

## INNERVATION OF THE ABDOMINAL INTERSEGMENTAL MUSCLES IN THE GRASSHOPPER

### I. AXON COUNTS USING UNCONVENTIONAL TECHNIQUES FOR THE ELECTRON MICROSCOPE

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#### INTRODUCTION

It is now well known from physiological evidence that many insect muscles receive a 'fast', a 'slow' and, in some cases, an inhibitory motor axon (see reviews by Hoyle, 1965; Bullock & Horridge, 1965; Aidley, 1967; Usherwood, 1967). A recent physiological investigation has shown, however, that single fibres of the cockroach posterior coxal levator muscle are innervated by six axons, four of which are excitatory and two inhibitory (Pearson & Bergman, 1969). Tyrer (1968) found nerves which contained either seven or eight axons supplying the intersegmental muscles of the abdomen in embryos of *Schistocerca gregaria*. Since these muscles have the relatively simple function of shortening the abdomen it is difficult to understand why so many axons are required. This problem has been investigated in the abdomen of adults of the grasshopper *Melanoplus differentialis*. A description is given here of the detailed anatomy of the innervation of the four median internal dorsal muscles and the median external dorsal muscle (Snodgrass, 1935), which will be referred to collectively as intersegmental muscles. A functional analysis of the innervation is described in a subsequent paper (Tyrer, 1971).

Axon counts in the nerves to these muscles could not be made reliably with the light microscope because of the small diameter ( $0.5\text{--}1.0\ \mu\text{m}$ ) of some of the axons and because of the small separation between axons. Even with the electron microscope consistent counts in the same nerve could not be obtained from single sections and it was necessary to make serial sections. Two unconventional techniques have been devised which make it considerably easier to examine long lengths of nerve by serial sectioning. First, the block of tissue embedded in Araldite was cut into very thick sections (*c.*  $50\ \mu\text{m}$ ) which were examined in the light microscope. Details of the muscles and the nerve bundles to them could be resolved. These sections could then be re-mounted on the face of the block and sections were cut for examination with the electron microscope. Secondly, a method was developed for examining serial sections up to  $0.5\ \mu\text{m}$  thick with a conventional electron microscope operated at 75 kV.

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## I. INITIAL INVESTIGATION METHODS

Young adult male and female *Melanoplus differentialis* were obtained from a breeding colony maintained in the laboratory. The paths of the nerves to the muscles of the fourth abdominal segment were traced by dissection in Usherwood's locust saline (Usherwood, 1968) using leucomethylene blue to stain the nerves (Pantin, 1964). After the blue stain had faded further details of the nerve branches could be revealed by dissecting after staining with 2% aqueous eosin for 10 min. Nine males and sixteen females were investigated in this way. Drawings and photographs of the dissections were made.

For all subsequent investigations, with both the light microscope and electron microscope, material was treated in the following way. The abdomen was cut off and opened to one side of the dorsal mid line. The body wall was pinned out rapidly on Plasticine in a Petri dish with the body laterally extended to straighten the dorsal nerves. The dish was flooded with ice-cold glutaraldehyde fixative containing 2.5% glutaraldehyde maintained at pH 7.0 in 0.05 M sodium cacodylate buffer with 0.17 M sucrose. After 2 h the gut was removed and the dorsal intersegmental muscles of the fourth segment were isolated by making cuts across the terga of the third and fifth segments and along the sternum of the fourth segment. After overnight washing in cold sodium cacodylate buffer containing 0.34 M sucrose the tissue was post-fixed in 1% osmium tetroxide buffered with sodium cacodylate at pH 7.0 for 1 h, dehydrated in an ethanol series and embedded in Araldite.

Serial sections were cut at 3  $\mu$ m in the horizontal plane through the whole of one block using a Porter-Blum MT 1 microtome and glass knives. The sections were floated on microscope slides in separate drops of warm water, dried down and stained with 1% methylene blue in 1% borax and mounted in neutral Canada balsam. In these sections it was possible to trace nerve branches which were difficult to dissect, and to check that there were no branches which had been missed in dissection. Although resolution was inadequate to make accurate axon counts, it was possible to make some estimate of the number of axons in the nerve branches in these sections.

To make accurate counts thin sections were cut from five different blocks using a Porter-Blum MT 2 microtome and glass knives. Gold sections 'stained' with uranyl acetate and lead citrate were examined in a Hitachi HU 11E electron microscope.

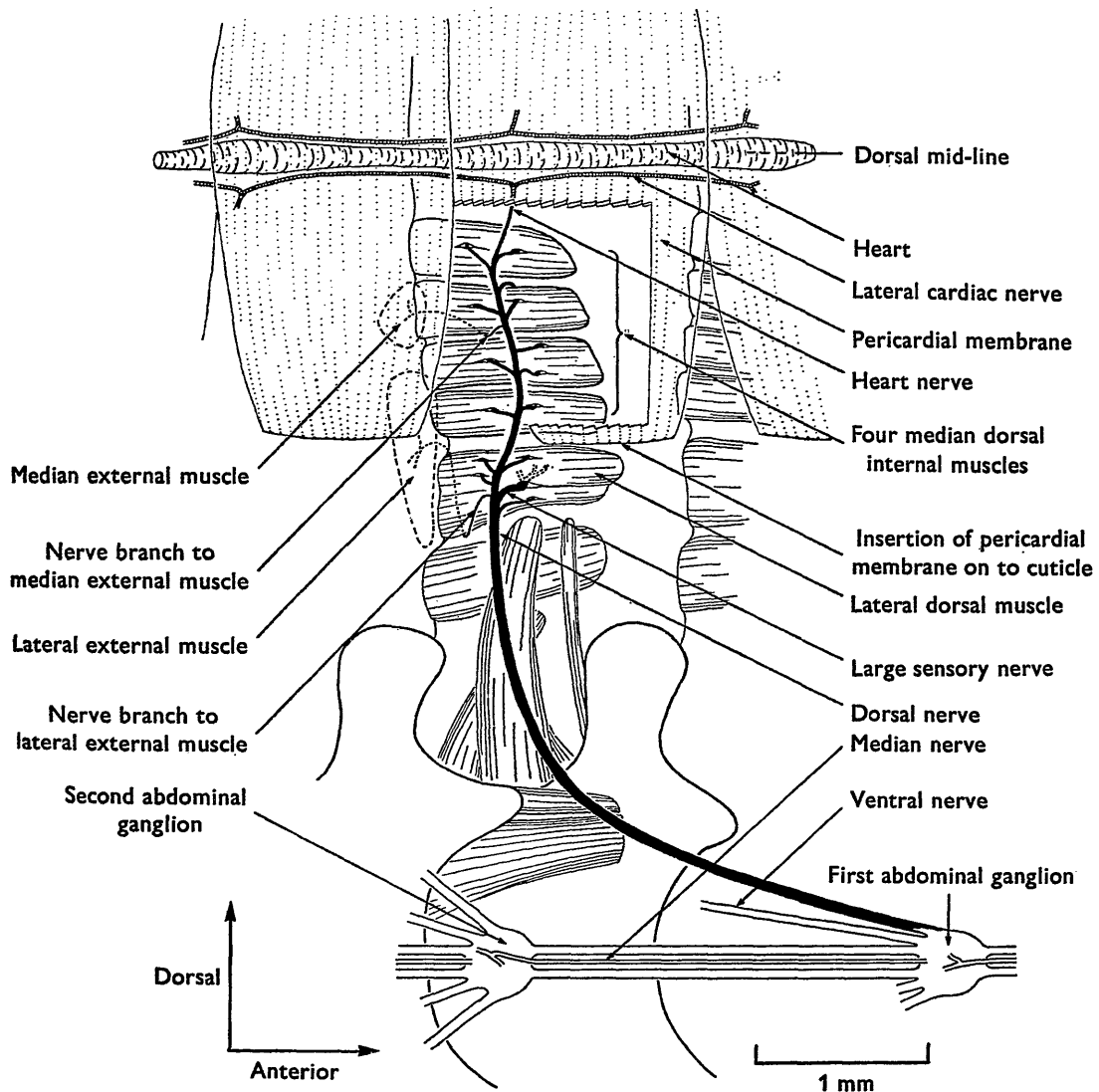
*Results**(a) Dissections*

A diagram of the fourth abdominal segment is shown in Text-fig. 1. The innervation of the abdominal muscles in *Melanoplus differentialis* is essentially the same as that for *Dissosteira marginalis* (Schmitt, 1954).<sup>\*</sup> Considerable individual variation was found in the details of the finer branches of the nerves even though the major branches were consistent from animal to animal. I shall describe only those features relevant to the median dorsal muscles.

In the tergal region the main branch of the dorsal nerve runs dorsally from the mid-line to join the lateral cardiac nerve. A large sensory branch runs from the cuticle and penetrates the lateral dorsal internal muscle to join the main branch. Distal to

<sup>\*</sup> A detailed map of the innervation of the abdomen will be supplied to anyone who requests it.

this junction two to four branches run from the main dorsal nerve to each of the four median dorsal internal muscles. The number and distribution of these branches show considerable individual variation. Each branch runs over the outside of the muscle for a short distance and then plunges between the fibres and into the muscle. Some leucomethylene blue preparations showed the bundle splitting into many branches within the muscle. Distal to the median muscles the dorsal nerve continues as the heart nerve, a fine bundle which joins the lateral cardiac nerve running longitudinally close to the heart (Text-fig. 1).



Text-fig. 1. A diagram of the fourth abdominal segment, showing the dorsal muscles and their innervation in relation to other features of the segment.

The external muscles are also innervated from the dorsal nerve by two branches, one to the lateral and one to the median muscle (Text-fig. 1). An interesting feature not shown in the figure is that all the nerve branches follow the course of the tracheal branches closely.

(b) *Light microscopy of 3  $\mu\text{m}$  sections stained with methylene blue*

Examination of serial horizontal sections confirmed the paths of the main nerve branches traced by dissection. The large sensory branch from the cuticle consists largely of axons less than 1  $\mu\text{m}$  in diameter. The dorsal nerve distal to this contains fibres 3–10  $\mu\text{m}$  in diameter which send branches to the intersegmental muscles and also a bundle containing axons less than 3  $\mu\text{m}$  in diameter which continues up to the lateral cardiac nerve without branching. It was confirmed that the branching patterns of the nerves and of the tracheae correspond closely.

(c) *Electron microscopy of nerve sections*

Axon counts from single sections varied considerably in different individuals and even axon counts made on the same nerve in the same animal were inconsistent. There were three problems in estimating the number of axons in a nerve:

(1) Some axons follow a tortuous course so that several profiles of the same axon may appear in one section (Text-fig. 2; cf. Plate 1 A, B).

(2) Some axons have folds in their walls (see Plate 1) and so two profiles of the same axon may appear in one section.

(3) It is sometimes difficult to identify an axon profile in transverse section. For example, there are axons containing neurosecretory granules in the heart nerve close to the heart, similar to those found in the lateral cardiac nerve (Johnson & Bowers, 1963; Johnson, 1966; Bowers and Johnson, 1966). In addition there are cells filled with similar granules surrounding the heart nerve which in transverse section are indistinguishable from axons (Plate 1 C). Serial transverse sections and sections longitudinal to the nerve show that many of these cells are roughly spherical in shape and cannot be axons. Glial folds may sometimes also closely resemble small axon profiles (Plate 1 B).

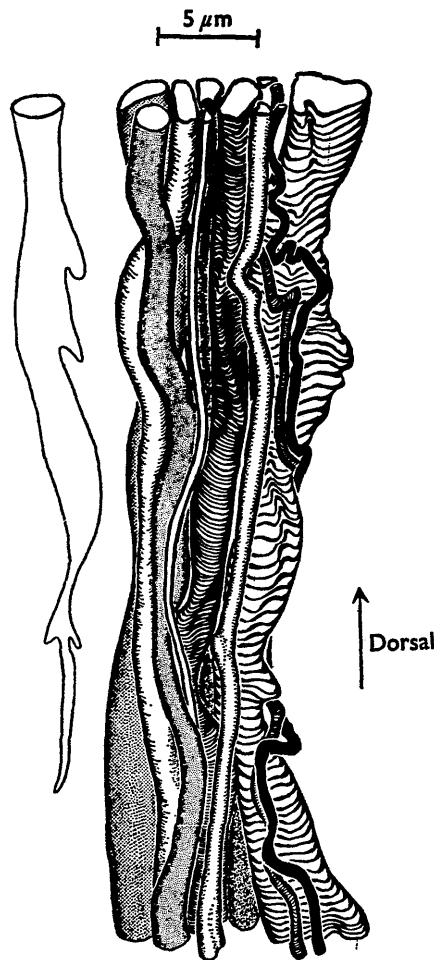
All of these problems can be solved by reconstructing the course of the axons from serial sections of the nerve bundle. Sometimes, however, 10–20  $\mu\text{m}$  of the nerve had to be reconstructed before the axon number could be determined with confidence. To do this for a number of nerve branches using conventional serial sectioning techniques for electron microscopy was very slow. For this reason special methods were developed for rapid serial sectioning.

## II. AXON COUNTS: SPECIAL METHODS

The fourth abdominal segment was examined in two male and two female individuals. The tissue was fixed and embedded in Araldite as described above. Horizontal sections were cut on a Porter–Blum MT 1 ultramicrotome with glass knives. A detailed examination was made of the dorsal nerve in all four specimens in four different regions of the segment (Text-fig. 3). Very thick sections were cut to find the region where a detailed examination was to be carried out and the detailed examination was made on 0.5  $\mu\text{m}$  sections with the electron microscope.

*50  $\mu\text{m}$  sections*

The face of the block was trimmed smooth using a glass knife. The knife was advanced 50  $\mu\text{m}$  and a very thick section was cut very slowly. Chipping from the top of the block was minimized by pressing down on top of the block with an applicator stick as the cut was made. The section was picked up with watchmaker's forceps, floated on a drop of water on a microscope slide and dried down on a hot plate at 50 °C.



Text-fig. 2. A reconstruction from serial 0.5  $\mu\text{m}$  sections examined in the electron microscope of the heart nerve in region D (see Text-fig. 3 and Plate 2). The main nerve consists of eleven axons which follow a tortuous course and have irregular infoldings of their walls. The reconstruction has been somewhat simplified by eliminating many of the infoldings. Alongside the main nerve is an axon (drawn unshaded) running in the same sheath which ends in several blunt processes. Some of these processes contain an amorphous electron dense material.

To cut a second section, a new part of the knife was selected and the face of the block was again trimmed smooth. Another section was cut as before and then dried down alongside the first section on the slide. These sections were examined with the light microscope without staining and without mounting medium. Details of the muscles and the nerve branches to them could be resolved. Sometimes the technique

was used only to reach a particular region of the segment and a more detailed examination was carried out on  $0.5\ \mu\text{m}$  sections cut from the original block. On other occasions, the thick sections were carefully lifted from the microscope slide, remounted on the block with '5 minute epoxy' resin (Devcon R. 205) and  $0.5\ \mu\text{m}$  sections cut from them.

#### *$0.5\ \mu\text{m}$ sections*

For examination in the electron microscope four  $0.5\ \mu\text{m}$  sections at a time were cut, flattened with chloroform vapour and picked up on  $2 \times 1\ \text{mm}$  slot grids coated with parlodion and carbon films. These sections were examined without staining in a Hitachi HU 11E electron microscope at 75 kV. One section on each grid was photographed so that a profile of the nerve was obtained approximately every  $2\ \mu\text{m}$ . Portions of the dorsal nerve were reconstructed from these photographs (Text-fig. 2). Although resolution was not as good as that obtained with conventional thin sections it was quite adequate for resolving the membranes of even the smallest axons ( $0.15\ \mu\text{m}$  diameter in the sensory nerve). It was possible to section the block at a rate of  $50\text{--}100\ \mu\text{m/h}$  and the sections were ready for examination with the electron microscope immediately.

In each of the four regions, sections were made for a distance of  $30\text{--}100\ \mu\text{m}$  from all four blocks. In addition shorter series of  $20\text{--}30\ \mu\text{m}$  were cut and axon counts were made on 17 branches to the intersegmental muscles.

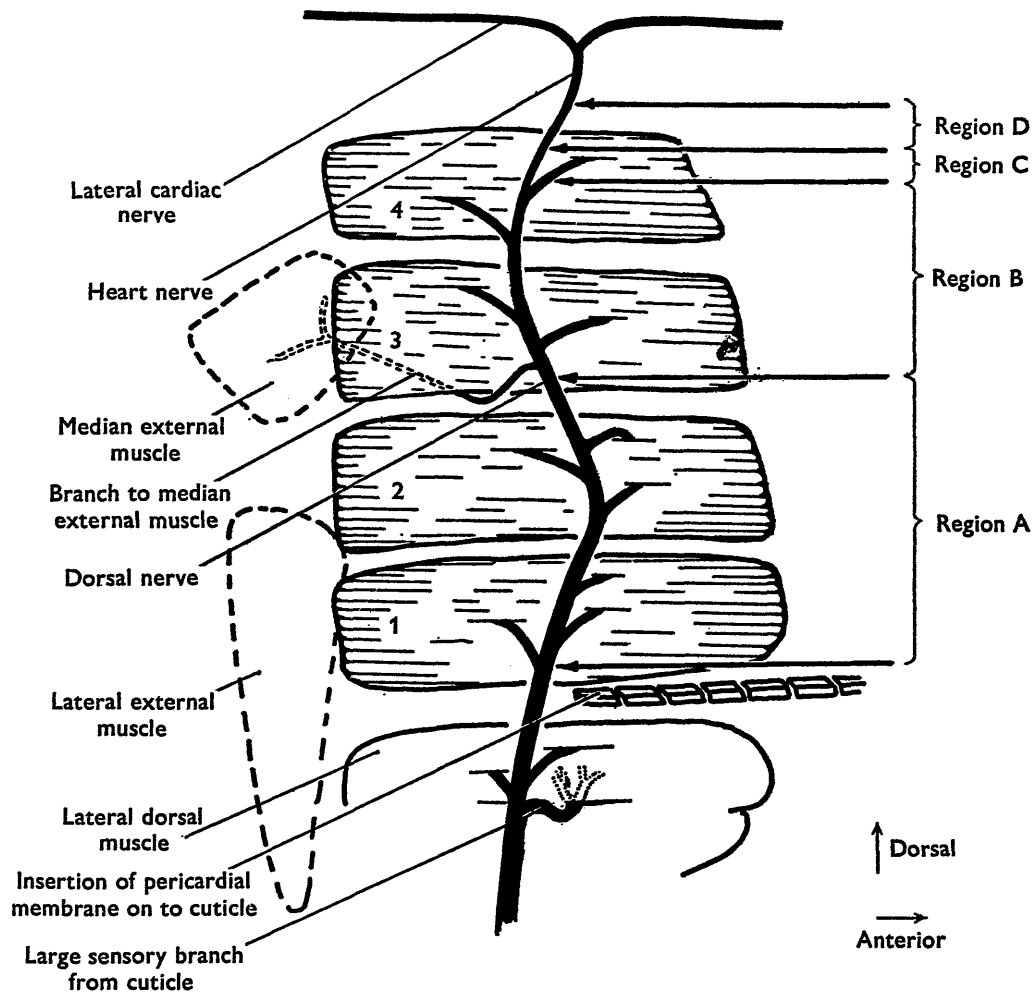
#### *Results*

The four regions sampled are defined in Text-fig. 3. Axon counts in these regions are given in Table 1 and examples are shown in Plate 2. The dorsal nerve in region A contains 21 axons. The largest of these are eight axons which innervate the median dorsal muscles. The nerves to the muscles contain branches of each of these eight axons. Proceeding distally, the diameters of the eight axons in the dorsal nerve are reduced as each branch leaves (see Plate 2). Usually each of the branches also contain eight axons. Of 21 branches examined, 18 contained eight axons, one contained seven and two contained six.

In region B two axons to the median external muscle leave the dorsal nerve. Beyond this point the dorsal nerve contains 19 axons, until the last branch of eight axons leaves for muscle four in region C. Distal to this the dorsal nerve contains only 11 axons which join the lateral cardiac nerve. It is not clear whether these axons run to or from the heart. Since their diameter is somewhat greater close to the heart ( $2.5\text{--}0.5\ \mu\text{m}$ ) than in the region of insertion of the pericardial membrane ( $1.5\text{--}0.3\ \mu\text{m}$ ) it seems likely that they run from the heart. Throughout the portion of the dorsal nerve examined the 11 heart axons follow a tortuous course and it is these which were particularly confusing when making axon counts. Dorsal to region D the heart nerve is surrounded by irregular cells containing granules of variable electron density (Plate 1C). Some of the axons in the heart nerve branch in this region. One of these branches was followed in serial sections and was found to end in several blunt processes alongside the main nerve (Text-fig. 2). Some of these processes contained an amorphous electron-dense material.

In any one individual the branches to the dorsal intersegmental muscles were not

all the same size. Typically, however, the branches were about  $10\ \mu\text{m}$  in diameter and contained 2 axons  $3\text{--}4\ \mu\text{m}$  in diameter, 2 axons  $2\text{--}3\ \mu\text{m}$  in diameter, 2 of  $1\text{--}2\ \mu\text{m}$  and 2 of about  $0.5\ \mu\text{m}$ . (Plate 2). Sometimes one of the large axons was larger than the other, and sometimes there were only three size categories: 2 fibres of about  $4\ \mu\text{m}$ , 4 of about  $2\ \mu\text{m}$  and 2 of  $1\text{--}0.5\ \mu\text{m}$ .



Text-fig. 3. The innervation of the four median dorsal internal muscles and the median external muscle. Axon counts in the dorsal nerve, shown in Table 1 and discussed in the text, have been made in each of the regions A to D.

Table 1

Region	Muscle	Axon no. in branch to muscle	Axon no. in the dorsal nerve
A	1	8	21
	2	8	
B	Median external	2	19
	3	8	
	4	8	
C	4	8	11
D	—	—	11

There was individual variation in the sizes of the axons in the dorsal nerve. Table 2 gives the diameters of the eight axons to the internal muscles, and the large axon to the medium external muscle, in region A, for the four individuals examined. This is the point at which external recordings were made in subsequent electrophysiological experiments (Tyrer, 1971).

Table 2. *Sizes of large axons in the dorsal nerve in region A close to the percardial membrane insertion*

Individuals	Size of axon ( $\mu\text{m}$ )				
	2-3	3-4	4-5	5-6	6-10
Male 1	2	0	5	2	0
Male 2	2	2	3	1	1
Female 1	2	2	3	0	2
Female 2	2	3	2	1	1

#### DISCUSSION

Many insect muscles are innervated by only a few axons (see review by Bullock & Horridge, 1965). The metathoracic extensor tibiae muscle of the locust, for example, receives only three (Hoyle, 1955). However, not all insect muscles are as simple as this. Some cockroach muscles have been described which have as many as seven or eight axons (Dresden & Nijenhuis, 1958; Becht, 1959). Many of the neck muscles of the locust receive innervation from six or more different axons and some from as many as 13-15 (Shepherd, 1969). Here I have demonstrated that some abdominal muscles, which might be presumed to be simple, are innervated by eight axons. The considerable variation in axon size in different individuals shown in this investigation has important consequences for physiological experiments. Kennedy & Takeda (1965), recording extracellularly from abdominal nerves in crayfish, were able to identify axons regularly according to the size of the potentials recorded. The individual variations in axon size in the locust abdomen means that units cannot be recognized easily by spike size in extracellular records.

It is interesting that the 11 heart axons follow a much more tortuous course than the axons to the intersegmental muscles. Even in dissections it is apparent that their character is different. A similar phenomenon has been noted in the cockroach segmental heart nerves by Alexandrowicz (1924), who observed that in dissections they had an uneven and varicose appearance. The character of these nerves may be a consequence of their embryological history or their physiological function.

An anatomical technique has been developed specifically because of the problems of making axon counts in a nerve which contains tortuous axons. Even where this problem is not encountered, however, the technique should be valuable for reconstruction of nerve bundles because of its speed, simplicity and high resolution.

#### SUMMARY

1. The course of the dorsal nerve in the fourth abdominal segment of the grasshopper *Melanoplus differentialis* is described.
2. Unconventional techniques of making serial sections of the dorsal nerve for

examination with the electron microscope are described in detail. The method permits a higher resolution than the light microscope but lower than more conventional electronmicroscope techniques.

3. It has been demonstrated that the median dorsal internal muscles are all innervated from eight axons in the dorsal nerve.

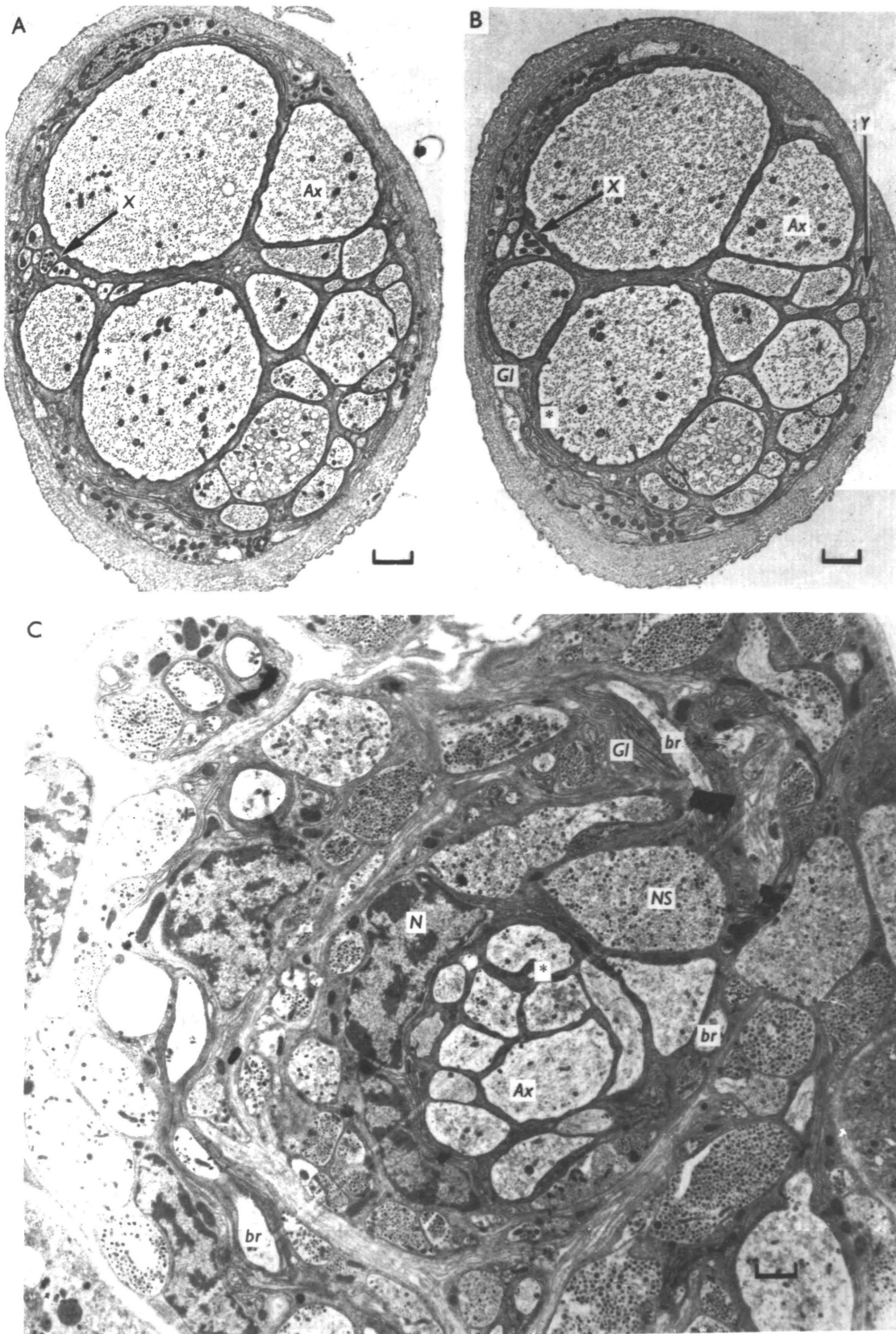
4. Although there is individual variation in the size of these axons there is some indication that they can be divided into four pairs according to their size.

5. Of the remaining 13 axons in the dorsal nerve, two supply the median external dorsal muscle and 11 are concerned with the innervation of the heart.

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