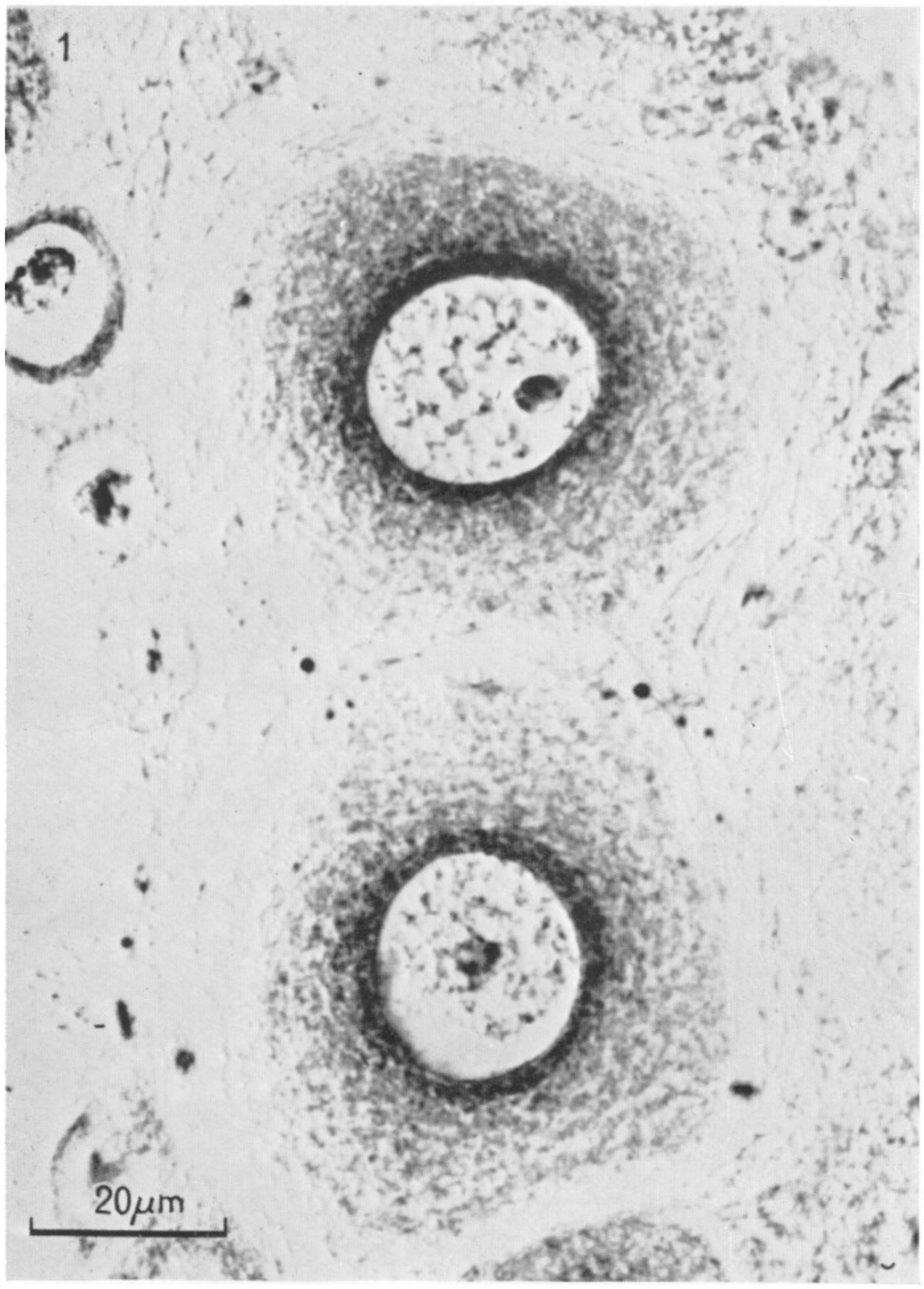

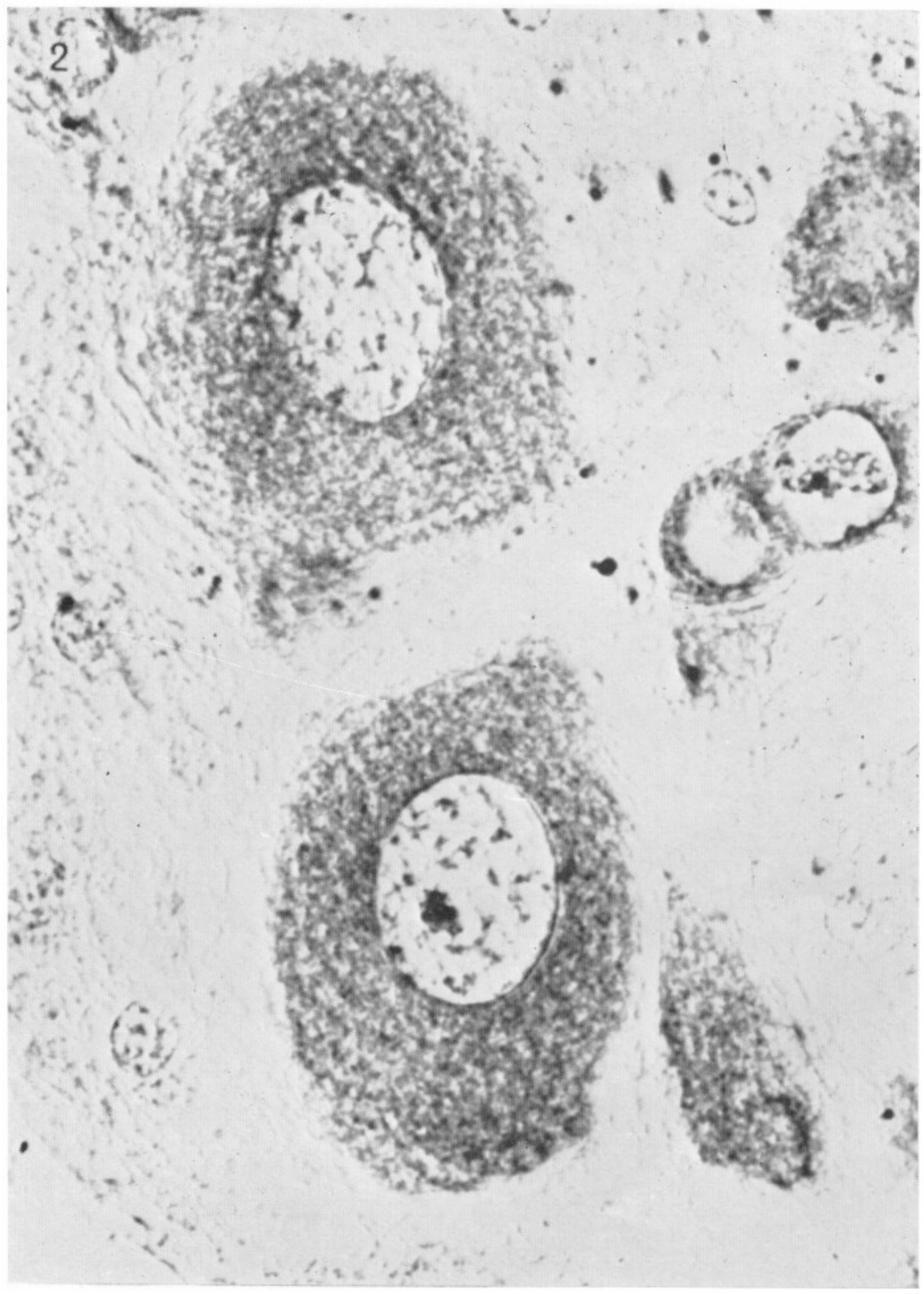

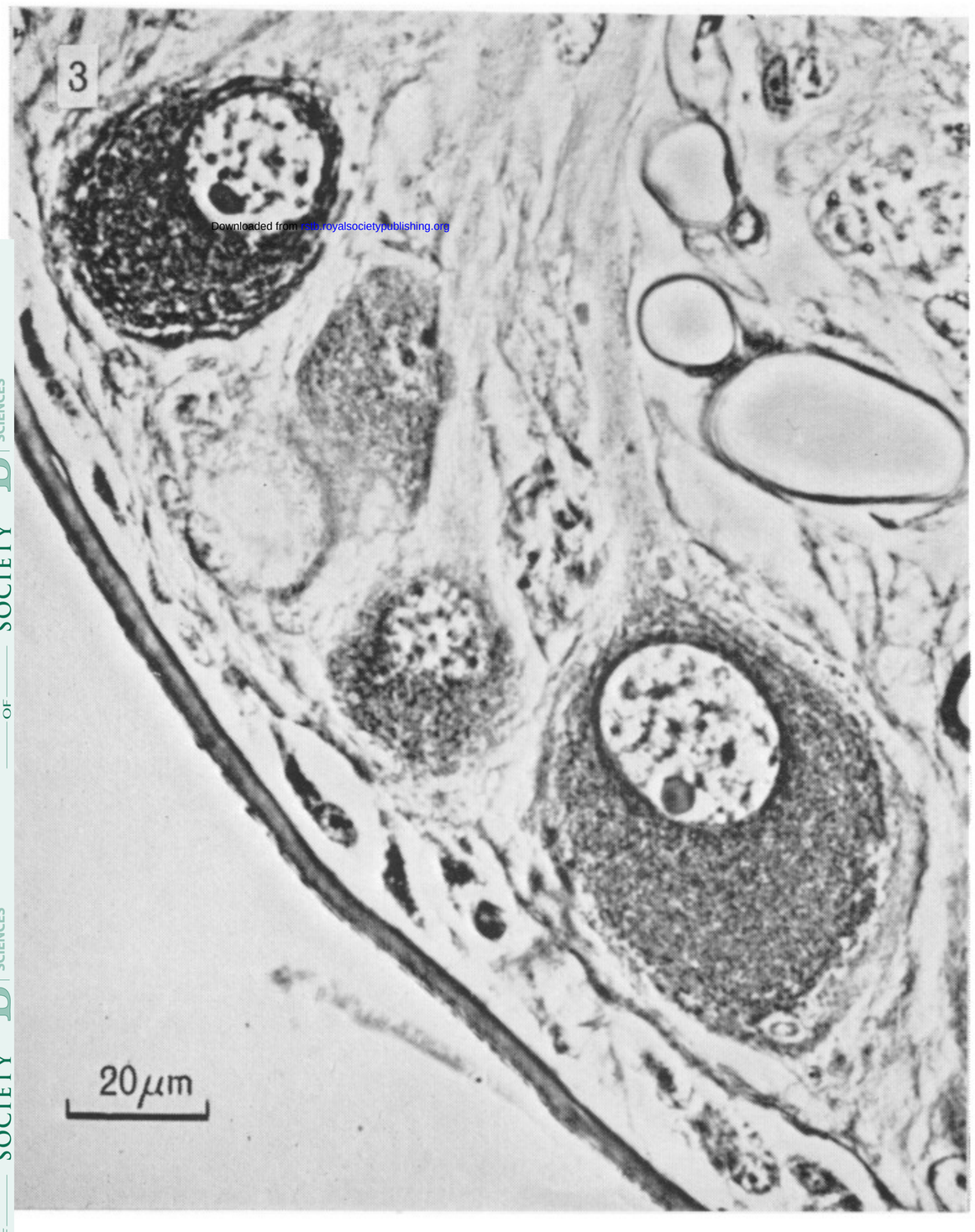

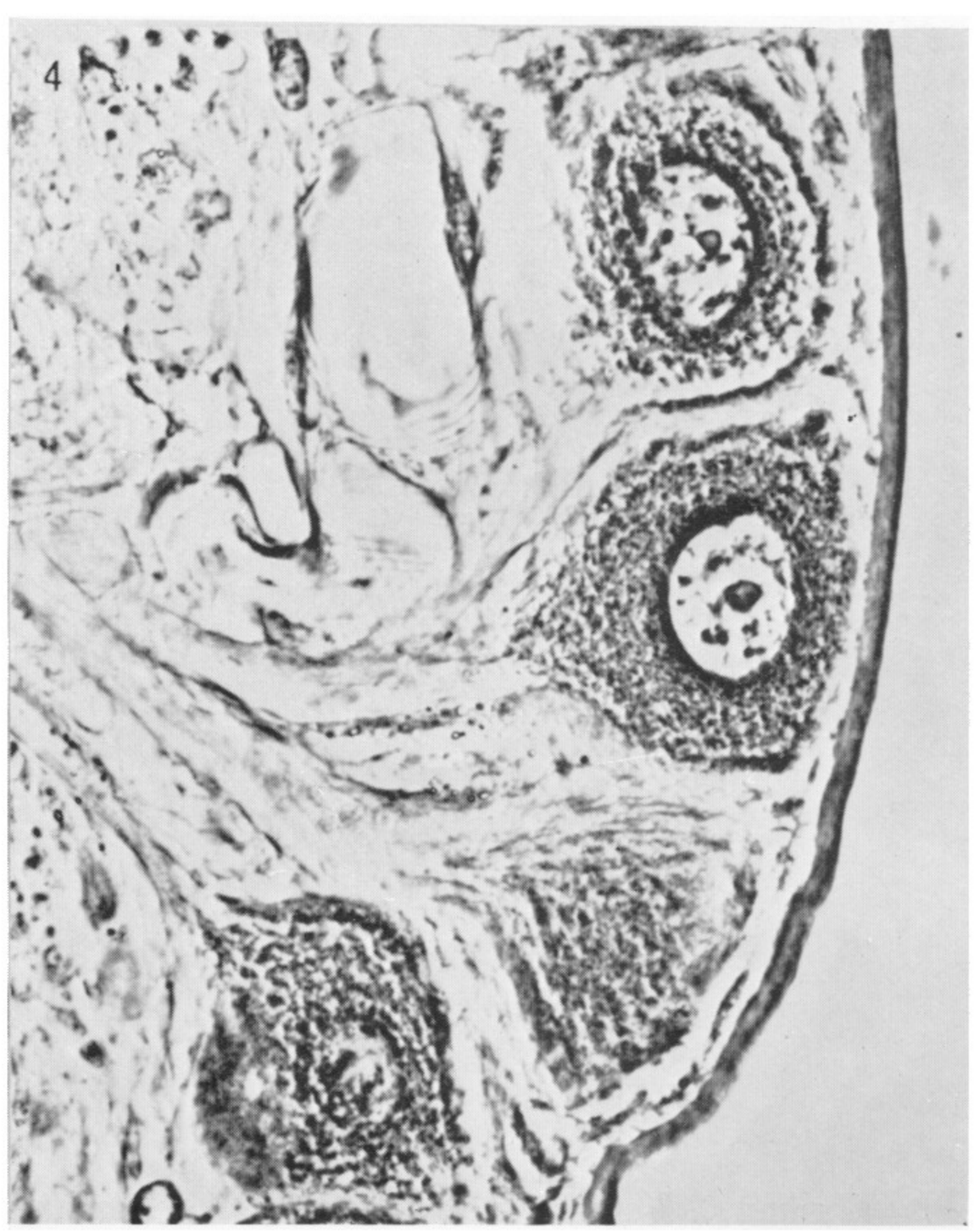

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FIGURES 1 to 4. For legends see facing page.

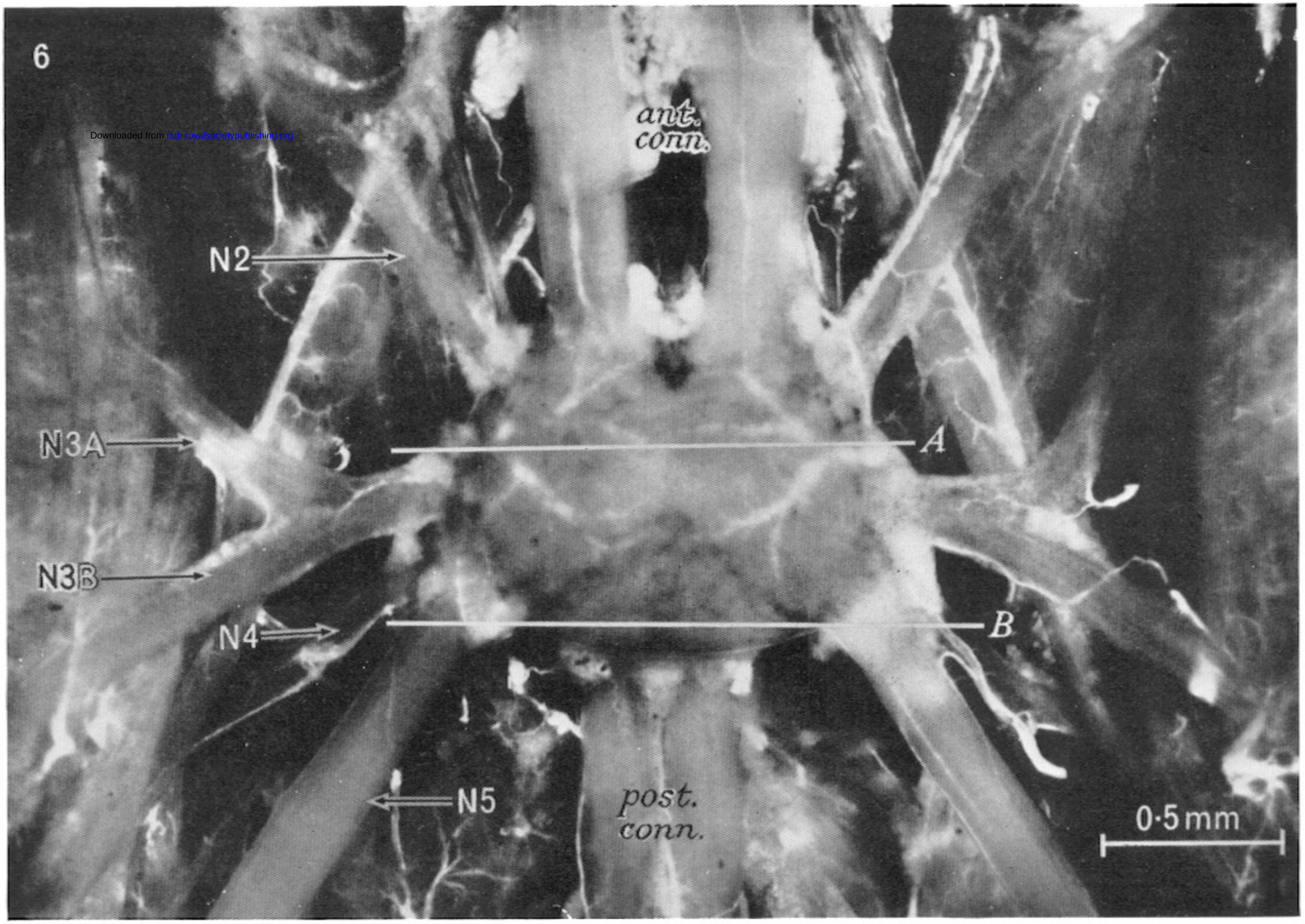
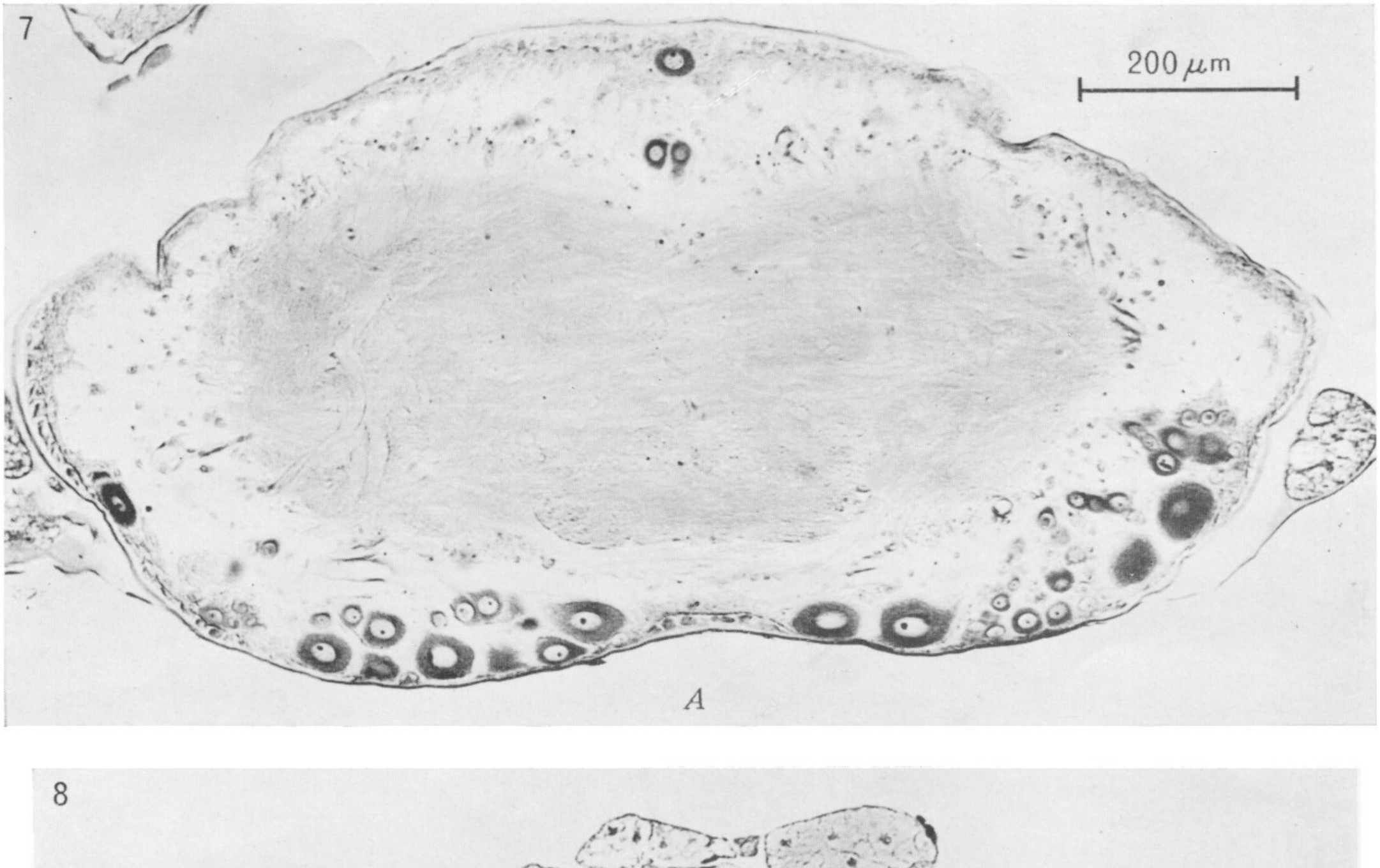


FIGURE 6. Ventral view of an unfixed metathoracic ganglion *in situ*. The large superficial tracheal trunks have been removed to show the peripheral nerve trunks. Labels are the same as in figure 5. The parallel white lines indicate the plane of the sections shown in figures 7 and 8.



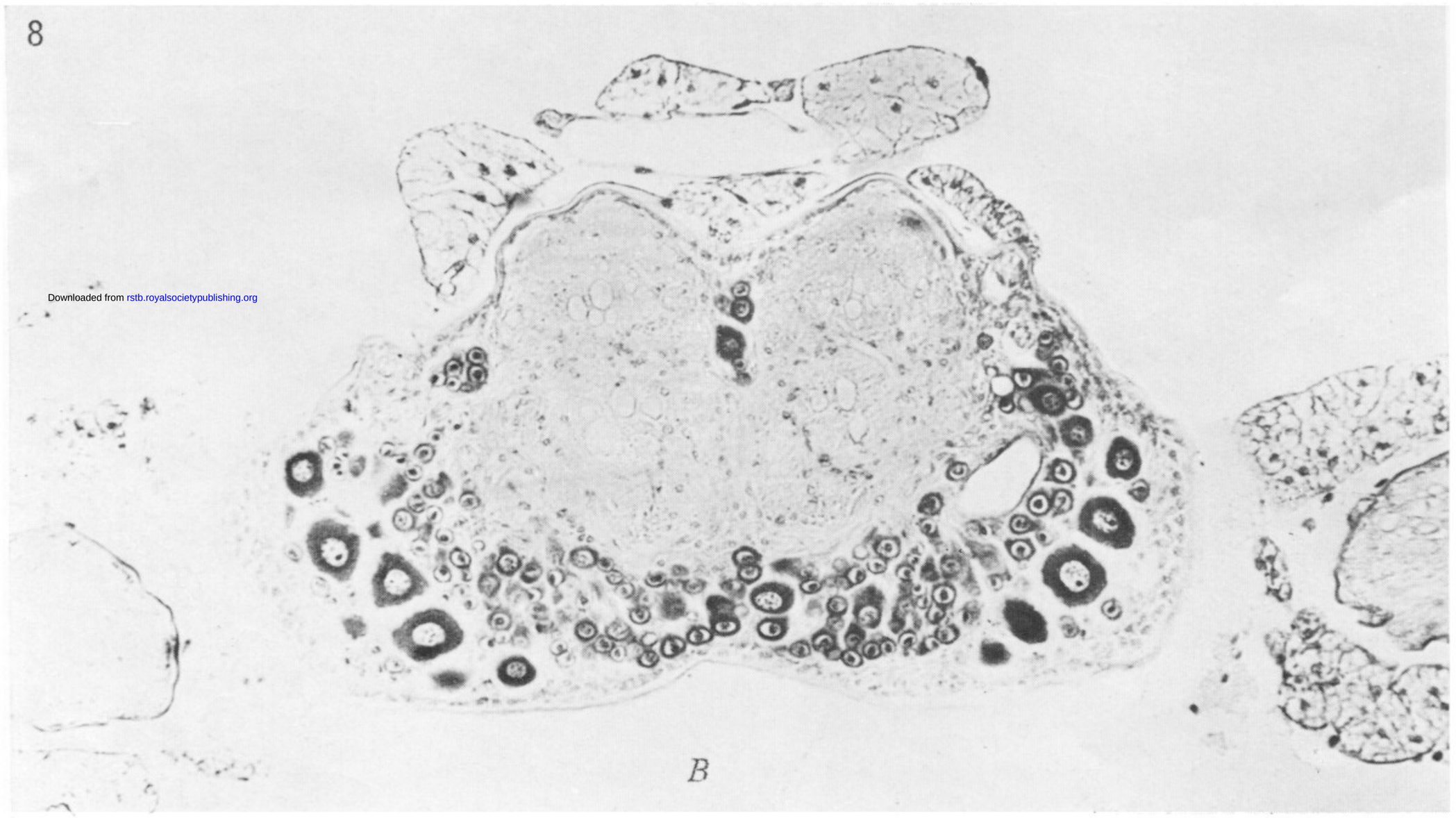


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FIGURE 7. Transverse section through a metathoracic ganglion in the plane indicated by line *A* in figures 5 and 6. Dorsal is toward the top of the page; stain is pyronine-malachite green. The matched pair of cells on either side of the mid-line are cells 11 as indicated in the maps of figures 5 and 9. Cell 10 is seen just to the right of cell 11 on the right side of the photograph.

FIGURE 8. Transverse section through a posterior portion of the same ganglion as in figure 7. The large cells stepped on either side of the section are cells of the '50' group indicated in the maps of figures 5, 9 and 10. The plane of section is indicated by line *B* in figures 5 and 6. Magnification same as figure 7.

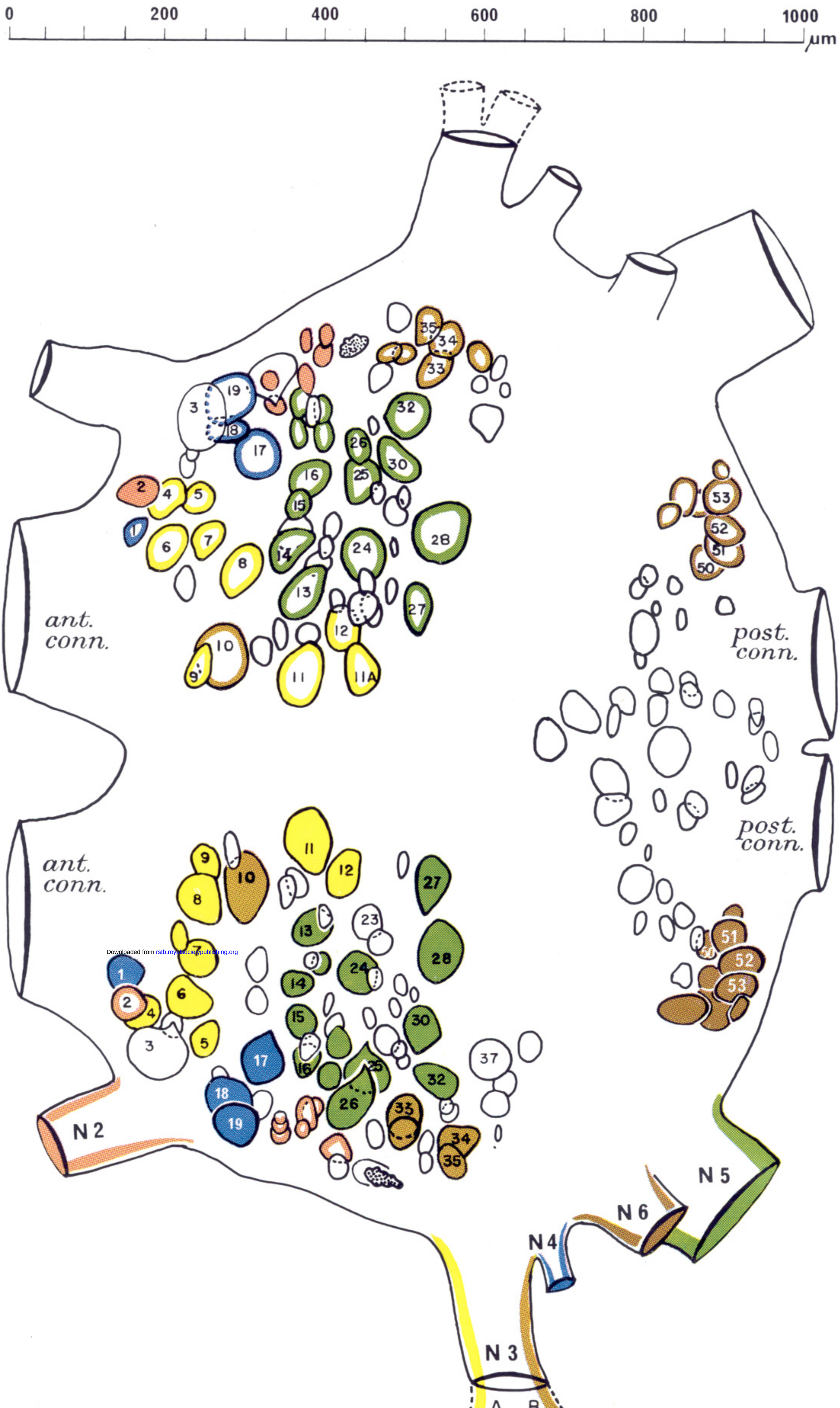

FIGURE 9. For legend see facing page.

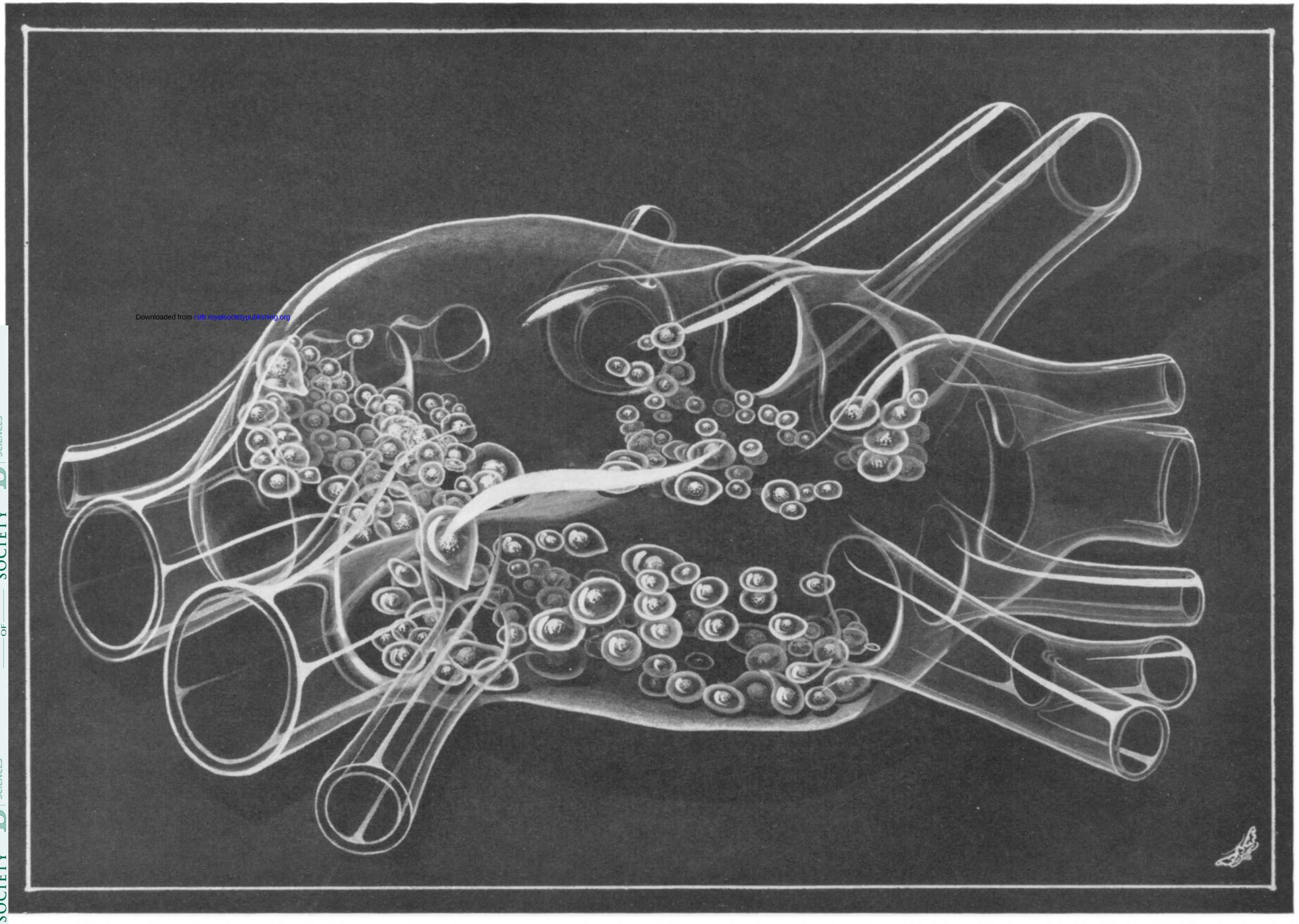

FIGURE 10. A three-dimensional representation of the same ganglion that is mapped in figure 9. The overlay is labelled to indicate the nerve trunks and some of the key cell bodies shown in figure 9. The scale is the same as in figure 9.

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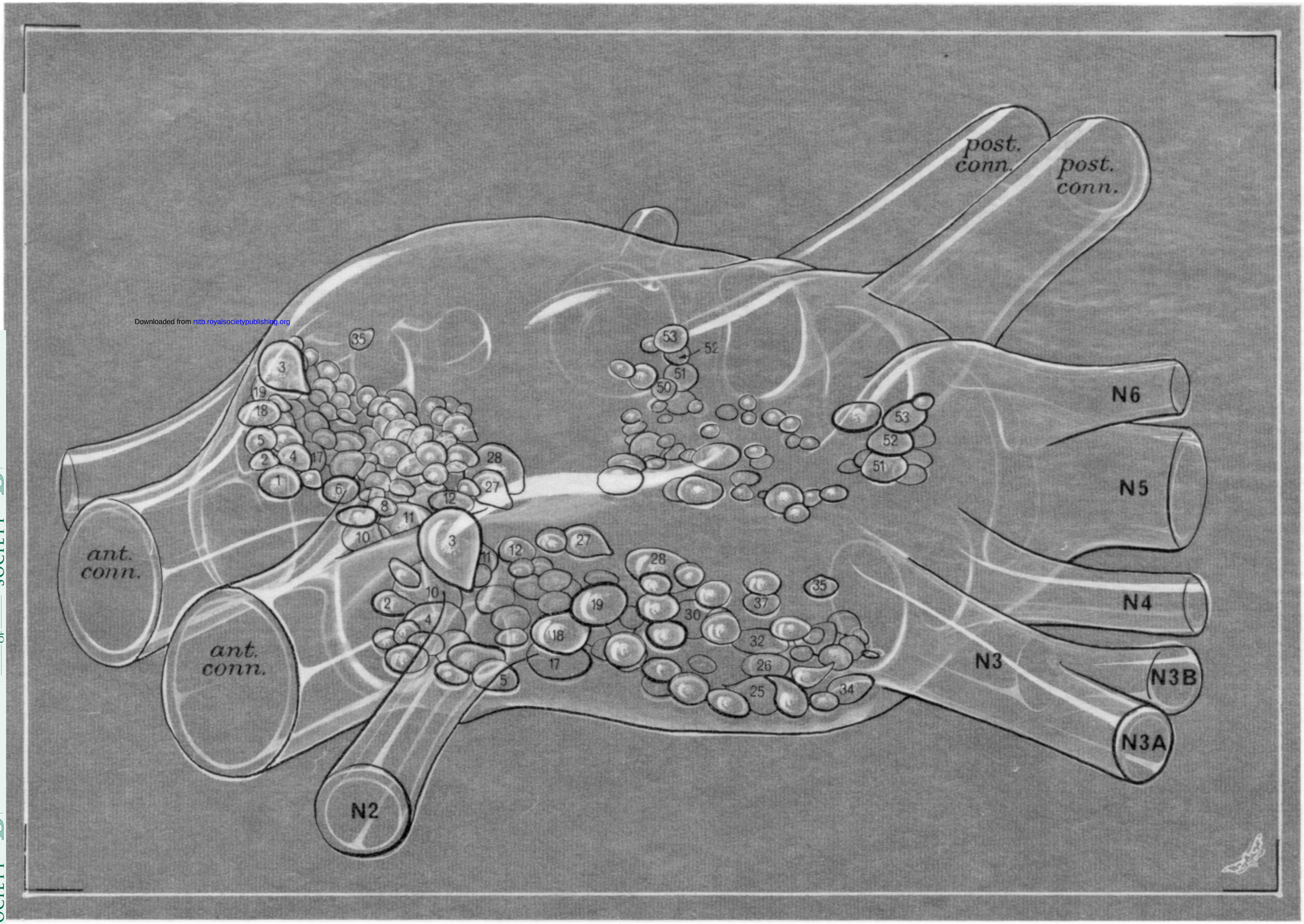

FIGURE 10. A three-dimensional representation of the same ganglion that is mapped in figure 9. The overlay is labelled to indicate the nerve trunks and some of the key cell bodies shown in figure 9. The scale is the same as in figure 9.